Bir Bahadur · Manchikatla Venkat Rajam
Leela Sahijram · K.V. Krishnamurthy
Editors

Plant Biology and Biotechnology

Volume II: Plant Genomics and Biotechnology

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Foreword

While writing this Foreword, I was reminded of a quote attributed to Mahatma Gandhi: "The expert knows more and more about less and less until he knows everything about nothing." The quote illustrates the great dilemma that all of us face in modern times: but this is especially acute for those engaged in the pursuit of science. Compared to the times of Archimedes or Leonardo da Vinci or Antonie Philips van Leeuwenhoek, whose range of interests covered several disciplines (they looked at the world in its entirety), most of us have now become narrow specialists of one kind or another, knowing less and less about the wider world. Thus, edited monographs, proceedings of seminars and the like have become absolutely essential to keep us informed and engaged in research and teaching more meaningfully (such publications allow summarizing of recent researches at a more advanced level than is possible in ordinary textbooks).

Turning to plant sciences, the *Annual Review of Plant Biology,* started in the middle of the last century, continues to be an invaluable source of information on the broad advances of plant biology. Yet, it is necessary to have a more inclusive look at advances over a somewhat longer period and also have this information in a way more organized than the format of annual reviews allows. Thus, Prof. Bir Bahadur and his colleagues deserve our grateful thanks on undertaking an incredibly difficult task of summarizing advances on the very broad front of plant biology – the topics cover not only fundamental aspects of plant biology but also plant biotechnology, which is now growing almost as a separate discipline. I welcome their style of a historical approach (nearly every article follows this style). This approach is often neglected by specialists, but the fact is that this is the *only way* to genuine understanding and for a non-expert to easily discern major advances or milestones. This unity in overall planning and laying out the style has obviously been possible due to the fact that two of three co-editors are in fact former pupils of the senior editor (Prof. Rajam, the senior most of them, was, in a sense, a colleague while I was at Delhi University). Understandably, in the combined work on Volumes I and II, Prof. Bir Bahadur is author of nearly ten chapters and Prof. Rajam author of five chapters. Their two other colleagues Dr. Leela Sahijram and Prof. Krishnamurthy have also contributed several chapters. Nonetheless, the work has very valuable contributions also from several national and international contributors (in Volume 2, there are around

ten authors from outside India), which has immensely added to the value of this work.

I think that on the whole, a very laudable contribution has been made. The editors have managed to include almost all topics which are significant in modern plant biology. In Volume 1, I was delighted to see several chapters close to my interest, such as those relating to polyploidy, photosynthesis, apomixis and flower development. But in Volume 2, there is special emphasis on genomics and plant biotechnology, and there are many other chapters of current interest. Space is not adequate to mention all the chapters or their topics, but to me, those on genetic markers, doubled haploids, plant genomes and genomics (there are several on these topics), epigenetic mechanisms, bioinformatics and systems biology were of special interest. Also, I am very delighted that Volume 2 starts with an excellent chapter on *Arabidopsis thaliana*. Inspired by a lecture on Langridge's work by Prof. Arthur W. Galston, I undertook in 1960s to 'tame' a wild Indian strain of *Arabidopsis* by raising in vitro cultures. However, despite the fact that *Arabidopsis* is now the principal material for basic research in plant biology, there are many who have never seen a live *Arabidopsis* plant, and surely, the opening chapter of this volume will be valuable for all.

Although ably aided by his pupils, Prof. Bahadur remains the chief architect of this endeavour. And I am struck with the expanse of his canvas and the breadth of his interest – it seems to me that in part, it is due to his early association with Prof. J.B.S. Haldane, F.R.S., whose own interest covered many disciplines, from mathematics, biochemistry and genetics to animal and plant biology. The topics he and his colleagues cover are of both fundamental and applied interest. I have to admit that many of us in universities are a bit distant from fields and sometimes unfamiliar with the full potential of fundamental discoveries for biotechnological applications. This work will help focus due attention of readers on both aspects of plant biology.

When the chapters were first sent to me, I noticed many typographic mistakes than are normally present in finished manuscripts – it is true that English is not the mother tongue of many of us in India, but I hope these mistakes have been rectified.

Once again, I wish to congratulate Prof. Bir Bahadur and his colleagues for a very unique monograph and insight in modern plant biology.

Satish C. Maheshwari

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Preface

The human population is increasing at an alarming rate and is expected to reach 11 billion by 2050. As there is a big gap between population growth and food production, food security for an ever-increasing population poses a major challenge for the present and future times. In fact, it will become necessary in the coming two decades or so to double food production with available arable land; else, it may precipitate great famines in some parts of the world. This is not achievable with just conventional strategies like plant breeding. However, the projected increase in food production may be achieved if traditional breeding methods are coupled with biotechnological approaches as the latter can offer novel ways for increasing productivity and quality of crops as also for producing an array of useful compounds including pharmaceuticals and biofuels. Indeed, during the past couple of decades, dramatic progress has been made in the field of plant genomics and biotechnology. Therefore, a need was felt for updating scientific developments in these areas.

Plant Biology and Biotechnology – Volume 2 was planned to present state-of-the-art scientific information on various basic and applied aspects of plant genomics. This volume comprises 37 chapters spanning various aspects of plant genomics and biotechnology and provides comprehensive and updated information on a wide variety of topics including *Arabidopsis* as a wonderful model system for plant research, plant–fungus interactions, microalgae in biotechnological applications, genetic markers and marker-assisted breeding, doubled haploids in breeding, DNA fingerprinting for plant identification, nuclear and organellar genomes, functional genomics, proteomics, epigenomics, bioinformatics, systems biology, applications of tissue culture in crop improvement and conservation of plant genetic resources, genetically modified crops for production of commercially important products and engineering abiotic and biotic stress tolerance, RNAi and microRNAs in crop improvement and environmental, marine, desert and rural biotechnologies. The book can serve as a good reference for plant molecular geneticists, plant biotechnologists, plant breeders, agricultural scientists and food scientists. Besides, it will also serve as a reference book for post-graduate students, researchers and teachers besides scientists working in agri-biotech companies.

Contributors of these volumes were selected from a wide range of institutions for introducing a diversity of authors. At the same time, these authors were selected based on their vast expertise in specific areas of their choice to

match the diversity of topics. These authors have a deep understanding of their subject to enable them not only to write critical reviews by integrating information from classical to modern literature but also to endure an unending series of editorial suggestions and revisions of their manuscripts. Needless to say, this is as much their book as ours.

We hope that these books will help our fellow teachers and a generation of students enter the fascinating world of plant genomics and biotechnology with confidence, as perceived and planned by us.

Hyderabad, Telangana, India — Bir Bahadur
New Delhi, India — Manchikatla Venkat Rajam
Bangalore, Karnataka, India — Leela Sahijram
Bangalore, Karnataka, India — K.V. Krishnamurthy

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We wish to express our grateful thanks to a number of friends and colleagues for their invaluable help in many ways and for their suggestions from time to time during the evolution of the two volumes. We also thank research scholars of Prof. M.V. Rajam (University of Delhi South Campus) – Shipra Saxena, Meenakshi Tetorya, Mahak Sachdeva, Bhawna Israni, Mamta, Manish Pareek, Anjali Jaiswal, Jyotsna Naik, Sneha Yogindran and Ami Choubey for their help in many ways.

We wish to express our appreciation for the help rendered by Ms. Surabhi Shukla, Ms. Raman, Mr.N.S. Pandian and other staff of Springer for their cooperation and invaluable suggestions. Above all, their professionalism, which made these books a reality, is greatly appreciated.

We wish to express our grateful thanks to our respective family members for their cooperation.

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Dr. Bir Bahadur, born 5 April 1938, studied at City College, Hyderabad, for 5 years including an Intermediate Course (Osmania University), graduated from Nizam College and postgraduated from University College, Osmania University, both in the first division. He obtained his Ph.D. in Plant Genetics from Osmania University. He was closely associated with late Prof. J.B.S. Haldane, F.R.S., a renowned British geneticist who encouraged him to study heterostyly and incompatibility in Indian plants, a subject first studied by Charles Darwin.

He made significant contributions in several areas, especially heterostyly, incompatibility, plant genetics, mutagenesis, plant tissue culture and biotechnology, morphogenesis, application of SEM in botanical research, plant asymmetry, plant morphology and anatomy and lately the biofuel plants Jatropha and castor.

He served as Lecturer and Reader at Osmania University, Hyderabad, and as Reader and Professor at Kakatiya University, Warangal. He also served as Head of Department; Chairman, Board of Studies; Dean, Faculty of Science; and Coordinating Officer/Dean, UGC Affairs at Kakatiya University. He has over 40 years of teaching and over 50 years of research experience. He has supervised 29 Ph.D. students and 3 M.Phil. students in both these universities and has published about 250 research papers/reviews, which are well received and cited in national and international journals, textbooks and reference books.

He was a postdoctoral fellow at the Institute of Genetics, Hungarian Academy of Sciences, Budapest, and worked on mutagenesis and chromosome replication in *Rhizobium.* He is a recipient of the direct award from the Royal Society Bursar, London. He also worked at Birmingham University (UK). He was conferred with the title of Honorary Research Fellow by the Birmingham University. He studied species differentiation in wild and cultivated solanums using interspecific hybridization and the enzyme-etched seeds technique in combination with scanning electron microscopy to assess the relationship among various *Solanum* species. At the invitation of the Royal Society, he visited Oxford University, Leeds University, Reading University and London University, including the Royal Botanic Gardens, Kew, and various research labs. He was invited for international conferences by the US Science Foundation at the University of Missouri, St. Louis, at the University of Texas, Houston (USA), and at the SABRO international conference at Tsukuba, Japan. He has extensively visited most countries of Eastern and Western Europe as well as Tanzania and the Middle East.

He has authored/edited ten books. One of his important books is entitled *Jatropha, Challenges for a New Energy Crop*, Vol. 1 and 2, published by Springer, New York, USA, 2013, jointly edited with Dr. M. Sujatha and Dr. Nicolas Carels. These books are considered significant contributions to bioenergy in recent times. He was Chief Editor, *Proceedings of Andhra Pradesh Akademi of Sciences*, Hyderabad, and Executive Editor, *Journal of Palynology* (Lucknow).

He is the recipient of the Best Teacher Award by the Andhra Pradesh Government for mentoring thousands of students in his teaching career spanning over 40 years. He was honoured with the Prof. Vishwambhar Puri Medal of Indian Botanical Society for his original contributions in various aspects of plant sciences. He has been honoured with the Bharat Jyoti Award at New Delhi for outstanding achievements and sustained contributions in the fields of education and research. He has been listed as one of the 39 prominent alumni of City College, a premier institution with a long history of about 90 years as per the latest update on its website. He has been chosen for distinguished standing and has been conferred with an honorary appointment to the Research Board of Advisors by the Board of Directors, Governing Board of Editors and Publications Board of the American Biographical Institute, USA.

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Dr. Manchikatla Venkat Rajam is currently Professor and Head, Department of Genetics, University of Delhi South Campus, New Delhi, India. He obtained his Ph.D. in Botany (1983) from Kakatiya University, Warangal, India. He was a postdoctoral fellow at the prestigious Yale University, New Haven (1984–1985), and also worked at BTI (Cornell University, Ithaca) for a couple of months as a visiting research associate. At Yale University, his work led to the discovery of a new method for the control of fungal plant infections through selective inhibition of fungal polyamine biosynthesis. This novel method has been adapted by several research groups globally for the control of a variety of fungal infections, and a large number of research articles have been published in this line of work. He returned to India to join as Pool Officer (CSIR) and worked for about 2 years (1986–1987) at Kakatiya University. Subsequently, he joined the University of Delhi South Campus, where he has been on the faculty since 1987. He had worked in ICGEB, New Delhi, for 6 months as a National Associate of DBT (1994). He made several short visits to various countries including France, Italy, China and Indonesia under the collaborative projects supported by the EU and Indo-French. He is a Fellow of the prestigious Indian National Science Academy (FNA); National Academy of Sciences, India (FNASc); National Academy of Agricultural Sciences (FNAAS); Andhra Pradesh Akademi of Sciences (FAPAS); and Association of Biotechnology and Pharmacy (FABAP) and is an elected member of the Plant Tissue Culture Association, India, since 1995. He was awarded the Rockefeller Foundation Biotech Career Fellowship in 1998 (but could not avail it); the 'Shiksha Rattan Puraskar' by the India International Friendship Society in 2011; Department of Biotechnology National Associateship in 1994; and National Scholarship for Study Abroad (Government of India) in 1984 and for Research in 1985 by the Rotary International Club of Hyderabad. He is serving as an Associate Editor and member of the editorial board of several reputed journals including *BMC Biotechnology* and the OMICS journal *Cell and Developmental Biology* and is a member of the advisory or other committees of some universities, institutions as well as other bodies. He has guided 28 Ph.D. students, 7 M.Phil. students and over 22

postdoctoral fellows and has published over 120 papers (80 research articles in peer-reviewed journals, 15 review articles, 20 book chapters and general articles). He has one Indian patent to his credit. He has vast experience in plant biotechnology and RNA interference and has handled over 22 major projects in these areas.

Dr. Leela Sahijram

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Her team pioneered the micropropagation of banana (globally, the leading tissue culture–propagated fruit crop), which has spawned a multibillion-dollar industry worldwide. In 1990, she successfully demonstrated over 20 choice clones of banana from across India to be 'micropropagatable', including cultivars of the Cavendish Group. She was member of the Task Force for the rehabilitation of Nanjangud Rasabale (Pride of Karnataka) syn. Rasthali, 'Silk' group – a clone threatened with extinction. She has also worked extensively on micropropagation and 'specific-pathogen-free' (SPF) plantlet production through meristem culture/micrografting in crops like citrus, caladium, bougainvillea and chrysanthemum besides bananas and plantains. She specializes in hybrid embryo rescue in perennial horticultural crops (intergeneric/interspecific/intervarietal crosses), particularly in fruit crops, namely,

mango, seedless grapes/citrus, banana and papaya. In 2000–2001, she pioneered hybrid embryo culture and *ex vitro* grafting in controlled crosses of mango.

She was conferred with the Dr. Vikram Govind Prasad Award 1999–2000 for research on molecular diagnostics of viruses in micropropagated bananas. She was also honoured with the Horticultural Society of India Award 2006–2007 for research on hybrid embryo rescue in seedless grapes and with the Rashtriya Samman Award 2007 for developing biotechnologies for horticultural crops. She has been editing the *Journal of Horticultural Sciences*, an international journal, for the past 9 years as a Founder Editor. She has also edited a book entitled *Biotechnology in Horticultural and Plantation Crops*. She has several book chapters in national and international publications to her credit. She is the author of many technical and semi-technical popular articles and a laboratory manual besides having trained hundreds of personnel from development departments for setting up commercial plant tissue culture laboratories. She has travelled widely.

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Dr. K.V. Krishnamurthy is currently an Adjunct Professor at the Institute of Trans-Disciplinary Health Science and Technology (IHST), Bangalore, India, and offering consultancy services in Ayurvedic Pharmacognosy. He obtained his M.Sc. in Botany with University First Rank from Madras University, Chennai, in 1966 and his Ph.D. in Developmental Plant Anatomy from the same university in 1973. After a brief stint in government colleges in Tamil Nadu, he joined the present Bharathidasan University, Tiruchirappalli, in 1977 and became a Full Professor in 1989. He has an overall teaching and research experience of more than 47 years and has guided 32 Ph.D. scholars, more than 50 M.Phil. scholars and hundreds of master's degree holders. He has published more than 180 research papers and 25 books including *Methods in Cell Wall Cytochemistry* (CRC Press, USA) and a textbook on biodiversity (Science Publishers, USA), *Bioresources of Eastern Ghats: Their Conservation and Management* (with Bishen Singh Mahendra Pal Singh, Dehradun). His major research areas include plant morphology and morphogenesis, biodiversity, wood science, cytochemistry, plant reproductive

biology and ecology, tissue culture and herbal medicine and pharmacognosy. He has operated more than 15 major research projects so far. He has been a Fulbright Visiting Professor at the University of Colorado, Boulder, in 1993 and has visited and lectured in various universities in the UK in 1989. His outstanding awards and recognitions include the following: INSA Lecture Award 2011; Prof. A Gnanam Endowment Lecture Award 2010; President 2007, Indian Association for Angiosperm Taxonomy; Prof. V. Puri Award 2006 by the Indian Botanical Society; Rashtriya Gaurav Award 2004 by India International Friendship Society, New Delhi; Scientist of the Year Award 2001 by the National Environmental Science Academy, New Delhi; Tamil Nadu State Scientist Award 1997–1998 in the Field of Environmental Science; Dr. V.V. Sivarajan Gold Medal Award by the Indian Association for Angiosperm Taxonomy for Field Study in the year 1997–1998; Prof. Todla Ekambaram Endowment Lecture Award, Madras University, 1997; Prof. G.D. Arekal Endowment Lecture Award, Mysore University, 1997–1998; Prof. V.V. Sivarajan Endowment Lecture Award, Calicut University, 1997; Prof. Rev. Fr. Balam Memorial Lecture Award, 1997; 1984 Prof. Hiralal Chakraborty Award instituted by the Indian Science Congress in recognition of the significant contributions made to the science of botany, 1960; Dr. Pulney Andy Gold Medal awarded by Madras University as University First in M.Sc. Botany, 1966; Dr. Todla Ekambaram Prize awarded by Madras University for standing first in M.Sc. Plant Physiology, 1966; Maharaja of Vizianagaram Prize awarded by Presidency College, Madras, for outstanding postgraduate student in science, 1965–1966; and Prof. Fyson Prize awarded by Presidency College, Madras, for the best plant collection and herbarium, 1965–1966. He has been the following: Fellow of the National Academy of Sciences of India (FNASc); Fellow of the Linnean Society, London (FLS); Fellow of the Indian Association for Angiosperm Taxonomy (FIAT); Fellow of the International Association of Wood Anatomists, Leiden; Fellow of the Plant Tissue Culture Association of India; and Fellow of the Indian Botanical Society. He has been the Editor and editorial member of many journals in and outside India and has also been reviewer of research articles for many journals. He has also served in various committees, the major funding organizations of India and several universities of India. He has been the Registrar and Director, College and Curriculum Development Council; Member of Syndicate and Senate; Coordinator of the School of Life Sciences and Environmental Sciences; Head of the Department of Plant Sciences; and a Visiting Professor in the Department of Bioinformatics at Bharathidasan University, Tiruchirappalli, before assuming the present job after retirement.

Arabidopsis thaliana: A Model for Plant Research

1

R. Sivasubramanian, Nitika Mukhi, and Jagreet Kaur

Abstract

Arabidopsis thaliana, a small, flowering, self-pollinating weed, has been developed into an elegant model system. Concerted effort from the plant research community has led to development of extensive genomic resources, tools, and techniques. Advances in high-throughput (omics-based) approaches and their application in *Arabidopsis* research have provided ample understanding of basic biological processes in plants. Further, bioinformatics platforms allow for integration of the multiple "omics" data, thus, enhancing our appreciation of biological interactions at an organismal level. Taken together, *A. thaliana* has emerged as an excellent reference source for functional and comparative genomic analysis. In this chapter, we summarize advances made in the field of *Arabidopsis* research and resources, tools, and technologies available to the plant scientific community. In addition, we briefly discuss ways in which knowledge gained from this model system can be harnessed for effective deployment in crop improvement.

Keywords

Arabidopsis thaliana • Model organism • Forward and reverse genetics • Functional genomics • Community resources • Crop plants • Plant biology

1.1 Passage to Glory: From "Tiny Weed" to a "Model Plant"

Mendel's seminal work on *Pisum sativum* (pea) and, later, *Zea mays* (maize) brought the two plants into the main foray as ideal systems for studying crop genetics. Maize, a major crop plant suitable for cytogenetic studies, played an instrumental role in providing valuable insights into

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various facets of plant biology. Horticultural plants like tomato (*Solanum lycopersicum*) and petunia (*Petunia hybrida*) were the other models being used by plant geneticists for studying biological processes. Despite being extensively used in plant biology, these crops failed to develop into ideal model systems for studies on molecular genetics. A major drawback with most of these crop plants being their long generation time and complex genomes. *Arabidopsis thaliana*, a dicot flowering weed belonging to the Brassicaceae family, was not given much importance until Friedrich Laibach included it in his search to identify a plant which had fewer numbers of large chromosomes suitable for cytogenetic analysis. But, due to the small-sized chromosomes, *Arabidopsis* was left out and was not mentioned in plant research for long (Meyerowitz 2001). Laibach refocused his attention on *Arabidopsis* in 1943 and proposed it as a genetic model owing to its short generation time, small size, large progeny, and self-pollinating lifestyle with possibility of outcrossing (Fig. 1.1). Laibach, along with Albert Kranz, further contributed to *Arabidopsis* research by collecting a large number of natural accessions (750) from around the world. George Redei, another plant geneticist, extensively worked towards standardizing mutagenesis protocols for *Arabidopsis* and generated a collection of X-ray induced mutants. Langridge, in 1955, described the first auxotrophic mutant in higher plants. Thereafter, the use of *Arabidopsis* mutants to dissect physiological and biochemical pathways underlying various biological processes gained momentum. Maarten Koornneef's group at Wageningen Agricultural University in Netherlands also started using *Arabidopsis* mutants in a major way and constructed its detailed genetic map, further facilitating research in *Arabidopsis* genetics (Koornneef and Meinke 2010). Around the same time, Estelle and Somerville (1986) used *Arabidopsis* mutants to characterize important biochemical processes like photorespiration, further emphasizing usefulness of this plant in genetic analysis. Pruitt and Meyerowitz (1986) demonstrated that *Arabidopsis* had a small genome relative to other crop models, thereby making mapping and gene cloning comparatively convenient. The next big step in *Arabidopsis* research was the discovery of

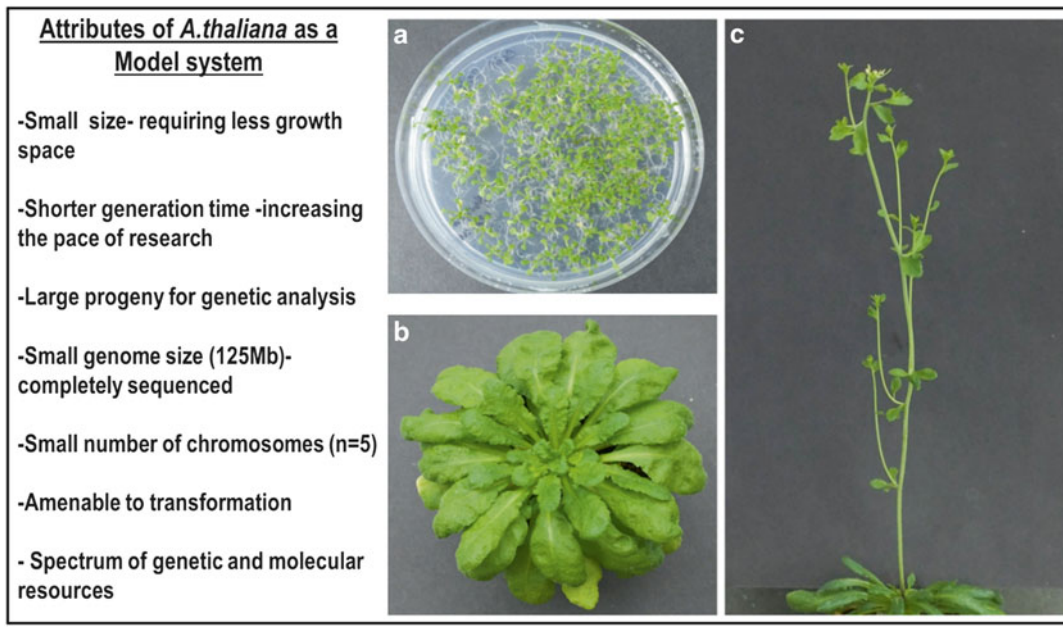

Fig. 1.1 (**a**) Large number of seeds can be grown on 90 mm petri plate. (**b**) Rosette of 4-week-old *Arabidopsis* (**c**) a 5-week-old *Arabidopsis* plant with the inflorescence flowers

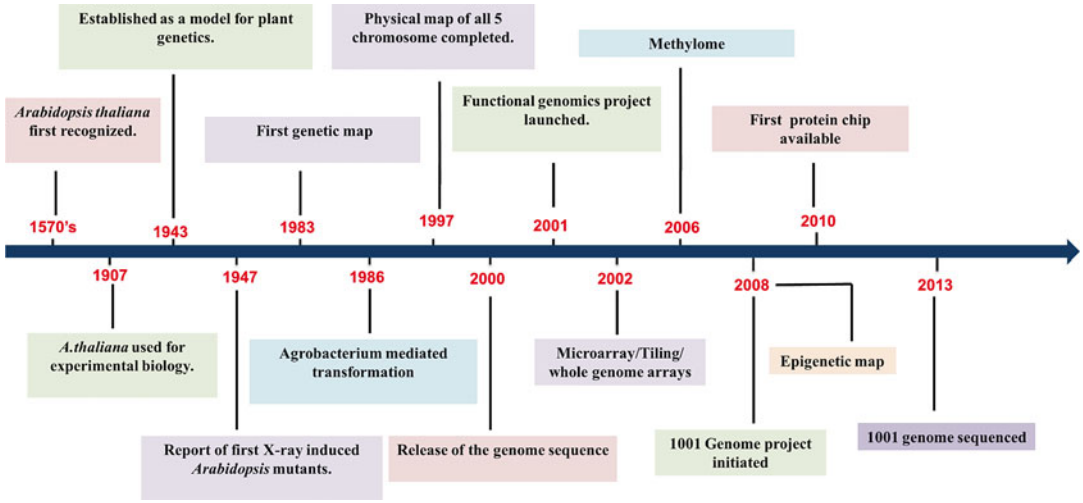

Fig 1.2 Milestones in *Arabidopsis* research

a simple, convenient *Agrobacterium tumefaciens*-mediated transformation of germinating seeds which opened the floodgates for developing various tools for genetic analysis (Feldmann and Marks 1987). Clough and Bent (1998) further simplified plant transformation by devising the "floral dip" method. All these advances, together, brought this weed into limelight as a model plant in the field of plant genetics (Fig. 1.2).

1.2 The *Arabidopsis* Genome: "Catalyst" for Plant Research

A relatively smaller genome size (approx. 125 Mb) was the simple reason *Arabidopsis* was chosen as a subject for the first plant genome sequencing project. By contrast, the genome size of related *Brassica napus* (rapeseed mustard) and *Brassica juncea* (Indian mustard) is about ten times that of *Arabidopsis*. Similarly, the genome of important cereals like rice, maize, and wheat is much more complex and roughly about 3×, 45×, 100×, respectively, compared to *Arabidopsis*. It was the first plant genome to be completely sequenced in the year 2000 under an international Arabidopsis Genome Initiative (AGI). Analysis of the genome using various gene-finding algorithms, along with supporting data from vast experimental evidences like EST sequences, MPSS tags, cDNA clones, etc. predicts about 33,000 gene models (TAIR 10). The genome analysis also revealed that it is enriched for genes with an average size of 5 Kb (Bevan et al. 1998). It was also observed that there is very little repetitive DNA compared to any other higher plant, which facilitates molecular studies and map-based cloning. Release of its genome sequence acted as a catalyst for commencement of various projects on functional genomics, leading to generation of a vast stockpile of resources discussed hereunder (Fig. 1.2).

1.3 Genetic Resources for Functional Genomics

Properties of a living organism are determined mainly by its genetic constitution and its interaction with the environment. With the ever-expanding wealth of genomic data produced by genome sequencing projects, the next essential step is to decipher the gene function. Multiple tools and techniques have been developed for *Arabidopsis* with a focus on dissecting and defining its gene function and interactions in a given biological process (Table 1.1).

Table 1.1 List of resources available for the *Arabidopsis* research community

	Resource name	Web address	Reference
Phenome	Arabidopsis Biological Resource Center (ABRC)	http://www.arabidopsis.org/	Lamesch et al. (2012)
	Nottingham Arabidopsis Stock Center (NASC)	http://arabidopsis.info/	Scholl et al. (2000)
	SENDAI Arabidopsis Seed Stock Center (SASSC)	http://sassc.epd.brc.riken.jp/	
	Versailles Arabidopsis Stock Center (VNAT)	http://publiclines.versailles.inra.fr/	
	SeedGenes Project Database	http://seedgenes.org/index.html	Meinke et al. (2008)
	CSHL Trapper DB	http://genetrap.cshl.org/	Sundaresan et al. (1995), Martienssen (1998)
	SALK Lines	http://signal.salk.edu/tabout.html	
	GABI-KAT Lines	http://www.gabi-kat.de/	Rosso et al. (2003)
	AGRIKOLA RNAi	http://www.agrikola.org/	Hilson et al. (2004)
	Activation tagging and FOX hunting system	https://database.riken.jp/sw/en/Home/cria301u1i/	Ichikawa et al. (2006)
	RARGE II	http://rarge-v2.psc.riken.jp/	Akiyama et al. (2014)
Genome	Genome Browsers	http://www.arabidopsis.org/tools/index.jsp	
		http://plants.ensembl.org/Arabidopsis_thaliana/Info/Index	
	1001 Genomes project	http://1001genomes.org/	
	The SIGnAL *Arabidopsis* SNP, Deletion and SFP Database	http://signal.salk.edu/cgi-bin/AtSFP	Borevitz et al. (2007)
	POLYMORPH tool	http://polymorph.weigelworld.org/	
	QTL mapping tools for MAGIC population	http://mus.well.ox.ac.uk/magic/	
	Multiple SNP Query Tool (MSQT)	http://msqt.weigelworld.org/	Warthmann et al. (2007)
Transcriptome	ArrayExpress	https://www.ebi.ac.uk/arrayexpress/	
	Arabidopsis Transcriptome Genomic Express Database	http://signal.salk.edu/cgi-bin/atta	Laubinger et al. (2008)
	The PLANt co-EXpression database	http://planex.plantbioinformatics.org/	Yim et al. (2013)
	MPSS	http://mpss.udel.edu/	Nakano et al. (2006)
	AtGenExpress	http://www.weigelworld.org/resources/microarray/AtGenExpress/	
	CressExpress – Co-expression analysis	http://cressexpress.org/	Srinivasasainagendra et al. (2008)
	At-TAX (Tiling Array Express)	http://www.weigelworld.org/resources/microarray/at-tax	
	MAPMAN tool	http://mapman.gabipd.org/	Thimm et al. (2004)
	ATTED-II	http://atted.jp/	Obayashi et al. (2014)
	Arabidopsis Small RNA Project	http://asrp.danforthcenter.org/	
	eFP browser for Arabidopsis	http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi	Winter et al. (2007)

Proteome	SubCellular localization Database for *Arabidopsis* proteins (SUBA3)	http://suba.plantenergy.uwa.edu.au/	Tanz et al. (2013)
	MASC-P	http://www.masc-proteomics.org/mascp/index.php/Databases	
	Plant Proteome DataBase (PPDB)	http://ppdb.tc.cornell.edu/	Sun et al. (2009)
	AtProteome Database	http://fgcz-atproteome.unizh.ch/	Baerenfaller et al. (2008)
Metabolome	PMN- Plant Metabolite Network	http://www.plantcyc.org/	
	Arabidopsis Metabolomics Consortium	http://plantmetabolomics.vrac.iastate.edu/ver2/	Bais et al. (2010)
	KEGG	http://www.genome.jp/kegg/	Kanehisa et al. (2012)
	MetaCrop	http://metacrop.ipk-gatersleben.de/	Schreiber et al. (2012)
	UniPathway	www.grenoble.prabi.fr/obiwarehouse/unipathway	Morgat et al. (2012)
Epigenome	EPIC (Epigenomics of Plants International Consortium)	https://www.plant-epigenome.org/	
	The EPIC-CoGe Epigenomics Webbrowser	https://genomevolution.org/wiki/index.php/EPIC-CoGe_Tutorial	
	Epigenetic Natural Variation browser	http://chromatin.cshl.edu/cgi-bin/gbrowse/epivariation/	
Systems biology	*Arabidopsis thaliana* Protein Interactome Database (AtPID)	http://www.megabionet.org/atpid/webfile/	Cui et al. (2008)
	Arabidopsis thaliana protein interaction network (AtPIN)	http://bioinfo.esalq.usp.br/atpin	Brandao et al. (2009)
	Arabidopsis protein interaction network analysis (ANAP)	http://gmdd.shgmo.org/Computational-Biology/ANAP	Wang et al. (2012)
	AIP	https://www.araport.org/	
	AGRIS (Arabidopsis Gene Regulatory Information Server)	http://arabidopsis.med.ohio-state.edu/	Davuluri et al. (2003)
	Systems biology tool kit	http://www.garnetcommunity.org.uk/resources/systems-biology-tool-kit	
	Virtual Plant	http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/	Katari et al. (2010)
	iPlant Collaborative – a cyberinfrastructure for plant sciences	http://www.iplantcollaborative.org/	Goff et al. (2011)

1.3.1 Forward Genetic Tools for Functional Analysis

Forward genetic approach is the classical phenotype-based approach for screening mutants in a biological pathway or process of interest. Large-scale forward genetic screens have provided a basis for the discovery of a multitude of new genes and pathways fundamental to various aspects of plant biology. Gene disruption is the most robust and direct approach to address the biological function of a gene. Various libraries of mutants for forward genetics generated in *A. thaliana* are available as a public resource and are discussed below.

1.3.1.1 EMS Mutagenesis

Ethyl methane sulfonate (EMS), a known and commonly used chemical mutagen (alkylating agent), induces point mutations which vary from complete knockouts to hypomorphic mutations, thus allowing isolation of a series of allelic variants of a given gene (Bowman et al. 1991). Isolation of weak alleles is advantageous especially when characterizing genes involved in essential cellular functions. EMS treatment has been successfully used for generating a high frequency of irreversible, randomly distributed mutations across the *Arabidopsis* genome (Greene et al. 2003). To dissect any biological process, a saturated mutagenized population is screened for a desired phenotype, and the classical positional cloning approach is used for identifying the causal mutation/gene (Fig. 1.3). This approach requires the mutant to be crossed to an *Arabidopsis* accession significantly polymorphic at the DNA level to generate a segregating F_2 mapping population. The mapping is done in a biphasic manner, where coarse mapping with a few, well-dispersed genome-wide markers is fol-

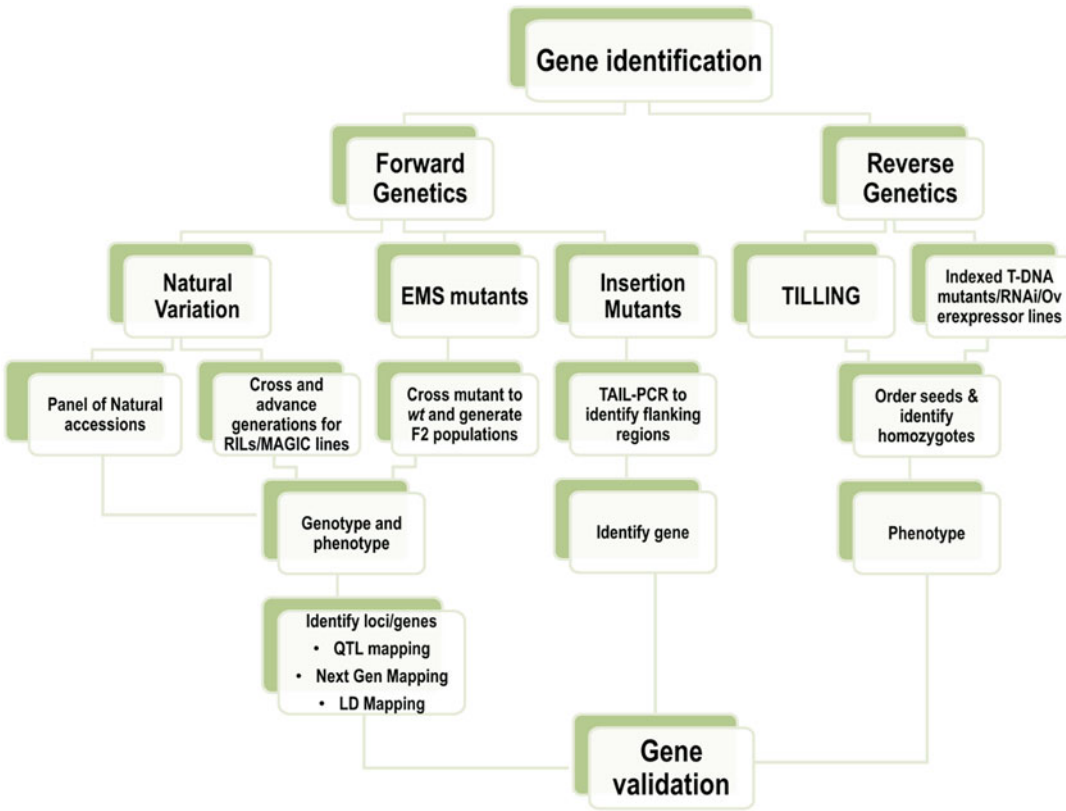

Fig. 1.3 An overview of gene identification methodology in *A. thaliana*

lowed by fine mapping with a large number of region-specific markers. A large number of molecular markers have been used for mapping in *Arabidopsis*, viz., simple sequence length polymorphism (SSLP), insertions and deletions (Indels), cleaved amplified polymorphic sequences (CAPS), and single nucleotide polymorphisms (SNPs). Mapping resolution depends upon (i) the number of molecular markers employed and (ii) the number of meiotic recombinants analyzed. Mutations that interfere with pattern formation during embryogenesis (Souter and Lindsey 2000), branching pattern (Schmitz and Theres 1999), flower morphology (Komaki et al. 1988), flowering time (Putterill et al. 1995), response to hormones, and many cellular/physiological processes have been identified in simple, forward EMS mutant screens. With the advent of "next generation sequencing" (NGS), the traditional positional-cloning approach can be replaced by direct identification of mutations by whole-genome sequencing. Multiple studies have successfully used NGS to identify directly the mutation of interest (Schneeberger et al. 2009; Austin et al. 2011; Schneeberger and Weigel 2011; Uchida et al. 2011).

1.3.1.2 Insertional Mutagenesis and Its Modification

Since the classical positional cloning is an extensive and a long-drawn exercise for identifying a corresponding gene responsible for a phenotype of interest, insertional mutagenesis – an alternate tool for gene disruption – was developed. Integration/insertion of T-DNA/transposable element (TE) into the genic region causes disruption of the gene. Since the mutant genes are tagged with T-DNA inserts, the gene can be easily identified by isolating the sequences flanking the insertion sites. Success of this approach depends upon (i) an easy and efficient transformation system and (ii) the ability of T-DNA and transposable elements to integrate randomly into the host genome (Galbiati et al. 2000). Efficient and simplified *Agrobacterium*-mediated floral dip transformation method has helped in generating exceptionally large numbers of insertional mutants and a near-saturation mutagenesis of the *Arabidopsis* genome (Clough and Bent 1998). Four major T-DNA mutant collections available at TAIR collectively encompass 95 % of predicted *Arabidopsis* genes: (a) SALK lines (Alonso et al. 2003), (b) GABI-Kat lines (Rosso et al. 2003), (c) Syngenta Arabidopsis Insertion Library (SAIL) (Sessions et al. 2002), and (d) INRA/Versailles lines (Samson et al. 2002). These stocks are in the public domain and are available from ABRC and NASC stock centers. These are popular resources for both forward and reverse genetic approaches for functional analysis. Similar to EMS mutants, insertion mutants too have been used for unraveling molecular mechanisms underlying various biological processes, viz., meiotic recombination in plants (Reddy et al. 2003; Kerzendorfer et al. 2006), embryo development (Stacey et al. 2002), organ development (Dievart et al. 2003), and systemic acquired resistance signaling (Maldonado et al. 2002).

1.3.1.2.1 Trap lines

Traditionally, gene identification relied on disruption of a gene function leading to a recognizable phenotype. But most of the genes in *Arabidopsis* and other crop plants are members of multigene families and can act redundantly, which makes it difficult to characterize them using the classical approach. In addition, some phenotypic characters are hard to be detected unless the mutated gene is studied in a certain mutant background which reveals its loss-of-function phenotype. Yet another class of genes not amenable to classical genetic studies is the ones that function at multiple developmental stages and whose loss of function may lead to lethality at early developmental stages. Modification of the insertional mutagenesis tool kit has led to the development of an alternate powerful strategy that permits gene identification based on their expression pattern, thus eliminating the need for a mutant phenotype (Sundaresan et al. 1995; Springer 2000). The basic principle underlying this strategy is to randomly integrate into the genome a promoterless reporter construct (gene/promoter trap) or a reporter construct with a minimal promoter (enhancer trap) close to

the end of the insertional element (T-DNA or TE). The expression of the reporter gene is activated when an endogenous cis-acting promoter or transcriptional enhancer is present at the site of integration. Bacterial *uidA* encoding for β-glucuronidase (GUS) is a commonly used reporter since endogenous β-glucuronidase activity in plants is absent (Jefferson et al. 1987). Alternatively, light emitting bacterial protein (lux) and luciferase (luc) enzyme from the fire fly have been used as reporters for nondestructive screens. Gene traps have been extensively used to unravel genes involved in various developmental processes like lateral root formation (Malamy and Benfey 1997), female gametophyte development (Springer et al. 1995), embryo development (Topping and Lindsey 1997), and floral organ development (Nakayama et al. 2005). Trap lines have also been used to identify stress responsive genes (Alvarado et al. 2004). Besides gene identification, several organ-, tissue-, cell-, and stage-specific markers have been identified which are useful tools in developmental biology studies. Additionally, the promoter traplines provide a direct access to highly specific promoters. For example, to tackle the problem of drought stress in crop plants engineering stomatal activity is an attractive idea. Guard cells control the influx of CO_2 for photosynthesis and water loss during transpiration, and the signaling cascade involved in these responses are well dissected (Schroeder et al. 2001). Francia et al. (2008) screened gene trap and promoter trap lines to isolate stomata-specific genes and promoters for biotechnological applications. This approach has also been extended to other crops like rice to identify cell-type-/tissue-, stage-, and/or conditionally specific regulatory elements (Yang et al. 2004).

1.3.1.3 Natural Variation and Association Mapping

Extensive genotypic and phenotypic variations have been documented in natural accessions of *A. thaliana*. Natural variation is the basis for traditional linkage mapping/quantitative trait loci (QTL) mapping aimed at identifying genes governing a trait of interest. F_2 populations and recombinant inbred lines (RILs) have been used as experimental populations for QTL mapping. RILs allow higher mapping resolution as compared to F_2 populations. Over 60 RIL populations have been developed and are available to the research community through stock centers. The wide range of intraspecific diversity (wild accessions) available in *A. thaliana* makes it well suited for association mapping (Fig. 1.3). Association mapping is based on linkage disequilibrium (LD) and offers very high resolution in comparison to traditional linkage mapping, since it takes advantage of historic recombination events accumulated over several generations. Linkage disequilibrium (LD) in *Arabidopsis* on an average extends over 5–10 kb, thus offering nearly single-gene resolution (Kim et al. 2007b). Array-based re-sequencing of 20 maximally diverse natural accessions of *A. thaliana* has led to development of a genotyping array (AtSNPtile1) containing probe sets for 2,48,584 SNPs (Kim et al. 2007b). Given the small size of the genome (~125 Mb), this array provides, on average, 1 SNP for every 500 bp, sufficient enough for genome-wide association mapping. There have been several reports of genome-wide association studies (GWAS) in *A. thaliana* (Aranzana et al. 2005; Atwell et al. 2010; Brachi et al. 2010; Chan et al. 2011; Nemri et al. 2010). The SNP chip has been used for genotyping around 1,307 accessions, and the data is available to the public (Horton et al. 2012). Several software and web-based platforms have been developed for GWAS in plants, viz., TASSEL (Bradbury et al. 2007), GAPIT (Lipka et al. 2012), GEMMA (Zhou and Stephens 2012), Matapax (Childs et al. 2012), and the GWA-portal (Seren et al. 2012). A major drawback of association mapping is the confounding caused due to population structure and the consequent increase in number of false positives. In addition, identification of epistatic loci continues to be a major challenge in GWAS. A new mapping design that combines advantages of classical QTL mapping and association mapping, known as nested association mapping (NAM), has been pioneered in maize wherein experimental populations derived from crosses of several founder lines are used (McMullen et al. 2009; Yu et al.

2008). In *Arabidopsis*, two such mapping populations have been developed, viz., AMPRIL (Arabidopsis Multiparent RIL) populations (Huang et al. 2011) and MAGIC (Multiple Advanced Generation Intercross) populations (Kover et al. 2009) (Fig. 1.3). The MAGIC population is derived from a heterogeneous stock of 19 inter-mated accessions which have been completely sequenced, and tools required for QTL/association mapping in these populations are freely available (Table 1.1). Alternatively, association mapping can be combined with QTL mapping in several independent RIL populations to retain statistical power and, yet, not compromise on resolution of mapping. Though the array-based re-sequencing effort led to identification of around 250K SNPs, it also revealed that the reference accession Col-0 lacks a substantial portion of genes present in other accessions. A 1001 genome project for *A. thaliana* was announced in 2007 to sequence genomes of other accessions which would contain sequences not present in the reference genome (Weigel and Mott 2009) (Table 1.1). This multinational effort would not only shed light on local polymorphism patterns and chromosomal-scale differences but be directly useful in QTL and association mapping as well. Since many of the accessions sequenced are parents of RIL populations, availability of the genome sequence may identify polymorphisms responsible for various QTLs detected so far. Complete-genome sequences will not only help identify the causal allele directly in GWAS but also assist in predicting activity differences between causal alleles and tackling problems of allelic heterogeneity and rare variants.

1.3.2 Reverse Genetic Tools for Functional Genomics

The genome annotations for Arabidopsis have been refined overtime and there are more than 30,000 genes predicted, but the role of majority of these genes in various biological processes is yet to be elucidated. This poses a major challenge and has made it essential to carry out systematic genome-wide functional analysis. As emphasized above, cloning the genes based on phenotype requires tremendous labor and time; therefore, complementary reverse genetic approaches are developed to directly investigate the gene function in specific pathways of interest. Some of the genetic resources commonly used in the *Arabidopsis* functional analysis are discussed below (Fig. 1.3).

1.3.2.1 Sequence-Indexed Insertion Mutants

As a part of the extensive effort to experimentally validate the function of all the predicted genes, high-throughput thermal asymmetric interlaced-PCR (TAIL-PCR) in combination with sequencing has made it possible to index all the insertion mutants available. The gene indexed T-DNA mutant library is a valuable resource for several reverse genetic studies. The ultimate aim of the project was to identify at least two genetically stable loss-of-function mutations in all the predicted *Arabidopsis* genes. Using simple PCR-based screening, the mutations can be confirmed and one can select for lines that are homozygous for the mutation. Recently, Bethke et al. (2014) attempted to dissect the role of multiple members of the *Arabidopsis* pectin methyl esterases (AtPME) (a 66-member gene family), in pattern-triggered immunity and immune responses to *Pseudomonas*, *Botrytis*, and *Alternaria brassicicola*. They identified T-DNA mutants in multiple members of AtPME gene family and analyzed single and combinations of multiple mutations to address their role in plant defense.

1.3.2.2 Targeting Induced Local Lesions in Genome (TILLING)/ EcoTILLING

EMS mutagenesis can create a higher frequency of broad-spectrum mutations as compared to the insertion mutagenesis approach. The major drawback of EMS is that the eventual cloning of the mutation is tedious. Colbert et al. (2001) developed a PCR-based high-throughput strategy for identifying a SNP in the gene of interest from a mutagenized F_2 population. TILLING (Targeting Induced Local Lesion in Genome) combines the robustness of random EMS-induced mutagenesis with high-throughput PCR-based screening to

identify mutations in the gene of interest. The PCR products from the gene of interest are denatured and reannealed to form heteroduplex which is preferentially digested by the mismatch-specific CEL1 endonuclease, and the cleaved products are analyzed on a denaturing polyacrylamide gel. Initially, this reverse genetic strategy was used to identify numerous mutations in *Arabidopsis* mutagenized populations (Enns et al. 2005). The use of this technique was further extended for identifying naturally occurring genetic variations in the available accessions and has been named as EcoTILLING (Comai et al. 2004). A high-throughput and cost-effective TILLING approach has been employed by the *Arabidopsis* TILLING project (ATP) to provide series of allelic point mutations for the general *Arabidopsis* community. This strategy can be extended to any crop plant for functional genomics and even crop improvement (Slade et al. 2005; Cooper et al. 2013).

1.3.2.3 RNA Interference (RNAi) for Targeted Mutagenesis

Although insertional mutagenesis is an effective method for generating loss-of-function mutants, the method falters when dealing with lethal genes or those genes which are functionally redundant. Targeted gene silencing via antisense, co-suppression, posttranscriptional gene silencing, and most recently RNA interference (RNAi) has emerged as a powerful alternative reverse genetic approach for attenuating the gene expression. In this approach, stable transformants expressing double-stranded RNA (under a constitutive or inducible promoter) against target genes are generated, and the effect of knockdown of the gene expression is analyzed in terms of the phenotype. In comparison to T-DNA mutagenesis, RNAi lines typically show a wide spectrum of gene expression, from no reduction to complete shutdown (Waterhouse and Helliwell 2003). To date, experimental proof of function for only 10 % of the predicted genes is available. The AGRIKOLA project (Arabidopsis Genomic RNAi Knockout Line Analysis) aims to create targeted gene knockdown lines via RNAi for all the predicted genes in *Arabidopsis* (Hilson et al. 2004). In this project, about 150–600 bp long gene-specific tags (GSTs) have been designed for approx. 25,000 genes which were used to construct dsRNA-expressing vectors. These RNAi constructs have been transformed into wild-type *Arabidopsis* plants to generate a library of knockdown transformants. These knockdown lines will be an invaluable source for determining the function of individual *Arabidopsis* genes and, by extrapolation, function of orthologous genes in other crop plants as well.

1.3.2.4 Gain-of-Function Systems for Functional Analysis

Activation tagging is another addition in the arsenal of gene identification tools available to the plant scientific community. This is a popular gain-of-function approach where the overexpression of the gene results in a novel phenotype. Gene activation tagging systems have been established in *Arabidopsis* using either T-DNA vectors or transposon-based vectors carrying multimers of the 35S CaMV enhancers (Walden et al. 1994; Weigel et al. 2000; Nakazawa et al. 2003). *Agrobacterium-mediated* genome-wide random integration of the activation construct results in upregulation of the gene present in the vicinity of the integration site. Ectopic upregulation of the gene can result in an observable phenotype. Since the gene is tagged, identification by inverse PCR or TAIL-PCR can be carried out rapidly. Activation tagging approach has been instrumental in deciphering the function of a number of genes including ADR1 in defense response (Grant et al. 2003; Aboul-Soud et al. 2009), BAK1 in brassinosteroid signaling (Li et al. 2002), and FT in floral transition (Kardailsky et al. 1999).

FOX hunting system (full-length overexpression of cDNA for gene hunting) is an alternative approach to activation tagging where the ease of transformation of *Arabidopsis* and the availability of the full-length cDNA sequences have been exploited. As opposed to activation tagging, genes responsible for the overexpression phenotype can be easily identified. Using a normalized full-length cDNA library, Ichikawa et al. 2006 have developed 30,000 independent *Arabidopsis*

FOX lines which are available from RIKEN. As a further extension of this, full-length rice cDNA clones have been transformed into *Arabidopsis* with an aim of screening for rice functional genes in a high-throughput manner (Sakurai et al. 2010).

1.4 Omics: A High-Throughput Tool for Deciphering Gene Function

Functional genomics is a genome-wide approach that attempts to use the ever expanding wealth of data produced through various high-throughput analysis for defining the gene function and its interactions in a given biological process.

1.4.1 Transcriptomic Resources for *A. thaliana*

Transcriptomics, a comprehensive study of the whole-genome expression, is an informative approach towards functional gene analysis. The spatial and temporal expression of a gene to a certain extent is reflective of its activity within the cell. This section discusses the various publically available resources for gene expression analysis and their application in functional genomics.

1.4.1.1 Expression Profiling Provides Insights into Gene Function

To get a glimpse of the transcriptional activity within the cell, a large-scale expressed sequence tag (EST) sequencing project was undertaken in *Arabidopsis*. The data generated in the EST sequencing was useful in gene discovery as it helped in annotating the expressed regions in the genome besides providing information about the gene expression. Serial analysis of gene expression (SAGE) and its various modifications like micro SAGE and mini SAGE were commonly used by various research groups to identify large number of differentially expressed transcripts present in different tissues/conditions. More recently, the NGS-based massively parallel signature sequencing (MPSS) has gained the edge. In this approach, short sequence tags are generated for a cDNA library by sequencing approx. 20–25 bp from the 3′ side of cDNA. Besides discovering novel transcripts, MPSS also provides a robust method for assessing the transcript abundance. Additionally, the MPSS platform has been used to identify a large number of small RNAs.

The hybridization-based approaches for acquiring large-scale gene expression profiles have also been established for *Arabidopsis* and are being continuously improved. The traditional microarrays used for analyzing the transcriptional profiles were biased towards the known and predicted genes. With the whole genome sequence available, it became possible to develop tiling arrays (TA) and whole genome arrays (WGA). These arrays cover the entire genome with probes either at regular interval (TA) or probes that are overlapping along the entire length of the genome (WGA). These arrays have not only been used for estimating the transcript levels but also play a significant role in identifying novel transcripts, various alternate transcripts, and polymorphisms (Mockler et al. 2005). Using the WGA, Zeller et al. (2009) studied the stress-induced changes in the *A. thaliana* transcripts in response to various abiotic stresses like salt, osmotic, cold, and heat. They identified several novel stress-induced genes which were missed in the earlier classical microarray experiments. Thousands of microarray experiments that have been conducted in different laboratories with *A. thaliana* now form a part of large quantitative data on gene expression in different tissue and in response to different treatments and experimental conditions. Similarly, a comprehensive expression atlas for *A. thaliana* has been developed based on WGA and is available at *A. thaliana* Tiling Array Express (At-TAX) (Laubinger et al. 2008). The utility of the tiling array has been further extended by combining this platform with immunoprecipitation methods for detecting chromosomal locations at which protein-DNA interaction occurs across the genome (Wang and Perry 2013). Further, Zhang et al. (2006) generated an

extensive *Arabidopsis* methylome data by coupling tiling arrays with methylcytosine immunoprecipitation methods. Most of the microarray data has been deposited in the public databases such as NASC and TAIR, and this data can be accessed for analysis either directly from these sites or through the various available links/tools like Genevestigator (Zimmermann et al. 2004) and MAPMAN (Thimm et al. 2004) (Table 1.1).

1.4.1.2 Co-expression Analysis: Guilt by Association

Since an extensive set of microarray expression data across multiple experiments is available, the attention has now shifted to exploring the correlated expression of the entire genome with fine focus on defining specific pathways in a biological process. These correlation studies are a powerful tool to identify new genes which could be functionally related. Several co-expression analysis interfaces like ATTED-II and CressExpress have been developed that use the comprehensive collection of the publicly available transcriptome data sets to identify co-regulated genes (Nakashima et al. 2009). The PLAnt co-Expression database (PLANEX) is a genome-wide database which includes publicly available GeneChip data obtained from the Gene Expression Omnibus (GEO). A comparative transcriptomic analysis using meta-analysis approach for drought (abiotic) and bacterial (biotic) stress response in rice and *Arabidopsis* identified several genes, common to both the stresses (Shaik and Ramakrishna 2013). This identification of master regulatory genes that act in biotic and abiotic stress response would be the potential candidates for manipulating stress tolerance in crop plants as well.

Though co-expression analysis proves to be a powerful tool to identify new genes which could be functionally related, one needs to keep in mind that the co-expression analysis is a reflection of regulation at the mRNA level only. The analysis would gain weightage if any known protein–protein interaction information can be integrated into the co-expression analysis.

1.4.1.3 Small RNA Database and Tools for Functional Genomics

The regulation of gene expression occurs at multiple levels including mRNA stability. It has become evident that small RNAs are one of the major players in regulating gene expression during growth, development, and stress responses in plants. The cost-effective next generation sequencing has made it possible to discover hundreds of small RNA from *Arabidopsis* and other plants. This extensive data is available through the web interface –the Arabidopsis Small RNA Project (ASRP) which also integrates the community-wide resources related to small RNA and various bioinformatic tools for mi- and siRNA identification (Backman et al. 2008). The *Arabidopsis* model system has been useful in dissecting the small RNA component of genetic and epigenetic regulation in plant development, growth, and disease resistance. Palatnik et al. (2003) showed the involvement of microRNA in controlling leaf morphogenesis. Auxin signaling responses were also shown to be regulated via small RNA. Several auxin response factors (ARFs) were predicted to be targets for miRNA. ARF10 and ARF17 contain potential sites for miR160 (Jover-Gil et al. 2005). Overexpression of miR160 resistant version of ARF17 led to higher accumulation of ARF17. These changes in expression correlate with the pleotropic morphological abnormalities and reduced fertility observed in the transgenic suggesting the regulation of ARF17 by miR160 (Mallory et al. 2005). Similarly, another miR393 also plays a significant role in integrating the environmental cues to auxin signaling pathway (Windels and Vazquez 2011).

Additionally, using the basic principle of small RNA-directed gene silencing, virus induced gene silencing (VIGS), hairpin-based RNA interference (RNAi), and artificial microRNA (amiRNA) tools have been developed to regulate targeted gene expression. The use of these strategies has been further extended for selectively regulating gene expression in crop plants as well.

1.4.1.4 Tools for Regulatory Sequence Analysis

The control of gene expression is pivotal to all cellular processes, and one of the major challenges in biology is to unravel the mechanisms that regulate gene expression. The gene function is directly linked to its spatial and temporal expression which is regulated by a network of transcription factors, the key regulatory proteins. The cues for gene regulation are hard wired into the promoter region which is formed by cis-regulatory elements. These cis-regulatory elements are recognized by specific transcription factors. Therefore, in order to understand the gene expression and thereby the gene function, basic information on the transcription factors and their binding sites is important. *A. thaliana* encodes more than 1,500 transcription factors which are classified into 40–50 families based on the sequence similarity (Riechmann et al. 2000). Arabidopsis Gene Regulatory Information Server (AGRIS) is the interface that hosts AtcisDB, AtTFDB, AtRegNet, and ReIN databases (Table 1.1), which provide a catalogue of cis ad trans factors involved in gene regulation.

1.4.2 Epigenomic Resources

Epigenetics is the study of changes in the regulation of gene expression that do not involve a change in the DNA sequence. Epigenetic changes/modifications include methylation of the DNA, chemical modification of the histones, and alternative histone variants. These modifications are known to play important roles during development and in responses to different environmental cues. An integrated epigenome map of *Arabidopsis* was published in 2008, which describes interactions between the methylome, transcriptome and the small RNA transcriptome, and their effect on gene regulation (Lister et al. 2008). Further, the epigenetic variation between the different accessions of *Arabidopsis* and its dependence on the genetic variation between the accessions has been reported (Schmitz et al. 2013). DNA methylations and their effect on the transcriptional activity have been studied in response to biotic stress (Dowen et al. 2012). The Epigenomics of Plants International Consortium (EPIC) provides a list of epigenetic resources in the form of databases and tools, which are available to the research community (Table 1.1). Also, The EPIC-CoGe Epigenomics Webbrowser provides a user-friendly interface to scan and search for epigenetic marks in the genome (Table 1.1).

1.4.3 Proteomics Resources

Transcriptional analysis in *Arabidopsis* using the whole genome arrays and tiling arrays suggested that the transcriptional capacity of the *Arabidopsis* genome is far more than predicted by genome annotation. Many non-annotated intergenic regions were transcribed and a lot of antisense transcripts were identified. These findings prompted the scientists to look at the global translational products and their posttranslational modifications which are known to play a key role in many cellular processes like cell signaling, regulation of gene expression, protein degradation, and protein–protein interactions. "Proteomics" is the study and characterization of the complete set of proteins present at a given time in the cell and organelle using gel- and mass spectrometry-based high-throughput techniques. Proteome profile of *Arabidopsis* has led to the identification of several organ-specific biomarkers for different developmental stages, organs, and undifferentiated cell cultures (Baerenfaller et al. 2008). A proteome map of different root cell types using *Arabidopsis* root marker lines and fluorescence activated cell sorting (FACS) was recently released (Petricka et al. 2012). Proteome profiling of *Arabidopsis* under diverse abiotic stresses such as cold, drought, salinity, hypoxia, etc. (Kosova et al. 2011; Ghosh and Xu 2014) determined the role of several families of transcriptional factors (TFs) and protein phosphorylation/dephosphorylation events in mediating stress response. For

instance, analysis of *Arabidopsis* nuclear proteome and its variation under cold stress identified several differentially expressed transcriptional factors (Bae et al. 2003). A comprehensive and well-annotated database of transcription factors like Stress Responsive Transcription Factor Database (STIFDB) (Shameer et al. 2009) and *Arabidopsis* Stress Responsive Gene Database (ASRGDB) (Borkotoky et al. 2013) provide a useful resource to study gene regulatory pathways. MASC-P is a web-based interface which contains a comprehensive set of databases containing proteome profile of not only *Arabidopsis* but also other plant species as well.

Proteomics has proved to play a significant role in characterizing virulence and pathogenicity factors produced by pathogens. Exoproteome of phytopathogenic fungus *Sclerotinia sclerotiorum* identified several cell wall degrading enzyme, in addition to the uniquely identified α-arabinofuranosidase, formerly not been detected by EST studies, thus highlighting the importance of proteomic studies (Yajima and Kav 2006; Mehta et al. 2008). With respect to plant responses, proteomic studies on *Arabidopsis–A. brassicicola* pathosystem identified the role of different pathogenesis-related proteins PR4, glycosyl hydrolases, and glutathione-S-transferases in mediating defense response (Mukherjee et al. 2009). Proteome analysis of *Arabidopsis* plants challenged with *Pseudomonas syringae* pv. tomato (Pst) identified several phosphorylated glutathione-S-transferases and peroxiredoxins involved in redox signaling (Jones et al. 2006). Cecconi et al. (2009), using the same pathosystem, addressed the role of nitric oxide (NO) in hypersensitive response. They identified several proteins undergoing tyrosine nitration during an incompatible interaction. Most of these proteins were enzymes associated with important cellular processes like photosynthesis, glycolysis, and nitrate assimilation, indicating their importance in mediating plant hypersensitive response. A high-throughput proteomic study thus presents a new way to gain deeper insights into organismal biology.

1.4.4 Metabolomics Resources

Plants respond to changes in the environmental conditions by altering their metabolism. Transcriptome and proteome analyses provide useful insights into the metabolic network that contributes to a biochemical reaction, but these techniques are not all-encompassing methodologies. Metabolomics hence can be used as an additional tool to characterize the degree of impact of environmental perturbations. The basic requirement for metabolomics study is to quantitatively and qualitatively analyze all the metabolites in a given sample in a truly unbiased manner. To date, "metabolite profiling" has mainly been performed using NMR, Fourier transform infrared (FT-IR) spectroscopy, or MS techniques. Besides relative quantification, metabolomics also help in *de novo* identification of unknown metabolites whose presence has been demonstrated. The metabolic response of plants to various biotic (Balmer et al. 2013) or abiotic (Kaplan et al. 2004) stresses has recently received attention. Metabolite profiling of *Arabidopsis* plants subjected to high salinity revealed the role of polypropanoid pathway and glycine betaine biosynthesis in mediating salt stress response (Kim et al. 2007a). Similarly, molecular basis of freezing tolerance in different *A. thaliana* accessions has also been addressed using metabolomic approach. Enhanced freezing tolerance in accessions originating from Scandinavia to the Cape Verde Islands has been found to be associated with downregulation of photosynthesis and hormonal responses and induction of flavonoid metabolism (Hannah et al. 2006). Metabolomics has also emerged as a functional genomics tool for elucidating the functions of many unknown genes. For instance, genes associated with glucosinolate, flavonoid, and sterol biosynthesis have successfully been functionally annotated using the metabolomic approach (Hirai et al. 2004; Morikawa et al. 2006; Schauer and Fernie 2006; Tohge et al. 2005). Till date, approximately 1,800 metabolites have been detected in *Arabidopsis*. Many publicly available databases like AtMetExpress and Plant Metabolic Network (PMN) have been developed to provide the

scientific community with access to the metabolomics data along with tools to allow for its interactive analysis.

1.4.5 Systems Biology: "Integrating the Omics"

The omics approaches have allowed us to sneak a glance at each step of translation of the genetic information to the trait, and in this process, a large quantity of high-quality data has been generated. Systems biology integrates the omics data and allows us to study the interactions among various biological components such as genes, metabolites, proteins, and regulatory elements. The study of interactions involves majorly four types of networks: (i) gene–metabolite networks, (ii) transcriptional regulatory networks, (iii) gene regulatory networks, and (iv) protein–protein interaction networks. Gene–metabolite networks have been used to study the responses of the plant during sulfur and nitrogen starvation (Hirai et al. 2004) and during biotic stress (Goossens et al. 2003). Also, gene expression data has been used in the context of metabolic pathways to dissect out the interaction of sugar and circadian rhythm signaling during the diurnal cycles (Blasing et al. 2005; Gibon et al. 2006). Diverse biological processes within a cell are mediated by multiple protein complexes consisting of individual proteins organized by their protein–protein interactions (PPI). Several large-scale protein interaction studies have been carried out in *Arabidopsis* (Braun P et al. 2011; Mukhtar et al. 2011), and multiple databases with PPI data have been established. Apart from PPI, genetic interactions have also been mapped for many genes in *Arabidopsis*, and data from both genetic and protein–protein interactions have been consolidated to define an interactome of *Arabidopsis*. Some of the databases that contain curated PPI and genetic interaction data include Biological General Repository for Interaction Datasets (BioGRID) (Stark et al. 2006, 2011), IntAct Molecular Interaction database (Aranda et al. 2010), *Arabidopsis thaliana* Protein Interactome Database (AtPID) (Cui et al. 2008), Biomolecular Interaction Network Database (BIND) (Bader et al. 2003), the Molecular INTeraction database (MINT) (Ceol et al. 2010), and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (Jensen et al. 2009; Szklarczyk et al. 2011) (Table 1.1). Knowledge from these interactions has been used extensively to study and understand various complex cellular signaling pathways. In *Arabidopsis*, this data has been used to define the interfaces of various pathways involved in retrograde signaling (Glasser et al. 2014), to study how WRKY transcription factors interact with themselves and other proteins to regulate various biological processes (Chi et al. 2013), and to understand how the JAZ proteins in the jasmonate signaling cascade interact with other transcription factors and proteins from other hormone signaling pathways to regulate male fertility, trichome development, and other biological processes (Pauwels and Goossens 2011). Transcriptional regulatory networks describe the interactions between transcription factors and their downstream targets/genes. Transcriptional regulatory networks have been used to dissect out the regulatory network of glucosinolate biosynthesis and identify major transcription factors regulating glucosinolate biosynthesis (Hirai et al. 2007). Transcriptional regulatory networks have also aided in elucidating the regulatory mechanisms controlling dehydration, cold stress response, and systemic acquired resistance (SAR) (Yamaguchi-Shinozaki and Shinozaki 2006). Gene regulatory networks define gene–gene interactions during a biological process and reflect the dynamics of a biological system. Gene regulatory networks have been mostly described for developmental processes that are well studied in *Arabidopsis* such as flower development (Espinosa-Soto et al. 2004) and guard cell size (Li et al. 2006). Several large-scale studies have been carried out involving various omics technologies such as genomics, proteomics, transcriptomics, interactomics, and metabolomics. Datasets generated from such studies need to be integrated to derive models for describing biological interactions at the organismal level.

1.5 Translating Weed Power: From *Arabidopsis* to Crops

Fourteen years after the release of the whole-genome sequence, *Arabidopsis* research is more vibrant and vigorous than ever. There has been an enormous development of new high-throughput tools, techniques, and a parallel surge of advancement on the bioinformatics front enabling the researchers to handle and analyze the available data. The studies on *Arabidopsis* have generated a wealth of new fundamental insights in all areas of biology. Large genetic and genomic resources and extensive set of expression, proteome, and metabolome data covering a range of environmental conditions and various developmental stages have made *A. thaliana* an excellent reference source for functional and comparative genomic analysis in other crop plants. The translation of this knowledge from *Arabidopsis* to crop plants has been multipronged. One approach has been to look for orthologues in other crop species to identify genes regulating agronomically important traits. The other approach is to the ectopically overexpress the *Arabidopsis* genes in crop species for improving the traits. Additionally, the *Arabidopsis* system has proved to be a useful and time-saving testing ground for components discovered in crops.

1.5.1 *Arabidopsis*: A Reference for *Brassica* Genomics

The genus *Brassica*, within Brassicaceae family, is agronomically the most important genus since it is a rich source for oil seeds, vegetable, and fodder crops. Six different *Brassica* species are extensively cultivated all over the world, out of which three are diploids (*B. rapa*, *B. nigra*, *B. oleracea*) and three are amphidiploids (*B. juncea*, *B. napus*, *B. carinata*). The *Brassica* and *Arabidopsis* lineages have diverged approximately 14–20 million years ago. Previous studies have shown that the present diploid species in the *Brassica* genus have descended from a common hexaploid ancestor (Lagercrantz and Lydiate 1996). Comparative mapping between *Arabidopsis* and *B. nigra* and *B. oleracea* indicate that the *Brassica* diploid genomes have evolved through extensive duplication followed by frequent fragmentation and rearrangements (Lagercrantz 1998; Town et al. 2006). Comparative mapping in the amphidiploid *B. napus* with *Arabidopsis* revealed that there are 21 syntenic blocks shared between them which can be duplicated and rearranged to represent 90 % of the *Brassica napus* genome (Parkin et al. 2005). The *Arabidopsis* genome can be represented as gene blocks (from A to X) based on its synteny with the *B. rapa* and *B. nigra* genomes (Schranz et al. 2006). These gene blocks can be rearranged to model genome structures of other crucifer species where genome information is limited.

Flowering time and its control are of importance in agriculture as well as to the understanding of plant development. In *Arabidopsis*, the pathway that promotes flowering in response to photoperiod changes is well studied and characterized (Mouradov et al. 2002). Some of the major players involved in the control of flowering time are CO (daylength response), FLC (repressor of flowering and vernalization responsive), FRI (vernalization responsive), FT, and SOC1 (flowering time). Homologues of CO were found to be collocated with loci that regulate flowering time in *B. nigra* (Lagercrantz et al. 1996). Similarly, QTLs responsible for vernalization-responsive flowering time in *B. napus* (Osborn et al. 1997; Long et al. 2007) *B. rapa* (Schranz et al. 2002; Lou et al. 2007) and *B. oleracea* (Okazaki et al. 2007) co-localized with homologues of the FLC gene of *Arabidopsis*. Identification of QTLs for other economically important traits such as oil composition, glucosinolate content, and disease resistance has also been expedited by the information from *Arabidopsis*. QTL studies for oil components such as oleic acid and erucic acid have identified orthologues of *Arabidopsis* FAD2, FAD3, and FAE1 gene, respectively, as the genes controlling variation for the traits (Fourmann et al. 1998; Hu et al. 2006). Similarly, genes for clubroot resistance were identified in *B. rapa* through comparative mapping with *Arabidopsis* (Suwabe et al. 2006).

1.5.2 Insights into Crop Developmental Biology

Various aspects of plant development and physiology have been deciphered using *Arabidopsis*, and the insights gained have improved our understanding of similar biological processes in the crop counterparts. Fruit ripening in horticultural crops is one such important trait as inefficient ripening or overripening results in unpalatable horticultural products. The phytohormone ethylene is known to play an important role in fruit ripening. A lot of effort is spent in controlling the ethylene effects to save the products especially under postharvest storage conditions. The basic knowledge regarding the mode of perception and action of ethylene has been elucidated in depth mainly from the work done in *Arabidopsis*. A classical genetic screen of EMS-mutagenized population for a triple-response phenotype led to isolation of mutants which were either insensitive to ethylene or showed enhanced ethylene responses even in the absence of ethylene (Bleecker et al. 1988). The ensemble of advanced molecular genetic tools developed in *Arabidopsis* facilitated the rapid identification of several components of the complex ethylene signaling pathway like the ethylene receptors ETR1, ETR2, ESR1, and EIN4 (Stepanova and Ecker 2000). ETR1 belongs to a family of transmembrane proteins. Orthologues of ETR1 have been found in other plant species. A dominant negative mutation of the *Arabidopsis* ethylene receptor etr1-1 when heterologously expressed in tobacco, tomato, or petunia resulted in ethylene insensitive transgenics (Wilkinson et al. 1997). Similarly, Constitutive Triple Response1 (CTR1) identified in *Arabidopsis* as a negative regulator of the ethylene response pathway has homologues in other crop plants, and some of them can complement the weak Atctr1-18 mutants thereby suggesting functional similarity as well (Adams-Phillips et al. 2004). These observations indicate that the mechanism of ethylene perception and response defined in the model plant is largely conserved in other crop species as well. Another integral component of the ethylene signaling pathway EIN2 when mutated results in a complete block of the ethylene responses (Bisson and Groth 2011). Based on the sequence similarity, two rice orthologues of EIN2 were identified and reported to be positive regulators of ethylene signaling similar to the *Arabidopsis* counterpart (Jun et al. 2004). Since the ethylene signaling pathway is highly conserved in crop plants, manipulating the ethylene production and response at the molecular level has been explored as an efficient strategy to prolong the shelf life of fruits and ornamentals. Floral longevity of petunia and carnation flowers has been extended by either overexpressing the etr1-1 dominant mutant under fbp1, a floral specific promoter, or by suppression of ein2 (Wilkinson et al. 1997; reviewed in Stearns and Glick 2003).

The root system plays a diverse and crucial role in the overall growth of the plant. The root system not only provides anchorage and mechanical strength but also determines the amount of water and nutrient uptake from the soil. In addition, it is the major site of interaction with symbiotic microorganisms. In light of the diverse role the underground system has in the overall plant growth, breeders have focused their attention on this system for improving crop productivity. *A. thaliana* has been instrumental in providing insights into several aspects of root development such as description of cellular structure of the root (Dolan et al. 1993). Fitz Gerald et al. (2006), using *Arabidopsis*, identified a QTL involved in regulating size and plasticity of the root system. Comparative analysis of genome-wide transcriptional changes associated with the process of lateral root development in the model plants *Arabidopsis* and maize has helped identify a core set of genes putatively conserved between both species. This helps researchers extrapolate knowledge gained from *Arabidopsis* to crop species for improvement of root architecture.

1.5.3 Engineering Stress Tolerance

The ever-increasing demand for agricultural products due to population growth, coupled with the impending climate change, has imposed immense pressure on agricultural production.

Minimizing yield losses from various biotic and abiotic factors through development of stress-tolerant crops is, therefore, essential for sustainable food production.

1.5.3.1 Abiotic Stress

Abiotic stress responses well studied in *Arabidopsis* include salt, cold, and drought stress tolerance. Membrane transport proteins (transporters) are involved in transport of minerals, sucrose, and water and are implicated in various pathways mediating abiotic stress tolerance. The vacuolar ion transporter, AtNHX1 in *Arabidopsis*, has been shown to confer tolerance to salt stress in *B. napus* (Zhang et al. 2001) and tomato (Zhang and Blumwald 2001). Also, AtNHX1 orthologues have been isolated from various other crop species such as rice (Fukuda et al. 1999), buckwheat (Cheng et al. 2007), and sugar beet (Xia et al. 2002). Drought tolerance, as a trait, has received much attention due to its direct implication in agriculture, and multiple pathways involved in drought-stress tolerance have been elucidated in *Arabidopsis*. Certain families of transcription factors such as the AP2/ERF family, DREB (dehydration responsive element binding), and NAC family have been largely implicated in mediating drought tolerance. DREB/CBF proteins identified in *Arabidopsis* have been ectopically expressed in wheat, rice, and tomato to confer drought-stress tolerance (Wang et al. 2003; Pellegrineschi et al. 2004; Oh et al. 2005). *Arabidopsis*, being easily amenable to genetic transformation, has also been used for testing the function of genes isolated from various crop species. Constitutive overexpression of CBF orthologues from rice and maize has resulted in drought tolerance in *Arabidopsis* (Dubouzet et al. 2003; Qin et al. 2004). Also, PI4K (phosphatidylinositol 4-kinase) gene from wheat has been ectopically overexpressed in *Arabidopsis* to confer drought tolerance (Liu et al. 2013).

1.5.3.2 Biotic Stress

Crops suffer heavy losses due to diseases caused by a range of phytopathogens. Improving disease resistance has been an important mandate in most of the crop breeding programs. For devising new crop protection strategies, it is fundamental to understand the biology of the plant responses to pathogen invasion. *Arabidopsis* has been established as a model for molecular investigations of resistance to different crop pathogens like *Albugo* sp., *Hyaloperonospora* sp., *Colletotrichum* sp., *Plasmodiophora brassicae*, plant viruses, *Ralstonia* sp., etc. *Arabidopsis* research has not only contributed immensely to delineating various defense responses to biotrophic and necrotrophic pathogens but also yielded insights into conserved plant responses across different classes of pathogens. "R" genes identified in *Arabidopsis* can be used to engineer resistance into crop plants. The *Arabidopsis–Albugo candida* pathosystem has led to identification and molecular characterization of white rust resistance 4 (WRR4), a TIR-NB-LRR gene. WRR4 confers broad-spectrum resistance against *Albugo* sp. and has the potential for use in *Brassica* crops. Recently, transgenic *B. juncea* and *B. napus* expressing AtWRR4 from *Arabidopsis* have been reported to show improved resistance against multiple races of *A. candida* (Borhan et al. 2010). Various research groups are using the natural genetic variation existing in *Arabidopsis* accessions for identifying novel resistance genes via association mapping approaches. Bouwmeester et al. (2011), in a similar approach, identified *Arabidopsis* Lectin Receptor Kinase 1.9 (AtLecRK 1.9) which is capable of recognizing *Phytophthora infestans*, a notorious pathogen of potato. Interfamily transfer of the gene AtLecRK 1.9 to potato and tobacco could confer resistance against *P. infestans* and, hence, can be used in breeding strategies to develop durable late-blight resistant *Solanaceous* crops (Bouwmeester et al. 2014). Similarly, NPR1 (non-expressor of PR1), a master regulator of the SA pathway, plays a critical role in systemic acquired resistance (SAR). Overexpression of AtNPR1 has been shown to confer resistance against various pathogens in several crop plants including rice (Chern et al. 2001; Quilis et al. 2008), tomato (Lin et al. 2004), wheat (Makandar et al. 2006), carrot (Wally et al. 2009), and cotton (Parkhi et al. 2010).

It is equally important to understand the mechanism used by the pathogen to successfully colonize and reproduce on its host plant, as this could also provide leads for developing novel resistance strategies. *Arabidopsis* has also been used for investigating genomics and effectromics of different oomycetes like *A. candida*, *Hyaloperonospora arabidopsidis*, and *P. infestans*, to name a few.

1.6 Moving Beyond the Model System

Knowledge gained from studies in the dicot model *Arabidopsis* has facilitated understanding of various aspects of plant growth, development, and responses to environmental cues. Genes involved in core cellular processes like cell cycle, transcription, and translation tend to be conserved throughout eukaryotic evolution (Koonin et al. 2004). Although homologues of genes regulating key biological pathways have been identified in various crop species, there is a growing awareness that regulation of these pathways differs considerably between species, thus rendering translation of knowledge from the model plant into a crop inefficient. A prime example of this comes from the well-studied flowering-time pathway, wherein homologues of flowering-time genes such as FT and CO have been found in many crops such as rice and wheat, but transcriptional and posttranscriptional regulation of these genes varies considerably in response to external and internal cues (Ballerini and Kramer 2011). Also, it has been shown that responses of *Arabidopsis* and *B. napus* to *Leptosphaeria maculans* infection differ significantly (Sasek et al. 2012; Larkan et al. 2013). A recent commentary discusses inadequacies of *Arabidopsis* as a model system for understanding complex networks of responses that have evolved through years of host–pathogen coevolution (Rouxel and Balesdent 2013). Therefore, the model system may be used to define a set of genes involved in a particular biological process, but regulation and interactions need to be resolved in a context-specific manner. There is also a need to develop other model systems more closely related to the target crops that are amenable to cultivation under laboratory conditions. One such emerging model system is *Brachypodium distachyon*, of the grass subfamily Pooideae, which is closely related to many cereal crops including wheat, barley, rice, sorghum, and maize. The emergence of affordable high-throughput sequencing technologies has culminated in many crop genomes being sequenced. This, in turn, has led to development of crop-specific resources, thus paving the way for unraveling gene-trait correlations directly in the crop species itself. Though there have been many developments recently in the field of crop genomics, we have a long way to go before this knowledge can be used directly for crop improvement. Till then, model systems would continue to be workhorses in plant biology research.

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Microalgae in Biotechnological Application: A Commercial Approach

2

Nilofer Khatoon and Ruma Pal

Abstract

Microalgae are used as food, feed, and fodder and also used to produce a wide range of metabolites such as, proteins, carbohydrates, lipids, carotenoids, vitamins, fatty acids, sterols, etc. They are able to enhance the nutritional content of conventional food and feed preparations and hence positively affect humans and animal health including aquaculture animals. They also provide a key tool for phycoremediation of toxic metals and nanometal production. The use of microalgae in nanotechnology is a promising field of research with a green approach. The use of genetically modified algae for better production of different biotechnological compounds of interests is popular nowadays. Microalgal biomass production for sustainable biofuel production together with other high-value compounds in a cost-effective way is the major challenge of algal biotechnologists. Microalgal biotechnology is similar to conventional agriculture but has received quite a lot of attention over the last decades, because they can reach substantially higher productivities than traditional crops and can use the wastelands and the large marine ecosystem. As history has shown, research studies on microalgae have been numerous and varied, but they have not always resulted in commercial applications. The aim of this review is to summarize the commercial applications of microalgae.

Keywords

Microalgae • Nutrition • Metabolites • Phycoremediation • Nanometals • Nanotechnology • Biofuel

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2.1 Introduction

Algae including cyanobacteria are the most primitive plants that appeared on the earth's surface at least 3500 Ma ago. They are chlorophyll-bearing photosynthetic organisms which built up the oxygen in the atmosphere. The algae comprise one of the most diverse plant groups and constitute a species range of 40,000–10,000,000, with majority being the microalgae (Hawksworth and Mound 1991; Metting 1996). Traditionally, "algae" include the prokaryotic cyanobacteria and other eukaryotic members of Chlorophyta, Rhodophyta, Heterokontophyta, etc. Therefore, in general, algae include photosynthetic prokaryotic and eukaryotic organisms as well as the heterotrophic members. On the basis of molecular phylogeny, many of these groups are widely scattered on the "tree of life." As a result, the term "algae" refers to a polyphyletic, artificial assemblage of organisms. But in recent terminology "algae" excludes cyanobacteria. They are described as "lower" plants that never have true stems, roots, and leaves, and they are normally capable of photosynthesis. Plant bodies are thalloid, and there are members with coccoid, capsoid, amoeboid, palmelloid, colonial, plasmodial, filamentous, and parenchymatous (tissue like) organizational levels. The size of algae ranges from tiny single-celled species to gigantic multicellular organisms (few micron to few meters). Algae of different sizes and shapes not only occupy all aquatic ecosystems but also occur in almost all other habitats. Algae are one of the earth's most important natural resources that contribute to approximately 50 % of global photosynthetic activity. The first use of microalgae by humans dates back 2,000 years to the Chinese, who used *Nostoc* to survive during famine. However, microalgal biotechnology only really began to develop in the middle of the last century, and establishment of *Spirulina* production plant in Mexico can be considered as benchmark of microalgal biotechnology (Borowitzka 1999). Particularly during the past two or three decades, algal biotechnology grew steadily into an important global industry with a diversified field of applications, and more and more new entrepreneurs began to realize the potentiality of microalgae in biotechnological exploitation. Nowadays, about 10^7 t of microalgae are harvested each year by different industries for various purposes. Today's commercial algal biotechnology is still a non-transgenic industry that basically produces food, feed, and feed additives, cosmetics, and pigments. Presently active researches are going on for the development of genetically modified algae and for biotechnological exploitation. Due to the marine and aquatic applications, algal biotechnology is sometimes also called blue biotechnology.

Commercial large-scale cultivation of microalgae started in the early 1960s in Japan with the culture of *Chlorella* by the company Nihon *Chlorella* (Taipei, Taiwan) (Borowitzka 1999; Iwamoto 2004). It was followed in the early 1970s by the establishment of an *Arthrospira* culturing and harvesting facility in Lake Texcoco by Sosa Texcoco S.A. (Mexico City, Mexico) (Borowitzka 1999). The first use of algae in aquaculture appeared in the 1970s (Pulz and Scheibenbogen 1998). By 1980, there were 46 large-scale factories in Asia producing more than 1,000 kg of microalgae (mainly *Chlorella*) per month. The commercial production of *Dunaliella salina*, as a source of β-carotene, became the third major microalgal industry when production facilities were established by Western Biotechnology (Hutt Lagoon, Australia) and Betatene in 1986 (Whyalla, Australia, now Cognis Nutrition and Health). These were soon followed by other commercial plants in Israel and the USA. Together with these, the large-scale production of cyanobacteria (blue-green algae) began in India at about the same time. More recently, several plants producing *Haematococcus pluvialis* as a source of astaxanthin have been established in the USA and India (Biotechnological and Environmental Applications of Microalgae [BEAM]). Thus, in a short period of about 30 years, the algal biotechnology industry has grown and diversified significantly. Nowadays, the microalgal biomass market produces about 5,000 t of dry matter/year and generates a turnover of approximately US$ 1.25×10^9/year.

The aim of this review is to focus on the latest status of multidisciplinary research in the area of algal biotechnology mainly related to industrial applications of algae.

2.2 Microalgae in Human and Animal Nutrition

The microalgal market as food is dominated by *Chlorella* and *Spirulina* (Becker 2004; Pulz and Gross 2004), mainly because of their high protein content and nutritive value. The biomass of these algae is marketed as tablets, capsules, and liquids (Table 2.1, Fig. 2.1). Microalgae can act as a nutritional supplement or represent a source of natural food colorants (Apt et al. 1996; Borowitzka 1999; Soletto et al. 2005). The commercial applications are dominated by four strains: *Arthrospira*, *Chlorella*, *Dunaliella salina*, and *Aphanizomenon flos-aquae*. *Arthrospira* is used in human nutrition because of its high protein content and its excellent nutritive value (Radmer 1996; Rangel-Yagui et al. 2004; Soletto et al. 2005; Desmorieux and Decaen 2005). In addition, this cyanobacterium has various possible health-promoting effects, like alleviation of hyperlipidemia, suppression of hypertension, protection against renal failure, growth promotion of intestinal *Lactobacillus*, suppression of elevated serum glucose level, etc. (Vílchez et al. 1997; Yamaguchi 1997; Liang et al. 2004).

The world's largest producer of *Arthrospira* – the Hainan Simai Enterprising – is located in the Hainan province of China. This company has an annual production of 200 t of algal powder, which

Table 2.1 Major microalgae commercialized for human nutrition

Microalga	Major producers	Products	Production (t/year)
Spirulina (*Arthrospira*)	Hainan Simai Pharmacy Co. (China)	Powders, extracts	3,000
	Earthrise Nutritionals (California, USA)	Tablets, powders, extracts	
	Cyanotech Corp. (Hawaii, USA)	Tablets, powders, beverages, extracts	
	Myanmar *Spirulina* factory (Myanmar)	Tablets, chips, pasta, and liquid extract	
Chlorella	Taiwan *Chlorella* Manufacturing Co. (Taiwan)	Tablets, powders, nectar, noodles	2,000
	Klötze (Germany)	Powders	
Dunaliella salina	Cognis Nutrition and Health (Australia)	Powders	1,200
		B-carotene	
Aphanizomenon flos-aquae	Blue Green Foods (USA)	Capsules, crystals	500
	Vision (USA)	Powder, capsules, crystals	

Adapted from Pulz and Gross (2004), Viskari and Colyer (2003), Spolaore et al. (2006), Hallmann (2007)

Fig. 2.1 Algal food used in human and animal nutrition

accounts for 25 % of the total national output and almost 10 % of the world output. Their *Arthrospira*-based products (tablets and powder) are distributed in over 20 countries around the world. Many other companies sell a wide variety of nutraceuticals made from this microalga. For example, the Myanmar *Spirulina* Factory (Yangon, Myanmar) sells tablets, chips, pasta, and liquid extract, and Cyanotech (a plant in Kona, Hawaii, USA) produces products ranging from pure powder to packaged bottles under the name Spirulina Pacifica.

For human consumption, Cognis Nutrition and Health, the world's largest producer of this strain, offers *Dunaliella* powder as an ingredient of dietary supplements and functional foods. According to many research studies, used alone or in combination with other nutraceuticals and natural food products, *A. flos-aquae* promotes overall good health (Jensen et al. 2001; Pugh and Pasco 2001; Benedetti et al. 2004).

Many evaluations have shown the suitability of algal biomass as a feed supplement (Becker 2004). Mainly, the microalgae *Spirulina* and, to some extent, *Chlorella* are used in this domain for many types of animals: cats, dogs, aquarium fish, ornamental birds, horses, poultry, cows, and breeding bulls (Spolaore et al. 2006). All these members are able to enhance the nutritional content of conventional feed preparations, and hence, they positively affect the physiology of these animals. Even very small amount of microalgal biomass can positively affect the physiology of animals by improving the immune response, resulting in growth promotion, disease resistance, antiviral and antibacterial action, improved gut function, probiotic colonization stimulation, as well as by improved feed conversion, reproductive performance, and weight control (Harel and Clayton 2004).

The external appearance of the animals may also be improved, resulting in healthy skin and a lustrous coat, for both farming animals (poultry, cows, breeding bulls) and pets (cats, dogs, rabbits, ornamental fishes, and birds) (Certik and Shimizu 1999). Since feed corresponds to the most important exogenous factors influencing animal health and also the major expense in animal production, the use of alternative high-quality protein supplements replacing conventional protein sources is encouraged. In fact, 30 % of the current world algal production is sold for animal feed applications (Becker 2004).

2.3 Aquaculture Feed

From an economic perspective, the importance of using algae in aquaculture has grown over the years as it can be utilized as an unconventional source of protein alternative to the much used fish meal. Since 1940s, microalgae became more and more important as live feed in aquaculture (shellfish or fish farming). Microalgal feeds are currently used mainly for the culture of larvae and juvenile shell and finfish as well as for raising the zooplankton required for feeding of juvenile animals (Fig. 2.2) (Benemann 1992; Chen 2003). They are required for larval nutrition during a brief period, either for direct consumption in the case of mollusks and penaeid shrimp or indirectly as food for the live preys, mainly rotifers, copepods, and *Artemia nauplii*, which in turn are used for crustaceans and fish larvae feeding (Brown

Fig. 2.2 Algal feed for aquaculture

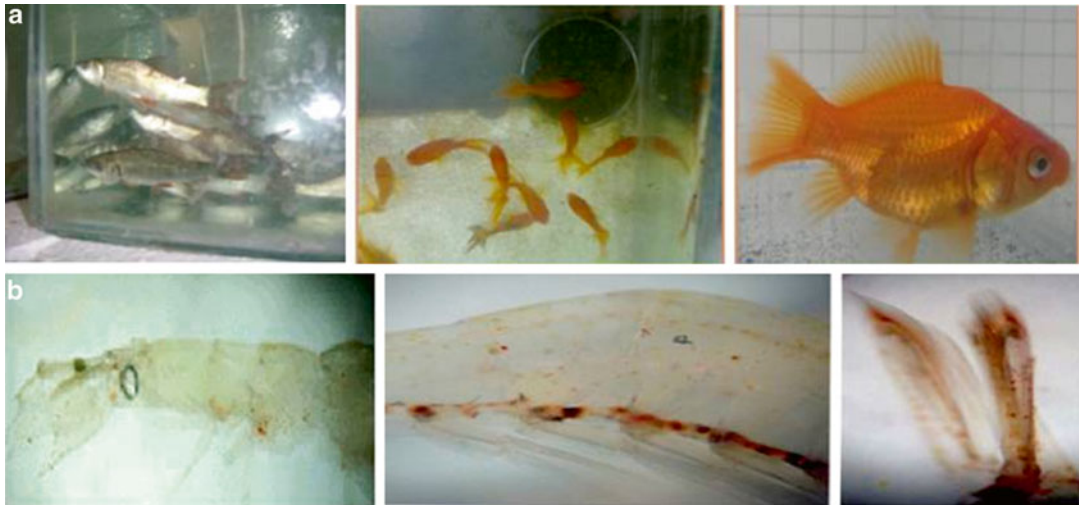

Fig. 2.3 Improved growth and color after feeding on algal feed (**a**) rohu and goldfish and (**b**) prawn

et al. 1997; Duerr et al. 1998; Muller-Feuga 2000). In 1999, the production of microalgae for aquaculture reached up to 1,000 t (62 % for mollusks, 21 % for shrimps, and 16 % for fish) for a global world aquaculture production of 43 × 106 t of plants and animals (Muller-Feuga 2000). The most frequently used microalgae in aquaculture are *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nanno-chloropsis*, *Skeletonema*, and *Thalassiosira* (Yamaguchi 1997; Borowitzka 1997; Apt and Behrens 1999; Muller-Feuga 2000). Microalgae contain essential nutrients which determine the quality, survival, growth, and resistance to disease of cultured species. To support a better balanced nutrition for animal growth, it is often advised to use mixed microalgae cultures, in order to have a good protein profile, adequate vitamin content, and high polyunsaturated fatty acids, mainly EPA, AA, and DHA, recognized as essential for survival and growth during the early stages of life of many marine animals (Volkman et al. 1989). One of the beneficial effects attributed to adding algae is an increase in ingestion rates of food by marine fish larvae which enhance growth and survival as well as the quality of the fry (Naas et al. 1992). Aquatic species, such as salmonids (salmon and trout), shrimp, lobster, sea bream, and koi carp, under intensive rearing conditions need a supplementation of carotenoids pigments in their diet to attain their characteristic muscle color. In addition to pigmenting effects, carotenoids, namely, astaxanthin and canthaxanthin, exert benefits on animal health and welfare, promote larval development, and provide growth and performance. A positive metabolic role of carotenoids in the nutrition of larval fish and survival of young fry was also discussed by Reitain et al. (1997), Planas and Cunha (1999), and Lazo et al. (2000).

The present group reported the use of nonconventional algal feed with improved results in growth, carcass composition, and pigmentation of goldfish (Fig. 2.3) (Khatoon et al. 2010a), rohu (Khatoon et al. 2010b), and prawn (Khatoon et al. 2009).

2.4 Chemicals and Pharmaceuticals

A large number of studies have demonstrated the benefits and potential of algae as a source for biologically active (Table 2.2; Fig. 2.4) compounds (Hirayasu et al. 2005; Bansemir et al. 2006) and their antioxidant activity (Yuan et al. 2005).

There are at least 30,000 known species of microalgae among which only a handful is currently of commercial significance (Table 2.3). Algae provide a largely untapped reservoir of

Table 2.2 Biomass composition of microalgae expressed on a dry matter basis (Um and Kim 2009; Sydney et al. 2010)

Strain	Protein	Carbohydrates	Lipid
Anabaena cylindrica	43–56	25–30	4–7
Botryococcus braunii	40	2	33
Chlamydomonas reinhardtii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	41–58	12–17	10–22
Dunaliella bioculata	49	4	8
Dunaliella salina	57	32	6
Dunaliella tertiolecta	29	14	11
Euglena gracilis	39–61	14–18	14–20
Porphyridium cruentum	28–39	40–57	9–14
Prymnesium parvum	28–45	25–33	22–39
Scenedesmus dimorphus	8–18	21–52	16–40
Scenedesmus obliquus	50–56	10–17	12–14
Scenedesmus quadricauda	47	–	1.9
Spirogyra sp.	6–20	33–64	11–21
Spirulina maxima	60–71	13–16	6–7
Spirulina Figurensis	42–63	8–14	4–11
Synechococcus sp.	63	15	11
Tetraselmis maculata	52	15	3

Fig. 2.4 Algal compounds in the form of cosmetics and medicines

novel and valuable compounds. Current exploitation mainly aims to utilize fatty acids, pigments, vitamins, and other bioactive compounds. In the 1980s, the use of microalgae as a source of common and fine chemicals was the beginning of a new trend (de la Noue and de Pauw 1988).

Higher plants and animals lack the requisite enzymes to synthesize polyunsaturated fatty acids (PUFAs) of more than 18 carbons (Lee 2001; Sancho et al. 1999). Thus, they have to get them from their food. Currently, algal docosahexaenoic acid (22:6 ω3, 6, 9, 12, 15, 18) produced from the dinoflagellate member, *Crypthecodinium cohnii*, is the only commercially available ω3-polyunsaturated fatty acid producer (Walker et al. 2005), but others like γ-linolenic acid from *Spirulina*, arachidonic acid from *Porphyridium*, and eicosapentaenoic acid from *Nannochloropsis*, *Phaeodactylum,* or *Nitzschia* have already demonstrated as potential for industrial production of their high-value compounds (Spolaore et al. 2006). Other fatty acids or lipids are isolated from *Phaeodactylum tricornutum* as a food additive; from *Odontella aurita* for pharmaceuticals, cosmetics, and baby food; and from *Isochrysis galbana* for animal nutrition (Pulz and Gross 2004).

Polysaccharides are isolated from *Chlorella* spp. for dietary supplements (Walker et al. 2005) and from *Porphyridium cruentum* for pharmaceuticals, cosmetics, and nutrition (Pulz and Gross 2004). Extracts from the cyanobacterium

Table 2.3 Commercial companies selling algae or algal products (Hallmann 2007)

Company	Products
Algatech, Israel	Astaxanthin, microalgae-derived products
Algisa, Compania Industrial de Alginatos S.A., Chile	Alginates
Bluebio Bio-Pharmaceutical Co., Ltd., China	Biomass (*Chlorella*, *Spirulina*)
Cognis Nutrition and Health, Australia	β-carotene
Dainippon Ink and Chemicals, Japan	Pigments
Exsymol S.A.M., Monaco	Cosmetics
Far East Bio-Tec Co., Ltd., Taiwan	Biomass (*Chlorella*, *Spirulina*), microalgae extracts, health care products, cosmetics
Martek Biosciences Corporation, USA	Fatty acids
Mera Pharmaceuticals, USA	Astaxanthin
Penglai Dengzhou Seaweed Co., Ltd., China	Alginates, mannitol, iodine
Phycotransgenics, USA	Transgenic microalgae (*Chlamydomonas*)
Qingdao Richstar Seaweed Industrial Co., Ltd, China	Food additives, pharmaceutical chemicals
Spectra Stable Isotopes, USA	Stable isotope biochemicals
Subitec GmbH, Germany	Fatty acids
Nature Beta Technologies, Israel	β-carotene
Necton S.A., Portugal	Biomass (microalgae)
Codif Recherche et Nature, France	Cosmetics

Lyngbya majuscula are used as immune modulators in pharmaceuticals and nutrition management (Pulz and Gross 2004). Components of algae are frequently used in cosmetics as thickening agents, water-binding agents, and antioxidants.

Cosmetics companies claim benefits on the skin or health in general from contents like carrageenan, other algal polysaccharides, algal proteins or lipids, vitamin A, vitamin B1, iron, phosphorus, sodium, copper, magnesium, calcium, or other elements; some companies promise that algal extracts inhibit oxidative degeneration of collagen and hyaluronic acid and that they have antiaging properties. From a scientific point of view, many of the promised effects have to be judged as not scientifically proven and unsubstantiated. Typical species that are used for cosmetics are *Spirulina platensis*, *Nannochloropsis oculata*, *Chlorella vulgaris*, and *Dunaliella salina*.

From a commercial point of view, the carotenoids and the phycobiliproteins seem to be the most important. In addition to their role in coloration, carotenoids act as provitamin A and as biological antioxidants, protecting cells and tissues from the damaging effects of free radicals and singlet oxygen. Therefore, carotenoids are utilized in pharmaceuticals, health food, and dietary supplements, cosmetics, and as a feed additive. The carotenoids including β-carotene are produced in large amounts from the halophilic green microalga *Dunaliella salina*. To a lesser extent, carotenoids are isolated from *Dunaliella bardawil* (Walker et al. 2005). The most prominent xanthophyll is astaxanthin, which is extracted in large scale from the green microalga *Haematococcus pluvialis* (Pulz and Gross 2004). Further utilized xanthophylls are lutein, canthaxanthin, and zeaxanthin. Phycobiliproteins consist of proteins with covalently bound phycobilins, which are tetrapyrrole structures with pyrrole rings that are laid out linearly. For efficient photosynthesis, phycobiliproteins capture light energy at certain wavelengths and pass it on to the chlorophylls. The phycobiliproteins-phycoerythrin and phycocyanin are isolated from *Spirulina* and *Porphyridium* and are utilized for health food, pharmaceuticals, and cosmetics (Becker 2004; Pulz and Gross 2004; Spolaore et al. 2006). Phycobiliproteins are not only used as pigments but have also been shown to have health-promoting properties. They are also used in research laboratories as labels for biomolecules (Spolaore et al. 2006). The first and most important application of phycocyanin is as food pigment, replacing current synthetic pigments (Becker 2004). Dainippon Ink & Chemicals (Sakura) has developed a product called "Lina blue" which is used in chewing gum, ice sherberts,

popsicles, candies, soft drinks, dairy products, and wasabi. They also sell another form of this pigment for natural cosmetics like lipstick and eyeliners (Yamaguchi 1997; Viskari and Colyer 2003). Their global market was estimated at more than US$50 million in 1997.

2.5 Biofertilizers

Biofertilizer contains microorganisms which promote the adequate supply of nutrients to the host plants, ensure their proper development of growth and regulation in their physiology, and protect plants from soil-borne diseases to a certain degree. The need for the use of biofertilizer has arisen, primarily for two reasons: first, because increase in the use of fertilizers leads to increased crop productivity, and second, because increased usage of chemical fertilizer leads to damage in soil texture and raises other environmental problems. Therefore, the use of biofertilizer is both economical and environment friendly. Among the microalgae, cyanobacteria have drawn much attention as prospective and rich sources of biologically active constituents that can be used as biofertilizers (Fish and Codd 1994; Schlegel et al. 1999). In early times, several authors like De (1939) and Gupta and Shukla (1967) studied the algal influence on growth, yield, and protein content of rice plants and showed that presoaking rice seeds with BGA cultures or extracts enhances germination, promotes the growth of roots and shoots, and increases the weight and protein content of the grain. Beneficial effects of cyanobacterial inoculation were reported, not only for rice but for other crops as well such as wheat, soybean, oat, tomato, radish, cotton, sugarcane, maize, chili, bean, muskmelon, and lettuce (Venkataraman 1972; Rodgers et al. 1979; Arif et al. 1995; Thajuddin and Subramanian 2005; Saadatnia and Riahi 2009; Maqubela et al. 2008; Karthikeyan et al. 2007). Efficient nitrogen-fixing blue green algal strains are utilized for rice production. Several reasons have been proposed for beneficial effects of cyanobacteria on the growth of different plants. The capacity for enhanced biosynthesis of growth-promoting substances such as auxins, amino acids, sugars, and vitamins (vitamin B12, folic acid, nicotinic acid, and pantothenic acid) by algalization was reported by Misra and Kaushik (1989b) that can enhance the growth of plant. Cyanobacteria play an important role in fixing atmospheric nitrogen due to the presence of heterocysts and buildup of soil fertility, consequently increasing rice growth and yield as a natural biofertilizer (Song et al. 2005). Additionally, cyanobacteria excrete complex organic carbon compounds that bind to the soil particles and improve soil aggregation, hence improve soil structure, soil permeability, and water-holding capacity of soil (Kaushik 2007). Cyanobacteria are capable of abating various kinds of pollutants and have advantages as potential biodegrading organisms (Subramanian and Uma 1996). Blue-green algal inoculation with composite cultures was found to be more effective than single culture inoculation. Both free-living andas well as symbiotic cyanobacteria (blue-green algae) have been harnessed in rice cultivation in India. A composite culture of BGA having heterocystous *Nostoc*, *Anabaena*, *Aulosira*, etc. is given as primary inoculum in trays, polythene-lined pots, and later mass multiplied in the field for application as soil-based flakes to the rice-growing field at the rate of 10 kg/ha. At many sites where algal inoculation was used for three to four consecutive cropping seasons, the inoculated algae establish well and the effect persisted over subsequent rice crop. The blue-green algal inoculum may be produced by several methods, viz., in tubs, galvanized trays, and small pits and also in field conditions.

However, the large-scale production is advisable under field condition which is easily adopted by farmers. Nowadays, liquid fertilizers are in use. There are many advantages of liquid biofertilizer over the conventional carrier-based biofertilizers. As they have greater potentials to fight with native population; cost saving on carrier material, pulverization, neutralization, sterilization, packing, and transport; easy and quick quality

Fig. 2.5 Algal-based biofertilizers

control protocols; better survival on seeds and soil; and very much easy to use by the farmer. The increasing demand for the biofertilizers and the awareness among farmers and planters in the use of biofertilizers have paved way for the fertilizer manufactures and new entrepreneurs to get into biofertilizer production. A number of biofertilizer production units have been started recently particularly in the southern states of our country (Fig. 2.5).

The application of BGA biofertilizer in rice popularly known as "algalization" helps in creating an environment-friendly agroecosystem that ensures economic viability in paddy cultivation while saving energy-intensive inputs. The technology can be easily adopted by farmers for multiplication at their own level.

2.6 Biofuel

Algae, particularly green unicellular microalgae, have been proposed for a long time as a potential renewable fuel source (Benemann and Oswald 1996; Oswald and Golueke 1960; Brennan and Owende 2010). Microalgae have long been recognized as potentially good sources for biofuel production because of their high oil content and rapid biomass production (Table 2.4). In recent years, the use of microalgae as an alternative biodiesel feedstock has gained renewed interest from researchers, entrepreneurs, and the general public. As with plant-derived feedstocks (Table 2.5), algal feedstocks can be utilized directly or processed into liquid fuels and gas by a variety of biochemical conversion or thermochemical conversion processes (Amin 2009; Brennan et al. 2010). Dried algal biomass may be used to generate energy by direct combustion (Kadam 2002), but this is probably the least attractive use for algal biomass. In addition, hydrogen can be produced from algae by biophotolysis (Melis 2002). One of the attractions of microalgae as a biofuel feedstock is that they can be effectively grown in conditions which require minimal freshwater input unlike many plant-based biofuel crops and utilize land which is otherwise nonproductive to plant crops, thus making the process potentially sustainable with regard to preserving freshwater resources. There has therefore been significant interest in the growth of microalgae for biofuels under saline conditions (Rodolfi et al. 2009; de la Noue et al. 1992).

Table 2.4 Oil content of microalgae (Chisti 2007)

Microalga	Oil content (% dry weight)
Botryococcus braunii	25–75
Chlorella sp.	28–32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16–37
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis suecica	15–23

The rise in world oil prices led to a sharp increase in biofuel production around the world. High oil species of microalgae cultured in growth-optimized conditions of photobioreactors have the potential to yield 5,000–15,000 gal of

Table 2.5 Comparison of microalgae with other biodiesel feedstocks (Teresa et al. 2010)

Plant source	Seed oil content (% oil by wt in biomass)	Oil yield (l oil/ha year)	Land use (m^2 year/ kg biodiesel)	Biodiesel productivity (kg biodiesel/ha year)
Corn/maize (*Zea mays* L.)	44	172	66	152
Hemp (*Cannabis sativa* L.)	33	363	31	321
Soybean (*Glycine max* L.)	18	636	18	562
Jatropha (*Jatropha curcas* L.)	28	741	15	656
Camelina (*Camelina sativa* L.)	42	915	12	809
Canola/rapeseed (*Brassica napus* L.)	41	974	12	862
Sunflower (*Helianthus annuus* L.)	40	1,070	11	946
Castor (*Ricinus communis*)	48	1,307	9	1,156
Palm oil (*Elaeis guineensis*)	36	5,366	2	4,747
Microalgae (low oil content)	30	58,700	0.2	51,927
Microalgae (medium oil content)	50	97,800	0.1	86,515
Microalgae (high oil content)	70	136,900	0.1	121,104

Fig. 2.6 Algal biofuel

microalgal oil per acre per year. The most significant distinguishing characteristic of algal oil is its yield and hence its biodiesel yield. According to some estimates, the yield (per acre) of oil from algae is over 200 times the yield from the best-performing plant/vegetable oils (Becker 1994). They can complete an entire growing cycle every few days. Microalgae are very efficient solar energy converters, and they can produce a great variety of metabolites (Chaumont 2005). A major current problem for the commercial viability of biodiesel production from microalgae is the low selling price of biodiesel. Biodiesel from microalgal oil is similar in properties to the standard biodiesel (Huang et al. 2010). The algae that are used in biodiesel production are usually aquatic unicellular green algae. These types of algae are a photosynthetic eukaryote characterized by high growth rates and high population densities. Under good conditions, green algae can double its biomass in less than 24 h. Additionally, green algae can have huge lipid contents, frequently over 50 %. This high yield, high density biomass is ideal for intensive agriculture and may be an excellent source for biodiesel production (Fig. 2.6).

Algal oil contains saturated and monounsaturated fatty acids. The fatty acids were determined in the algal oil in the following proportions: 36 % oleic (18:1), 15 % palmitic (16:0), 11 % stearic (18:0), 8.4 % iso-linoleic (17:0), and 7.4 % linoleic (18:2). The high proportion of saturated and monounsaturated fatty acids in this alga is considered optimal from a fuel quality standpoint, in that fuel polymerization during combustion would be substantially less than what would

occur with polyunsaturated fatty acid-derived fuel (Chaumont 2005; Gao et al. 2010). After oil extraction from algae, the remaining biomass fraction can be used as a high-protein feed for livestock (Schneider 2006). This gives further value to the process and reduces waste. Algae biomass can play an important role in solving the problem between the production of food and that of biofuels in the near future (Chisti 2007).

2.7 Bioremediation

Organic pollutants and heavy metals are considered to be a serious environmental problem for human health. Lead, cadmium, and mercury are the most frequent water- and soil-polluting heavy metals. Industrial processes, including plastic manufacturing, electroplating, Ni-Cd battery production, mining, and smelting industries, continuously release substantial amounts of heavy metals into the environment. The contamination of soils and aquatic systems by toxic metals and organic pollutants has recently increased due to anthropogenic activity. Phycoremediation is the use of microalgae for the removal or biotransformation of pollutants, including nutrients and xenobiotics from wastewater and CO2 from waste air with concomitant biomass propagation (Olguín et al. 2003, 2004; Mulbry et al. 2008; Moreno-Garrido 2008). Phytoremediation technologies are becoming recognized as cost-effective methods for remediating sites contaminated with toxic metals at a fraction of the cost of conventional technologies, such as soil replacement, solidification, and washing strategies (Chaney et al. 1997; Flathman and Lanza 1998; Munoz and Guieysse 2008). Recent studies have shown that microalgae can indeed support the aerobic degradation of various hazardous contaminants (Munoz et al. 2008; Safonova et al. 2004). Since few decades the potential of microalgae in metal biosorption process has been studied extensively due to their ubiquitous occurrence in nature (Gekeler et al. 1998). Many algal genera are found to have capabilities to accumulate heavy metals, thereby reducing their toxic effects (Huang et al. 1990; Kuyucak and Volesky 1990; Schiewer and Volesky 2000; Satıroğlu et al. 2002; Arica et al. 2004; Selatnia et al. 2004). There has been a growing interest in using algae for biomonitoring eutrophication, organic pollutants, and inorganic pollutants. There are numerous processes of treating water, industrial effluents, and solid wastes using microalgae aerobically as well as anaerobically. Many algae synthesize phytochelatins and metallothioneins that can form complexes with heavy metals and translocate them into vacuoles (Oswald and Golueke 1960). The growth of microalgae is indicative of water pollution since they respond typically to many ions and toxins. Blue-green algae are ideally suited to play a dual role of treating wastewater in the process of effective utilization of different constituents essential for growth leading to enhanced biomass production. Secondly, the role of microalgae is the accumulation and conversion of wastewater nutrients to biomass and lipids. Microalgae are efficient in the removal of nutrients from wastewater. Unicellular algae have shown great efficiency in the uptake of nutrients and have been found to show dominance in oxidation ponds (Pittman et al. 2010). *Chlorella*, *Ankistrodesmus*, and *Scenedesmus* species have been already successfully used for the treatment of olive oil, mill wastewaters, and paper industry wastewaters (Abeliovich 1986; Narro 1987; Tilzer 1983). The algae have many features that make them ideal candidates for the selective removal and concentration of heavy metals, which include high tolerance to heavy metals, ability to grow both autotrophically and heterotrophically, large surface area/volume ratios, phototaxy, phytochelatin expression, and potential for genetic manipulation (Cai et al. 1995). Mei et al. (2006) suggested that *Platymonas subcordiformis*, a marine green microalgae, had a very strontium uptake capacity, although high concentrations of strontium cause oxidative damage, as evidenced by the increase in lipid peroxidation in the algal cell samples and the decrease in growth rate and chlorophyll contents. *Caulerpa racemosa* var. *cylindracea* as a low-cost biomaterial could be used for the removal of boron species from aqueous solution (Bursali et al. 2009). *Dunaliella salina*, a green

microalgae, have high tendency for zinc accumulation followed by copper and cobalt, the lowest tendency was for cadmium, and this may be due to the importance of zinc as hydrogen transferring in photosynthesis (Liu et al. 2002). The present group also reported several algae like *Oscillatoria*, *Anabaena*, *Rhizoclonium*, and *Chara* that accumulated lead, cadmium, and chromium from wastewater (Chakraborty et al. 2011). Arsenic-resistant genera recorded from the contaminated area were *Oscillatoria princeps*, *Oscillatoria limosa*, *Anabaena* sp., *and Phormidium laminosum* (Bhattacharya 2011).

The biomass resulting from the treatment of wastewaters can be applied for different aims, including the use as additives for animal feed, the extraction of added value products like carotenoids or other biomolecules, or the production of biofuel. The accumulation of heavy metals by algae provides an advantage for phytoremediation over other methods which are more costly and not environmental friendly. Therefore, there is a need to improve the possibilities of accumulation of heavy metals in algae.

2.8 Phyconanotechnology

Nanotechnology and biotechnology are the two most fascinating technologies of the twenty-first century, and unification of these two results in the development of nanobiotechnology which deals with the synthesis of nanostructures using biological organisms. Reliable and eco-friendly synthesis of metallic nanoparticles is an important goal of nanotechnology. Nanomaterials are gaining so much of interest due to their unique optical, chemical, photoelectrochemical, and electronic properties which are absent in the bulk material because of the quantum-size confinement imposed by nano-size (Kumar et al. 2003).

Nanoparticles are synthesized by a number of chemical and physical procedures. Several manufacturing techniques are in use that employs atomistic, molecular, chemical, and particulate processing in a vacuum or a liquid medium (Daniel and Astruc 2004). But most of these techniques are costly, as well as inefficient in materials and energy use. Therefore, second-generation nanotechnology is focused toward clean technologies that minimize possible environmental and human health risks associated with manufacture and fabrication; there is an ever-growing demand for the development of clean, nontoxic, and environmentally benign synthesis procedures. Green synthesis of nanoparticles is a significant process for the production of precious metals such as gold, silver, platinum, and palladium due to their various applications in material sciences. A number of microorganisms and higher plants have already been found to be competent to serve as eco-friendly nanofactories for the synthesis of gold nanoparticles (GNP) and silver nanoparticles (SNP). Cyanobacteria and microalgae having the reducing power are already designated as potential bioreagents for nontoxic nanoparticle synthesis. They are effective in nanoparticle synthesis, as they grow rapidly in eco-friendly atmosphere producing large amount of biomass in a very short time and can accumulate high quantity of metals followed by subsequent reduction. Their production costs are also negligible (Nair and Pradeep 2002; Lin et al. 2005; Lengke et al. 2006a, b). The properties of nanogold change with varying sizes and shapes of the particles and are suitable for applying in diverse fields, starting from decorative coating paints to different fields of science like electronics, catalysis, pollution control, cancer therapy, drug delivery, biomedical assay, biosensor, bioimaging, etc. (Xu et al. 2004; Huang et al. 2006; Hauck et al. 2008). The "green" route for nanoparticle (NP) synthesis is therefore of great interest for economic prospects and applications. The green chemistry synthetic route can be employed for both GNP and SNP syntheses. Moreover, nanoproducts, synthesized from algae, are expected to be biocompatible, therefore minimize environment and human health risks. The use of SNPs and GNPs in drug delivery systems might be the future thrust in the field of medicine. Further research is needed to screen the most suitable algal taxa for large-scale controlled synthesis of gold and silver nanoparticles.

The synthesis of nanogold was first reported in 1995 in *Chlorella vulgaris* (Ting et al. 1995) and

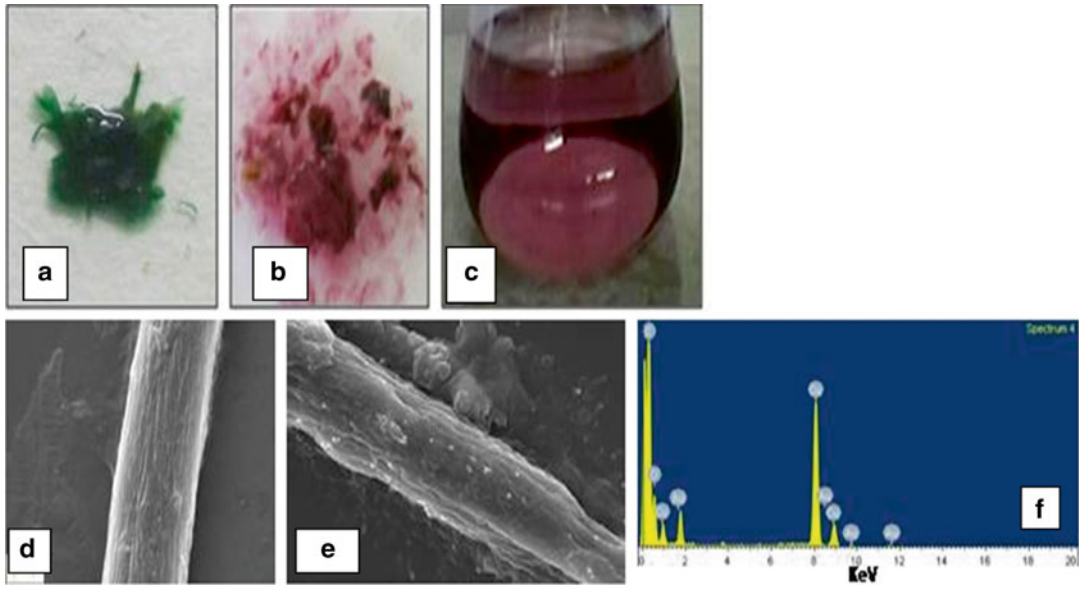

Fig. 2.7 Algae showing (**a**) control filament, (**b**) gold nanoparticle synthesis, and (**c**) extracellular cold nanoparticles in the media. SEM of (**d**) control, (**e**) gold-treated algae, and (**f**) X-ray spectroscopy showing nanoparticles synthesis

afterwards, Lengke et al. (2006a, b) reported it in *Plectonema boryanum*. In the same year, the present group reported cyanobacteria- and algae-based systemic process of gold and silver nanoparticle synthesis and published a series of papers on biosynthesis of gold nanoparticle by cyanobacteria (such as *Lyngbya majuscula* and *Spirulina subsalsa*), green algae (*Rhizoclonium hieroglyphicum* and *R. riparium*), Spirogyra and diatoms (*Nitzschia obtusa* and *Navicula minima*) (Chakraborty et al. 2006, 2009; Nayak et al. 2006a; Roychoudhury and Pal 2014). Screening for different algae as bioreagent of GNP production and biosynthesis of gold nanorods by *Nostoc ellipsosporum* was done by Parial et al. (2012); Parial and Pal (2014) and of silver nanoparticles by Roychoudhury et al. (2014) (Fig. 2.7).

2.9 Genetic Engineering and GM Algae

Algal genome research is needed to get a new level of efficiency in biotechnological applications. Algal species are targets of genetic engineering to improve productivity, broaden environmental tolerance limits, or increase pest or pathogen resistance. DNA sequences are introduced into the algal cells with the goal of modifying biochemical pathways. Among the biotechnologically important biomolecules, some are with high value but required in small quantity like medicinal compounds, but some are low-value product and are required in large amount like algal biodiesel. Therefore, the aim of the algal biotechnologists is always to reduce the cost of the required products. Sometimes the cost of microalgal biomass production becomes so high that ultimately the production cost of the required compound becomes exorbitant therefore unusable. For this reason, biotechnologists tried to manipulate genetically many commercially viable microalgae to get suitable combinations of genes in the required strain for successful low-cost production or to get the required compounds – called as molecular farming. But till date, most of the results related to GM algae production are laboratory confined. The propagation of GM algae is in question as algae are the lower group of plants which frequently propagate through vegetative propagation like binary

fission resulting in the uncertain distribution of the altered genetic combination.

There are several microalgal genome projects (Table 2.6), of which the most advanced projects are those for the red alga *Cyanidioschyzon merolae*, the diatom *Thalassiosira pseudonana*, and the three green algae *Chlamydomonas reinhardtii*, *Volvox carteri*, and *Ostreococcus tauri*. Cyanobacteria are preferable source materials in plant genetic engineering studies due to genetic homology of chloroplasts in eukaryotic plants, prokaryotic genome organization, short generation time, and fast growth rate. Most studies have therefore been made on commonly called as 'green yeast'. *Chlamydomonas reinhardtii*. Apart from *Chlamydomonas*, cyanobacterial strains of *Synechocystis* sp., *Synechococcus* sp., and *Anabaena variabilis. Synechocystis* sp. and *Synechococcus* sp. are naturally transformable with exogenous DNA. The green alga *Haematococcus pluvialis* naturally accumulates large amounts of astaxanthin when exposed to unfavorable growth conditions. The gene coding for the enzyme that converts ß-carotene into astaxanthin has been identified from *H. pluvialis* and cloned into *Synechococcus*. After the transformation, both zeaxanthin and the attractive compound astaxanthin were produced (Sahu et al. 2012). Tremendous success has been achieved in establishing functional association between plant seedling/calli/protoplast cultures and N_2-fixing cyanobacterial filaments through the application of basic tissue culture technique. *Nostoc*, *Anabaena*, and *Plectonema* are among the few N_2-fixing cyanobacterial species successfully practiced in these studies. The use of transgenic cyanobacterium to control insect is a new technology. Various combinations of genes were introduced in *Anabaena* which showed toxicity against mosquito larvae. Further studies of genetic engineered algae and bioremediation have been performed on the green algae on *Chlamydomonas reinhardtii*. In the last few years, successful genetic transformations of ~25 algal species have been demonstrated; most of these were achieved by nuclear transformation. Ten species of green algae have been transformed; stable transformation have been shown for seven of them, one of which was the unicellular model organism *Chlamydomonas reinhardtii* (Debuchy et al. 1989; Kindle et al. 1989); and transient transformation was demonstrated in the other three. All of these green algae are unicellular species except for *Volvox carteri*, for which stable transformation has been shown (Schiedlmeier et al. 1994). Many species of cyanobacteria, e.g., *Spirulina*, *Anabaena*, or *Synechocystis*, can be transformed by electroporation or conjugation (Koksharova and Wolk 2002). Algae have also demonstrated suitability

Table 2.6 Biotechnologically utilized transgenic algal species

Algal genera	Utilization	Reference
Chlamydomonas reinhardtii	Biomass from transgenics for animal health and feed, bioremediation, environmental monitoring, production of recombinant proteins	Walker et al. (2005)
Dunaliella salina	β-carotene and other carotenoids for health food, dietary supplements, cosmetics, and feed	Pulz and Gross (2004)
Haematococcus pluvialis	Astaxanthin for health food, pharmaceuticals, and feed additives	Pulz and Gross (2004)
Chlorella vulgaris	Biomass for health food, dietary supplements, and feed surrogates; extracts for cosmetics	Pulz and Gross (2004)
Phaeodactylum tricornutum	Lipids and fatty acids for nutrition	Pulz and Gross (2004)
Odontella aurita	Fatty acids for pharmaceuticals, cosmetics, and baby food	Pulz and Gross (2004)
Nannochloropsis oculata	Extracts for cosmetics	Spolaore et al. (2006)
Spirulina Figurensis	Phycocyanin, phycoerythrin, and biomass for health food, pharmaceuticals, feed, and cosmetics	Becker (2004), Pulz and Gross (2004)
Lyngbya majuscula	Immune modulators for pharmaceuticals and nutrition	Pulz and Gross (2004)

for synthesizing vaccines. In this regard, stable expression of the hepatitis B surface antigen gene has been shown in *Dunaliella salina* (Sayre et al. 2001; Geng et al. 2003; Sun et al. 2003). A further project aims at the application of antigen producing algae in the fish industry. It is intended to use an alga-produced antigen to vaccinate fish against the infectious hematopoietic necrosis virus (IHNV) which causes an infectious disease that kills 30 % of the US trout population each year; vaccination is realized simply by feeding the fish with the algae (Banicki 2004).

For further biotechnological exploitation of algae, several researchers are screening extracts from a multiplicity of algal species in order to find effective organic components like secondary metabolites (Kopecky et al. 2000; Lubián et al. 2000), antifungal or antibacterial biomolecules (Piccardi et al. 2000), algal toxins (Piccardi et al. 2000), or active pharmaceutical ingredients as drug candidates (Skulberg 2000).

2.10 Cultivation

Algae have an important role to play in the current world economy with an approximate turnover of US$5 billion per year. The biotechnological basis for the most efficient production of microalgal biomass is a key issue for the future impact of these organisms. The large-scale cultivation of microalgae and the practical use of its biomass as a source of certain constituents were probably first considered seriously in Germany during World War II. This initial research was taken up by a group of scientists at the Carnegie Institution of Washington, who summarized their experiences in the classic report *Algal culture: from laboratory to pilot plant* (Burlew 1953). In the course of time, the continuous cultivation of algae under partially or fully controlled conditions has become an important development, with various economic possibilities. Techniques for the cultivation of algae on a large scale and processes for their utilization have been successfully developed in several countries (Table 2.7), and an attempt in this direction is worthwhile in some of the developing countries (Benemann and Oswald 1996; Venkataraman and Becker 1985).

Table 2.7 Present state of microalgal production (Pulz and Gross 2004; Ratledge 2004; Hejazi 2004)

Alga	Annual production	Producer country
Arthrospira	3,000 t dry weight	China, India, USA, Myanmar, Japan
Chlorella	2,000 t dry weight	Taiwan, Germany, Japan
Dunaliella salina	1,200 t dry weight	Australia, Israel, USA, China
Aphanizomenon flos-aquae	500 t dry weight	USA
Haematococcus pluvialis	300 t dry weight	USA, India, Israel
Crypthecodinium cohnii	240 t DHA oil	USA

Most algal species are obligate phototrophs and thus require light for their growth. The requirement for light, coupled with the high extinction coefficient of chlorophyll in these organisms, has necessitated the design and development of novel systems for large-scale growth. A few algal species are capable of heterotrophic growth, and for these organisms, conventional fermentation technology can be used for large-scale cultivation (Fig. 2.8).

2.11 Phototrophic Systems

Commercial growth of photosynthetic algae has been achieved in different ways: (1) open cultivation using natural sunlight (Oswald and Golueke 1960), (2) closed cultivation using natural sunlight, and (3) closed cultivation using artificial illumination. Each system has advantages and disadvantages, and the choice of system depends on the degree of parameter control needed to produce the desired product and on the value of the product. A common limitation to all these systems is the need to supply light to the culture, making it advantageous to maximize the surface-to-volume ratio of the culture. Many configurations of open cultures using natural sunlight have been proposed and constructed (Oswald 1988; Chaumont 1993; Pushparaj et al. 1997). These systems are generally large, open ponds or raceways, and the principle advantage

Fig. 2.8 Algal cultivation in (**a**) photobioreactor, (**b**) open tank, and (**c**) open raceway pond

of these configurations is that the light energy is free (Pulz and Scheibenbogen 1998). However, this advantage is more than offset by several significant disadvantages. In open systems, it is very difficult to prevent contamination of the algal culture by other organism (i.e., algae and other microorganisms). This problem has been addressed by culturing algae that require or tolerate unique growth conditions that would exclude contaminating organisms. Open cultures attain cell densities leading to the need to process large quantities of water to harvest the algae. Outdoor phototrophic growth systems are also subject to daily and seasonal variations in light intensity and temperature, making it difficult to control or reproduce specific culture conditions. Nevertheless, for specific algal products, this technology has proven very successful, producing many thousands of tons of dried biomass per year (Lee 1997). This is especially true for *Spirulina*, which is extensively cultured in the USA, Mexico, Thailand, and China (Metting 1996; Li and Qi 1997; Vonshak 1997). Several different closed systems using natural sunlight have been described (Richmond et al. 1993; Qiang and Richmond 1994; Molina Grima et al. 1995; Spektorova et al. 1997). In these systems, the algae are enclosed in a transparent material (either glass or plastic) and the vessels placed outdoors for illumination. The closure of the vessels minimizes contamination by other algal species. Closed, outdoor systems are still subject to variations in light intensity and temperature that make cultivation reproducibility problematic. In addition, a major problem with closed systems is the removal of oxygen from the culture and the provision of adequate temperature control. Although both of these issues can be resolved, the cost of doing so can more than offset the cost advantage of using natural sunlight. As with the outdoor systems, numerous designs have been constructed for the indoor, closed culture of algae using electric lights for illumination (Ratchford and Fallowfield 1992; Wohlgeschaffen et al. 1992; Iqbal et al. 1993; Lee and Palsson 1994). These vessels are often referred to as photobioreactors, and in principle, they are similar to conventional fermentor, the major difference being that they are driven by light rather than by an organic carbon source. These vessels provide the ability to control and optimize culture parameters, and, coupled with the closure that they provide, photobioreactors are suitable for culturing many different types of algae (Ratchford and Fallowfield 1992).

2.12 Heterotrophic Systems

The most significant advance in closed culture systems is the adaptation of fermentation technology that allows for the heterotrophic growth of microalgae and eliminates the problem of light limitation (Barclay et al. 1994; Kyle 1996; Chen 1997). A significant number of microalgae are capable of heterotrophic growth and potentially suitable for growth in fermentors (Droop 1974; Gladue and Maxey 1994). The basic principle of fermentor growth is to provide highly controlled optimal growth conditions to maximize productivity. In these, the biomass levels are at least tenfold higher than those achieved by photosynthesis-based culture systems (Radmer and Parker 1994). The high biomass levels also greatly decrease the volume of water that must be pro-

cessed during harvesting. The effectiveness of large-scale cultures and the production of high biomass levels can make the cost of fermentative growth an order of magnitude less expensive than photobioreactors (Radmer and Parker 1994). Larger-scale production of the dinoflagellate *Crypthecodinium* by fermentative growth for the production of the polyunsaturated fatty acid DHA has been under way for several years (Kyle 1996). Production of *Crypthecodinium* begins with a certified seed stock that was cryopreserved under liquid nitrogen conditions to maintain genetic stability. *Crypthecodinium* has been reported to produce approximately 30 % of their dry weight as the total fatty acid (Kyle et al. 1992), with DHA making up close to 50 % of the total fatty acid (Behrens and Kyle 1996). *Chlorella* is also extensively grown in large quantities by fermentation techniques. Production levels in Japan are estimated to exceed 500 T per year, accounting for 50 % of the country's total production (Lee 1997). Plans have also been announced in Korea to begin the production of heterotrophically grown *Chlorella* at a level exceeding 1,000 T per year (Lee 1997). Milking of microalgae is the most advanced technique for biotechnologically important compounds (Hejazi and Wijffels 2004).

These systems have to be evaluated in their various configuration concepts regarding their potential productivity and economic feasibility. The most important and most obvious differences in microalgal production systems are the exposure of the microalgal culture to the environment.

2.13 Conclusions

More basic research needs to be performed before algal biotechnology would reach a capacity to compete with other systems. But since many physiological, morphological, biochemical, or molecular characteristics of algae are quite different from higher plants or animals, algae can meet several requirements that other systems cannot sufficiently accomplish. This is one reason why algal systems gain more and more influence in the production of substances of economic, industrial, and pharmaceutical importance. However, microalgae are still not a well-studied group from a biotechnological point of view. The genetic improvement of algal strains is also a present challenge. The use of transgenic microalgae for commercial applications has not yet been reported but holds a significant promise. Among the algal species that are believed to exist, only a few thousand strains are kept in collections, a few hundred are investigated for chemical content, and just a handful are cultivated in industrial quantities (i.e., in tons per year). The combination of the exceptional nutritional value of microalgae with coloring and therapeutical properties, associated with an increase demand of natural products, makes microalgae worth exploring for utilization in the future in feed, food, cosmetic, and pharmaceutical industries, with recognized advantages comparing with the traditional ingredients.

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Application of Biotechnology and Bioinformatics Tools in Plant–Fungus Interactions

3

Mugdha Srivastava, Neha Malviya, and Thomas Dandekar

Abstract

Fungi have been recognized to be a major cause of disease in immunocompromised hosts: moreover, the loss of food and fodder crops through fungi has been unmatched since the last decade. Fungi colonize the plant cell and organs by modulating the host defense response. A number of different methods have been recently used to understand host–fungus interactions. With the advent of HiSeq approaches, more fungal genomes and transcriptomes are now sequenced, and their bioinformatics analyses have enriched and assisted our knowledge of the interplay between plant and fungi. The present chapter reviews the current biotechnological and bioinformatics approaches for the study of plant–fungus interactions.

Keywords

Plant–fungi interactions • Host–pathogen interaction • Systems biology • Fungal infection

3.1 Introduction

Fungi have coevolved with other eukaryotes including plants and animals. They can be either saprophytic or parasitic. The plant-parasitic fungi are strictly host specific, and the infection in plants is mediated by the enzymes used in the breakdown of cell walls and toxins that inhibit or reduce the activity of host cells. Fungal infection in plants is also caused by disturbance in the hormonal equilibrium of the plant cell, causing disruptions to the growth and differentiation of the cells and tissues (Ludwig-Müller 2000). The mode of infection in fungi can be necrotrophic, biotrophic, and hemibiotrophic. Necrotrophic infection leads to dysfunction of the tissue and eventually death of the plant. Biotrophs take nutrients and growth-regulating substances from the plants, but do not kill it, while hemibiotrophs initiate infection with a period of biotrophy, followed by a necrotrophic phase, and they possess

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properties of both groups (Meinhardt et al. 2014). Significant yield losses due to fungal attacks occur in most of the agricultural and horticultural species. After insects, fungal diseases are rated as the second most important factor contributing to yield losses in our major cereal, pulse, and oilseed crops (Grover and Gowthaman 2003). Penetration of fungi into plant cells occurs through diverse invasion strategies. Infection structures such as "Spitzenkörper", an organizing center for hyphal growth, are produced, which enable the fungus to penetrate different types of plant cell walls (Mendgen et al. 1996). These fungi penetrate and degrade the cell wall and produce toxins for their effective infection (Mendes-Giannini et al. 2005). Often N-terminal type II signal peptides containing fungal AVR proteins facilitate the fungal virulence by suppressing pathogen-associated molecular pattern-triggered immunity and induce effector-triggered immunity in plants containing cognate resistance proteins (Luderer et al. 2002).

The different modes of infection include:

3.1.1 Infection Structures

Spores or hyphae of fungi penetrate through wounds of the epidermis or open stomata. For example, fungi imperfecti secrete a terpenoid (fusicoccin) that increases the influx of potassium into the cells of the stomata and induce thereby a permanent opening of the stomata and cause a high loss of water which may lead to death. Thus fusicoccin is also known as a wilting toxin (Arntzen et al. 1973).

3.1.2 Cell Wall Degradation

There is a range of polysaccharide-degrading enzymes and glycosidases such as *alpha-galactosidase*, *beta*-galactosidase, *beta-xylosidase*, and *alpha*-arabinosidase produced by phytopathogenic fungi. These enzymes are able to degrade walls of plant tissues (Albersheim and Valent 1974).

3.1.3 Toxin Production

2-Hydroxycyclopropyl-*alpha*-D-galactopyranoside (helminthosporoside) has been identified in fungus-infected leaves of sugarcane with the help of nuclear resonance analysis and mass spectroscopy (Strobel and Hess 1974). Further detailed analysis revealed that it was a mixture of three isomer sesquiterpene–alkaloids that contain digalactosyl residues at both ends of the molecule. Helminthosporoside resembles *alpha*-galactosidases which are present in plant tissues and are taken up by the plant cells actively. These toxins attach at the plasma membrane, to the same binding site as these sugars, but in contrast to the sugars are not transported into the cell. Instead, binding to the toxin increases the activity of the potassium/magnesium pump with the uptake of potassium. This causes an increase in the uptake of water, a bigger osmotic pressure, damage of the membrane, and finally the disintegration of the cell (Strobel and Hess 1974). Further, the study of signal transduction pathways in virulent fungi is particularly important in view of their putative role in the regulation of pathogenicity (Mendes-Giannini et al. 2005).

3.2 Biotechnological Approaches for Plant–Fungus Interactions

The study of fungal–host interplay is essential for developing alternative protection and resistance strategies. With the emergence of plenty of functional data from genomics, transcriptomics, and metabolomics, the study of plant–fungus interactions has been promoted. Functional genetic studies are divided into forward or reverse genetics screens. In the forward genetic study, genes are mutated at random. After identification of a mutant phenotype, the mutations must be mapped, a process which is usually time-consuming and nontrivial. Conversely, reverse genetic approaches involve the disruption of a gene of interest, so as to determine its function and/or involvement in a pathway (Silva et al. 2004).

3.2.1 Reverse Genetics Approaches

Reverse genetics approaches such as targeted gene disruption/replacement (knockout), gene silencing (knockdown), insertional mutagenesis (transposon and T-DNA mutagenesis), or targeting induced local lesions in genomes (TILLING) are now used to analyze the functional information from the large number of genome projects (González-Fernández et al. 2010). With the advent of "reverse genetics," the functions of genes are discovered which contribute in the molecular mechanisms of fungal development, nutrition, and interaction with host plant (Bhadauria et al. 2009). Targeted gene replacement leads to the study of the phenotypes of mutants in which a genomic locus has been altered by insertion (gene disruption) or replacement (gene replacement) with heterologous DNA (Reski 1998). Homologous recombination between a target gene and the introduced DNA carrying its mutant allele results in targeted gene knockout. This approach was first pioneered for the budding yeast *Saccharomyces cerevisiae* to decipher gene function (Winzeler et al. 1999). Gene knockout can be performed by the simple disruption of the gene of interest by a resistance cassette, by alteration of the expression of the candidate gene rather than its deletion, by overexpression or mis-expression of a gene, and by gene replacement, which combines the second and third approaches (Bhadauria et al. 2009). A crucial feature that makes this technology feasible is the ability to introduce linear DNA into a large number of cells simultaneously (Melissa and Jeff 2002). However, targeted gene disruption is again time-consuming. A faster, simpler, and cheaper alternative of attenuating gene function in a sequence-specific manner has emerged in the form of antisense RNA technology, ribozymes, and, more recently, RNA interference (RNAi) (Silva et al. 2004). In this technique double-stranded RNA (dsRNA) triggers the degradation of a homologous mRNA, thereby diminishing or abolishing gene expression. RNA-mediated gene silencing methods that block the expression of genes at the posttranscriptional level have been identified in a few economically important phytopathogens such as *Magnaporthe oryzae* (Kadotani et al. 2003) and *Phytophthora infestans* (West et al. 1999; Latijnhouwers et al. 2004; Whisson et al. 2005). Generally, RNAi causes only a partial reduction in gene expression and not a complete loss. This is a major disadvantage of this method (Bhadauria et al. 2009). Insertional mutagenesis is performed by chromosomal integration of transforming DNA. The presence of a selectable marker in the transforming DNA is used to establish linkage between the insertion and the observed phenotype and to recover DNA representing the mutated allele for cloning and subsequent analyses (Brown and Holden 1998). Targeting induced local lesions in genomes (TILLING) or chemical mutagenesis is a new reverse genetic strategy for plants that combines the efficiency of ethyl methanesulfonate (EMS)-induced mutagenesis with the ability of denaturing high-performance liquid chromatography (DHPLC) to detect base pair changes (G/C to A/T transition) by heteroduplex analysis (McCallum et al. 2000; Chen et al. 2014). It has been evolved as an influential tool for functional genomics of phytopathogens. Lamour and colleagues (2006) employed TILLING to isolate gene-specific mutants in *Phytophthora* spp. Single-cell uninucleated spores like zoospores of *Phytophthora* spp. are best for TILLING mutagenesis (Bhadauria et al. 2009). This technique has also helped to improve waxy quality trait in the polyploid wheat plant (Slade et al. 2005).

All the reverse genetics tools described above have their own merits and drawbacks, and any one of them may be more effective for a particular phytopathogen and case study while less suitable for others.

3.3 Gene Expression Analysis

3.3.1 Real-Time Polymerase Chain Reaction (qPCR) and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The advantage of real-time PCR is that the process of amplification is monitored in real time by using the fluorescence technique (Wilhelm and

Pingoud 2003). This technique has been used for rapid quantitative assessment of the expression of genes involved in powdery mildew disease development using the powdery mildew pathogen *Golovinomyces orontii* on a set of hypersusceptible and resistant host plant *Arabidopsis thaliana*. The technique was found to be rapid, reproducible, and useful for quantitative estimation of gene expression in other fungi as well (Webling and Panstruga 2012). The semiquantitative RT-PCR is currently the common technique for gene expression analysis, although an accurate normalization is essential to control for experimental error (Huggett et al. 2005). In this relative analysis method, expression of the gene of interest is compared with expression of a reference gene (an internal control). The technique has been used for different fungal plant studies such as coffee rust fungus *Hemileia vastatrix* (Vieira et al. 2011) and *Verticillium dahliae* in potato (Pasche and Mallik 2013).

3.3.2 Direct Fluorescent In Situ RT-PCR

Fungal interaction with host tissue and the understanding of the subsequent events have been studied by using direct fluorescent in situ RT-PCR. This technology combines PCR and in situ hybridization which is employed for mRNA localization in cell organelles or tissue sections. Digoxigenin-labeled nucleotides (digoxigenin-11-dUTP) are incorporated in the PCR product after reverse transcription and subsequently detected with an anti-digoxigenin antibody conjugated with alkaline phosphatase. Further modifications can be done using fluorescent probes and fluorescence immunocytochemical labeling (Lossi et al. 2011). This technology has been used to study the symbiotic relationship of arbuscular mycorrhizal fungi (Seddas-Dozolme et al. 2010), as well as for the identification of an infection gene in hemibiotrophic plant pathogen *Colletotrichum lindemuthianum* (Tollot et al. 2009). Further applications include identification of *Blumeria graminis* spores and mycelia on barley leaves (Bindsley et al. 2002) and others. The disadvantage of this method is that it is a time-consuming technique due to the use of hybridization step and technically demanding procedures such as light microscopy (Capote et al. 2012).

3.3.3 TRAC Analysis

There are a number of techniques for the analysis of gene expression; TRAC (transcript analysis with aid of affinity capture) analysis is a new and advanced technique for expression analysis of genes in strains producing foreign proteins. It is an efficient technique for the analysis of large numbers of samples (Nowrousian 2014). In this technique, the cells are directly lysed, and gene expression is measured without using RNA purification or RT-PCR (Rautio et al. 2008). A set of fluorophore-labeled probes are used for the recognition of the gene of interest. One of the most often used probes is biotin oligo-dT, which is used to capture targets from their polyA tails. By the use of TRAC method, 100–200 genes of the filamentous fungus *Trichoderma reesei* and the yeast *Saccharomyces cerevisiae* have been identified with supposed importance in different biological pathways (Rautio et al. 2007). These evaluated genes show potential in many physiological effects such as growth, protein production rate, and availability of nutrients and oxygen (Rintala et al. 2008).

3.3.4 Suppression Subtractive Hybridization

Suppression subtractive hybridization (SSH) technique is used for distinguishing two closely related DNA samples. They can be used both for cDNA subtraction and genomic DNA subtraction (Rebrikov et al. 2004). In this technique only one round of subtractive hybridization is needed where the subtraction step excludes the common sequences between target and driver population. This increases the probability of obtaining low-abundance differentially expressed cDNA and simplified analysis of subtracted library (Diatchenko et al. 1999). This has been used to

identify the upregulated genes in the establishment of mycorrhiza between *Medicago truncatula* and *Glomus mosseae* (Weidmann et al. 2004) in comparison with an infected plant and the pathogen in axenic culture. Other examples analyzed are genes associated with phytoplasma in Chinese jujube (Liu et al. 2014) and plants infected with a wild-type isolate compared to plants infected with mutants (Horwitz and Lev 2010). SSH can also identify fungal genes against a background of the uninfected host, for example, *Alternaria brassicicola* on *Arabidopsis* (Cramer and Lawrence 2004), genes of the white rot fungus *Heterobasidion parviporum* expressed during colonization of Norway spruce stems (Yakovlev et al. 2008), and in symbiosis for arbuscular mycorrhizae (Brechenmacher et al. 2004). It is even possible to isolate differentially expressed mycoparasite genes in antagonistic fungal–fungal interactions (Carpenter et al. 2005; Morissette et al. 2008).

3.3.5 Laser Microdissection

Infection structures are formed in fungi to extract nutrition from plants. Laser microdissection (LM) of plant tissues infected with a fluorescent protein-tagged fungus is a useful method for obtaining samples highly enriched in fungal RNA for downstream analysis such as hybridization to a microarray, RT-PCR, and others (Tang et al. 2012). The technique can be used to isolate both fungal and host plant cells after pathogen infection. The samples are carefully fixed to maintain the integrity of the cell and later on dissected by laser. RNA isolation is further used for gene expression analysis (Fosu-Nyarko et al. 2010). By using this technique, host–fungus interaction studies have been done in many cases, such as in *Arabidopsis*, where the epidermal cells of leaves infected with powdery mildew (*Erysiphe cichoracearum*) were isolated for gene expression studies (Inada and Wildermuth 2005), and fungal haustoria could be separated from infected host plant cells (van Driel et al. 2007). This technique is utilized for the study of biochemistry and structure of hyphal cells and fungal interaction with host tissues (Fosu-Nyarko et al. 2010).

3.3.6 Biochemical Methods

Biochemical methods are powerful tools for investigating mechanisms of gene expression. These techniques consist of Western blot analysis, protein purification using epitope-tagged fusion protein, protein immunoprecipitation (IP), and chromatin immunoprecipitation (ChIP) assays. Immunoblot analysis, also called Western blot, is a commonly used method to detect specific proteins in tissue homogenates or protein extracts. Protein purification is also an approach routinely used to study the protein activity and to identify protein complexes. Immunoprecipitation (IP) is a method to specifically concentrate the protein of interest by using a specific antibody, which is widely used to study protein–protein interaction (PPI). ChIP assay is used to investigate the association of a protein of interest with specific DNA regions (Collas 2010). These methods can be modified under different growth conditions and in different filamentous fungi.

3.3.7 Proteome Profiling and Mass Spectrometry Tools

Study of proteome changes in response to fungal invasion is crucial to understanding the molecular mechanisms underlying plant–pathogen interaction and pathogenesis. Several approaches are involved for identifying large numbers of proteins expressed in cells and also for globally detecting the differences in levels of proteins in different cell states. These techniques involve:

3.3.7.1 Electrophoresis

One-dimensional (1D) SDS polyacrylamide gel electrophoresis (PAGE) is a widely used tool for the separation of total protein extracts as well as protein fractions; two-dimensional electrophoresis (2DE) and two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) are

protein separation techniques widely used in fungal proteomics.

3.3.7.2 Mass Spectrometry (MS) Tools

The major invention in MS tool is the time-of-flight (TOF) MS which is a relatively nondestructive method used to convert proteins into volatile ions. A typical MS consists of an ion source, a mass analyzer, and a detector. Matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) have made it possible to volatilize and ionize large biomolecules, such as peptides and proteins (Yates 1998; Godovac-Zimmermann and Brown 2001; Mann et al. 2001). The mass analyzers currently used in proteomics research are ion trap, TOF quadrupole, and Fourier transform ion cyclotron (FT-MS). MALDI is usually coupled to TOF analyzers that measure the mass of intact peptides (Chalupová et al. 2014). A new matrix-assisted laser desorption/ionization TOF/TOF MS with the novel laser-induced fragmentation technique (MALDI LIFT-TOF/TOF MS) provides high sensitivity for peptide mass fingerprints (PMF).

3.3.7.3 Multidimensional Protein Identification Technology (MudPIT)

In this method, multidimensional liquid chromatography is coupled with tandem mass spectrometry (MS) and database searching by the SEQUEST algorithm (Washburn 2004). MudPIT was first applied to the fungal proteome of the *Saccharomyces cerevisiae* strain BJ5460 grown to mid-log phase and yielded the largest proteome analysis to date. A total of 1,484 proteins were detected and identified (Washburn et al. 2001).

3.3.8 Next-Generation Sequencing

Next-generation sequencing (NGS) technologies are continuing to rise with the fall in its costs. This enables researchers to conduct whole-transcriptome sequencing (RNAseq) studies of interactions between plants and pathogenic fungi (Wang et al. 2009). The application of NGS in plant–fungus interaction research shortens the overall time of development of molecular genetic information necessary for functional and translational studies. Zhuang et al. (2012) reported novel transcriptome sequence information from the pea–*S. sclerotiorum* interaction obtained by 454 pyrosequencing. Illumina's next-generation sequencing technology sequencing by synthesis (SBS) is a powerful tool to rapidly sequence genomes and transcriptomes and is not all that expensive (Venu et al. 2011).

3.3.9 Genetic Engineering: An Approach for Plant Disease Resistance

Genetic engineering is an artificial technique used for transferring genes from one organism to other. This technique is used to manipulate the genetic makeup of a cell to produce new characteristics in an organism (Zhu et al. 2013). Plants produce different secondary metabolites, for the prevention of growth of fungi. Transgenic plants are developed either by increasing or decreasing the expression of genes that are naturally present in an organism or by transferring genes between individuals of the same or different species. Pathogenesis-related (PR) proteins expressed by the host plant are induced by pathogen attachment or other stresses (Antoniw and Pierpoint 1978; Van Loon et al. 1994); these are not only accumulated locally in the infected leaf but are also induced systemically, associated with the development of systemic acquired resistance (SAR) against further infection by fungi, bacteria, and viruses. PR protein genes appear to be a good potential source as candidate genes for engineering fungal resistance in plants. Induction of PR proteins has been found in many plant species belonging to various families. The genes which encode for chitinases and glucanases (PR proteins) are the most extensively used for developing fungal resistance in plants by their overexpression. Another important hydrolytic enzyme is beta-1,3-glucanase which inhibits fungal growth in plants by catalyzing the degradation of beta-1,3-glucan abundantly present in plant cell

wall of many filamentous fungi (Wessels and Sietsma 1981). The detailed information on the engineering fungal resistance in plants by expression of PR protein and other antifungal genes has not been given here as it is covered in another chapter in this book.

3.3.10 Signal Transduction Pathway Activation

Systemic acquired resistance (SAR) is an inducible system that becomes activated by pathogen exposure and leads to a broad range of resistance against diverse pathogens. After being exposed to a pathogen, the plant cell activator molecules interact with signal molecules such as H_2O_2, salicylic acid (SA), jasmonic acid (JA), and ethylene and trigger defense responses. H_2O_2 has been shown to directly inhibit pathogen growth and to induce PR proteins, SA, and ethylene, as well as phytoalexins (Mehdy 1994). Overexpression of H_2O_2 in transgenics of cotton, tobacco, and potato reduced disease development caused by a number of different fungi, but higher level of overexpression was phytotoxic (Murray et al. 1999). Salicylic acid (SA) is also one of the signal molecules involved in defense response. Overexpression of SA in transgenic tobacco plants enhances PR protein production and provides resistance to fungal pathogens (Verberne et al. 2000). R genes (resistance genes) have evolved in plants whose products allow recognition of specific pathogen effectors, either through direct binding or by recognition of the effector's alteration of a host protein (Jones and Dangl 2006). R gene products control a broad set of disease resistance responses whose induction is often sufficient to stop further pathogen growth and spread.

Fungal infection in plants has been associated with reduced photosynthetic rate, nutrient mobilization at the site of infection, and formation of patches called green islands (Walters and McRoberts 2006). Cytokinins have the ability to mobilize nutrients toward sites of application and delay senescence which has led to the speculation of involvement of cytokinin in green island formation. Spores of some fungi such as rust and powdery mildew are known to contain high levels of cytokinins (Király et al. 1967), and it has been suggested that the nutrient mobilization and green island formation are due to cytokinin produced by the fungus. It has also been proposed that cytokinin-induced increases in invertase activity occurring early on in the pathogen–host interaction lead to carbohydrate accumulation and the downregulation of photosynthetic metabolism. These events indicate that cytokinins play a crucial role in allowing the biotrophic pathogen to establish and grow in the host (Walters and McRoberts 2006). However, cytokinins have been shown to increase immunity in plants against bacteria (Naseem et al. 2014). Systems biology studies have been performed to understand the interplay of auxin and cytokinin (Naseem et al. 2012; Naseem and Dandekar 2012) in bacteria and host plants as well as the effect of cytokinin on jasmonate–salicylate antagonism in *Arabidopsis* immunity (Naseem et al. 2013).

3.3.11 RNA Silencing

RNA silencing is used as a reverse tool for gene targeting in fungi. Homology-based gene silencing induced by transgenes (co-suppression), antisense, or dsRNA has been demonstrated in many plant-pathogenic fungi. This includes *Cladosporium fulvum* (Hamada and Spanu 1998), *Magnaporthe oryzae* (Kadotani et al. 2003), *Venturia inaequalis* (Fitzgerald et al. 2004), *Neurospora crassa* (Goldoni et al. 2004), *Aspergillus nidulans* (Hammond and Keller 2005), and *Fusarium graminearum* (Nakayashiki 2005); whether it is suitable for large-scale mutagenesis in fungal pathogens remains to be tested. Nakayashiki (2005) were successful in silencing 70–90 % of transformants *mpg1* and polyketide synthase gene. The *mpg1* gene is a hydrophobin gene that is essential for pathogenicity, as it acts as a cellular relay for adhesion and a trigger for the development of appressorium (Talbot et al. 1996).

3.4 Bioinformatics and Systems Biology Approaches

Biological problems often deal with different numbers of factors which are interrelated and lead to the whole behavior or function. This organized complexity has recently introduced systems biology as a new age science. Systems biology studies in plant science have increased our knowledge about circadian rhythms, multigenic traits, stress responses, and plant defenses. Plants continually face biotic and abiotic stress and respond with sophisticated biochemical and structural defenses. Various external stimuli are differentially perceived by plants and lead to distinct cascades of molecular responses and signaling networks at the cellular level. A detailed biochemical understanding of pathogen action and plant responses would enable development of disease control strategies, a challenging goal of plant pathology. "Omics" research approaches have produced copious data such as genomics, proteomics, transcriptomics, interactomics, and metabolomics which can be exploited in order to better understand biological interactions.

3.4.1 Brief Introduction to Network Reconstruction and Modeling

The interactions of genes, proteins, metabolites, or other components are illustrated by networks. Network reconstruction and analysis is one of the most common approaches for describing biological systems. Both component integration and interactions are key features of systems biology. Networks can be either static or dynamic, and of different types, including gene-to-metabolite networks, protein–protein interaction networks, transcriptional regulatory networks, and gene regulatory networks (Yuan et al. 2008). In metabolic networks, each reaction is a network edge connecting nodes that represent reaction substrates and products. The nodes connected by an edge are also associated with values indicating the quantity or concentration of a reaction participant (Pritchard and Birch 2011). The values at the nodes vary over time, representing the ebb and flow of material that is created or destroyed. The network edges are said to carry a flux, the rate at which material "flows" from one node to another, and flux is also represented by a value. There are two approaches for network reconstruction: top-down approach and bottom-up approach (Shahzad and Loor 2012). Top-down approach provides a broad overview of the system where the regions can be gradually filled with detailed information of factors affecting the dynamics of the network. A bottom-up approach is a detailed model where the output of the module affects processes described by other modules at a higher level of organization. A bridge or bottleneck is a pathway of communication, the breaking of which can split the network into noncommunicating parts. A bottleneck is a potential point of fragility in a network. However, biological networks are error tolerant and hence follow a "power law" with the loss of a single edge rarely leading to a global disconnection in the network. Hubs are the nodes which are directly connected to many other nodes. In protein–protein interaction networks, the number of network hubs varies by organism but is always small. In biological cells we expect a few highly connected "hubs" to play critical roles in signal transduction, regulating transcription, and mediating metabolic flux across the network. Such central protein nodes with many interactions to other proteins (so-called hub nodes with a large degree) are thus candidates for effector targets.

3.4.2 Systems Biology of Plants

Advances in plant systems biology studies are expected to support our current requirements of crop adaptation for food, feed, biofuels, and industrial and pharmaceutical production. Plants have sessile lifestyle and phototrophic nutrition, and in order to accommodate this behavior, they have a unique genetic and metabolic repertoire (Morsy et al. 2008). However network modeling and experimental validation in plants can be executed in a fast and reiterative way owing to their relatively short generation times and their

capability to produce enough offspring for genetic analyses.

3.4.3 Different Plant–Fungus Studies

Human pathogenic fungi such as *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, and *Encephalitozoon cuniculi* have been studied for some time now; plant–fungus interactions have not been studied much. However the complete sequencing of the *Magnaporthe oryzae*, a causal agent of blast disease in rice, has shown that multi-locus genes are concordant with host preference leading to the segregation of a new species of *Magnaporthe oryzae* from *Magnaporthe grisea* (Gudimella et al. 2010). This example indicates horizontal transfer of gene clusters in human pathogenic fungi in accordance with host preference. The interolog mapping approach has been used to transfer interaction information from animal model organisms such as *Drosophila*, *Caenorhabditis elegans*, and human to the model plant *Arabidopsis*. In *Arabidopsis*, approximately 10,000 orthologs have been detected in at least one reference species (i.e., in yeast, *Drosophila*, or humans) (Morsy et al. 2008). Structure modeling and docking approaches have also been used to develop a peptide which can competitively inhibit the crucial glyoxylate pathway in fungi (Srivastava et al. 2010).

3.4.4 Computational Techniques that Can Aid Studies on Host–Fungus Interactions

Different computational techniques which can help in the study of host–fungus interaction are enlisted below with a brief description:

3.4.4.1 Analogies from Mammal–Pathogen Interactions

Of the 1.5–5.1 million fungal species, an estimated 270,000 species are associated with plants, and 325 are known to infect humans (Blackwell 2011; Hawksworth and Rossman 1997; Robert and Casadevall 2009; Woolhouse and Gaunt 2007; Gauthier and Keller 2013). A small subset of plant pathogens such as *E. rostratum* can cross kingdoms and infect humans. These crossover pathogens include fungi from Ascomycota and Mucoromycotina phyla. The human host infected by crossover fungi is generally immunocompromised. Gauthier and Keller (2013) enlist the fungi which can cause disease in both plants and human. Fungi have different methods of infecting plants and animals; however, they produce the same mycotoxins and secondary metabolites, assimilate similar nutrients such as iron, and have similar MAPK, cAMP-PKA, and G-protein signaling pathways. Targeted mutagenesis allows characterization of fungal pathogenesis according to the loss of function. The mutagenesis relies on either random or targeted insertion of a selectable marker. The targeted genes are identified by comparative genome analysis between pathogen and non-pathogen and/or by large-scale transcriptome analyses. PHI-base (Pathogen–Host Interaction Database) catalogs the phenotypes resulting from mutations in defined genes of both plant and animal pathogens (Sexton and Howlett 2006). The genomic resources which can be exploited for orthologous studies of plant hosts are rice and *Arabidopsis thaliana* as well as non-mammalian hosts such as *Drosophila melanogaster* and *Caenorhabditis elegans*.

3.4.4.2 Predicted Interactomes

Two types of interactome have widely been recognized: genetic and physical. A genetic interactome is a network of genes characterized on the basis of genetic interactions serving to elucidate gene function within physiological processes (Yuan et al. 2008). Genetic interactions can be predicted using a systematic genetic analysis (SGA)-based method. In this approach a query mutation is crossed to an entire mutation array set to identify any genetic interaction, yielding functional information of the query gene and the gene it interacts with. The SGA-based genetic interactome has also been applied successfully in the nematode *Caenorhabditis elegans*, the fungi *Cryptococcus neoformans*, the virus *Neisseria*

meningitidis, and many other physiological processes and protein families. SGA in plants is however complicated because of polyploidy. As compared with genetic interactions, physical interactions among proteins are relatively easier to characterize in plants. Different protein–protein interaction methodologies have been enlisted by Morsy et al. (2008). The computational technique which has been used to define the protein interactions is "interologs." Interologs are the predicted interactive protein pairs, based on orthologous proteins that are known to interact in the reference species such as yeast and human. The interactome of *Arabidopsis* was predicted by Geisler-Lee et al. (2007) using confidence scoring based on the number of observations and experimental approaches that detected the interaction, together with subcellular location and co-expression data, to indicate the reliability of the interaction. Using the interolog method, 11,674 PPIs among 3,017 *M. grisea* proteins were also inferred from the experimental PPI data in different organisms (He et al. 2008). The potential of interactomes in understanding the systemic regulation of biological processes is unquestionable. However, the techniques to build the interactome should be used carefully.

3.4.4.3 Metabolic Modeling

Fungal infection of plants leads to changes in the secondary metabolism to boost defense programs. It also affects primary metabolism affecting growth in plants. Charting the metabolic changes can lead to better understanding of fungal infections in plants. A set of linear pathways often cannot capture the full complexity and behavior of a metabolic network (Schuster et al. 2000). However once the genome of the intended microbe has been annotated, the entire metabolic map representing the stoichiometry of all the metabolic reactions taking place in the cell can be constructed. Metabolic networks have been restructured for different microbes such as *Mycoplasma pneumoniae* (Schuster et al. 2002), *Listeria monocytogenes* (Schauer et al. 2010), *Staphylococcus aureus* (Cecil et al. 2011; Liang et al. 2011), *Staphylococcus epidermidis* (Cecil et al. 2011), and *E. coli* (Jain and Srivastava 2009); however, the metabolic models for fungi are still in their infancy. With the increasing number of sequencing projects and increasing amounts of metabolic data, the metabolic model for plant–fungus interactions will soon increase.

3.4.4.4 EST Mining and Functional Expression Assays

Microbial effectors are small secreted proteins that are capable of manipulating the microbial biochemistry by triggering a defense response. They can act out of the host cell or can enter the living host cell by specialized pathogen-derived structures such as type III secretion systems in bacteria; haustoria in fungi, oomycetes, and parasitic plants; and the nematode stylet. PexFinder, an algorithm for automated identification of extracellular proteins from EST data sets, was developed and applied to 2147 ESTs from the oomycete plant pathogen *Phytophthora infestans*. The program identified 261 ESTs (12.2 %) corresponding to a set of 142 nonredundant Pex (*Phytophthora* extracellular protein) cDNAs, of which 78 (55 %) Pex cDNAs had no significant matches in public databases (Torto et al. 2003). This appears to be a promising approach to identify the microbial effectors in other fungal pathogens.

3.4.5 Fungal Pathogen Genomics

The advances in genomics and sequencing techniques have allowed the analysis of gene expression changes at the whole-genome level. Different sequencing projects are ongoing. A fungal genome initiative (FGI) was initiated in 2000 to promote the sequencing of medically and industrially important fungi (http://www.broad.mit.edu/annotation/fgi). The Genome Online Database (GOLD) contains a number of draft and complete sequence project for plant fungi (Pagani et al. 2012). With the abundance of sequencing and genomics data, data analysis will become the rate-limiting step. Most gene prediction programs have been based on humans and do not automatically work for fungi. For the correct structural prediction of pathogenic genes, it will

be more realistic to train the gene-finding software EST infection libraries (Soderlund 2009). In closely related plant-pathogenic fungi, comparative analysis can be used to improve de novo gene prediction and identify genes involved in host range determination, infection-related morphogenesis, and virulence (Xu et al. 2006). For pathogen–host studies, genes that are not found in abundance in the non-pathogens but are found in pathogens are natural candidates for pathogenicity. Functional analysis of genes is possible by testing the effect of mutated genes on pathogenicity. The Plant-Associated Microbe Gene Ontology (PAMGO) Consortium has collaborated with the Gene Ontology (GO) Consortium to define GO terms to describe the biological processes common to plant and animal microbes (Xu et al. 2006).

3.4.5.1 Tools, Techniques, and Resources for Plant–Pathogen Studies

While the number of symbiotic and pathogenic fungi with genome-based research is limited, the resources available and subsequently genes sequenced are increasing. The ESTS, genome sequence data, microarray data, in silico mutation models, and protein interaction databases are available to use for the organism of interest. Although all these resources have their limitations and should be used in combination with in vitro and in vivo experiments, their availability is capable of saving all kinds of expenses. The work of the Human Proteome Organization Proteomics Standards Initiative (HUPO–PSI; http://www.psidev.info/) has greatly improved the ability to combine or compare interaction data from diverse sources. In addition, HUPO–PSI has published guidelines outlining the minimum information required for reporting a molecular interaction experiment (MIMIx) (Mitchell et al. 2003). Some useful weblinks are listed in Table 3.1.

3.5 Future Challenges in Plant–Fungus Studies

Network modeling approaches are often based on assumptions and generalizations and hence are not capable of completely representing the actual biological scenario; however, the predictions

Table 3.1 Repositories useful for plant–fungus interaction studies

Name	Function	Weblink	Reference
Phytopathogenic Fungi and Oomycete EST Database	EST database with EST data from eighteen plant-pathogenic fungi, two species of oomycetes, and three species of saprophytic fungi	http://cogeme.ex.ac.uk/	Soanes and Talbot (2006), Soanes et al. (2002)
IntAct	Interaction data from literature curation and user submission	http://www.ebi.ac.uk/intact/	Orchard et al. (2014)
Molecular Interaction Database (MINT)	Experimentally verified protein–protein interaction mined from scientific literature	http://mint.bio.uniroma2.it/mint/Welcome.do	Licata et al. (2012)
Arabidopsis thaliana Protein Interactome Database (AtPID)	Information pertaining to protein–protein interaction networks, domain architecture, ortholog information, and GO annotation in the *Arabidopsis thaliana* proteome	http://www.megabionet.org/atpid/webfile/	Li et al. (2011)
Database of Interacting Protein (DIP)	Experimentally determined interactions between proteins	http://dip.doe-mbi.ucla.edu/dip/Main.cgi	Salwinski et al. (2004)
Biomolecular Interaction Network Database (BIND)	Biomolecular interaction and complex and pathway information	http://baderlab.org/BINDTranslation	Bader et al. (2003)

(continued)

Table 3.1 (continued)

Name	Function	Weblink	Reference
Biological General Repository for Interaction Datasets (BioGRID)	Interaction data through comprehensive curation	http://thebiogrid.org/	Stark et al. (2006)
Pathogen–Host Interaction Database (PHI-base)	Catalogs experimentally verified pathogenicity, virulence, and effector genes from fungal, oomycete, and bacterial pathogens, which infect animal, plant, fungal, and insect hosts	http://www.phi-base.org/	Winnenburg et al. (2008)
Comprehensive Phytopathogen Genomics Resource (CPGR)	Comprehensive plant pathogen genomics and annotation resource	http://cpgr.plantbiology.msu.edu/	Hamilton et al. (2011)
Genomes Online Database (GOLD)	Information regarding genome and metagenome sequencing projects	http://genomesonline.org/cgi-bin/GOLD/index.cgi	Pagani et al. (2012)
Plant Expression Database (PlexDB)	Unified gene expression resource for plants and plant pathogens	http://www.plexdb.org/	Dash et al. (2012)
Fungal Secretome KnowledgeBase (FunSecKB)	Resource of secreted fungal proteins, i.e., secretomes, identified from all available fungal protein data in the NCBI RefSeq database	http://proteomics.ysu.edu/secretomes/fungi.php	Lum and Min (2011)

ought to improve as more interactome data become available about plants and thus will serve as powerful tools for generating hypotheses for future experimental investigations.

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Genetic Markers, Trait Mapping and Marker-Assisted Selection in Plant Breeding

4

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Abstract

Genetic markers have long been used for characterization of plant genetic diversity and exploitation in crop improvement. The advent of DNA marker technology (during 1980s) has revolutionized crop breeding research as it has enabled the breeding of elite cultivars with targeted selection of desirable gene or gene combinations in breeding programmes. DNA markers are considered better over traditional morphology and protein-based markers because they are abundant, neutral, reliable, convenient to automate and cost-effective. Over the years, DNA marker technology has matured from restriction based to PCR based to sequence based and to eventually the sequence itself with the emergence of novel genome sequencing technologies. Trait mapping has been the foremost application of molecular markers in plant breeding. Genomic locations of numerous genes or quantitative trait loci (QTLs) associated with agronomically important traits have been determined in several crop plants using linkage or association mapping approaches. Plant breeders always look for an easy, rapid and reliable method of selection of desirable plants in breeding populations. Conventionally, desirable plants are selected based on phenotypic observations. The phenotypic selection for complex agronomic traits is difficult, unpredictable and challenging. Once the marker-trait association is correctly established, the gene- or QTL-linked markers can be used to select plants carrying desirable traits, the process called marker-assisted selection (MAS). Marker-assisted backcrossing (MABC) has been widely used for transferring single major gene or combination of major genes into the background of elite cultivar; the process

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refers to gene pyramiding. Marker-assisted recurrent selection (MARS) and genome-wide association analysis (GWA) are considered potential MAS strategies for improvement of complex traits but still remain as theoretical possibilities in plant breeding. Though molecular markers and MAS have promises for improved plant breeding process, the marker-trait associations or QTLs are statistical associations, which are influenced by several factors such as trait heritability, phenotyping methods, marker density, population type and other experimental conditions that might lead to false positives. Therefore, a cautiously optimistic approach is necessary to consider MAS in crop breeding programmes. In this chapter, the potentials of genetic markers in plant breeding are described.

Keywords

Morphological markers • Isozymes • Cytological markers • DNA markers • Restriction fragment length polymorphism • Random amplified polymorphic DNA • Inter-simple sequence repeats • Amplified fragment length polymorphism • Microsatellites • Simple sequence repeats • Single nucleotide polymorphism • Gene-targeted and functional markers • Genotyping by sequencing • Trait mapping • Gene tagging • Gene mapping • Quantitative trait loci • Mapping populations • Single marker analysis • Simple interval mapping • Composite interval mapping • Multiple interval mapping • Linkage disequilibrium • Association analysis • Marker-assisted selection • Gene pyramiding • Marker-assisted backcrossing • Foreground selection • Recombinant selection • Background selection • Marker-assisted pedigree selection • Marker-assisted recurrent selection • Genomic selection • Genome-wide selection • Genotyping platforms • Cultivar improvement

4.1 Introduction

Historically, plant breeding has been augmented with novel tools and methods from time to time. In recent years, molecular marker technology (especially DNA-based markers) has become popular among plant breeders. Molecular markers are widely used for characterizing genetic diversity in germplasm collections, mapping and identification of genes associated with important traits, mining of superior alleles and marker-assisted selection (MAS) of desirable genes/traits in breeding programmes. DNA markers are more attractive compared to conventional morphology and protein-based markers as they meet the characteristics of an ideal genetic marker system: unlimited in number, insensitive to environmental conditions, highly reliable and can easily be automated for convenience and cost-effectiveness. During the last three decades, DNA marker technology has progressed tremendously; a variety of marker types has been developed, which basically differ in their ability to detect DNA polymorphisms. The recent advent of next-generation sequencing (NGS) technologies and bioinformatics tools has enabled high-throughput development of sequence-based markers with extraordinarily less cost and time.

Genetic enhancement for agronomically important traits is challenging because most of them are genetically complex, controlled by multiple genes and influenced by environment. Classical breeding methods and tools are inadequate to deal with complex traits. Molecular markers provide means to precisely identify the number of genes controlling complex traits, to

locate them on linkage maps and to quantify their effects thereby enabling breeders to selectively manipulate complex traits in breeding programmes. In this chapter, the developments in genetic marker technology, its application in trait mapping and MAS for crop improvement are briefly described.

4.2 Genetic Markers

Genetic markers are the biological features, i.e. traits, enzymes/proteins and fragments of DNA that are inherited from parent to progeny following the Mendelian segregation. These features are used to keep track of an individual or a gene, hence called 'markers'. Genetic markers can be classified into four categories, viz. morphological markers, cytological markers, protein/isozymes and DNA markers. As the knowledge on biology improves, the marker system has evolved from using mere morphological features as markers to the level of using variation at DNA itself. More detailed description of different types of markers is given in the following sections.

4.2.1 Morphological Markers

Historically, plant breeders use easily observable morphological traits such as leaf shape, flower colour, pubescence, pod/seed colour, seed shape, and others to distinguish individuals as well as to use as a proxy in selection, when they are linked with other agronomic traits. Morphological markers have been reported in several crops, viz. rice, wheat, soybean, tomato and corn. For example, Khush (1987) has listed more than 300 morphological traits that can be used for genetic analysis in rice. A few associations/linkages between morphological and agronomic traits have been successfully used for selection in breeding populations. As early as 1923, Karl Sax found an association between seed colour and seed size in *Phaseolus vulgaris* that helped for selecting plants with large seeds. The other examples include linkage of semid-warfism with yield in rice and wheat, dwarfism with male sterility in wheat, which were extensively used in rice and wheat improvement during 1990s. Morphological markers are still relevant for genetic and breeding applications, if they show discrete variation in the study materials and the trait is not affected by developmental stage of the plant and environment.

4.2.2 Cytological Markers

The variations in banding patterns of the chromosome are called 'cytological markers'. They are used for chromosome characterization, detection of mutations and studying taxonomical relationships. The distribution of euchromatin and heterochromatin is the basis for variations in the banding pattern. Generally, the variations are visualized in terms of colour, width, order and position of the bands created by staining the chromosomes. Based on the stains used, different banding techniques were developed that include Q-banding, G-banding, R-banding, C-banding, NOR-banding and T-banding. In recent times, fluorescent in situ hybridization (FISH) is used to detect and localize the presence or absence of specific DNA sequences on chromosomes using fluorescence-labelled DNA or RNA as probes. Cytological markers were widely used in physical mapping and linkage group identification. Orellana et al. (1993) have mapped the genes controlling seed storage proteins to chromosome 1R of rye using cytological markers. Santos et al. (2006) reported a translocation-based cytological marker that is associated with winter hardiness in oat. However, similar reports on linkage/association of cytological markers with traits of importance are very minimal perhaps due to rare occurrence and laborious procedures for detection.

4.2.3 Protein Markers

Some of the enzymes are present in multiple forms in an individual and still carry out the same function. Such variants of enzymes are called 'isozymes' or 'allozymes' and used as genetic

markers. The isozymes are enzyme variants that are the products of different genes and thus represent different loci, whereas enzyme variants produced by different alleles of the same gene are called allozymes. Isozymes were the first set of molecular markers. Enzyme variants differ in the amino acid sequences causing differences in size and charge, which can be visualized by gel electrophoresis. This class of markers was widely used in population genetic analysis during 1980s. However, protein-based markers are not stable across tissues, organs and developmental stages, and the number of informative marker loci is too small to use in gene mapping studies.

4.2.4 DNA Markers

Technological advances have enabled us to visualize the differences in DNA sequences itself across genotypes, which have led to the advent of DNA-based markers. DNA markers are the variations observed in a particular portion of the DNA among the individuals of a species. These variations may be due to different mutations such as insertions, deletions and substitution or errors in replication of tandemly repeated DNA. Since 1980, several DNA marker technologies have been developed (Table 4.1), which differ on the basis of polymorphism and the methodology to

Table 4.1 List of different DNA markers in chronological order of their publication

Nomenclature[a]	Acronym	Reference
Restriction fragment length polymorphism	RFLP	Grodzicker et al. (1975)
Short tandem repeats	STR	Hamada et al. (1982)
Variable number tandem repeats	VNTR	Jeffreys et al. (1985)
Allele-specific oligonucleotides	ASO	Saiki et al. (1986)
Allele-specific polymerase chain reaction	AS-PCR	Landegren et al. (1988)
Oligonucleotide polymorphism	OP	Beckmann (1988)
Inverse PCR	IPCR	Triglia et al. (1988)
Single-stranded conformational polymorphism	SSCP	Orita et al. (1989)
Sequence-tagged sites	STS	Olsen et al. (1989)
Randomly amplified polymorphic DNA	RAPD	Williams et al. (1990)
Arbitrarily primed polymerase chain reaction	AP-PCR	Welsh and McClelland (1990)
Sequence-tagged microsatellite sites	STMS	Beckmann and Soller (1990)
Restriction landmark genome scanning	RLGS	Hatada et al. (1991)
DNA amplification fingerprinting	DAF	Caetano-Anolles et al. (1991)
Expressed sequence tags	EST	Adams et al. (1991)
Cleaved amplified polymorphic sequence	CAPS	Akopyanz et al. (1992)
Strand displacement amplification	SDA	Walker et al. (1992)
Simple sequence length polymorphism	SSLP	Dietrich et al. (1992)
Degenerate oligonucleotide primer-PCR	DOP-PCR	Telenius et al. (1992)
Simple sequence repeats	SSR	Akkaya et al. (1992)
Multiple arbitrary amplicon profiling	MAAP	Caetano-Anolles et al. (1993)
Sequence characterized amplified region	SCAR	Paran and Michelmore (1993)
Microsatellite-primed PCR	MP-PCR	Meyer et al. (1993)
Inter-simple sequence repeats	ISSR	Zietkiewicz et al. (1994)
Selective amplification of microsatellite polymorphic loci	SAMPL	Morgante and Vogel (1994)
Single nucleotide polymorphisms	SNP	Jordan and Humphries (1994)
Random amplified microsatellite polymorphisms	RAMP	Wu et al. (1994)
Single primer amplification reactions	SPAR	Gupta et al. (1994)
Amplified fragment length polymorphism	AFLP	Vos et al. (1995)
Allele-specific associated primers	ASAP	Gu et al. (1995)
Site-selected insertion PCR	SSI	Koes et al. (1995)

(continued)

Table 4.1 (continued)

Nomenclature[a]	Acronym	Reference
Cleavase fragment length polymorphism	CFLP	Brow et al. (1996)
Inverse sequence-tagged repeats	ISTR	Rohde (1996)
Directed amplification of minisatellite DNA-PCR	DAMD-PCR	Bebeli et al. (1997)
Sequence-specific amplified polymorphism	S-SAP	Waugh et al. (1997)
Multiplexed allele-specific diagnostic assay	MASDA	Shuber et al. (1997)
Retrotransposon-based insertional polymorphism	RBIP	Flavell et al. (1998)
Anchored simple sequence repeats	ASSR	Wang et al. (1998)
Single feature polymorphism	SFP	Winzeler et al. (1998)
Inter-retrotransposon amplified polymorphism	IRAP	Kalendar et al. (1999)
Retrotransposon-microsatellite amplified polymorphism	REMAP	Kalendar et al. (1999)
Miniature inverted-repeat transposable element	MITE	Casa et al. (2000)
Three endonuclease-AFLP	TE-AFLP	van der Wurff et al. (2000)
Inter-MITE polymorphisms	IMP	Chang et al. (2001)
Sequence-related amplified polymorphism	SRAP	Li and Quiros (2001)
Diversity arrays technology	DArT	Jaccoud et al. (2001)
Target region amplification polymorphism	TRAP	Hu and Vick (2003)
Promoter anchored amplified polymorphism	PAAP	Pang et al. (2008)
Conserved DNA-derived polymorphism	CDDP	Collard and Mackill (2009)
Start codon targeted polymorphism	SCoT	Collard and Mackill (2009)
Inter-primer binding site	iPBS	Kalendar et al. (2010)
Inter-SINE amplified polymorphism	ISAP	Seibt et al. (2012)

[a]Some of the markers are very similar or synonyms

detect it. They can be categorized into dominant or codominant types based on their ability to distinguish heterozygous from homozygous individuals. Dominant markers are bi-allelic and multi-locus, whereas codominant markers are either bi-allelic or multi-allelic and locus specific (Fig. 4.1). Some of the widely used DNA markers based on hybridization and PCR techniques are described below.

4.2.4.1 Restriction Fragment Length Polymorphism (RFLP)

The RFLP uses the technique of Southern blotting, a nucleic acid hybridization technique developed by Southern (1975). It was first used in human linkage mapping (Botstein et al. 1980). The basis for polymorphism is the mutation occurring in or between restriction sites causing differences in the restriction products. RFLP markers are locus specific, mostly codominant and highly reproducible. It is a robust marker system for synteny analysis across different species. It does not require sophisticated equipment, but large amount of high-quality DNA and technical expertise are paramount. Its throughput is very low, hence not widely used especially in plant breeding applications.

4.2.4.2 Random Amplified Polymorphic DNA (RAPD)

The RAPD was first demonstrated in the 1990s and is the simplest marker system to date. This is PCR-based technique, which requires only a single, short, random primer sequence to amplify DNA segments arbitrarily (Williams et al. 1990; Welsh and McClelland 1990). RAPD can detect several marker loci simultaneously in the form of presence or absence of the bands. The basis for polymorphism is the changes in DNA sequence that inhibit primer binding. RAPD was the most widely reported molecular markers till a decade ago because it is inexpensive and can be performed in any laboratory with limited infrastructure. But, it suffers from the problem of reproducibility, and many consider it as inappropriate for any applications. However, it may

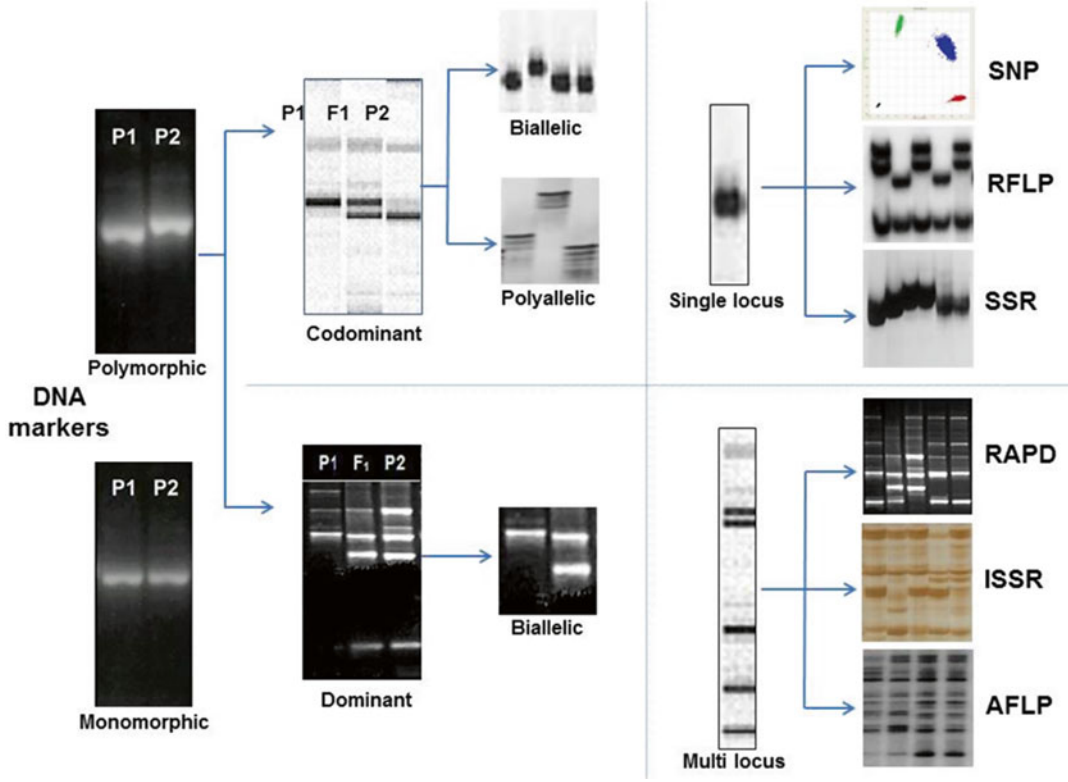

Fig. 4.1 Properties of different DNA markers

be still useful for certain applications such as looking at overall diversity of a set of samples or confirmation of hybrids. When useful RAPD band is identified, this could be converted to other types of PCR-based markers, like sequence characterized amplified region (SCAR) to improve the specificity (Michelmore et al. 1991; Martin et al. 1991). There are several variants of this technology such as arbitrarily primed PCR (AP-PCR) and DNA amplification fingerprinting (DAF).

4.2.4.3 Inter-Simple Sequence Repeats (ISSR)

The ISSR technique uses a single primer designed based on the complimentary sequences of a microsatellite as core and few selective nucleotides as anchor at the 3′ end to amplify the sequences between two adjacent microsatellites that are oppositely oriented. The basis of polymorphism is the differences in length of the sequences between two adjacent microsatellites. The major advantage of the ISSR is that the primers can be synthesized without the prior sequence information. It is a simple technique and a single run can produce multiple bands. Like RAPD, ISSR is also a dominant marker and suffers from reproducibility and the possibility of occurrence of similar-sized nonhomologous fragments. This marker system has potential applications in fingerprinting and genetic identity tests.

4.2.4.4 Amplified Fragment Length Polymorphism (AFLP)

The AFLP is generated by digesting the genomic DNA with a pair of restriction enzymes and selective amplification of restriction fragments using PCR. The basis of polymorphism is the same as that of RFLP, but this technique allows visualization of several polymorphisms simultaneously. The throughput is high. A typical AFLP run would produce about 100 bands, and about

70–80 % of them would be polymorphic depending on the nature of samples. This technique can be used for any organism without much initial investment in primer or probe development and sequence analysis. AFLPs are extremely useful as tools for DNA fingerprinting, studying diversity and germplasm characterization. However, the process is laborious and expensive. As that of RAPD, it is also a dominant marker though claims for codominant inheritance are also made based on band intensity.

4.2.4.5 Microsatellites

Microsatellites are tandemly repeated units of short nucleotide motifs of about 1–6 bp long. They are also known as simple sequence repeats (SSR), short tandem repeats (STR), simple sequence length polymorphisms (SSLP) or sequence-tagged microsatellite sites (STMS). The copy number of the repeats varies among individuals, and therefore, the polymorphism is observed in terms of variation in sequence length based on the number of repeats. Microsatellites are the markers of choice in most areas of molecular genetics because they are hyper-variable, highly reproducible, codominant, locus specific, abundant and widely distributed across the genome. Microsatellite analysis requires low amount of DNA and can be easily automated for high-throughput screening. They are highly transferable between populations, and the data can be exchanged easily across laboratories. It is necessary to have DNA sequence information to develop SSR markers. In the past, the development costs of SSRs were high due to genomic or cDNA library construction and sequencing. With the advent of high-throughput sequencing techniques, it is possible now to develop microsatellite markers for any organism with little investment in time and money.

4.2.4.6 Single Nucleotide Polymorphism (SNP)

A SNP is a single nucleotide base difference between DNA sequences of individuals, which occur within coding/non-coding regions of genes or in the intergenic regions. They are numerous and occur at different frequencies in different regions of the genome. SNP becomes the most preferred marker system nowadays due to the abundance (two to three polymorphic sites every kilobase), high level of polymorphism, high-throughput capability and cost-effectiveness. The SNP markers may be a better choice for the crop species especially legumes and oilseeds, wherein the microsatellite diversity appears to be very low. Even though the possibility of using SNP as marker was demonstrated in the 1990s, it has become popular only in the recent times due to the advent of NGS technologies. The NGS technologies have drastically increased the speed at which the DNA sequence can be generated, while reducing the costs by several folds. Thus, the discovery of SNPs has become very simple and inexpensive, but the development of genotyping assays for SNPs is still hindered by the low conversion rate and high cost. Several SNP genotyping options are available which ranges from simple cleaved amplified polymorphic sequence (CAPS) analysis to very high-throughput, array-based methods. The choice of the method largely depends on the number of SNPs to be used for genotyping. A detailed description on several methods of SNP typing has been provided by Bagge and Lübberstedt (2008).

4.2.4.7 Gene-Targeted and Functional Markers

The markers described above may belong to either transcribed or non-transcribed regions of the genome and are generally developed without the prior knowledge of their function. Hence, they are called ‘anonymous’ or ‘random markers’. Advancement in genomic studies now provides opportunities to specifically target genic regions of the genome to develop markers, which are termed ‘gene-targeted markers’. Examples of such markers include IRAP, REMAP, SRAP, TRAP, PAPP, CDDP, SCoT, iPBS and ISAP (Table 4.1). Furthermore, when DNA sequence information from fully characterized genes is available, markers can be developed from polymorphic sites within such genes causing the phenotypic variation. These markers are called ‘functional markers’. The Functional markers possess a specific advantage that it can be used in

the segregating population without the issue of recombination. The candidate genes for plant height (*Dwarf8*), plant stature (*tb1*), flowering time (*Dwarf8*), vernalization requirement (*VFR2*), fruit size (*fw2.2*) and grain quality (*GBSS*) have been used to develop functional markers in rice, maize, tomato and rapeseed. The potentials and prospects of gene-targeted and functional markers have been reviewed by Andersen and Lubberstedt (2003) and Poczai et al. (2013).

4.2.4.8 Genotyping by Sequencing

Historically, genotyping was done by surveying only a portion of the genome using any of the marker technologies. Due to reduction in the cost of whole genome sequencing, newer approaches have been developed to use sequencing itself for routine genotyping for genetic studies and breeding applications. These approaches are called 'genotyping by sequencing (GBS)'. In this method, a reduced representation library is developed from the genomic DNA of each sample and sequenced using any one of the NGS platform. The variants such as SNPs and InDels are scored across the genome among the samples. The GBS procedure has been demonstrated with maize and barley recombinant inbred populations, where roughly 200,000 and 25,000 sequence tags were mapped, respectively (Elshire et al. 2011).

4.2.5 Choice of Markers

In general, there is no perfect marker. Markers vary in their attributes such as abundance, genomic distribution, level of polymorphism, basis of polymorphism, technical requirements, cost, etc., and the choice depends mainly on the application. Suitability of different markers for selected applications is given in Table 4.2. Other factors influencing the selection of markers include accessibility, technical expertise, turnaround time and level of polymorphism in the study material, DNA quantity and quality requirement, transferability between laboratories and populations and cost.

4.3 Trait Mapping

Trait mapping has been the foremost application of molecular markers in plant breeding (Paterson 1996). It refers to determining number, genomic locations and effects of genes associated with target traits (qualitative or quantitative) using molecular markers either through linkage or association analysis. The information on genes and the linked markers would help breeders to design suitable strategies for marker-assisted breeding.

4.3.1 Mapping Major Genes

Traditionally, when classical genetics analysis suggests that the trait is under the control of major genes, a series of near isogenic lines (NILs) carrying different genes are created by repeated backcrossing with a common recurrent parent. Subsequently, the NILs and the recurrent parent are analysed for polymorphic markers. The linkage of polymorphic markers with the target gene is established using segregating populations (Yoshimura et al. 1993). But the construction of NIL is time-consuming and tedious. However, this limitation has been overcome by an alternative strategy called bulked segregant analysis (BSA) developed by Michelmore et al. (1991). The BSA involves the use of phenotypic extremes of a segregating population, instead of NIL, for tagging genes with the following steps: development of segregating populations (F_2 or F_3) by crossing two parents that differ for target trait (for instance, resistant or susceptible), identification of polymorphic markers between parents, phenotyping of segregating populations and identification of phenotypic extremes (resistant or susceptible progenies), making of DNA bulks of phenotypic extremes (usually about ten DNA samples for each phenotype category) and comparison of polymorphic markers between DNA bulks of phenotypic extremes along with the DNA of parents. The marker alleles that are

Table 4.2 Suitability of popular markers for different applications

Applications	Markers	Suitability
Genetic diversity analysis	RAPD	Suitable, easy and inexpensive system
	RFLP	Suitable but expensive and low throughput for this application
	AFLP	Provide more data point per run and ideal for this application but slightly expensive and nonrandom in its genomic distribution in some species
	ISSR	Provide more data point per run and easy to perform but may be nonrandom in its genomic distribution
	SSR	Ideal because of its hyper-variability and multi-allelic nature but would be low throughput unless automated and less polymorphic in certain species
	SNP	Highly suitable because it can survey the entire genome, but assay development is expensive
Comparative mapping and synteny analysis	RAPD	Not suitable due to its randomness
	RFLP	Highly suitable because conserved gene sequences could be used as probes
	AFLP	Not suitable
	ISSR	Not suitable
	SSR	A fraction of the markers might be useful
	SNP	Not suitable
Genetic mapping	RAPD	Random and not locus specific, not suitable
	RFLP	Suitable but tedious and low throughput
	AFLP	Not ideal as they are not locus specific and dominant
	ISSR	Not ideal as they are not locus specific and dominant
	SSR	Highly suitable because they are locus specific and codominant and have good genomic distribution
	SNP	Highly suitable because of its abundance and codominant nature
Hybrid confirmation	RAPD	Easy and inexpensive system, if stringent PCR conditions are maintained
	RFLP	Expensive and cumbersome process
	AFLP	Expensive and cumbersome process
	ISSR	Suitable and easy system if agarose gel is used for detection
	SSR	Highly suitable
	SNP	Expensive unless low-cost assays like KASP are used
Marker-assisted selection	RAPD	The target band is to be converted to SCAR
	RFLP	Not suitable due to tedious process and low throughput
	AFLP	Not suitable
	ISSR	Not suitable as tracking is difficult
	SSR	Highly suitable because of its codominant nature and transferability across labs
	SNP	Highly suitable when low-cost genotyping assays like KASP are used
Genomic selection	RAPD	Not suitable in terms of reproducibility
	RFLP	Not suitable in terms of throughput and number
	AFLP	Not highly suitable in terms of throughput and tracking
	ISSR	Not highly suitable in terms of throughput and tracking
	SSR	Not highly suitable in terms of throughput
	SNP	Highly suitable in terms of number and distribution

detected in the resistant parent as well as in the resistant DNA bulk are further tested on the large segregating population for its linkage with the resistance gene. The putative location of target gene can be determined by mapping the linked marker on a reference linkage map.

4.3.2 Mapping Polygenes/QTLs

'QTL' is an acronym for 'quantitative trait loci'. Most of the agronomically important traits are quantitative in nature and are controlled by multiple genes with variable effects. The 'QTLs' are genomic regions that are associated with a quantitative trait, which may be found on different chromosomes. The process of locating QTLs on genome using linked genetic markers is called 'QTL mapping'. This can be achieved by statistical methods that evaluate the association between marker genotype and the variation in trait value (phenotype) of individuals of a population. QTLs are detected based on the co-segregation of marker loci with the phenotypic trait. The co-segregation between the marker genotype and the trait (phenotype) is expected, if the marker loci and the genes responsible for the trait are in linkage disequilibrium. The earliest report of QTL analysis dates back to the 1920s. Sax (1923) presented the first evidence for linkage between a morphological marker (seed colour or colour patterns) and a quantitative trait (seed size) in *Phaseolus vulgaris*. Later, Thoday (1961) presented the methods for locating QTL on chromosomes based on their linkage relationships to Mendelian marker loci. He postulated that the process of QTL identification is limited only by the non-availability of sufficient number of genetic markers. During the last couple of decades, the limitation on number of markers has been overcome by the advances in molecular biology. At present, it is possible to find and use millions of markers for any species. Hence, the probability of finding the linkage between the genes and the marker loci is very high. Because of this opportunity, several attempts have been made to identify QTL in many organisms especially human, animals and plants. QTL mapping has enormous applications in crop improvement (Paterson 1998; Nelson et al. 2013). The immediate utility of QTL analysis is to know the genetic architecture of a trait based on the number, genomic positions and effects of QTLs that explain the variation in the phenotypic trait. It may also indicate if the trait is controlled by many genes of small effect or by a few genes of large effect. Once the association between the genomic region(s) and the trait is established, the candidate genes underlying the trait can be identified by high-resolution mapping followed by sequencing the target region(s).

4.3.2.1 Steps in QTL Mapping

A typical QTL mapping experiment involves the following major steps:

1. Developing/assembling a mapping population
2. Genotyping the mapping population using molecular markers
3. Construction of linkage map for linkage-based QTL analysis
4. Phenotyping the mapping population
5. Statistical analysis of genotypic and phenotypic data

4.3.2.1.1 Mapping Population

The genetic material used for QTL mapping could be a set of either related or unrelated individuals, but the individuals should widely differ for the trait under study. The populations used for QTL analysis can be broadly classified into two categories, viz. (i) populations derived from controlled crosses of two or more parents and (ii) natural population.

Several types of populations can be created by crossing two or more parents in a defined fashion. Among them, the populations produced from two parents (biparental mapping populations) that include F_2s, backcross population (BC), recombinant inbred lines (RIL), doubled haploids (DH), test cross, half sibs, full sibs, introgression lines (IL) and near isogenic lines (NIL) are widely used for QTL mapping. However, biparental populations have inherent limitations that they provide low recombination and it is possible to study only two alleles. To overcome these limitations, multi-parent-based mapping populations such as

nested association mapping (NAM) population (Yu et al. 2008) and multi-parent advanced generation intercross (MAGIC) population (Cavanagh et al. 2008) are used in the recent times.

A natural population of unrelated individuals from the germplasm collection can also be used for QTL analysis based on the principle of linkage disequilibrium (LD). It is assumed that the unrelated individuals in the germplasm may still contain very small chromosomal segments that are identical by descent, which leads to nonrandom association of alleles across the genome and can be captured through genome-wide marker analysis, which is popularly called 'association mapping' or 'LD mapping'. An unstructured natural population is more ideal for association mapping because the presence of subpopulations can result in spurious marker-trait associations. However, the concern of population structure or family relatedness has been overcome with the recently developed statistical methods.

The genetic model for different populations are based on the genotype constitution of the population. Each of the above-mentioned populations has their own advantages and disadvantages. Large number of markers may be needed to identify the QTL in natural population compared to population from controlled crosses due to high LD decay in natural populations. However, the accuracy and mapping resolution will be high in natural population. The early generation populations such as F_2 or $F_{2:3}$ provide the opportunity to estimate the effect of additive and dominance gene actions at specific locus, whereas only additive gene action can be studied in homozygous RILs and DH. However, RIL and DH being immortal populations, they could be used in replicated trials across locations and seasons. Hence, the traits that are highly influenced by environment can be better studied using RIL.

Therefore, the choice of population should be based on the nature of trait, timeframe of the project as well as the availability of resources. The size of the population depends on the type of population, nature of the trait and objective of the study. Several simulation and empirical studies indicated that about 500 individuals are required for mapping QTL of small effects, whereas population of 200–300 individuals would be sufficient for identifying QTL with large effects.

4.3.2.1.2 Genotyping of Mapping Populations

Any type of markers can be used for QTL analysis. The choice of markers depends on the availability of the marker system in the species, ease with which the genotyping can be performed and the available budget. For QTL identification through linkage analysis, it may be sufficient to have a marker at every 10 cM interval (Kearsey 1998). The minimum number of markers for genome-wide association analysis mainly depends on the extent of LD in the species (Kruglyak 1999) and the specific population under study. For example, it has been estimated that about 275,000 markers are required for genome-wide association analysis in sorghum, whereas just 2,000 markers would be sufficient for *Arabidopsis*. Even for the same species, the required number of markers differs based on the level of recombination. For example, association analysis in a collection of maize landraces would require 750,000 markers, whereas only 50,000 markers might be sufficient, if the population consists of only elite inbreds of maize. For candidate gene-based analysis, only markers in the particular genomic regions, viz. promoters, introns, exons and 5′/3′ UTRs, would be required.

4.3.2.2 Phenotyping of Mapping Populations

The accurate and precise measurement of the trait is the critical step in QTL analysis. The success of any QTL mapping greatly relies on efficient and effective phenotyping of large population. Measuring the quantitative traits is a cumbersome process because the genetic architecture of these traits often involves many loci of small effect that may interact with each other as well as with the environment. The trait must be evaluated in replicated trials under different environments using a reliable screening methodology. If possible, each trait is to be dissected into component traits, and more objective measurements are to be taken. There is concern that

several hundred QTLs have been reported in publication, but only a handful of them are in use. One reason for this situation could be the reporting of false-positive QTLs detected using flawed phenotypic screens. The traits may not be amenable for QTL mapping if proper methods for quantification are not available.

4.3.2.2.1 QTL Detection Through Statistical Analysis

QTL mapping heavily relies on the statistical model used in the analysis (Lander and Botstein 1989; Churchill and Doerge 1994; Zeng 1994; Doerge et al. 1997; Zeng et al. 1999; Barton and Keightley 2002; Zou and Zuo 2006). Different analytical methods have been evolved, but the use of a particular procedure largely depends on the genetic architecture of the trait and the genomic information available at the point of time. The QTLs could be of 'main-effect QTLs' or 'epistatic QTLs' based largely on the presence or absence of epistasis. Main-effect QTLs are the ones showing clear additive or dominant effect on allelic substitution and are denoted by the specific allele of the locus. Such QTLs could be identified by single marker analysis or through 'interval mapping', if linkage information is available. The epistatic QTLs are detected by analysing the interactions between alleles at two or more loci through epistatic models.

Single Marker Analysis

In this method, the individuals are grouped based on the marker genotypes. The difference in the phenotypic value among the group is statistically tested (t-test, ANOVA, regression) (Haley and Knott 1992). A significant difference in the group means indicates the possible linkage/association of the marker (used to group the individuals) to the trait. If the backcross population is used, the individuals can be grouped into two classes based on the marker genotypes. Then, the difference among the group means can be tested by simple t-test. If F_2 or other early generation intercross population is used, the number of genotypic groups will be more than two. In that case, ANOVA can be used to assess the significance in the mean difference. Single marker analysis is the simplest form of QTL analysis. It can be performed even when there is no linkage map available. However, this method has limitations that QTL location cannot be determined and their effects may be underestimated.

Interval Mapping

Linkage map is essential for this analysis. A QTL is assumed in the region flanked by two linked markers and tested for its likelihood based on the phenotypic variance. The likelihood is calculated for QTL position and its effect, based on the observed phenotypic data and marker genotypes. The significance is tested using a likelihood ratio test. Likelihood for a single putative QTL is assessed at each location on the genome. The limitation in this approach is that QTLs located elsewhere on the genome can have an interfering effect compromising the power of detection and accuracy of effects (Jansen and Stam 1994).

Composite Interval Mapping (CIM)

This is the most widely used method. In this method, interval mapping is performed using a subset of marker loci as covariates. These markers serve as proxies for other QTLs to increase the resolution of interval mapping, by accounting for linked QTLs and reducing the residual variation. The key problem with CIM concerns the choice of suitable marker loci to serve as covariates.

Multiple Interval Mapping (MIM)

This method uses multiple marker intervals simultaneously. QTL models are built using all the QTLs identified initially through CIM. Alternative models are evaluated based on the information content and the best fit is chosen. This method allows simultaneous detection and estimation of additive, dominance and epistatic effects (Kao et al.1999).

LD-Based Association Analysis

There are two major strategies for mapping QTL using association analysis: (i) genome-wide association analysis and (ii) candidate gene approach. Genome-wide association analysis uses anonymous markers from across the genome. In this approach, no assumptions are made as to what the genes might be, and markers are chosen sim-

ply to scan the whole genome for marker-trait association. Genome-wide approach requires a large number of markers for genotyping the samples. Hence, it is difficult to apply for crops with a large genome or with limited genomic resources.

In the candidate gene approach, a list of genes presumed to be involved in the trait is assembled and markers are chosen within those genes. The focus is the search for alleles at these candidate genes that result in variation in the trait of interest. Here, association mapping can be used as a validation method based on candidate genes or markers linked to QTL previously detected by classical methods or to mine novel and superior alleles from unexplored genetic materials.

Software

There are several software available for QTL mapping. The most widely used software include Mapmaker-QTL, QTL Cartographer, Win-QTL Cartographer, PLABQTL, R/QTL, MapQTL, QGene, MQTL, MapManager/QTX and QTLNetwork. Among these, Mapmaker-QTL, QTL Cartographer, Win-QTL Cartographer, PLABQTL and R/QTL are command-line software. Except MapQTL, all others are free software. Mapmaker-QTL is the oldest software and can perform only simple interval mapping. QTL Cartographer is the most versatile software and has options for performing most of the reported QTL mapping methods. R/QTL is a very flexible command-line software that offers Bayesian (hidden Markov model) method of QTL analysis. There are specific software for QTL analysis in nonconventional populations. The software 'HAPPY' performs Multipoint QTL Mapping in genetically heterogeneous stocks. TASSEL is the most widely used software for marker-trait association in natural population (Bradbury et al. 2007).

4.3.3 New Developments in QTL Mapping

Now, the QTL mapping paradigm has extended beyond the trait measured at whole organism level. The level of expression of the gene as the amount of RNA transcript or the amount of protein produced is considered as quantitative traits (Damerval et al. 1994). The gene expression phenotypes are mapped to particular genomic loci by combining microarray or next-generation sequencing analyses of expression profiles and conventional QTL mapping of the same segregating population. This approach is called expression QTL (eQTL) mapping (Michaelson et al. 2009). The 'eQTL mapping' is used to determine the genomic regions involved in the regulation of transcription and to study the impact of polymorphisms within these regions. Several eQTL studies have been reported in crops like eucalyptus (lignin-related genes), rice (stress-regulated gene expression with QTL for osmotic adjustment) and maize (degree of heterosis).

4.3.4 Caveats in QTL Analysis

The possibility of detecting the QTL is largely dependent on the heritability of the trait being mapped, the precision with which the trait can be measured and the size and type of the mapping population. Accurate phenotyping of a large population is crucial but seldom achieved. Even though the number of markers is rarely a limiting factor in QTL mapping experiments nowadays, identification of QTLs located in certain regions like centromere would still be difficult due to recombination suppression in those regions. The QTLs identified in a particular cross may be missing in a different cross. Even the same cross may reveal different QTLs, if the measurements are done in different environments. At first, QTL is a mere statistical entity. It will not be useful, unless a biological meaning is associated with the QTL. But, our ability to move from QTL to gene is not an easy task. The QTL is a large chromosomal segment potentially harbouring several genes, and all of them may not have connection to the observed phenotype. Pinpointing the gene responsible for the trait will be a time- and cost-intensive process. It is to be noted that the biological processes are oversimplified in QTL analyses (Arunachalam and Chandrashekaran 1993). The current statistical models do not take into account allelic heterogeneity and epigenetic

inheritance. Epitasis is considered only as the portion of the genetic variance that cannot be explained by the marginal effects of individual loci in the hierarchical genetic effect model. Irrespective of the shortfalls, there are several success stories of QTL mapping, as reported by Price (2006). The advent and rapid evolution of genomic technologies provides tremendous opportunity for QTL mapping. However, QTL mapping should be performed with prudence, and it is not a simple click and play with software, as perceived by many researchers.

4.4 Marker-Assisted Selection

Plant breeders always look for an easy, rapid and reliable method of selection of desirable plants in breeding populations. Conventionally, desirable plants are selected based on phenotypic observations. The phenotypic selection has long been successful for qualitative and simply inherited traits. However, in most cases, the agronomic traits are quantitative, controlled by polygenes and influenced by environment, which makes the selection difficult, unpredictable and challenging. DNA markers provide alternative selection tool for efficient selection of desirable genotypes in a breeding programme. Marker-assisted selection (MAS) refers to selection of desirable plants based on the presence or absence of a DNA marker that is associated or linked to the gene contributing for the target trait. During the last three decades, enormous progress has been made in genetic mapping and identification of molecular markers for selection of genes/QTLs governing agronomic traits in crop plants. The MAS provides an excellent opportunity for plant breeders to have several ways to improve the efficiency and genetic gains in their breeding programmes (Collard and Mackill 2008; Moose and Mumm 2008).

4.4.1 When Is MAS Preferred in Breeding?

The MAS is preferred in breeding to address the following critical issues that are often encountered by the plant breeders.

4.4.1.1 Proxy to Phenotypic Selection

The MAS improves the effectiveness and reliability of selection when the phenotyping is particularly expensive, laborious, time-consuming, destructive and growth stage and environment dependent. For instance, genotypes with high-oleic trait are selected by analysing seeds using biochemical methods such as gas chromatography. Though highly accurate, this method is laborious, time consuming and destructive. But marker assays can be carried out using DNA from young seedlings to predict high-oleic trait before harvesting of the seeds, and also phenotype of F_1 or F_2 plants can be predicted, while analysis of individual seeds is not possible by the conventional biochemical methods. However, it is important to confirm the phenotype of the predicted plants at every stage of the breeding programme. Therefore, it must be kept in mind that MAS is only a surrogate method and not a substitute for phenotypic selection.

4.4.1.2 Shortening the Breeding Cycle

The MAS helps the breeders to select plants carrying desirable genes at heterozygous state itself; therefore, selfing can be avoided and a season can be saved. This is especially important for traits controlled by recessive genes as well as crops that are highly season bound. Conventional backcrossing will not give any clue about the genome status of the backcross progenies. Based on a simulation study, Frisch et al. (1999a) reported that ~1 % of the backcross progenies may have the maximum of recurrent parent genome. When MAS is applied, those progenies (with ~97 % of recurrent parent genome) can be identified with one or two generations fewer than that would be required in conventional backcrossing.

4.4.1.3 Pyramiding of Multiple Genes

It has been possible to transfer only one or two genes into a single cultivar background through conventional breeding methods, especially for simply inherited traits as well as in some cases for pest and disease resistance owing to the availability of pathotypes or biotypes (Khush and Brar 1991). Those cultivars that carried one or two genes succumbed to pest pressure soon after the

release for the cultivation. Therefore, pyramiding of more genes in the background of cultivars is warranted to improve the durability of resistance. Pyramiding of multiple genes is almost impossible if pathotype or biotype information is not available because resistance genes cannot be differentiated at phenotype level. MAS will be extremely useful for gene pyramiding when the information on the genes and the linked markers are available for the donor genotypes.

4.4.1.4 Breaking Undesirable Linkages

Sometimes, 'linkage drag' and 'repulsion linkages' may cause serious hurdles for finding desirable recombinants in a breeding programme. Linkage drag refers to the undesirable effects that come along with the target trait while introgressing genes from wild species, which happens when genes for undesirable traits are closely linked with the target gene. In backcrossing programme, it is possible that a large segment of wild chromosome is fixed in the plant selections. For instance, *Ty1* and *Ty3* genes conferring resistance to tomato yellow leaf curl disease are part of a large region (~32 cM) from *Solanum chilense*, and the lines homozygous for this region may show deleterious effects (Verlaan et al. 2011). Based on a simulation study, Frisch et al. (1999b) reported that even after 20 rounds of backcrossing, the expected linkage drag around the target locus would be around 10 cM, when assumed that the target locus is at the centre of 100 cM chromosome. Since MAS allows selection of plants which are heterozygous for target gene region, it is possible to have recombination in the region so that size of the linkage drag can be reduced further. A repulsion linkage refers to linkage of desirable allele of a target gene with undesirable allele of another target gene. For example, when resistance allele of a gene 'A' is closely linked with susceptible allele of a gene 'B', during the selection of resistance allele 'A', the susceptible allele 'b' also gets selected automatically. Tomato breeders have observed that the *Ty1*/*Ty3* alleles are linked to susceptible allele *mi-1* of root-knot nematode (*Meloidogyne incognita*) resistance gene *Mi-1* and *Ty-2* linked to susceptible allele *i-2* of the *Fusarium* wilt resistance gene *I-2* (Peter Hanson, AVRDC-The World Vegetable Center, Personal Communication, 2010). Crossovers between the alleles linked in repulsion phases are rare but do occur; hence, by genotyping a large population, the desirable recombinants can be obtained.

4.4.1.5 Rapid and Cost-Effective Selection

Plant breeding is a number game. The probability of finding an ideal plant improves when a large number of segregating populations are generated and phenotyped rigorously. When 'genotypic information' of the parental lines or progenies are available to breeders before hand, the undesirable progenies can be rejected timely, and only a small number of useful progenies can be followed up, which can save substantial amount of time and cost of a breeding programme. For instance, a detailed economic analysis by Kuchel et al. (2005) on the use of MAS in an Australian wheat breeding programme showed that MAS enhanced the genetic gain over phenotypic selection as well as reduced the overall cost by 40 %. It is also important to note that MAS methods for conversion of maize inbred lines for a single dominant gene at International Maize and Wheat Improvement Center (CIMMYT) were not particularly advantageous over phenotypic selection in terms of cost-effectiveness (Morris et al. 2003). Phenotypic selection was less expensive but slow, whereas MAS was expensive but rapid. Therefore, according to Morris et al. (2003), MAS will be the best breeding method when the operating capital is abundantly available; otherwise, the conventional phenotypic selection will be the best option.

4.4.2 Strategies of MAS

Traditional breeding methods such as backcross, pedigree and recurrent selection can be improved with the integration of MAS. With the advent of genomic technologies, genome-wide MAS strategies are also emerging. The commonly known MAS strategies are briefly described below.

4.4.2.1 Marker-Assisted Backcrossing (MABC)

Backcrossing is a commonly used breeding method to incorporate one or a few major genes into an adapted or elite variety. Conventionally, the elite variety (recurrent parent) will be crossed with donor parent for the target trait. The backcross progenies will be screened phenotypically for the target trait, and a few promising progenies will be selected and crossed back to recurrent parent. A few backcrosses will recover the elite variety with the desirable trait transferred or introgressed from the donor parent. The whole process can be efficiently managed if molecular markers are integrated with backcross breeding programme. Collard and Mackill (2008) have outlined the strategy of MABC, which is described briefly here.

1. Foreground selection (stage I) – refers to selection of a subset of plants that carry desirable allele from the donor parent. This will be done by genotyping of BC_1F_1 plants for target gene or QTL using linked marker. A single marker or flanking markers that are tightly linked to the target locus can be used in foreground selection. The use of single marker may be risky because the recombination between the marker and the target locus may lead to wrong selection of allele. Therefore, the use of flanking markers may reduce the probability of losing the target alleles.
2. Recombinant selection (stage II) – refers to further selection among those of BC_1F_1 plants, which show heterozygosity in the target gene or QTL region. The BC_1F_1s that have fixed alleles may not have recombination in that region so that the size of the introgression cannot be reduced. Hence, the BC_1F_1 progeny that is heterozygous for the target locus will be selected.
3. Background selection (stage III) – refers to final selection of BC_1F_1s that carry maximum proportion of recurrent parent genome. This will be done by genotyping the selected backcross progenies using genome-wide markers.

The above steps may be repeated for two or three times till an ideal backcross plant, which carries the target locus in heterozygous genomic region and having the maximum of recurrent parent genome, is recovered. The selected backcross plants may be selfed for two or more generations to fix the agronomic traits before taking up field trials.

4.4.2.2 Marker-Assisted Pedigree Selection (MAPS)

Pedigree method of breeding involves combining of multiple desirable genes from two elite parents for the development of improved breeding materials and/or new cultivars. When the number of genes to be pyramided is many, or the genes are tightly linked in repulsion, it is practically impossible to recover the desirable recombinants in a single generation using a realistic population size; therefore, repeated selection at several generations is required. The availability of markers tightly linked to the desirable genes makes it possible to conduct effective individual selection at early generations (F_2 or F_3). This reduces the number of lines tested in the later generations and increases the desirable genotype frequency in the selected progeny (Ye and Smith 2008).

4.4.2.3 Marker-Assisted Recurrent Selection (MARS)

Recurrent selection is a cyclic breeding system for improvement of complex traits, wherein superior genotypes are identified and intermated to accumulate a large number of favourable alleles (Hallauer 1992). MARS uses QTL-linked markers to identify ideal genotypes for crossing in every selection cycle; thereby, favourable QTL alleles from diverse parental sources are accumulated within a short span of time (Peleman and Van Der Voort 2003; Ribaut and Ragot 2007).

MARS programme may involve at least two or more superior parents. A MARS scheme with two superior parents aims to identify the most favourable QTLs from two parents and accumulate them in a single genotype. Ribaut et al. (2010) provide a detailed scheme of a biparental MARS programme, which is briefly described below. The two selected parents will be crossed, and F_3-derived populations will be developed and advanced to F_4 generation through single seed descent method. The F_3 plants will be genotyped

using molecular markers, and the F_4 progenies will be phenotyped for target traits at multiple locations. QTL analysis will be carried out based on the F_3 genotypic data and F_4 phenotypic data, and a set of key QTLs will be identified. A subset of F_3 progenies will be chosen based on the QTL information and the phenotypic performance. Their corresponding F_4 plants will be grown and genotyped using the nearest marker to the QTL peak or flanking markers to select the desirable plants for recombination cycle. For example, if eight progenies are chosen, they will be crossed as pairs (first recombination cycle), the F_1s will be intercrossed as pairs (second recombination cycle) and the final two F_1s (third recombination cycle) will be subsequently selfed for two or more generations to fix the desirable alleles. The resulting lines will be genotyped using QTL-linked markers, and ideal genotypes that carry the most of the desirable QTLs will be identified. When multiple sources of parental lines are involved in MARS, the source parents will be crossed as pairs (several biparental combinations), F_1s will be intercrossed as pairs and the final two F_1s will be used to start the first selection cycle. Compared to biparental MARS, multi-parental MARS scheme will save time and resources and permit rapid accumulation of favourable alleles from diverse parental lines at once.

During the MARS process, the selection of an individual will be based on a molecular score or selection index, which is derived from genotypic and phenotypic data of the individual. In case a single marker is used for selection, the molecular score of an individual for use in recurrent selection is obtained as the estimate of the statistical association between marker genotype and phenotype. In cases where multiple markers are used in selection, genotype effects of all the markers are summed up to a single molecular score. It will be ideal to consider both molecular score and phenotypic information of the individuals to obtain maximum genetic improvement (Dekkers and Hospital 2002; Eathington et al. 2007).

4.4.2.4 Genomic or Genome-Wide Selection (GWS)

Genomic selection (GS) or genome-wide selection (GWS) is considered a new strategy of MAS for plant breeding (Jannink et al. 2010; Nakaya and Isobe 2012). In GS, selection of desirable individuals will be based on genomic estimated breeding values (GEBVs). Conventionally, breeding value is predicted using phenotypic data from family pedigrees. GS uses an innovative method for the estimation of breeding value based on genome-wide dense DNA markers. In the first step, significant relationships between phenotypes and genotypes are predicted using a subset of a population (called training population). In the second step, breeding population is genotyped using the informative markers, GEBV for each individual is derived by summing the marker effects and desirable plants are selected based on GEBVs. Genomic selection is expected to address small-effect genes that cannot be captured by traditional MAS.

4.4.3 Critical Considerations for an Effective MAS Programme

The following issues must be kept in mind before embarking on a MAS programme.

4.4.3.1 Choice of MAS Strategy and Statistical Issues

A suitable strategy of MAS must be determined based on the goals of a breeding programme, heritability of a trait, the number of genes governing a trait, strength of linkage between target locus and marker, population size and availability of resources. Based on simulation studies, Visscher et al. (1996), Frisch et al. (1999a, b) and Prigge et al. (2009) provide the theoretical possibilities for the minimum marker distance, marker density and population size required for selecting at least one desirable individual when a single gene is transferred through

MABC. According to Frisch et al. (1999b), if the goal is to select a BC_1F_1 plant that is heterozygous for a target gene and homozygous for recurrent parent alleles at both flanking markers with 99 % probability, minimum number of BC_1F_1 plants required would be about 4,066 when the target locus is at the distance of 5 cM from the flanking markers. In case if the goal is to select a BC_1F_1 plant that is heterozygous for a target gene and homozygous for recurrent parent allele at one flanking marker and heterozygous at the other with 99 % probability, the minimum number of BC_1F_1 plants required would be about 100 when the target locus is at the distance of 5 cM from the flanking markers. Ishii and Yonezawa (2007) have outlined various schemes for pyramiding two or more genes. For instance, one of the schemes involves crossing of multiple donors (~4) with a single recipient parent followed by separate backcrosses. MAS will be applied at each backcross generation to select the plants carrying target locus (heterozygous state) for further backcrossing. After three rounds of backcrossing, crosses between selected plants will be made so that the final population is expected to contain a genotype having all genes in homozygous condition. Bernardo and Charcosset (2006) and Bernardo and Yu (2007) discuss several issues concerning MARS and GWS for breeding applications based on simulation studies.

4.4.3.2 Genotyping Platforms for MAS

Simple, cost-effective and breeder-friendly genotyping platforms must be available depending upon the type of markers, the scale of a MAS programme and the resources. Gel-based genotyping platform would be enough and effective when markers such as sequence-tagged sites (STS), sequence characterized amplicon regions (SCAR), simple sequence repeats (SSR) and cleaved amplified polymorphic sequences (CAPS) are used and only a few genes or a few hundred samples are handled in a MAS programme. Single nucleotide polymorphism (SNP) markers are ideal for achieving high-throughput and automation. A variety of SNP genotyping assays, viz. high-resolution melting (HRM) (Terracciano et al. 2013), KBiosciences Competitive Allele-Specific PCR (KASPar) (Luciana Rosso et al. 2011), Golden Gate (Thomson et al. 2012), etc., are available. The use of high-throughput marker system may reduce the cost per genotypic data point (Herzog and Frisch 2011).

4.4.3.3 Phenotyping Platforms for Trait Validation of MAS Progenies

Effects of gene/QTL may be influenced by the genetic background (QTL x genotype interaction) and the environments (QTL x environment interaction) where it needs to be introduced. For example, Vadez et al. (2011) reported that effects of stay-green QTLs on water extraction, transpiration efficiency and seed yield varied in different genetic backgrounds. There are numerous examples to suggest that QTL effects vary across environments. Hence, prioritization of genes/QTLs for MAS work must be made after a thorough validation across genetic backgrounds and target environments to avoid false positives. This would demand an effective phenotyping network across target environments.

4.4.3.4 Integrated Breeding Platform

The fact that MAS has been highly successful in private sector suggests its efficacy. However, Delannay et al. (2011) observed that the public sector in the developing countries has very limited progress in MAS due to severe constraints, viz. resource-limited breeding programmes, shortage of trained manpower, inadequate high-throughput capacity, poor phenotyping infrastructure, lack of information and data handling systems/tools. Delannay et al. (2011) advocate an integrated breeding platform that connects the above-listed components for the successful implementation of MAS programmes.

4.4.4 Cultivar Improvement Using MAS

Globally, substantial efforts have been made to develop improved cultivars using MAS in public as well as private plant breeding programmes

(Eathington et al. 2007; Brumlop and Finckh 2011). The list is exhaustive and a few prominent examples are provided below.

4.4.4.1 Enhancing Yield Potential

Improvements of *per se* yield potential in varieties and hybrids have stagnated in many crops. It is envisaged that further improvement in yield potential might require integrated approaches involving traditional breeding and MAS to select a crop ideotype for a given environment (Francia et al. 2005). MAS for yield enhancement in a cultivar has been demonstrated in barley (Schmierer et al. 2004). Yield-enhancing QTLs from wild species appear promising (Swamy and Sarla 2008) but are yet to be exploited for cultivar development.

4.4.4.2 Resistance to Biotic Stresses

In India, popular rice varieties Samba Mahsuri and Pusa Basmati 1 and parental lines of rice hybrid PusaRH10 (PRR78 and Pusa6B) have been improved for resistance to bacterial blight disease through MABC. Samba Mahsuri has been pyramided with bacterial blight resistance genes, viz. *xa5*, *xa13* and *Xa21* (Sundaram et al. 2008). Pusa Basmati 1 has been improved with bacterial blight resistance genes, *xa13* and *Xa21*, along with blast resistance genes, viz. *Piz-5*, *Pi-Kh*, *Pi-1, Pi-ta*, *Pi-b*, *Pi-5* and *Pi-9*. PRR78 and Pusa6B have been pyramided with *xa13* and *Xa21* along with *Piz-5* (Prabhu et al. 2009). A highly cultivated wheat variety in India, PBW343, has been improved for leaf rust resistance by pyramiding *Lr24*, *Lr28* and *Lr48* genes (Prabhu et al. 2009). Jairin et al. (2009) improved a Thai aromatic rice cultivar 'Khao Dawk Mali 105' (KDML105) by transferring *Bph3* for resistance to brown plant hopper (*Nilaparvata lugens*) from a donor variety, Rathu Heenati. Tomato breeding lines pyramided with multiple *Ty* genes, conferring resistance to tomato yellow leaf curl virus, are spearheading private tomato breeding programmes in the Asia-Pacific region (Peter Hanson, AVRDC-The World Vegetable Center, Personal Communication, 2010).

4.4.4.3 Tolerance to Abiotic Stresses

Several popular cultivars have been improved for abiotic stress tolerance by transfer of major QTLs through MABC. For instance, mega rice varieties of India, viz. Swarna, Samba Mahsuri, IR64, TDK1 and CR1009, have been improved for submergence tolerance using the *Sub1* QTL (Neeraja et al. 2007; Septiningsih et al. 2009). Similarly, Chin et al. (2011) improved Indonesian rice varieties for high yield under phosphorus-deficient conditions by transferring *Pup1* QTL. In Vietnam, Huyen et al. (2012) and Cuc et al. (2012) improved Vietnam elite rice variety ASS996 for tolerance to salt with *Saltol* QTL and submergence with *Sub1* QTL.

4.4.4.4 Enhancement of Quality Traits

Development of quality protein maize hybrids using the *opaque-2* gene has been a commendable success of MAS worldwide. In India, Vivek Maize Hybrid 9, a popular single-cross hybrid, has been improved for its protein quality through MAS. The improved QPM hybrid, Vivek Quality Protein Maize (QPM) 9, showed 41 % increase in tryptophan and 30 % increase in lysine over the original hybrid. The Vivek QPM9 has been released for cultivation in India during 2008 (Gupta et al. 2013). In rice, Yi et al. (2009) improved cooking quality traits in Myanmar rice cultivar Manawthukha by transferring genes for fragrance coupled with intermediate level of amylase content from the donor variety, Basmati 370, through MABC. Chu et al. (2011) reported improvement of a peanut variety 'Tifguard' for high oleic-linoleic (O:L) ratio along with resistance to root-knot nematode (RKN) through MABC. The O:L ratio of the improved 'Tifguard' was about 25–30 % compared to <5 % in the original 'Tifguard'. Molecular markers associated with a mutation causing high oleic level (~70–75 %) have been derived from the *FAD-2* gene family members in sunflower (Schuppert et al. 2006) and safflower (Liu et al. 2013). The potential of these markers for development of high-oleic cultivars in sunflower and safflower remains to be tested.

4.5 Conclusions

Molecular plant breeding has been recognized as the foundation for crop improvement in the twenty-first century to increase crop production (Moose and Mumm 2008; Tester and Langridge 2010). The MAS is an important tool for breeding that would certainly empower plant breeders to make their breeding programmes highly efficient and productive.

Many of the features of the markers like cost, time and expertise have changed over time due to technological developments. The advent of NGS has removed the divide between the so-called 'orphan' and 'well-bestowed' crops in terms of availability of markers. Using NGS technologies, the complete genome or the transcriptome of a set of representative genotypes of useful gene pool can be sequenced, and genome-wide genetic variants such as SSRs, InDels and SNPs can be rapidly identified and easily converted into genetic markers for any crop. Hence, there are multiple options available for the geneticists and breeders, and the selection of marker is mainly driven by the genetic materials in hand and the intended application.

Among the MAS strategies, MABC has been extensively used to improve widely adopted mega cultivars in important crops such as rice, wheat, maize and tomato particularly for traits that are controlled by major genes or major QTLs. Information on agronomically important genes/QTLs in orphan crops is very much limited, which might perhaps change due to the advent of new cost-effective, high-throughput genomic tools. The challenge of breaking the yield plateau would require manipulations of several complex traits, which are low heritable and controlled by a large number of minor genes/QTLs. There is a concern that most of the reported QTLs are false or not accurate because the numbers of QTLs are plenty but their use in breeding is scanty. The accuracy of small QTLs can be improved by designing phenotypic screens that improve the heritability, improve understanding of genotype by environment interaction and refine map positions through candidate gene approach (Price 2006; Xu and Crouch 2008). MAS for complex traits would warrant extensive application of genome-wide MAS strategies such as MARS and GWS, which still remain as theoretical possibilities in crop breeding.

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Doubled Haploid Platform: An Accelerated Breeding Approach for Crop Improvement

5

Salej Sood and Samresh Dwivedi

Abstract

Haploid plants provide an excellent example of cellular totipotency as they develop from either the male or female gametes without fertilization. Haploids can be induced in vivo and in vitro. Androgenesis is the development of haploids from male gametes. The two main techniques that are employed to generate androgenic plants in vitro are (a) anther culture and (b) microspore culture. The application of anther culture is widespread. Different developmental pathways of microspore embryogenesis have been reported in different plant species and in response to different stress treatments and its microspore developmental stage. Genotype or physiology of the donor plant, microspore developmental stage, stress pretreatment, culture medium and culture conditions affect the androgenesis process. Each crop has individual optimal pretreatment regimes and in vitro culture requirements. In vivo haploid induction techniques involve parthenogenesis and wide hybridization followed by chromosome elimination. In maize (*Zea mays* L.), haploids are commonly produced by in vivo haploid induction system and the method has been commercially exploited by both public and private sector organizations. Wide hybridization method is limited to potato and cereals. Wheat, barley and potato are excellent examples of wide hybridization followed by chromosome elimination system of haploid induction. The regenerated plants particularly in androgenesis need ploidy analysis to distinguish haploids from non-haploid plants. Regardless of the mode of development, doubling of haploid plants either spontaneously or by chemical means leads to a

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homozygous doubled haploid (DH) individual with two identical copies of each chromosome. Doubled haploids have been used in breeding programmes to produce homozygous genotypes in a number of important species and the number is increasing. DH populations are ideal for genetic mapping and allow the development of high-density marker maps that can then be exploited in quantitative trait locus identification. Genetic mapping using DHs had an impact in locating gene control traits for yield, quality, agronomy, abiotic and biotic stress. DHs now feature in cultivar production in a number of crops, and breeding time is considerably reduced. Hence, worldwide use of DH technology as an accelerated approach to crop improvement has become routine by many breeding companies and laboratories leading to the development of almost 300 new varieties.

Keywords
Androgenesis • Haploids • Doubled haploids • Plant breeding • Wide hybridization • High-density marker maps • Quantitative trait loci

5.1 Introduction

Doubled haploid technology is one of the revolutionary techniques in accelerating the plant breeding process. Haploids occur spontaneously in nature or are induced artificially. The spontaneous haploids were first reported in *Datura stramonium* (Blakeslee et al. 1922). Subsequently, the reports came in other crop plants (Forster et al. 2007). However, the frequency of naturally occurring spontaneous haploids is very low and therefore is of limited use. It was the in vitro induced haploids from *Datura* anthers (Guha and Maheshwari 1964, 1966) which opened the doors for haploids in basic plant research. Later, in vitro haploids were successfully produced in tobacco through anther culture (Nataka and Tanaka 1968; Nitsch and Nitsch 1969). Success was also achieved from microspores in rice via haploid callus (Niizeki and Oono 1968). Subsequently, the reports came in wheat, barley, maize and other crops, but the rate of success was far below the levels found in Solanaceae particularly in tobacco. Slowly, the number of crop species increased, and even new methods of haploid induction wide hybridization followed by chromosome elimination were also reported (Kasha and Kao 1970).

Haploid is a general term used to designate a plant containing gametic chromosome number (n). In a diploid species, the haploid may also be called as monoploid ($n=x$) as it contains only one set of chromosomes. The haploids of a polyploid are called polyhaploids because they contain more than one set of chromosomes. The dihaploid term is thus used for haploid of a tetraploid species where the gametic chromosome number is $n=2x$. It has often been observed that the word dihaploid is commonly used for a plant where the chromosome number is doubled and which actually should be termed as doubled haploid (DH), not a dihaploid.

Rapid strides in research on conversion of sterile haploids into fertile doubled haploids led to the further development of chromosome doubling techniques to produce homozygous DH plants (Jensen 1974). Tobacco, rapeseed and barley are currently the most responsive species and model species for doubled haploid production.

5.2 Haploid Induction Methods

The two broad systems of haploid induction are:

In vitro haploid induction:	Androgenesis and gynogenesis
In vivo haploid induction:	Induced parthenogenesis
	Wide hybridization followed by chromosome elimination

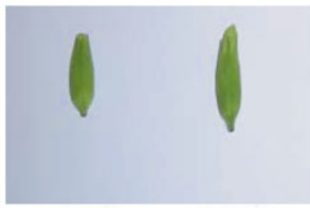
The common method used for producing haploids is anther and microspore culture which has been commercially exploited in many major crops e.g. tobacco, brassica and rice. In maize, haploid inducer stocks are used to produce in vivo haploids. In wheat and barley, wide hybridization followed by chromosome elimination is the preferred method for large-scale haploid production. Gynogenesis is the only practical method in onion. Palmer and Keller (2005) differentiated parthenogenesis from gynogenesis whereby the former involves 'the normal development of endosperm and embryo formation occurs in vivo', whereas in the case of gynogenesis, endosperm degenerates with the passage of time and there is a need to rescue the embryo in the laboratory conditions. In our chapter, we will be discussing androgenesis, induced parthenogenesis and wide hybridization in haploid induction, and ploidy determination methods, chromosome doubling and application of doubled haploids in plant breeding.

5.2.1 Androgenesis

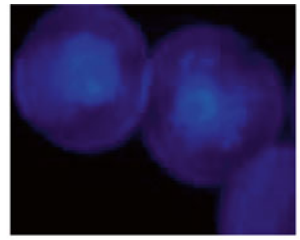
During sexual reproduction in plants, the gametes are formed with half the chromosome number, which reunites to restore the normal ploidy level. The process of microsporogenesis results in the reduction of chromosomes through meiosis in vegetative cells to give rise to microspores. The normal fate of microspores is to develop into pollen grains, but microspores can be induced to undergo a developmental process that leads to the formation of haploid embryos. The haploid embryos subsequently develop into haploid plants. This process is called androgenesis or microspore embryogenesis. Thus, androgenesis is the process of induction and regeneration of haploids and double haploids from male gametic cells. It is often the method of choice for doubled haploid production in crop plants (Sopory and Munshi 1997). Anther culture requires good aseptic techniques but is generally simple and applicable to a wide range of crops (Maluszynski et al. 2003).

Guha and Maheshwari (1964) first successfully discovered that the immature pollen could be induced to bypass normal development within the anther to produce haploid plants in *Datura inoxia* Mill. Since then considerable efforts were made to extend the technique to other species. Still many agronomically important crops are recalcitrant to androgenesis. Concerted efforts on many different plants have resulted in the documentation of the microspore embryogenesis process in over 250 species (Maluszynski et al. 2003; Dunwell 2010). However, success with androgenesis is still restricted to mostly annuals and non-woody plants.

The two main techniques that are employed to generate androgenic plants in vitro are the anther culture and the microspore culture. The application of anther culture is widespread, where the excised immature anthers are dissected out from flower buds and cultured on suitable growth media under appropriate in vitro conditions. Various stages of anther culture in tobacco are shown in Fig. 5.1. In microspore culture, microspores are isolated by mechanical means such as crushing of anthers and release into medium by magnetic stirring. They can also be isolated as naturally shed microspores in the culture medium after pre-culture of anthers. Although microspore culture is technically more demanding and is less efficient in some plant species than the culture of whole anthers, it has several advantages over anther culture. In the anther culture method, plants regenerate from somatic tissue of the anther wall also rather than from the haploid microspore cells. This problem does not arise in microspore culture because of isolation of microspores from anthers before culture. As individual



Buds selection for culture



DAPI stained uninucleate microspores

Anthers plated on Nutrient media

Globular embryo

Small plantlets 4 weeks after culture

In vitro colchicine treatment

Hardening of colchicine treated plants

Haploids/doubled haploid plants in greenhouse

Fig. 5.1 Anther culture in tobacco

plants develop from separate pollen cells, the chance of chimaera production is also low. Further, the culture of individual microspores makes possible the tracing of events that occur from microspore initiation through to embryogenic development, by cell tracking studies allowing for the greater understanding of the process of androgenesis in plants. In the case of anther culture, certain factors responsible for microspore embryogenesis are provided by the anthers which need to be supplemented externally for microspore culture. However, in some plant species, microspores require the parent environment during initial culture period, which is available only inside the anther wall.

5.2.2 Modes of Androgenesis

The initial steps of microspore embryogenesis are unusual to any other embryogenic system, since multicellular structure (MCS) formation takes place inside the exine wall of microspores (Mordhorst et al. 1997). During the initial phase of microspore embryogenesis, several patterns of cell division have been identified to take place inside the exine wall. The asymmetric division of the microspore nucleus resulting in a generative and a vegetative cell characterizes the A-pathway. In the A-pathway, MCSs are formed from repeated divisions of the vegetative cell, while the generative cell or its derivatives

degenerate and die (Sunderland 1974). This is the most widely spread mechanism of MCS formation during androgenesis and it has been described in several plant species, including most cereals (Raghavan 1986). In the B-pathway, it is the symmetric division of the microspore nucleus that gives rise to MCSs (Sunderland 1974). The B-pathway is known to play a major role in rapeseed (*Brassica napus* L.), potato (*Solanum tuberosum* L.), tobacco (*Nicotiana tabacum* L.) and wheat (*Triticum aestivum* L.) microspore embryogenesis (Zaki and Dickinson 1991; Rihova and Tupy 1999; Touraev et al. 1996; Indrianto et al. 2001), and it has been also described among barley MCSs (Sunderland and Evans 1980). An alternative route to androgenesis is defined by the independent divisions of the generative and vegetative cells, giving rise to MCSs with heterogeneous compositions. This modified version of the A-pathway, also termed E-pathway (Raghavan 1986), has been described in barley, maize, tobacco and pepper (*Capsicum annuum* L.) MCSs (Sunderland et al. 1979; Pretova et al. 1993; Touraev et al. 1996; Kim et al. 2004). Conversely to the known A- and B-pathways, in which the roles of the generative and vegetative cell are clearer, in this modified version of the A-pathway, both generative and vegetative cell appear to contribute equally to embryo formation (Raghavan 1986). What leads microspores to give preference to a particular division sequence is not yet clear. Different developmental pathways have been reported to vary according to plant species and in response to different stress treatments and the microspore developmental stage (Raghavan 1986; Sunderland et al. 1979; Kasha et al. 2001b; Kim et al. 2004).

Irrespective of the above early pattern of microspore divisions, the embryogenic pollen grains ultimately become multicellular and burst open, gradually assuming the form of a globular embryo. This is followed by the normal stages of postglobular embryogeny until the development of plant. This is called direct androgenesis. Examples are *Datura* and *Nicotiana*. Alternatively, the multicellular mass liberated from the bursting pollen grain proliferates to form a callus which may later differentiate into whole plants either on the same medium or on a modified medium. This is called indirect androgenesis and is common in most of the species, caused by complex media. The callus-derived plants also result in genetic variations.

5.3 Factors Affecting Androgenesis

5.3.1 Genotype and Physiological Conditions of the Donor Plants

The androgenic response is genotype specific; different varieties and genotypes respond differently to anther/microspore culture. The number and the vigour of microspores within the anther, the nutritional status of the tissues of the anther and the endogenous levels of growth regulators may to some extent be influenced by the physiological age and growth condition of the donor plant, which in turn will have a bearing on microspore embryogenic competence. Differences in competence of the cultured microspores for embryogenic development have been observed from field-grown and pot-grown plants (Datta 2005; Silva 2010) and crop grown under different seasons (Foroughi-Wehr and Mix 1979). The natural flowering conditions are normally the best environment for donor plants to produce anthers for successful regeneration experiments (Wang et al. 2000). We also observed similar results where androgenetic response from field-grown plants was higher in comparison to the pot-grown greenhouse plants in the case of *Nicotiana tabacum* (unpublished data). Infected, diseased and unhealthy plants should be avoided as donors as they will reduce the response in androgenesis. In general, the anthers from the first or early flush of flowers have been found to be highly responsive to embryogenic development rather than the anthers from buds in later stages or tillers of the plant, and the androgenic response can be improved by carefully nurturing of donor plants and identifying the favourable environmental conditions.

5.3.2 Developmental Stage of Anthers/Microspores

The microspore development stage is a major factor bearing strong influence on the success of anther culture. The exact microspore development stage that responds to inductive treatment varies with the species, but is restricted to a relatively narrow developmental window. There are slight differences in the developmental stage of microspores inside the anthers; thus, even the microspores inside an anther will vary with regard to their embryogenic competence. Therefore, only a percentage of microspores within a single anther would shift their normal gametophytic pathway to sporophytic development. For many species, the most amenable stage is either the uninucleate stage of the microspore or at or just after the first pollen mitosis, which is the early binucleate stage. At this time, the transcriptional status of the microspore may still be proliferative and not yet fully differentiated (Malik et al. 2007). Lessons learnt from model crops suggest that mature microspores are considered irreversibly committed to gametogenesis when the vegetative cytoplasm of binucleate pollen starts to accumulate starch (Touraev et al. 1997a).

It is necessary in anther culture to identify the flower buds that need to be harvested, containing microspores inside the anthers at the correct stage appropriate for embryogenesis. In many species, a maturity gradient is observed in flower buds within the inflorescence that displays either an acropetal or basipetal developmental succession. Therefore, a series of buds taken from different positions on the inflorescence or buds of different sizes in the case of individual flowers need to be pre-examined in order to identify the stage of microspore development within their anthers. This is done by squashing anthers to release pollen and staining with acetocarmine to observe the nuclei under the light microscope. DAPI (4′, 6-diamidino-2-phenylindole) may be also used to stain the nuclear DNA, which can then be visualized using a fluorescent microscope. Such pre-examination will be very useful to establish a correlation between easily observable morphological traits (e.g. the petal to sepal length ratio in tobacco as reported by Sunderland et al. (1974) and Sood et al. (2013) and size and appearance of buds in pepper by Ozkum-Ciner and Tipirdamaz (2002)) with the pollen development stage. This can be used as a quick guide to identify flower buds containing anthers/microspores suitable for androgenesis.

5.3.3 Stress Pretreatment

In androgenesis, a physical or chemical preculture treatment is applied to excised flower buds, whole inflorescences or separated anthers to trigger the induction of the sporophytic pathway, thereby preventing the normal gametophytic pathway of pollen development (Hosp et al. 2007a). A variety of microspore pretreatment stresses have been tested and found to enhance pollen embryogenesis, although the type, duration and time of application of these pretreatments may vary with the species or even the variety (Shariatpanahi et al. 2006). It has been shown that stress treatment to anthers also enhances the haploid regenerants in crop plants. With regard to different explants, the type, levels and duration of pretreatments are different, and regeneration efficiencies vary as well. Stress pretreatments such as high or low temperature, carbohydrate/nitrogen starvation, high osmotic pressure and colchicine treatment are most often used at the time of culture initiation and have shown to play a vital role for the reprogramming of microspores. During the plant life cycle, stress in general seems to be a common trigger for phase change. First, stressed microspores undergo a transition towards a more dedifferentiated state. This transition is exhibited in changes in their overall morphology, cellular enlargement, typical ‘star-like’ vacuolization and cytoskeletal rearrangements resulting in the migration of the nucleus towards a more central position (Touraev et al. 1997a; Raina and Irfan 1998). Second, the degradation of cellular components and certain proteins takes place and is considered

a prerequisite for developmental reprogramming in many organisms (Wang et al. 1999; Maraschin et al. 2005). Third, in tobacco and rapeseed, the stress-treated microspores undergo a cell cycle arrest, which is relieved when microspores are transferred to non-stress conditions (Zarsky et al. 1995; Binarova et al. 1997). Some reports described that stress pretreatment is not only needed for switching the development fate but also influence of divisions and embryos, green plant production and spontaneous doubling efficiency (Kasha et al. 2001a; Oleszczuk et al. 2006). The mechanisms underlying the stress-induced switch from gametophytic to sporophytic pathway on cellular, biochemical and molecular processes taking place during transition to the embryogenic state have been reported by Shariatpanahi et al. (2006).

The mechanisms of androgenesis for induction and embryo formation process are correlated to cell cycle change with different biological systems. Wang et al. (2000) mentioned that during androgenesis, the haploid microspore divides and develops into an embryo and subsequently into a new haploid plant. Maraschin et al. (2006) showed that the embryogenic development cell cycle changes in barley during androgenesis is divided into three main characteristics, e.g. (i) acquisition of embryogenic potential, (ii) initiation of cell division and (iii) pattern formation of embryos. Touraev et al. (1996) described that after the asymmetric division, the vegetative cell become arrested in the G1 phase of the cell cycle, while the generative cell progresses into mitosis and divides again to produce two sperm cells. Induction of androgenesis by stress is able to overcome the developmentally regulated cell cycle arrest, as the vegetative cell re-enters S-phase during stress treatment, and microspores progress into G2/M transition in culture.

Specific microspore developmental stages, combination of stress pretreatment and proper density of microspores are very essential for the cell development to embryo formation (Heberle-Bors 1989; Hoekstra et al. 1997). It has been mentioned that the type, duration and time of application of stress pretreatments may vary with the species (Datta 2005). After stress pretreatment, a rapid loss of chlorophyll and degeneration of tapetum and anther wall of cells were observed, and these types of cells are apparently most sensitive to stress and enter a cell death pathway (Wang et al. 1999). The death of these cells may release signals that are able to trigger susceptible microspores to enter a sporophytic development pathway. Corral-Martínez et al. (2013) also showed that the commitment of microspores to embryogenesis is associated with both massive autophagy and excretion of the removed material. It is also hypothesized that autophagy would be related to the need for a profound cytoplasmic cleaning and excretion would be a mechanism to avoid excessive growth of the vacuolar system.

5.3.4 Culture Medium and Culture Conditions

Successful induction of embryogenesis from cultured microspores or anthers depends to a large extent on the composition of the culture medium. The requirements of a given species are identified mainly through an empirical process of trial and error. The source of carbon, macronutrients (particularly the form in which nitrogen is supplied in the medium) and micronutrients may determine if embryogenesis will be initiated or not. The type and concentration of the growth regulators, particularly the auxins and cytokinins, as well as their interactive presence can be the deciding media factor that would influence pollen embryogenesis. Solidifying agent should be considered an integral part in the case of solid culture medium (Dunwell 1985). Standard media have been developed for different species although specific genotypes may have their individual requirements. The more widely used basal media for anther culture are the Nitsch and Nitsch (1969), MS (Murashige and Skoog 1962), N6 (Chu 1978) and B5 (Gamborg et al. 1968). These media are used often in their original form

but sometimes modified by supplementing or subtracting one or more components to better suit a given plant species or genotype.

It is well known that the culture atmosphere affects the yield of microspore embryos (Dunwell 1979). The temperature at which the cultures are incubated, light/dark conditions, the density of anthers or microspores in a culture vessel and other such post culture environmental conditions may have a subtle effect on the success of microspore embryogenesis, depending on the plant species or genotype. The temperature at which anther cultures are incubated has been shown to be an important factor in rice (Okamoto et al. 2001). However, very few investigations have been carried out to manipulate culture temperatures for enhanced anther culture efficiency. Light is another environmental factor that has a bearing on anther culture success because it is a stimulus that influences in vitro pollen morphogenesis (Reynolds and Crawford 1997). Generally, anther or microspore cultures are maintained in the dark during the initial phase of culture, particularly when regeneration occurs through callus, and transferred to illuminated conditions at a later stage. For some plant species, diurnal alternation of incubation for several hours in the light and then darkness has been beneficial (Germana et al. 2005; Sunderland 1971). However, optimal conditions need to be determined for each system.

Microspore culture has provided a wealth of information about the mechanisms underlying microspore embryogenesis. Studies of microspore cultures led to the identification of the stress in various forms that converts microspores from the gametophytic to the sporophytic pathway or from a cell with limited developmental potential into a totipotent or embryonic stem cell (Shariatpanahi et al. 2006). Functional genomics approaches that determine genes associated with androgenic induction revealed that a collection of genes are up-regulated and down-regulated during the switch in developmental pathways from gametophyte to sporophyte development (Hosp et al. 2007b). Several genes have been isolated and characterized as being involved in the reprogramming of microspores from the gametophytic to the sporophytic phase, making microspore embryogenesis an interesting system in which to study cellular reprogramming, phase change and totipotency in higher plants (Hosp et al. 2007a, b; Tsuwamoto et al. 2007).

Although the application of anther culture is widespread, the processes involved are poorly understood. There is a need to study, understand and manipulate microspore embryogenesis to develop genotype-independent methods. There is also a need to ensure direct embryogenesis to eliminate an intermediary callus phase that can promote gametoclonal variation among regenerants.

5.4 In Vivo Haploid Induction

5.4.1 Induced Parthenogenesis in Maize

In maize (*Zea mays* L.), DH lines are commonly produced by in vivo maternal haploid induction (Bordes et al. 1997; Prigge and Melchinger 2012). After pollinating the source germplasm with pollen inducers, a fraction of 8–10 % of the developing seeds have a haploid embryo (Rober et al. 2005). At present, discrimination of haploid seeds from diploid F_1 seeds resulting from normal cross-fertilization relies largely on the dominantly inherited marker gene R1-nj carried by the inducer. The marker gene causes purple coloration of the scutellum and aleurone of diploid seeds (Nanda and Chase 1966; Neuffer et al. 1997). Thus, it can be used as an embryo- and endosperm-specific marker to distinguish haploids from diploids seeds as well as seeds originating from unintended outcrossing. The use of the R1-nj marker system for haploid detection in combination with colchicine treatment of haploid seedlings for chromosome doubling has paved the way for routine application of the DH technology in private and public maize breeding programmes during the past decade (Seitz 2005; Smith et al. 2008). The visual scoring of the R1-nj marker expression is very labour intensive.

Moreover, variable colour intensity of the embryo marker hampers an unequivocal classification of seeds. The classification system fails if the source germplasm harbours inhibitor alleles such as C1-I (Paz-Ares et al. 1990), which suppress the expression of the R1-nj marker (Coe 1962). For rapid and reliable discrimination of haploid from diploid seeds, an alternative method based on differences in oil content stemming from pollination with high oil inducers has been developed (Melchinger et al. 2013). This technology has applicability to any maize germplasm and is amenable to automated high-throughput screening.

5.4.2 Wide Hybridization Followed by Chromosome Elimination for Haploid Induction

Wide crossing between species has been shown to be a very effective method for haploid induction and has been used successfully in several cultivated species. It exploits haploidy from the female gametic line and involves both interspecific and intergeneric pollinations. Haploid embryos can be produced in plants after pollination by distantly related species as shown in barley (Kasha and Kao 1970) and wheat (Laurie et al. 1990).

In barley, wide hybridization between cultivated barley (*Hordeum vulgare*, $2n = 2x = 14$) as female and wild *H. bulbosum* ($2n = 2x = 14$) as the male results in haploid production. The double fertilization takes place normally and hybrid zygote is formed. However, during early embryogenesis, chromosomes of the wild species are eliminated leaving a haploid embryo. The rapidly dividing endosperm also suffers chromosomal elimination and usually aborts early in seed development. The haploid embryos are excised and grown in vitro. The 'bulbosum' method was the first haploid induction method to produce large numbers of haploids across most genotypes. The method was quickly adopted and improved by researchers and barley breeders worldwide (Devaux 2003). Maize pollen could also be used for the production of haploid barley plants at lower frequencies.

Wheat is another crop where wide hybridization has been used for haploid production; chromosome elimination has been studied and investigated to a great extent and is being utilized for large-scale practical applications. Barclay (1975) was the first person who recovered wheat haploids in wide crosses between the wheat variety Chinese Spring and *Hordeum bulbosum*. The technique was, however, genotype specific due to the presence of the dominant crossability inhibitor genes *Krl* and *Kr2* in wheat, which are expressed in many wheat varieties. Later, wheat × maize system for haploid induction was reported (Laurie and Bennett 1987), which was genotype nonspecific because of the insensitivity of maize pollen to the action of *Kr1* and *Kr2* genes, thereby rendering this system more efficient and of practical value.

In wheat × maize hybridization, hybrid embryo develops after pollination, but, in the further process, the maize chromosomes are eliminated and haploid embryos are formed. The endosperm aborts and haploid embryos cannot develop normally. Such haploid embryos are supplemented in situ with growth regulator 2,4-dichlorophenoxyacetic acid to maintain the stage suitable for embryo isolation and in vitro culture. This method enables the production of large numbers of wheat haploids from any genotype. Pollination with maize is also effective for inducing haploid embryos in several other cereals, such as barley, triticale (× *Triticosecale*) (Wedzony 2003), rye (*Secale cereale*) (Immonen and Tenhola-Roininen 2003) and oats (*Avena sativa*) (Rines 2003). Several species, for example, *H. bulbosum*, teosinte, sorghum and pearl millet, have been tested as pollinators, but currently the most popular pollen donor is maize (*Zea mays*).

Although the wheat × maize system of haploid production is quite successful, the maize crop needs to be grown under controlled conditions so as to coincide flowering with wheat, thereby increasing the cost of haploid production. Therefore, wheat × *Imperata cylindrica* system was shown to be an efficient alternative to wheat × maize system for obtaining the high frequency of haploid and doubled haploids in wheat and triticale (Chaudhary et al. 2005; Chaudhary 2007,

2008a, b, 2010). *I. cylindrica* is a winter season plant and coincides well for flowering with that of wheat and triticale under natural conditions. It doesn't require repeated sowings and available under natural conditions in almost all parts of the world where wheat is cultivated. Moreover, *I. cylindrica* is also genotype nonspecific for hybridization with any variety of wheat, triticale or their derivatives, and the pollen of *I. cylindrica* is readily available in abundance during the wheat hybridization period.

A similar system operates in potato (*Solanum tuberosum*) (De Maine 2003). In this case, cultivated potato, *S. tuberosum*, is tetraploid ($2n = 4x$) crossed with the diploid relative *Solanum phureja*. In these crosses, both sperm nuclei can fuse with the central cell of the ovule to form a functional endosperm that initiates and supports the parthenogenic development of the unfertilized egg (De Maine 2003). The tetraploid female *S. tuberosum* produces an embryo sac containing one egg cell and two endosperm nuclei, all with the genetic constitution $n = 2x$, while the diploid pollinator *S. phureja* produces two sperms of the genetic constitution $n = x$ or $2x$. After pollination, dihaploid embryos can develop from unfertilized egg cells, which are supported by a $6x$ endosperm formed by the fusion of polar nuclei with both reduced sperm cells. The frequency of seed containing DH embryos is low, but these can be easily differentiated from hybrid seed by exploiting a colour marker gene carried by the pollinator line. Dihaploid potatoes can be used for breeding purposes, including alien germplasm introgression or selection at the diploid level, but such plants are not homozygous. Haploids have a significant role in potato breeding programmes of quite a few companies, since they enable interspecific hybridization, which would not otherwise be possible due to differences in ploidy levels and endosperm balance numbers. The gene pool of potato can be broadened, and certain valuable traits, such as disease resistance characters from the wild solanaceous species, can be more efficiently introgressed into cultivated potato (Rokka 2009).

It has advantages in that the methods involved (emasculation, pollination, embryo culture) are familiar to breeders, cross combinations can be manipulated to maximize haploid embryo production by eliminating genotypic dependency on the maternal side, and there is no seedling mortality caused by albinism (a major problem in microspore-derived haploids of cereals). However, flowering times of both parents need to be matched (seasonal limitations) and additional chemical treatments, such as colchicine, are required to double up the chromosome complement of haploid plants. In species other than cereals, inefficient chromosome doubling is considered to be the main bottleneck of this technology.

5.4.3 Ploidy Determination of Regenerants

Non-haploid embryos and plantlets have been observed in anther culture of different genotypes (Dunwell 2010). Therefore, ploidy determination is of highest importance in haploid induction programmes. Three are two methods for detection of haploid and non-haploids.

5.4.4 Direct Method

This involves mitotic or meiotic cell chromosome counting of induced plants. Root tips are the most convenient source of mitotic cells where the chromosome counting is easier and faster. When the roots are not available, young floral buds, leaves or callus can be used. Basic principles for handling the mitotic chromosomes of all plant species are similar and consist of material collection, fixation and staining. However, slight modifications are required depending upon the plant species. The chromosome preparation varies with the plant species and chromosome size. For example, tobacco chromosome spread is difficult in comparison to rice and wheat spread.

5.4.5 Indirect Method

There are a number of indirect methods for ploidy determination. These include flow cytometry, stomatal size, chloroplast number of the guard cells and morphological observations. Among these methods, stomatal size and chloroplast number are simple, inexpensive and less labour intensive, while recording morphological observations like plant height, leaf dimensions and flower morphology requires long time.

A large population of haploids need to be screened for use in plant breeding studies. This requires an efficient high-throughput ploidy analysis system. Flow cytometry has emerged as a powerful tool for large-scale speedy genome analysis. Flow cytometry provides a rapid and simple option for large-scale ploidy determination as early as in the in vitro culturing phase (Bohanec 2003). It also enables the detection of mixoploid regenerants and the determination of their proportion. We have analyzed tobacco haploids and doubled haploids plants using flow cytometry (Fig. 5.2).

Several markers can be used for assessing the origin of diploids, depending on their availability for a particular plant species. In the past, evaluation of regenerants mainly relied on phenotypic markers, progeny testing after self-pollination and isozyme analysis. Nowadays, DNA molecular markers, such as AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA), SCAR (sequence characterized amplified region) or SSR (simple sequence repeat), are commonly used for homozygosity testing and assessment of plant origin. There is a considerable difference in the interpretation between dominant or codominant electrophoretic profiles. Codominant molecular markers, as well as isozyme markers, have the advantage that a single locus, when heterozygous in donor plants, might be used for homozygosity determination. In contrast, a more complex profile is analyzed with dominant markers. In such a case, bands missing from the donor profile indirectly indicate homozygosity.

5.4.6 Chromosome Doubling of Haploids

To use haploid plants in breeding programmes, it is necessary to have high chromosome duplication rates of haploid plants. Haploids are generally smaller, less vigorous, more sensitive to disease and stress sources and, most importantly, sterile. Duplication of the chromosome complement is therefore necessary. Conversion of sterile haploid plants into fertile homozygous doubled haploid plants requires spontaneous or induced chromosome doubling.

5.4.7 Spontaneous Chromosome Doubling

In anther-/microspore-derived plants, spontaneous duplication of chromosomes may occur in

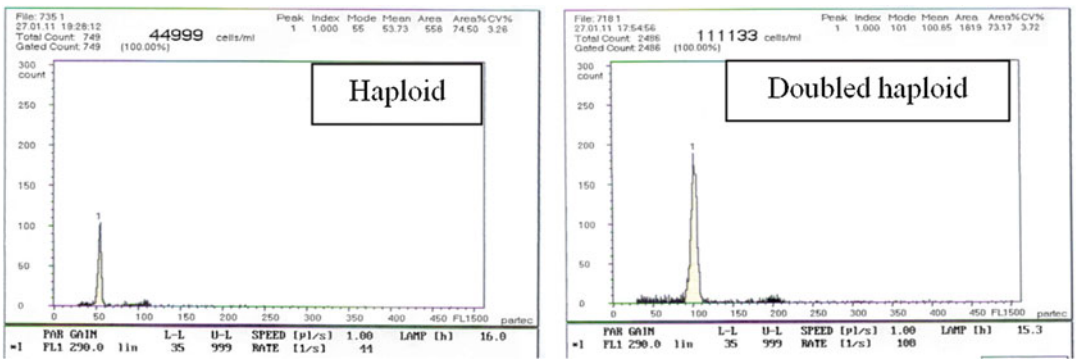


Fig. 5.2 Detection of haploids and doubled haploids using flow cytometry analyses

cultures, often in a sufficient proportion, thus eliminating the need for chemically induced doubling. Spontaneously doubled plants are sometimes preferred because of the fear that the duplication process might induce undesired mutations. This percentage may be largely influenced by the in vitro conditions used to induce androgenesis. However, we as well as other authors (Kasha 2005; Kasha et al. 2006) feel that such a process seems far from being spontaneous, as many different in vitro or ex vitro factors may be influencing duplication. As pointed out by Henry (1998) and Kasha (2005), the microspore stage at the time of anther/microspore inoculation, the stress treatments or the culture conditions used to induce androgenesis do affect the frequency of chromosome doubling. Several factors, like duration of inductive conditions, temperature (heat and cold), mannitol pretreatments and colchicine and other antimitotic drugs, used to induce androgenesis, are known to influence the frequency of genome duplication (Zhao and Simmonds 1995; Henry 1998; Kasha et al. 2001a; Zhou et al. 2002a, b; Kasha 2005; Shim et al. 2006). The use of plant hormones for in vitro cultures has also been directly related to DNA duplication events (Joubes and Chevalier 2000; Magyar et al. 2005). Even the type of explant used to induce androgenesis can have an impact. A striking example can be found in the work on *Brassica rapa* of Sato et al. (2005), who found that microspore culture yields up to a threefold higher rate of duplication than anther culture. Similar results have been obtained in *B. napus* (Lichter et al. 1988) and *B. oleracea* (Wang et al. 1999). With respect to androgenesis, endoreduplication has long been suggested as a mechanism to explain the spontaneous chromosome doubling and polyploidy in certain species (for details of endoreduplication in androgenesis, see Seguí-Simarro and Nuez 2008). However, it has been inferred that the early stages of androgenesis induction and microspore embryogenesis do not match many of the characteristics of cells typically undergoing endoreduplication (Segui-Simarro and Nuez 2008). The evidences in favour of nuclear fusion during androgenesis have increased in the last few years. However, most of the reports demonstrating nuclear fusion do not exclude the possibility of endoreduplication (Kasha 2005; Shim et al. 2006). But in some species, mainly cereals, evidence for nuclear fusion is conclusive (González-Melendi et al. 2005; Shim et al. 2006).

Because of the spontaneous chromosome doubling occurring in the haploid calli and embryos, ploidy level analysis cannot always identify pollen-derived plants. In fact, when in vitro anther or ovary cultures are performed, diploid plants can be homozygous doubled haploids or heterozygous diploids produced by the somatic tissue. For example, anther culture can be employed to obtain somatic embryos and clonal plant propagation in many genotypes (Germana 2003, 2005, 2011). Isozyme analyses, RAPD markers, sequence characterized amplified region (SCAR) and simple sequence repeats (SSRs) have been utilized to assess homozygosis and to determine the gametic origin of callus and regenerated plantlets, irrespective of their ploidy.

5.4.8 Induced Chromosome Doubling

There are always a number of induced embryos which do not undergo doubling and finally become a haploid plant. This number of individuals may oscillate enormously among species. Within a species, there are differences among genotypes as well. For maternally derived haploid plants, the rate of spontaneous doubling is often much lower or entirely absent, so a doubling procedure is essential. The most frequently used application is treatment with antimicrotubule drugs, such as colchicine (colchicine is a natural alkaloid extracted from several species of the genus *Colchicum*, especially *C. autumnale*), which inhibits microtubule polymerization by binding to tubulin. Although colchicine is highly toxic, used at a millimolar concentration and known to be more efficient in animal than in plant tissues, it is still the most widely used doubling agent. Other options are oryzalin, amiprophosmethyl (APM), trifluralin and pronamide, all of which are used as herbicides

and are effective in micromolar concentrations. The method of application for effective duplication varies depending on the genotype.

Colchicine concentration and treatment duration seem to have a key role (Caperta et al. 2006). The right combination of time and concentration is critical for the promotion of either c-metaphase arrest or chromosome doubling followed by cell cycle progression. Short-term exposures to colchicine seem to work better than prolonged treatments in terms of doubling frequency and avoidance of polyploidies or embryo abnormalities (Chen et al. 1994; Zhou et al. 2002a, b). Genotype is another factor that influences the final action of colchicine. The most common stages to apply the treatment are (1) application to parts or the whole haploid plant, once regenerated, (2) application to developing microspore-derived haploid embryos and (3) application during androgenesis induction as a component of the induction medium. Haploid plants can be totally sunk into colchicine solutions for varying times, never longer than a few hours. However, this method yields poor efficiency of doubling and may carry a significant amount of plant abnormalities and/or death. An additional drawback is the generation of a considerable volume of toxic waste that must be properly disposed of. An alternative is to apply colchicine to specific plant organs such as roots, axillary buds or apical meristems. These in planta methods are easy but seem not to be an efficient wide-range strategy.

Colchicine can also be applied in vitro to developing androgenic embryos (Pintos et al. 2007) or calli (Wan et al. 1989; Ouyang et al. 1994), but it implies low survival rates and chimaerism. However, what seems to be more efficient is to directly apply colchicine as soon as possible during the early stages of haploid embryogenic development. When applied before embryo/callus formation, the generation of chimaeric individuals can be greatly avoided. Besides, early application is much more economic due to the small amount of reagent used and the reduced volume of waste generated. In wheat, the incorporation of colchicine during the first hours of anther or microspore culture improved the doubling rate (Hansen and Andersen 1998; Soriano et al. 2007) and colchicine concentrations of 1 mM for 24 h or 0.3 mM applied for 48 or 72 h during the first hours of microspore culture, resulting in pronounced increase in the proportion of doubled haploids in triticale (Wurschum et al. 2012). However, in the case of melons, in vivo colchicine application has been found better in terms of enhanced doubling efficiency in comparison to in vitro treatments (Yetisir and Sari 2003; Solmaz et al. 2011). In vitro trifluralin treatment to microspores of *Brassica napus* resulted in higher doubling efficiency in comparison to in vivo and in vitro colchicine and in vitro oryzalin application (Klima et al. 2008). Trifluralin has been suggested as more efficient, cheaper and less toxic substitution of widely used antimitotic agent colchicine for production of doubled haploid lines in *Brassica* sp. (Abraha et al. 2008). N_2O has been used to induce chromosome doubling in a variety of species, mostly cereals. However, N_2O is considered to be less effective than colchicine in dicotyledons.

5.5 Applications of Doubled Haploids

Haploids and double haploids have very important applications in the field of plant breeding, genetic and molecular studies. Some of these applications are detailed below.

5.5.1 Development of Cultivars

Conventional breeding programme involving pedigree method of selection normally takes 7 years to produce a variety in self-pollinated crops (excluding the testing period). Doubled haploids overcome many limitations of conventional breeding (Fig. 5.3). It is the fastest route to homozygosity and can be performed in any generation in breeding programme. Since DH lines are homozygous, there are no dominance effects on selection. Thus, DH breeding accelerates the breeding programmes and shortens the time up to 3–5 years to release the cultivars. These DH lines

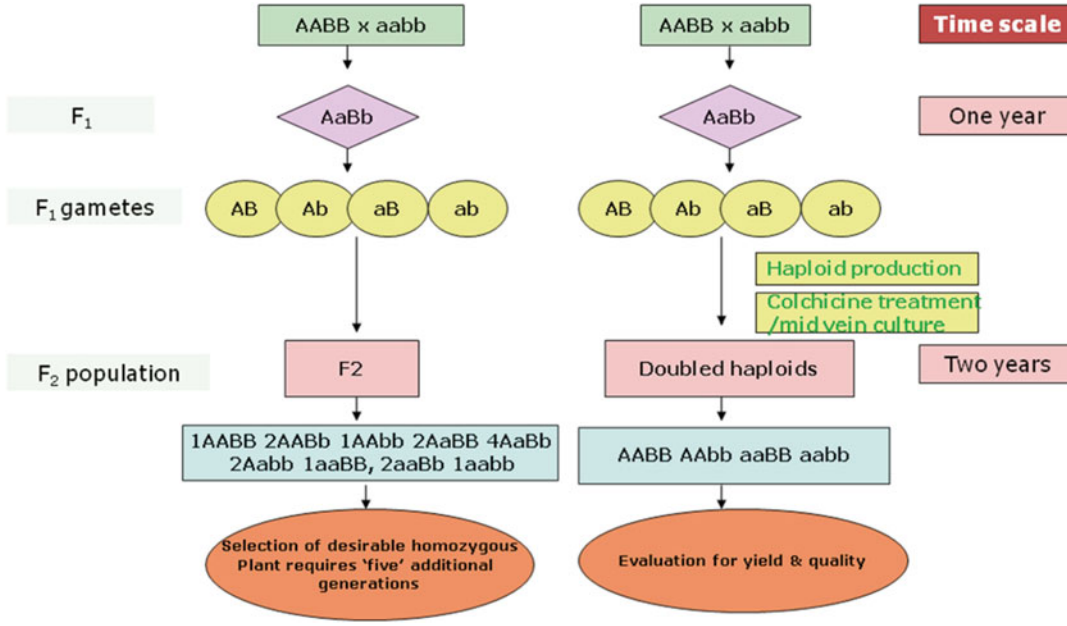


Fig. 5.3 Reduction in breeding cycle-conventional vs DH breeding

can be directly released as a variety for general cultivation and/or used as parents in breeding programmes to develop inbred lines for hybrid seed production in the case of cross-pollinated species. The DH lines can also serve as useful germplasm in generating new cross combinations.

There is currently much interest in expanding DH for F1 hybrid production to high value crops such as medicinal and aromatic plants. Because breeding has been neglected in these species, DH for F1 production has the potential to make significant advances in providing high, stable and predictive yields of the raw biochemicals processed by pharmaceutical and nutraceutical industries (Ferrie 2006). Furthermore, DH is an indispensable tool to obtain homozygosity in species suffering from inbreeding depression (implying therefore that they cannot easily produce fertile homozygous lines by self-pollination), such as rye (*Secale cereale*) (Immonen and Anttila 1996) and forage grasses *Festuca* and *Lolium* (Nitzche 1970), as well as in perennial woody species, generally characterized by a long juvenile period, a high degree of heterozygosity, large size and, often, self-incompatibility (Germana 2006, 2009) and generally in plants with barriers to repeated selfing (e.g. dioecy and self-incompatibility), where it is not possible to obtain homozygous breeding lines through conventional methods. In *A. officinalis*, a dioecious crop species, an inbred population is produced through sib crosses between pistillate and staminate plants which yield 50 % males and 50 % females. However, the commercially desirable features of this crop are uniform male population with spears having low fibre content. Anther culture was used to produce haploids of this species and this was diploidized to raise homozygous males. These are called as super-males.

The first crop plant released by DH technology was the rapeseed (*Brassica napus*) cultivar Maris Haplona (Thompson 1972), followed by the cultivar Mingo in barley (*Hordeum vulgare*), obtained in Canada in the late 1970s (Ho and Jones 1980). Worldwide, the use of DH technology has become routine by many breeding companies and laboratories involved in the improvement of a wide range of crops (Tuvesson et al. 2007), resulting in the development of almost 300 new varieties (these data are probably underestimated because of the scarce information

from some parts of the world and from some private companies) (Thomas et al. 2003; Dunwell 2010). Most of the DH varieties are in barley (96), followed by rapeseed (47), wheat (20) and the rest in pepper, rice, tobacco, eggplant, melon, triticale and asparagus. DH cultivars are now a feature in many crop species; for barley (*H. vulgare*) it is estimated that 50 % of contemporary cultivars in Europe are produced via DH (Forster et al. 2007). In vegetable crops, DHs are used prominently as parents for F1 hybrid seed production.

However, a number of comparisons have shown that DH populations were comparable to pedigree breeding and single-seed descent-derived populations. The technology has not been routinely used in public breeding programmes. The main reason for non-inclusion of DH as primary technology for cultivar release universally could be its resource intensiveness, lack of skilled workers and high initial setup cost. However, the commercial plant breeding sector has included the DH programme especially in hybrid breeding.

5.5.2 Backcross Breeding

Backcross conversion is a standard plant breeding method for improving an elite line defective in a particular trait like disease resistance, which is transferred usually from a hardy or wild genotype (donor) to a high-yielding but susceptible genotype (recipient) of the same species. This is done by crossing donor and recipient parents during the first year and recurrent backcrossing of F1 hybrid to recipient parent to reconstitute/restore the genes of high yield in new cultivar. It takes 5–6 years to recover the genes of recipient parent. Another problem in backcross breeding is identification of gene of interest or disease resistance at each generation particularly if disease resistance is controlled by recessive gene. In this case we have to self each BC generation to generate recessive homozygotes for gene expression. This takes double time as compared to dominant gene. Contrary to the problems associated with conventional backcross method, DH technology combined with molecular markers not only solves the problems mentioned above but also decreases the product development period significantly. A molecular marker is used to identify a gene of interest in a backcross generation and DH is developed to get the homozygosity in a single generation. The method has been used successfully to introgress stripe rust resistance in barley (*H. vulgare*) (Chen et al. 1994; Toojinda et al. 1998). Backcross inbred lines (BILs) are produced by backcrossing, and making DH from this backcross is very useful and ideal for gene mapping. Hence, double haploidy combined with marker-assisted selection (MAS) increases the efficacy and precision of selection of desirable traits. It is also helpful in stacking resistance genes.

5.5.3 Mutant Selection

The use of haploid systems for mutant induction and selection has been listed among the most important applications of haploid development technologies (Kasha 1974). On an average the isolation of a homozygous mutant line in seed-propagated crop takes three to four generations, whereas the DH system can shorten the process by half. All mutated traits are expressed in the first generation after mutagenic treatment, both at haploid and doubled haploid levels. DH mutant lines selected on the basis of their phenotype are genetically fixed and do not segregate in the next generation. The frequency of desired mutant in DH population will be much higher than in conventional M_2 generation due to homozygous condition for mutants. Haploids seem to be a good platform for selection of mutants under in vitro conditions. The population of haploid cells can provide an opportunity of identifying a rare mutation event by screening a large mutagenized population in vitro. Two approaches of applying mutagenesis to haploids in in vitro culture have been suggested by Maluszynski et al. (1996). In the first case, haploid cells or tissues can be directly given the mutagenic treatment, and in the alternative approach, seeds are treated with mutagen, and

M1 plant gametes are used as donors for the haploid culture.

Microspores offer an interesting alternative for mutation treatment. By targeting the mutation treatment at single gametic cells and then inducing embryogenesis and DH plant production, it is possible to create a population of homozygous mutant lines directly (Szarejko and Forster 2006) which provide the possibility to screen both recessive and dominant mutants in the first generation. DH also increases the selection efficiency of desired mutants due to the gametic segregation ratio (1:1) and lack of chimaerism. However, the disadvantage is that homozygous lethal mutations would be eliminated which otherwise are retained in the heterozygous condition.

5.5.4 Reverse Breeding and Male Sterility

DH plants also play an integral part in reverse breeding, a recently developed technology. The protocol for 'reverse breeding' was proposed by Wijnker et al. (2007). This technology involves the recovery of DHs from microspores of plants in which no or limited recombination has occurred. First, the superior hybrid genotypes are identified among the segregating population. Then using genetic transformation, a gene for induced suppression of meiotic recombination is introduced. The resulting recombinant inbred populations can be screened via molecular markers to identify those with complementary combinations of chromosomes to allow an original heterozygous parent of the DH to be reconstructed by hybridizing the two individuals. Consequently, different parents with different chromosome constitutions can be identified to reconstruct existing F1 hybrids.

Reversible male sterility and doubled haploid production have been used in tobacco for F1 hybrid production. The technology has been developed based on metabolic engineering of glutamine in developing tobacco anthers and pollen (Ribarits et al. 2007, 2009). Cytosolic glutamine synthetase (GS1) was inactivated in tobacco by introducing mutated tobacco GS genes fused to the tapetum-specific TA29 and microspore-specific NTM19 promoters. Pollen in primary transformants aborted close to the first pollen mitosis, resulting in male sterility. A non-segregating population of homozygous doubled haploid male-sterile plants was generated through microspore embryogenesis. Fertility restoration was achieved by spraying plants with glutamine or by pollination with pollen matured in vitro in glutamine-containing medium. The combination of reversible male sterility with doubled haploid production results in an innovative environmentally friendly breeding technology. This technology precludes the release of transgenic pollen into the environment.

5.5.5 Doubled Haploids in Quantitative Genetics Studies

DH lines are valuable and the most important material for quantitative inheritance studies as these are completely homozygous and there is no heterozygosity present in these lines. This makes them best fit for studying 'quantitative inheritance' (Touraev et al. 2009; Mochida et al. 2004). Important components of quantitative inheritance like number of genes controlling a quantitative trait, gene interactions among different genes, gene linkages and chromosome locations can be studied using DH lines (Choo 1981a; Jui et al. 1997). The number of genes controlling a polygenic trait can be determined by taking the square of deviation of extreme DH plants from sample mean and dividing it by genotypic variance or additive variance of DHs (Choo 1981b). For linkage studies, DH lines from F1 and F2 generations are developed, and means, variances and skewness are compared for both types of DH lines produced which gives the idea about linkage among the genes. This method was proposed by Snape and Simpson (1981). They also concluded that if skewness of a DH population is zero, it means there is no gene interaction present. If skewness is greater than zero, complementary gene interaction is present and less than zero

means duplicate gene interaction is present. So we can detect additive gene interaction and multiple gene action by this analysis.

5.5.6 Doubled Haploids in Genetic Mapping and Genomics

DHs are being routinely used for basic research in areas such as genomics, gene expression and genetic mapping (Ferrie and Caswell 2011). DH populations are ideal for genetic mapping due to their complete homozygosity, ability of repeating the experiment on several times and at different labs and less time required to produce a large DH population. Map construction is relatively easy using a DH population derived from a F_1 of two homozygous parents due to simple segregation ratio (1:1) similar to backcross (Snape 1976). Moreover, using DH lines for QTL mapping provides an opportunity to score the polymorphism in complete homozygous conditions in several environments in different years (Young 1994; Paterson 1996). DHs have been a key feature in establishing genetic maps in a range of species, notably barley (*H. vulgare*), rice (*Oryza sativa*), rapeseed (*B. napus*) and wheat (*T. aestivum*) (Forster and Thomas 2005). In out-pollinating species, genetic analysis can be simplified by using at least one DH parent in the initial cross to produce a segregating population – this has been successful in vegetables such as the *Brassica oleracea* complex, namely, cabbage, cauliflower, broccoli and Brussels sprouts (Sebastian et al. 2002). Marker maps provide a platform for trait mapping, which is of particular interest to plant breeders. The development of molecular marker map in DH populations led to a rapid deployment in QTL studies. The first QTL studies in barley were published within a year of their respective marker maps (Heun 1992; Hayes et al. 1993). For complete homozygous lines (like DH lines), an MQTL software has been developed for QTL analysis at multi-locations (Tinker and Mather 1995). In responsive crop species, such as barley, the process of producing a segregating DH population from an F_1 hybrid to find marker-trait associations has become routine (Tuvesson et al. 2006).

In rice, DH population has been used for QTL mapping, and QTLs have been found for yield; quality; agronomic, developmental traits; and biotic stress (Forster and Thomas 2005). Similarly, the QTLs have also been detected in wheat, barley, maize, mustard, rapeseed, flax, pepper, etc. (Forster and Thomas 2005).

DHs are also ideal in establishing marker-trait associations in bulked segregant analysis (BSA), a technique that compares individuals from two extremes of a population distribution for a given trait, for example, disease resistance and susceptibility. As with quantitative trait mapping, accuracy in BSA is dependent upon robust phenotyping; because DHs can be repeatedly tested, reliable data, including field data from trials over several seasons, can be obtained. DNA from the selected DHs can then be genotyped progressively with a range of marker systems. BSA and DH analysis has been successful in establishing marker-assisted selection for several breeding traits, mainly disease and pest resistance, but also quality traits (Michelmore et al. 1991).

DHs play an important role in genomics. Expressed sequence tags (ESTs) are used for searching of candidate genes. These ESTs can be mapped for their position relative to QTLs, and their physical chromosomal location can be identified via BAC libraries. DH populations play a vital role in integrating genetic and physical maps, thereby providing precision in targeting candidate genes. In rice, ESTs have been found to be co-located with major genes and QTLs for resistance to major diseases in the IR 64*x* Azucena DH map (Wang et al. 2001). Induced mutation provides another method of linking genes to phenotypes. The mutant populations for TILLING (Targeting Induced Local Lesions in Genomes) have been developed in several species for forward and reverse genetics. It is important that mutant populations are derived from an inbred or a homozygous line preferably DH line to avoid the detection of false positives owing to inherent variation in the starting material.

5.5.7 Production of Transgenic Plants

Transgenes can be incorporated into the haploid microspore system to produce homozygous transgenic plants. Both haploid and DHs can be used for transformation of desirable gene, and thus, transgenic plant having stability are produced which can be used for transfer of a desirable gene, genetic studies like mapping and gene identification (Forster et al. 2007). Although the primary target is the unicellular microspore, cells or explants at all stages from microspore embryogenesis and regeneration have been used as recipients for gene delivery, the choice being determined by regeneration potential after treatment. Many transformation techniques have been tried, including microinjection, electroporation, particle bombardment and *Agrobacterium tumefaciens-mediated* transformation (Touraev et al. 2001). Jahne et al. (1994) were the first to produce homozygous transgenic plants using biolistic bombardment of barley microspores. In tobacco, particle bombardment has been directed at embryogenic and non-embryogenic microspores and immature pollen (Stoger et al. 1995; Touraev et al. 1997b). In wheat, DH transgenic plants were produced for drought tolerance using the transgene (*HVAI*), and transgenic plants had higher tolerance to simulated water stress. The transgene was stably integrated and expressed over generations (Chauhan and Khurana 2011). The problem with transformation process is that once a transgene is transferred, transgenic plants need to be stabilized for homozygosity to study the phenotypic effects of transferred gene. This requires additional time and resources. Thus, combining DH with transgenic technology can enhance the efficiency and speed up the production of transgenics.

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Plant Molecular Biology Applications in Horticulture: An Overview

6

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Abstract

Horticultural crops are important for nutritional and livelihood security of farmers in the developing countries. Generating improved varieties adapted to modern production, transportation and storage requirements can make a significant impact on these economies. Compared to progress achieved in cereals and grasses, genetic improvement in horticultural crops has lagged behind. A significant review of the status and availability of genomic resources in these crops can be utilised efficiently to conserve and explore existing genetic diversity, to understand relationship between genotype-phenotype and to accelerate breeding. Recent advances in automation and high-throughput sequencing can be used in decoding as yet uncharacterized, complex genomes. In this review, we explore the various tools available under genomics, with emphasis on underutilised tropical and subtropical horticultural crops.

Keywords

Genome sequencing projects • Next-generation sequencing • Expressed sequence tags • Transcriptome • Comparative genomics • Molecular markers • Marker-assisted breeding • Quantitative trait loci • Linkage maps • Phenotyping

6.1 Introduction

The importance of horticulture in improving productivity of land, generating employment, improving economic conditions of the farmers and entrepreneurs, enhancing exports and, above all, providing nutritional security to the people is widely acknowledged. Horticulture sector, which includes fruits, vegetables, spices, floriculture,

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plantation crops and medicinal and aromatic crops, is an important component of the agriculture sector. Advances made in the field of molecular biology are being effectively used in horticultural crop improvement programmes. With particular reference to fruit crops, we provide herewith advances made in tropical and subtropical crops. Genomic resource development, in the form of whole genome sequencing, expressed sequence tags (ESTs), genomic survey sequences (GSS) and high-throughput genome sequences (HTGs), is helping growth and genetic improvement of these crops. Resources such as a large number of ESTs, large-insert genomic libraries, plenty of molecular markers and high-density genetic maps are being used for sequencing and annotation/identification/mapping and cloning of genes or quantitative trait loci (QTLs) and marker-assisted selection (MAS) in important horticultural crops. Availability of next-generation sequencing (NGS) technologies like FLX-454, Illumina, SOLiD and PacBio has accelerated the pace at which genomic resources are being generated for economically important horticultural crops (Van et al. 2013).

6.2 Genome Sequencing Projects

Whole-genome sequencing and annotation projects have significantly improved our knowledge of genome composition, organisation and evolution. Our understanding of plant origin and evolution through genome duplication, ancestral rearrangements and unexpected polyploidization events has made rapid progress in recent years due to the analysis of plant genomes. Many sequencing projects have been initiated in several horticultural crops of importance. Some of the most noticeable projects are tomato genome sequencing project (www.sgn.cornell.edu/about/tomato), potato genome sequencing consortium (www.potatogenome.net), papaya genome sequencing project (www.asgpb.mhpcc.hawaii.edu/papaya/) and grape genome sequencing project (www.vitaceae.org). Genome Database for Rosaceae (GDR) provides centralised access to Rosaceae genomics, genetics and breeding data, and analysis tools include apple, peach, strawberry, rose and other crops of Rosaceae family and many more.

Papaya whole genome sequencing project has been completed and published the genome sequence in early 2008 (www.asgpb.mhpc.hawaii.edu/papaya) (Ming et al. 2008). Transgenic female SunUp variety was sequenced to 3× coverage using whole genome shotgun (WGS) with Sanger sequencers. Genome size of papaya is 372 Mbp, about three times the size of *Arabidopsis*, with which it shared a common ancestor about 72 million years ago (Wikström et al. 2001). Some interesting features emerged from genome sequencing data, like papaya having about 25 % fewer genes than *Arabidopsis*, with reduction across most gene families and biosynthetic pathways. The lower gene number is believed to be due to the absence of the recent genome wide duplication of papaya genome, unlike in *Arabidopsis* (Ming et al. 2008). The much awaited banana genome, based on double-haploid *Musa acuminata* cv. Pahang, was published in June 2012. The genome was sequenced to a depth of 20× using a combination of 454 and Sanger sequencing and 50× using Illumina short-read sequencing technology. The genome is organised into 11 pseudomolecules, covering 332 Mbp accounting for 63 % of the estimated total banana genome size of 523 Mbp. An additional 140 Mbp of unanchored scaffolds and contigs (33 % of the estimated total genome size) are also included in the current genome release (D'Hont et al. 2012). Among citrus species, the draft genome of sweet orange cv. valencia has been recently published (Xu et al. 2013), while the genome of clementine orange is unpublished and still not fully assembled. The genome of haploid clementine has been developed by in situ parthenogenesis of Clemenules clementine induced by irradiated pollen of fortune mandarin, followed by direct embryo germination in vitro by the International Citrus Genome Consortium (ICGC http://www.citrusgenome.ucr.edu/). Using Sanger whole genome shotgun approach, 301.4 Mbp of genome spread over 1,398 scaffolds with 2.1 % gap at 7× coverage has been

generated. Over 96 % of the assembly is accounted for by the nine chromosome pseudomolecules. Similarly, a double-haploid (dihaploid) line derived from the anther culture of valencia sweet orange has been used to reduce the complexity of the sequenced genome (Xu et al. 2013). In addition to this, transcriptome sequencing of four representative tissues using shotgun RNA sequencing (RNA-seq) to capture all transcribed sequences and paired-end-tag RNA sequencing (RNA-PET) to demarcate the 5′ and 3′ ends of all transcripts has been conducted. On the basis of DNA and RNA sequencing data, characterisation of the orange genome for its gene content, heterozygosity and evolutionary features has been accomplished.

Availability of the full genome sequence of several crop species is helping plant biologists to answer many questions about the evolution of species, genome structure and genetics. Also, it helps plant breeders conserve and explore biodiversity. In order to illustrate how knowledge about genomes is directly impacting crop research, we can consider the examples in rice, maize, tomato, potato, sugar beet and apple. In rice, once the genome sequence information became available, it was immediately used to help elucidate a major QTL for rice grain production, which was found to be a cytokinin oxidase (Ashikari et al. 2005). Later, a transcription factor controlling the expression of the gene was identified as DST (Li et al. 2013). The re-sequencing of many accessions within a species is enabling high-throughput genotyping to identify a large array of SNPs and subsequently to produce haplotype maps (Barabaschi et al. 2012). In maize, this technique has been used to develop powerful haplotype maps using genome information (Gore et al. 2009), and also QTLs for biomass and bioenergy using whole genome and metabolic prediction have been identified (Riedelsheimer et al. 2012). The tomato genome has elucidated the esterase responsible for differences in volatile ester content in different tomato species (Goulet et al. 2012). A gene underlying the uniformity ripening locus has also been identified to be Golden 2-like transcription factor which is responsible for chlorophyll distribution in unripe fruits (Powell et al. 2012). The cultivated tomato genome along with the draft genome of its wild relative *S. pennellii* has shown the evolution of terpene biosynthesis in this genus (Matsuba et al. 2013). The information generated will undoubtedly help in breeding tomatoes with improved quality traits. The genome of another member of Solanaceae family potato has been used for identification of transcription factors involved in the regulation of plant maturity and life cycle (Kloosterman et al. 2013). The sugar beet genome has been used to identify the biological factors controlling flowering time in order to create a 'winter beet' which would be able to avoid bolting during winter thereby giving more yields (Pin et al. 2010). Among fruit crops, in apple, 27 cultivars representing worldwide breeding germplasm have been re-sequenced at low coverage with Illumina Genome Analyzer II. These sequences were aligned with the whole genome sequence of 'Golden Delicious' to identify a total of 2, 113,120 SNPs. Applying stringent filtering criteria, SNPs exhibiting even distribution across the apple genome and a range of minor allele frequencies were chosen giving rise to a panel of 7,867 apple SNPs for International RosBREED SNP Consortium (IRSC) apple 8K SNP array. Of these, 5,554 were reported to be polymorphic in segregating families and the germplasm collection (Kumar et al. 2012). This genomic resource is proving helpful in marker-locus-trait association discovery, description of genetic architecture of quantitative traits, investigation of genetic variation and genomic selection in apple.

6.3 EST Resources in Horticultural Crops

Expressed sequence tags (ESTs) are short (200–800 nucleotide base in length), unedited, randomly selected single-pass sequence reads derived from cDNA libraries. EST and complementary DNA (cDNA) sequences provide direct evidence for all the sampled transcripts.

These are the most important resources for transcriptome exploration. ESTs enable gene discovery, complement genome annotation, aid in gene structure identification, establish viability of alternative transcripts, characterise single nucleotide polymorphism (SNP) and facilitate proteome analysis (Jongeneel 2000; Rudd 2003; Dong et al. 2005). High-throughput ESTs can be generated at reasonably low cost from either 5′ or 3′ end of a cDNA clone to gain insight into the transcriptionally active regions in any organism. In plants having a large genome size, it is cumbersome to sequence the whole genome; but, EST data sets can be utilised to complement or serve as an alternative to genome sequencing, earning the label 'poor man's genome'(Rudd 2003). However, ESTs are subject to sampling bias resulting in underrepresentation of rare transcripts, often accounting for only 60 % of an organism's genes (Bonaldo et al. 1996). ESTs have been developed in several plant species, and this DNA sequence information has been deposited in online databases. In several fruit crops, sequence data for several fully characterised genes and full length cDNA clones have been generated. At the National Centre for Biotechnology Information (NCBI) EST database (dbEST; www.ncbi.nlm.nih.gov/dbEST/), there are 77,528, 44,565 and 234,474 ESTs of papaya, *Musa* and sweet orange, respectively. These fruit crops are of worldwide economic importance; therefore, they are being studied extensively at the genomic level.

With the advent of NGS, it is expected that the number of ESTs will significantly increase, since these sequences form an important resource for functional genomics. ESTs derived from various kinds of tissues, including tissues of organisms in a range of developmental stages or under stress, could significantly facilitate gene discovery as well as gene structure annotation, large-scale expression analysis and the design of expressed gene-oriented molecular markers and probes for microarrays until the whole genome is sequenced (Zhang et al. 2004; Kawaura et al. 2006).

6.4 Transcriptome/Gene Expression Studies

In fruit crops with large EST collections, the next logical step is expression analysis of genes using techniques like SAGE, microarray, tilling array and RNA-seq. Once available, the microarray can be exploited to study genes involved in disease resistance, fruit ripening, origin and evolution of reproductive organs, etc. The transcriptome can also be used as a proxy for a complete genome and sequenced using NGS technology at a lower cost, as the transcriptome size can be a fraction of the total genome size as demonstrated in maize which has 2.3 GB genome, but the size of annotated genes is a mere 97 Mb or 4 % of the genome (Schnable et al. 2009). A representation of a majority of all genes in de novo assembly can be obtained through sampling of several core tissues with diverse biological functions, e.g. root, leaf, flower and fruit at lower cost.

Several studies have been made on economically important fruits like papaya, banana, citrus, avocado and pineapple. In papaya, analysis of ripening-related genes has been carried out using a cross-species (XSpecies) microarray technique based on phylogenetic proximity between papaya and *Arabidopsis thaliana* (Fabi et al. 2012). Transcriptome analysis resulted in the identification of 414 ripening-related genes. An expression of some of these genes was validated using qPCR. The transcription profile was compared to tomato and grape, which revealed several similarities between ripening in papaya and tomato, especially with respect to primary metabolism, regulation of transcription, biotic and abiotic stress and cell wall metabolism. In another study, the identification of genes responsible for sex determination in papaya, namely, male, female and hermaphrodite plants, was done using the digital gene expression analysis by Ht-SuperSAGE (Matsumura et al. 2010) which resulted in the identification of a large number of tags by sequence analysis using SOLiD3 which were mapped on papaya primitive sex chromosome.

In avocado, transcriptome sequencing studies have been undertaken in a big way to discover candidate genes. Identification of defence-related genes after infection with *Phytophthora cinnamomi* which causes root rot in avocado (Mahomed and Van den Berg 2011) and *Colletotrichum gloeosporioides* responsible for anthracnose disease, using Roche 454 pyrosequencing (Djami-Tchatchou and Straker 2012), has been done. Floral transcriptome of avocado is of interest to biologists since it is considered as a basal angiosperm, with weakly differentiated floral organs. Using expression profiles of flowers at different stages of development, it has been shown that overlapping transcriptional programmes characterise the floral transcriptome of avocado compared to *Arabidopsis* where floral gene expression domains are typically organ-specific (Chanderbali et al. 2009). Transcription analysis is also useful for the development of molecular markers like the SNP and SSRs. In an unpublished work by John Ohlrogge of Michigan State University, a large EST library has been prepared for 'Hass' genotype of avocado from different tissues like leaves, flowers, mesocarp, etc. using Roche 454 sequencing. This is being used as a reference transcriptome, while RNA from leaves and flowers of other varieties like 'Bacon', 'Simmonds' and 'Tonnage' has been pooled and sequenced using Illumina GAII. These reads have been aligned to 'Hass' reference to develop single-nucleotide polymorphisms and indels (Arias et al. 2012).

Musa (which includes both bananas and plantains) is an important genus for global export and local markets. *Musa paradisiaca* (ABB group, plantain) has been shown to possess superior stress resistance (drought, chilling, diseases, etc.) compared to *Musa acuminata* (AAA group, banana). The de novo transcriptome assembly has been used to gain a comprehensive overview of plantain transcriptome to identify resistance genes/proteins for use in banana breeding programmes. Fungal infections like *Fusarium oxysporum* f. sp. *cubense* (*Foc*) cause devastating disease in this crop. Recently, efforts have been made to understand the mechanism of *Foc* infections (Wang et al. 2012) and the genetic basis of resistance to *Fusarium oxysporum* f. sp. *cubense* tropical race 4 by transcriptome profiling of resistant and susceptible Cavendish banana roots (Li et al. 2012). Using RNA-seq and digital gene expression (DGE) technique, these studies have shed light on the transcriptional changes in roots following infection and large differences in the transcriptome profile of resistant somaclonal variant and its susceptible wild type. The characterisation of tolerance to *Fusarium oxysporum* f. sp. cubense infection in banana using suppression subtractive hybridization and gene expression analysis along with host response, pathogen diversity, current understanding of biochemical and molecular changes which occur during host-pathogen (*Foc*) interaction and strategies to develop resistant genotypes has been reviewed (Swarupa et al. 2013, 2014).

In pomegranates, the fruit peel is a rich source of bioactive compounds, and work has been done on transcriptome analysis of peel at different stages of fruit development to unravel the genes and pathways involved in the synthesis of these compounds; also, genic SSR markers have been developed in this study (Ono et al. 2011). Recently, in Litchi, de novo assembly and characterisation of fruit transcriptome has been done to identify differentially regulated genes in fruit in response to shading. This study also sheds light on the molecular mechanism underlying severe fruitlet abscission process under the shade which leads to very low yield and significant economic losses for growers (Li et al. 2013).

In citrus, extensive gene expression studies have been conducted on different species as listed by NCBI (January 2013). A maximum number of studies (13) have been carried out on sweet orange (*Citrus sinensis*) due to its commercial importance, followed by *C. reticulata* (4), *C. paradisi* (3), *C. limona* (2), *C. clementia* (2), *C. trifoliata* (2) and one each in *C. medica*, *C. maxima*, *C. aurantifolia* and *C. limon*. These studies cover a wide range of problems like differential gene expression due to infection with pathogens in response to peel wounding, water deficit, etc. Comparative transcript profiling has been done

for seedless and wild-seeded orange, *C. reticulata* Blanco and stylar canal cells of *C. clementia*, to identify candidate genes implicated in self-incompatibility.

With the goal of discovering genes controlling consumer and grower traits in mango, gene discovery project has been started by the Queensland Primary Industries and Fisheries (Holton 2010). Traits like fruit quality and tree architecture are being focussed on using a multidisciplinary approach of sequencing expressed genes via ESTs and serial analysis of gene expression (SAGE), next-generation sequencing producing low-pass genome coverage, identifying candidate genes for fruit quality (aroma and colour) and tree architecture. Recently, the leaf transcriptome and chloroplast genome of mango have been characterised using NGS (Azim et al. 2014). A total of 24,593 annotated unigenes (80 % of total) has been reported and *C. sinensis* identified as the closest neighbour with 37 % matched sequences. The chloroplast genome in mango was reported to contain 139 genes, out of which 91 are coding protein.

In other crops like pineapple, persimmon, etc., few transcriptome studies have been done, while such resources and studies are yet to be taken up for several other horticultural crops of interest. Because of the various key functionalities of transcriptome-based gene discovery studies, it is essential to integrate these with other studies, like diversity analyses and combining transcript expression with metabolite levels and other 'omics' fields. By doing this, transcriptomics can become more useful than a descriptive gene cataloguing exercise and can aid in identifying key genes in a pharmaceutically/nutritionally relevant pathway (Hirsch and Buell 2013).

6.5 Molecular Markers: Marker-Assisted Selection (MAS) and Genetic Diversity

Molecular marker techniques (employing RFLP, RAPD, microsatellites, AFLP, SNP and other kinds of marker systems) represent a suite of powerful research tools for characterisation and management of genetic polymorphism (variation and diversity) in plant breeding and germplasm characterisation programmes. Where they are available and cost-effective, molecular markers can have a wide number of applications in plant breeding, the most commonly considered being marker-assisted selection (MAS). The FAO-BioDeC suggests that the earliest generation forms of DNA-based molecular markers (RAPD, RFLP) are more widely used than the more recently developed types of molecular markers (microsatellite and AFLP markers).

MAS is based on identification and use of markers which are linked to the gene(s) controlling the trait of interest (FAO 2003). By virtue of that linkage, selection may be applied to the marker itself. The advantage consists in an opportunity for speeding up the application of selection procedure. For instance, a character which is expressed only at the mature-plant stage may be selected at the plantlet stage if selection is applied to a molecular marker. Also, selection may be applied simultaneously to more than one character, and selection for a resistance gene can be carried out without needing to expose the plant to the pest, pathogen or deleterious agent. Finally, when there is linkage between a molecular marker and a quantitative trait locus (QTL), selection may become more efficient and rapid. The construction of detailed genetic and molecular maps of the genome of the species of interest is a prerequisite for most forms of MAS. However, the current cost of the application of these techniques is significant, and the choice of one technique rather than others may be dictated by cost factors. There are still very few examples of crop varieties in farmers' fields which have been developed based on MAS, largely because of the currently prohibitive cost of incorporating large-scale MAS into the budgets of most plant breeding programmes.

In addition to MAS, molecular markers can be used in germplasm characterisation. Compared to morphological and protein markers, DNA-based genetic markers are often considered to be the most useful for genetic diversity studies because they are highly polymorphic and heritable (their expression is not affected by

environmental variability). In recent years, different molecular markers (RAPD, RFLP, AFLP, SSRs and ISSR) have been employed for the investigation of cultivar origins and taxonomic relationships of several plant species (Manoj et al. 2010). Molecular approaches, such as RAPD for the estimation of genetic diversity (Chen et al. 2007; Feria-Romero et al. 2009), ALFP for genetic characterisation of genotypes (Hernández-Delgado et al. 2007) and SSRs for cultivar identification and linkage mapping (Risterucci et al. 2005), have been reported in crops such as guava, papaya, pomegranate, mango and several others. The FAO-BioDeC indicates that molecular markers are being extensively used in Latin America with 93 trials and 165 molecular marker projects at the research phase in nine countries. The assessment of the genetic diversity of Andean local roots and tubers in Latin America using molecular markers is an interesting development. Species reported to be included in molecular marker programmes in these countries are sugar cane, rice, cocoa, banana, bean and maize. The survey also indicates that most countries in Asia are undertaking a wide spectrum of crop research using molecular markers. Molecular marker-related research activities in Africa are reported to be underway in only a few of the countries, such as Ethiopia, Nigeria, South Africa and Zimbabwe; the range of Africa crops under study with molecular markers, however, is very wide: from traditional commodities to tropical fruits.

6.6 Linkage Maps and Association Studies

Conventional fruit breeding is primarily based on phenotypic selection with the help of morphological markers, but this takes years in case of woody species which have a long generation time and are mostly cross-pollinated. Also, the morphological markers are influenced by epigenetics and environmental factors. Traits of economic importance like fruit quality, yield, and precocity and disease resistance are polygenic and complex in nature. Therefore, advanced genetic tools like molecular markers (which can be used on any tissue at any time of plant growth, overcoming limitations of traditional methods) are being identified and used in the breeding/varietal identification of fruit trees. The most popular markers developed from genomic resources like ESTs include SSRs, SNPs and conserved ortholog set (COS) markers. SNPs are becoming more popular than SSRs as genetic markers in linkage analysis because these are more abundant and suitable for automation (Novelli et al. 2004; Selmer et al. 2009). In temperate fruit crops like the apple, Illumina Infinium SNP assay containing 8,000 SNPs is being used as a crucial tool for application of a novel selection strategy called genomic selection (GS) in breeding programmes. Availability of this assay has resulted in dramatic reduction in genotyping costs for large-scale genotyping of apple seedlings (Chagné et al. 2012). SNPs have been developed only for a few tropical fruit species like banana, where a small number of 20 SNP markers are available with NCBI. At present, the cost of developing and testing SNPs is still higher than that for developing SSRs. Nonetheless, SNP discovery is actively being pursued in crops like avocado (Arias et al. 2012), mango (Kuhn 2014; Sherman et al. 2014) and longan, under a USDA-sponsored project on genomics and single nucleotide marker discovery in horticultural fruit crops. In fruit trees, where the reference genome sequence exists, like papaya, clementine and sweet orange and banana, genotyping- by-resequencing using next-generation sequencing will allow rapid and efficient identification of SNPs and other types of polymorphism that are related to structural variations such as copy-number variations (CNVs), insertion-deletion polymorphisms (IDPs) and presence-absence variants (PAVs) between genomes. These can be used for genome-wide and candidate gene-based association mapping studies to identify QTL-trait associations (Khan and Korban 2012). Such studies have been made in cereal crops like maize, where extensive structural variation (including hundreds of CNVs and thousands of PAVs) has been discovered and implicated in important biological phenomena like heterosis (Springer et al. 2009).

A number of approaches have been developed to reduce the complexity of the genomes for cost-efficient and simplified discovery of SNP markers using NGS, like RNA-seq, complexity reduction of polymorphic sequences (CRoPS), restriction-site-associated DNA sequencing (RAD-seq) and genotyping-by-sequencing (GBS) (Davey et al. 2011). Among these, RAD-seq and GBS are proving to be particularly efficient in rapid and mass discovery of SNPs in highly heterozygous species. This is expected to help develop ultrahigh density maps and association mapping. Together with NGS, these techniques help to discover SNPs by sequencing a large set of restriction fragments. DNA fragments from one individual are ligated to a modified adapter containing a barcode. Fragments from many individuals can, therefore, be pooled together and sequenced on a single lane. The resulting reads can be separated bioinformatically by identifying the barcode. By sequencing a family or other populations of interest this way and comparing the tags to phenotypes of individuals, many biologically relevant SNPs and genetic loci can be identified in a single experiment. If each genotype of a mapping population has been characterised phenotypically for traits of interest and has an individual barcode, then RAD and GBS markers can be used routinely for linkage mapping and QTL analysis (Rowe et al. 2011). Using RAD-seq, Chutimanitsakun et al. (2011) recently constructed an RAD linkage map in barley and performed QTL analysis; Scaglione et al. (2012) discovered about 34,000 SNPs and 800 indels in globe artichoke; and Stölting et al. (2013) revealed the patterns of divergence and gene flow between ecologically divergent species of poplar. GBS information for hundreds of cultivars and few wild relatives has been used for development of a catalogue of segregating SNPs in cassava (Prochnik et al. 2012). Among these two markers, GBS holds more promise because of its ability to allow simultaneous marker discovery and genotyping with low-cost and simple workflow (Kumar et al. 2012). In grape, under the project VitisGen, GBS is being employed for a coordinated marker discovery and application using centralised phenotyping and map development (Davidson and Lance 2012). Under this project, over 5,000 breeding lines are being genotyped each year to track alleles introgressed from 11 *Vitis* species by mapping 20,000 SNP per population, including alleles not present in *V. vinifera* reference genome. It is expected that a marker set of at least 30 markers will be generated from this project for alleles controlling traits of interest for current and future grape breeding programme.

While SNP markers are the markers of choice for well-studied plant species, these may not address the needs of researchers working on crops with limited genomic information available. In such crops, microsatellite markers have been used in various areas like conservation of genetic resources, the establishment of core germplasm, population genetics, molecular breeding and fingerprinting of varieties (Ellegren 2004). The wide range of application of these markers is due to their codominant, multiallelic and highly reproducible nature; high resolution, amenability to high-throughput and polymerase chain reaction (Oliveira et al. 2006). These markers have been discovered traditionally by constructing genomic libraries enriched for a few, targeted SSR motifs and sequencing of clones containing SSRs. However, such library-based approaches are expensive, labour- and time-consuming and can isolate only the targeted, enriched SSR motifs (Zalapa et al. 2012). The various methods of genome-wide marker discovery (SSR and SNPs) and genotyping using NGS like reduced-representation sequencing using reduced-representation libraries (RRLs) or complexity reduction of polymorphic sequences (CRoPS), restriction-site-associated DNA sequencing (RAD-seq) and low-coverage genotyping can be used in both model crops with high-quality reference genome sequence and in non-model species with no existing genomic data. Low-coverage genotyping by pyrosequencing has been used to develop 171 highly polymorphic markers (60 % markers with PIC value >0.5) in pomegranate which will help in the breeding and conservation of this speciality fruit crop (Ravishankar et al. communicated, unpublished as of date).

Sequences generated by pyrosequencing were also mined to identify five conserved miRNAs in pomegranate (Kanupriya et al. 2013). Available genomic resources like ESTs are also used for isolating SSRs using bioinformatics tools. However, EST-derived markers are generally less polymorphic; thus, they are not very useful for developing linkage maps and for DNA fingerprinting (Kalia et al. 2011).

Linkage maps are used to show the position of known genes or genetic markers relative to each other in terms of recombination frequency, rather than a specific distance along each chromosome. These maps are important for the development of physical maps, mapping QTLs and association studies. In high-density maps, the markers are placed very close to each other throughout the genome (Tanksley et al. 1992). A large number of SSR/SNP markers and mapping population is required for developing a high-density linkage map. Availability of such maps along with phenotypic data is useful for predictive breeding since it helps in identifying the QTL regions associated with traits of interest. A physical map of a chromosome or a genome on the other hand shows the physical locations of genes and other DNA sequences of interest. These maps facilitate in the identification and isolation of genes by positional cloning. In the past decade, linkage maps have been prepared for a few fruit crops using various types of mapping populations and markers. QTL mapping has not been widely used due to the perennial nature of most fruit crops, because of which, establishing and maintaining biparental crosses and progenies of fruit trees in multiple locations are difficult and costly (Rikkerink et al. 2007). Also, the F1 progeny derived from full-sib crosses between two outbred parents is used for QTL mapping which gives a low resolution of QTLs. The QTL region has been shown to span ~ 5–10 cM, with likelihood of presence of hundreds of genes within this region (González-Martínez et al. 2007). Therefore, utilising QTLs in MAB without a fine mapping is not advocated due to genetic drag effects and/or rapid decay of linkage disequilibrium in most fruit trees (Khan and Korban 2012).

Association mapping (AM) has been proposed as a viable alternative to overcome the above limitations. This mapping technique has the potential to identify a single nucleotide polymorphism within a gene that is responsible for phenotypic differences. It involves searching for genotype-phenotype correlations between unrelated individuals. It has high resolution due to historical recombination accumulated in natural populations and collections of landraces, breeding materials and varieties (Flint-Garcia et al. 2003). Although AM presents clear advantages over linkage mapping, they are often applied in conjugation. The low-resolution linkage analysis is used to identify large candidate regions (1–10 Mb) within which fine mapping is used for identification of causal locus. Linkage maps therefore must be developed in fruit crops to validate the associations identified by AM, thus reducing spurious associations/false positives (Kover et al. 2009). An example where three maps have been used in conjugation is found in peach where classical linkage, physical map and association genetics analyses were combined to find alleles of SSR markers suitable for marker-assisted selection (MAS) of the flat peach character at both the parent and progeny levels (Picañol et al. 2013). Phenotypic characterisation of germplasm is a prerequisite for association mapping analyses. However, it is cumbersome and expensive for perennial plant species. Therefore, characterisation could be more efficient if focused on a reasonably sized subset of accessions or so-called core collection, reflecting the geographic origin and variability of the germplasm (El-Bakkali et al. 2013). Hence, there is an urgent need for identification of core collections in many of the horticulturally important fruit crops using markers.

6.7 Comparative Genomics

Comparative genomics is the study of the relation between structural and functional attributes of genomes across species generally within a family. Sequence information on several fruit

crops deposited in public databases can be utilised for both basic and applied research in related genera. This approach has been used mainly across families like Rosaceae, Brassicaceae and Myrtaceae. Comparative genomic studies have shown that genome evolution within family results from chromosomal rearrangements, leading to syntenic colinearity among large chromosomal regions. As a result, chromosomal regions where the marker order is highly conserved have been utilised in *Malus*, where primer pairs have been designed from conserved exon sequences flanking predicted intron-exon junctions in *Malus* cDNA sequences. Transferability of these primers to other rosaceous genera was found to be high, with primer pairs representing 85 % of genes amplifying products from *Fragaria* and primer pairs representing 85 % of genes, amplifying products from *Prunus* genomic DNA. These primers were screened in *Fragaria* and *Prunus* mapping bin sets, and 38 % of the genes were successfully located on both maps (Sargent et al. 2009). This approach has also proved useful for construction of first genetic linkage map of loquat (*Eriobotrya japonica*) by using SSR markers derived from *Malus* (Gisbert et al. 2009). Whole genome of *Eucalyptus grandis*, which is a member of Myrtaceae family, has been recently sequenced (Myburg et al. 2011). This sequence is currently in use as a reference to identify homologies in species belonging to the same family like guava (Ritter 2012). Development of various genomic tools such as genetic map, physical map, transcript map and map-based genome sequence, markers, in model/important crop of one family can be exploited in improvement of related crops, for example, mango, cashew and pistachio belong to Anacardiaceae, while in Moraceae family breadfruit, jackfruit, mulberry and fig are found. Whole genome sequencing of one of the members of these families will help in developing genomic resources for others (Ravishankar et al. 2013) making this as one of the most cost-effective methods in the development of genomic resources.

6.8 Database Resources

Large-scale genotype and phenotype projects along with the advent of next-generation sequencing technology have increased the amount of data generated and the scale at which analysis can be performed. There is many a software available for sequence analysis, creation of database and curation of data for plant genome. These databases have become an essential resource for experimental and computational biologists allowing users to take advantage of diverse data sets and provide a platform for comparative genomics. These resources are organised around a specific crop or related genera. Table 6.1 shows resources generated in fruit crops and provides curated records that have detailed and updated information. Such records provide researchers the essential genomic resources for molecular understanding of biological properties and for application of this knowledge. Such database resources are widely used in many horticultural crops like tomato (Iquebal et al. 2013; Suresh et al. 2014). The large resources of SNPs/SSR markers, especially in the genic region, QTLs, genes and miRNAs, would immensely benefit both breeders and the molecular biologist to utilise the natural trait reservoir and functional aspects of genes for crop improvement. It is therefore the development of an integrated database of SSRs, genes, miRNAs, known QTLs, SNPs and their location on chromosome which would greatly help crop biologists in understanding a crop and their efforts at crop improvement.

6.9 Phenotyping

The plant phenotype is the set of structural, physiological and performance-related traits of a genotype in a given environment. Phenotyping is the act of determining the quantitative or qualitative values of these traits. In recent years, with increasing availability of high-density genotypic information, our understanding of the components

Table 6.1 Database resource for fruit crops

Database	Crop	Resource	Institute
Genome Database for Rosaceae http://www.rosaceae.org/	Rosaceae species	Genes, genotypes, germplasm, maps, markers, phenotype, publications, QTL/MTL, species, unigenes	Supported by NSF Plant Genome Programme, the USDA Specialty Crop Research Initiative, the Washington Tree Fruit Research Commission, Clemson University and Washington State University
Citrus Genome Database http://www.citrusgenomedb.org/	Orange, grapefruit, mandarin, tangerine, lemon, lime and pummelo	Genomic tools like linkage maps, BAC libraries, physical maps, EST libraries, microarray platforms, and whole genome sequencing of sweet orange (heterozygous diploid) and mandarin (haploid)	Washington State University, University of Florida, Clemson University, Boyce Thompson Institute for Plant Research
Tree Fruit Genome Database Resource http://tfgdr.bioinfo.wsu.edu/	Rosaceae and *citrus*	Designed to facilitate the discovery of genes underlying important agricultural traits and the development of markers for genomics-assisted-breeding and enhance critical decision-making by apple, cherry, peach, strawberry and citrus breeders and growers	USDA NIFA Specialty Crop Research Initiative Programme
Grape Genome Browser http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/	Grape	Annotated genes, protein sequences, markers	Genoscope, INRA, Institute of Applied Genomics and a consortium of Italian groups
Kiwifruit Genome Database http://bioinfo.bti.cornell.edu/cgi-bin/kiwi/home.cgi		Annotated genes, protein sequences, markers	International Kiwifruit Genome Consortium (IKGC)
Carica papaya Genome Database http://www.plantgdb.org/CpGDB/	Papaya	Provides a convenient sequence-centred genome view for *Carica papaya*, with a narrow focus on gene structure, annotation	NSF-funded project

(continued)

Table 6.1 (continued)

Database	Crop	Resource	Institute
Banana Genome Hub http://banana-genome.cirad.fr/	Banana	Genome sequence along with gene structure, gene product information, metabolism, gene families, tanscriptomics (ESTs, RNA-seq), genetic markers (SSR, DArT, SNPs) and genetic maps	Cirad, Bioversity International, South Green
Cocoa Genome Database http://cocoagendb.cirad.fr/	Cocoa	Phenotypic, genetic and genomic data on cocoa	USDA, The University of Reading, BCCCA
TropGeneDB http://tropgenedb.cirad.fr/tropgene/JSP/index.jsp	Tropical crops: banana, cocoa, coffee, cotton, oil palm, rice, rubber, sugarcane	Manages genomic, genetic and phenotypic information	CIRAD

of phenotypic variation that are due to genetics has improved considerably. However, the genotype-phenotype relationship is expected to be more dependent on the availability of high-quality phenotypic and environmental information. Unlike genotyping, which is now highly mechanised, automated and uniform across organisms, phenotyping is still a species-specific, labour-intensive and environmentally sensitive methodology which requires integrated efforts of specialists from disciplines such as functional, quantitative and computational genetics/genomics, bioinformatics, modelling, physiology and computer science (reviewed by Cobb et al. 2013).

Several approaches are available for phenotyping, destructive or noninvasive measurements, in or ex situ, at a spatial resolution stretching from the subcellular level (nm-scale precision) to canopy stands (metre-scale precision) and temporal resolutions ranging from seconds to entire growing seasons. At the macroscopic level, shoot structure, growth, physiological status and photosynthetic performance can be measured in a range of crop species using optical methods (2D imaging and 3D reconstructions in the visible and invisible regions of the electromagnetic spectrum) and non-optical methods (e.g. microwaves). Phenotyping for complex traits is aided by analytical chemistry such as mass spectroscopy, high performance liquid chromatography, inductively coupled plasma spectroscopy, etc. Several 'omics' technologies (transcriptomics, metabolomics, ionomics, proteomics, methylomics, etc.) are also being used for this purpose. These are all highly automated, and useful, due to their high-throughput rates and accuracy (Cobb et al. 2013). Choice of the phenotyping approach depends on several factors like the biological question the researcher is trying to answer, the size of the population in question [e.g. less than ten individuals for precise physiological experiments to a moderate number of plants (200–400) for mapping studies or GS training populations or a large number of plants (400–1,000?) for association studies] and the heritability of the phenotype, controlled environment testing, tractability of the phenotype and resource availability (Cobb et al. 2013).

Examples of high-throughput phenotyping in tree species can be found in eucalyptus, poplar and apple. In eucalyptus and poplar, phenotypic assessment methods of physical and chemical properties of wood using near-infrared reflectance (NIR) together with the classical measurements of growth and wood quality tree properties have been reported by Poltri et al. (2011). In apple, fruit quality is an important trait, and large-scale standardised phenotyping using sensory and instrumental methods has been developed under RosBREED to enable data pooling across locations/institutes (Evans et al. 2011). Texture analysis in an apple progeny through instrumental, sensory and histological phenotyping has been reported by Gálvez-López et al. (2012) and high-throughput metabolic phenotyping by McGhie and Rowan (2012). From these experiences, we need to evolve methodologies for traits of interest in each tropical fruit tree crop through institutional collaborations.

6.10 Conclusions

Technologies available in the present day, like next-generation sequencing, are helping biologists decipher genome information in a short span of time and apply it to generate genomic information for non-model horticultural crops. NGS has led to sequencing of whole genomes and transcriptomes providing opportunity for discovery of new and useful information on the genetic control of important traits. Converting these into genetic tools like markers would enhance the efficiency and accelerate the breeding of these crops compared to conventional phenotype-based approaches. Currently, the application of these technologies is much slower in horticultural crops compared to others like cereals.

First, we need to employ these technologies in the discovery of markers like SSR and SNPs in large numbers in non-model crops. Also, generally, the laboratories involved in researching second-tier crops and non-model species have limited technical and financial resources; therefore, it is desirable that marker assays developed

should be easy enough to be implemented in laboratories with basic equipments. Such resources will aid in the development of linkage maps provided that large population with a known family structure is available for traits of interest. Another useful application of markers will be in identification and conservation of core group of individuals or genotypes which can be used for association studies. The NGS technologies will also help in sequence-enabled allele mining and exploration and exploitation of naturally occurring allelic variations in candidate genes controlling key traits for pre-breeding. This approach is expected to help identify new and useful haplotypes and guide development of allele-specific markers to be used in marker-assisted selection. Secondly, the NGS technology is also proving to be an important tool in de novo transcriptome assembly providing rapid, robust, inexpensive and informative assessment of transcriptome content, transcript abundance and diversity. A transcriptome can serve as a proxy for the genome and can be generated at significantly lower cost by whole transcriptome shotgun sequencing (RNA-seq method). High-throughput sequencing methods can also help identify known/novel small RNAs involved in the regulation of gene expression. Thirdly, comparative genomics, using genomic data sets from phylogenetically related species, can help assemble and interpret genomes and transcriptomes of desired plant species. Finally, there is a need to develop strong international collaborations and industrial and academic interactions to ensure adequate public and private funding of projects. This has been demonstrated in other crops like tomato, apple, maize and rice where major achievements have been made with regard to crop improvement.

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A History of Genomic Structures: The Big Picture

7

Nicolas Carels

Abstract

We present a comprehensive compilation of genomic structure evolution that should help the reader who is not familiar with genomics to understand the mechanisms that are shaping its structures over time. We believe that this understanding is essential to work with genomics in the sense that it should help to formulate productive hypothesis for new original works. We believe that the mechanism by which the extant genomic structures arose is more important than the shape of these structures since evolution is continuously at work. In addition, taking genomics under the evolution perspective gives the possibility to release a unified picture of its unlimited natural complexity to the reader without fair to be incomplete. It is amazing to realize how fast complex biological structures arose in the early time of Earth, and it has been our aim to try to give a constructive view of the life journey to the reader.

Keywords

Nuclear genome • Genome structure evolution • RNA world • Origin of codons • Regulatory sequences • Noncoding RNAs • LUCA • DNA repair pathway • Genome size • DNA methylation • Synthetic biology

7.1 Introduction: How Did Life Start? Understanding the Origin of Modern Genomic Structures

Life's major transitions were classified as the (i) compartmentalization of replicating molecules in the first cells, (ii) coalescence of replicating molecules to form chromosomes, (iii) use of DNA and proteins as the fundamental elements of genetic code and replication, (iv) consolidation

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of symbiotic cells to generate the first eukaryotic cells containing chloroplasts and mitochondria, (v) sexual reproduction involving the production (by meiosis) and fusion of haploid gametes, (vi) evolution of multicellular organisms from unicellular ancestors, and (vii) establishment of social groups composed of discrete multicellular individuals. All these transitions had consequences for the organization of genomes and led to the development of specific strategies among lineages. In every major transition, selection favoring increased levels of biological complexity has been opposed by genetic conflicts acting within and across levels of biological organization (see in Grosberg and Strathmann 2007). It is the management of these conflicts across successive generations that led cells to display the genome structure diversity observed in extant organisms. This diversity is so large and displays such intricated functions that it would not make sense to describe it without reference to its evolutionary context. Here, we aimed to review in a comprehensive way the challenges that drove the emergence of genomic structures during their evolution from prebiotic chemistry to complex multicellular organisms, with particular focus on plants.

7.1.1 Prebiotic Chemistry and the RNA World

The hypothetical emergence of RNA might have occurred during the period referred to as *prebiotic chemistry*, approximately 0.5 billon years after the formation of the solar system, i.e., *ca*. 4.5 billion years ago (Gya), by which time Earth's ocean had condensed (*ca*. 4.4 Gya). Many chemical and biochemical studies pertaining to the chemical origin of life, which were performed under simulated prebiotic conditions (Miller 1987), showed that cyclic autocatalytic systems mediated by small organic molecules might have undergone Darwinian evolution in agreement with the local conditions prevailing under prebiotic chemistry (Davies et al. 2009). This chemical activity is expected to have set the foundations for the emergence of a molecule that was able to encode information and catalyze functions in a similar way to that used by modern ribonucleotide acid (RNA). This model offers a solution to the difficulties linked to the synthesis of RNA (Bernhardt 2012). Dissection of fundamental innovations in the molecular machinery of translation revealed that translation appeared after the discovery of a large number of metabolic functions but before the enzymes that are necessary for DNA synthesis. The catalysis of a glycosidic linkage between nitrogenous bases and ribose according to a putative prebiotic chemistry has been recognized as problematic (Ricardo et al. 2004). If some prebiotic routes for the synthesis of both pyrimidine and purine nucleotides could be found (Glaser et al. 2007; Powner et al. 2009), then the abiotic synthesis of RNA polymers via oligomer condensation might have been sufficient to trigger the emergence of a replicating system based on RNA (Cheng and Unrau 2012). It is this inference that is actually expected from the *RNA world hypothesis* (coined by Gilbert 1986). According to this hypothesis, "the most critical event in life emergence is the apparition of a self-replicating molecule with catalytic function that can both copy itself and evolve by mutation. It is more parsimonious to conceive of a single type of molecule replicating itself than to posit that two different molecules (a nucleic acid and a protein capable of replicating that nucleic acid) were synthesized by random chemical reactions in the same place at the same time" (Gilbert 1986).

7.1.2 Peptides First?

However, peptides might also have preceded RNA emergence, or, at least, RNA and proteins might have coevolved. This hypothesis is known as the *proteins (or peptides) first hypothesis* (Andras and Andras 2005). As shown by Oba et al. (2005), peptides with catalytic activity could accumulate and participate in the multiplication of primeval proteins by *pseudo-replication* in a process of repeated drying-heating cycles; thus, without the need of an RNA-based translation system. Primeval proteins might have possessed the catalytic activity to promote the

formation of cyanide and purines from amino acids (McGlynn et al. 2010). The route of nucleotide formation from HCN in aqueous solution by physico-chemical means is relatively simple (Glaser et al. 2007; Roy et al. 2007; Powner et al. 2009), and it is thought to have contributed to the abiotic synthesis of RNA-like polymers via oligomer condensation to trigger the emergence of a replicating system based on RNA (Cheng and Unrau 2012). Interestingly, peptides are synthesized today by a complex and apparently universal protein machinery termed *non-ribosomal peptide synthetase* (NRPS) (Strieker et al. 2010) that does not involve RNA molecules and NRPS proteins are more ancient than the ribosomal proteins (Bernhardt 2012). It is unlikely, however, that protein synthesis occurred before the advent of the ribosome (Bernhardt and Tate 2010). However, after coding emerged, the sequences of noncoded proteins might have needed to be recapitulated by coded proteins. Thus, the phylogenetic signal can only go back to the point of recapitulation, which draws a veil over the history of the protein before the recapitulation point. The situation is different for noncoding RNAs such as ribosomal RNA and tRNA because these molecules were able to replicate prior to the evolution of ribosomal protein synthesis (Bernhardt 2012). With phylogenetic trees in hand, it is now possible to infer the sequences of ancestral proteins as they might have been 3 Gya and to study their enzymatic activity in the laboratory using recombinant DNA technology. Such reconstruction makes sense because successful adaptations tend to persist on large time scales. Evolutionary descent follows a path involving finite steps, but each descendant is continuously linked to its antecedents by individual practical changes in a genetic text (Yarus 2011).

7.1.3 The Primordial Replicator

In the context of prebiotic chemistry, it is interesting to note that a mineral replicator might have preceded the rise of RNA (Wächtershäuser 1988; Orgel 2000; Martin and Russell 2003). Montmorillonite clay might have participated to vesicle formation, thereby creating a pathway for the prebiotic encapsulation of catalytically active surfaces within membrane vesicles (Wächtershäuser 1988; Cavalier-Smith 2001). These vesicles can grow and divide upon agitation to give daughter protocells carrying newly replicated nucleic acids (Hanczyc and Szostak 2004). Some form of encapsulation is likely to have been a key early step in life. Encapsulation can protect a genome from degradation and predation and allows the cellular concentration of useful small molecules, thus enabling natural selection (Cech 2012).

Even if one considers that RNAs were coopted by proteins, it is likely that at least some of the cofactors now used by proteins came directly from the RNA world, in which they played a similar role in assisting catalytic functions. Actually, many naturally occurring RNA riboswitches are able to bind protein enzymes as cofactors (Bernhardt 2012). An appeal of the RNA world hypothesis is that it shows that the roles of genotype/replicator and phenotype/catalyst might have been played by the same molecule. The primordial replicator is expected to have given way to RNA ~4 Gya; therefore, the immediate ancestor must have been chemically related to RNA by a plausible transformation involving a few atoms (Yarus 2011). The earliest replicating polymers should have satisfied three primary conditions: (i) initial polymers should have had an intrinsic mechanism to facilitate their replication either abiotically or biotically, (ii) monomers should have been easily synthesized and polymerized abiotically, and (iii) the resulting polymers should have been able to promote a wide range of chemical reactions (Fig. 7.1). Systematic investigation of naturally occurring nucleic acid analogs led to the identification of threose nucleic acid (TNA) (Wächtershäuser 1988; Eschenmoser 2004). TNA pairs with itself and with RNA and is much more stable in water than RNA. Therefore, TNA might potentially serve as a template in nonenzymatic template-directed RNA oligomerization. Thus, a transition from a TNA world (based on a primitive replicating system, with relatively simple chemistry involving a stable phosphodiester backbone) to an RNA world is theoretically

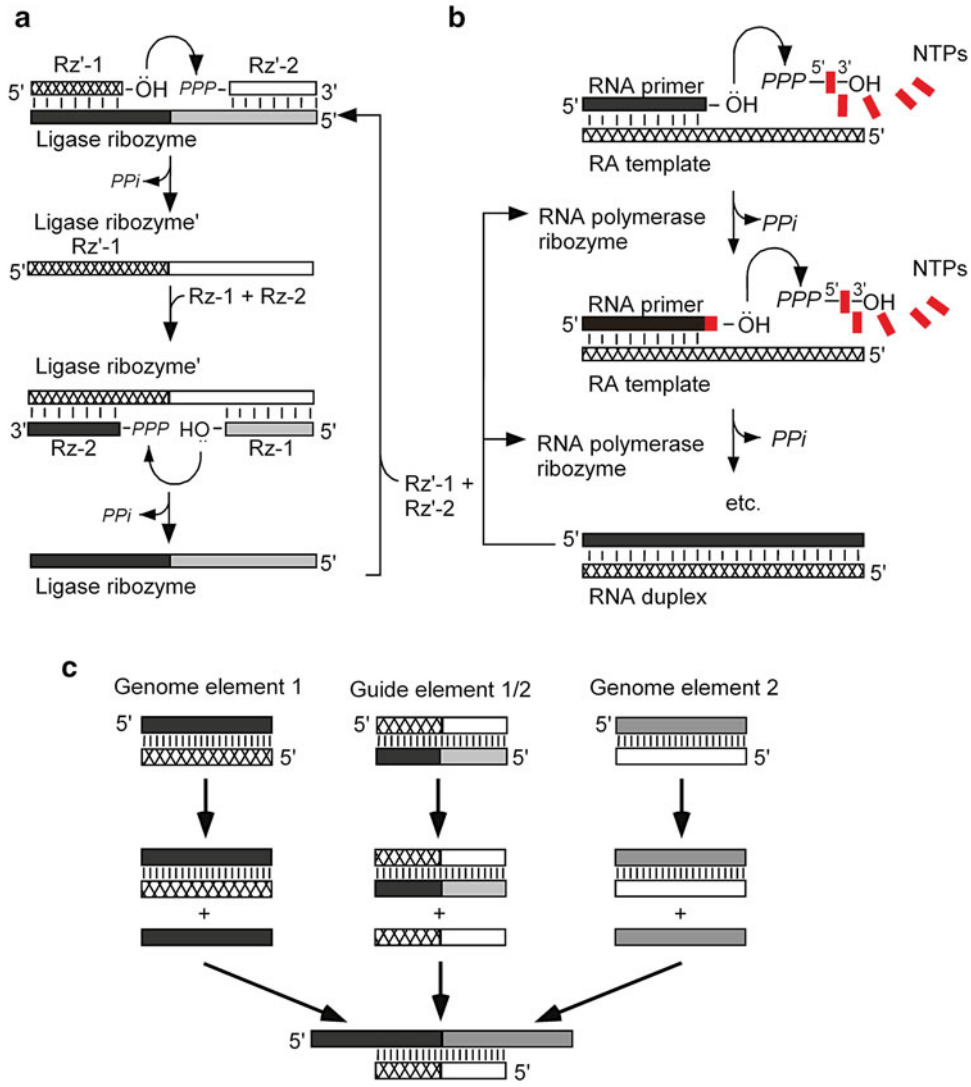

Fig. 7.1 Model of the putative primordial replicator. (**a**) Cross-replication of RNA ligase ribozymes: a ligase ribozyme (*gray and black*) catalyzes the ligation of *white* and *crossed* oligonucleotides (Rz′−1 and Rz′−2) to generate a ligase ribozyme that catalyzes the ligation of *black* and *gray* oligonucleotides (Rz−1 and Rz−2) to regenerate the first ligase ribozyme. (**b**) Ribozyme-catalyzed RNA polymerization. The complete extension of an RNA primer (*black*) according to the sequence of a template (*crossed*) by an RNA polymerase produces a RNA duplex. (**c**) The genome replication, i.e., short double-stranded elements of RNA (*left* and *right*), is guided by mixed priming (*center*). The asymmetrical transcription of either strand of the duplex genomic element results in the synthesis of multiple genomic element copies together with an excess of one strand (Modified from Cheng and Unrau (2012))

possible (Srivatsan 2004). Glycerol-derived nucleic acid analogs, peptide nucleic acids (PNAs), and other nonsugar compounds were also proposed as ancestral polymer backbones that could have acted as molecular supports for information (Benner et al. 2012). PNA forms stable double helices with RNA or DNA, and information can be transferred from PNA to RNA in a template-directed fashion. Polymers of N-(2-aminoethyl)-glycine (AEG) have also been hypothesized as possible backbones of PNAs because they form in spark-tube experiments and

can spontaneously polymerize at 100 °C. In addition, AEG polymers are synthesized by Cyanobacteria (Banack et al. 2012). Cyanobacteria are photosynthetic cosmopolitan bacteria that are supposed to have strongly contributed to the oxygenation of the atmosphere and occupy extreme habitats such as geothermal pools or hypersaline waters that arose early during Earth's history. The earliest evidence of Cyanobacteria, in Australian fossils, date from ~3.5 Gya (Schopf 1993). However, the catalytic potential of PNA, TNA, and other possible RNA analogs has not yet been explored in detail (Srivatsan 2004).

7.1.4 What Does RNA Do Today?

In contemporary biology, RNA acts as (i) a primer in DNA replication; (ii) a messenger that carries genetic information to the translation machinery (mRNA); (iii) a small noncoding RNA (snRNA) in spliceosomal intron splicing, which requires the collaboration of ~200 proteins and five snRNAs; (iv) self-splicing introns (groups I and II); (v) a primer for the telomerase in telomere extension; (vi) metabolite signaling molecules (riboswitches bind small metabolites; they use the binding energy to switch from one RNA structure to another and might affect gene expression in bacteria and eukaryotes); (vii) defenses against pathogens (RNAi); (viii) an endonuclease RNA (RNase P) that specifically promotes tRNAs maturation; (ix) a transcription modulator by switching off gene expression and attracting proteins that modify chromatin structure (the effect may affect one or few genes or can even spread over an entire chromosome as with *Xist RNA*); (x) a component (7SL RNA) of the signal recognition particle (SRP) responsible for the delivery of proteins to cellular membranes; (xi) a ribozyme (virtually all redox reactions in modern organisms can be catalyzed by RNA molecules by directed evolution, as with a NAD+-dependent redox-active ribozyme mimicking alcohol dehydrogenase activity. Ribozymes are also involved in functions of information processing, such as editing, ligation, polymerization, and replication); (xii) a modulator of gene expression (small double-stranded RNAs, e.g., 21-bp small interfering RNAs and microRNAs regulate the stability or translatability of mRNAs); (xiii) an active factor in the highly condensed state of heterochromatin at chromosome centromeres; (xiv) weapons (siRNA) to circumvent host defense by viruses or to manipulate host cellular machinery; (xv) a peptidyl transferase; (xvi) a mRNA start site; (xvii) codon–anticodon interaction agents; and (xviii) decoding centers in ribosomal operation.

7.1.5 SELEX: A Synthetic RNA World

It is generally considered that to be an acceptable model, the RNA world must support Darwinian evolution in the laboratory (Benner et al. 2012). One of the questions regarding the RNA world asks which mechanisms might have acted as a foundation for a primeval replication system suitable for Darwinian evolution. Guide RNAs, having the reverse complement of some target sequence, could have been very useful early in evolution and might have directed RNA ligase ribozymes to specifically ligate short RNA fragments in a sequence-specific fashion. Precisely, RNA genetic material might have been formed of RNA exons held together by self-splicing RNA introns. Intron out-splicing would have tied the exons together to make functional ribozymes (Fig. 7.2). This view (i) provides a way of distinguishing information storage (genome) from chemical reaction catalysis (ribozyme), (ii) provides a mechanism for enhanced illegitimate recombination by the RNA synthetase occasionally jumping from one molecule to another during replication and thus creating recombinant products (Darwinian evolution) (in addition, two introns surrounding an RNA exon can cut across the exon and construct a transposon that can then enter an intron in some other molecule – Gilbert and de Souza 1999), and (iii) allows the reduction of combinatorial space to be screened by looking for complex RNA functions among sequences constructed from the association of short RNA exons (30–40 bases long).

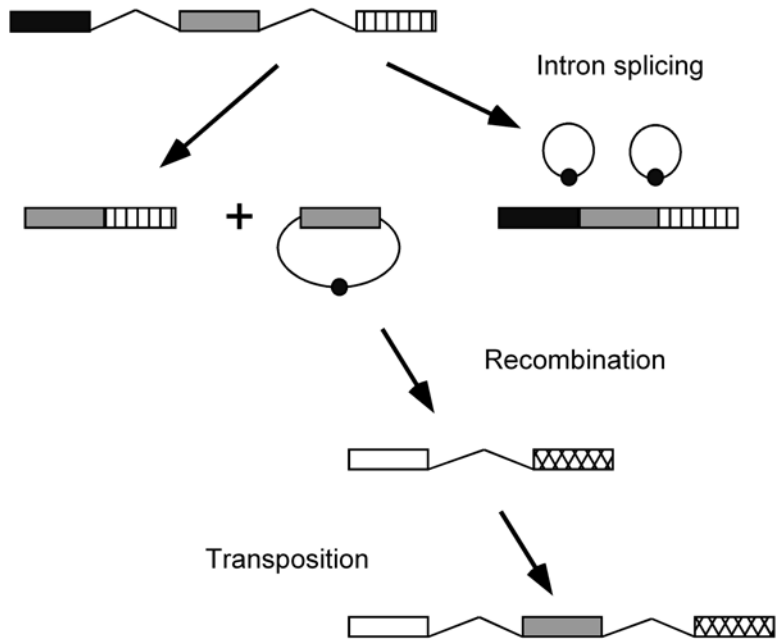

Fig. 7.2 Model of putative ancestral RNA gene with intron–exon structure. Type I introns might splice out to produce a ribozyme or might splice together to carry an intervening exon to a new position in a novel gene, i.e., transpose (Modified from Gilbert and de Souza (1999))

By analogy with proteins, a RNA enzyme (ribozyme) might need to be larger than 300 nucleotides to carry out a complex function. Considering 300-base ribozymes, a selection process for a given catalytic activity would require more than 4^{300} molecules to be screened. One way to circumvent this problem is to hypothesize that the enzymatic activity is the result of the combinatorial effect of short RNA pieces following an intron–exon structure (Gilbert and de Souza 1999). Currently, it is considered that if the best molecules in a library are present at a frequency of 10^{-9}–10^{-13} or less, a starting random library must be of the order of 10^{14}–10^{15} molecules (Gold et al. 2012). In vitro RNA selection provides the opportunity to explore the repertoire of RNA-catalyzed reactions and has been successfully used to isolate many new ribozymes. These ribozymes include catalysts for glycosidic bond formation in nucleotide synthesis, RNA polymerization, the aminoacylation of transfer RNA, peptide bonds, the Diels–Alder reaction, hydroxyl phosphorylation, and the Michael reaction (see in Srivatsan 2004). Laboratory-induced ribozyme evolution using the *systematic evolution of ligands by exponential enrichment* (SELEX) technique (Wright and Joyce 1997) led to the isolation of both pyrimidine nucleotide and purine nucleotide synthase ribozymes (Lau et al. 2004). Catalytic RNA activities that might have been needed in an RNA world, but have not been found in the RNA pool of contemporary organisms, are generally already present in large combinatorial libraries of RNA sequences and can be discovered using SELEX (Cheng and Unrau 2012). Ribozymes are comprised of sensing ligands and a catalytic platform. The sensing ligands are termed "aptamers" (derived from the Greek word aptus, "to fit") and are the output of the SELEX process (Gold et al. 2012).

Due to the establishment of the RNA world, improvements in RNA polymerization might have enabled the gradual concatenation of RNA genomic elements into longer fragments. This process only requires two replication activities: RNA ligation and RNA polymerization to transcribe and replicate short RNA genomic elements. Because the phosphodiester chemistry mechanisms of RNA ligation and polymerization are related, their emergence from an abiotic system might be evolutionarily linked and thus provide a simple route to biological replication (Cheng and Unrau 2012). Initial efforts during the 1990s led to the isolation and structural characterization of the highly efficient class I ligase ribozyme (Bartel and Szostak 1993) and,

subsequently, DSL ligase (Voytek and Joyce 2007). A synthetic ribozyme polymerase (B6.61) that is able to extend a primer-template duplex by 20 nucleotides (Zaher and Unrau 2007) was then developed in the laboratory. A ribozyme replicase that is able to replicate RNA stretches of 95 nucleotides has also been created in the laboratory (Wochner et al. 2011). Replication of nucleic acid polymers always involves the synthesis of sense and antisense strands following canonical base-pairing rules.

Fundamentally, replication is a copying process whose symmetry is broken during transcription where a single strand is over-copied in several fragments to allow gene expression. A significant advantage of dsRNA over ssRNA or folded RNAs is that the dsRNA duplex has a uniform and predictable double helix that makes it easily recognizable as a genetic component (genome) by replicative enzymes, thus forming a basis for the evolution of a repairing system. Thus, a synthetic genomic system would involve the combination of transcriptional asymmetry and the replication and ligation of short RNA genomic elements that would produce a self-consistent system of RNA replication capable of simultaneous expression of metabolic ribozymes (Cheng and Unrau 2012). One of the aims of the investigations described above was to create cellular cooperative systems that are capable of artificial RNA evolution (Bartel and Unrau 1999) for use in validating the RNA world hypothesis. If such a system was constructed in the laboratory, it would be much easier to understand the challenges associated with the very earliest steps of the complex metabolic systems that now dominate the Earth (Cheng and Unrau 2010). For now, RNA molecular interactions can be inferred from in silico simulations (Tinoco et al. 2010) because the prediction of RNA secondary and tertiary (3D) structures has improved dramatically in recent years (Mathews et al. 2010).

7.1.6 Protocells

Ancestral to the *last universal common ancestor* (LUCA), a primeval organism based on RNA would already contain proteinaceous material in the form of oligopeptides to support RNA enzymes. It is now clear that the formation of peptide bonds in modern cells is catalyzed by RNA moieties of the ribosome (Moore and Steitz 2002). Therefore, the emergence of the ribozyme ancestor of today's ribosomes was a crucial transitional step in the history of the RNA world, together with the establishment of an earliest version of the present genetic code (Forterre 2005). Peptide synthesis would have begun with a few amino acids and a single transfer/activating RNA. A simple hairpin duplication–ligation mechanism has been proposed for the ancestor of tRNA by Di Giulio (1992). The number of amino acids would have extended to 20 during the transition from an RNA world to an RNA–protein world (Gilbert and de Souza 1999). Gradually, protein chains would have increased in complexity and replaced ribozymes in a number of biochemical processes. Even if amino acids and short peptides were already present in prebiotic times, large self-folding proteins could only have appeared after the emergence of a primeval ribosomal system.

RNA clearly preceded DNA because multiple enzymes are dedicated to the biosynthesis of the ribonucleotide precursors of RNA, whereas deoxyribonucleotide biosynthesis is a derivative of ribonucleotide synthesis and requires only two additional enzymatic activities (thymidylate synthase and ribonucleotide reductase). DNA took over the role of genome approximately 1 Gya. The LUCA already contained a DNA genome and carried out biocatalysis using both protein-based and ribonucleoproteic (RNP) enzymes (such as the ribosome) and ribozymes (Cech 2012). Life radiation would have been impossible without the cellular confinement of metabolism. Several membrane-related proteins were already present in the LUCA, and this can be considered a definitive argument that the LUCA was formed by cellular organisms. The RNA world should be seen as a biosphere containing cells with RNA genomes (RNA cells); this world ended when all of their cellular descendants were eliminated by Darwinian competition from newly emergent cells with DNA genomes (DNA cells). Selective pressures would have induced organisms to modify their genomes to protect them

against RNAses from hostile competitors. The higher stability of DNA (compared to RNA) would have allowed the genome size to increase during evolution, in turn allowing the evolution of new metabolic functions in DNA cells beyond those that existed in RNA cells (Forterre 2005).

7.2 The Genetic Code and Genes

7.2.1 Origin of Codons

Proteins are chains of amino acids that constitute most parts of the cell machinery. Together, 20 amino acid types are present in proteins, and a typical protein comprises a string of several hundred amino acids. Each amino acid is encoded by a three-base nucleotide sequence termed a codon. With four different letters in the DNA alphabet, $4 \times 4 \times 4 = 64$ different codons are possible; 61 of these specify amino acids, and three represent stop. The question of how this genetic code originated in a primordial world remains open.

A curious observation made during the application of SELEX is that aptamers selected to bind arginine (Arg) in different laboratories using different protocols are predominantly composed of Arg codons (Knight and Landweber 1998). This finding, together with the finding that experiments simulating the early Earth's atmosphere (Miller 1987) yielded as many as ten different natural amino acids, lays the foundation for a theory that the genetic code evolved under primordial conditions (Wächtershäuser 1988; Orgel 2000) still dominant in modern proteins (Carels and Ponce de Leon 2015).

Alanine (Ala) and glycine (Gly), the most abundant amino acids resulting from Miller's experiments, are encoded today by the complementary triplets GCC and GGC, respectively; this finding suggested the hypothesis that complementarity and thermostability were important for the evolution of the triplet code (Eigen and Schuster 1977, 1978a, b). Presumably, the earliest minigenes carried messages encoding miniproteins based on two independent alphabets: a Gly alphabet encoding Gly strands and an Ala alphabet encoding Ala strands. At a later stage, minigenes fused together in longer sequences, and the resulting sequences had a mosaic structure comprising short patches of the two alphabets (Trifonov 2004). AGE aptamers recognizing the amino acids contained in the prebiotic soup, as defined by Miller's experiment, and a primeval code of 3 AGE letters might have been the starting point of a pre-RNA world genetic system. As seen above, the enzymatic activity of AGE or any other type of polymer easily catalyzed under primeval Earth conditions remains to be demonstrated. If such polymers do, like ribozymes, have enzymatic activity, they might have been ancestral candidates for a pre-RNA world genetic code. Riboswitch-like molecules would progressively have captured more and more amino acids from the prebiotic soup and evolved together with their substrate: the primeval genetic code. Ribozyme-like molecules could have carried out the peptide synthesis of these primeval proteins. A GNC code (G for guanine, N for any of the four nucleotides, and C for cytosine) is sufficient to encode the four amino acids Gly, Ala, aspartate (Asp), and valine (Val) able to form globular, water-soluble proteins with α-helix, β-sheet, and β-turn secondary structures (Ikehara 2009) that are capable of catalytic activities (Oba et al. 2005). According to Ikehara (2009), this primitive code would have evolved first to a code linking 16 codons and 10 amino acids, the so-called SNS (S for strong: G or C), and then the RNY (R for purines, Y for pyrimidines) ancestral codons (Carels and Frias 2013).

The chronology of amino acid–codon pair association would have followed the order: Gly, Ala, Asp, Val, proline (Pro), serine (Ser), glutamate (Glu) [leucine (Leu), threonine (Thr)], Arg [isoleucine (Ile), glutamine (Gln), asparagine (Asn)], histidine (His), lysine (Lys), cysteine (Cys), phenylalanine (Phe), tyrosine (Tyr), methionine (Met), and tryptophan (Trp) ("[" and "]" identifies the amino acids that appeared at the same time). The genetic code would have evolved toward higher complexity by successive amino acid capture according to successive transition steps in Gly-strand and Ala-strand modes. A purine transition at the 2nd position of the Gly codon GGC would have successively led to GAC

and GAG to capture Asp and Glu, respectively. Arg would have been captured by the CGC codon, which is complementary to the Ala codon GCG on the Ala strand. Up to this point, the Gly strand would have encoded Gly, Asp, Glu, and Arg, whereas the Ala strand would have encoded Ala and Val. The most frequent transitions (purine to purine and pyrimidine to pyrimidine) would have left the N-purine-N and N-pyrimidine-N triplet structures unchanged. All later stages in codon evolution would have required changes at the third position and, consequently, in the first positions of the codons on the complementary strand. The two alphabets Gly, Asp, Glu, Arg, Ser, Gln, Asn, His, Lys, Cys, Tyr, Trp (the Gly alphabet) and Ala, Val, Pro, Ser, Leu, Thr, Ile, Phe, Met (the Ala alphabet) would have successively appeared in descending order of thermostability (Trifonov 2004). Tryptophan and cysteine (as well as selenocysteine and pyrrolysine) codons were likely borrowed from a putative UGN repertoire of terminators (Trifonov 2004).

Modern proteins follow an amino acid bias that is led by physicochemical constraints (hydropathy and secondary structures) on proteins, highly correlated with a linear regression with thymidine in the 2nd codon position (T2). The choice of secondary protein structures for a given catalytic activity depends on the energy available in the biological system because the energy cost of secondary protein structures follows the series: strand > helix > turn. In contrast, the amino acid choice is optimized in such a way that the minimal energy cost (the number of chemical bonds in the amino acid residue) of amino acid synthesis is associated with a larger purine content in the 1st position (R1) than in codon positions 2 or 3 (Ponce de Leon et al. 2014, Fig. 7.3). Thus, one can conclude that the RNY (or Rrr, Carels et al. 2009) ancestral codon reflects selective pressures on protein functionality, energy costs and thermodynamic constraints on the translation machinery (Ponce de Leon et al. 2014). Protein-coding DNA sequences can be distinguished from nonprotein-coding DNA

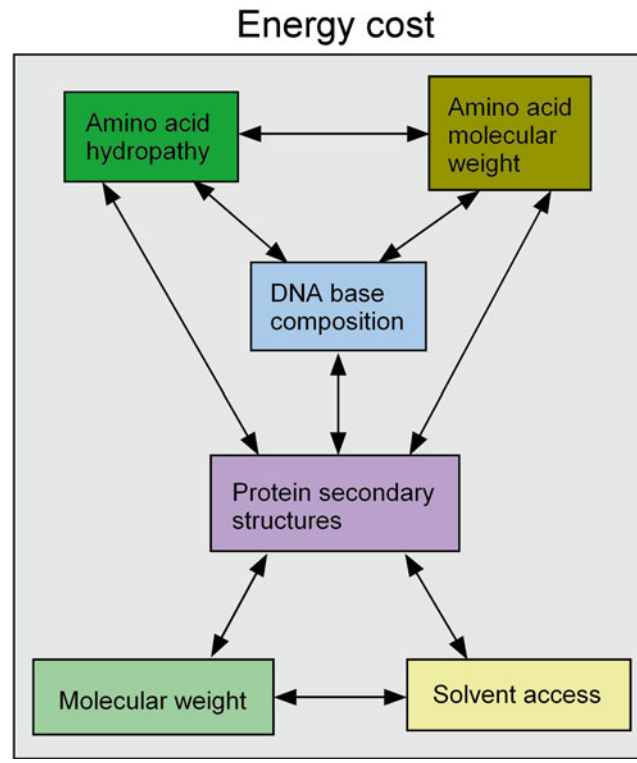

Fig. 7.3 Physicochemical properties of proteins that affect nucleotide composition in the three positions of codons

based on the purine bias (Rrr) across codons (Carels and Frias 2009, 2013). Because the codon structure of CDSs is a universal pattern across the biosphere, CDSs are the easiest DNA motifs to classify; approximately 95 % of folding proteins are larger than 100 amino acids (300 bp), corresponding to the minimal size required to detect a coding frame among the six possible frames with a statistical consistency of approximately 95 % (Carels and Frias 2009, 2013).

7.2.2 A Definition of Genes

The current understanding of a gene is that it represents a basic unit of genetic information that controls a specific aspect of a phenotype. According to this definition, which is operational in the field of selective breeding, one must consider the 5′ and 3′ noncoding regions as part of a gene at the molecular level because these regions are involved in the regulation of CDS transcription and translation (see Gerstein et al. 2007). Given the complexity of the gene regulation and expression processes, a full explanation of the molecular consequences of the gene concept can be eventually confusing; Fig. 7.4 that depicts how genes are translated into phenotype effects.

In many cases, the multiple alignments of protein sequences sharing a given function across the three superkingdoms (Bacteria, Archaea, and Eukarya) show a patchwork of similar and dissimilar stretches suggesting that similar stretches are homologous, but dissimilar stretches are not. This observation suggests that modern genes considered homologous today might have been assembled from shorter primeval polyphyletic elements during the formation of the LUCA by a process of mini-gene trans-splicing (Di Giulio 2008). According to this hypothesis, the translation of mRNAs from mini-gene patchworks resulted in proteins whose genes (DNA) would have evolved much later, i.e., after the divergence of the three superkingdoms (Di Giulio 2006). In this way, one can explain why multiple alignments of the same protein from organisms belonging to the three domains of life can be obtained and why they apparently define a monophyletic origin of genes. The hypothesis of a polyphyletic origin of genes is consistent with Gilbert's proposition (1986), presented above, that genes evolved as a former patchwork of exon/introns; thus, the appearance of large coding sequences (CDS) was only enabled by intron excision, a phenomenon still largely exploited by eukaryotes. It would not be surprising that trans-splicing (still in operation in protozoa) would have been a major player in the RNA world and that CDSs from exon shuffling might have been easily exchanged among protocells (Hanczyc and Szostak 2004) as plasmids are still exchanged among bacteria through conjugation.

7.2.3 Introns and Spliceosome Machinery

Introns are noncoding intervening sequences that are not part of the coded protein message and must be removed from RNA transcripts to produce functional mRNAs. Active mRNAs are comprised of exons concatenated into CDSs by splicing out introns from pre-mRNAs.

Group I and II introns are found in some bacterial and organelle genomes, and group I introns

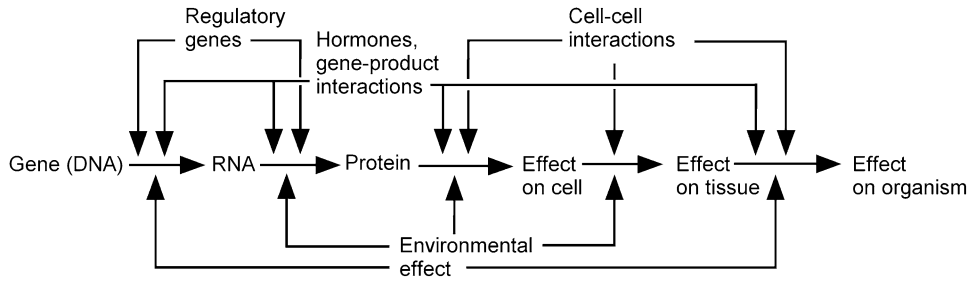

Fig. 7.4 The pathway from gene to phenotype expression

are also found in the ribosomal RNAs (rRNAs) of protist and fungal nuclei. These two groups have distinct RNA structures that facilitate their self-splicing activity. These introns may also contain internal ORFs, facilitating both their splicing from RNA transcripts and propagation to intronless sites by reverse transcription. In total, approximately 1,500 group I and 200 group II introns have been identified. The original invasion of self-splicing Group II introns, presumably originating from the mitochondrial endosymbiont, into the genome of the emerging eukaryote might have led to alternative splicing, a major contribution to the biological complexity of multicellular eukaryotes (Rogozin et al. 2012). Spliceosomal introns are present in the nuclear genomes of all characterized eukaryotes but absent in prokaryotes. Their numbers and lengths vary widely between species, from tens of bases in some protists to hundreds of kilobases in mammals. The spliceosome is a complex (Ritchie et al. 2009) that comprises five small nuclear RNAs (snRNAs) and hundreds of proteins. Each snRNA is associated to proteins to form a small nuclear ribonucleic particule (snRNPs). Surprisingly, two spliceosomal complexes emerged before the origin of multicellularity. The *major spliceosome*, a complex RNA–protein machine containing the U1, U2, U4/U6, and U5 snRNPs, splices the classic set of introns that usually start with GT and end with AG (Fig. 7.5). By contrast, the *minor spliceosome* involves four specific snRNPs and the U5 snRNP, which is shared by both spliceosomes. The minor spliceosome processes a different set of introns, many of which start with AT and end with AC, that is, introns that are ~300 times less frequent than those processed by the major spliceosome (Steitz et al. 2008).

Prokaryotes experienced a massive intron loss since the LUCA; it has been proposed that this loss is one of the many outcomes of a deterministic trend toward the reduction of the number of rate-limiting factors in gene expression that is determined by a strong selection pressure for rapid reproduction and/or survival at higher temperatures (Rodríguez-Trelles et al. 2006). In eukaryotes, 20–68 % of introns are specific to each species; 25 % of human introns are found at the same positions as those in orthologous genes from *Arabidopsis*. It is also clear from the homologous comparison of genes in maize and *Arabidopsis* that GC-poor and GC-rich genes are under different selective constraints (Carels and Bernardi 2000a). The bias of GC-poor genes toward high intron number has been attributed to the fact that the regulatory functions of intron sequences depend on specific AT constraints for correct splicing efficiency (Carels 2005a). Such constraints would justify why position, but not size (correlated to genome size), is conserved between distant species. The classical view is that intron sequences are subject to weak purifying selection at best or evolve in a regime that is indistinguishable from neutral evolution. However, as noted by Rogozin et al. (2012), "this

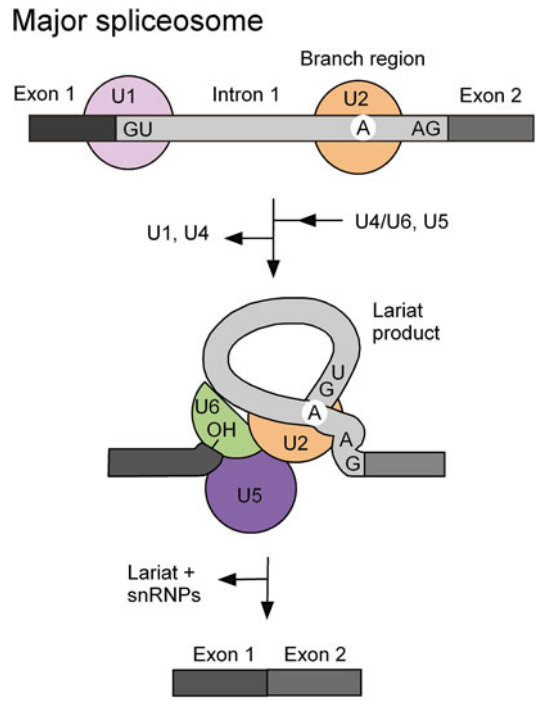

Fig. 7.5 Splicing of pre-mRNA by the major spliceosome. Conserved motifs are found at the 5′ and 3′ intron extremities as well as at the branching region (A). Stepwise assembly of U1, U2, U4/U6·U5 snRNPs with pre-mRNA introns at conserved regions forms the spliceosome, which catalyzes sequential transesterification reactions leading to the intron splicing out as a Lariat product and the assembly of contiguous exons in a coding sequence

(nearly) neutral background of intron evolution does not rule out the possibilities that, first, the very presence of introns affects the regulation of expression of the respective genes (presumably through the interaction with the splicing machinery) and hence their function, and second, that many introns harbor specific functional elements. Indeed, there is abundant evidence that introns are often functional at both levels." These authors reviewed the functions associated with (i) splicing (splicing and mRNA export are directly coupled), (ii) alternative splicing and exon shuffling, and (iii) genes nested within introns (for instance, miRNA or lncRNA genes – Yang et al. 2012; Geisler et al. 2012).

7.2.4 Regulatory Sequences

Eukaryotes employ diverse mechanisms to regulate gene expression, including chromatin condensation, DNA methylation, transcriptional initiation, alternative RNA splicing, difference in mRNA stability, translational controls, several forms of post-translational modification, intracellular trafficking, and protein degradation. Genes encoding regulatory proteins possess some of the most complex expression profiles. In metazoans and metaphytes, most such genes are expressed in several distinct domains (see in Wray et al. 2003). Although the transcription profiles of housekeeping genes are generally much simpler, most genes are transcribed at different levels among different cell types.

7.2.4.1 Transcription Factors and Their DNA Targets

The function of a promoter is to integrate information about the status of the cell and to tune the rate of transcription of a single gene or operon accordingly. The core promoter is a ~100 bp region whose function is to provide a docking site for the transcription complex and to position the start of transcription relative to CDSs (Lee and Young 2000). The organization of promoters is much less regular than that of CDSs, and no consistent sequence motifs exist for the promoters of protein-coding genes in eukaryotes. However, two functional features are always present: a core promoter and a collection of diverse transcription factor binding sites that tune specificity and the level of transcription.

Eukaryotic genes that encode proteins are transcribed by the RNA polymerase II holoenzyme complex, which is composed of 10–12 proteins (Lee and Young 2000). Core promoter sequences differ among genes. For many genes, the critical binding site is a TATA box, usually located approximately 25–30 bp 5′ of the transcription start site. However, most genes lack a TATA box, although some of these may contain an initiator element spanning the transcription start site or neither a TATA box nor an initiator element. Some core promoters may also contain additional protein binding sites for general transcription factors (Fig. 7.6). A gene may have more than one basal promoter, each of which initiates transcription at a distinct position, and both TATA and TATA-less basal promoters can be associated with alternate start sites of the same gene (Goodyer et al. 2001).

The complement of active transcription factors within the nucleus differs in response to environmental conditions during development across regions of the organism and among cell types. This changing array of transcription factors provides nearly all of the control over when, where, at what level, and under what circumstances a particular gene is transcribed (Wray et al. 2003).

Most transcription factor binding sites span 3–8 bp. Given that there are many different transcription factors and that each may bind many different short sites, every kilobase of genomic DNA contains many potential transcription factor binding sites on the basis of similarity (Stone and Wray 2001). However, many of these consensus matches do not bind protein in vivo and have no influence on transcription. Due to DNA looping between proteins that are associated with DNA sequences at distant binding sites, the physical extent of cis-regulatory regions varies by a few hundred base pairs to ~100 kb. The position of transcription factor binding sites relative to the

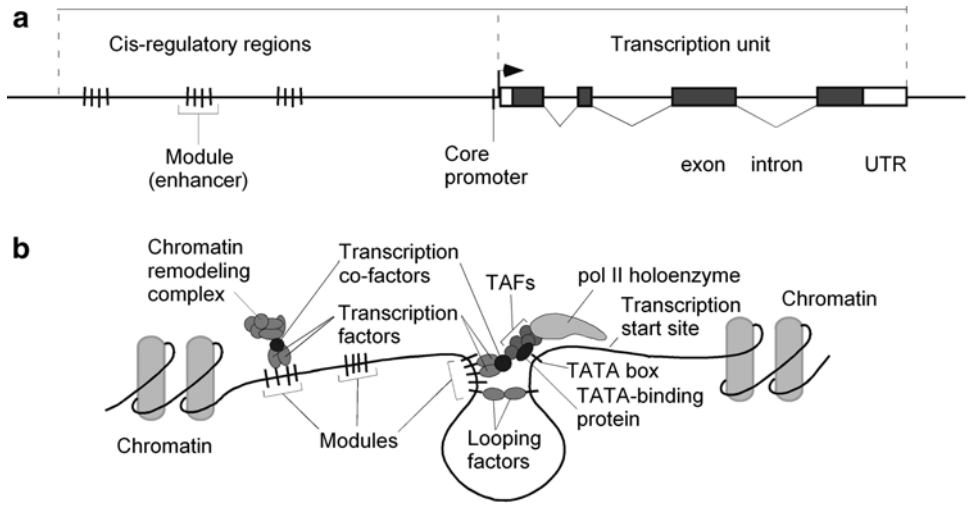

Fig. 7.6 Promoter structure and function in eukaryotic genes. (**a**) Relative position of the transcription unit, basal promoter region (*black box*), and transcription factor binding sites (*bars*). (**b**) Model of a promoter in operation. The initiation of transcription requires several dozens of interacting proteins, including the RNA polymerase II holoenzyme complex, TATA-binding protein, general transcription factor proteins (TAF), transcription factors, transcription cofactors, and chromatin remodeling complexes (Modified from Wray et al. (2003))

transcription unit also differs enormously among genes. Transcription factor binding sites often lie within a few kb 5′ of the core promoter.

All proteins that regulate transcription directly or indirectly influence the frequency with which the polymerase II complex assembles onto the basal promoter. This influence occurs through a wide variety of protein–protein interactions. Some transcription factors maintain local chromatin in a decondensed state whereas others condense it (Chen and Courey 2000). Chromatin remodeling is highly dynamic and is apparently regulated on spatial scales as small as promoters or even regions within a promoter. Condensed chromatin impairs the docking of the RNA polymerase II complex and blocks transcription in the absence of specific transcription factors. Because transcription is inactivated by default, all promoters contain binding sites for transcription activators, but only some contain binding sites for negative regulators (see in Wray et al. 2003).

Clusters of approximately 6–15 binding sites for 4–8 different transcription factors may operate as functionally coherent segments termed enhancers (Arnone and Davidson 1997). The terms enhancer, booster, activator, insulator, repressor, locus control region, upstream activating sequence, and upstream repressing sequence all refer to various types of regulatory sequences with specific features. Activators and repressors can function as Boolean (off/on) or scalar (quantitative) elements whose interactions have predictable, additive effects on transcription (Tuch et al. 2008). Deleting one of these regulatory sequences often eliminates a specific aspect of the expression profile without disrupting the remainder. In addition, predictable artificial expression profiles can be constructed by combining modules from different promoters. Promoters integrate multiple diverse inputs and produce a single scalar output, i.e., the rate of transcriptional initiation. In many promoters, signal integration occurs at the basal promoter through specific interactions between bound transcription factors and components of the RNA polymerase II enzyme complex (Lee and Young 2000).

Most transcription factors directly regulate a few percent of the genes. Genetic networks are therefore highly connected, and each node that is represented by a transcription factor is linked to many other nodes. This high degree of connectivity may be responsible in large part for the classical genetic phenomena of epistasis, polygeny,

and pleiotropy. The expression profile of a gene is a system property; thus, even if a mutation in a promoter region alters transcription, the network of functionally interacting genes and gene products may modulate this effect. Feedback loops are rather common components of gene networks (Lee et al. 2002) and may mask some functionally significant mutations in promoters. The number of functional noncoding nucleotides is approximately equal to the number of protein-coding nucleotides, and approximately half of all functional or phenotypical molecular evolution involves non-CDSs. Thus, a substantial fraction of a eukaryotic genome is devoted to extracting information from itself. Because transcriptional regulation conditions the way in which genotype is converted into phenotype, functional genetic variations in promoter sequences within populations are sorted by selection. Many mutants that have emerged from genetic screens involve quantitative effects on transcriptional regulation and are constituents of quantitative trait loci (QTL).

The gradual modification of transcription circuits over evolutionary timescales is an important source of the diversity of life. Natural selection may operate on CDS expression and phenotype morphology by promoting sequence variations at promoter sites through (i) negative (purifying) selection; (ii) positive selection, where some promoter alleles appear to be under directional selection; and (iii) overdominant selection, where similar clusters of transcription factor binding sites are sometimes present in the promoters of orthologous genes of species that diverged up to 10^7–10^8 years ago (Tümpel et al. 2002). In contrast, promoter sequences can also diverge extensively among even relatively closely related species by including gains or losses of multiple binding sites and changes in the position of regulatory sequences relative to the transcription start site. Regulatory sequences inserted into promoters through transposition may exert an influence on transcription. Through exonization, retroposition can create novel genes that are subsequently expressed (Wang et al. 2002). Genes that arise through retroposition are often expressed in tissue-specific patterns similar to those of a parent locus (Bétran et al. 2002).

7.2.4.2 Noncoding RNAs

Noncoding RNA (ncRNAs) genes produce transcripts that function as RNA, such as ribosomal RNA (rRNA) and transfer RNA (tRNA), rather than encoding proteins. On the basis of their length and genomic locations, ncRNAs can be further classified as (i) small ncRNAs including miRNAs and small interfering RNAs (siRNAs), (ii) natural antisense transcripts (NATs), (iii) long intronic noncoding RNAs (incRNAs), and (iv) long intergenic noncoding RNAs (lincRNAs). RNAs in the last three categories are at least 200 nucleotides (nt) or longer, and they are referred to as long noncoding RNAs (lncRNAs). Several lncRNAs have biological function as regulators of gene expression both transcriptionally and posttranscriptionally. Similar to most mRNAs transcribed by RNA polymerase II, lncRNAs are both capped and polyadenylated. It has been proposed that lncRNAs are used as a means to tightly maintain repression at inducible genes and that efficient clearance of the lncRNA by decapping through DCP2 (decapping proteins) is vital for robust gene activation (Geisler et al. 2012). In plants, thousands of lncRNAs were identified in *Arabidopsis*. A large number of these lncRNAs are responsive to abiotic stresses. Genomic loci of many lncRNAs are associated with histone modifications and DNA methylations suggesting an epigenetic regulation of these loci. In addition, some sense and antisense double-stranded RNAs involving lncRNA partners are processed by the RNA interference machinery into siRNAs (Jin et al. 2013).

The category of small ncRNAs (smRNA) encompass ~20–30 nt RNA molecules that have emerged as critical regulators in the expression and function of eukaryotic genomes including chromatin structure, chromosome segregation, transcription, RNA processing, RNA stability, and translation. Short interfering RNAs (siRNAs) and microRNAs (miRNAs) act to regulate endogenous genes and to defend the genome from invasive nucleic acids (virus, mobile elements). The effects of small RNAs on gene expression and control are generally inhibitory, and the corresponding regulatory mechanisms are referred to as RNA silencing. smRNAs serve

as specificity factors that direct bound effector proteins to target nucleic acid molecules via base-pairing interactions. The core component of the effector machinery is a member of the ARGONAUTE protein superfamily that mainly functions in eukaryotes. Single-cell organisms such as the green algae *Chlamydomonas reinhardtii* also produce miRNAs (Molnar et al. 2007). The common features of miRNAs and siRNAs in algae and protists indicate that complex RNA-silencing systems evolved before multicellularity and were a feature of the LUCA (Makarova et al. 2009). From the three main categories of smRNA – siRNAs, miRNAs, and piwi-interacting RNAs (piRNAs) – only siRNAs and miRNAs are present in plants. These smRNAs are only known to be present in eukaryotes, although the ARGONAUTE proteins (or their CRISPR protein homologues) can also be found scattered in prokaryote species. Thus, the boundaries between the various small RNA classes are becoming increasingly difficult to discern. siRNAs and miRNAs are the most broadly distributed in both phylogenetic and physiological terms and are characterized by the double-stranded nature of their RNA precursors (dsRNA). They trigger the silencing of genes with homologous sequences through RNAi.

A transcript may encode clusters of distinct miRNAs, or it may encode a miRNA and a protein. The latter type of transcript is organized in such a way that the miRNA sequence is located within an intron (see in Carthew and Sontheimer 2009). Given the small size of intronic sequences in plants, intronic miRNAs in rice and *Arabidopsis* are coexpressed with their host genes. Exonic miRNAs are mostly found in retrotransposon exonic sequences and participate to their silencing (Yang et al. 2012). A transcript is then processed into 20- to 25-nt siRNAs or microRNAs (miRNAs) by RNase III-like enzymes called Dicers. These siRNAs bind to effector protein complexes, termed RNA-induced silencing complexes (RISC), to mediate degradation of cognate mRNA, translational repression, or transcriptional silencing (Fig. 7.7). RISCs contain several proteins, including a member of the AGO clade of ARGONAUTE proteins, which has a smRNA binding domain (PAZ) that provides slicing activity to RISCs and programs it to guide sequence-specific inactivation of complementary RNA or DNA (see the pathway in more details in Voinnet 2009).

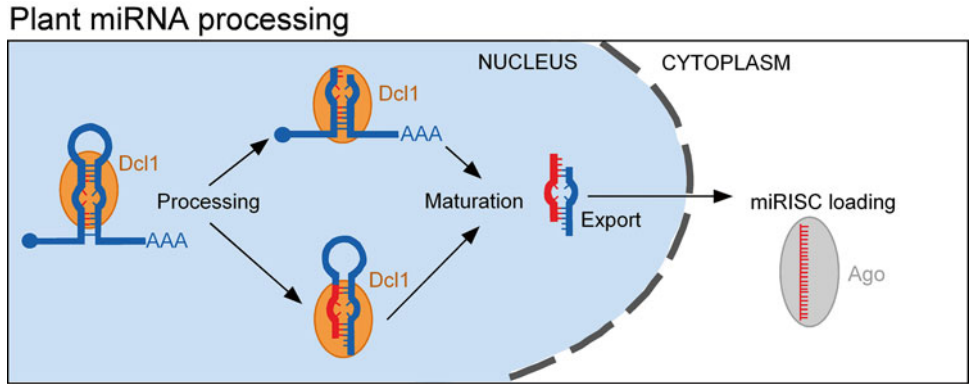

Fig. 7.7 Biogenesis of miRNAs and assembly into miRISC in plants. Nuclear transcription leads to capped and polyadenylated pri-miRNAs. Dcl1 processes the RNA in succession. The terminal loop may first be excised or it might be the flanking segments that are cleaved first. The second processing step by Dcl1 yields a mature miRNA/miRNA* duplex that becomes methylated and exported from the nucleus to the cytoplasm and assembled into miRISC. Only one strand of the duplex is stably associated with a miRISC complex, and the miRISC* is degradated (Modified from Carthew and Sontheimer (2009))

7.2.5 Ancestral Metabolism

Protein networks are commonly used in biology to represent molecular mechanisms occurring in the cell, from gene regulation (regulatory networks) to enzymatic reactions (metabolic networks) and protein–protein interactions (signaling networks) (Mazurie et al. 2010).

The description of *regulatory networks* remains elementary because the annotation of regulatory elements in genomes has lagged behind the analysis of coding regions. Coding regions can be readily assessed by comparing genome sequences with cDNA sequences; in contrast, regulatory regions are still identified primarily on a gene-by-gene basis. Even with the use of powerful tools, such as whole-genome ChIP-seq and the Encode project, the functional significance of binding sites found by large-scale screening can only be definitively tested by measuring the effect of the loss of binding due to mutation on gene expression (Gronostajski et al. 2011).

Currently available *metabolic pathway* databases contain hundreds of separate metabolic maps describing different parts of general metabolism (Kanehisa and Goto 2000; Maltsev et al. 2006). Metabolism started in the prebiotic era and was optimized during the biotic era to exploit a wide variety of energy sources. The metabolism processed by extant enzyme networks results from the evolutionary optimization of an enormous variety of modules of similar chemistry. Major transitions during evolution occur only when energy or some other growth-determining resource is abundant because the extra costs involved in developing something new are low, and pioneering variants have a chance to develop their fitness-improving functions under low selective pressure (Schuster 1996). Pathways processing similar enzymatic functions are likely to have similar topologies. This observation has motivated the construction of a phylogenetic tree based on metabolic pathways to represent the evolutionary changes in both genetic content and metabolism (Mithani et al. 2009, 2010). The organization of metabolism is affected by the species' lifestyle and phenotype. Comparison of metabolic networks across species is a key methodology in understanding how evolutionary pressures shape these networks. Metabolic networks differ among the Archaea, Bacteria, and Eukarya due to natural selection (Wagner 2009). Species that evolved to accommodate lifestyles that are more complex developed metabolic networks that are not only bigger but also more structured. Communication between pathways is typically increased by the addition of hubs (highly connected pathways that convert metabolites to obtain a broader range of input and output pathways) and switchboards (centrally connected pathways that capture a large fraction of all metabolite traffic). However, specialization niches and extreme environments tend to decrease the modularity of the metabolic networks of taxa (Mazurie et al. 2010). An example of pathway that has been acquired during angiosperm evolution is that of terpenoids. Terpenoids constitute the largest class of plant secondary metabolites and play important roles in plant ecological interactions. Sesquiterpene synthases are involved in the production of floral scents used to attract pollinators and are present in dicots, monocots, and Magnoliaceae but absent in non-seed plants, gymnosperms, and *Amborella* (*Amborella* Genome Project 2013).

Signaling pathways are the primary means of regulating cell growth, metabolism, differentiation, and apoptosis of cells to manage their activities. The sensing and processing of extracellular stimuli are mediated by signal transduction cascades: molecular circuits that seek to detect, amplify, and integrate information to generate responses such as changes in enzyme activity, the activation/deactivation of transcription factors, gene expression, or ion-channel activity. An extracellular signal is transmitted through a series of molecular modifications (e.g., phosphorylation, dephosphorylation, acetylation, and methylation) and interactions (e.g., protein–protein interactions and protein–DNA interactions). A signaling pathway can be defined as a specified group of genes that have a coordinated association with a phenotype of interest. Signal transduction pathways have the potential to branch at many steps of a cascade. The specificity of cel-

lular responses can be achieved by a small number of mechanisms, which may act in combination: (i) the same receptor can activate different intracellular transducers; (ii) differences in the kinetics of the ligand or receptor might generate distinct cellular outcomes; (iii) combinatorial activation by signaling pathways might result in the regulation of specific genes; and (iv) cells that express distinct transcription factors might respond differently when exposed to the same signals. Two types of general network architecture might account for robustness: positive- and negative-feedback loops. In positive-feedback loops, the formation of the ligand is often enhanced, thereby amplifying, stabilizing, or prolonging signaling. Negative-feedback loops are used to inhibit and/or limit signaling (PiresdaSilva and Sommer 2003).

7.3 The LUCA

The *last universal common ancestor* (LUCA) of all extant life forms is regarded as the most basal node of the tree of life and is believed to have been a cellular entity. However, the LUCA cannot be part of a formal lineage because its cellular status was most likely attained progressively from a gradually evolving community of primordial organisms. The LUCA's complexity arose by the stepwise elaboration of genetic and cellular structures. A large number of simpler intermediate forms must have existed before the LUCA, but these forms have not left any descendants.

Life is thought to have arisen on Earth by ~3.8 Gya based on evidence provided by carbon isotope data relating to biological CO_2 fixation in sedimentary rocks of that age (Ueno et al. 2002). Stromatolites are the oldest (~3.5 Gya) fossils indicative of biological material deposition by photosynthetic prokaryotes. By ~1.5 Gya, microfossils of unicellular organisms from eukaryotic algae became reasonably abundant, and exceptionally well-preserved fossils from multicellular organisms, most likely those of red algae, were laid down approximately 1.2 Gya in sedimentary rocks (Butterfield 2000).

The LUCA is thought to have been a hyperthermophilic prokaryote because of (i) the GC-rich composition of primordial codons (Trifonov 2004), (ii) the shape of the rRNA tree (Woese 1998), (iii) the small genomes and sparse growth requirements of primeval organisms, and (iv) the hot conditions prevailing on the early Earth (Di Giulio 2001). However, the hyperthermophilic nature of the LUCA has not been confirmed (Forterre 2001), and many hyperthermophiles have genomes with relatively low guanine plus cytosine (GC) contents. Salts and compatible solutes are present at high concentrations in the cytoplasm of hyperthermophiles, stabilizing DNA at high temperatures (Santos and da Costa 2002).

7.3.1 The LUCA and the Three Superkingdoms of Life

As just commented above, which of the three superkingdoms (Archaea, Bacteria, and Eukarya) evolved from the LUCA first is the subject of heated debate. Phylogenetic artifacts can be responsible for the varied depictions of the tree of life (Fig. 7.8). When evolution is understood not only as a biological process but also as a general thermodynamic process, it becomes evident that the quest for the LUCA is unattainable by sequence comparison alone. Ambiguities in alignments are unavoidable because the driving forces and paths of evolution cannot be separated from each other. Thus tracking down life's origin is by nature a non-computable task (Koskela and Annila 2012). One can list factors promoting phylogeny artifacts as (i) a high pace of sequence change, (ii) differential evolutionary rates among organismal lineages, (iii) non-orthologous gene displacement, (iv) difficulty in identifying homology by sequence alignment, and (v) horizontal gene transfer (Kim and Caetano-Anollés 2011). Although comparative genomics has revealed that gene transfer occurs frequently during genome evolution, a core of *informational genes* (those involved in information processing and expression) appears more resistant to transfer.

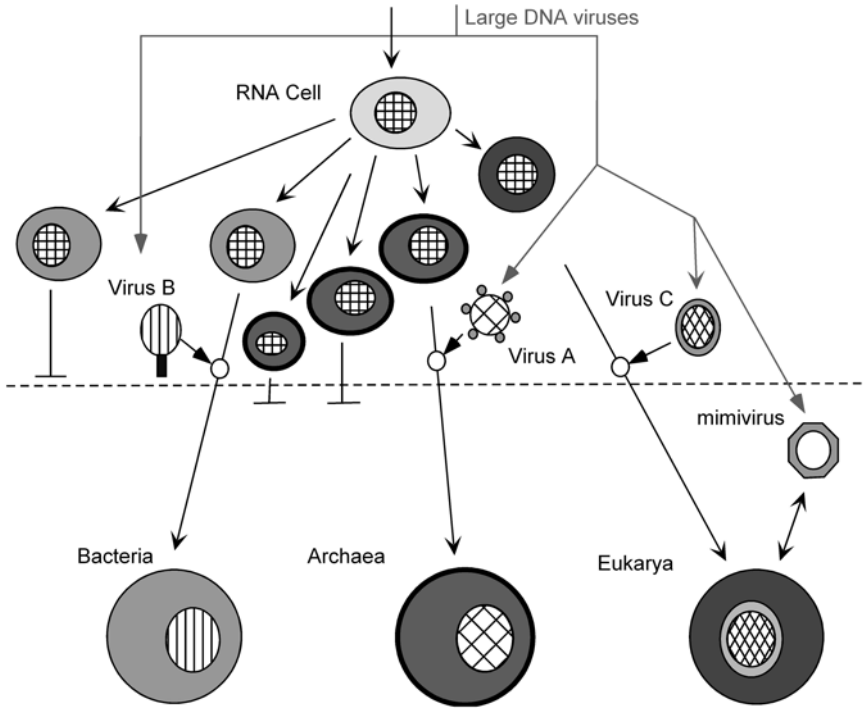

Fig. 7.8 Model of putative formation of the three superkingdoms of life by independent viral DNA transfers to RNA cells. Many lineages of RNA cells (*square crossed*) with various types of membranes (*bold*, *thin* for archaeal-like and bacterial-like lipids, respectively) and translation apparatus with different canonical patterns (*light gray, dark gray*) diverged from the RNA LUCA during the second age of the RNA world. In three of them, the RNA genomes were replaced independently by DNA genomes from different large double-stranded DNA viruses (**a**–**c**). The DNA viruses and the RNA cells at the origin of Archaea and Eukarya shared more similar features in their informational proteins, but the RNA cells at the origin of Bacteria and Eukarya shared similar lipids. The descendents of the three ancestors of the present-day domains eliminated all RNA cell lineages by Darwinian competition. The *dashed line* indicates the time of genome transition from RNA to DNA (Modified from Forterre 2005)

These genes testify to the existence of a close relationship between archaeal and eukaryal informational processes (Forterre 2001). By contrast, the *operational genes* of eukaryotes (those involved in metabolic and biosynthetic pathways) reflect eubacterial ancestry (Walsh and Doolittle 2005).

A phylogenomic study of protein domain structures based on highly conserved fold superfamilies (FSF) and an iterative approach was used to reconstruct upper and lower bounds for the LUCA's proteome of approximately 220 FSFs. The functional composition of the putative LUCA's proteome represents only 5–11 % of the 1,420 FSFs of extant proteomes. Trees of proteomes reconstructed directly from FSFs showed that the LUCA's proteome is always placed at their base and have shown that the tree of life is rooted in Archaea (Kim and Caetano-Anollés 2011). Rooting the tree of life in Archaea implies that bacterial and archaeal lineages would have evolved from a primordial eukaryotic-like lineage by reductive loss, as confirmed by phylogenomic analysis (Wang et al. 2007). The relatively small number of FSFs (SCOP – http://scop.mrc-lmb.cam.ac.uk/scop/ – currently defines approximately 2,000 FSFs) present in nature indicates that protein structures are much more conserved (three to ten times) than their primary sequences (Illergård et al. 2009). Protein structures are therefore good phylogenetic markers for deep events in evolutionary history; however, these

markers are limited in use to periods after the LUCA because complex folded proteins are not expected to have existed before it.

According to a taxonomic system based on the three superkingdoms as outlined above, an individual FSF can be unique to one of the superkingdoms Archaea (A), Bacteria (B), or Eukarya (E) or common to all three superkingdoms (ABE) if it was present in the LUCA. The putative LUCA's proteome was defined in such a way that it only contained ABE domains. Domains common to ABE were found ubiquitously and are highly represented in modern proteomes. The case where a FSF is shared by two phyla (AB, BE, or AE) implies horizontal transfer of the character considered during the formation of those phyla.

The informational functions encompassed by the putative LUCA's proteome include DNA replication/repair, transcription, RNA processing, and translation, i.e., ribosomal proteins that are crucial for protein synthesis act as landmarks of ribosomal evolution. Ribosome evolution occurred in two steps before 3 Gya, suggesting that the earliest start of organismal diversification occurred sometime after 2.9 Gya (Kim and Caetano-Anollés 2011), during planetary oxygenation. The potential for the emission of oxygen by green plant photosynthesis existed at least by 2.7–2.1 Gya, and atmospheric oxygen first began to be available in relatively large quantities most likely by approximately 1.2 Gya before present. Functions related to nuclear structure and chromatin structure that are essential for developing the eukaryotic cell were absent in the putative LUCA's proteome and only developed as life diversified into the three superkingdoms.

Considering *operational functions*, FSFs related to metabolism were commonly distributed in coenzyme metabolism and transport (m/tr), amino acid m/tr, other enzymes, carbohydrate m/tr, transferases, polysaccharide m/tr, redox, secondary metabolism, energy, and storage. FSFs for photosynthesis, cell envelope m/tr, electron transfer, nitrogen m/tr, and lipid m/tr were completely absent from the putative LUCA's proteome. The subcategories of intracellular processes including protein modification, transport, proteases, and ion m/tr were present in the putative LUCA's proteome, whereas cell cycle functions related to apoptosis, phospholipid m/tr, cell motility, and trafficking/secretion were absent. Regarding regulatory functions, FSFs present in the lower bound of the putative LUCA's proteome were related to kinase/phosphatase activities and DNA binding, whereas additional FSFs involving receptor activity and other regulatory functions only appeared later (Kim and Caetano-Anollés 2011).

7.3.2 The LUCA's Emergence

Presumably, molecular oxygen from the photodissociation of water vapor might have threatened the highly reduced nucleic acids of early self-replicating cellular systems. To protect themselves from oxidation, cells eventually evolved toward mechanisms to produce ATP that utilized visible solar energy absorbed by chlorophyll-like porphyrins, replacing earlier mechanisms in which ATP was synthesized by vacuolar proton translocating pyrophosphatase (V-H+PPase) proteins that are localized in the acidocalcisomes of extant organisms from bacteria to higher eukaryotes (Motta et al. 2009). The energy demands of the LUCA in the environments of early Earth might have been met by a combination of polyP hydrolysis, the use of a H+ gradient, and the regeneration of ATP and PPi. The multifunctionality of polyP makes it a unique link between living organisms and the inorganic world (Seufferheld et al. 2011). With the energy released from light-produced ATP, atmospheric hydrogen might have been used to reduce CO_2. The production of increasing amounts of oxygen by photosynthesis must have been deleterious for organic compounds, and obligate anaerobes would have found progressively fewer niches. Ultimately, all life might have become dependent on visible light photosynthesis. Genes in the respiratory pathway might have been selected to transfer hydrogen to oxygen rather than nitrogen or sulfate (Sagan 1967).

7.3.3 The Birth of Eukaryotes and Green Plant Emergence

In the *Margulis' hypothesis* referred to as *serial endosymbiosis*, mitochondria and photosynthetic plastids were once free-living prokaryotic cells (Sagan 1967). Classical mitosis evolved in protozoan-type cells millions of years after the evolution of photosynthesis. During the course of the evolution of mitosis, photosynthetic plastids would have been acquired symbiotically more than 1.5 Gya by some of these protozoans engulfing a cyanobacterium (Martin and Russell 2003) to form eukaryotic algae and green plants. It is now widely accepted that an α-proteobacterium was the ancestor of mitochondria (Dyall et al. 2004).

Terrestrial conquest by simple rootless plants lacking vascular tissue and producing spores rather than seeds such as bryophytes (liverworts, hornworts, mosses) occurred at the Ordovician by ~450 million years ago (Mya) and was followed by vascular plants with one or few cells in the surface layer of the apical meristem such as lycopods by ~415 Mya during the Silurian. New vegetal groups (ferns and horsetails) appeared at around 385 Mya (Devonian) together with first arthropods (scorpions). Terrestrial vertebrates appeared at ~360 Mya successively with dipneusts, amphibians, and reptiles at ~340 Mya (Carboniferous). The first gymnosperms appeared at ~320 Mya and the first angiosperms between 180 and 140 Mya (Lower Jurassic) with their fast radiation (≤5 million years) during the Lower Cretaceous into 97 % of extant species, i.e., more than 250,000 (Bell et al. 2010; Soltis et al. 2009). Orthologs of developmental genes most likely existed long before the establishment of their specific role in seed plant (Graham et al. 2000; Becker et al. 2003; Dolan 2009; Huang et al. 2014). The gene set of the *last angiosperm common ancestor* (LACA) was inferred to contain at least 14,000 protein-coding genes with 1,179 (including genes for flowering, MADS box, wood formation, responses to environmental stress) being more specifically associated to angiosperm divergence. Notable examples of genes involved in response to external stimuli include those that drove floral and insect coevolution in angiosperms. Herbivory has also been proposed as a significant environmental constraint in angiosperm evolution, particularly in Poaceae (*Amborella* Genome Project 2013). Most of small interfering RNAs (siRNAs) and microRNA (miRNA) families and their homologous targets were also likely present in the LACA.

7.3.3.1 The Origin of the Nucleus

In contrast to mitochondria and plastids, the origin of the cell nucleus has not yet been fully elucidated. The nucleus is a complex structure, and therefore, it is expected to have arisen only once during evolution; the underlying reasons cannot be simple, and most models take this into account. The most widespread and familiar model for the origin of nucleus is the concept that the endomembrane system of eukaryotes, to which the nucleus and endoplasmic reticulum belong, is derived from invagination of the plasma membrane of a prokaryote (Cavalier-Smith 2002, 2004). The invagination model postulates that a prokaryote lost its cell wall and evolved phagocytosis. An example of natural endocytosis still exists in prokaryotes, i.e., endospore formation by extant bacteria. Another striking example of prokaryotic endomembranes that superficially resemble those of the eukaryotic nucleus is found in the planctomycetes (Lindsay et al. 2001). Discrete membrane-bound compartments have been described in two planctomycetes of the genera *Gemmata* and *Pirellula* (Fuerst and Sagulenko 2013).

Another attractive hypothesis is that the cell nucleus could have evolved from viruses. Viruses could have evolved by parasitic reduction from ancient cellular lineages that were out-competed in the Darwinian selection process before the LUCA and thus could only survive as parasites in the winners of this competition (Forterre 2005). Nuclei and viruses have several features in common:

(i) The cell nucleus is functionally equivalent to a selfish DNA virus; it replicates its DNA using cellular metabolism.

(ii) Cell nuclei and viruses lack protein- and lipid-producing pathways within their boundaries.
(iii) Both contain linear chromosomes, whereas most bacterial chromosomes are circular.
(iv) Both disassemble their boundaries during replication.
(v) Both transcribe DNA but do not translate mRNA within their boundaries. As they replicate within a cell, some poxviruses even form a membrane around their DNA using the endoplasmic reticulum of the infected cell. The eukaryotic cell uses the same material to surround its nucleus.
(vi) Some viral DNA strands have primitive telomeres, which act as protective DNA sequences that are found at the ends of eukaryotic chromosomes.

An astonishing virus that largely overlaps with the world of cellular organisms, both in terms of size and genome complexity, is the giant *Acanthamoeba polyphaga*, a eukaryotic nucleocytoplasmic large dsDNA virus (NCLDV). This NCLDV has a genome of 1.2 Mb representing 911 genes with little evidence of horizontal transfer (Raoult et al. 2004).

According to the *viral hypothesis*, the viral transfer of DNA replication apparatus to the LUCA's cells would have occurred independently three times (Fig. 7.8), thus promoting the foundations of the three ancestral cell lineages for Archaea, Bacteria, and Eukarya (Forterre 2005). With this viral hypothesis, it would be possible to explain (Forterre 2006):

(i) Why there are three superkingdoms instead of a single continuum between the LUCA and extant organisms.
(ii) The existence of three canonical ribosomal patterns.
(iii) The critical differences exhibited by the otherwise similar eukaryotic and archaeal replication machineries. Cellular DNA informational proteins are often found in only one or two (not three) versions and exist in different analogous families. For instance, there are six known analogous families of cellular DNA polymerases: one version in Bacteria, one in Archaea, and several in Eukarya (Filée et al. 2002).
(iv) Why eukaryote lipids are bacterial-like, but ribosomes are archaeal-like.

The type of combinatorial evolution just outlined is integral to many current views of evolution, and it is obvious that horizontal transfers have occurred continuously during genome evolution. The relationship between Eukaryotes and Archaea/Bacteria bears witness to a long common history of multiple interchanges between phyla that is difficult to decipher (Walsh and Doolittle 2005), as is the issue concerning the origin of the nucleus. However, it is now thought that the molecular machinery involved in information storage and retrieval in eukaryotes shares much greater similarity in terms of overall design and sequence conservation to archaeal counterparts than to bacterial ones (Anantharaman et al. 2002). Comparative genome analyses have shown that the *last eukaryote common ancestor* (LECA) possessed much of the complexity of extant eukaryotes now seen in the replisome, the spliceosome, the nucleoli, and the endocytic system, as well as the machineries necessary for meiosis and phagotrophy.

Multinuclearity and duplicated cistrons or entire genomes (polyploidy) may have been early mechanisms to distribute greater amounts of DNA evenly to daughter cells because the use of multiple copies greatly increases the probability that each daughter cell will contain at least one copy of the genome. However, the inefficiency of polyploidy and the selective advantage of linkage groups over polyploidy have been demonstrated (Gabriel 1960). Many lower eukaryotes that still follow poliploidy (*premitosis*) as a mean of genetic material transmission to their progeny have the general features of eukaryotic cells (mitochondria, nuclear membranes, etc.), but their aberrant division figures often lack the spindles and centrioles of typical eumitosis (as seen in *Amoeba, Euglena, Tetramitus,* etc.). This system is ancestral to meiosis and fertilization. The evolution of mitosis must have taken millions of years. Based on the abundance of fossil eukaryotes, this event must have occurred before the dawn of the Cambrian. Hence, the most likely

period for the evolution of mitosis is between 1.2 and 0.6 Gya (Sagan 1967).

7.3.3.2 Chromosomes

Eukaryotic chromosomes may differ in size, shape, number, and DNA composition. The size of metaphase chromosomes varies from less than 1 μm to more than 10 μm, but upper and lower tolerance limits for chromosome size are apparently determined by the genome size, chromosome number, and karyotype structure of a given species. The shape of a chromosome is determined by the position of the primary constriction (the centromere), and its size can be altered by reciprocal translocation, sister chromatid exchange, crossover, and replication slipping (Schubert 2007). Telomeric sequences may move to interstitial positions by translocation or inversion and become hot spots of chromosome breakage. According to the *minimum interaction hypothesis* (Imai et al. 1986), karyotype evolution generally tends toward an increasing number of acrocentric chromosomes, thereby minimizing the risk of deleterious rearrangements. The reduction of chromosome number and the formation of metacentric chromosomes are considered rare and randomly generated events that are tolerated or favored when they provide a short-term advantage. The nucleolus-organizing region (NOR), which is the site of 45S repetitive DNA (rDNA), may mark a chromosome either at a terminal or an interstitial position by giving rise to a secondary constriction (Fig. 7.9). The positions of NOR and 5S rDNA are highly polymorphic and potentially mobile within a genome. Most chromosome rearrangements occur within regions of extended repeats and are the result of preferential mis-repair by ectopic recombination between nonallelic repeats (Schubert et al. 2004).

Linear chromosomes are a prerequisite for the accumulation of DNA because dispersed, repetitive sequences tend to recombine ectopically during DNA repair, often with deleterious consequences for circular, but less so for linear, DNA molecules (Schubert 2007). Chromosomes evolve by rearrangements and ploidy alterations subsequent to interspecific hybridization and/or mitotic or meiotic errors. Chromosome rearrangements are due to the erroneous ligation of DNA strands during repair processes. Transposition, errors during replication, unequal recombination, and insertions and/or deletions

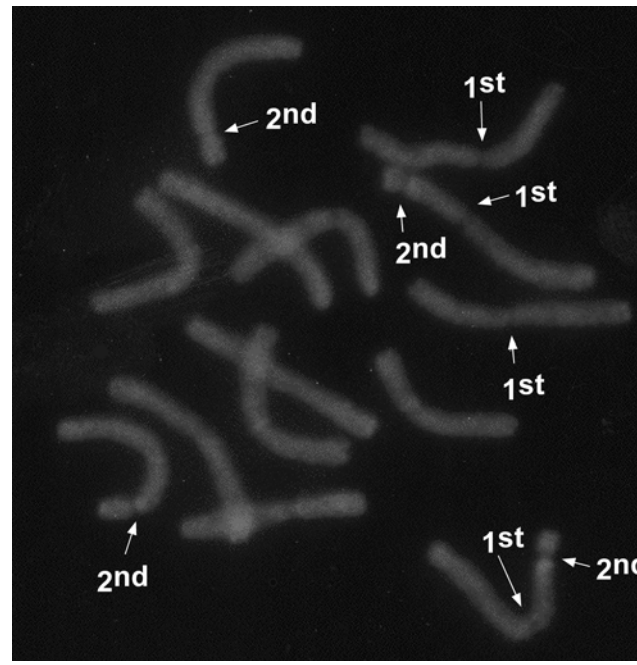

Fig. 7.9 A karyotype of maize chromosomes showing primary (1st) and secondary (2nd) constrictions

during double-strand break repair via recombination might also contribute to chromosome evolution by promoting their shrinkage or expansion.

7.3.3.3 Centromere

Centromeric repeats comprise the most rapidly evolving DNA sequences in eukaryotic genomes. Satellite changes occur through a variety of mutational processes including (i) replication slippage, (ii) unequal exchange, (iii) transposition, and (iv) excision. The fast sequence changes observed in the centromere appear paradoxical in comparison to the stable inheritance of this structure. Indeed, the chromosome segregation machinery is highly conserved across all eukaryotes. Despite the lack of universal sequence motifs, repeat unit length can be remarkably similar between organisms and is typically between 100 and 200 bp. It is thought that incompatibilities between rapidly evolving centromeric components may be responsible for the reproductive isolation of emerging species. Proteins from the kinetochore are only found at centromeres. The kinetochore is a proteinaceous structure that assembles on centromeric chromatin and connects the centromere to spindle microtubules. However, only constitutive proteins are candidates for directly maintaining centromeres. The model states that a H3-like histone exists that is specific to the centromere region. The sequence of the variable core of this histone coevolves with that of centromeres across eukaryote species. This H3-like histone is only present at native centromeres and neocentromeres, is absent from mutated or inactivated centromeres, and is expected to maintain favorable interactions with centromeric satellites.

The asymmetry of the meiotic tetrad provides an opportunity for chromosomes to compete for inclusion into the oocyte nucleus by attaining a preferable orientation at meiosis. This competitive process might drive adaptive changes, thus providing an explanation for fast centromere evolution, which is critical for such an essential function. Deleterious mutation by transposon insertion might promote satellite accumulation representing centromeric relics surrounding functional centromeres. Parents in hybrids are compatible when the two sets of centromeric satellites and the two alleles encoding the centromeric histone are balanced. Thus, infertility and distortion of the sex ratio are expected symptoms displayed by hybrids bringing together incompatible combinations of satellite vs. H3-like histones and eventually reproductive isolation or speciation (Henikoff et al. 2001).

7.4 DNA Repair Machinery

In response to continuous hydrolytic and oxidative DNA damage, cells must have an effective network of repair systems that recognize, remove, and rebuild the injured sites. DNA repair is critical for the maintenance of genome integrity and replication fidelity in all cells and therefore was arguably of major importance in the LUCA as well. Like Bacteria, Archaea have minimal sets of genes involved in all of the major cellular information transfer processes compared with Eukarya, which have highly paralogous and redundant sets of genes for DNA replication, repair, and recombination.

Repair mechanisms can be classified into several distinct major pathways: (i) direct damage reversal (DDR), (ii) base excision repair (BER), (iii) nucleotide excision repair (NER), (iv) mismatch repair (MMR), and (v) recombination repair (RER, Fig. 7.10). The main protein functions involved in these repair systems are (i) endo- and exonucleases and glycosidases, (ii) DNA helicases, (iii) ATPases (other than helicases) that are involved in events such as strand migration and the loading of multi-protein repair complexes onto DNA, (iv) DNA ligases, (v) DNA polymerases and nucleotidyltransferases, (vi) DNA-binding domains, (vii) adaptors (protein–protein interaction domains that glue together diverse proteins in repair complexes and link to other cellular components, such as eukaryotic chromatin), and (viii) ATPases (Jensen et al. 2011).

Repair systems are modeled by selective pressures on the fidelity of transmission of genetic information and the need for evolvability. Repair

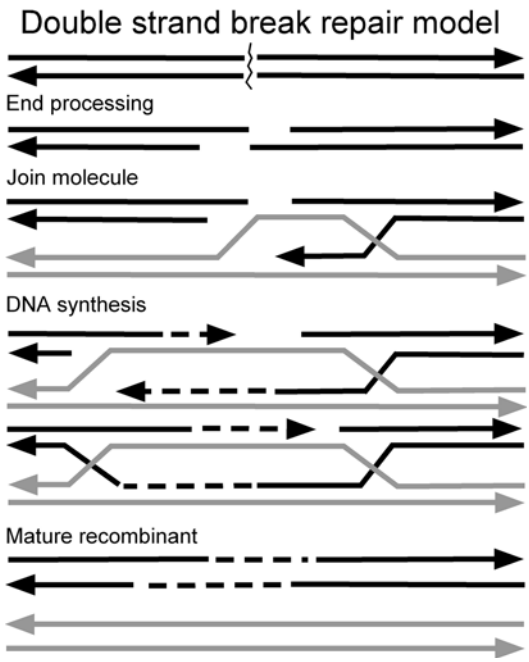

Fig. 7.10 Repair of double-strand breaks by homologous recombination in eukaryotes

systems are striking for their poor representation of universal components, which contrasts with the case of translation machinery (Farnell 2011). The repair machinery shows considerable variability, in terms of the genes that are present or absent, even in closely related bacteria. Error-prone repair or lesion bypass synthesis refers, here, to specialized DNA polymerases that are able to progress through DNA lesions. This pathway, termed *translesion repair,* is inherently mutagenic because of the miscoding nature of most damaged nucleotides (DiRuggiero and Robb 2004). A highly efficient repair is mandatory in free-living organisms, which are subject to rapid changes in the environment and thus employ more sophisticated repair regulation at the transcription level by specialized regulators. In free-living organisms, genomic variation is obtained via error-prone repair mechanisms, such as those induced by translesion repair. Eukaryotes have a higher-order chromatin structure that is more complex than that of their prokaryotic counterparts. The evolution of these structures placed additional barriers to the interaction of repair enzymes with damaged DNA and led to the concomitant evolution of specific adaptor proteins that connect the repair machinery and chromatin (Aravind et al. 2000).

The LUCA is expected to have encoded a recombinase and several helicases and nucleases, including approximately ten types of repair protein domains (universal families). Most of these nuclease and helicase families have members with RNA substrates, and the most common nucleic acid-binding module in repair proteins, HhH, exists in both RNA-binding and DNA-binding versions, suggesting that DNA repair evolved from systems that already provided functions in the RNA world. The number of orthologous or even clearly functionally equivalent repair proteins that are shared by all three superkingdoms is very small. In contrast, a much larger number of repair proteins are conserved in one or two superkingdoms. There appear to be only a few known repair proteins, such as RecA/RadA recombinase, that have an identical domain arrangement that is conserved among Bacteria, Archaea, and Eukarya (Aravind et al. 2000).

7.5 The Mutation Bias in Bacteria as a Model for Eukaryotes

The genomic GC content of bacteria varies dramatically, from less than 15 % (McCutcheon and Moran 2010) to more than 70 % (Hildebrand et al. 2010). It has been shown that genomic GC content is correlated with a number of factors including genome size, environmental features, aerobiosis, nitrogen utilization, and temperature, which may be subject to selective pressure for tuning GC content (see in Hildebrand et al. 2010). Genomic GC variation is generally ascribed to differences in the pattern of mutation toward adenine plus thymine (AT) or GC. There are two patterns of mutation bias, i.e., global or transcript-specific, each of which is derived from a different mechanism. The former is attributable to DNA replication and global repair (see above), and the latter mainly results from transcription-coupled repair (Tornaletti 2009). Deficiencies in

mutator genes can dramatically increase the mutation rate. For example, in the absence of both *mutY* and *mutM*, thousandfold increases in GC-to-AT mutation bias were observed; the same magnitude of mutations is evident in *mutT-deficient* strains, but resulting in a bias toward GC (Horst et al. 1999).

It has been demonstrated that the DNA polymerase III α subunit and its isoforms participate in either replication (polC) or SOS mutagenesis/translesion synthesis (dnaE2) and play dominant roles in determining GC variability (Wu et al. 2012). The early phase of the SOS response is mostly dominated by accurate DNA repair, whereas the later phase is characterized by elevated mutation levels caused by error-prone DNA replication. SOS mutagenesis is largely the result of the action of DNA polymerase V (pol V), which has the ability to insert nucleotides opposite to various DNA lesions via translesion. Pol V, encoded by the *umuDC* operon, is largely responsible for the approximately 100-fold increase in chromosomal mutation induced by DNA damages. The mutations that occur may kill many cells; however, if replication is successfully restarted, the lucky cells will survive (Patel et al. 2010).

A correlation exists between variation in GC content and the dimeric combinations of DNA polymerase III α subunits, showing that eubacteria can be grouped into different GC variable groups: (i) the full GC spectrum or dnaE1 group, (ii) the high GC content or dnaE2–dnaE1 group, and (iii) the low GC content or polC–dnaE3 group (Zhao et al. 2007). The existence of dnaE2 and polC is associated with higher GC (>50 %) and lower GC (<50 %) contents, respectively. The DNA polymerase III α subunit and its isoforms participate either in replication (polC) or in SOS mutagenesis/translesion (dnaE2), playing a dominant role in producing GC variations that can be classified into three basic spectra: GC variable, high-GC, and low-GC groups. For example, the presence of dnaE2 is an indication of a higher GC content. On average, aerobic bacteria exhibit higher GC contents than anaerobic bacteria because aerobes tend to carry the GC-enriching polymerase whereas anaerobes tend to carry the AT-enriching polymerase. Concerning the relationship between genomic GC content and optimal growth temperature, thermophiles most frequently use the AT-enriching polymerase, whereas mesophiles or psychrophiles most frequently use the GC-enriching polymerase (Wu et al. 2012). Thus, the loss or gain of specific mutator genes is expected to alter the GC content of bacterial genomes (Akashi and Yoshikawa 2013). A similar process is expected to occur in eukaryotes (Touchon et al. 2004). Experimental evidence is given in the following section to show that selective processes also affect GC content in eukaryotic genomes.

7.6 Correlations Between GC Content and Genomic Features in Eukaryotes

The universal inter-genomic correlation shows that CDSs from prokaryote or eukaryotic genomes that are homogeneous regarding GC content follow a regression line (D'Onofrio et al. 1999). This relationship also holds when considering the intra-genomic correlation of GC in 2nd position of codons (GC2) vs. GC in 3rd position of codons (GC3) within genomes of heterogeneous eukaryotes such as humans and rice (Carels and Frias 2013). In uncharacterized species, GC2 and GC3 can be easily obtained from transcriptome data (Carels and Frias 2013). Genome size and GC content are easily obtained using flux cytometry. CsCl sedimentation remains the only technique able to faithfully report the whole-genome statistics of large DNA fragments >100 kb (Bernardi 2000; Clay et al. 2001; Carels 2005a). However, the DNA sequencing of large DNA fragments has become more consistent due to the technology developed by Pacific Bioscience (Eid et al. 2009). Next-generation sequencing of large DNA fragments is expected to increase in the future due to the development of emerging sequencing technologies such as that developed by Oxford Nanopore (Clarke et al. 2009).

CsCl sedimentation of large DNA fragments has allowed Bernardi's group to investigate

genome evolution in vertebrates (Clay et al. 2003) and plants (Carels 2005a). By analyzing the DNA fragment (>100 kb) distribution of large eukaryotic genomes, it has been shown that GC transitions occurred independently in birds and mammals (two warm-blooded vertebrate lineages that evolved independently from cold-blooded vertebrates) and in plants (Poaceae, Fig. 7.11). Interestingly, the platypus is also surprisingly GC-rich (Costantini et al. 2009) as is *C. reinhardtii*, indicating that compositional transitions toward GC have occurred independently several times in eukaryotes. Transitions toward AT also exist, for example, in unicellular parasitic eukaryotes (Costantini et al. 2013); however, these transitions were investigated to a much lesser extent. The compositional characterization of unicellular eukaryotes also shows a mosaic of DNA fragments of different GC compositions, indicating that such a compositional patchwork already existed in the LUCA, which would not be surprising given that modular genetic structures were inherited from the RNA world. Correlations between the GC content of genomes and selective pressures associated with species' living conditions are difficult to investigate due to the inherent large variability associated with ecological variables and independent cellular strategies to cope with the environmental selective pressure. The search for correlations relating to GC content among genomic structures (especially in eukaryotes) or even nuclear compartments was much more productive (see the pioneering investigations conducted by Saccone et al. 2002 in warm-blooded vertebrates).

The first concept investigated was that of isochores (typically found in human Pavlicek et al. 2002; Costantini et al. 2006), i.e., large genomic regions (>>300 kb) that are somewhat homogeneous in GC (GC variation ≤4 %, Bernardi 2000; Costantini et al. 2009) and are present in large heterogeneous genomes such as those of mammals (ranging from 30 to 60 % GC in humans, Zoubak et al. 1996) and birds. In further developments, correlations between the CDS distribution and the GC level of isochores were sought. Genes

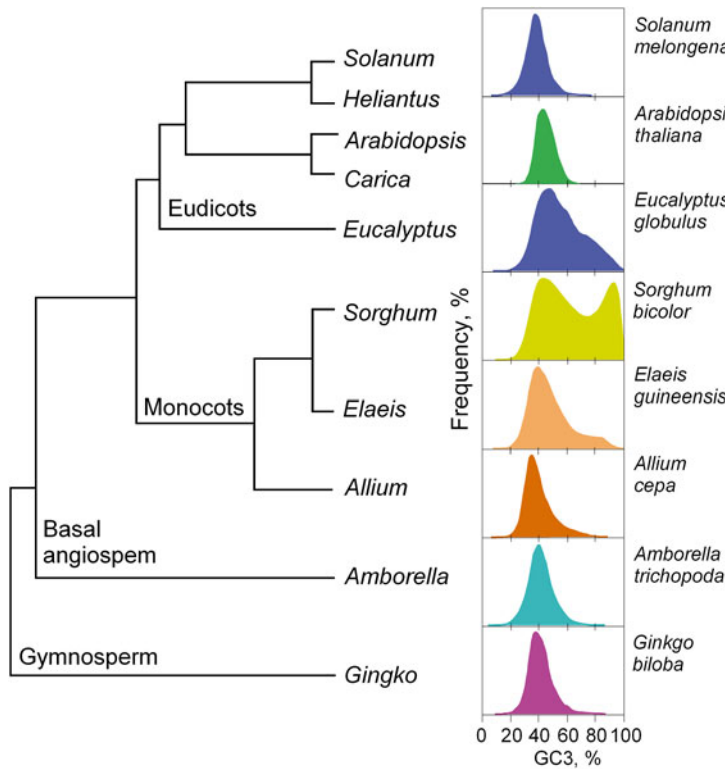

Fig. 7.11 Taxonomic associations for eight plant species with their corresponding GC3 distributions. Sorghum (Poaceae) show a clear GC transition toward high GC3 contents compared to *Arabidopsis*, taken as an example. Another partial compositional transition can be observed in Eucalyptus (Modified from Serres-Giardi et al. (2012)). Other transitions should appear as sequencing efforts proceed

were found to be concentrated in a *gene space* whose GC level is larger than the mode of DNA fragment distribution according to GC content (Fig. 7.12). Such a CDS distribution bias according to GC level led to the introduction of the concept of gene deserts, which are associated with GC-poor genomic fragments in which genes are rare (Bernardi 2012). Sets of genes can also be classified as GC-poor or GC-rich according to their intron number and size (Carels and Bernardi 2000b). A similar correlation has been found in humans by Duret et al. (1995), but we verified that this is much less striking than the correlation in plants. Interestingly, the GC level of exons is generally larger than that of introns and intergenic sequences in vertebrates (Costantini and Bernardi 2008) and in plants (Carels et al. 1998; Carels and Bernardi 2000a, b; Carels 2005b). According to the classification of genes as GC-poor or GC-rich, we also verified that promoters of GC-rich genes in Poaceae match the definition of *CpG islands*, which is not the case for GC-poor genes, suggesting that different types of gene regulation apply to either GC class (Carels 2005a). In humans, a positive correlation is observed between GC and CpG (Varriale and Bernardi 2010); however, even in the GC-rich compartment, the ratio of CpG observed over the expected level is generally lower than 1 (Costantini et al. 2009), although it is larger or equal to 1 in rice (Carels 2005a), showing that CpG targets remain unmethylated in the GC-rich compartment of Poaceae despite the more complex methylation pattern observed in plants. The resistance of plant CpG islands against GC erosion in GC-rich genes of plants demonstrates an

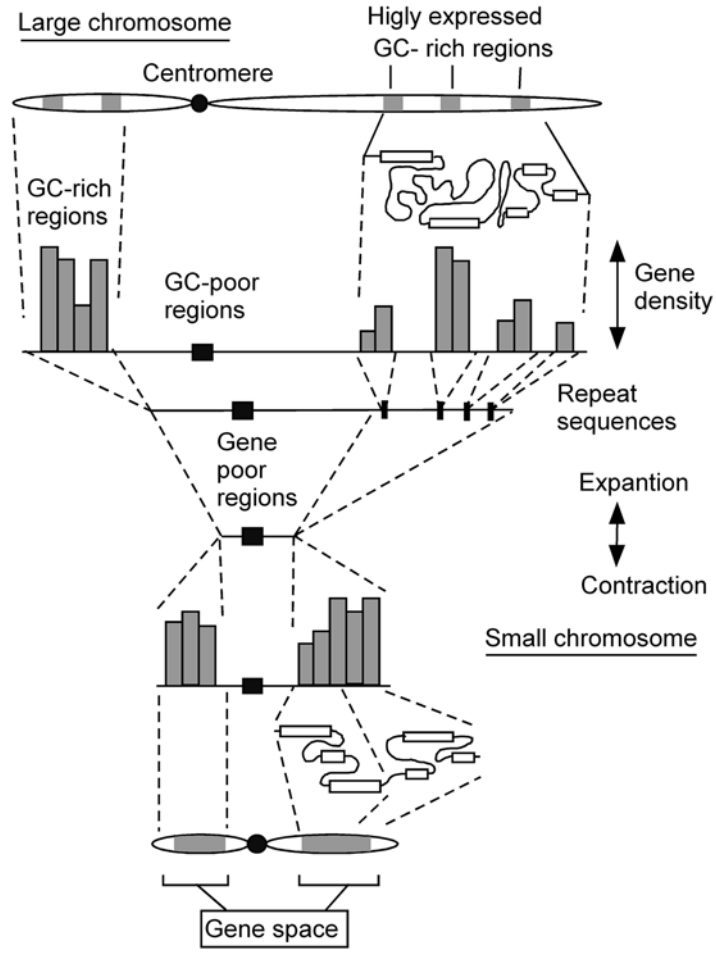

Fig. 7.12 Relationship between genome size, gene expression, physical distribution of genes and repeated sequences in plants

active maintenance against the CpG erosion into TpG due to the mutation of 5′-*CpG-3′ (5mC) to 5′-TpG-3′. In humans, genes in the gene desert compartment that are essentially formed by AT-rich isochores appear to be subject to a much more sophisticated regulation machinery (Nobrega et al. 2003) than genes from GC-rich isochores; this is somewhat analogous with plant organization with respect to the difference in gene regulation between GC-poor and GC-rich compartments, as outlined above. The gene desert typically matches Giemsa+ bands in vertebrates whereas the gene space lies in Giemsa- bands (Costantini et al. 2007a) where chromatin is more open and the recombination rate is higher (see in Carels 2005a; Tian et al. 2009; Nam and Ellegren 2012; Flowers et al. 2012). In maize (Carels 2005b), the situation is somewhat different because all genes (except those encoding storage proteins) that cover the same GC3 interval as all human genes (30 % < GC3 <90 %) are grouped into a gene space of 4 % GC, i.e., an interval that is equivalent to the GC variability of DNA fragments belonging to a single isochore family (the human genome includes five isochore families, Bernardi 1989). Although it occurs to a lesser extent, the difference in gene density between GC-poor and GC-rich chromosomal compartments is observed even in such a compact genome as that of *Arabidopsis* (Carels and Bernardi 2000a). In wheat, the gene distribution is organized as islands of higher density (Sandhu and Gill 2002; Erayman et al. 2004).

In humans, the isochore GC level has been found to correlate with the 3D organization of the nucleus in interphase in such a way that GC-poor sequences are on the nuclear periphery and stack onto the nuclear membrane. In contrast, GC-rich isochores loop toward the center of the nucleus (Saccone et al. 2002). This 3D organization suggests that GC-rich and GC-poor isochores are involved in very different functions. In plants, GC-rich genes include genes for carbohydrate transport and metabolism, cell wall synthesis, extracellular structures, and secondary metabolite synthesis; these genes are essential for energy management, physical structure, and defense, i.e., for the relationship between a cell and its environment. In contrast, GC-poor genes mainly address information functions, such as cell mechanics, and general functions such as cell identity (Carels 2005a).

Isochore families are also found in cold-blooded vertebrates, although usually within a more homogeneous context (Costantini et al. 2009) with a similar preference for the gene space to be associated with GC-rich isochores (Bucciarelli et al. 2009); this shows that the GC-rich compartments experienced the compositional transition as seen in its most expressive form in birds and mammals (Costantini et al. 2007b). Thus, the gene landscape (Cruveiller et al. 2004) is a sophisticated feature that might have emerged with compositional heterogeneity in large genomes. A selective advantage associated with such a sophisticated organization of chromosomal territories might be found in the regulation system, allowing genes to be regulated spatially and temporally under different compositional contexts (different promoter and enhancer motifs) (Bernardi 2012). Differential levels of gene expression between the GC-poor and GC-rich compartments have been observed in humans (Arhondakis et al. 2004, 2011) and plants (Carels and Bernardi 2000a, b).

7.7 Genome Size

The regional structure of heterogeneous genomes may have been promoted by the interplay of (i) an increase in genome size by polyploidization (Fig. 7.13), which is rather common in plants (Matassi et al. 1991; Tate et al. 2005); (ii) isopycnic retrotransposon integration (Rynditch et al. 1998); and/or (iii) a mutational bias toward GC under selective pressure, possibly on the regulation of gene expression. In animals, polyploidization events also exist in metazoa (Gregory and Mable 2005), such as fish (the tetraploidization event in the stem lineage of ray-finned fish resulted in >20,000 living species), amphibians, and reptiles (lizards). Diploid–triploid mosaics are known in humans, cats, mice, rabbits, cattle, pigs, mink, and chickens, but these are usually malformed or at least sterile

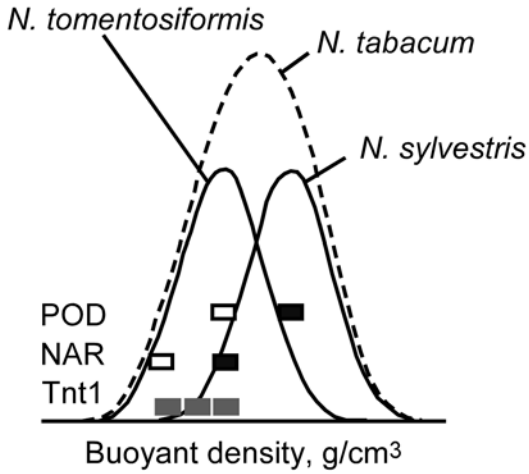

Fig. 7.13 Gene and DNA fragment distributions in the amphidiploid genome of tobacco. Genes (NAR = nitrate reductase; POD = lignin-forming peroxidase) located in the GC-poor and GC-rich compositional fractions of *N. sylvestris* are shown as *black boxes* while the distribution of the same genes in *N. tomentosiformis* are shown as *white boxes*. The compositional distribution of *Tnt*-1 transposon is shown by *gray rectangles*. This experiment shows how interspecific hybridization may affect the regional organization in plant nuclear genomes. DNA fragment distribution was obtained by CsCl sedimentation and gene distribution by Southern hybridization with Cs_2SO_4 sedimentation fractions (Modified from Matassi et al. (1991))

(Fechheimer et al. 1983). The South American red viscacha rat *Tympanoctomys barrerae* has twice the DNA content and number of chromosomes as its closest relatives, providing the first example of a naturally occurring tetraploid mammal (Gallardo et al. 2004). Two rounds of entire genome duplications would have occurred early during vertebrate evolution, the evidence of which can still be observed as duplicated blocks in the human genome (Van de Peer and Meyer 2005).

In plants, polyploidy seems to have been decisive for the success of angiosperm diversification and radiation among many habitats, life forms, and biotic interactions; it has been estimated that 70 % of all angiosperms had experienced one or more episodes of polyploidy in their ancestry. Subfunctionalization of gene duplicates has been shown to be a prominent molecular evolutionary force for coping with whole-genome duplication in both flowering plants (Jiao et al. 2011) and ray-finned fish. The Hawaiian flora has the highest incidence of polyploidy known, and most Hawaiian species are paleopolyploid most likely because dispersal into volcanic archipelagoes involves the colonization of habitats that change dramatically over relatively short periods of time. Molecular investigations of polyploid genome have indicated that intra-genomic rearrangement and altered gene regulatory relationships can contribute to evolutionary flexibility (see in Soltis et al. 2009). Several polyploidization events were documented in plants, i.e., ζ in the seed plant common ancestor, ε in the LACA, σ and ρ in monocotyledons, γ in the eudicot ancestor, and α and β in Brassicaceae (Soltis et al. 2009; Jaio et al. 2011; *Amborella* Genome Project 2013, Fig. 7.14). A compilation of 2,802 angiosperm species has shown that haploid genome sizes range from ca. 120 Mb to over 148 Gb (Michael and Jackson 2013) with ~50 % of the flowering plants analyzed to date having genome sizes in the interval 120 Mb and 3.5 Gb (Bennett and Smith 1976; Leitch et al. 1998). Familiar examples are rice, sorghum, potato, tomato, maize, barley, and wheat whose genome size are 450, 750, 850, 950, 2,500, 5,000, and 16,000 Mb, respectively (Arumuganathan and Earle 1991; Lukas et al. 2009; The Potato Genome Consortium 2011; The Tomato Genome Consortium 2012; Michael and Jackson 2013). Gymnosperms have highly repetitive genome of large (1–40 Gb, Ohri and Khoshoo 1986; Kovach et al. 2010; Burleigh et al. 2012), but rather homogeneous size (~40-fold variation compared to ~1,000-fold variation for angiosperms).

Interestingly, genome reduction appears to be the dominant mode of evolution. Most lineages were apparently dominated by extensive loss of genes (Sankoff et al. 2010) and introns, and two distinct evolutionary phases can be distinguished: a short, explosive, innovation phase that led to an abrupt increase in genome complexity, followed by a much longer reduction phase (Fig. 7.15), which encompassed either a neutral ratchet of genetic material loss or adaptive genome streamlining (Wolf and Koonin 2013). An example of plant genome reduction is sorghum whose

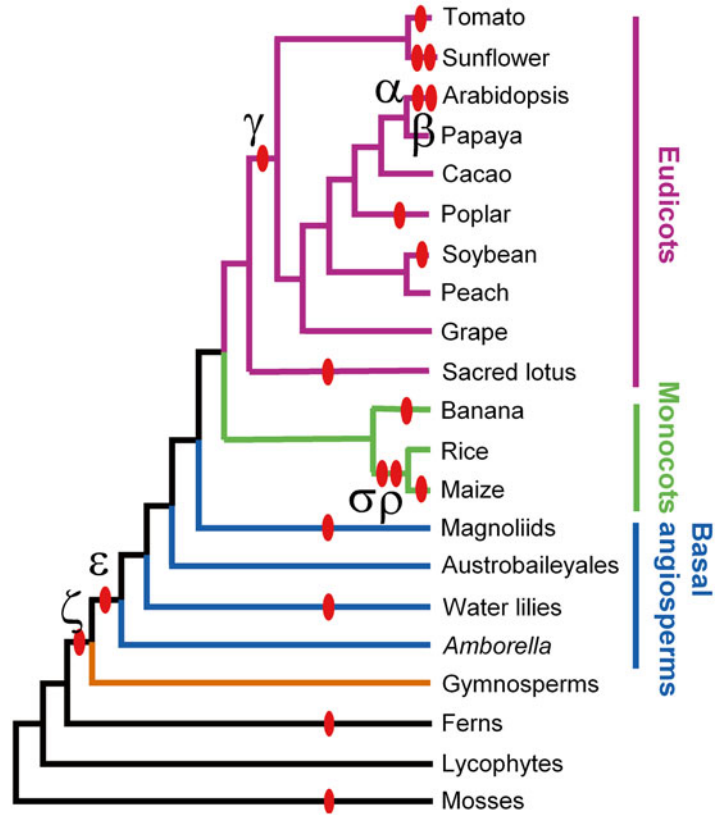

Fig. 7.14 Overview of land plant phylogeny showing the relationships among major lineages of angiosperms. Putative polyploidy events in land plant evolution are indicated with *red ellipses*. The first whole-genome duplication (WGD) most likely occurred in the common ancestor of seed plants and is known as ζ. The next polyploidization events are known as ε and γ and occurred in the LACA and common ancestors of eudicots, respectively. Several other polyploidization events occurred more recently in Brassicaceae (α, β) and Poaceae (σ, ρ) independently as well as in other lineages (Modified from *Amborella* Genome Project (2013))

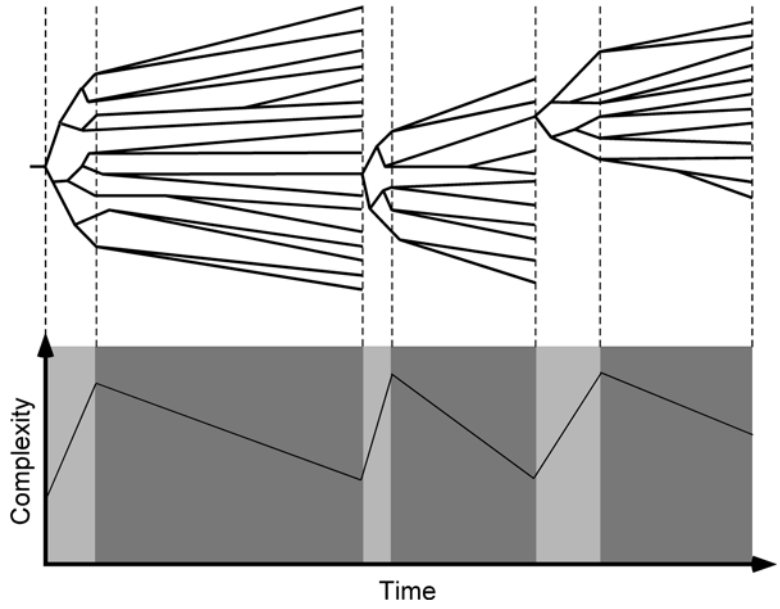

Fig. 7.15 Model of punctuated evolution of genomes. *Top*: alternance of species radiation burst over short time periods and long phases of diversification stasis in the history of a particular lineage. *Bottom*: the complexity profile of punctuated evolution shows sudden increased in genome complexity matching species radiation alternating with long period of slow genome reduction (Modified from Wolf and Koonin (2013))

chromosome number has been reduced from 10 to 5. Similarly, the ancestor of *A. thaliana* (~120 Mb) went through three phases of genome reduction through chromosomal condensation to reach the final chromosome number of five. The ancestor of rice as well went from allotetraploid to diploid through genome shuffling and reduction. Considering extant birds and lizards, their most recent common ancestor is expected to have lost nearly 20 % of its DNA content. Indeed, most of the correlations between recombination rate and genome contraction are also seen in the human genome (Nam and Ellegren 2012).

Concerning isopycnic integration (Rynditch et al. 1998), nested retrotransposon families of different GC composition were remarkably well described in maize (SanMiguel et al. 1996; Meyers et al. 2001; Martienssen et al. 2004). Like maize, humans also contain 70 % repeat sequences (Pavlicek et al. 2001; Costantini et al. 2012). Based on their structure, retrotransposons are divided into two groups: those that are flanked by long terminal repeats (LTRs) and those that are not flanked by LTRs (non-LTR retrotransposons) such as the long interspersed nuclear elements (LINEs). LTR retrotransposons are further divided into two groups: Ty1 or copia (Peterson-Burch and Voytas 2002) and Ty3 or gypsy (Kidwell 2005). The major structural difference between the copia and gypsy groups is the order of the reverse transcriptase (RT) and integrase domains in their pol genes. In plants, Ty1/copia elements were first identified as insertions near maize genes, whereas the highly repetitive Ty3/gypsy elements exhibit a preference for insertion into or near other repetitive elements (Bennetzen 1996). Gypsy group elements exhibit similarities to retroviruses. Copia group sequences have been found in diverse species, including single-cell algae, bryophytes, gymnosperms, and angiosperms. Gypsy-like elements have been reported in major taxonomic plant groups (pine, lily, maize, tomato, pineapple, rice, several angiosperms and gymnosperms; see refs. in Friesen et al. 2001). In addition to these elements, short interspersed elements (SINEs) are nonautonomous retroposons that also have successfully spread within the genome of almost all eukaryotes. SINEs are ancestrally related to functionally important RNAs, such as tRNA, 5S rRNA, and 7SL RNA; possess an internal promoter that can be recognized by the RNA polymerase III (pol III) enzyme complex; and are usually organized in a monomeric or dimeric structure. Monomeric tRNA-related SINE families are present in the genomes of species from all major eukaryote lineages, and this organization is by far the most frequent. These elements comprise a 5′ tRNA-related region and a central region of unknown origin, followed by a stretch of homopolymeric adenosine residues or other simple repeats with the opposite organization. SINEs likely retropose by target site-primed reverse transcription (TPRT) using the enzymatic machinery of LINEs. Pol III SINE transcription is induced by cellular stresses such as heat shock, treatment with cycloheximide, DNA-damaging agents, and viral infections (see Pélissier et al. 2004 and refs. therein). The dispersion of retrotransposons follows the master sequence model (Deragon et al. 1994); i.e., only a small number of loci that match the master sequences are responsible for the amplification of the family, whereas the majority of the members are inactive on the evolutionary timescale. The consensus sequence of each family represents the starting point of the process and is copied in the genome in a great number of inactive retroposons. Each member of the subfamily diverges according to compositional constraints (Paces et al. 2004) after its dispersion from the master sequence. In contrast to retrotransposons, transposons (class II), such as miniature inverted-repeats (MITEs) (Jiang et al. 2004), Touristes, Ac, Dc, and Mu, have a low rate of amplification (approximately 100 copies per genome). The amplification of class II transposons relies on DNA, and they are associated with genes.

7.8 Genome Collinearity

Despite tremendous variability in genome size and chromosome number in angiosperm, comparative genetic mapping has revealed genome collinearity (gene order conservation) between

species of the same family. Thus, the map-based cloning of genes of a crop plants with a large genome size can be made easier by comparing it with a species of the same family but having a small genome. Comparative genetic mapping between species of the Poaceae revealed extensive genome collinearity (Fig. 7.16), but collinearity has also been observed in Brassicaceae, Solanaceae, pines, legumes (see in Gale and Devos 1998), and Euphorbiaceae (Chauhan and Sood 2013). If gene order tends to be conserved among species of the same family, it is not true for intergenic sequences, and micro-collinearity (collinearity at DNA level) can be hard to find. Numerous small rearrangements, insertions/deletions, duplications, inversions, and translocations have been detected. Duplications and subsequent gene loss might have occurred during the evolution of the genomes being compared. The probability of these event increases with the phylogenic distance but may also be consistent within the same family. Only five chromosomal inversions are needed to explain differences in marker organization between the 12 tomato and potato chromosomes. In contrast, collinear regions span only few centimorgans in the comparison of *A. thaliana* and *Brassica nigra* because of about 90 chromosomal rearrangements that occurred since the divergence of these two species. In distantly related species, it can be difficult to detect orthologous genes, and noncollinear positioning of transposon sequences can increase the difficulty of collinear segment detection. Size differences in intergenic regions are not always explained by retroelement sequences, and lack of collinearity can be due to different processes such as chromosomal rearrangements, unequal crossing-over, DNA slippage, point mutations, or gene conversions (Schmidt 2002). The rate of chromosome rearrangements detected by comparative map-

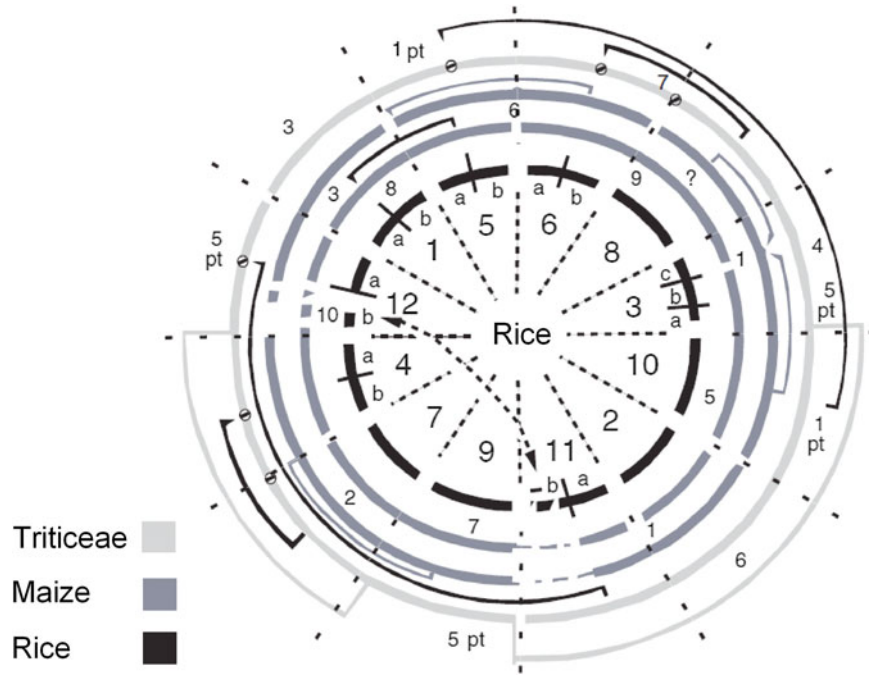

Fig. 7.16 Genome collinearity in Poaceae. The ancestral genome of Poaceae is represented by 19 linkage groups from rice aligned with the wheat and maize collinear regions (*circles*). The genome segments corresponding to chromosomes are themselves divided by *dashed lines* to indicate the linkage groups that are homologous among the three species. The numbers are for chromosomal locations. The alignment is based on the genetic map of the D genome of wheat. The linkage groups that form a part of wheat chromosome 5 are referred to by "pt" and linked by bridges. The chromosomes where a linkage group is inserted within another are indicated by *arrows* showing the insertion point (Modified from Bennett (1996))

ping of species pairs belonging to different families has been calculated to be 0.14 (+0.06) structural mutations per chromosome and per million years of divergence on average. By extension, it was estimated that 43–58 % of chromosomal tracts of ≤3 cM should remain collinear over a period of 130–200 million years. According to these predictions, distantly related species, such as those of monocotyledon and dicotyledon that diverged about 130–200 Mya, should share small collinear chromosome segments, which is actually the case (Paterson et al. 1996). Collinearity has also been shown between the genomes of grape, peach, and cacao (*Amborella* Genome Project 2013).

Comparing sea anemone (*Nematostella vectensis*) and human genomes, which evolved independently during approximately the last 0.7 Gya, Putnam et al. (2007) found that more than 30 % of conserved linkage groups accounted for a large fraction of the putative ancestral eumetazoan set. Typically, 40–50 % of genes from large-scale conserved collinearity have anemone or human counterparts and vice versa. Based on the genes conserved among anemones and humans, the LECA genome was not very different from ours. The putative LECA genome was intron-rich and contained the nearly complete toolkits for animal biochemistry and development (which can now be recognized as pan-eumetazoan), as well as the core gene set required to perform sophisticated neural and muscular functions. The common ancestor had blocks of linked genes that remain together in the modern human and anemone genomes. An ancestral linkage group of particular interest includes the human Hox clusters of homeobox transcription factors that regulate anterior–posterior identity in bilaterians (see in Putnam et al. 2007).

7.9 Multicellularity

Many requirements for multicellular organization (cell adhesion, cell–cell communication and coordination, programmed cell death) likely evolved in ancestral unicellular organisms. However, the evolution of multicellular organisms from unicellular ancestors may be opposed by genetic conflicts that arise when mutant cell lineages promote their own increase at the expense of the integrity of the multicellular organism. There are only three lineages that produced complex organisms, all from the eukaryote superkingdom: plants, animals, and fungi. Although the transition to multicellularity may be relatively easy, obstacles to the evolution of multicellular complexity are less well understood. Size initially favored can still promote the persistence of multicellularity. Increased size may have been favored to (i) provide storage reserves when nutrients are limiting, (ii) expand feeding opportunities, (iii) generate an internal environment protected by an external layer of cells, (iv) allow novel metabolic opportunities, or (v) enhance motility for dispersal or foraging (see in Grosberg and Strathmann 2007). In addition, some key metabolic processes cannot concurrently take place within a single cell. Division into germ and soma in Volvocacean green algae indicates benefits from size increasing in the course of evolution. The advantages of multicellularity depend upon cooperation among cells, but cooperation also leads to conflicts between genetically distinct cell lineages, some of which may be devoted to efficacy and others to selfish proliferation (for instance, in cancers). The price to pay for multicellularity is a sophisticated molecular machinery to manage complexity that relies on homeobox (Hox) genes, signaling pathways, and DNA methylation.

7.9.1 Homeobox Genes and Body Development

In animals, tetrapods have four Hox clusters that arose by duplication on the vertebrate stem (HoxA, HoxB, HoxC, and HoxD). Hox genes are typically found together in a single complex on a chromosome. Hox genes promote the identity of body parts or segments along the anterior–posterior axis of the embryo in the same order in which they lie on the chromosome. Most Hox genes interact with each other to maintain proper expression domain boundaries by both positive

and negative regulation (Miller et al. 2001). In general, a more posterior Hox gene will suppress the expression or the function of a more anterior one (Garcia-Fernàndez 2005). Genes within Hox clusters show temporal collinearity in addition to spatial collinearity (anterior genes are expressed earlier than posterior genes) and are regulated by long-range controlling enhancer elements grouped into *islands* acting on several neighboring Hox genes in a coordinated manner. The various islands contact each other and form megabase-scale regions of chromatin interactions (*topological domains*). As outlined above, such *regulatory landscape* was identified within gene deserts and displays intricate expression patterns (Montavon and Duboule 2013). From an evolutionary perspective, Hox genes are important because Mendelian inheritance of mutations in regulatory genes and nonlethal mutations in the recessive state may explain the punctuated evolution observed in fossil records (Schwartz 1999).

In plants, Hox genes range from 6 to 148 in number according to the species considered among flowering plants, *Selaginella*, and mosses (Figs. 7.17 and 7.18). Plant Hox genes can be unambiguously classified by sequence evolutionary analysis into 14 distinct classes that are also characterized by their conserved intron–exon structure and by unique co-domain architectures that were already differentiated in the last common ancestor of mosses and vascular plants 420 Mya (Dolan 2009) and evolved subsequently with tunned function in ferns (Huang et al. 2014), gymnosperms (Becker et al. 2003), and angiosperms (Kater et al. 2006). The total number of Hox genes found among plant genomes reflects the complex patterns of genome duplication and gene duplication or loss that have characterized the evolutionary histories of these species and the higher developmental and organizational complexity in angiosperms than in mosses and unicellular algae (Mukherjee et al. 2009).

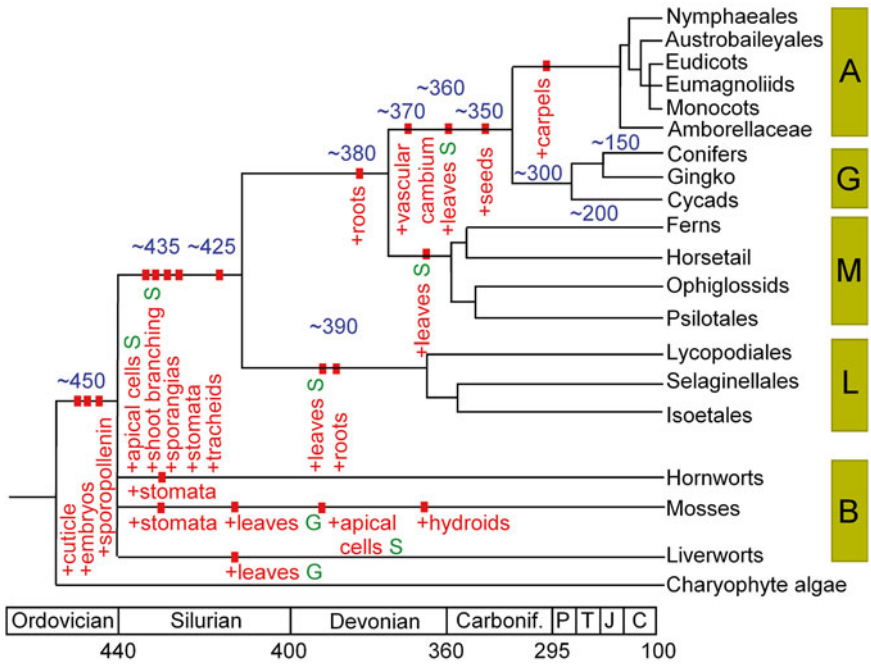

Fig. 7.17 Land plant phylogeny showing predicted developmental trait acquisition at each node (green "S" and "G" for sporophyte and gametophyte, respectively). The approximate time of divergence is given at each node in blue. The *bar* on the *right* indicates class divisions, i.e., *A* angiosperms, *G* gymnosperms, *M* monilophytes, *L* lycophytes, *B* bryophytes (Modified from Langdale and Harrison (2008))

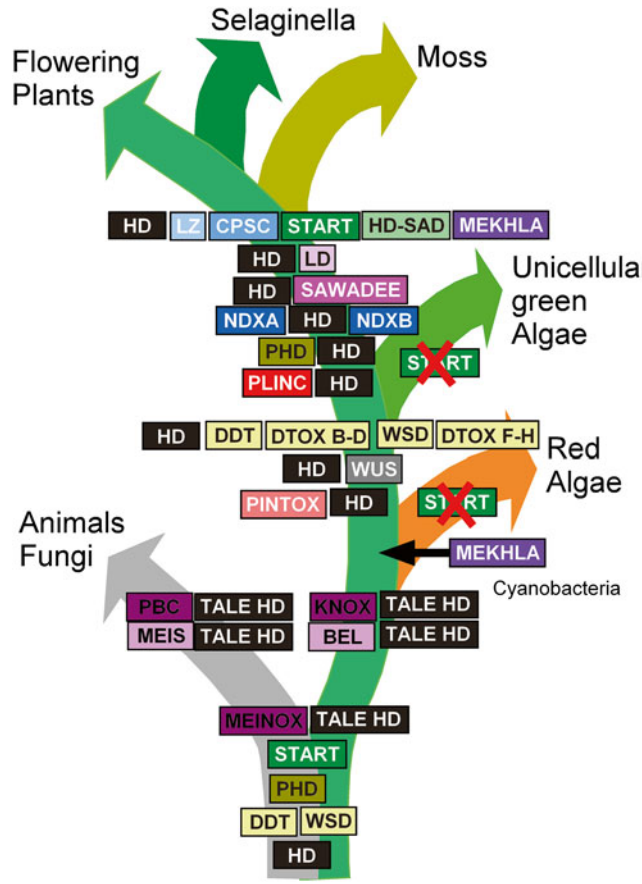

Fig. 7.18 Model of the putative evolutionary history of plant homeobox gene classes and co-domains. A class or motif represented in a parental branch indicates that the same class/motif is also present in all of its child branches unless otherwise indicated. The HD-ZIP I to IV classes are represented by the single domain architecture HD-LZ-CPSC-START-HD-SAD-MEKHLA. Loss of the START domain in the genomes of unicellular green algae and red algae is represented by the domain crossed in *red*. The *arrow* indicates the MEKHLA domain acquisition through Cyanobacteria and chloroplasts (Modified from Mukherjee et al. (2009))

In contrast to animals, plants entail the continuous development of new organs as time progresses (Geuten and Coenen 2013), which has implications for the type of regulatory elements used by Hox plant genes compared to animals. A number of genes that encode proteins involved in determining and maintaining the identity of shoot, root, vascular, and flower meristem cells have been identified in plant systems:

(i) *Plant apical growth*. Angiosperm shoot apical meristems (SAM) are layered; slowly dividing stem cells are surrounded by a peripheral morphogenetic zone of more rapidly dividing cells that are recruited for the differentiation of stem and leaf tissues. Growth is sustained through the activity of the *WUSCHEL* (*WUS*)/*CLAVATA* (*CLV*) pathway. The apical dominance is under a complex gene balance. In addition to the *KNOX* inhibitory effect and regulation by miRNAs, *AtMBD9* controls gene expression by chromatin structure modification through histone acetylation and by decreasing whole DNA methylation level. *AtMBD9* inactivation leads to altered expression of a number of genes related to the axillary branching and flowering pathways (Yaish et al. 2009).

(ii) *Leaves*. In angiosperms, leaf growth is finite and usually exhibits an adaxial/abaxial asymmetry whose opposing developmental programs coexist at the shoot apex. Class I *KNOX* genes are downregulated in the leaf primordia at the periphery of the SAM by members of the myb-domain protein family including *ASYMMETRIC LEAVES1* (*AS1*) in *A. thaliana*. Leaf blade polarity is established by the coordinated expression of a number of different transcriptional regulators. In *Arabidopsis*, factors such as *AS1* and *AS2* myb-factors, zipperIII (HD-ZIP III)

transcription factors, and *PHABULOSA* (*PHAB*), *PHAVOLUTA* (*PHAV*), and *REVOLUTA* (*REV*) genes confer adaxial identity while *YABBY* and *KANADI* genes confer abaxial identity (Friedman et al. 2004).

(iii) *Vascular system*. The activity of the two transcription factors VASCULAR RELATED NAC-DOMAIN6 (VND6) and VND7 is sufficient to induce transdifferentiation of various cells into protoxylem or metaxylem vessel elements in Arabidopsis.

(iv) *Roots*. The developmental program associated with root apical meristems (RAM) is similar to that of SAM. There is a reiteration of the receptor-like kinase pathway in RAM, and ligands of the *CLV3/ESR*-related (*CLE*) gene family are involved. A homologous *WUS*-like homeodomain gene, *WUSCHEL-related homeobox5* (*WOX5*), is also expressed in *A. thaliana* roots. Two members of the GRAS-transcription factor family, SCARECROW (SCR) and SHORTROOT (SHR), are required for the establishment of the endodermal layer in both roots and shoots (Friedman et al. 2004).

(v) *Flowers*. The growth of the flower's individual organs has been modeled using the *ABC model* with the aim to assign homeotic genes as determinant of organ's identity in order to describe the biological basis of the developmental process. This model was then extended with D and E. Each letter depicts the pattern of floral organ development. Floral primordia are arranged into successive whorls of sepals, petals, stamens, and carpels, all these organs being homologous to leaves. The A genes act as determinants of sepal–petal identity and contribute to the repression (*APETALA1* and *APETALA2*) of B, C, and E genes. The B (*APETALA3* and *PISTILLATA*) and C (*AGAMOUS*) genes establish the identity of stamens and carpels. The D (*FLORAL BINDING PROTEIN7* and *FLORAL BINDING PROTEIN1L*) genes specify the identity of ovules as a separate reproductive feature compared to carpels. The E genes function as a physiological requirement necessary for the development of the three innermost whorls. When D function is lost, the structure of ovules becomes similar to that of leaves, and when E function is lost, all verticil structures are similar to leaves.

The A, B, C, and D floral genes are MADS-box genes, which encode transcription factors involved in controlling all major aspects of development. Animal and fungal genomes generally possess only around one to five MADS-box genes while angiosperm genomes have around 100 MADS-box genes. The MADS-box family has been divided into two main groups. Type I includes *ARG80/SRF-like* genes of animals and fungi, also designated as M type genes in plants, while type II includes *MEF2-like* genes of animals and yeast as well as MIKC-type genes of plants. It is proposed that an ancestral duplication before the divergence of plants and animals gave rise to these groups. The MIKC-type genes are also characterized by the presence of the K domain that could have evolved after the divergence of these lineages. Type II genes have been categorized into MIKCC and MIKC* based on structural features. MIKCC genes have been further classified into 14 clades based on phylogeny. Type I genes have also been categorized into M (α, β, γ, δ) and N types based on their protein motifs as well as on their phylogenetic relationships (Arora et al. 2007). Members of the plant class I MADS-box domain subfamily from *Arabidopsis* includes 61 genes from which only *PHERES1*, *AGAMOUSLIKE80* (*AGL80*), *DIANA*, *AGL62*, and *AGL23* were functionally characterized and revealed important roles for these genes during female gametophyte and early seed development. A complete atlas of their expression patterns in *Arabidopsis* revealed that the expression of class I MADS-box genes is predominantly observed in the central cell, antipodal cells, and chalazal endosperm (Bemer et al. 2010). Seventy-five rice MADS-box genes were also characterized for expression profiling by microarray and were mapped along rice chromosomes showing some regional segregation between MIKC and M types (Arora et al. 2007). In seed plants, the MIKCC shows considerable

diversity, but the MIKC* has only two subgroups, P and S clades, which show conserved expression in the gametophyte. All three MIKC* genes are expressed late in pollen development (Liu et al. 2013). The absence of class I MADS-box genes in gymnosperms suggests that they were recruited in basal angiosperms for the evolution of the double fertilization process (Bemer et al. 2010).

In contrast to A, B, C, and D genes, E genes belong to miRNAs. *miR156* and *miR172* act as the main players in the regulation of developmental timing in flowering plants. In early stages of development, *miR156* levels are high and, then, decrease during plant development, while *miR172* shows the opposite pattern. These two microRNAs contribute to both the juvenile–adult phase transition and the transition to flowering through their sequential and antagonistic actions. *miR156* represses targets of the *SQUAMOSA PROMOTOR BINDING PROTEIN-LIKE* (*SPL*) gene family and maintains juvenile features. When miR156 levels decline, the level of SPL proteins increases, *miR172* is activated, flowering genes are activated, and adult leaf features are induced. When *miR172* levels are increasing, the *APETALA2-like* (*AP2-like*) genes are progressively silenced and adult leaf traits as well as flowering are induced (see in Geuten and Coenen 2013). More miRNAs are being described as important players in plant development as expected from their early evolution (Fig. 7.19).

Interestingly, extensive RNA silencing can be generated, in plants, through ncRNA diffusion from cell to cell through plasmodesmata and from cell to organs over long distances through the vascular phloem. For example, the trans-acting siRNA (ta-siRNA) silencing activity of *miRNA165/166* over a short distance is crucial for the regulation of the HD-ZIP III transcription factor in a dosage-dependent manner in roots and is also involved in xylem patterning. The extent of silencing through plasmodesmata is limited to as few as 10–15 cells beyond the site of initiation. Silencing over greater distances may be achieved by an amplification relay. A signal initiated in a few cells of a source leaf can be actively spread over longer distances through phloem to induce silencing in sink leaves as is the case for disease

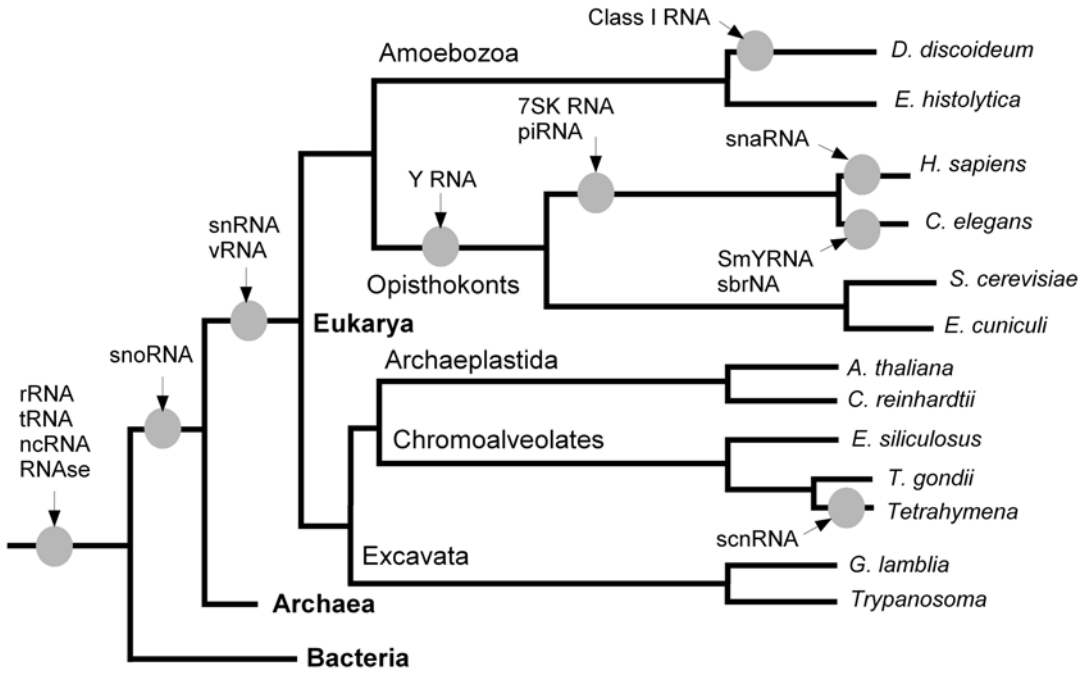

Fig. 7.19 An overview of the emergence of some important ncRNAs in eukaryotes (Modified from Avesson (2011))

control by grafting the shoot of one species on the root system of another (see in Uddin and Kim 2011).

Signaling pathways evolved by gene duplication and subsequent protein sequence divergence. Plants have unique features that evolved in response to their environments and ecosystems. The protein–protein interaction (PPI) patterns in *Amborella* are generally consistent with those in other angiosperms and show clear differences from those in gymnosperms. Evolutionary shifts in PPI patterns after gene duplications along with changes in gene sequence and expression patterns have likely been crucial for functional innovations in the regulatory network for reproductive organ development and the flower origin as well as for functional diversification of the many floral forms among lineages of angiosperms (*Amborella* Genome Project 2013). The complete *Arabidopsis* protein–protein interactome, excluding isoforms, is estimated to be 299,000 ± 79,000 binary interactions. Despite the fact that the *Arabidopsis* interactome is estimated to be larger than those of yeast, worms, or humans (Brown and Jurisica 2005), the number of interactions per possible protein pair is similar among all four species (5–10 per 10,000). The dynamic rewiring of interactions following gene duplication events appears as an evidence that evolution acted upon interactome networks. The difficulty to unveil network interactions in plants is because more than 60 % of the protein-coding genes of the model plant *A. thaliana* remain functionally uncharacterized (*Arabidopsis* Interactome Mapping Consortium 2011).

7.9.2 DNA Methylation

Dynamic DNA methylation is important in maintaining the epigenomic plasticity to enable efficient and coordinated responses to developmental signals and environmental stresses. DNA methylation refers to the covalent addition of a methyl group (–CH3) to the base cytosine (C) in the dinucleotide CpG to form 5mC. Most CpG dinucleotides in the human genome are methylated. However, unmethylated CpGs are not randomly distributed but are usually clustered together in *CpG islands* in the promoter region of many genes.

A relatively high degree of nuclear DNA methylation is a specific feature of plant genomes. Targets for cytosine DNA methylation in plant genomes are CG, CHG, and CHH (H is A, T, C) sequences. DNA methylation in plants is species, tissue, organelle, and age specific; it is involved in the control of all genetic functions including transcription, replication, DNA repair, gene transposition, and cell differentiation. DNA methylation is engaged in gene silencing and parental imprinting; it controls the expression of transgenes and foreign DNA in cells. Plants have a much more complicated and sophisticated system of genome methylations compared to animals. The level of methylation ranges from 3 to 8 % of cytosines in vertebrates and from 6 to 30 % in plants. Considering *Arabidopsis*, about 19 % of the entire genome sequence is methylated. As expected, extensive DNA methylation is found in the heterochromatic centromeric and pericentromeric regions, reflecting the dense methylation of transposons and other repetitive sequences. Consistent DNA methylation is also found in euchromatic regions, both in intergenic regions and genes, but promoter methylation seems to be more important for downregulating gene expression than CDS methylation. The relative distribution of 5mC residues between different sequence contexts is 55 % in CG, 23 % in CHG, and 22 % in CHH. Thus, nearly half of all cytosine methylated sites throughout *Arabidopsis* genome are non-CG ones. Most CG sites are either unmethylated or highly methylated (80–100 %), CHG sites are unmethylated or partially (between 20 and 100 %) methylated, and CHH sites are either unmethylated or lowly methylated (about 10 %). All three methylation types are found in repeat-rich pericentromeric regions, whereas gene bodies contain almost exclusively CG methylation. Among all expressed genes, more than ~60 % appeared to be entirely unmethylated and about ~5 % are methylated at their promoter regions (within 200 bp upstream of transcription start site). About 33 % of genes are methylated in their CDSs and unmethylated in

their promoter regions (body methylated). The 5′ and 3′ flanking sequences of most body-methylated genes tend to be relatively hypomethylated. Body-methylated genes tend to be highly and constitutively expressed; many genes coding for enzymes occur in this category. Genes with methylated promoters tend to be expressed at a relatively lower level, often in a tissue-specific fashion (see in Vanyushin and Ashapkin 2011).

In *Arabidopsis,* establishing methylation in all sequence contexts is entirely dependent on the de novo methyltransferase activity by *DOMAINS REARRANGED METHYLTRANSFERASE* (*DRM*) (similar to the mammalian *Dnmt3* family), which functions through the RNA-directed DNA methylation (RdDM) pathway. The *Arabidopsis* MET1 (a homologue of the *Dnmt1* that is responsible for maintenance of CG methylation in mammals) is controlling CG methylation and also likely acts in the maintenance of non-CG methylation. *MET1* is responsible for 60 % of methylation. CHG methylation is mainly maintained by the plant-specific *CHROMOMETHYLASE 3* (*CMT3*) and *KRYPTONITE* (*SUVH4*). These enzymes are important to warrant the *maintenance* of preexisting methylation patterns after DNA replication (see in Zhang et al. 2010), which is referred to as the *passive mode* of cytosine removal. In the active mode of demethylation, the methyl mark is removed by DNA glycosylases (DME and ROS1) that are directed toward methylated cytosines even in the absence of DNA replication.

Methylation levels change during plant development with the highest methylation levels being observed in senescence tissues. The methylation pattern is largely stable over multiple generations in plants and is consistent with genomic imprinting. Genomic imprinting refers to the parental specific expression of a gene. This process evolved independently in flowering plants and mammals. In mammals and flowering plants, imprinting occurs in the embryo and its nourishing tissues, i.e., placenta and endosperm, respectively. Imprinted genes are differentially marked in the gametes before fertilization, which makes maternal and paternal chromosomes functionally different. Imprinting is mainly accomplished by the active removal of methylated cytosines by DNA glycosylases, rather than through sequential genome-wide erasure followed by the targeted de novo methylation of paternal alleles at the imprinted loci as is the case in mammals. Maternal DME activity is required for development of viable seeds, which reflects its expression specificity or imprinting in the central cell of the female gametophyte. In plants, imprinting might have evolved as a by-product of a defense mechanism destined to control transposon activity in gametes. A selective advantage of this process might have been that imprinted genes control the nutrient flow from the mother to the offspring (see in Köhler and Weinhofer-Molisch 2010). Mechanistically, methylated cytosines are capable of attracting methyl-binding proteins (MBD), which in turn recruit histone deacetylases and chromatin remodeling proteins to form a complex, which hinder the binding by transcription factors. At least six *Arabidopsis* MBD genes (*AtMBD1, 2, 4, 5, 6,* and *7*) exhibited specific binding activity for methylated CG sequence in vitro (Zhang et al. 2010). The tendency to be inherited through multiple generations seems to be one of the major differences between DNA methylation and other epigenetic modifications, such as histone modifications and chromatin remodeling. Adaptive responses to environmental factors may be conferred by a local change in DNA methylation and be stable for multiple plant generations. However, many regions of an artificially demethylated genome gradually regain initial methylation in few generations. A classical example of adaptive epimutation is a naturally occurring flower mutant of *Linaria vulgaris* changing flower symmetry from bilateral to radial. This mutation was first described by Linnaeus but only recently was shown to be a result of an epigenetic modification. This epimutation rarely reverts to wild-type flowers. Thus, an epigenetic mutation may be heritable for hundreds of generations and play a significant role in evolution. Other examples relative to disease and salt resistance were also given (Vanyushin and Ashapkin 2011).

Comparison of methylated genome sequences and known collections of *Arabidopsis* smRNA

sequences showed that the majority of siRNAs correspond to heavily methylated DNA sequences, which is not surprising since siRNA in plants is preferentially associated to intergenic sequences such as retrotransposons and other repetitive elements. In wild-type plants 85.4 % smRNA loci contained, at least, one 5mC. Overall half of all cytosines are methylated at these loci, whereas about 4 % of all cytosines are methylated at non-smRNA loci. However, only a third of all methylated cytosines are associated with smRNA loci (see in Vanyushin and Ashapkin 2011). The 24 nt siRNAs cause epigenetic silencing by directing de novo cytosine methylation through the RdDM pathway, where the siRNAs are generated by the action of the putative DNA-directed RNA polymerase Pol IV, RDR2 (RNA-dependent RNA polymerase 2), and DCL3 (Dicer-like 3). The siRNAs are then loaded into AGOs to direct cytosine methylation by the de novo DNA methylase DRM2 (see in Zhang et al. 2010).

7.10 Synthetic Biology

Synthetic biology is an emerging field focused on the engineering of biomolecular and cellular systems for a variety of medical or industrial applications for product development (Ruder et al. 2011); this field came into reality with genetic engineering in the 1970s. After plasmid cloning, signals of a newly emerging era were seen in artificial chromosomes, which were first constructed in budding yeast (YAC, Murray and Szostak 1983) and *Escherichia coli* (BAC, O'Connor et al. 1989). BACs and YACs have proven invaluable for the sequencing, mapping, and characterization of many large genomes due to their utility in cloning large DNA fragments. In plants, telomere-mediated truncation coupled with the introduction of site-specific recombination cassettes has been used to produce minichromosomes consisting of little more than a centromere. The idea is to use synthetic chromosomes to add multiple transgenes, multigene complexes, or entire biochemical pathways to plants to change their properties for agricultural applications or to use plants as factories for the production of foreign proteins or metabolites (Gaeta et al. 2012).

Synthetic biology is now moving into an era of whole-genome synthesis. The genome of *Mycoplasma mycoides* has been synthesized in vitro based on in silico data, assembled in yeast, and reintroduced into recipient cells, thereby giving rise to a self-replicating cell line (Gibson et al. 2010). The cell lines behaved normally in culture and had a relatively stable phenotype, although they contained some mutations introduced during their development. This study demonstrated that genetically functional living cells can be produced from computer-designed genome annotations.

The applications of synthetic genomics now extend beyond the engineering of genes to include whole metabolisms, regulatory networks, and even ecosystems. Wild cells use genetic circuits comprising interacting genes and proteins to implement functions including growth and division, signaling, and differentiation. A major goal of synthetic biology is to develop biological design by building circuits that are not able to survive in the natural world but that have biotechnological applications through their ability to perform processes at low energy cost. Engineered organisms are expected to change our lives in the near future, leading to cheaper drugs, biofuels, biomaterials, and targeted treatments against infective diseases and physiological dysregulation, such as cancer (Khalil and Collins 2010). One can already often modify (i.e., *rewire*) parts of wild circuits, thus providing insights into the design principles of native architectures. Rewiring and building autonomous and integrated circuits de novo will become one of the primary tools to understand, control, envision, and create new biological systems for biotechnological or medical purposes (Nandagopal and Elowitz 2011; Ruder et al. 2011). Synthetic construction methods have applied to genetic switches, memory elements, and oscillators, as well as to other electronics-inspired genetic devices, including pulse generators, digital logic gates, filters, and communication modules (Khalil and Collins 2010).

The boot-up and propagation of synthetic information involves its transcription, translation, replication, and regulation to produce the desired effect or phenotype (Shimizu et al. 2001), which has ultimately led to the encapsulation and expression of DNA in artificial cells (Amidi et al. 2011; Hosoda et al. 2008; Kuruma et al. 2009; Murtas 2009). Artificial cell engineering (Noireaux et al. 2011; Noireaux and Libchaber 2004) has led to the encapsulation of entirely reconstituted *E. coli* translational machinery (the PURE system, Shimizu et al. 2001) inside vesicles (Luisi et al. 2010; Pereira de Souza et al. 2009). Consequently, the need to develop methods for the propagation of DNA and vesicles has led to the production of an advanced protocell that links the process of DNA replication to the multiplication of encapsulating vesicles (Kurihara et al. 2011). However, synthetic genomics may involve a variety of DNA templates from a variety of different organisms, thus complicating the process. This creative endeavor requires the assistance of software, which is being progressively written (Xia et al. 2011), to assist the entire genome design process (Khalil and Collins 2010). The task of synthetic genome design is conceptually similar to the task of computer program writing, involving version management, tracking of dependencies, metadata management, and tools that promote code reusability.

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Organellar Genomes of Flowering Plants

8

Ami Choubey and Manchikatla Venkat Rajam

Abstract

Arguably, the two most important organelles of the eukaryotic cell are not its original inhabitants. Mitochondria and chloroplasts have evolved by endo symbiosis and have a prokaryotic ancestry. This is reflected perfectly in their genome organization and the basic functioning of their genetic system. Over the long course of evolution, they have given away most of their genes. The genes were either lost because they were no longer needed or were relocated to the nucleus. Nuclear transfer of their DNA is an ongoing process, and the two organellar genomes are still evolving. The DNA sequences are in a constant state of motion because of interorganellar and horizontal gene transfer within and between different plant species. In contrast to the chloroplast genomes, which are much conserved and do not generally exhibit any structural or functional anomalies, mitochondria are notorious for their lack of synteny, frequent DNA rearrangements, constantly varying intergenic regions and accumulation of heterologous DNA sequences. Since the nucleus cannot work in isolation, all the random and mischievous genetic activities of organellar genomes have had a direct or indirect impact on nuclear genome evolution. They act as one of the major mechanisms by which genetic novelty is brought about in the nuclear genome. As they say, to produce good music, both black and white chords have to work in harmony. Similarly, irrespective of individual nature of the three genomes in a cell, these three always work in harmony for optimum functioning of the plant cell.

Keywords

Endosymbiosis • Nuclear genome • Chloroplast genome • Mitochondrial genome • Genome evolution • Interorganellar and horizontal gene transfer • DNA rearrangements • Heterologous DNA sequences

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8.1 Introduction

From freedom to captivity,
From securing a home to losing their identity,
They were destined to live in servitude.
Yet they fought, fought hard for their genomic treasure,
Came out winners and then stayed on with pleasure

The lines above broadly summarize the origin of mitochondria and chloroplast by endo symbiosis and their evolution as semi-autonomous organelles in the modern eukaryotic cell. The mitochondria arrived first, followed by the plastids. Their genomes have undergone a sea change from their free-living ancestors to the present day, highly reduced and tightly regulated organellar genomes of the much evolved flowering plants. Both the organelles had to lose a major share of their genes to the nucleus, yet they managed to retain some of the most essential ones in their genome compliment. They have also evolved a complex transcriptional and translational machinery to coordinate their gene expression with the nuclear genome. The field of organellar genome research has accelerated a lot in the past two decades with the availability of whole genome sequences of various plant species. It has helped in the better understanding of their genomic structure, organization, gene contents, sequence evolution and the genetic exchanges between the nucleus and organelles and also amongst the organelles.

Comparative analyses of the chloroplast genomes suggest that over the course of evolution, they have remained much conserved in their structure, gene composition and gene order (Barbrook et al. 2010). It's just the opposite in the case of plant mitochondrial genomes. They exhibit a highly variable genome, show drastic size differences, contain many introns and repeat elements and experience frequent genomic rearrangements due to recombination at the repeat sequence sites (Knoop 2012). Also, the mitochondrial intergenic regions receive promiscuous DNA originating from both nuclear and chloroplast genomes. Such anomalies and their evolutionary significance continue to baffle the geneticists and the evolutionists alike.

So, in the same chronological order in which they had first appeared in their eukaryotic host cell, we will discuss in brief various aspects of the two organellar genomes, their complexities and their motive for still surviving inside the organelles.

8.2 Endosymbiotic Evolution of Organelles and Their Genome

Plastids and mitochondria are semi-autonomous organelles in a eukaryotic cell which perform the bioenergetic functions of photosynthesis and respiration, respectively. Till two billion years ago, they lived as free-living prokaryotes. So, they have not been together forever. The concept of their endosymbiotic origin in eukaryotic cells from the prokaryotic ancestors was laid more than a hundred years ago by a Russian botanist von C. Mereschkowski (1905) in his published work *Über Natur und Ursprung der Chromatophoren im Pflanzenreiche* (eventually translated in English by Martin and Kowallik in 1999). It went unnoticed and unappreciated for a long time. In fact it took almost another 70 years for the idea to be rediscovered by Margulis (1970) and for the evidences to show up in support of the theory. It began when Lynn Margulis first saw cytoplasmic incorporation of labelled DNA precursors in amoeba (unicellular eukaryotes) but could not immediately relate her observations with the organelles. As the similarities between the organelles and bacteria became more apparent, she got convinced that the hypothesis was true and that is how the theory of endo symbiosis was rediscovered and presented with more conviction the second time (Wells 2005).

Endosymbiotic theory states that mitochondria and chloroplasts have originated from free-living prokaryotes (endosymbionts) – α-Proteobacteria and Cyanobacteria, respectively – when they entered into a symbiotic relationship with a host cell (Gray 1999). This was

followed by a drastic reduction in their genome size. From harbouring thousands of genes in their prokaryotic ancestors, they were reduced to a few essential genes in their newly evolved organellar genomes. In fact in mitochondria, the number has sunk further down to below a hundred (Unseld et al. 1997).

It is now an accepted fact that inception of the two organelles had occurred independently of each other, with mitochondria preceding chloroplasts. As far as the nature of the host cell is concerned, it is still controversial. Although the classical endosymbiotic theory suggests that it was an amitochondriate eukaryotic cell which played as a host to our mitochondrial ancestor α-Proteobacterium, there is no evidence yet which proves the existence of an amitochondriate phase in eukaryotic evolution. So, it might be possible for them to have a concurrent origin (Gray and Archibald 2012). As for the chloroplasts, the host was a eukaryotic phagotroph (Douglas 1998).

Although there is an overwhelming molecular data to help understand explicitly even the most intricate details of the endosymbiotic theory of organellar evolution, some fundamental questions pertaining to the earliest events leading to the establishment of organelles in the hypothetical eukaryotic host cell are still unanswered (Gray and Archibald 2012).

8.3 Organellar Genome

8.3.1 Mitochondrial Genome

In spite of the recent advances in next-generation sequencing methodologies, sequencing plant mitochondrial genomes (also known as plant chondromes) has not been as successful as their plastid counterparts in terms of the number of species whose chondromes have been completely sequenced. As per the NCBI organelle genome resources database, around 94 plant chondromes have been sequenced so far of which flowering plants account for just 37 (Shearman et al. 2014). Plants have a very large and complex mitochondrial genome that exhibits enormous variation in its size and structure and has long-endured frequent genomic rearrangements in its DNA. All these factors, along with a large number of repetitive sequences accumulated by the plant chondromes, made the sequencing of plant mitochondrial genomes very difficult (Zhang et al. 2011). It is because of these technical difficulties that it took 12 years for the first angiosperm mitochondrial genome (*Arabidopsis*) to be sequenced (Unseld et al. 1997) after the first chloroplast genome (*Nicotiana tabacum*) was sequenced in 1985.

Plant chondromes are characteristically peculiar in many ways. Firstly, in contrast to their metazoan counterparts that average just about 16 kb in size, plant chondromes are enormously large double-stranded (ds) DNA molecules showing dramatic size variation, ranging between 200 and 2,900 kb in land plants, though their coding capacity (~50–60 genes) is surprisingly very low (Unseld et al. 1997; Marienfeld et al. 1999; Handa 2003; Sugiyama et al. 2005). The enormous genome size is partly due to the accumulation and duplication of repetitive sequences and partly due to DNA intrusion from the intracellular nuclear and plastid genomes (Kitazaki and Kubo 2010). It may also receive DNA sequences by horizontal gene transfer (HGT) (Bergthorsson et al. 2003). Other features include extensive intramolecular recombination leading to frequent genomic rearrangements and presence of a number of interrupted genes that require trans-splicing at the RNA level (Knoop 2012). Above all, they are still reeling under the ongoing evolutionary process of DNA transfer to the nucleus. Combined with the high dose of RNA editing, trans-splicing, low mutation rates and the fact that it has a rapidly evolving genomic structure yet the slowest in terms of sequence evolution amongst the three plant cell genomes (Palmer and Herbon 1988; Knoop 2004), plant chondromes make for an interesting study subject to trace phylogeny of early land plants (Knoop 2004). These striking features, in short, make plant mitochondrial genome alluring for studying the dynamics and organellar genome evolution inside its eukaryotic host cell.

8.3.1.1 Structural Organization

The decades' long quest for decoding the in vivo structure of mitochondrial DNA (mtDNA) paints a hazy picture. Akin to chloroplast genome, the general opinion about mitochondrial genomes was that they were circular DNA molecules, but there could be other structural forms present as well. The linear mtDNA molecules, which would often come up in the isolated mtDNA preparations, were dismissed as artefactual degraded products of circular mitochondrial genomes. Over the years, numerous attempts of recovering large populations of intact circular mtDNA have failed due to the predominance of linear and branched structures which show up in most of the electrophoretic and microscopic analyses of mitochondrial DNA (Bendich 1993). There is a sufficient evidence to support both the circular and the linear, multibranched forms of mitochondrial DNA, yet the idea of mtDNA as circular DNA molecules has persisted (Mower et al. 2012; Sloan 2013). Deep sequencing analyses in recent years have proven to be the myth busters with overwhelming evidences to prove that the linear forms not merely exist but exist in good percentage. In fact in the case of maize CMS-S cytotype, the mitochondrial genome exists mainly as multiple linear molecules (Schardl et al. 1984; Allen et al. 2007).

Mower et al. (2012) have precisely defined the paradox – 'Circular maps continue to be presented in genome sequencing publications because they are convenient indicators of genome content and sequencing completion'. So, irrespective of what the real structure is, the mitochondrial genome is conveniently described as a 'master circle' which is a single circular DNA molecule that carries a complete set of genes (Lonsdale et al. 1984). Hence, the name was given 'master'. In spite of having a defined physical map of their genome, their structure in vivo is still way complicated and difficult to comprehend. They may also sometimes appear larger than their size (Backert et al. 1997). It is because they carry repeat sequences, ranging from 6 bp to 14 kb, dispersed throughout the genome and undergoing active recombination, that they have a multipartite genomic organization and exhibit isomeric structural forms (Fauron et al. 2005). Frequent homologous recombination events within a pair of direct repeat sequences of the master circle may lead to 'looping out' of smaller circles called sub-circles (Kitazaki and Kubo 2010). Similarly, large inverted repeats in mitochondrial genome may also recombine to give rise to isometric genome structures. Both the isometric and sub-circular forms are subgenomic structural isomeric forms of the master circle genome (Chang et al. 2013). Sometimes, illegitimate recombination may occur at short repeat sequences that give rise to substoichiometric DNA molecules called sublimons. They differ in sequences, and hence only one of the forms survives in subsequent divisions (Kitazaki and Kubo 2010; Gabay-Laughnan and Newton 2012).

8.3.1.2 Genome Size and Its Genetic Make-Up

8.3.1.2.1 Genome Size

Plant mitochondrial genomes show a wide size variation, ranging from 200 kb to up to 2,900 kb (Fig. 8.1). In the family Cucurbitaceae alone, genomes range from an estimated 390 kb in *Citrullus lanatus* (watermelon) to an unprecedented 2.9 Mb in *Cucumis melo* (muskmelon) (Ward et al. 1981) which is even bigger than the genomes of many free-living bacteria (Moran 2002). This extreme inflation in the mitochondrial genomes of Cucurbitaceae family members is mainly due to the accumulation of large amounts of plastidial sequences, proliferation of dispersed short repeat sequences, expansion of existing introns and accumulation of large amounts of heterologous DNA sequences from various sources like nuclear and chloroplast genomes, viruses and bacteria (Alverson et al. 2010, 2011a). Ever since their inception in the eukaryotic cell, mitochondrial genomes of all plant species have undergone enormous size expansion in their intergenic regions and are still expanding. This is in much contrast to the metazoan mitochondrial genomes which have progressively become smaller and more compact in size. Although their sequences are known to be still evolving, the gene number, their arrangement and the overall genome size have remained

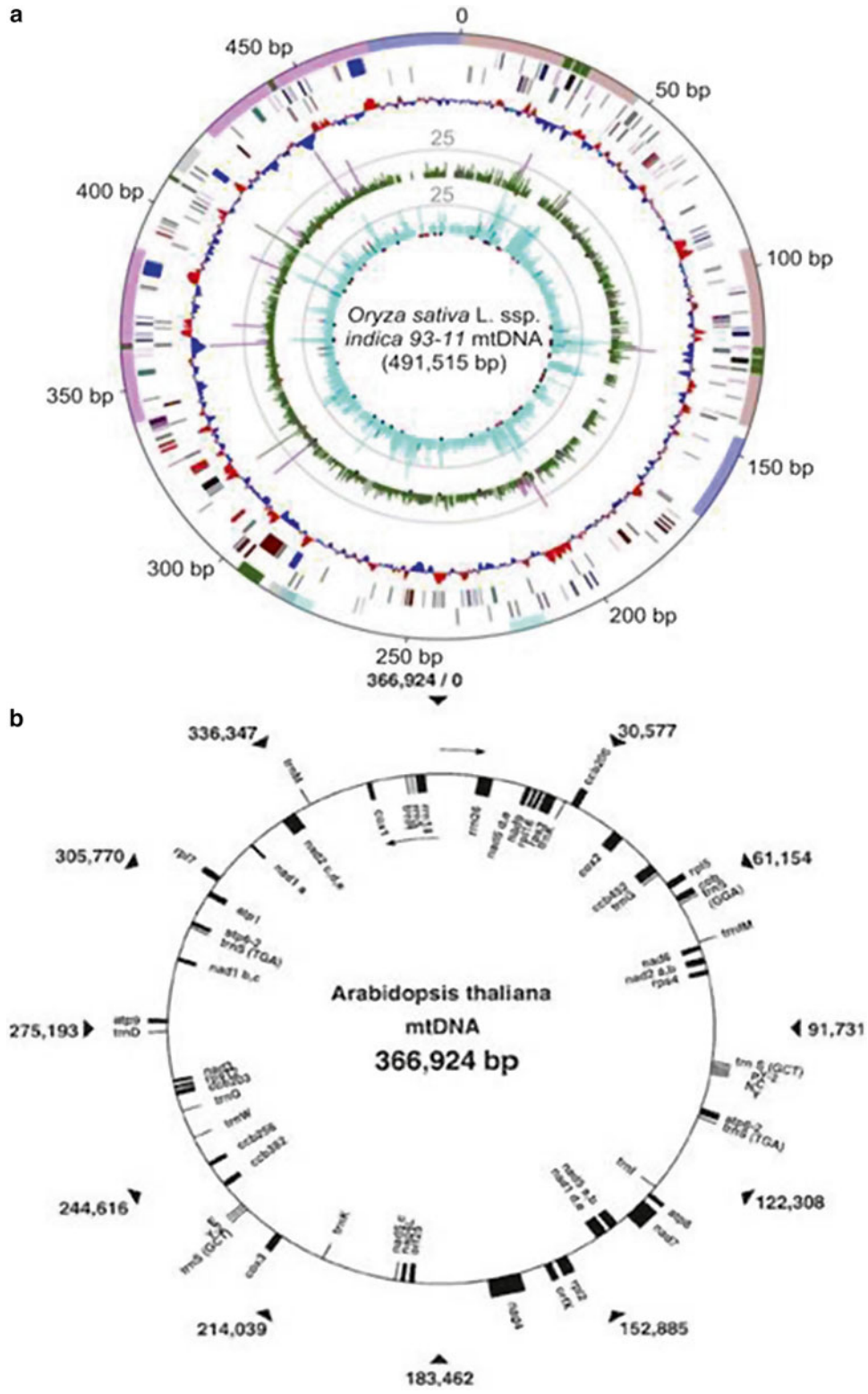

Fig. 8.1 Circular DNA maps of two mitochondrial genomes: (**a**) Rice mitochondrial genome (Tian et al. 2006) and (**b**) *Arabidopsis* mitochondrial genome. Genes annotated outside the *circle* are transcribed clockwise, and those inside are transcribed anticlockwise (Dombrowski et al. 1998)

constant over the long periods of evolutionary time (Boore 1999). True for all plant mitochondrial genomes, despite the overall increase in their genomic content, the coding regions have remained conserved and limited.

Both nuclear and plastid sequences contribute to size expansion of the mitochondrial genomes. Nucleus-derived mobile genetic elements, especially retrotransposons, integrate and propagate in the intergenic regions. Until we have the information on more nuclear genome sequences, it puts a constrain on identifying new sequences of nuclear origin in the mitochondrial genomes. Plastid-derived sequences are also prominently found in the mitochondrial genome. Apart from the sequences from these two known sources, most of the sequences in the intergenic region are of unknown origin (Kubo and Newton 2008).

Most of the intergenic regions are not even conserved between any two plant species (Kubo and Mikami 2007). Much of this diversity is due to the differences in the accumulation and activity of repetitive sequences. Repeats of diverse size and number are found in the mitochondrial genomes which also lead to the expansion of intergenic regions. The repeat sequences affect their structural diversity by giving rise to subgenomic circles on the account of undergoing inter- and intramolecular recombination (Alverson et al. 2011b).

8.3.1.2.2 The Genetic Make-Up

During evolution, most of the essential mitochondrial genes have been transferred to the nucleus where they are transcribed, and their products are transported right back into the mitochondria either in the RNA or in the protein form. Some protein-coding genes though are retained in the mitochondria and require mitochondrial transcriptional, translational machineries for their expression (Unseld et al. 1997). Unlike the chloroplast genome which has retained a full set of their RNA genes like rRNA, tRNA and RNA components of ribonucleoproteins, mitochondria do not contain the complete set of tRNA genes sufficient to read all its codons (Marienfeld et al. 1999). Consequently, it has to import the cellular tRNAs and aminoacyl tRNA synthetases to compensate for their missing function in mitochondria (Duchêne et al. 2009). Then, there are a few 'chloroplast-like' tRNA genes in mitochondria which are plastid genome derived and code for mitochondrial tRNAs (Maréchal-Drouard et al. 1993). This way, the mitochondrial genomes contain both native and immigrant types of information. The native class comprises of those genes which have always been in the mitochondria, 'the original occupants' of the mitochondrial genome. This class includes the genes for respiratory chain functions, ribosomal protein genes, incomplete set of tRNA genes, proteins involved in cytochrome c biogenesis and some conserved open reading frames. The immigrant class includes transposon sequences from the nucleus and tRNA genes from the chloroplast (Marienfeld et al. 1999).

Mitochondrial genome may also contain pseudogenes – evolution's misplaced witnesses (Bensasson et al. 2001) – scattered throughout the genome. They either represent the remnants of genes which have got transferred to the nucleus rendering their mitochondrial copies redundant and nonfunctional or they could be the nonfunctional versions of functional mitochondrial genes arisen by the gene duplication event. Another prominent feature of mitochondrial genes is the presence of introns which all belong to the category of group II introns with the only exception of horizontally acquired group I intron in *cox1* gene of mitochondria (Kitazaki and Kubo 2010). Group II introns include both *cis*-splicing and *trans*-splicing introns. Although group II introns are known to act as both catalytic RNAs and mobile genetic elements (Lambowitz and Zimmerly 2004), none of the plant mitochondrial introns have been shown to undergo self-splicing in vitro. In fact, they seem to use a complex splicing machinery, consisting of numerous nucleus-encoded factors, for their efficient excision (Bonen 2008).

8.3.1.3 Genomic Rearrangements

The mitochondrial genomes of angiosperms are amongst the largest organellar genomes ever known (Ward et al. 1981). They may show so much variation in their size and structure that

even within an organism mtDNA could be present in a heteroplasmic state, defined as 'mitotypes' (Kmiec et al. 2006). The coding sequences in mitochondria are highly conserved. It is the intergenic sequences which house many repeat sequences that bring about structural diversities amongst different mitochondrial genome populations. Long and short repeat sequences act as the substrates for extensive intra-genomic and inter-genomic recombinations (Palmer and Shields 1984). These recombination events lead to dynamic changes in the mitochondrial genome, from creating chimeric genes, pseudogenes, duplication and deletions to multicellular mitochondrial genomes (Conklin and Hanson 1994). Generally, recombinations create new copies of the large sequence repeats which further act as catalysts for more recombinations. Intramolecular recombination between direct repeat sequences leads to reshuffling of the gene order, whereas intermolecular recombinations may lead to large deletions and duplications. Drastic structural changes are usually brought about by homologous recombination in large (>1 kb)- and intermediate-sized repeats (0.1–1 kb) (Arrieta-Montiel et al. 2009). If the repeats are in inverted orientation, it leads to genomic inversions, whereas if they are in direct orientation, it can create subgenomes, each containing only a portion of the total genetic information (Abdelnoor et al. 2003).

Intragenic ectopic recombination events can also occur at short repeat sites which results in the generation of novel gene chimaeras, possibly associated with mutant phenotypes like cytoplasmic male sterility, plant variegation and other aberrant phenotypes (Abdelnoor et al. 2003). High-frequency recombinations take place in large repeat sites which give a number of recombination products that remain in stoichiometric equilibrium, whereas recombination across short repeat sequences tends to be asymmetric. What follows is a preferential recovery of only one of the two recombination products that are formed in the subsequent cell divisions leading to a phenomenon known as 'substoichiometric shifting'. This may eventually lead to the replacement of the original predominant mitochondrial form by the rare recombinant form (Gabay-Laughnan and Newton 2012). In the nutshell, genomic rearrangements are arguably the biggest driving force behind evolutionary changes in the mitochondrial genome organization and structural diversities.

8.3.2 Plastid Genome

Plastids are an integral part of a plant cell. Chloroplasts (the green plastids) are endowed with the function of photosynthesis which they carry out in a separate genetic compartment using proteins that are both endogenously made and imported from the cytoplasm. Plastids begin their life journey as undifferentiated organelles (proto-plastids) in the meristematic cells that later differentiate into different forms (chloroplasts, chromoplasts, leucoplasts, amyloplasts and etio-plasts) depending upon the tissue type they are going to be functional in. And just like mitochondria, chloroplasts also maintain their own genetic organization which reflects upon their cyanobacterial ancestry.

A major breakthrough in the chloroplast genome research came up in 1986 with the sequencing of whole plastid genomes of two land plants *Nicotiana tabacum* (Shinozaki et al. 1986) and *Marchantia polymorpha* (Ohyama et al. 1986). The latest count till date, as per NCBI organellar genome database, of the number of chloroplast genome sequences available across all plastid-containing eukaryotes is 524. Land plants including green algae account for 470 sequenced genomes. The extensive knowledge of plastomic sequences has helped us better understand the intricacies of plastome/genome organization, plastid genome evolution and its structural and functional contribution to nuclear and mitochondrial genomes.

8.3.2.1 Structural Organization

A generalized account of the plastid genome organization can be given because they are widely conserved amongst the seed plants. In the evolutionary perspective, they have undergone very little change in terms of their gene content

and organization (Barbrook et al. 2010). Chloroplast DNA (cpDNA) exists as a negatively supercoiled double-stranded molecules ranging in size between 37 and 217 kb with most of the photosynthetic organisms lying in the range of 115–165 kb.

The literature describes cpDNA as double-stranded circular DNA molecules. Though the statement is true, but it is not completely true. In fact as it turns out, just like their mitochondrial counterparts, only a small fraction of the total cpDNA may be present as circular entities (Bendich 2004). When the structure and organization of a large number of intact cpDNA was analyzed from plants like *Arabidopsis*, tobacco and pea, it was found that only 25–45 % of the total cpDNA were circular DNA molecules, and the rest were in linear form (Lilly et al. 2001). Circular molecules can undergo multimerization (head to tail genomic concatemers), sometimes raking in up to even ten genome equivalents in rare cases. Generally, they are present as dimers. They even undergo isomerization with respect to the orientation of single-copy sequences sandwiched between the two inverted repeat (IR) regions. The two forms are usually present in equimolar concentrations (Palmer 1983).

Chloroplast genome has a bipartite structure with two copies of large inverted repeat regions (IRA and IRB) dividing the genome into one large and one small single-copy regions (SCRs) (Palmer 1985). Many of the plastid genes in higher plants are organized into operons, a trait that suggests their prokaryotic origin. The genes with related functions are usually clustered together in an operon, but it may not always be necessary (Sugita and Sugiura 1996). The genes in an operon are transcribed as polycistronic transcript which undergoes further processing and modifications like splicing and editing to yield a functional transcript.

8.3.2.2 The Genetic Make-Up

As mentioned earlier, cpDNA of seed plants contains an average of 120 genes, all essential. The gene content and the gene order amongst the seed plants are generally conserved, especially within a group of organisms (Fig. 8.2). The numbers may vary occasionally if there are gene duplications in the inverted repeats. The plastid genome maintains a core of basic set of genes required for photosynthesis and its gene expression. The genes can broadly be classified into three categories: one that codes for the photosynthetic genes like *rbcL*, *ndhA*, *ndhB*, *psaA*, *psbA*, etc. and the second that includes genes required for the gene expression like *rRNA*, *tRNA* genes and those coding for the subunits of plastid-encoded RNA polymerase. The third category consists of conserved ORFs-*ycfs* (h**y**pothetical **c**hloroplast OR**Fs**) and other potential protein-coding genes like *matK* (Ravi et al. 2008). Some of the chloroplast genes, ~20, are interrupted by introns. Introns are of group II type which have lost the self-splicing capacity and are dependent on nucleus-encoded pentatricopeptide repeat proteins (PPR) for their splicing.

8.3.2.3 Plastid Genome Packaging

Plastid DNA is packaged into a higher-order structure with the help of DNA-binding proteins to form highly conserved, discrete nucleoprotein complexes called plastid nucleoids. They form the structural and functional unit of plastid genome. This chromatin dense area is not surrounded by any membrane and remains attached to the intraplastidial membranes (Sakai et al. 2004). Their number, location and size may vary depending upon the plastid type (Miyamura et al. 1986), and this variation is reflected in the gene expression at different stages of chloroplast development (Melonek et al. 2012).

In green plants and many green algal groups, the nucleoids exist as small, uniformly dispersed entities with a diameter of approximately 0.2ϰ. Depending on the plant species, tissue type and developmental stage, the number of plastid DNA copies per nucleoid may range between10 and 20 (Kuroiwa 1991; Sato et al. 2003). Packaging of cpDNA into condense units also serves the purpose of ease in vegetative segregation of plastid DNA during plastid divisions (VanWinkle-Swift 1980). Nucleoids do not exist as isolated groups. In fact, they form a dynamic group of nucleoproteins, sensitive to the environmental and developmental cues, and are involved in the regulation of

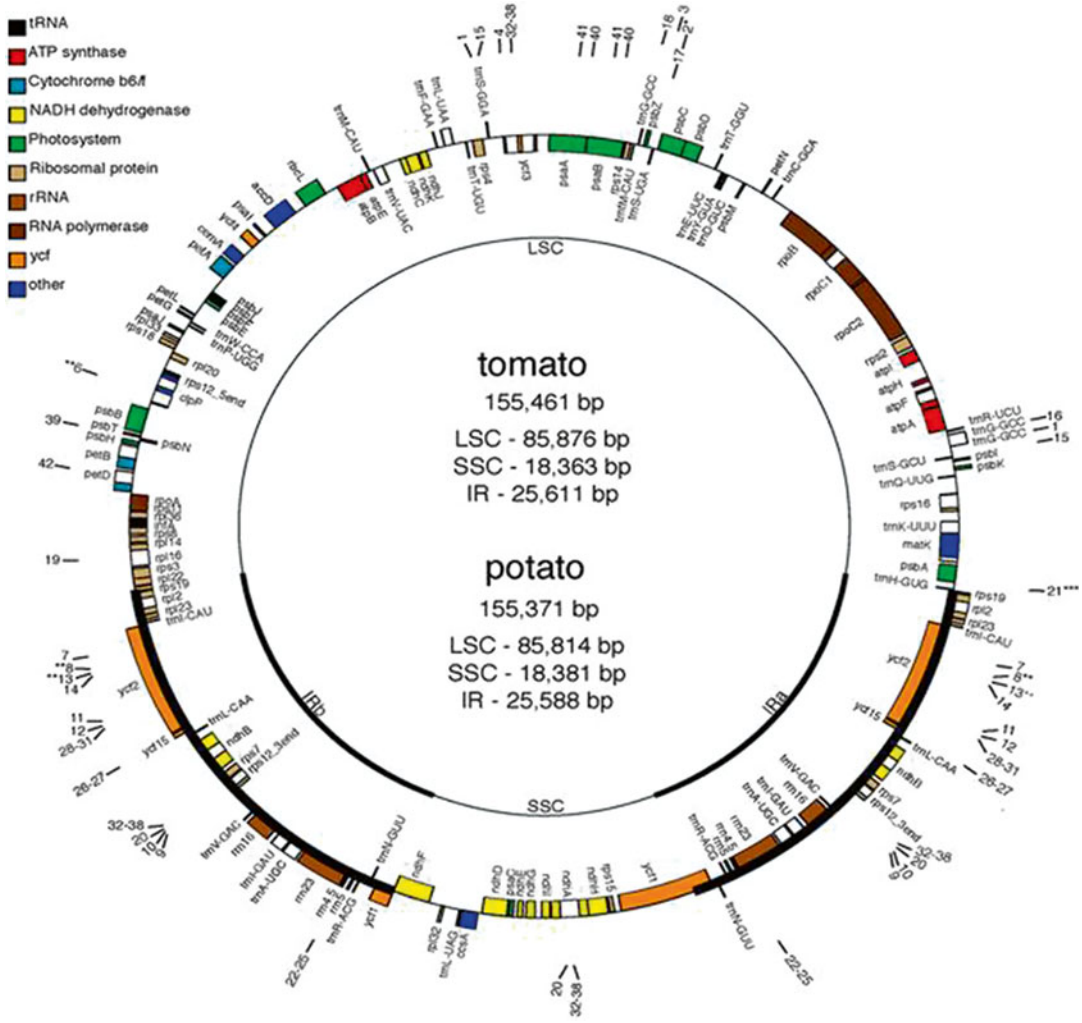

Fig. 8.2 Circular DNA maps of plastid genomes: genome map of tomato and potato chloroplast genomes. The *thick lines* indicate the two inverted repeats. Genes annotated outside the *circle* are transcribed clockwise, and those inside are transcribed anticlockwise (Daniell et al. 2006)

gene expression, plastid function and differentiation.

A wide range of proteins can be found in a nucleoid (Yagi and Shiina 2014), but this composition may vary at different time points as nucleoid is not an isolated structure, and its protein pool, which shows much flexibility, could only be defined depending upon the functional state a nucleoid is in (Kuroiwa 1991). The proteins are tightly bound to the DNA in the central region, whereas they are loosely bound to the DNA fibrils in the peripheral nucleoid space (Hansmann et al. 1985). This pseudo-compartmentalization gives the plastid nucleoid a layered structure (Sakai et al. 2004). The core of the nucleoid is tightly organized and has all the necessary proteins required for the active transcription and replication of plastid genome, while the peripheral regions are endowed with the task of translation and complex assembly. Many proteins have been identified and characterized from the enriched nucleoid fractions of various plant species like *Arabidopsis*, mustard, spinach, maize, pea, etc. (Phinney and Thelen 2005; Pfalz et al. 2006; Steiner et al. 2011; Melonek et al. 2012; Majeran et al. 2012). The plastid nucleoid associated

proteins (ptNAPs) can be classified into two categories based on their functions – first is of enzymatic ptNAPs that contribute to the DNA replication, DNA repair and transcriptional and translational activities of plastome. This category includes plastid-encoded RNA polymerase (PEP), PEP-associated proteins (PAP) and pentatricopeptide repeat proteins (PPR proteins) (Pfalz et al. 2006). 70S ribosomes, ribosome assembly factors and many other proteins are associated with DNA replication, DNA repair and mRNA splicing, editing and translation (Yagi and Shiina 2014). The second category is of architectural ptNAPs – those involved in membrane anchoring and DNA compaction like PEND, PD1, PD3, MFP1, TCP34, pTAC16, SiR, CND41, YLMG1 and SWIB (Krupinska et al. 2013).

8.3.2.4 Plastid DNA Methylation

Methylation of nuclear DNA is of common occurrence in eukaryotes and plays a pivotal role in modulating chromatin structure, regulating gene expression and in epigenetic inheritance (Suzuki and Bird 2008). However, whether or not cpDNA methylation occurs, it is controversial.

The first report for cpDNA methylation dates back to 1972 when Sager and Lane (1972) provided the first ever evidence of chloroplast methylation and demethylation during the life cycle of unicellular green alga *Chlamydomonas* and its role in maternal inheritance of chloroplast genome. This report sparked off the quest for similar methylation marks in higher plants including tomato (Ngernprasirtsiri et al. 1988a; Kobayashi et al. 1990) and maize (Ngernprasirtsiri et al. 1989; Gauly and Kossel 1989). The basis for detecting methylation then was to use a pair of isoschizomer restriction endonucleases of which one would be methylation sensitive and the other would be methylation dependent so that they gave two different restriction patterns of the sample DNA depending upon whether one of them is methylated or not. Accordingly, when DNA methylation of plastid DNA from fully ripened fruits, green mature fruits and green leaves of tomato was compared (Ngernprasirtsiri et al. 1988a), the isoschizomeric endonucleases produced different restriction patterns of plastid DNAs in tomato fruits and tomato leaves. Different methylation patterns could also be seen for the genes that were expressed differentially in the chromoplasts. Further examination of methylation status of the genes during transitional conversion of chloroplasts to chromoplasts in ripening tomato is correlated with their expression patterns making methylation one of the possible mechanisms for regulating gene expression in plastids (Kobayashi et al. 1990).

Similarly in maize, tissue-specific differential expression of plastid-encoded genes was attributed to a regulatory mechanism mediated by DNA methylation (Ngernprasirtsiri et al. 1989; Gauly and Kossel 1989). However, these observations were refuted by subsequent studies in which no such stage-specific methylation pattern could be detected during tomato fruit ripening (Marano and Carrillo 1991). Another study around the same time on barley chloroplasts also failed to detect any methylation footprints on the plastid genes. Restriction patterns of leaf plastid DNA from different stages of growth and development were indistinguishable, although they could clearly see differential gene expression and protein synthesis at different stages (Tomas et al. 1992). Same year, Ohta et al. (1991) had reported significant amount of cytosine methylation at most of the CC(A/T)GG sites and some of the GATC sites in the cpDNA of *Pisum sativum*. But, the methylation pattern could not be correlated with the gene expression as opposed to another report by Ngernprasirtsiri et al. (1988b) in which they had claimed that nonphotosynthetic plastid (amyloplast) DNA is heavily methylated which results in the transcriptional suppression of chloroplast genes, whereas non-methylated plastid DNA in photosynthetically active cells has transcriptionally active genes.

Palmgren et al. (1991) observed varied levels of DNA methylation in different tissue types in carrot but failed to derive any conclusive correlation between the age, type and the differentiated stage of plant tissue with its levels of DNA methylation. Almost a decade later, methylation of cpDNA in higher plants was again challenged by Fojtova et al. (2001) who, by performing

bisulphite genomic sequencing, failed to detect any methylated cytosine residues in tobacco plastome.

In the midst of all the controversies surrounding DNA methylation of plastid genome, *Chlamydomonas* remains arguably the best and the only example where the C-residues in cpDNA have been shown to be methylated (Umen and Goodenough 2001). In *Chlamydomonas*, methylated DNA has a function in that only the methylated maternal cpDNA is transmitted to the daughter cells. There are two mating types in *Chlamydomonas*: a heavily methylated maternal mating type plus, mt +, and a non-methylated paternal mating type, mt- (Burton et al. 1979). There are two theories to explain the inheritance of methylated maternal cpDNA. One is the methylation-restriction system, wherein upon successful mating, non-methylated mt- cpDNA is selectively degraded allowing for the uniparental inheritance of the maternal cpDNA (Sager and Grabowy 1983; Nishiyama et al. 2002). Another proposed mechanism is based on selective DNA replication of methylated genome. In an elegant experiment carried out by Umen and Goodenough in 2001, they demonstrated that methylation of maternal cpDNA is not for its protection from methylation sensitive nucleases but to enhance its replication in early zygotic stages. Probably, a different mode of cpDNA replication is adapted in germinating zygotes which selectively amplifies intact and properly methylated cpDNA molecules.

The ambiguous status of cpDNA methylation in higher plants notwithstanding, experiments have been carried out to ectopically express adenine and cytosine DNA methyltransferases in the chloroplast genome of tobacco (Jaffe' et al. 2008; Ahlert et al. 2009) by generating stable transplastomic lines. It was shown that the plastid genomes were efficiently methylated, yet they were phenotypically normal and viable, and the maternal inheritance was also not affected. More importantly, there were no alterations in plastid gene expression (Ahlert et al. 2009) which effectively dismissed any idea of transcriptional regulation of plastid gene expression by DNA methylation.

In conclusion, although DNA methylation of cpDNA in tissue-specific or developmental stage-specific manner cannot be ruled out with surety, it's not yet clear so as to what functional role it may have in the chloroplast genome. A direct link with the transcriptional regulation of gene expression can clearly be ignored.

8.3.3 Organellar Inheritance

Unlike nuclear genome, organellar genomes in angiosperms follow a non-Mendelian, maternal pattern of inheritance (Birky 2008). It was first discovered independently by Carl Correns in *Mirabilis jalapa* (Correns 1909) and Erwin Baur (1909) in *Pelargonium*. Through carefully designed reciprocal crosses, Correns showed that the leaf colour of the progeny was always determined by the phenotype of the maternal parent and not the paternal parent. Similar results echoing the cytoplasmic inheritance pattern were found in another set of reciprocal crosses between green and variegated plants of *Pelargonium zonale* by Baur who, in this case, demonstrated the biparental organellar transmission. Correns thought that there were certain cytoplasmic factors which were responsible for the 'diseased' cytoplasmic state that confers variegated phenotype, and the transmission of the diseased versus healthy cytoplasm depends upon the cytoplasmic state of the maternal parent. But, Baur could correctly realize that it was the genetic factors in plastids that were randomly segregated and had given the observed phenotypes (Birky 2008). On the basis of the actual explanations given by the two scientists and for the overall understanding of the mechanism, only Erwin Baur can be credited as the founder of the theory of plastid inheritance (Hagemann 2010). These two pioneering discoveries, though, marked the beginning of an exciting era of organellar genetics.

Although uniparental mode of inheritance is the predominant mode of inheritance, it may extend beyond maternal inheritance to leaky paternal transmission or even biparental inheritance (McCauley 2013). Considerable research has been done in this field in order to study the

inheritance pattern across numerous plant species, deviations from the obvious and the mechanisms behind different modes of organellar inheritance. For example, *Pelargonium*, in which non-Mendelian inheritance was first discovered by Baur (1909), exhibits all the three modes of organellar inheritance (Weihe et al. 2009).

In angiosperms, maternal inheritance of plastids accounts for about 80 % of cases, while the rest are known to be biparental (Corriveau and Coleman 1988). Deviation from a strictly maternal inheritance to a strictly paternal inheritance is of rare occurrence. *Actinidia* (Testolin and Cipriani 1997) is the only known example till date to exhibit an exclusive paternal inheritance of plastids. Uniparental paternal inheritance or biparental inheritance can be found mostly in interspecific crosses as a means to rescue defective plastid genomes in hybrids with nuclear plastid incompatibility (Zhang and Sodmergen 2010). Examples include *Passiflora* (Hansen et al. 2007), *Oenothera* (Chiu et al. 1988), *Larrea* (Yang et al. 2000), *Turnera* (Shore et al. 1994), *Pelargonium* (Weihe et al. 2009), *Chlorophytum comosum* (Liu et al. 2004), etc.

Several mechanisms have been proposed for maternal inheritance that prevent paternal plastids from getting transmitted to the progeny. These include both pre- and post-fertilization preventive measures. First is the physical exclusion of plastid organelles from the generative cell. It can occur during pollen mitosis I or from the maturing generative cells by enucleated cytoplasmic bodies. Post-fertilization mechanism involves differential rates of DNA replication between maternal and paternal plastid genomes (Nagata 2010).

Many instances of paternal leakage of mitochondrial genomes have also been reported. One of the initial reports that hinted at the possibility of paternal mtDNA inheritance was in alfalfa (*Medicago sativa*) in which large mtRNAs were found to be paternally inherited (Fairbanks et al. 1988). In fact paternal inheritance of mtDNA in gymnosperms is known since the 1970s (Neale et al. 1989), and it is the most commonly found inheritance pattern in gymnosperm species. Amongst angiosperms, the prominent examples include rapeseed (Erickson and Kemble 1990), banana (Faure et al. 1994) and cucumber (Havey 1997). Otherwise, paternal leakage and heteroplasmy normally occur in interspecific crosses. Barring these few examples, mitochondrial genome inheritance in flowering plants occurs predominantly via maternal transmission. One of the classical examples of maternally inherited mitochondrial trait is the cytoplasmic male sterility (CMS). Frequent genomic rearrangements in mtDNA can create chimeric open reading frames (ORFs) whose expression can result in defective mitochondrial functions which ultimately lead to pollen abortion (Hanson and Bentolila 2004).

One of the reasons for a persistent uniparental mtDNA inheritance across the evolutionary timeline is the need to maintain organellar homoplasmy. A biparental inheritance would mean mixing of maternal and paternal mitochondrial genomes giving rise to a heteroplasmic state (McCauley 2013). Such a genetic heteroplasmy of organelles is usually not tolerated by the cells, and in the subsequent divisions, they get rid of the mitochondrial genome type which is less competent of the two. Since mitochondrial genomes can undergo fusion, even in plants (Arimura et al. 2004), mtDNA from the two sources may recombine and cause unwanted genetic variations and heterogeneity in mtDNA sequences.

8.3.4 Plant DNA Barcodes Based on Organellar Genome

DNA barcoding uses short orthologous DNA sequences for species identification and for decoding its evolutionary history (Ali et al. 2014). Phylogenetic analysis using DNA barcoding has revolutionized the traditional disciplines of taxonomy and phylogeny. Molecular phylogenetic investigation relies upon the heritable differences at the molecular level to establish phylogenetic relationship between different organisms. The main aim of DNA barcoding is to establish a shared community resource of DNA sequences which will be unique for each species so that it can be used for organismal identification

and taxonomic clarification (Hollingsworth et al. 2011).

DNA barcoding surely offers many benefits. Apart from correctly identifying the known species, it has been instrumental in the identification of a number of probable cryptic species which were earlier thought to be single species based on morphological characters (Dasmahapatra and Mallet 2006).

For a locus, coding or noncoding, to be considered as a barcode, the foremost requirement is that it should easily get amplified using a universal set of primers so that it can be used on a large sample size. More importantly, it should have an outstanding discriminating power, i.e. it should be able to make a clear distinction between different plant species (Hollingsworth et al. 2011). In animals, mitochondrial gene cytochrome *c* oxidase I (*COI*) is routinely used as the standard barcode for bio-identification (Hebert et al. 2003). But in the case of plant mitochondrial gene markers, they are highly conserved sequences with a very low incidence of single nucleotide substitutions. This translates into their low discriminating powers making them unfit to be used as plant barcodes. Also, due to extreme genomic rearrangements in the intergenic regions of mitochondrial genome (Palmer et al. 2000) and the fact that sequence information is available for only a limited number of plant species, mitochondrial genome in plants cannot provide enough suitable candidates for selecting a plant barcode (Hollingsworth et al. 2011). Using plant nuclear genes as barcodes may also be not very useful as they can be highly variable across different plant species making the design of universal primers difficult. Hence, a universal barcode is most likely to come from the plastid genome in plants. Initially, seven candidate plastid DNA regions, *atpF-atpH* spacer, *matK* gene, *rbcL* gene, *rpoB* gene, *rpoC1* gene, *psbK-psbI* spacer and *trnH-psbA* spacer (Pennisi 2007), were considered, and on the basis of the selection criteria of recoverability, sequence quality and its species discrimination power, a 2-locus combination of *rbcL*/*matK* was eventually finalized as the universal plant barcode (CBOL Plant Working Group 2009). *matK* consistently shows a high level of discriminancy amongst angiosperm species, but because of high sequence variability and the presence of mononucleotide repeats in the gene (Hilu and Liang 1997), it is inherently difficult to amplify and sequence. On the other hand, *rbcL* is easy to sequence and amplify but shows an average discriminancy power. However, to circumvent the problem of amplifying *matK* gene, new universal primers have now been designed with fairly strong amplification (93.1 %) and sequencing (92.6 %) successes in the species tested (Yu et al. 2011).

The use of plant barcodes in species identification is still in its initial stages. A compilation of the studies which have used DNA barcoding for plant identification has been illustrated in Table 8.1 (Hollingsworth et al. 2011).

Barcoding offers many advantages like species identification in cases where only small amounts of tissue from sterile, juvenile or fragmentary materials are available, and morphological identification is not possible or if it involves geographically restricted sample sets, such as studies focusing on the plant biodiversity of a given region or local area (CBOL Plant Working Group 2009).

Although DNA barcoding is an effective phylogenetic tool, no single locus can be conserved in all the species and still has enough discrimination power to work across all species (Li et al. 2015). Moreover, the species identification is based on a single DNA sequence which can create artefacts in the case of plants as sequences from a totally unknown source can reach a given species by horizontal transfer of DNA sequences.

8.4 Genetic Crosstalk Between Nucleus, Chloroplasts and Mitochondria

It is known for a fact that ever since their inception in eukaryotic host cell as subcellular organelles, mitochondria and chloroplasts have undergone massive reduction in their genome complexity with most of their genes being syphoned into the nuclear genome only to reimport the products with the help of transit peptides and

Table 8.1 Applications of DNA barcoding in plants

Application	Barcode markers used	Notes
Identification of cryptic orchid species	*matK*	
Identification of cryptic *Conocephalum* (bryophyte) spp.	*rbcL*	
Identification of cryptic *Herbertus* (bryophyte) species	*rbcL*+*matK*+*trnH-psbA*+nrITS	*matK* and nrITS rated as the best performing regions
Identification of cryptic *Taxus* species	nrITS, *trnL-F*, *trnH-psbA*, *matK*, *rbcL*	nrITS and *trnL-F* rated as the best performing regions
Identification of seedlings in tropical forest plots	nrITS, *trnH-psbA*, *rbcL*, *matK* (and other regions)	*trnH-psbA* was rated as the best performing region
Community phylogenetics of tropical forest plot	*rbcL*+*trnH-psbA*+*matK*	
Identification of Chinese medicinal plants: Polygonaceae	*trnH-psbA*	
Identification of Chinese medicinal plants: Fabaceae	nrITS2, *matK*	
Identification of *Phyllanthus* species in herbal medicines	*trnH-psbA*	
Identification of *Actaea* species in herbal supplements	*matK*	
Identification of medicinal plant species	nrITS2, nrITS, *matK*, *rbcL*, *pcbA-trnH* (and other regions)	nrITS2 rated as the best performing marker
Identification amongst berry species in foods	nrITS	*matK* and *trnH-psbA* did not show enough variation
Assessing the plant components of honey	*trnL* PG loop	
Identification of invasive species (*Cardamine*)	nrITS, *trnL* intron, *trnL-F*	
Identification of invasive species (*Hydrocotyle*)	*tmH-psbA*	*matK* proved troublesome to amplify so it was discarded
Molecular identification of roots in grassland communities	*rbcL*	
Identification of *Osmunda* gametophytes	*rbcL*	
Identification of an aquatic fern gametophyte	*rbcL, trnL intron* (and other regions)	*trnL intron* is less informative due to low coverage in reference database
Identification of traded fern species	*rbcL* (and other regions)	
Identification of CITES listed ramin timber and products	*matK* (SNP assay of barcode sequence)	
Identification of poisonous plants	*matK*, *trnH-psbA* and other regions	*matK* preferred (along with 2 single-copy nuclear regions)
Identification of plant components of herbivore diet	*trnL* PG loop	
Identification of plant components of herbivore diet	*trnL* intron	
Identification of plant components of herbivore diet	*trnH-psbA*	
Proof of concept for application to forensics	*trnH-psbA*, *trnL-F*	

protein import machinery. This means that they still have the full quota of proteins but not the genes (Martin 2003). In addition to the organellar DNA influx into the nucleus, nuclear DNA fragments have also found their way into the mitochondrial genome. In fact, plastid sequences might also contribute to some plant mitochondrial genes (Wang et al. 2012a). As far as plastid

genome is concerned, there is always a one-way movement of genes away from the plastid into the nucleus or mitochondria.

As we will see in the next section, establishing an organellar gene in the nucleus is a very demanding task. So, what was the need for moving the organellar genes into the nucleus during the course of organellar evolution? No matter how straightforward it seems, it is a very subjective question.

From the point in an evolutionary timeline wherein the two free-living prokaryotes – α-Proteobacterium and Cyanobacterium – were sequentially engulfed by their eukaryotic host cell, they were in the continuous process of evolving into the present-day organelles, mitochondria and chloroplast, respectively. They had been assigned a specific function and could not possibly have continued to live as an independent entity as earlier. That made it imperative for the newly evolved organelles to share their genetic load with the host cell nucleus. This meant giving the nucleus a firm authority to regulate their functions, thus embarking on a long-lasting symbiotic relationship. This clearly indicates an evolutionary pressure to centralize genetic information in the nucleus (Daley and Whelan 2005).

Splitting of the organellar genes also means a well-co-ordinated gene expression between the nucleus and the organelles, ensuring more co-operations in cellular functions and a better regulation of their own function with respect to the internal and external cues as perceived by the nucleus.

In pure genetic terms, there are many proposed hypotheses to explain the nuclear movement of the organellar genes: the need (1) for a more compact organellar genome that can naturally be selected for rapid replication (Selosse et al. 2001), (2) to avoid Műller's ratchet which is the accumulation of deleterious mutations in asexually propagating nonrecombining organellar genomes by recombination in nuclear genome (Lynch 1996) and (3) to select for a better fit nuclear copy of the organellar gene (Blanchard and Lynch 2000).

Having discussed all the possible reasons for the nuclear transfer of organellar genes, we will see to what extent the genes have been transferred to the nucleus and why some genes are still retained in the organellar genome.

8.4.1 Promiscuous Plastid and Mitochondrial DNA in Nucleus

Boogaart et al. (1982) reported the presence of a gene sequence in nuclear genome that was homologous to the mitochondrial DNA sequence in *Neurospora crassa*. It was the first ever report of its kind. Later that year, John Ellis (1982) for the first time used the term 'promiscuous DNA' for the chloroplast genes inside the plant mitochondrial genome. This was followed up by a burst of reports on promiscuous DNA across many systems, like in maize (Stern and Lonsdale 1982), yeast (Farrelly and Butow 1983), fungus (Wright and Cummings 1983), locust (Gellissen et al. 1983) and so on. An event which was previously thought as a rare event had suddenly become a 'rampant process'.

The large-scale DNA transfer from the organelles to the nucleus has significantly contributed to the nuclear genome evolution. The transferred DNA sequences in nucleus are called nuclear integrants of organellar DNA, norg, which is classified into nupt, for 'nuclear plastid DNA', and numt, for 'nuclear mitochondrial DNA (Leister 2005). Most of these segments are generally less than 1 kb in length (Richly and Lester 2004a, b).

Experimental attempts have been made to reconstruct the functional gene transfer from the chloroplast to nucleus and to show that the transferred genes become functional in the nucleus when followed by suitable genomic rearrangements in the nuclear genome. Another finding of these experiments was that mitochondrial and chloroplast DNA transfer to the nucleus occurs at relatively high frequency (Huang et al. 2003; Stegemann et al. 2003). Stegemann and Bock in 2006 employed a simple and effective experimental design in which they showed functional activation of a chloroplast marker gene in the nucleus. Stable transplastomic lines (chloroplast transformed) were raised using a cassette containing *npt*II gene under nuclear CaMV 35S

promoter upstream of the chloroplast marker *aadA* gene cloned under a chloroplast-specific promoter, *Prrn*. Spectinomycin (spec)-resistant cells were then challenged with kanamycin (kan) in culture, and many resistant lines could be recovered implying a nuclear transfer of the *npt*II gene. The lines were outcrossed to replace the transgenic chloroplast with the wild types. The kan-resistant and spec-sensitive lines were again screened for spectinomycin resistance to select for the lines in which the chloroplast gene had become functional in the nuclear genome. Again, the desired lines could be obtained implying the activation of *aadA* gene in the nucleus. But this could happen only at the cost of the associated nuclear gene whose segments were deleted to acquire the CaMV35S promoter.

Transfer of the organellar DNA to the nucleus is an ongoing evolutionary process. Relocation of an organellar DNA segment to the nucleus does not guarantee a functional acquisition in the nucleus. There are many steps involved in the transfer process, from the actual movement of DNA towards nucleus to the final attainment of a function (Brennicke et al. 1993; Adams and Palmer 2003). The first step is to escape the organellar genome, i.e. gene escape. The DNA needs to be freed from the parent location and become available for the nuclear integration. DNA segments can escape the organelles by various mechanisms. In the past, it has been suggested to have occurred during the early stages of organellar evolution when the endosymbiont division was not synchronized with the host cell division, resulting in unequal partitioning. During those early divisions, some cells might have lysed to release their genomic contents in the cytoplasm which somehow managed to reach the nucleus and got integrated into the nuclear host genome (Barbrook et al. 2006). Other mechanisms include escape during disruption of organellar membranes which might occur at various occasions like during digestions of organelles by lysosomes or vacuoles (Adams and Palmer 2003) or during environmental stress conditions (Wang et al. 2012b).

The next task would be to transfer the escaped DNA to the nucleus. But in what physical form does it travel all the way to the nucleus? Is it DNA, RNA or cDNA? It could be any of the three. Large-scale DNA transfers have been shown experimentally as well as observed naturally which clearly implies that DNA escapes in its native double-stranded form and reaches the nucleus. Integration occurs via illegitimate, non-homologous DNA repair of double-stranded breaks (Noutsos et al. 2005) that can cause genomic rearrangements around the integrated sites. In fact there are many examples of ongoing endosymbiotic gene transfer occurring multiple times and independently in different angiosperm lineages. For example, mitochondrial gene *rps10*, which codes for a protein component of the small ribosomal subunit, was found to be present in the mitochondrial genomes of some plants but absent in others (Knoop et al. 1995). The cases where it was missing from the mitochondrial genome, it had already migrated to the nucleus (Adams et al. 2000).

Some studies have shown that the transfer can take place via an RNA intermediate. Many organellar genes may contain introns and are processed by splicing or may undergo editing before giving rise to the mature transcripts. Such edited versions of the genes can be found as it is in the nuclei which suggest that transfer might have occurred via a reverse-transcribed RNA (Schljster and Brennicke 1987; Nugent and Palmer 1991; Henze and Martin 2001).

The last step is the activation, the expression and the acquisition of signal peptides by the transferred gene. The transferred genes must acquire the regulatory elements for their proper expression followed by the organelle targeting signal peptide so that it reaches the destination it is meant to be functional in (Adams and Palmer 2003). Once a transferred gene becomes functional in the nuclear genome, there is a transitional phase where both the nuclear and the organellar copy might be expressing simultaneously. This is exemplified by the nuclear and mitochondrial *cox2* genes in several legumes where both the genes are transcribed albeit at varying levels (Adams et al. 1999). Following the co-expression, the next logical step is the gene silencing and the loss of organellar copy of the

gene. The nuclear copy is rarely ever lost, unless the gene product is toxic to the cell, outside its organellar location or is not transported back to the organelle.

But, it is not always that a coding region has to be relocated. Many a times, a large chunk of non-coding organellar sequences are also transferred, and they remain devoid of any functions in the nuclear genome. The best possible example would be that in rice, where some very large insertions of cpDNA (>100 kb) have been found in the nuclear genome (The Rice Chromosome 10 Sequencing Consortium 2003). Another example is 620 kb large mtDNA insertion in the chromosome 2 of *Arabidopsis* (Stupar et al. 2001).

Not all the organellar DNA fragments that get transferred into the nucleus can become functional. As they move to the new nuclear environment, they are vulnerable to accumulating mutations: single nucleotide insertions/deletions (indels), point mutations or more commonly 5-methylcytosine hypermethylation which appears to be the most prominent mechanism of mutational decay of organellar DNA (Huang et al. 2005). Also, the integration and elimination of organellar DNA remains in an equilibrium, i.e. DNA which is transferred to the nucleus may get thrown out of the nuclear genome after much tailoring and shuffling in due course of time, say in a million years. Large organellar DNA fragments preferentially localize to the pericentromeric region of the chromosomes, where integration and elimination frequencies are markedly higher (Matsuo et al. 2005). In summary, the transfer of organellar DNA into the nucleus is a dynamic evolutionary process, but for it to become successful, it needs to undergo various adjustments, genetic alterations before it gets fixed and become functional in the nuclear genome.

8.4.1.1 Foreign DNA Invasion in Plant Mitochondrial Genome

Mitochondrial genomes of flowering plants are prone to taking up foreign DNA from various sources. They can receive DNA from nucleus and plastids within the cell or from other mitochondrial and plastid genomes via horizontal gene transfer. Two peculiarities which make mitochondria more receptive to horizontal transfer of genomic material are firstly, they can easily undergo fusions and secondly, genetically distinct mitochondrial genomes can recombine speeding up the exchange process (Bock 2009).

Plastid DNA sequences contribute 1–11 % of the total mitochondrial genome (Lloyd et al. 2012). These plastid-derived mtDNA sequences are abbreviated as mtpt sequences (Wang et al. 2007). The transfer of cpDNA sequences to mitochondria is estimated to have commenced at least 300 million years ago with the oldest predicted mtpt being *trnV*(*uac*)-*trnM*(*cau*)-*atpE-atpB-rbcL* stretch of cpDNA. The first mtpt sequences discovery was made over three decades ago in maize genome by Stern and Lonsdale (1982) in which a 12 kb mtDNA sequence was identified as identical to a part of the IR of maize chloroplast genome. Since then, large chunks of plastid DNA have been detected in the mitochondrial genome of various angiosperm species. As the more number of mitochondrial and plastid genomes are getting sequenced, more of such examples are expected to come up across the land plants.

Notable examples of mtpt sequences include *Phaseolus* mitochondrial genome that harbours a promiscuous cpDNA fragment form *trnA* gene intron which was acquired via a horizontal gene transfer event. This was the first report of a plastid sequence undergoing horizontal transfer to the mitochondrial genome of higher plants (Woloszynska et al. 2004). In silico studies had been carried out by Wang et al. (2007) to identify informative chloroplast gene clusters in mitochondrial genome and to search for the pseudo/functional protein-coding genes within the selected mtpt sequences. They even tried to look for particular regions in cpDNA, if any, that are regularly transferred to the mitochondrial genome so that DNA transfer hotspots can be identified. In their findings, which was in accordance with the previous reports (Notsu et al. 2002; Clifton et al. 2004), Wang et al. (2007) reported that all the protein-coding mtpt sequences in angiosperms degenerate to become nonfunctional. Nevertheless, some chloroplast-derived tRNAs were shown to be functional in mitochondria

(Miyata et al. 1998). As far as the source of chloroplast sequences in the plastid genome is concerned, the transfer can occur randomly from any part of the genome (Wang et al. 2007).

In a new finding by Wang et al. (2012a), some mtpt sequences have been shown to be contributing codons to unrelated mitochondrial protein-coding sequences. For example, in *Vitis vinifera* (grapevine), *Silene latifolia* (campion) and *Nicotiana tabacum* (tobacco), mtpt sequences at 3′ end of mitochondrial *ccmC* gene contribute four additional amino acids at carboxyl terminus of the polypeptide that are putatively involved in the mitochondrial mRNA maturation. Similarly, *mtpt* sequences at 3′ end of mitochondrial gene *atp4* in *Zea mays* which provides the stop codon and mtpt sequences at 5′ end of the gene *nad9* in *Triticum aestivum* (wheat) are all examples of mtpt sequences participating in mitochondrial functions. The plastid-derived sequences may remain dormant for years before they become functional again.

Nuclear DNA sequences can also get stably integrated into the mitochondrial genome and contribute to its size expansion. Many of these sequences are homologous to retrotransposons (Kubo and Mikami 2007) as exemplified by *copia*-, *gypsy*- and LINE-like retrotransposon fragments in the mitochondrial genome of *Arabidopsis thaliana* (Knoop et al. 1996) that make up ~5 % of the total genome size. Similar findings are resonated in the mitochondrial genomes of other plant species also: 19 TEs (16 retrotransposons and three DNA transposons) from rice mitochondrial genome (Notsu et al. 2002), 4 small fragments (50–277 bp) with similarity to known retrotransposons in maize (Clifton et al. 2004), 21 retrotransposon-like sequences in sugar beet (Kubo et al. 2000) and 5 retrotransposons in wheat mitochondrial genome (Ogihara et al. 2005). However, until we have the complete knowledge of nuclear and mitochondrial genome sequences of other plant species, it is difficult to estimate the share of nuclear sequences in mitochondrial genome (Kubo and Mikami 2007).

Horizontal gene transfer has played a significant role in eukaryotic genome evolution. HGT represents transfer of genetic material between organisms (or even organelles) by asexual means. Mitochondria can also take up DNA by HGT. It can go beyond the species barrier. In fact the donor organism need not always be a plant. It can be as diverse as viruses or non-plant eukaryotes or even prokaryotes (Bock 2009). It happens more frequently in mitochondrial genomes resulting in massive DNA transfers even between distantly related flowering plants. It occurs strictly via a DNA-level intermediate, but the exact mechanism largely remains unknown (Mower et al. 2010). HGT is commonly seen in parasitic plants (Davis and Wurdack 2004; Bock 2009) or in cases of plant-pathogen interactions where a cell-to-cell contact is established between the infecting agent (fungus, insect, nematode, etc.) and the host plant. In fact the endosymbiotic origin of chloroplasts and mitochondria and the ensuing large-scale genetic transfer are in itself the most striking examples of HGT. It is an extreme case wherein the entire genome of the endosymbionts has got transferred to the nuclear genome (Martin et al. 2002).

As already mentioned, amongst the three cellular genomes, mitochondrial genome is the most amenable to foreign DNA invasion by HGT. It is evident by the occurrence of massive mitochondrial gene transfers between parasitic flowering plants and their host plants (Davis and Wurdack 2004). For example, in *Rafflesia cantleyi* (Rafflesiaceae) and its close relatives, 24–41 % of their mitochondrial genome is derived from their host via HGT (Xi et al. 2013). Gene flow can also happen from the parasitic flowering plants to their host plants (Mower et al. 2004). Large-scale HGT can also be seen in *Amborella trichopoda* in which 1 or 2 copies of 20 of their 32 protein-coding mitochondrial genes have been acquired from other plants. Interestingly, most of the transferred genes have retained their function in the *Amborella* mtDNA (Bergthorsson et al. 2004). Another prominent example of HGT in mitochondria is that of highly invasive group I intron in angiosperm mitochondrial gene, *cox1*, coming from a fungal donor using double-stranded break repair pathway. It was first revealed in *Peperomia polybotrya* (Vaughn et al. 1995) and later detected in over 640 angiosperms

(Cho et al. 1998; Sanchez-Puerta et al. 2008). It appears to have been acquired via 70 separate horizontal transfer events. Since fungal-plant transfer of mitochondrial introns does not happen too frequently, a chance transfer event from a fungal donor might have been followed by its lateral transfer from one angiosperm to another or by illegitimate crossing over (Sanchez-Puerta et al. 2008).

The transferred foreign DNA in mitochondria can have four different fates. First, they could be rendered totally nonfunctional by frameshift mutations or indels (Lloyd et al. 2012). This happens most often. Second, few sequences might retain the intact reading frames so that they are efficiently transcribed and remain functional in their new host (Miyata et al. 1998). In the third possibility, if the sequence similarity is very high, some of the invading genes can even replace the native mitochondrial genes via homologous recombination leading to gene conversions and generating mitochondrial diversity (Mower et al. 2010). The native gene can either get completely replaced or only a part of it gets replaced resulting in a 'chimeric' gene that is still functional (Hao and Palmer 2009). In the fourth type, novel mitochondrial genes can be created by the transferred DNA segments, unrelated to the functions they previously had, making them evolutionary potent sequences (Wang et al. 2012a).

8.4.1.2 Why Not Get Rid of the Organellar Genome Altogether?

The modern-day organelles, chloroplasts and mitochondria, have a highly reduced genome. Their ancestors had thousands of genes in their genome, yet they themselves code for hardly a hundred genes. Some of the ancestral genes were dumped due to intracellular redundancy, while others were simply relocated to the nucleus. So far, the eukaryotic cell has failed to get rid of its mitochondrial and plastid genomes, retaining a minimum set of genes even in nonphotosynthetic, holoparasitic flowering plants, heterotrophic algae and apicomplexan parasites (Wolfe et al. 1992; Nickrent et al. 1997; Wilson 2005; Barbrook et al. 2006).

This raises the following fundamental questions: Why is the evolutionary transfer of organellar genes to the nucleus not yet over? Why carry the additional load? What is stopping it? Is it the prokaryotic nature of their genes? Is it the codon usage? But that did not stop the other genes from moving out. So what is it?

Many hypotheses have been put forth to crack this riddle. First, the 'hydrophobicity' hypothesis (von Heijne 1986) which suggests that the genes which were left behind are the ones whose products, being hydrophobic in nature, are difficult to transport back to the organelle from the cytosol. Gunnar von Heijne reasoned that mitochondrial proteins indeed contain long hydrophobic stretches which are not generally found in nuclear-encoded mitochondrial proteins. If these proteins were to synthesize in the cytosol, hydrophobic regions would serve as targeting signals for export across the endoplasmic reticulum, and the proteins could never reach the target organelle or the hydrophobicity could make them toxic for the cell when expressed in the cytosol (Oca-Cossio et al. 2003). Many bioinformatic studies and experimental evidences have favoured this hypothesis (Daley and Whelan 2005). However, there have been some relocation experiments which have countered this view along with the reports of successful import of many hydrophobic proteins like LHCII (chloroplasts) (Barbrook et al. 2006) and ADP-ATP carriers (mitochondria) (Allen 2003). Also, not all the genes in the organelles code for hydrophobic proteins. Despite these hiccups, hydrophobicity is still considered a major constrain in drawing the proteins back to the organelles.

Second, the 'CoRR' or Co-location for Redox Regulation hypothesis (Allen 1993a, b, 2003) states that there are genes which are directly regulated by the redox state of their gene products or that of their interacting partners. This necessitates the presence of such genes in the organelle itself so that any redox imbalance can be rectified instantly instead of delaying the response by first sending a signal to the nucleus and then waiting for the gene products to reach the organelle (Barbrook et al. 2006). But, there are flaws in this hypothesis too. Only few organellar genes have been shown to be under the

redox control, while there are many nuclear-encoded chloroplast and mitochondrial genes which are under redox regulation but are not coded by them (Pfannschmidt et al. 2003).

Next in line is 'code disparity hypothesis' (de Grey 2005). There are issues of genetic code disparity between nuclear and mitochondrial genome and codon modifications in some plastid genes which may hamper the functional gene transfer to nucleus (Barbrook et al. 2006). Code disparity would mean a different amino acid getting incorporated during translation in the cytosol, probably altering its function altogether. Then their transcripts also undergo RNA editing to 'correct' the codons before they are translated, which interestingly confers increased hydrophobicity to the proteins encoded (Jobson and Qiu 2008). Also, there are plastid genes that use non-canonical start codon such as GUG which is bound to be missed in the cytosolic translation. To compensate for these disparities, additional mutations would be needed in the nuclear DNA which only complicates the matter further (de Grey 2005; Barbrook et al. 2006).

Fourth would be 'essential tRNA hypothesis' (Barbrook et al. 2006). It says that tRNA encoded by the plastid gene *trnE* is unique in the sense that apart from its primary role in protein biosynthesis, it is also involved in tetrapyrrole biosynthesis (Kumar et al. 1996). So even if it is to be replaced by cytosolic tRNAGlu in protein biosynthesis, it cannot take over its function in tetrapyrrole biosynthesis. This makes the plastid copy indispensable and is forced to be retained by the plastid genome along with the machinery for its transcription (Howe and Smith 1991).

Fifth is 'limited transfer window' hypothesis (Barbrook et al. 2006). One of the proposed mechanisms by which organellar DNA had moved into the nucleus was by their lysis during endosymbiont divisions, releasing its genomic contents in the host cytoplasm. This spilled over DNA then got integrated into the nuclear genome of the host. So according to this hypothesis, organisms which have only one organelle per cell will have 'limited' (or effectively closed) opportunities to transfer their DNA to the nucleus as its breakdown will be essentially lethal to that particular cell. This hypothesis is supported by the fact that plastid DNA-derived sequences are much rarer in the nuclear genomes of single plastid holders like *Chlamydomonas* and *Plasmodium* (Richly and Leister 2004b).

'Work in progress'. It's not a hypothesis per se, but there could be a chance that we do not see all the organellar genes in the nucleus yet simply because that evolutionary process is still underway! We could just be witnessing the last stages of what must be a very slow evolutionary transfer of genes to the nucleus.

In conclusion, no one hypothesis is full proof and sufficient enough to defend the retention of only a few kinds of genes by the organellar genome. Each of them has their share of merits and demerits. There could be other reasons too that are not covered here, but in practical terms, it is not an individual but the combination of different probabilities that can explain this dilemma.

8.5 Conclusions and Future Perspectives

The knowledge of organellar genome sequences can be very resourceful in studying plant molecular ecology and evolution. The ever growing organellar genome database is definitely a boost to the organellar research as it helps unravel the great mysteries of the organellar genomes which are still holding up. The current knowledge of genomes across a diversified range of plant species clearly indicates that all the three kinds of cellular genomes are in a continuous state of evolution and maintain a very healthy interorganellar communication. DNA fragments of all sizes are often moving in and out of nucleus and organelles, undergoing mutational alterations and rearrangements, creating novel sequence combinations, disrupting genes on arrival or sometimes even creating new genes where they integrate.

Of the two organellar genomes, chloroplast genome is the one which is more stable and has a simpler genome compared to the mitochondrial genome. The quality of a chloroplast genome is such that it is most suitable to study the phylogenetic relationships amongst the major

clad1es of flowering plants. One of the biggest attractions of chloroplast genome research in the future will be the use of cpDNA-based plant barcodes in their taxonomical pursuits and to create a unique identification mark for each and every plant species on this planet.

On the contrary, the plant mitochondrial genome, being the most dynamic and with an 'always changing' attitude, makes for an interesting study from the evolutionary perspective. The animal mitochondrial genome, on the contrary, has remained docile and extremely stable with the relocation process of their mtDNA into the nucleus coming to an evolutionary halt. The plant mitochondrial genome is associated with so many peculiarities and jaw-dropping statistics that the more we think we know about this genome, the more mystical it appears. There are so many unanswered questions that we can only hope the sequencing of more number of genomes will help understand them better.

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DNA Fingerprinting Techniques for Plant Identification

9

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Abstract

Advances in knowledge about the molecular structure of plant genome and development of techniques to rapidly characterise genomic variations have established DNA fingerprinting as a powerful tool for identifying plant species, varieties, clones, individuals and even plant products. This article provides details of DNA polymorphism in plants and classical and more recent molecular marker techniques based on restriction fragment length polymorphism (RFLP), polymerase chain reaction (PCR), DNA microarrays and sequencing. The advantages and limitations of each technique are given along with some recent examples of their application in plant identification.

Keywords

DNA fingerprinting • DNA polymorphism • Molecular markers • Restriction fragment length polymorphism • PCR-based techniques • DNA microarrays • DNA sequencing • Plant identification

9.1 Introduction

The term 'DNA fingerprinting' has its origin in the method developed by Sir Alec Jeffreys and his colleagues in 1985 (Jeffreys et al. 1985b) whereby individual specific DNA fragment profiles are produced, thus providing an identification tool having the same application as conventional fingerprinting. This restriction fragment length polymorphism-based method, its variants and several other techniques have found wide application across the biological spectrum to address issues of identification, diversity, relatedness, phylogeny and breeding (Karp et al. 1996, 1998; Staub et al. 1996; Henry 2001; Weising et al. 2005; Jiang 2013; Nybom et al. 2014).

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9.2 DNA Polymorphism

DNA fingerprinting techniques are mostly targeted to locate polymorphism in the organisation of four nitrogenous bases adenine (A), guanine (G), cytosine (C) and thymine (T) which besides deoxyribose sugar and phosphate constitute the primary structure of DNA. These bases exist as single or low-copy-number coding sequences, promoters and regulatory sequences, introns and pseudogenes, moderately repeated interspersed and tandem repeats and highly repeated satellite DNA sequences (Heslop-Harrison and Schwarzacher 2011; Heslop-Harrison and Schmidt 2012; Pathak and Ali 2012).

9.2.1 Polymorphism in Single Nucleotides

Polymorphism in single nucleotides can originate through insertion or deletion (indel) or substitutions of single bases (single nucleotide polymorphisms – SNPs) between DNA sequences. SNPs are ubiquitous in eukaryotic genome and occur outside as well as within the coding regions (Gupta et al. 2001; Rafalski 2002; Ganal et al. 2009). In maize with a genome size of 2.3 Gbp, on an average one SNP occurs every 44–75 base pairs (Ganal et al. 2011). SNPs are discovered either by mining sequence databases (in silico methods) or by experimental methods of direct sequencing or microarray technology (see later in the chapter).

9.2.2 Interspersed Repeats

Interspersed repeats are moderately repeated DNA sequences present throughout the genome and comprised of transposons, elements that can change their position within the genome (Frost et al. 2005; Morgante et al. 2007). Transposons are ubiquitous within eukaryotic genomes and are of two basic classes.

9.2.2.1 Retrotransposons or Retroelements

These replicate through RNA intermediates which are reverse transcribed back at some random location in the genome. Four types of retrotransposons have been identified. Retroviruses have RNA genome which on entering host cells gets transcribed to form reverse transcriptase. The latter copies RNA into DNA which integrates into the host genome and replicates the viral genome to transcribe coat protein. Long terminal repeat retrotransposons (LTRs) are quite similar to retroviruses but have their coding sequences flanked on both ends by 300–500 bp direct repeats and lack protein-coding gene. Long interspersed elements (LINEs) and short interspersed elements (SINEs) do not bear terminal repeats but have poly (A) sequences at 3′ end. LINEs are several kilobases long and carry genes for their transposition, while SINEs are 100–500 bp long and are unable to transcribe on their own.

9.2.2.2 DNA Transposons

These comprise sequences of one to two genes flanked by short inverted repeats. They move by getting cut and inserted at a new location in the genome. Since their movement does not involve replication, their copy numbers in a genome unlike retrotransposons are small.

9.2.3 Tandem Repeats

Tandem repeats consist of repeated units of up to several thousand nucleotides arranged end to end. Depending upon unit length, number of copies and location in the genome, tandem repeats are classified into three different types.

9.2.3.1 Satellite DNA

Satellite DNA derives its name from the observation that it separates from the rest of DNA by buoyant density gradient centrifugation (Skinner et al. 1974). The basic sequence motif is generally of 100–300 bp length but can vastly vary, and the motif is repeated 1,000–100,000 times at a locus. Satellite DNA is localised in the centromeric and subtelomeric regions of chromosomes

and does not have much application for fingerprinting.

9.2.3.2 Minisatellites

Minisatellites have shorter repeat motifs (10–60 bp) than satellites, have lower number of repeats (in hundreds) but occur at thousands of loci in the genome (Jeffreys et al. 1985a; Durward et al. 1995). The number of minisatellite copies varies greatly among individuals; hence, these are also called as variable number of tandem repeats (VNTRs) (Nakamura et al. 1987).

9.2.3.3 Microsatellites

Microsatellites have 1–6 bp motifs repeated 10–20 times and are widely distributed all over the genome including within genes and chloroplast DNA, but rarely in plant mitochondrial DNA (Tautz and Renz 1984; Tautz 1989; Sharma et al. 1995). The repeat motif may be perfect (single uninterrupted array), imperfect (with one or more bases between repeat units) or compound (perfect and imperfect units combined). While microsatellites are randomly distributed in the genome, reports of their biased distribution are also available (Grover et al. 2007).

9.3 Molecular Markers

Several types of markers are used for characterisation of plants. Conventionally used morphological features like form and size of vegetative and reproductive organs suffer from a number of drawbacks. They are expressed at different growth stages and, hence, require extended periods of observation; many characters are affected by environmental variables thus making it difficult to separate genetic effect from environmental effect, and many morphological traits are controlled by more than one gene rendering genetic analysis of traits quite complex.

Cytological features like chromosome number, karyotype and chromosome banding patterns are highly reliable and have found much use in identification, classification and evolutionary relationships at species or higher levels (Heslop-Harrison and Schwarzacher 2011). However, these features are usually not variable enough to detect polymorphism at individual or population level.

Molecular methods encompass a wide range of techniques targeting variations in proteins and nucleic acids (Hillis and Moritz 1990; Karp et al. 1997, 1998; de Vicente and Fulton 2003; Spooner et al. 2005; Weising et al. 2005; Semagn et al. 2006; Agarwal et al. 2008; Nybom et al. 2014). Protein marker techniques generally measure the migration of constituent fragments through various kinds of gels, including starch and polyacrylamide, across an electric field. Isozymes, functionally similar but electrophoretically different forms of enzymes encoded by different genes, and their allelic forms (allozymes) have been widely used for analysis of genetic relationships and phylogeny in plant species (Gottlieb 1984; Hamrick and Godt 1997). But they are limited in number and much less polymorphic than nucleic acid markers, being products of functional component of the genome, generally housekeeping genes.

Nucleic acid, largely DNA, marker techniques are based on hybridisation and/or electrophoretic properties of DNA sequences. Digestion by restriction enzymes or in vivo amplification by polymerase chain reaction is used to isolate the targeted fragments. Though not required in all the fingerprinting techniques, the fragments are separated by gel electrophoresis using horizontal, vertical or capillary electrophoresis with agarose (agarose gel electrophoresis – AGE) or polyacrylamide (polyacrylamide gel electrophoresis – PAGE) as the medium and visualised by radio- or non-radioactive labels or by staining the gel with ethidium bromide or silver nitrate. Brief details of these techniques are given below.

9.3.1 Restriction Fragment Length Polymorphism (RFLP)

RFLP (Botstein et al. 1980) involves digestion of DNA with restriction enzymes, which cut DNA at precise locations, separating the fragments by gel electrophoresis, blotting the fragments to a filter and hybridising labelled DNA probes of

interest to the separated fragments (Southern 1975). Probes are short DNA fragments homologous to a section of the genomic DNA. Specific combinations of enzymes and probes give highly reproducible band patterns.

RFLP is a highly reliable technique with good reproducibility across laboratories. The markers are codominantly inherited; hence, genetic designation is unambiguous. Using different probe and enzyme combinations, discrimination at species, population or individual levels is possible. For example, the use of minisatellites and microsatellites as probes produces multilocus individual specific fingerprints. The variation arises from changes in the copy number of basic repeats, referred to as variable number of tandem repeats (VNTRs).

RFLP is used less frequently these days because of its cumbersome protocol, limited options for automation and requirement of large amount of high-grade DNA. However, in combination with PCR, microarray and sequencing, restriction-based polymorphism analysis continues to be popular.

9.3.2 PCR-Based Techniques

Polymerase chain reaction (PCR) (Saiki et al. 1988) involves enzymatic amplification of short DNA fragments to amounts that can be visualised after electrophoresis. The technique consists of denaturation of double-stranded genomic DNA under high temperature (~92° C) followed by DNA polymerase-catalysed synthesis of DNA fragments using short single-stranded DNA sequences as primers that bind at complementary sites on the denatured DNA. The newly synthesised DNA is reamplified 25–45 times through repeated PCR cycles, and the products are separated by electrophoresis. Depending upon the type of primers and other techniques used in combination, PCR-based marker techniques are grouped into different classes.

9.3.2.1 Arbitrarily Amplified Markers

Collectively known as multiple arbitrary amplicon profiling (MAAP) (Caetano-Anolles 1994), these techniques use arbitrary primers with no previous knowledge about the sequence of DNA being amplified. Random amplified polymorphic DNA (RAPD) (Williams et al. 1990) protocol uses one 10 bp primer and three PCR temperature cycles with a constant annealing temperature. Amplification products are separated on agarose gel and stained with ethidium bromide. DNA amplification fingerprinting (DAF) (Caetano-Anolles et al. 1991) uses shorter (5–8 bp) primers at higher concentration than RAPD and two temperature cycles. Generally, larger number of amplification products are obtained which are resolved on polyacrylamide gel and silver stained. Arbitrarily primed PCR (AP-PCR) (Welsh and McClelland 1990) uses 20 bp or higher sized primers and is conducted in three steps using different constituent concentrations and PCR stringencies. The products are separated on polyacrylamide gel and detected by autoradiography. In a modified protocol, arbitrary primers were used to amplify cDNA to locate individual specific polymorphism in RNA, a technique known as RNA fingerprinting by arbitrarily primed PCR (RAP-PCR) (Welsh et al. 1992; Menke and Mueller-Roeber 2001).

RAPD is by far the most widely used technique of MAAP class. The advantage of this and related techniques is that sequence information is not required, cumbersome blotting and hybridisation are avoided and the techniques are amenable to automation. On the other hand, these markers are dominant, there is no certainty that comigrating bands represent genetically same loci and, due to several intrinsic and extrinsic factors, undesirable variation in band intensity is often encountered.

9.3.2.2 Amplified Fragment Length Polymorphism (AFLP)

AFLP technique combines RFLP with PCR to selectively amplify DNA restriction fragments (Zabeau and Vos 1993; Vos et al. 1995). The DNA is digested with two different enzymes, one a rare cutter (6 bp recognition site) and the other a frequent cutter (4 bp recognition site). Double-stranded synthetic adapters are ligated to the DNA fragment ends thus generated. This

step is followed by two PCRs, the first a preselection PCR with 17–21 bp primers complementary to the adapter sequences and restriction sites. The primers have an additional nucleotide to amplify only a subset of fragments. The amplified fragments are subjected to a second PCR using two more additional nucleotides on the primer to make further fragment selection. The thus generated amplification products are separated by PAGE with silver staining or capillary gel electrophoresis with fluorescent dyes. AFLP of cDNA (cDNA-AFLP) displays differences in the expression of genes (Bachem et al. 1998).

AFLP combines the advantages of PCR and RFLP resulting in large number of highly reproducible bands. There is no requirement of sequence information and high level of polymorphism is detected by AFLP. On the other hand, AFLPs are dominant markers and the technique is more demanding than MAAP.

9.3.2.3 Sequence-Tagged Sites (STS)

STS markers (Olson et al. 1989) are developed by using primers based on some degree of genomic information. A number of STS techniques varying in the type of primers used have been developed.

9.3.2.3.1 Microsatellites or Simple Sequence Repeats (SSR) or Sequence-Tagged Microsatellite Sites (STMS) or Simple Sequence Repeat Polymorphism (SSRP)

As detailed earlier in the chapter, SSRs are 1–6 bp repeat sequences existing throughout the genome. Polymorphism in the number of repeat units is detected by PCR amplification using primers complementary to the flanking sequences of microsatellites (Saghai Maroof et al. 1994; Wenz et al. 1998). The amplified fragments are separated by AGE, PAGE or capillary electrophoresis and visualised by ethidium bromide, silver staining or fluorescent labelling.

SSR markers are highly variable, reproducible and codominant, due to which they have found wide application in characterisation and diversity analysis. However, the technique requires elaborate marker development effort involving construction of genomic libraries, enriching the libraries for the targeted SSR motifs, sequencing libraries for SSRs and flanking sequences and testing their amenability to PCR protocol (Sharma et al. 2007). On the other hand, a number of developments have greatly facilitated discovery and application of SSR markers. SSR markers have now been developed in many plant species and are publicly available. Several cases of transferability of SSRs across species have been reported (Kuleung et al. 2004; Grover et al. 2009; Park et al. 2010). Li and colleagues (2008) combined SSR with gene sequence amplification using multiplex PCR to fingerprinting potato cultivars. Increasing online availability of DNA sequence information, particularly concerning expressed sequence tags (ESTs) (Wolfsberg and Landsman 2001; Parkinson and Blaxter 2009), is also facilitating in silico development of SSRs (Louarn et al. 2007; Arya et al. 2009). Finally, next-generation methods have revolutionised DNA sequencing and consequently provided substantial boost to SSR development.

9.3.2.3.2 Intersimple Sequence Repeats (ISSR)

The technique involves DNA amplification using microsatellites as primers for PCR amplification (Gupta et al. 1994; Zietkiewicz et al. 1994). The primer sequences are generally 10–30 bp long comprising 2–5 repeats which are unanchored or anchored with 1–4 degenerate bases at 5′ or 3′ end. Accordingly, amplification of the intervening region of two identical microsatellites takes place. AGE or PAGE is used for separation of amplification products. Ye and colleagues (Ye et al. 2005) used one ISSR and one RAPD primer (R-ISSR) to detect new genomic loci in maize lines.

The advantage of ISSR is that it is as simple as RAPD; the markers are highly polymorphic and show higher reproducibility than RAPD. Dominant inheritance and doubts about genetic homology of comigrating bands are the drawbacks the technique shares with RAPD.

9.3.2.3.3 Sequence-Characterised Amplified Region (SCAR)

SCARs are locus-specific markers developed from MAAP markers (Paran and Michelmore 1993). The technique involves cloning MAAP fragments and sequencing the two ends to develop 24-mer PCR primers. SCARs are genetic markers, show dominant or codominant inheritance and are highly reproducible. While SCARs are often developed to tags traits, they have also been useful for genotype identification (Vidal et al. 2000; Polashock and Vorsa 2002).

9.3.2.3.4 Cleaved Amplified Polymorphic Sequence (CAPS) or PCR-RFLP

The technique combines the advantages of PCR and RFLP but eliminates the need for probe development and blotting (Williams et al. 1991; Bertea and Gnavi 2012). PCR products obtained by locus-specific amplification are subjected to restriction digestion followed by separation of the products by AGE or PAGE. CAPS markers are codominant and highly reproducible, but polymorphism depends on existence of variation in the restriction sites alone. A variant technique, derived CAPS (dCAPS), involves introduction of mismatches in a primer (Neff et al. 1998). The new PCR product is subjected to restriction digestion.

9.3.2.3.5 Random Amplified Microsatellite Polymorphism (RAMP)

RAMP involves amplification of genomic DNA with primers comprising labelled repeat sequences with a 5′ anchor and with or without a RAPD primer (Wu et al. 1994). PCR is conducted at two temperatures to allow proper melting and annealing. PAGE with label detection is used so that only bands obtained by anchored labels are detected.

9.3.2.3.6 Sequence-Related Amplified Polymorphism (SRAP)

SRAP technique targets polymorphism in the open reading frames (ORF) of genome (Li and Quiros 2001). Two sets of primers are used which have a core of 13–14 bases of which the first 10–11 are random sequences, but differ in reverse and forward primers, followed by CCGG sequence in the forward primer and AATT in the reverse primer. Three selection nucleotides are added at the end of core sequences. The first PCR cycle is carried out at lower annealing temperature than the rest. PAGE is used for fragment separation. SRAP markers are both dominant and codominant depending upon the type of mutation and are used for fingerprinting as well as gene tagging (Chen et al. 2010a; Yin et al. 2014).

9.3.2.3.7 Target Region Amplification Polymorphism (TRAP)

The PCR-based technique reveals polymorphism in candidate genes by targeting ESTs (Hu and Vick 2003). Two 18 nucleotide primers are used, one complementary to the EST sequence and the other a random primer with AT- or GC-rich core which can anneal with an intron or exon.

9.3.2.3.8 Single-Strand Conformation Polymorphism (SSCP)

SSCP detects differences in electrophoretic mobility of single-stranded DNA due to changes in number and type of bases (Hayashi 1991). Target sequences are amplified by PCR followed by denaturation of the double-stranded DNA to yield single strands. The latter acquire three-dimensional shapes which differ in electrophoretic mobility among sequences with even single-base differences.

9.3.2.3.9 Intertransposon Amplified Polymorphism (IRAP)

As implied by its name, the technique reveals polymorphism between LTRs (Kalendar et al. 1999). Pairs of primers complementary to 3′ end of one LTR and 5′ end of another LTR are developed and used for PCR amplification. Since LTRs can have same sequences at either end due to head-to-head or tail-to-tail orientation, single primers can also yield amplification products.

9.3.2.3.10 Retrotransposon-Microsatellite Amplified Polymorphism (REMAP)

REMAP targets polymorphism between LTRs and SSRs (Kalendar et al. 1999). Two primers, one complementary with the 5′ end of LTR and the other corresponding to SSR and anchored to 3′ end for a selective base are used for PCR amplification.

9.3.2.3.11 Sequence-Specific Amplification Polymorphism (S-SAP)

S-SAP is a modified AFLP technique to detect polymorphism in transposons (Waugh et al. 1997). Here the second AFLP amplification is carried out using Mse-I adaptor-specific and retroposon-specific primers.

9.3.2.3.12 Transposon Display (TD)

The technique involves use of primers corresponding to a restriction site and a transposon for AFLP so that polymorphism in the sequence comprising parts of transposon and specific restriction site is detected (Va et al. 1998; Jiang et al. 2003).

9.3.2.3.13 Retroposon-Based Insertion Polymorphism (RBIP)

In this technique a transposon-specific primer is used along with a primer complementary to the flanking region which generates a PCR fragment containing the insertion (Flavell et al. 1998). If the insertion is absent, primers specific to both the flanking regions will generate a fragment.

9.3.3 DNA Microarrays

Microarrays or 'chips' comprise hundreds of DNA samples (known as probes) immobilised on glass or nylon slides which are hybridised by different DNA samples (known as targets) (Kehoe et al. 1999; Galbraith and Edwards 2010). Hybridisation between probe and target is detected and quantified by appropriate labelling, detection and quantification techniques. Besides other applications, DNA microarrays are used to compare genomic constitution, detect SNPs and identify biological contaminants/adulterants in food or feed (Niu et al. 2011). Different microarray-based technologies are used for fingerprinting purposes.

9.3.3.1 Diversity Array Technology (DArT)

DArT involves restriction digestion of a gene pool, PCR amplification of selected fragments and cloning and spotting on a microarray (Jaccoud et al. 2001). The DNA of a sample to be identified is restricted with the same enzymes and hybridised to the array. The advantage of DArT is that it does not require sequence information, is robust, detects single-base differences and is highly automated. On the other hand, the technique is expensive and technology demanding.

9.3.3.2 Suppressive Subtractive Hybridisation Array (SSHA)

The method is based on suppressive subtractive hybridisation (SSH) which excludes sequences common to two samples under comparison and thus greatly increases the probability of capturing polymorphic DNA fragments which may be in low abundance (Li 2004; Lukyanov et al. 2007). The subtracted and PCR-amplified fragments are printed on microarrays and hybridised with target DNA fragments.

9.3.3.3 Subtracted Diversity Array (SDA)

In this technique, pooled genomic DNA from one set of samples is extracted from a pooled genomic DNA of other sets of DNA samples to obtain DNA unique to the second set (Jayasinghe et al. 2007). The latter is used as microarray probe to fingerprint a set of samples genetically closer to the second set.

9.3.3.4 Oligonucleotide and Gene-based Microarrays

Species-specific oligonucleotide sequences or gene sequences are used as probes. Sequences of target species corresponding to the probes are amplified, labelled and hybridised onto the oligo-

nucleotide array (Zhu et al 2008; Niu et al. 2011). Alternatively, target DNA is sheared, labelled and hybridised (Edwards et al. 2008).

9.3.4 DNA Sequencing

Till recently, the method developed by Sanger and colleagues was the most widely used one for DNA sequencing (Sanger et al. 1977; França et al. 2002). It involves selective addition of chain-terminating nucleotides during in vitro replication of template DNA. Electrophoresis is carried out to separate the amplified fragments from which the sequence of template DNA is read.

Sequencing is one of the steps in SSR, SCAR, SNP and sequence-based microarray protocols. In addition, sequence information of some specific genomic regions has been found to be quite useful in species differentiation (DNA barcoding), including identification of constituent species in mixtures (He et al. 2010; Chen et al. 2010b; Pang et al. 2011; Newmaster et al. 2013; Tripathi et al. 2013). In plants, nrITS, nrITS2, *accD*, *matK*, *ndhJ*, *rpoB*, *rpoC1*, *ycf5*, *atpF-H*, *psbK-1*, *rbcL*, *rbcLa*, *trnH-psbA*, *trnL* (*P6*) and UPA, singly or in combination, have been proposed as suitable barcodes (Hollingsworth et al. 2011). While the discovery of a universal barcode that could differentiate all the world's plants is yet to come true, the technology has been quite effective in differentiating species in some plant groups (Hollingsworth et al. 2009; Spooner 2009; Hollingsworth 2011).

A new generation of sequencing methods, next-generation sequencing (NGS), has emerged during the last few years which has vastly increased the capacity (up to one billion bases per run) and dramatically reduced the cost of sequencing (Egan et al. 2012; Kumar et al. 2012), the cost per mb having reduced from US$ 5,224 by Sanger technology to US$ 0.12–0.39 by Illumina (Zalapa et al. 2012). Three main methods, sequencing by synthesis, sequencing by ligation and single-molecule sequencing, have been used for NGS. Global sequencing efforts along with new bioinformatics tools have resulted in generation and analysis of massive amounts of sequence data from model as well as non-model plants (Bateman and Quackenbush 2009; Lee et al. 2011; Michael and Jackson 2013).

In plants where reference genomes are available, whole-genome resequencing (Huang et al. 2009) provides an excellent tool for analysing genome-wide diversity in an array of samples, including SNP discovery and genotyping (genotyping by sequencing) (Bekele et al. 2013; Sonah et al. 2013; Finkers 2014). To overcome the problems of too large a size of genome and relatively small informative regions, several approaches have been adopted to reduce complexity by enriching specific regions before sequencing. Transcriptome sequencing and restriction site-associated DNA sequencing (RADseq) are some such approaches (Miller et al. 2007; Priest et al. 2010).

9.4 DNA Fingerprinting for Plant Identification: Some Examples

In their publication, *DNA Fingerprinting in Plants: Principles, Methods, and Applications*, Weising et al. (2005) have elaborately covered the literature on the subject published till 2004. In Table 9.1, some subsequent publications exemplifying the applications of conventional and more recent fingerprinting techniques in plant identification are listed.

9.5 Which Markers to Use?

The choice of marker techniques for DNA fingerprinting will depend upon several factors: whether the purpose is to discriminate individuals, populations or higher taxonomic categories; is there a need for a highly robust method or can reproducibility be compromised for some other advantage; what is the required number of markers and their inheritance to yield results of acceptable confidence level; what is the availability of laboratory facilities, technical expertise and budget for consumables; and what is the scale of

Table 9.1 Recent examples of DNA fingerprinting for plant identification

Reference	Technique	Plants/products
Akbari et al. (2006)	DArT	Wheat cultivars
Akond et al. (2012)	SSR	Rose species and cultivars
Arya et al. (2009)	EST-SSR	Millet and pearl millet genotype identification
Bassil et al. (2009)	SSR	Hazelnut clones in gene bank
Ben-Ayed et al. (2012)	SSR	Authenticity of virgin olive oil
Bertea et al. (2006)	PCR-RFLP	*Salvia* species from green and dry samples
Bhatia et al. (2009)	ISSR	Clonal fidelity of micropropagated gerbera plants
Biswas et al. (2010)	RAPD, ISSR, IRAP, REMAP	*Citrus* genotypes
Caramante et al. (2011)	SSR	Tomato cultivars in food chain
Chen et al. (2006)	AFLP	*Oncidium* cultivars
Chen et al. (2010b)	DNA barcoding	Medicinal plant species
Cheng and Huang (2009)	SSR	Peach cultivars and landraces
de Souza et al. (2010)	RAPD	Intraspecific and interspecific hybrids in Annonaceae
Edwards et al. (2008)	Oligo-microarray	*Oryza* species
Faleiro et al. (2010)	RAPD	Mango cultivars
Ferrari et al. (2005)	AFLP	*Corylus avellana*
Figueiredo et al. (2013)	RAPD	*Olea europaea* L. ssp. *europaea* accessions
Fu et al. (2014)	EST-SSR	Celery cultivars
Grover et al. (2009)	SSR	Potato cultivars and lines
He et al. (2010)	DNA barcoding	Medicinal *Aconitum* species
Hu et at. (2005)	TRAP	Lettuce cultivars and wild accessions
Hu et al. (2007)	TRAP	Spinach commercial hybrids and accessions
Jiang et al. (2010)	SSR	Genotyping of indica and japonica rice varieties
Kacar et al. (2006)	SSR	Turkish sour cherry germplasm accessions
Kafkas et al. (2008)	AFLP	*Morus* accessions
Kashyap et al. (2005)	RAPD	*Fragaria* genotypes
Keshavachandran et al. (2007)	RAPD	*Piper nigrum* and *P. longum* cultivars
Kiani et al. (2012)	ISSR	*Tulipa* cultivars
Kim et al. (2012)	RBIP	Pear cultivars
Kjos et al. (2010)	AFLP	*Argyranthemum frutescens* cultivars
Korir et al. (2013)	RAPD	Tomato varieties
Kumar et al. (2013)	SSR	Mango varieties
Kwon et al. (2005)	TD	*Oryza* species and genotypes
Lamia et al. (2010)	AFLP, SSR	Apricot germplasm
La Mantia et al. (2005)	SSR	Sicilian olive germplasm
Lezar et al. (2004)	DArT	Closely related *Eucalyptus* trees
Li et al. (2006)	SSHA	*Dendrobium* species
Li et al. (2008)	Multiplex SSR and gene PCR	Potato cultivars
López et al. (2009)	SSR, AFLP	Grapevine cultivars
Louarn et al. (2007)	SSR	*Brassica oleracea* cultivars
Lu et al. (2009)	RAPD, ISSR	Broccoli and related cultivars

(continued)

Table 9.1 (continued)

Reference	Technique	Plants/products
Mantri et al. (2012)	SDA	Asterid species
Miñano et al. (2009)	RAPD	Chrysanthemum cultivars and somaclonal variants
Naito et al. (2006)	TD	Rice genotypes
Newmaster et al. (2013)	DNA barcoding	Detecting contamination and substitution in herbal products
Niu et al. (2011)	SDA	*Panax* species from dried samples
Olarte et al. (2013a)	SDA	*Salvia* species
Olarte et al. (2013b)	SDA	*Echinacea* species
Park et al. (2010)	SSR	Rose and strawberry cultivars
Pathak and Dhawan (2010)	ISSR	Clonal fidelity of micropropagated apple rootstock
Pang et al. (2011)	DNA barcoding	Rosaceae species
Peng et al. (2010)	PCR-RFLP, ITS sequencing	Distinguishing *Lonicera japonica* from other *Lonicera* species
Pooler and Townsend (2005)	AFLP	Elm clones and hybrids
Prakash et al. (2005)	SSR, AFLP	Indian robusta coffee germplasm
Riaz et al. (2008)	SSR	Muscadine grapes
Sarwat et al. (2008)	ISSR, RAPD	*Tribulus terrestris* accessions
Shiran et al. (2009)	AFLP	Iranian almond cultivars and wild species
Singh et al. (2007)	ISSR	Mango cultivars
Singh et al. (2010)	SSR	Rice genotypes
Somsri and Bussabakornkul (2008)	DAF	Sex identification in papaya
Spaniolas et al. (2006)	PCR-RFLP	Authentication of coffee
Syed et al. (2005)	SSAP	Cashew genotypes
Tiwari et al. (2009)	RAPD, ISSR	Brinjal cultivars
Tripathi et al. (2013)	DNA barcoding	Tropical tree species
Wang et al. (2010)	SNP	*Panax ginseng* cultivar
Xi et al. (2012)	ISSR	Lily mutants
Xuan et al. (2009)	SSR	Sweet and sour cherry
Yang et al. (2013)	SCAR	*Dimocarpus longan* varieties
Yu et al. (2012)	SSR	*Gossypium* species and genotypes
Zhang et al. (2013)	SCAR	*Sorghum halepense* genotypes
Zhou et al. (2005)	AFLP	*Populus* species, clones and cultivars
Zhu et al. (2008)	Oligo-microarray	Ginseng plants and drugs
Zhu et al. (2011)	ISSR	Wheat cultivars

operation including the number of participating laboratories and data sharing (Karp et al. 1997; Henry 2001; de Vicente and Fulton 2003; Spooner et al. 2005; Weising et al. 2005; UPOV 2010).

Table 9.2 provides a general comparison of the commonly used fingerprinting techniques with respect to their efficiency, ease and cost of application. A number of studies are also available that compare these techniques in specific groups of taxa (Powell et al. 1996; Jones et al. 1997; Pejic et al. 1998; McGregor et al. 2000; De Riek 2001; Archak et al. 2003; Sarwat et al. 2008; Lamia et al. 2010). In general, SSRs are the preferred markers because of their abundant variation among but relatively low variation within cultivars, high reproducibility across laboratories, codominant inheritance, primer availability in many crops and ease of experimental procedure. Several of these advantages are true of SNPs as well. In fact, due to their robustness,

Table 9.2 Comparison of some widely used DNA marker systems in plants

Marker description	RFLP	RAPD	AFLP	ISSR	SSR	SNP
Genomic abundance	High	High	High	Moderate	Moderate to high	Very high
Genomic coverage	Low–moderate	Whole genome	Whole genome	Whole genome	Whole genome	Whole genome
Inheritance	Codominant	Dominant	Dominant/codominant	Dominant	Codominant	Codominant
Level of polymorphism	Moderate	High	High	Moderate	High	High
Reproducibility	High	Low	High	Moderate	High	High
Effective multiplex ratio	Low	Moderate	High	Moderate	Medium/high	Moderate to high
Marker index	Low	Moderate	Moderate to high	Moderate	Moderate to high	Moderate
Quality of DNA required	High	Low	Moderate	Low	Moderate	High
Technical expertise required	High	Low	Moderate	Low	Low/moderate	High
Automation	Low	Moderate	Moderate/high	Moderate	Moderate/high	High
Cost per assay	Moderate to high	Low	Moderate	Low	Moderate to high	High

Based on Karp et al. (1997), de Vicente and Fulton (2003), Spooner et al. (2005), Semagn et al. (2006), Jiang (2013)

SSRs and SNPs are best suited for genotyping involving forensic and IPR issues (Testolin and Cipriani 2010; UPOV 2010). While marker development expertise and cost is an issue with many laboratories, with the NGS-driven high speed and reducing cost of sequencing and expanding databases and software tools, SNPs along with SSRs are becoming the preferred fingerprinting options for a wide range of laboratories.

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Functional Genomics

10

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Abstract

Physiological responses, developmental programs, and cellular functions depend on complex networks of interactions at different scales and levels. Systems biology unites biochemical, genetic, and molecular approaches to generate omics data that can be analyzed and used in computational models to uncover these global networks. Various approaches, including genomics, transcriptomics, proteomics, interactomics, and metabolomics, have been employed to obtain these data at the cellular, tissue, organ, and whole organism levels.

Keywords

Genomics • Functional genomics • Transcriptomics • Proteomics • Metabolomics • DNA microarrays • Comparative microarray analysis • Systems biology

10.1 Introduction

Large-scale genome projects produce a large amount of data that are used to build models to understand how biological systems manage information. In contrast to the static representation of structural genomics, functional genomics focuses on the dynamic aspects of genome expression. Functional genomics emerged as a field of molecular biology that involves high-throughput methods in genome-wide investigations. In a broad sense, functional genomics is dedicated to the understanding of the relationship between the

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genome of an organism and its phenotype. The fundamental goal of functional genomics is to understand how biological functions arise from the information encoded in a genome.

Briefly, the biological information necessary to manage a biological system is stored in the genome and transcribed into the transcriptome to be finally translated into the proteome. The proteome is the sum of signaling and metabolic networks. The interactome represents the signaling networks because the response of the cell to a specific situation is obtained by the activation of gene expression; gene expression depends on the activation of a signaling pathway, and the signaling pathway is activated through the interaction of a receptor with a specific signaling molecule. By contrast, the subsection of the proteome that is dedicated to enzymatic activities describes metabolic pathways and interactions with the metabolome. The metabolome encompasses not only molecules that are substrates and products of enzymatic reactions but also cofactors and non-peptidic organic signaling molecules. Ionome, the core of ions, gives information on the state of the system under specific environmental conditions. The genome, transcriptome, proteome, and metabolome are considered the four pillars of functional genomics (Salt et al. 2008). The dynamic response and the interaction of these four pillars define how a living system operates, which is currently one of the greatest challenges in science (Salt 2004).

The genomic revolution began with the reporting of the complete genome sequence of the bacterial pathogen *Haemophilus influenzae* (Fleischmann et al. 1995). For the first time, science was in possession of a complete DNA catalog with the genetic information for every phenotype. Two decades later, thousands of prokaryotic and eukaryotic genome sequences are available. The first plant genome fully sequenced was *Arabidopsis thaliana*, which was selected because of the small size (~150 Mb) of its genome (The *Arabidopsis* Genome Initiative 2000). Suites of standard protocols are now applied to each new genome that is being sequenced, and the released information is made publicly accessible through GenBank (Benson et al. 2006), the European Molecular Biology Laboratory (Cochrane et al. 2006), and the DNA Data Bank of Japan (Okubo et al. 2006). The data format that is covered by these three databases was initially dedicated to the representation of gene structure. However, these databases have been progressively evolving toward the representation of intergenic sequences on a chromosomal scale and integrating data from multiple sources such as functions, cellular levels, and ontologies. Many individual laboratory initiatives have since developed their own websites with a specific format and links to other databases and have adapted to the type of released information. By consequence, the annotation pipelines of these workflows are globally independent from the scientific team that conducted the genome sequencing, but specificities exist in the way the tools are assembled. From a post-genomics perspective, the pioneer annotation pipelines can be considered as the starting point that has facilitated the development of technologies for high-throughput structural and functional genomics.

Among numerous notable websites, the following websites are highlighted. (i) PlantGDB is a database of molecular sequence data for all plant species with significant sequencing investments. The database organizes EST sequences into contigs that represent tentative unique genes. Genome sequence fragments are assembled on the basis of their similarity. Contigs are annotated and, whenever possible, linked to their respective genomic DNA. The goal of the PlantGDB website is to establish the basis for identifying sets of genes common to all plants or specific to particular species. To achieve this goal, PlantGDB integrates a number of bioinformatics tools that facilitate gene predictions and cross-comparisons in species with large-scale genome sequencing investments. (ii) Open Sputnik is a database for the comparison of plant genomes. This website uses sequence resources to fill information gaps in non-sequenced plant genomes and provides a foundation for in silico comparative plant genomics (Rudd 2005). (iii) Mercator is a Web server for the genome-scale functional annotation of plant sequence data (Lohse et al. 2014). (iv) PlantTFDB 3.0 is a website for the functional

and evolutionary study of plant transcription factors (Jin et al. 2014). For additional database and bioinformatics tools, see Table 10.1 and Martinez (2011).

Functional genomics data can be subdivided into sequence and experimental datasets. The sequences are used to perform fundamental genetic analyses such as a homology search, nucleotide substitutions, indels search, SNP detection, nucleotide composition analyses, gene expression measure, and gene structure description. These processes are commonly performed genome-wide on sequence datasets using automated algorithms running in silico. For example, the principle of conserved operons (Overbeek et al. 1999) can be used to predict the function and

Table 10.1 Databases for functional genomics of plant resources

Types	URL
Genomic databases	
See a list on plant organelles database	http://podb.nibb.ac.jp/Organellome/bin/listLinks.php
Computational biology and functional genomics laboratory	http://compbio.dfci.harvard.edu/tgi/plant.html
Plant repeat databases	http://plantrepeats.plantbiology.msu.edu/downloads.html
TropGENE database	http://tropgenedb.cirad.fr/tropgene/JSP/index.jsp
Genomics of small grain crops	http://wheat.pw.usda.gov/GG2/index.shtml
Comparative grass genomics	http://www.gramene.org/
Plant genome database	http://www.plantgdb.org/
The Arabidopsis Information Resource (TAIR)	http://www.arabidopsis.org/
Rice genome project (RGP)	http://rgp.dna.affrc.go.jp/
SNPs	http://autosnpdb.appliedbioinformatics.com.au/
Insertional mutagenesis of *Arabidopsis thaliana*	http://signal.salk.edu/cgi-bin/tdnaexpress
The floral genome project	http://fgp.huck.psu.edu/tribedb/10_genomes/index.pl
Intron polymorphism markers	http://ibi.zju.edu.cn/pgl/pip/
Plant promoter database	http://www.softberry.com/berry.phtml?topic=plantprom&group=data&subgroup=plantprom
Plant cis-acting regulatory elements	http://bioinformatics.psb.ugent.be/webtools/plantcare/html/
Small RNA from cereals	http://sundarlab.ucdavis.edu/smrnas/
Plant microRNA knowledge base	http://bis.zju.edu.cn/pmirkb/
Plant microRNA database	http://bioinformatics.cau.edu.cn/PMRD/
Plant MPSS database	http://mpss.udel.edu/
Plant resistance genes	http://www.pathoplant.de/
Predictive RNA editor	http://prep.unl.edu/
Expression database	
See a list on: plant organelles database	http://podb.nibb.ac.jp/Organellome/bin/listLinks.php
At TAIR	http://www.arabidopsis.org/servlets/Search?action=new_search&type=expression
At TIGR	http://jcvi.org/arabidopsis/qpcr/search.php

(continued)

Table 10.1 (continued)

Types	URL
EBI: the array express database	https://www.ebi.ac.uk/arrayexpress/
GARNet	http://www.garnetcommunity.org.uk/resources/microarray-tool-kit
RMOS	http://cdna01.dna.affrc.go.jp/RMOS/index.html
Stanford microarray database	http://smd.princeton.edu/
Database resource for the analysis of signal transduction in cells	http://www.drastic.org.uk/
Crop plant expression	http://www.agbase.msstate.edu/
Crop plant ESTs	http://pgrc.ipk-gatersleben.de/cr-est/
PLEXdb	http://www.plexdb.org/
Proteomics	
See a list on: plant organelles database	http://podb.nibb.ac.jp/Organellome/bin/listLinks.php
Crop plant metabolism	http://metacrop.ipk-gatersleben.de/apex/f?p=269:7:0::NO::P7_PATHWAY_ID:49
Plant transcription factor database	http://plntfdb.bio.uni-potsdam.de/v3.0/
Systematic comparison of proteome data	http://salad.dna.affrc.go.jp/salad/en/

functional interactions of unknown open reading frames (ORFs). In eukaryotes, the concept of ORF is only useful to describe transcriptome sequences (see in Carels and Frias 2013) because genomic coding sequences (CDS) are interrupted by introns. To cope with the challenge of gene prediction on a genomic scale in eukaryotes, sophisticated hidden Markov models (HMMs) were designed to account for the conditional probability of exons, introns, splicing signals, and promoters for different contexts such as GC content (Stanke et al. 2004; http://bioinf.uni-greifswald.de/augustus/). Putative exons may even be aligned with external datasets or transcriptome data to increase the consistency associated with these predictions (Keller et al. 2011). However, these models still produce large error rates (Coghlan et al. 2008), and a large-scale homologous comparison between genomic and transcriptomic sequences is generally preferred when available.

Experimental datasets, however, are adaptations of established molecular biology protocols that are scaled up to become high throughput. For example, DNA microarrays are a miniaturization of standard Southern hybridization techniques to cover the whole genome.

While there is a clear separation between sequence and experimental datasets, the primary utilization of these datasets is identical: the large-scale prediction of functional interactions (Muley and Ranjan 2012). From a systems biology perspective, a functional interaction can be reduced to a binary construct between two proteins or genes. Although this representation is a drastic oversimplification, it allows both sequence and experimental datasets to be converted and combined into interaction sets. Interacting pairs of genes can be represented by "1" and noninteracting pairs by "0." Because this binary characterization of an interaction is only a model of the reality and, thus, characterized by an error rate, any interaction predicted using a functional genomics dataset must be experimentally verified and characterized according to spatial and temporal variables. Furthermore, the process of interaction characterization lacks consensus regarding the definition of the term functional interaction. The problem resides in the interpretation of the word interaction and, by extension, the purpose

of the modifier, functional. A functional interaction may refer either strictly to the proteins that are engaged in direct physical contact (Cusick et al. 2005) or broadly to all proteins that are involved in a response or pathway (Eisemberg et al. 2000). The difficulty in constructing a consensus definition for functional interaction is similar to the current controversy in defining the concept of species in bacteria (Cohan 2002; Moreno 1997).

Ontology determines whether categories are fundamental and considers in what sense an item can be considered to *be* (legitimately) in one of those categories. By extension, ontology is a representation (semantic model) of something we know. Thus, the ontology is useful only when it successfully represents the targeted object. Ontologies provide insight into the relationships that exist between a gene and the genome by reducing the set of possible interactive partners from the whole genome to a subset. Rather than represent specific pairwise functional interactions, ontologies have been developed to classify proteins into functional groups including those in the Clusters of Orthologous Groups (COGs) for prokaryotes (Tatusov et al. 2000), Eukaryotic Orthologous Groups (KOG) for eukaryotes (Hirakawa et al. 2013), Gene Ontology (GO) (Ashburner et al. 2000), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al. 2004), and Protein Families (Pfam) (Finn et al. 2006). Although these ontologies use independent schemes in their classifications, computer algorithms can convert the data from one ontology into another, strongly indicating that these ontologies are based on similar underlying concepts.

In the following section, we characterize the most common types of sequence and experimental datasets currently available.

10.2 Sequence Datasets

One of the major strengths of genomic sequence datasets is that they are based on a technology that is well established and that produces robust data available for further biological inferences. However, these procedures heavily rely on bioinformatics. In the design of bench experiments, bioinformatics models have to be verified at the experimental level prior to further investigation to avoid the unnecessary loss of time and money because of eventual errors in these models. Despite this error load, genomic data allow detailed sequence comparison across species; this cross-species analysis becomes increasingly powerful as the number of available genomes increases. Prokaryotic genomes are relatively small compared to eukaryote genomes, and bacteria therefore have the largest number of sequenced genomes. However, the number of eukaryote genomes that are completely sequenced is increasing rapidly, and there are already 50 complete genomes for plants (Michael and Jackson 2013).

The release of any given genome sequence is accompanied by an initial genome annotation. The first, and perhaps the most important, annotation step is the mapping of predicted CDSs on a genome. In prokaryotes, this step has been successfully performed with algorithms such as GLIMMER (Delcher et al. 1999); eukaryote algorithms are based on HMM trained with samples of true positives such as in TigrScan, Fgenesh++, and Augustus. True positive coding ORFs are ORFs that were proven to code sequences in experimental studies. Coding ORFs have a purine bias (Rrr) within the codon that is universal to all living organisms (Carels et al. 2009) and that results from selective constraints on physicochemical properties of the encoded proteins (Ponce de Leon et al. 2014). The Rrr is a feature that is specific to coding DNA and is detected by Markov models. In eukaryotic genomes, ORF prediction is considered accurate for sequences larger than 300 bp (Brent 2005; Wang et al. 2006), but a rate of ~15 % error between frames +1 and −1 is currently observed (Carels and Frias 2009; Carels and Frias 2013a). This error may however be reduced to 5 % if an additional filter is added to the common algorithm. This filter must contain the conditions that guanine in the first position of codons (G1) is larger than in the second position (G2) and that the adenine in the second

position of codons (A2) is larger than the thymine in the first position (T1). These conditions are true in 95 % of proteins that were crystallized (Carels and Frias 2013b). In eukaryotes, gene prediction is more difficult than ORFs because the coding sequence is interrupted by noncoding introns. Because the average enzyme size is ~400 amino acids (1,200 bp), most exons are below the 300 bp size threshold that is required to warrant statistical consistency of the prediction. For these genomes, the best solution is to extract coding sequences from the transcriptome because mRNAs are quickly processed to remove introns. Thus, cDNA is generally deprived of introns. Obtaining the cDNA allows an easy classification of the coding ORFs in the coding frame (Carels and Frias 2013b). The homologous comparison of coding ORFs from contigs of cDNAs with the genomic sequence also allows the retrieval of introns; thus, the coding ORFs form the objective training set of introns and exons used for the training of a HMM for the case under consideration. Examples of freely accessible HMMs for gene searches in eukaryotes are GeneZilla (Allen et al. 2006) and Augustus (Stanke et al. 2004). This procedure should allow the discovery of the majority of genes even for species with no previous information.

Each predicted ORF is then assigned a putative annotation based on various methods that rely on the search for homologous pairs between a sample of query sequences and a sample of subject sequences. Applying the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1997) to compare each ORF against a reference database of characterized genes allows the transfer of an annotation from the reference (subject) to the query when the score of the homologous comparison is above a given threshold to preserve statistical consistency. The p-value associated with the comparison decreases with increasing score values. Consequently, the confidence in the function that is transferred from subject to query also increases with decreasing p-values. Depending on the type of data within the reference database, other features can be deduced from the significant homologous comparisons between sequence pairs (e.g., comparing a sequence with KOGs). The association of unknown CDSs with known enzymes in KEGG (Kanehisa et al. 2004) may allow the attribution of a CDS to a particular pathway. Furthermore, the homologous identification of protein domains using the HMM profiles (Eddy 1998) using Pfam annotations may allow the classification of an unknown CDS within a given enzymatic family. Therefore, evolutionary roles may be inferred for that CDS.

In addition to the annotation of CDSs, the genome sequence is also analyzed for additional features including noncoding regulatory RNAs (Eddy 2002; Gottesman 2005; Schattner et al. 2005; Vogel and Sharma 2005; Winkler 2005) such as transfer RNAs (tRNA), ribosomal RNAs (rRNA), microRNAs (miRNAs), and small untranslated RNAs (suRNA). These analyses are considered by experimentalists as a starting point for the characterization of specific genes or genetic networks.

Plant genome datasets were constructed according to their specific features. For species associated with large-scale genome sequencing, PlantGDB provides genome browsing abilities that integrate all available ESTs and cDNAs for current gene models. A special section dedicated to *A. thaliana* is named AtGDB (http://www.plantgdb.org/AtGDB).

The MapMan BIN ontology is another useful bioinformatic tool in plant functional genomics. MapMan was initiated by Thimm et al. (2004) and has constantly improved over time (Usadel et al. 2009; Urbanczyk-Wochniak et al. 2006; Rotter et al. 2007; Sreenivasulu et al. 2008). The goal of the MapMan approach is to assign genes to as few functional categories as possible without losing information. The MapMan ontology is organized as a hierarchically structured tree that comprises 35 main biological categories that are named BINs. Currently, approximately 1,000 distinct enzymatic activities are associated with 1,906 BINs in the MapMan hierarchy. Complete transcriptome annotations were obtained for many different plant species using the MapMan

classification system and are publicly available via the MapMan website (http://mapman.gabipd.org/web/guest/mapmanstore).

10.2.1 Cofunctional Network Inference and Analysis

Advances in high-throughput technologies have allowed the generation of extensive gene expression datasets at a genome-wide scale. The observation that genes sharing a common function can be coordinated at the level of transcription (Stuart et al. 2003) has enabled these datasets to be analyzed to assign putative functional associations between genes. The evaluation of gene coexpression uses statistical metrics to establish correlations between gene expression profiles across many samples based on the principle of guilt by association (Usadel et al. 2009).

Systems biology coordinates high-throughput data from biochemical, genetic, and molecular approaches. These coordinated data can be analyzed through mathematical and computational modeling to uncover interactive networks on a global scale. The elucidation of coexpression networks is a top-down approach that relies on gene clustering from transcription data. A cluster whose annotated genes are overrepresented in a Gene Ontology (GO) category suggests that the biological process associated to that category (Ashburner et al. 2000) can also be extended to the other members of this cluster. Thus, uncharacterized genes within the cluster are potentially participating in the same biological process as the annotated genes. Similarly, genes directly connected to (or co-expressed with) known central regulators of a developmental process are potentially within the identical metabolic pathway. Coexpression networks created using various combinations of these approaches have led to the identification, in *Arabidopsis*, of previously uncharacterized genes involved in cell wall synthesis (Persson et al. 2005), flower development (Usadel et al. 2009), seed germination (Bassel et al. 2011), and plant growth (Mutwil et al. 2010).

10.2.2 Online Resources for Sequence Datasets

Prolinks (Bowers et al. 2004), PLEX (Date and Marcotte 2005), and STRING (von Mering et al. 2005) online resources combine methods that predict functional interactions in genomes. These databases contain precomputed predictions for a number of sequenced bacterial genomes and can be searched for specific proteins. In addition, these databases allow users to submit their own query sequences and obtain the calculated functional predictions for all three methods.

In plants, the effectiveness of functional genomics based on sequences increases with the number of completely sequenced genomes. In addition to the limited number of sequenced plants genomes, there is also a specific sequencing bias because annotations rely on a small number of model organisms. For example, the accuracy of the structure determined to be associated with a gene fusion (a hybrid gene formed from two previously separate genes) depends on how well the non-fused proteins that were involved in the fusion event are described.

10.3 Experimental Datasets

Complete genome sequences have enabled the development of technologies based on bench experiments. Whereas the largest variety of experimental technologies are currently being applied to model organisms such as yeast, mice, worms, and flies, a number of these technologies have been extended to plants. For example, proteomic datasets available for *A. thaliana* (Joshi et al. 2012) are based on DNA microarrays (Magrimi et al. 2008), chromatin immunoprecipitation chips (Kaufmann et al. 2010), localization studies (Ito et al. 2014), gene essentiality studies (Zhang and Lin 2009), and metabolomics (Zenobi 2013; Landesfeind et al. 2014). In this section, we will focus on the datasets of functional genomic data based on bench experiments available for plants.

10.3.1 Proteomics

The field of proteomics has rapidly evolved with the availability of whole genome sequences, and a number of techniques have been developed to probe the interactions that occur between proteins within a cell. The goal of proteomics is to provide evidence for protein–protein interactions that occur within cells. One of the most difficult challenges to this approach is the ability to extract and screen large numbers of proteins. In bacteria, plants, and animals, two major types of proteomic approaches have been applied for the genome-wide study of protein–protein interactions: yeast two-hybrid assays and protein profiling. Both approaches provide fundamentally different types of information about protein–protein interactions, and each method has been successfully utilized to characterize the protein interactions that occur within cells and their associations to biological functions. Several assays have been developed to identify and predict protein interactions in yeast, in cell suspensions, in plants, or in silico, and various public databases have compiled these interactions (Table 10.2).

Table 10.2 Freely accessible databases and tools for network analyses

Types	Tools
Databases and warehouses	
Gene expression	BAR, Genevestigator, ATTED, AT-TAX, TileViz
Protein expression	Pep2pro
Protein–protein interactions	ANAP, BAR, PAIR, IntAct, AtPIN, AtPID, AthPPI, Associomics, PRIN (rice), BioGRID
Protein localization	SUBA, Predotar, TargetP
Gene set analysis	BAR, AMIGO, DAVID, FuncAssociate, BiNGO, ATCOECIS, MapMan, AraNet, AgriGO
Comparative genomics	Plaza, OrthologID, DoOP
Gene regulation and promoter architecture	
Motif detection	BAR, ATCOECIS, TAIR patmatch, DoOP, STAMP, Weeder
Binding site analysis	PLACE, AthaMap, AGRIS
Data integration and network inference	LeMoNe, ENIGMA, CORNET, Ondex, AraNet, GeneMania
Generic computation and modeling environments	R, MATLAB, Systems Biology Workbench
Data visualization	AraCyc, PlantCyc, Cytoscape, MapMan, REACTOME

10.3.1.1 Yeast Two Hybrids

The yeast two-hybrid (Y2H) system, first proposed by Fields and Song (1989), remains one of the most applied techniques to detect direct interactions between proteins. In this approach, the direct interaction of two proteins is detected by the activation of a specific reporter in an organism such as yeast. The protein of interest, known as the prey protein, is fused to a DNA-binding domain of a specific transcription factor such as GAL4; a second protein, the bait protein, is fused to the activator domain of the same transcription factor. Both protein fusions are combined through the yeast mating system, and the interaction of both proteins causes the activation of the reporter transcription factor. Yeast colonies are plated on media that require the activation of the reporter gene through the successful interaction of both proteins. As a result, protein–protein interactions can be deduced from yeast growth. A high-throughput implementation by parallel sequencing of PCR products has been proposed (Calvenzani et al. 2012). In comparison with the Y2H, the yeast one-hybrid (Y1H) screen is designed to detect protein–DNA interactions, i.e., DNA-binding proteins that recognize a DNA sequence cloned upstream of yeast reporter genes.

10.3.1.2 Protein Profiling

In contrast to Y2H, in which individual protein–protein interactions are predicted, protein profiling serves to identify those proteins that are expressed by a cell under specific conditions. The ability to profile the protein output of an organism can reveal how the cell responds to certain environmental conditions and provide clues to the function of these expressed proteins. The detection of the full complement of proteins expressed by a cell is obtained by the combination of two-dimensional (2D) electrophoresis and mass spectrometry (MS). In this approach, a cell

is harvested for its proteins and separated using a 2D slab gel. This results in the separation of proteins based on their isoelectric point (the pH at which a protein displays no net charge) and molecular mass. MS is used to further identify the proteins separated by 2D electrophoresis. More recent advances in MS technology, such as matrix-assisted laser desorption ionization time of flight (MALDI-TOF), have further increased the ability to identify proteins extracted from a gel. Specific aspects of these technologies were summarized in Hamrang et al. (2013). The latest advances in MS have also allowed the emergence of the profiling of more than 1,000 proteins in one study through Orbitrap technology (Scigelova and Makarov 2006; McAlister et al. 2008). This technology allows the relative quantification of profile components through a Fourier transform combined with the qualitative determination of proteins from trypsin-digested samples (Finkemeier et al. 2011).

10.4 Large-Scale Transcriptional Regulatory Networks

Tightly controlled gene expression is a hallmark of multicellular development and is accomplished using transcription factors (TFs) and miRNAs. Although many studies have focused on identifying downstream targets of these molecules, less is known about the factors that regulate their differential expression. The mapping and analysis of the large-scale regulatory networks associated with TFs and miRNAs allows the inference of their properties (Long et al. 2008). A gene regulatory network (GRN) describes the interaction of regulatory factors and their targets. Two complementary ways have been described to map GRNs: TF oriented and target oriented.

Chromatin immunoprecipitation (ChIP) coupled with microarrays (ChIP-chip) or next-generation sequencing (ChIP-seq) is a TF-centered approach for mapping GRNs. This technique involves immunoprecipitation of a TF and the bound target DNA with subsequent identification of downstream targets. The ChIP approach results in a network that describes the in vivo binding targets of a single TF.

The Y1H assay is also a target-oriented approach that allows the identification of upstream TFs that bind to promoters or motifs of interest. This assay requires the initial development of a TF collection or cDNA library of prey TFs that are assayed against bait promoters of interest in yeast (Deplancke et al. 2004). By contrast, Y2H is TF oriented.

ChIP studies have focused on TFs that are well characterized and typically abundant. In plants, ChIP studies have been performed primarily in *Arabidopsis* but also in rice (*Oryza sativa*) and maize (*Zea mays*) (Fornalé et al. 2010; Moreno-Risueno et al. 2010). A protein expressed in a few cell types or developmental stages can be difficult to precipitate because of low abundance. Traditionally, this has been overcome by overexpressing and conditionally inducing the gene of interest by fusion to the glucocorticoid receptor or to a hormone-inducible promoter (e.g., the estrogen-inducible promoter). Although this approach provides the investigator with potential binding targets, this method obscures the developmental or tissue/cell type-specific context of the transcriptional regulation identified. Additionally, high-quality antibodies against TFs are required to achieve specific and sufficient immunoprecipitation. The use of TFs fused with a characterized protein (i.e., green fluorescent protein) or epitope allows the circumvention of the development of protein-specific antibodies. However, the tagged protein must be verified to ensure that its expression reflects the wild type and that it can functionally complement a loss-of-function phenotype.

10.4.1 DNA Microarrays

The analysis of gene expression data generated by high-throughput microarray transcript profiling experiments has shown that transcriptionally coordinated genes are often functionally related. Recently, several studies have demonstrated through the integration of gene homology and expression information how correlated gene

expression patterns can be compared between species. The incorporation of detailed functional annotations and experimental data describing protein–protein interactions, phenotypes, or tissue-specific expression provides an invaluable source of information to identify conserved gene clusters and translate biological knowledge from model organisms to crops.

The purpose of a microarray experiment is to compare the steady-state mRNA levels between two samples that have been exposed to different conditions, either environmental or genetic. Messenger RNA is prepared from both samples and converted to cDNA with a reverse transcriptase. The fluorescent dyes green cyanine (Cy3) and red cyanine (Cy5) are used to label the two samples, respectively. Both samples are then combined and hybridized to the identical microarray that typically contains thousands of regularly spaced DNA spots. The DNA in each spot contains the complement of a fragment of one ORF in the genome, and any gene that was actively transcribed in either sample will hybridize with the complementary DNA spot on the microarray. Because the two samples are labeled with different dyes, the relative steady-state mRNA levels of each gene can be measured for each sample. Further explanation of DNA microarray technology is available in Rhodius et al. (2002), Ray et al. (2010), Gibriel (2012), and Movahedi et al. (2012).

10.4.1.1 Comparative Microarray Analysis

The analysis of DNA microarrays typically revolves around the fundamental observation that genes sharing similar expression patterns from a collection of microarray experiments have a higher likelihood of being functionally related. Multiple experiments in a large number of model organisms have confirmed this hypothesis (Jansen et al. 2002). One of the first applications of DNA microarrays to bacteria was the comparison of two genetically identical populations under different environmental conditions. For example, analyses of the gene expression of *E. coli* have been conducted by comparing microarray data for cells in minimal vs. rich media (Tao et al. 1999) and for groups of *E. coli* containing small and large populations. Similar microarray experiments have also been reported for other bacteria under comparative conditions such as drug treatment in both *M. tuberculosis* (Shi et al. 2012) and *Streptomyces coelicolor* (Weber et al. 2005) and stress conditions in both *B. subtilis* (Ye et al. 2000) and *P. putida* (Puchałka et al. 2008). Additionally, genes associated with the pollen transcriptome in *A. thaliana* were described using this method (Becker et al. 2003). Thus, many studies have used microarrays to probe differential gene expression between two strains of the same bacteria or compare the difference in the plant life cycle due to alternations between diploid sporophytic and haploid gametophytic generations. In one type of experiment, a specific gene of interest is inactivated, and a comparative microarray analysis is conducted between the mutant strain and the wild type.

The widespread use of microarrays as a tool for the large-scale investigation of differential gene expression in different organisms has resulted in the accumulation of a substantial amount of publicly available microarray data. A number of online databases exist to house these data, such as the ArrayExpress (Brazma et al. 2006), the Gene Expression Omnibus (Davis and Meltzer 2007), and the Stanford Microarray Database (Hubble et al. 2009). The availability of a large number of microarray experiments under a wide variety of experimental conditions has stimulated the development of tools to analyze this abundance of data.

10.4.2 RNA-Seq and Ampliseq

RNA-Seq (for RNA sequencing) is the high-coverage sequencing of a transcriptome obtained by massive parallel sequencing (next-generation sequencing – NGS) to quantify mRNAs, i.e., the mRNA count for each expressed gene in a genome. The number of reads that corresponds to a particular exon normalized by length yields gene expression levels that correlate with those obtained through qPCR (Li et al. 2008). RNA-Seq does not necessarily correlate with proteome

profiles because of posttranscriptional gene regulation events (such as RNA interference); however, measuring mRNA concentration levels is still a useful tool to determine how the transcriptional machinery of the cell is affected in the presence of external signals (e.g., chemical treatment) or how cells differ between a healthy and a diseased state. The correlation between the RNA-Seq quantities of mRNAs and proteomic profiles is obtained by sequencing polysomal mRNA from ribosomes isolated by sedimentation in sucrose gradients (Kleene et al. 2010).

The mRNA composition of the transcriptome of a cell is continuously changing as opposed to the stable genome. High-coverage sequencing by NGS allows the quantification of mRNA with marginal frequencies such as those resulting from alternative transcript splicing (Eksi et al. 2013), posttranscriptional changes, gene fusion, mutations (indels, SNPs), and changes in gene expression (Maher et al. 2009). In addition to mRNA transcripts, RNA-Seq can address different RNA populations such as total RNA, small RNAs (e.g., miRNA), tRNA, and ribosomal RNA profiling (Ingolia et al. 2012). The limited coverage of microarrays increases the attractiveness of RNA-Seq. RNA-Seq data have been recently used to infer genes involved in specific pathways based on Pearson correlations with variance-stabilizing transformation approaches (Giorgi et al. 2013).

RNA-Seq is obtained by aligning millions of reads to CDSs. Several software packages exist for short read alignments, and specialized algorithms for transcriptome alignments have been proposed, such as Bowtie to align short reads (Langmead et al. 2009), TopHat to align reads to a reference genome to discover splice sites (Trapnell et al. 2009), Cufflinks to assemble the transcripts and compare/merge them with others (Trapnell et al. 2010), and FANSe (Zhang et al. 2012). These tools can also be combined to form a comprehensive system (Trapnell et al. 2012).

Ampliseq is the one-shot sequencing of a high number of mRNA amplicons, typically in the range of 400 or more (Towler et al. 2008). This approach requires a previous study to uncover the gene pool that is worthwhile to measure by this method. Ampliseq is a type of parallel PCR that measures the expression of a range of selected genes. This method has the advantage of being cheaper than RNA-Seq.

10.4.3 Gene Expression Mapping

The focus of the majority of microarray, RNA-Seq, and Ampliseq experiments is to determine the genes that are differentially expressed under specific conditions; however, identification of genes that are similarly expressed under the largest possible set of conditions is also worthwhile. Combining the differential gene expression patterns for an organism for a multitude of experimental conditions provides insights into genes whose expression is tightly correlated, which is a strong indication of their functional interaction. The first application of this approach was reported by Kim et al. (2001) for the model organism *Caenorhabditis elegans*. These authors obtained data from over 500 microarray experiments from a number of sources conducted over a wide range of experimental conditions and constructed a gene expression map. Bray (2004) studied changes in gene expression that can be used to model the response of a plant to environmental challenges. For instance, one study used microarrays to report the expression patterns of thousands of genes simultaneously to understand the types and quantities of RNAs present in cells in response to a water stress. The analysis of the resulting map showed that the genes exhibiting similar annotations were found to cluster together.

10.5 Induced Mutations and Gene Editing

Mutagenesis has historically been a classical method to stably modify plant genome characteristics by exposing seeds to X-rays, gamma rays (Ahloowali et al. 2004), or chemical mutagens such as ethyl methanesulfonate (EMS) and dimethyl sulfate (DMS). Mutagenesis has also been performed by transposon activation (insertional mutagenesis) or random T-DNA insertion

(Alonso et al. 2003). The maize system (typically, the suppressor-mutator (Spm) and activator/dissociation (Ac/Ds)) was introduced in several plants of interest such as tomato (Cooley et al. 1996). Because the transposon sequence is known, any modification to the tagged gene can be easily identified by PCR and sequencing. The current preferential methods to induce mutagenesis are experimental techniques that tag a particular gene of interest.

10.5.1 RNA Interference Technology

The first description of virus-induced gene silencing was performed in plants at the end of the last century (Lindbo et al. 1993; Kumagai et al. 1995). Some years later, RNA silencing was described in other eukaryotes and was referred to as RNA interference (RNAi) (Katoch and Thakur 2013). Plants and insects have evolved to use RNAi as a protection against virus infection (Obbard et al. 2009). RNAi silences genes through a process in which double-stranded RNA (dsRNA), also referred to as hairpin RNA (hpRNA), is broken into small interfering RNAs (siRNAs) by a RNase III named dicer. The siRNAs then assemble into a RNA-induced silencing complex (RISC) that uses the siRNAs as guides for complementary mRNA degradation. Plants possess specific RNAi machinery to degrade the dsRNAs produced by viruses, and the siRNAs produced may act as guides over long distances through phloem transportation (Voinnet and Baulcombe 1997). The introduction of long dsRNAs or siRNAs has now been adopted as a potential tool for inactivating gene expression in living organisms.

The development of RNAi technology has allowed the investigation of gene function and regulation by knocking out the specific gene. Metabolic pathways controlling notable traits were uncovered through this type of biological engineering. In plants, RNAi is induced by introducing expression vectors that transcribe a self-complementary dsRNA (Horiguchi 2004). The transformation method is one of the factors that affect the efficacy of gene knockouts in plants. The *Agrobacterium*-mediated transformation is generally the preferred transformation technique. In plants that are incompatible with *Agrobacterium* infection, the direct introduction of RNAi vectors via particle bombardment and electroporation has also been used (Katoch and Thakur 2013). The use of RNAi technology to (i) improve plant traits offers a huge potential for agricultural development. RNAi technology has been used to improve traits, including those related to nutritional quality, content of food allergens and toxic compounds, and defense against biotic and abiotic stresses; (ii) induce morphological variations, male sterility, and secondary metabolite synthesis; (iii) produce seedless plant varieties (Saurabh et al. 2014; Tables 10.3); and (iv) create resistance to pathogens such as viruses, nematodes, insects, bacteria, and fungi (Table 10.4).

10.5.2 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs)

Each CRISPR locus consists of a series of short repeats that are separated by non-repetitive spacer sequences derived from foreign genetic elements (Fig. 10.1). This conserved repeat-spacer-repeat architecture was originally observed in the *E. coli* genome in 1987 (Ishino et al. 1987), but the function of these repeats remained enigmatic until 2005. The spacer sequences in CRISPR loci are often identical to sequences in bacteriophage (virus) genomes and plasmids (Bolotin et al. 2005).

CRISPRs and their associated genes (*Cas*) are essential components of cellular defense systems against infections by foreign nucleic acids and are widespread in bacteria and archaea. Similar to the RNAi pathways in eukaryotes, CRISPRs rely on small RNAs for the sequence-specific degradation of invading nucleic acids such as the nucleic acids of viruses (Sorek et al. 2013). CRISPR differs from DNA restriction enzymes in the sense that being RNA guided, CRISPR may be programmed to degrade any RNA or DNA substrate. The guided RNA nuclease activity of CRISPR is now being explored to engineer

Table 10.3 RNAi for crop plant improvement

Plant	Gene targeted	Trait	Benefit	References
Arabidopsis	ACR2 gene	Increased arsenic uptake by the plant	Phytoremediation of heavy metal soils	Dhankher et al. (2006)
Arabidopsis	MS1	Degeneration of the tapetum	Male sterility	Wilson et al. (2001)
Coffee bean	CaMxMt 1 gene	Reduced caffeine content in the seeds	Decaffeinated coffee	Ogita et al. (2003)
Cotton	ghSAD-1 and ghFAD2-1 genes	Increased stearic acid and oleic acid content of seed oil	Useful for cooking applications without the need for hydrogenation	Liu et al. (2002)
Cotton	δ-Cadinene synthase gene	Reduction in gossypol	Health benefits	Sunilkumar et al. (2006)
Maize	Gene for starch-branching enzyme	Increase in amylase content	Maize quality improvement	Chai et al. (2005)
Maize	Ms45	Male sterility in maize	Male sterile lines	Cigan et al. (2005)
Maize	Opaque-2	Reduced leucine levels	Higher lysine content	Segal et al. (2003)
Oilseed rape	BP1 gene	Reduced or absent petals	Improved photosynthesis	Byzova et al. (2004)
Onion	Lachrymatory factor synthase (LFS)	Reduced lachrymatory factor	Tearless onion	Eady et al. (2008)
Petunia	Chalcone synthase (CHS) genes	Anthocyanin biosynthesis	Improved market value	Hirner et al. (2001)
Petunia	TAZ1	Degeneration of the tapetum	Male sterility	Kapoor et al. (2002)
Rice	Lgc1	Low glutelin content	Health benefits	Kusaba et al. (2003)
Tobacco	CHI gene	Flower coloration	Ornamental plants	Nishihara et al. (2005)
Tomato	1-Aminocyclo propane-1-carboxylate oxidase	Longer shelf life associated with slower ripening	Reduced ethylene sensitivity	Xiong et al. (2005)
Tomato	DET1 gene	Increased carotenoid and flavonoid content	Nutraceuticals and health benefits	Davuluri et al. (2005)
Wheat	SBEIIa and SBEIIb	High amylose	Improved bowel functions	Regina et al. (2006)

Adapted from Katoch and Thakur (2013)

nucleic acids in various model systems, including plants (Fig. 10.2).

10.5.3 Zinc Finger Nucleases (ZFN) as Precision Tools for Genetic Engineering

Most endonucleases, such as restriction enzymes, recognize short sequences of four to eight base pairs and will therefore cut the genome many times. By contrast, zinc finger nucleases are synthetic endonucleases that have larger recognition sites. These nucleases are produced in two halves (ZFN-A and ZFN-B). Each half binds a target sequence of 12–18 bases and contains a monomer of the endonuclease Fok I. When the two halves are close enough for the Fok I domains to dimerize, the endonuclease becomes active and cuts the intervening sequence (Fig. 10.3).

The requirement for such extended binding sites focuses the activity and restricts the binding to a single site in a genome. In mammalian cells, a double-stranded break in DNA can be repaired

Table 10.4 RNAi for pest and disease resistance

Trait	Pest/disease	Target gene	Host	References
Insect resistance	*Helicoverpa armigera*	CYPAE14	Cotton	Mao et al. (2007)
	Corn rootworm	V-ATPase A	Maize	Baum et al. (2007)
Virus resistance	Rice dwarf virus (RDV)	PNS12	Rice	Shimizu et al. (2009)
	Bean golden mosaic virus (BGMV)	AC1 gene	Bean	Bonfim et al. (2007)
	BYDV (barley yellow dwarf virus)	BYDV-PAV	Barley	Wang et al. (2000)
Nematode resistance	*Meloidogyne incognita*	Splicing factor and integrase 16D10	Tobacco	Yadav et al. (2006)
	Meloidogyne	Tis11	*Arabidopsis*	Huang et al. (2006)
	Meloidogyne javanica		Tobacco	Fairbairn et al. (2007)
Bacteria resistance	*Xanthomonas citri* subsp. citri (Xcc)	PDS and CalS1	Lemon	Enrique et al. (2011)
Fungus resistance	*Phytophthora infestans*	SYR1	Potato	Eschen-Lippold et al. (2012)
	Phytophthora parasitica var. nicotianae	GST	Tobacco	Hernández et al. (2009)
	Blumeria graminis f. sp. *tritici*	MLO	Wheat	Riechen (2007)

Adapted from Saurabh et al. (2014)

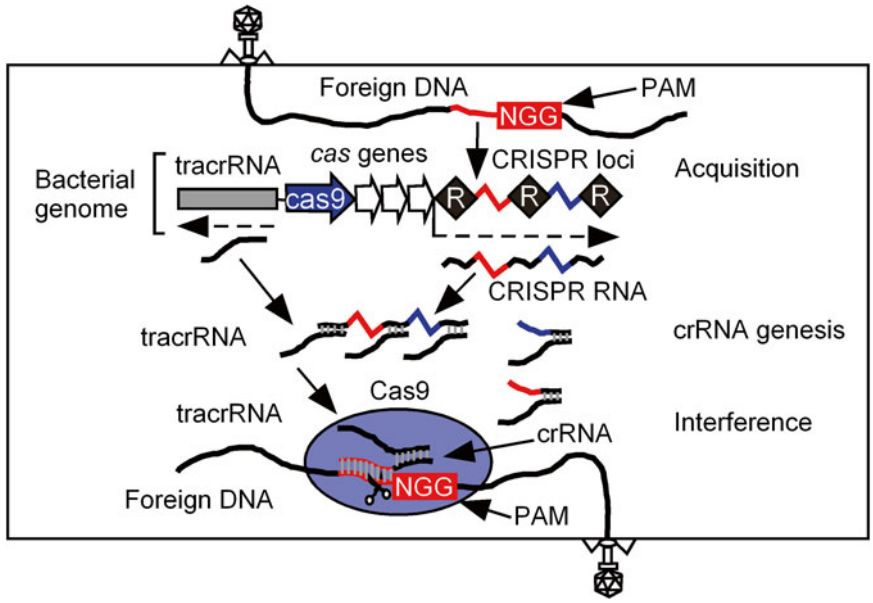

Fig. 10.1 CRISPR-Cas is a microbial adaptive defensive system that uses RNA-guided nucleases (Cas) to cleave foreign genetic elements. CRISPR system comprises a cluster of CRISPR-associated genes (*Cas*), noncoding RNAs, and a distinctive array of repetitive elements (direct repeats). These repeats (R, *black diamonds*) are interspaced by short variable sequences derived from exogenous DNA targets known as protospacers (*red* and *blue lines*), and together they constitute the CRISPR RNA (*crRNA*) array. Within the DNA target, each protospacer is always associated with a protospacer adjacent motif (*PAM*), which can vary depending on the specific CRISPR system. The CRISPR system II is composed of the nuclease Cas9, the crRNA array that encodes the guide RNAs, and a required auxiliary trans-activating crRNA (*tracrRNA*) that facilitates the processing of the crRNA array into discrete units. The exogenous DNA, typically from virus, is processed by proteins encoded by *Cas* genes into small elements (~30 base pairs in length), which are then inserted into the CRISPR locus of the bacterial genome. RNAs from CRISPR loci are constitutively expressed and are processed by tracrRNA and Cas proteins into crRNAs. Cas9 protein binds to the dsDNA/crRNA complex to cut the double DNA strands at the target motive (Adapted from Wilkinson and Wiedenheft (2014))

by nonhomologous end joining, leading to gene disruption, or by repair via a donor plasmid. Depending on the donor, this method can be used to correct mutations or to insert heterologous DNA. In recent years, ZFN technology has been used to create transgenic mammals, amphibians, fish, insects, and plants (Urnov et al. 2010).

10.5.4 Genome Engineering

Upon the double-stranded breakage induced by CRISPR or ZFN, a donor DNA identical to the wild-type sequence may be used to restore the DNA to the original sequence by homologous DNA repair (Ran et al. 2013). However, foreign DNA may also be used to introduce directed mutation such as single nucleotides (single nucleotide polymorphism – SNP), indels, or even whole gene replacement. The directed delivery of foreign DNA to specific locations in the genome driven by CRISPR RNA-guided nucleases (Fig. 10.2) or ZFN (Fig. 10.3) is being proposed as a gene therapy methodology to repair, modify, or replace defective genes.

CRISPR and ZFN allow the knockout of bi-alleles in plants or animals using a single-step process that is useful in hastening in vivo genetic studies in living systems (Chang et al. 2013; Hwang et al. 2013a, b; Nakayama et al. 2013; Li et al. 2013a, b). A notable feature of CRISPR and ZFN is the possibility of editing several genes in the identical genome or excising large genomic segments located between two different cleavage sites (Gratz et al. 2013). Multiplexing may be particularly useful for knocking out redundant genes or pathways (Wilkinson and Wiedenheft 2014).

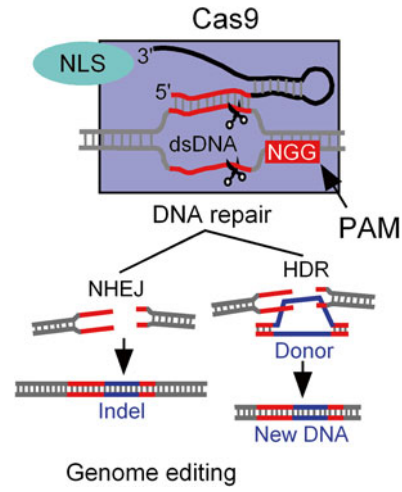

Fig. 10.2 Cas9 DNA targeting by PAM recognition and crRNA base pairing. The Cas9 nuclease recognize PAM and the pairing product of crRNA and target DNA. DNA-induced double-stranded breaks are repaired by nonhomologous end joining (*NHEJ*) or homology-directed repair (*HDR*). NHEJ is error-prone, resulting in insertions or deletions (indels) that disrupt the target site. HDR relies on a donor template that can be used to deliver foreign DNA at a specific location (Adapted from Wilkinson and Wiedenheft (2014))

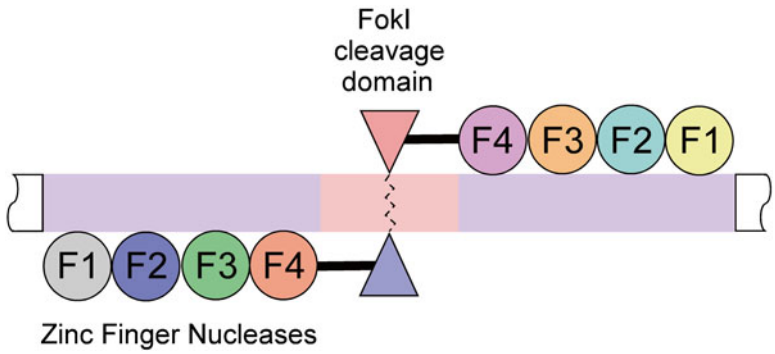

Fig. 10.3 Principle of DNA double break induction by ZFN. A dimer of four ZFs bounds to the target cleavage site. Each ZF recognizes 3 bp; thus, a chain of four ZF recognizes 12 bp. A combination of four ZFs is chosen to design a ZFN specific of each strand at the DNA motive to be broken. A spacer of 5–7 bp can be left between the break points on the + and − strands according to the application. Typically, the ZFN construct is brought together with a sequence to be inserted at the break point by homologous recombination

The simplicity in programming RNA-guided Cas9 nucleases has contributed to their fast implementation by the genome engineering community. Currently, a shared list of expression plasmids is available to the scientific community at Addgene.org (Wilkinson and Wiedenheft 2014).

10.6 Integration of Functional Genomics Datasets: Systems Biology

10.6.1 Metabolic Networks

Genome-scale metabolic network models have been built and applied to study a wide variety of topics, including adaptive evolution, network properties, and metabolic engineering (Feist and Palsson 2008). Genome-scale metabolic models in metabolic engineering have been used to couple growth rates with the overproduction of lactate (Fong et al. 2005) and L-Val (Park et al. 2011) in *Escherichia coli* and to prioritize alternative engineering strategies for overproducing ethanol in yeast (Bro et al. 2006). Although the majority of the work in metabolic network reconstruction and application has been performed in microbes, several advances in plant metabolic network reconstruction and modeling have been described recently. These advances pave the way for rational plant metabolic engineering.

Two types of plant metabolic networks have been produced: descriptive and predictive. Genome-wide descriptive metabolic networks have been obtained by referring to metabolic pathway databases that are being curated with experimental data from the literature (Caspi et al. 2012; Kanehisa et al. 2012). These genome-wide metabolic network representations are now available for several plant species, such as *Arabidopsis* (Zhang et al. 2010), *Populus trichocarpa* (Zhang et al. 2010), and grasses (Youens-Clark et al. 2011). Recently, the plant metabolic network group published online genome-wide predictive metabolic networks for maize, soybean (*Glycine max*), cassava (*Manihot esculenta*), and wine grape (*Vitis vinifera*) (www.plantcyc.org; Zhang et al. 2010). Most of these networks (except for *Arabidopsis*) are based on searches for homology with existing descriptive networks and are eventually not curated through extensive reference with experimental data from the literature; thus, caution should be used when interpreting these models. Descriptive and predictive networks were already used for a variety of studies, including the identification of novel genes in glucosinolate metabolism (Chan et al. 2011), the role of duplicated enzymes (Hanada et al. 2011), and metabolomic responses to UV-B (Kusano et al. 2011). Because they are descriptive, qualitative networks have been useful in many studies; however, they are not predictive and are therefore limited to the purpose of facilitating metabolic engineering.

The accumulation of a wide variety of different functional genomics datasets has resulted in the development of the field of systems biology (Kitano 2002; Hwang et al. 2005a). Systems biology attempts to decipher the complex behaviors exhibited by an organism by understanding how these behaviors are derived from the interactions of the underlying components. Although this is a broad statement, when viewed within the framework of functional genomics, this description can be reduced to the specific interactions that exist between proteins, proteins and metabolites, or proteins and DNA within cells. In essence, systems biology is a method for the interpretation of protein functions in the context of their interactions.

The view that functions can be encapsulated into objects of a genetic network represents a paradigm shift in the way we represent the genome of any living organism. The ability to quantify the inputs and outputs that exist for genes and proteins within and between objects represents a critical step in describing the function of a genome. Each genomic dataset represents one type of functional relationship that defines the objects and shapes the composition of genetic nodes in these networks. Systems biology strives to unify the contextual framework with their functional relationships through the integration of multiple algorithms and genomic datasets. The current generation of these algorithms

focuses on probabilistic integration, and the most recent algorithms have demonstrated the ability to recapitulate known pathways such as galactose utilization in yeast (Hwang et al. 2005b, c).

Because all functional genomics datasets may be used to predict functional interactions, the integration of these datasets is thought to provide a greater confidence in the individual predictions. In other words, an interaction that is predicted to occur between two genes in DNA microarray experiments combined with phylogenetic inference has a higher probability of being a significant interaction than if the interaction was predicted by only one of these methods. This postulate has gained wide acceptance within the systems biology community, and an ever-increasing number of new algorithms for the integration of functional genomic datasets are being reported (Joyce and Palsson 2006). Notably, the most profitable advances in the application of these algorithms are found in the integration of a large number of functional genomic datasets. Unsurprisingly, the majority of integrative algorithms are being reported in model organisms, such as yeast, mouse, fly, and Arabidopsis, in which a large number of functional genomic datasets are already available.

Several thousand expression microarray experiments performed on samples from *Arabidopsis* have been released to the public domain, and using these data to investigate metabolic networks in plants is now possible. With Web tools such as CressExpress (Srinivasasainagendra et al. 2008), users can identify clusters of genes that are co-expressed with one or more genes of interest. This method allows the identification of new members of metabolic pathways that are regulated at the level of mRNA abundance.

10.6.2 Functional Genomics as Genome Annotation

Functional genomics is an advanced form of genome annotation, i.e., the process of attributing biological definition to a DNA sequence. Annotation can be summarized as the application of two largely independent steps: the identification of a list of genetic elements on a genome (a process called gene finding or structural annotation) followed by the attachment of a biological definition to these elements (a process called functional annotation) (Zagursky and Russell 2001). Structural annotation is largely automated. Structural annotations are considered relatively stable. By contrast, functional annotations are dynamic and are subject to frequent changes as the body of experimental data grows or algorithms for gene predictions improve.

The functional annotation of a gene is traditionally assigned by one of two methods. In the first method, a novel annotation is produced through experimental investigations conducted on a specific gene, and the accumulated knowledge is assigned to that gene. The second method is annotation by homology, in which a functional annotation is assigned to one gene in a genome because the gene is homologous to a second gene that has been annotated in another organism. Unfortunately, annotation by homology will contribute to error propagation when the reference gene is not correctly annotated. The propagation of annotation error by homologous comparison is called genome rot (Galperin and Koonin 1998; Gerlt and Babbitt 2000). The annotation of genes through homology is routinely applied to newly sequenced genomes, and approximately 65 % of the predicted ORFs can be assigned with some annotation (Karaoz et al. 2004). The challenge is the assignment of a functional annotation to the remaining 35 %. The critical step is the confirmation of the annotation prediction obtained by high-throughput assignments. Thus, a long and tedious work of inference confirmation must be performed by experts in experimental biology to validate predictions.

The application of functional genomics to experimental research also results in the rapid accumulation of a large amount of data, and new methods are needed to manage and disseminate these data to the research community. As a result, data curation by experts is integral to the annotation process of any model organism's genome. Following the traditional curation model, a group of professional annotators attempt to synthesize

the vast amount of scientific knowledge for each model organism to annotate the genome (Stein 2001). Other types of curation processes are the party model, in which a community of scientists will meet periodically for the purposes of joint genome annotation, and the cottage industry model, in which experts within a field are recruited on a part-time basis to verify the consistency of the annotation.

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Translating the Genome for Translational Research: Proteomics in Agriculture

11

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Abstract

The increasing number of novel tools, methods and capacities to elucidate and understand the genome of various organisms is steadily leading the path for similar groundbreaking studies in downstream comprehension of the genomes. Platforms for analytical and functional transcriptomics have seen a similar surge in operational outputs. The cryptic complexity of the DNA in the genomes is manifested in various forms and functions of the RNA it codes for. Such complexity reaches much more elaborate intricacies in the translational protein products of the RNA. Although transcripts regulate a number of biological processes, proteins form the basic functional unit of most procedures that are evinced as a phenotype. Progressively increasing realization that there is limited correspondence between the level of a specific transcript and protein is only the beginning of appreciating that predictive biology would be less dependent on transcripts than on proteins. Principles of protein function that were explored much before the DNA and RNA paradigms are coming back to inform the omics world in the functional and regulatory capacity of proteins. Combined with the evolution of the high-throughput analytical capacity for proteins, these are clubbed into what is called proteomics, which is also riding the wave of computational and electronic revolutions in biology. Using such omic technologies for agriculture however lags behind compared to their use in medicine. Nevertheless, the recent appreciation of the need for food and feed security under the predicted climato-demographic scenarios is pushing the frontiers of agricultural research. The use of proteomics in particular is gaining ground rather quickly, and an increased understanding of crop phenotype as related to proteomics is

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being established. A general background of proteomics and its importance in agriculture is presented followed by a more detailed treatment of proteomics as applied to selected important crops in agriculture. A limited comparison is presented between plant and non-plant proteomics to highlight the gaps, and finally a perspective on the future utility of plant proteomics is presented.

Keywords
Proteomics • Crops • Agriculture • Food • Translational research

11.1 Introduction

Crops are the main source of human and animal food supply. Feeding the growing 7.1 billion world population, which is projected to increase to nine billion by the year 2040, is certainly of top priority for ensuring food security. Also, the use of arable land for commercial purposes and current climate change scenarios are also aggravating the food security issues. Though the challenge seems insurmountable, high-throughput platform technologies have had an evident impact in recent times in analysing and understanding biology at a speed never possible before. The chief example of such advances is the genome sequencing technologies, which have evolved at a rate outpacing Moore's law. Advances with computational biology for DNA sequence analysis have positively affected similar efforts in analysing other aspects of biology. This has indeed provided strong belief in the capability to meet the challenge. For the past 13 years, a rapid progress in the study of plant genomics is also evident. The completion of the genome sequencing projects of the model system such as *Arabidopsis thaliana* in 2000 (The *Arabidopsis* Genome Initiative 2000), followed by rice in 2002 (Goff et al. 2002; Yu et al. 2000; IRGSP 2005), poplar (Tuskan et al. 2006), castor (Lu et al. 2007), sorghum (Bedell et al. 2005), rape seed (Jaillon et al. 2007), soybean (Schmutz et al. 2010), tomato (http://solgenomics.net/organism/Solanum_lycopersicum/genome), maize (Wilson et al. 2009; Gore et al. 2009; Dolgin 2009), banana (http://banana-genome.cirad.fr/) and *Medicago truncatula* (http://www.medicago.org/) have paved the way for further intricate research using the advanced high-throughput *omic* technologies (Agrawal et al. 2011).

Much interest is now focussed on proteomics after steadily accumulating evidence of lack of a linear correlation between transcripts and functional protein expression levels, in an increasing number of genes (Hajduch et al. 2010). Such findings may be attributed to protein stability, in part due to the various post-translational modifications, certain signalling networks and protein-protein interactions. Earlier, genomics was considered as the primary source of information for functional biology. However with the advent of new technologies which allow for better understanding of different cellular activities, the protein complement is seen as highly relevant in predicting cellular/organismal function/phenotype. Differential modifications of the gene products occur due to various signals perceived by the cell. This in turn modulates the activity of genes per se. So what then is proteomics and what is its place in agriculture and food security?

11.2 What Is Proteomics?

Proteomics in a narrow sense is a high-throughput cataloguing of the repertoire of an entire set of proteins of a cell/tissue/organism at defined spatio-temporal points. It is based on a combination of several methods such as protein separation, mass spectrometry, genome sequencing/annotation and protein search algorithms (Thelen 2007). However, in a broader sense, proteomics is not only used to catalogue protein of a given biological material, but it is also utilized to monitor the reversible

PTMs such as phosphorylation, glycosylation, ubiquitination, acylation, prenylation, sulphurization, methylation, glycosylation, oxidation, nitrosylation, etc. A recent review takes account of the techniques for PTM analysis through proteomics (Remmerie et al. 2011). To date, more than 300 different types of post-translational modification have been characterized with the aid of proteomics. The rapid development of proteomics depends heavily on the dynamic advancement of mass spectrometry and bioinformatics as it elucidates protein function, domain structure, subcellular location, post-translational modifications, splice variants, quantities, biochemical structures and cellular functions of all proteins in an organism, organ or organelle across time and physiological state (Kenyon 2002, www.ncbi.nih.gov/pubmed/).

11.3 Historical Perspective of Proteomics

The vision of indexing human proteins started long before Human Genome Project was conceived. In the late 1970s, Norman G. Anderson and N. Leigh Anderson of the Department of Energy's Argonne National Laboratory developed the Human Protein Index. Its objective was to enumerate human proteins by separation on 2D gels (O'Farrel 1975), which was the only approach possible during those days. However, the effort was not sufficient because the technology on mass spectrometry and other suitable methods were not available. It was not until 1994 when an Australian PhD student, Marc Wilkins, coined the word proteomics while working on the concept of a protein complement. From then until now, the field of plant proteomics has flourished on different model systems and crops, to different levels of throughput, depending upon the crop or model-specific information and funding available. The extent of progress has been exceptional because the number of published research articles from a single paper in 1999 had reached to 3,806 in December 2013 using a single search term "plant proteome" in the PubMed database. Mostly studied are *Arabidopsis*, rice, maize, soybean and other emerging model crops.

11.4 Importance and Relevance

The field of proteomics is particularly important because most environmental and physiological responses of crops are manifested at the protein level. Consequently, proteomics seeks to directly correlate the involvement of specific proteins, protein complexes and their modification status to a given response.

Proteomics can singly serve as alternative to mRNA-based measurements because it can measure the actual protein levels, profile the expression dynamics of the cellular compartments and measure the large-scale protein modifications and their quantitative changes upon disturbance to the cell. Thus, it can determine the cellular response at the level of protein abundance and the downstream consequence in terms of gene expression changes (Cox and Mann 2007). In plants, proteomics was first used to annotate individual proteins to supplement genome and transcriptome information. However, today proteomics does not only characterize proteins but rather tries to understand the cellular response to environmental, and developmental change (Rakwal and Agrawal 2003).

11.5 Workflow of Proteomics

The process of proteomics can be carried out by first identifying a goal which could be to isolate, identify, characterize and/or measure the differential expression of a protein. This is followed by determining the tools for identification, characterization, analysis and validation. An attribute of a proteome study commences by extracting proteins from a sample followed by the classical method of 2D PAGE to separate proteins followed by high-performance liquid chromatography (HPLC) for amino acid analysis, peptide mass fingerprinting using mass spectrometry (matrix-assisted laser desorption/ionization, MALDI-MS, or electrospray ionization, ESI-MS) or sequence and sequence tags using post-source decay (PSD) for post-translational modifications as shown in Fig. 11.1.

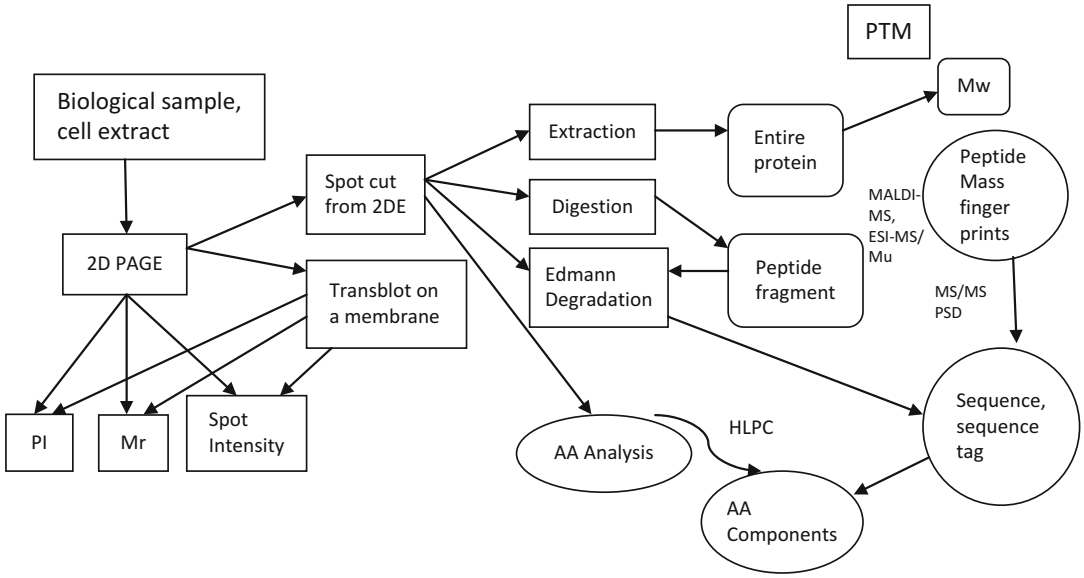

Fig. 11.1 Workflow of proteomics studies (Source: www.cn.mbnet.org)

11.6 Proteomic Avenues

The common approach towards a proteomic study was a focus-based approach that aimed to understand a specific protein from among the set of proteins found to be differentially expressed. However, with the swift progress in this field, a more global perspective now pervades proteomic studies. This now entails the need for a broader-based approach where the proteome as a whole is analysed and interpreted for its biological significance. The critical aspect in this strategy is to ask the correct biological question, choose the correct tissue at the correct stage in the life cycle and/or an environment of/for an organism and make use of the most advanced technologies. Table 11.1 shows the difference between the two approaches.

11.7 Frontiers and Methods Used in Crop Proteomics

After the first publication on proteomics in the year 1999 by Marc Wilkins, more than 300 papers were available at PubMed portal in the year 2009, covering various new techniques and discoveries on *Arabidopsis*, rice, maize, soybean and other major crops. After 4 years, about eight times (2,361 published papers) more publications are available on crop proteome studies.

Current crop proteomic methods in most labs still rely on gel and mass spectrometry (MS)-based methods. The gel-based method includes the classical strategy of conventional two-dimensional gel electrophoresis (2DE) and two-dimensional differential fluorescence-based gel electrophoresis followed by enzymatic digestion and identification of the proteins by mass spectrometric analysis. The improvements and results gained over time in proteomics research have shown that the behaviour and variability of proteins are more complex than had ever been imagined. Recent advances have led to gel-free technologies. The process now starts with direct digestion of the proteins of a complex mixture followed by protein identification also called as bottoms-up approach. A new top-down approach has also been used in some cases where the liquid chromatography is used directly on the complex mixture for the separation of the protein species followed by the identification of the protein species by mass spectrometry. The MS-based quantification methods consist of label-based and label-free

Table 11.1 Comparative description of broad- and focused-based approaches in proteomic study

	Broad-based approach	Focused-based approach
Basic steps	Identify an organism	Identify protein
	Identify sample type and preparation	Understand sample type and preparation
	Utilize a technology that is compatible with the same type	Isolate protein
	Bioinformatic analysis of the proteomic sample	Utilize analytical technology that is compatible
	Build a proteomic model	Bioinformatic analysis of the protein sample
		Model protein's function
Pros	Proteomic information about a specific tissue under certain condition can be gained	Inexpensive and results can be generated much quicker than a whole proteome study
	Relationships between many protein can be understood	Functional information about a protein can be determined
Cons	Extensive upfront planning	Protein information may not be valuable in a global proteomic perspective
	The study will cost more and last longer than a focused study	Hard to deduce any cross networks or other protein-protein interactions
	Proteomic data will be generated in the end	
Common technologies implemented	SDS-PAGE gel electrophoresis	SDS-PAGE gel electrophoresis
	2DE-DIGE	HLPC
	HLPC	Mass spectrometry
	Mass spectrometry	Molecular modelling tools (bioinformatics)
	Proteomic modelling tools (bioinformatics)	

protein quantification approaches (Nanjo et al. 2011) including MALDI-TOF (Sommerer et al. 2007), iTRAQ (isobaric tag for relative and absolute quantitation; Wiese et al. 2007), tandem mass tag approach (Thompson et al. 2003), MudPIT (multidimensional protein identification technology; Whitelegge 2002), SILAC (stable isotope labelling by amino acids in cell culture; Gruhler and Jensen 2008), ICAT (isotope-coded affinity tags; Lilley and Dupree 2005) and LOPIT (localization of organelle proteins by isotope tagging; Dunkley et al. 2004). Liquid chromatography-mass spectrometry (LC-MS) and label-free LC-MS are powerful tools that have facilitated the quantification and functional proteomic analyses of proteins. These tools and methods are used solely or in combination with other tools or with other "omics" techniques. However, no matter how much technological advancement improves or replaces the ones available, there will always be unique methods for each environmental stress the crops encounter because their utility depends on the objective of the researcher and each of these methods has their own advantages and disadvantages.

The readily available technologies are continuously improved for superior, more dependable results and afford deeper understanding on the phenomenon being studied. An example is the evaluation of 2D PAGE in rice caryopsis which demonstrated superior staining effect when silver staining protocol containing glacial acetic acid, sodium thiosulphate and Coomassie Brilliant Blue staining method was used. The other example was achieving higher resolution with IPG gel strip of pH 5–8 having an optimal loading of 130 μg and silver nitrate staining protocol (Liao and Huang 2011). Labelled LC-MS/MS has been used to establish cellular or subcellular proteome maps, characterize plant-pathogen interaction or stress response and profile protein patterns during developmental processes (Matros et al. 2010). The application of an automated approach for the derivatization of peptides for facilitated MS/MS de novo sequence determination in banana was reported, which

further concludes that the proteome does not always correspond to the presumed genome formula and that proteomics is a powerful tool to characterize varieties (Carpentier et al. 2010).

11.8 Crop Proteomics

11.8.1 Rice and Wheat

For more than a decade, rice has been the model crop plant for progress in establishing techniques for proteome studies of different tissues and organelles. The initial proteomic studies in rice progressed slowly. Research endeavours were mainly on constructing protein data files. Recently, dedicated groups have treaded the difficult path of systematically analysing the rice proteome at the cellular, tissue and whole-plant level (Rakwal and Agrawal 2009). From there on, research on rice tissues, organs and organelle under various abiotic stresses (drought, salt, oxidative, temperature, nutrient, hormone, metal ions, UV radiation and ozone) as well as various biotic stresses were performed. Dependable protein identification was facilitated by the combined power of MS technology and high-resolution 2D PAGE. The rice proteome was probed intensively through mapping PTMs, performing inter- and intraspecies comparisons, integrating proteomics with other "omics" technology-generated data and probing functional aspect of individual proteins (Agrawal et al. 2009).

Different abiotic stresses have been observed to yield different proteome responses in rice. Quantitative label-free shotgun proteomic analysis showed that 300 out of 850 proteins were found to be responsive to extreme temperatures and have unique expression either at 12 or 44 °C. In addition, there were novel response proteins that use an alternate sucrose pathway in response to extreme temperature (Gamulla et al. 2010, 2011). In terms of proteomic response of rice during salt stress, nano-LC-MS/MS and gel-based shotgun proteomic analysis revealed that ubiquitination of proteins in rice roots affected the protective mechanism of rice seedlings to resist salt stress (Liu et al. 2012b). For drought, label-free quantitative shotgun proteomics showed that there was a differential regulation of aquaporins. Similarly, proteins involved in signalling and transport showed differential regulation, suggesting a critical role in the overall response to drought (Mirzaei et al. 2012). These studies are just but few from among the many research highlights.

In durum wheat, heat stress was observed to upregulate proteins involved in glycolysis, carbohydrate metabolism and stress-related pathway. Laino et al. (2010) discovered that the wheat seed proteins comprised a subset of allergenic proteins. In response to flood stress, cell wall-associated proteins were differentially regulated. In addition, wheat seedlings respond to flooding stress by restricting the cell growth to avoid energy consumption (Kong et al. 2009). Drought stress elicits different defence mechanisms in wheat. Remobilization of carbohydrate from the wheat stem to the grain induces differential expression of proteins at 20 days after anthesis and upregulation of several oxidative stress-response proteins, which might suggest more effective ROS scavenging (Bazargani et al. 2011). Using 2DE and MALDI-TOF, drought stress-response proteins related to glycolysis and gluconeogenesis were identified (Caruso et al. 2009). Isobaric tags were used to follow changes in the relative protein abundance of Australian bread wheat that showed decrease in proteins involved in photosynthesis and Calvin cycle (Ford et al. 2011). The proteomic responses of wheat to winter and spring were different, as seen using 2D DIGE and MALDI-TOF/TOF. Proteins with higher abundance in the winter cultivar were observed to be involved in the regulation of stress response and development. Meanwhile in the spring cultivar, proteins involved in the restoration of cell division and plant growth were observed to be of higher abundance (Kosová et al. 2013). High temperature shortened the developmental programme of the endosperm of wheat without altering its sequential steps. Proteins active in biosynthesis and metabolism shifted to storage and protection against abiotic and biotic stresses (Hurkman et al. 2009, 2013).

Some important proteins of an elite Chinese cultivar known for having high yield, superior gluten quality and better biotic stress resistance, namely, H^+-ATPases, glutathione S-transferase, ferritin and triose phosphate isomerase, were found to be upregulated in the salt-tolerant lines (Gao et al. 2011). The differentially expressed proteins of wheat somatic seedlings were observed to be salinity/drought responsive but not cultivar specific (Peng et al. 2009). All these studies have been providing a base from where to build an integrative view of rice and wheat responses. Such an integrative view will, sooner rather than later, allow a cellular level understanding of response to developmental and environmental cues. That understanding in turn will allow identification of additional beneficial genes and alleles for crop improvement.

11.8.2 Soybean

Soybean is one of the most widely grown legumes in the world because it is an important source of vegetable oil and protein. However, it is also sensitive to abiotic stress. Some progress has been made through proteomics to understand how soybean can adapt to physiological and environmental stresses. One example of such proteomic studies showed that soybean roots had 32 proteins differentially abundant under drought (Mohammadi et al. 2011). Irrespective of the severity of the water stress, soybean exhibited tight control over carbon metabolism to meet the cells' energy demand for alleviating the effects of the stress (Hossain and Komatsu 2014). A comparative proteome analysis in soybean leaves against *P. sojae*, a soil oomycetes that causes root rot, showed that proteins involved in energy functions were the most abundant and the proteins involved in transport and transcription were the least abundant (Zhao et al. 2013). In response to salt stress, glyceraldehyde 3-phosphate dehydrogenase, glutathione S-transferase (GST9 and 10) and seed maturation protein PM36 were downregulated in the salt-tolerant soybean variety but were more abundant in the salt-sensitive variety (Xu et al. 2011a). A database has been created for soybean proteome data in response to stresses such as flooding, salinity and drought (Soybean Proteome Database; SPD; http://proteome.dc.affrc.go.jp/Soybean/). It is a repository for proteins from different organs, tissues and organelles as well as from plasma membrane, cell wall, chloroplast and mitochondrion. The data was obtained through 2D gel and non-gel methods (Ohyanagi et al. 2012). A recent review by Hossain and Komatsu (2014) highlights the achievements of proteomics in "untying the mechanism of flood and drought stress tolerance".

11.8.3 Maize

Recent advancement in proteomics study of maize has also considered various biotic and abiotic stresses. For the biotic stress of *Aspergillus flavus* pathogenesis, the proteomic analyses of young resistant rachis of maize were found to contain higher levels of abiotic stress-related proteins and proteins from phenylpropanoid metabolism, while susceptible ones contain pathogenesis-related proteins. When challenged with *A. flavus*, resistant rachis relied on constitutive defence systems, while susceptible rachis was more dependent on inducible defences (Pechanova et al. 2010). Proteome data combined with transcriptome and metabolome analysis for long-term nitrogen deficiency in maize at two developmental stages revealed major changes in photosynthesis and carbon metabolism. A number of these changes were similar to biotic and abiotic stress-mediated changes. However, such changes were different during the different developmental periods of N assimilation and grain filling. The integrated approach presented a useful set of results for improving the understanding of whole-plant nitrogen economics, potentially affecting breeding and agronomy decisions (Amiour et al. 2012). The effect of growing two transgenic lines in one location over three seasons against the control, as well as the effect of genotypes grown in different location in one growing season, showed great difference in the transcriptome, proteome and metabolome profiles of maize (Barros et al. 2010). Through

2DE and iTRAQ, the physiological responses to drought such as early stomatal closure of drought-sensitive genotypes were explained. Results showed that drought causes upregulation of protective and stress-related proteins (chaperones and dehydrins; Benesova et al. 2012). Maize proteomics has also been carried out to understand some other important aspect commercially more relevant to maize per se, such as heterosis (Marcon et al. 2013; Guo et al. 2013), starch biosynthesis and grain filling (Juarez-Garcia et al. 2013; Jin et al. 2013), C-4 photosynthesis (Zhao et al. 2013; Majeran et al. 2010), ear growth (Liao et al. 2012) and GM/non-GM comparison (Balsamo et al. 2011). A recent review captures the role of proteomics in providing an insight into the biology of maize (Pechanova et al. 2013).

11.8.4 Oilseed Crop Proteomics

Plant seeds are an important source of renewable of food feed and energy, such as seed oil. In oilseeds quantitative profiling of proteins expressed during seed filling in soybean, canola, castor and *Arabidopsis* has been studied through high-resolution 2D PAGE and MS approach. More than 500 proteins have been identified using MS with an aim to identify genes or gene families of allergens and silence their expression so that they have minimal effect on human and animal health (Thelen 2009). A recent technique used in studying seed allergens requires no gel or sample fractionation. Isotopically labelled synthetic peptides, called AQUA peptides, serve as synthetic mimics to proteotypic peptides and are used as internal standards at set concentrations. This approach allowed absolute quantification of proteins in complex biological mixtures (Stevenson et al. 2010).

Proteomic analysis of *Brassica napus* helped in revealing the dynamics of N remobilization through the action of several proteases that occur at the different stages of leaf senescence induced by N limitation/starvation (Desclos et al. 2009). Another comparative proteomic study in oilseed rape shed light on proteins involved in lateral root formation and maintenance of low phosphorus in the tolerant genotypes (Yao et al. 2011). Proteomics also provided important new insights into the resistance mechanisms within *B. napus* against *S. sclerotiorum* and also opened the way for novel engineering of new *B. napus* varieties with enhanced resistance and better management against the most serious disease of the oilseed rape (Garg et al. 2013). More recently, a combined approach of proteomics with genomics was used to gain insights into the genetic programmes that operate during embryo development in *B. napus L.* (canola). This study identified 20,183 genes and 2,607 proteins that are differentially expressed across different stages of embryo development and which share high degree of homology with Arabidopsis and display stage-specific and shared expression patterns associated with developmental and metabolic pathways (Venglat et al. 2013). Apart from oilseed rape, proteomics has also been used to study other oilseed crops. A nano-LC-MS/MS approach was used to study drought stress in the sunflower inbred lines. The productivity of sunflower, one of the most important oilseed crops, is severely affected by drought stress. Proteomics data indicated that changes in energy usage, water transport and ROS scavenging are important mechanisms for maintaining root growth as the soil dries (Ghaffari et al. 2013). Proteome analysis of *Jatropha curcas*, an important biodiesel crop, was performed on the seed oil bodies, and important differences are outlined in its oleosins (Popluechai et al. 2011). Oil body proteome determination methods were recently reviewed (Jolivet et al. 2013). *Jatropha curcas* seed oil, which can be utilized for biodiesel production upon transesterification (Kohli et al. 2009), is rich in phorbol esters in most genotypes (Popluechai et al. 2009). Phorbol esters are very toxic and are known to cause wide range of negative biological effects including cancer. An EASY nano-LC system coupled inline to an ESI-LTQ-Orbitrap Velos mass spectrometer was used to identify and study 1,103 proteins. This study led to a very important conclusion that suggested the plastids isolated from the endosperm of developing seeds do not synthesize phorbol esters (Pinheiro et al. 2013). The role of proteomics in

J. curcas was also reviewed (Raorane et al. 2013). Such studies provided insights into the major biosynthetic pathways for seed oil synthesis and certain unique features of the metabolic pathways at the whole proteome level (Hajduch et al. 2011).

11.8.5 Roots and Tubers Proteomics

Roots and tuber crops consist of beets, carrots, potatoes, sweet potatoes, cassava, yam, etc. Among these root crops, only potato and cassava are mostly studied using proteomics. Protein profiling in transgenic potato rich in protein suggested that proteome rebalancing might cause increased protein content in tubers (Chakraborty et al. 2010). In cassava, 2D PAGE and nano-electrospray quadrupole TOF tandem mass analysis identified 299 proteins, which were involved in processes related to energy, primary and secondary metabolism, disease and defence, destination and storage, transport, signal transduction, protein synthesis, cell structure and transcription to cell growth and division (Sheffeld et al. 2006). Lim and co-workers reported the first comprehensive proteomics analysis of the indirect mode of action of phosphite-based fungicides (Phi), demonstrating broad effects on plant defence and plant metabolism. The proteomics data and the microscopy study suggested that Phi triggered a hypersensitive response that was responsible for induced resistance of potato leaves against *Phytophthora infestans* (Lim et al. 2013). Tuber development in potato consists of a series of biochemical and morphological processes at the stolon tip. Proteomics was used to study development-related processes and to gain further insights into the proteins that show altered expression during in vitro potato tuberization (Yu et al. 2012). Furthermore, cassava has been fortified for iron and protein by reducing the cyanogen levels in the roots (Guerrero et al. 2012). Another root crop, Ginseng's root proteome analysis was done using the combination of internal amino acid sequencing using ESI QTOF MS (identified 91 proteins) and the expressed sequence tag (EST) database from *Panax ginseng* and the protein database of model plants such as *Arabidopsis thaliana* and *Oryza sativa* due to incomplete ginseng genome data (Kim et al. 2003).

11.8.6 Vegetable Crop Proteomics

Fruits and vegetables are extremely susceptible to decay and easily lose their commercial value within short periods of time after harvest. These post-harvest problems are due to various stresses such as attack of pathogens, heat treatment, cold storage and resistance mechanisms (Cook et al. 1999; Tian et al. 2001). Induced resistance can be caused by microbial, chemical and physical agents or natural compounds (Tian and Chan 2004). Proteomics and metabolomics revealed the molecular mechanisms of how fruits and vegetables respond to environmental stresses. However, extraction of the protein in fruits poses a challenge because of low protein content in the fruit tissues. Two successful extraction protocols in profiling fruit proteome were proposed, dubbed as the Homo and Phe methods (Chan et al. 2007, 2008). These were found to be better than the TCA-acetone method in profiling the protein of fruits. Another emerging technique is the multiple reactions monitoring (MRM) that was used to detect and quantify protein biomarkers in complex biological samples of loquat fruit (Marquez et al. 2013).

The unripe green and the ripe red tomatoes along with a ripening-inhibited (*rin*) mutant tomato were infected with *Botrytis cinerea* to assess postharvest reactions to the fungus at the proteome level. Both the fruit and fungal proteome were analysed 3 days postinfection (Shah et al. 2012). There were 186 proteins common among WT red ripe and *rin* red ripe-equivalent infected tomatoes. These proteins would thus be responsive to infection and not related to developmental aspects of ripening. However, the limited infection of the WT mature green fruit by *B. cinerea* resulted in 25 and 33 % fewer defence-related tomato proteins than in red and *rin* fruit, respectively. In contrast, the ripening stage of the infected fruit did not affect the secreted proteomes of *B. cinerea*. The composition of the set of proteins and their putative functions further argued for a role in plant-pathogen interactions.

2D DIGE approach revealed 52 differentially expressed proteins in peach fruits exposed to heat treatment. These proteins were proposed to play a role in plant metabolism, defence and stress response, cytoskeleton organization, primary metabolism, transcription and translation regulation and protein storage and catabolism. Additionally, through this study the small HSP family proteins in the heated peach were hypothesized to participate in the acquisition of tolerance against some chilling injury symptoms (Lara et al. 2009). Another study demonstrated that heat-treated peach fruit showed induction of ascorbate peroxidase, heat-shock proteins and an allergen protein (Zhang et al. 2011). Proteomic and metabolic profiling of mandarin citrus fruit using 2D PAGE (2-DE), gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) depicted that reactive oxygen species (ROS) and lignin play important roles in heat treatment induced fruit resistance to pathogens and physiological disorders (Yun et al. 2013). In citrus fruit, 28 differentially expressed proteins showed that the main affected categories were cell rescue, defence, virulence and metabolism. The activity of antioxidant enzymes was extensively changed upon heat treatment, including superoxide dismutases and alcohol dehydrogenases (Perotti et al. 2011).

Comparative transcriptomic and proteomic analysis of citrus fruit (Hirado Buntan pomelo) using digital gene expression profiling and 2DE revealed that calcineurin B-like protein (CBL), an interacting protein kinase complex, might be involved in signal transduction of low temperature stress. Meanwhile, fruit quality is likely regulated by sugar-mediated auxin and abscisic acid signalling. The accumulation of limonin, nomilin, methanol and aldehyde, together with the upregulated heat-shock proteins, COR15, and cold response-related genes have provided a comprehensive molecular view on the coordination of low-temperature stress responses in the fruits (Yun et al. 2012). 2DE and MS technology was used to identify the gene *Ptcorp*, which was induced by cold stress in citrus orange, which made it a well-known cold-tolerant fruit (Long et al. 2012).

In the fruit-ripening studies for papaya, six main categories such as cell wall synthesis, ethylene biosynthesis, climacteric respiratory burst, stress response, synthesis of carotenoid precursors and chromoplast differentiation were shown to be related to fruit ripening using 2DE-DIGE (Nogueira et al. 2012). Traditional 2D PAGE showed several cell wall-degrading enzymes also related to fruit ripening in papaya (Huerta-Ocampo et al. 2012). Another study on peach fruit highlighted the proteins involved in primary metabolism, ethylene biosynthesis secondary metabolism and responses to stress (Prinsi et al. 2011). A further study characterized the protein accumulation patterns in soft fruits of three peach and two nectarine varieties of melting flesh. This revealed that 164 of the 621 protein spots analysed displayed a differential accumulation associated with the softening process. Among them, only 14 proteins changed their accumulation in all the varieties assessed, including proteins mostly involved in carbohydrates and cell wall metabolism as well as fruit senescence (Nilo et al. 2012). In kiwifruit, proteomic analysis using 1D-SDS-PAGE and mass spectrometry identified 102 proteins during ripening. These were mainly involved in energy, protein metabolism, defence and cell structure (Minas et al. 2012).

11.8.7 Fibre Crop Proteomics

Almost all textiles are derived from cotton fibres and other natural fibres such as hemp, silk and wool. Like other crops, these plants are also subjected to physiological and environmental stresses, which is why proteomic studies were undertaken to understand adaptive mechanisms to abiotic and biotic stresses. An example of such studies is the comparative proteomic analysis of cotton, which revealed that certain proteins had putative functions for fibre differentiation and that ROS homeostasis may be the central regulatory mechanism for cotton fibre morphogenesis through post-transcriptional and post-translational regulation (Kang et al. 2012). Proteomic analysis of cotton at three developmental stages (5, 10 and 15 days post-anthesis) showed that 37 protein

spots changed significantly under low temperature stress. These proteins were involved in malate metabolism, cell wall loosening, cellulose synthesis, cytoskeleton, cellular response and redox homeostasis (Zheng et al. 2011). A global protein analysis using 2DE and tandem MS on flax seeds grown in an irradiated area identified 318 seed storage proteins, and out of it, 82 proteins were identified to be involved in central metabolism (Klubicoba et al. 2010). Proteins in hempseed which were suspected to have an important role in development, germination and storage were profiled using 2DE and LC-MS/MS. Results showed that 168 unique proteins are involved in regulation, metabolism, stress response, cytoskeleton, binding function and protein synthesis (Park et al. 2012). Recently, a proteomic approach with MALDI-TOF/TOF MS was used to study the proteome of 21 days post-anthesis green cotton fibre to understand the molecular mechanisms related to pigment biosynthesis (Li et al. 2013). Another similar comparative study with brown and white cotton, at different days post-anthesis, illustrated several aspects of pigment synthesis and fibre development (Li et al. 2013). Such studies demonstrated the presence of a complicated metabolic network and advanced the understanding of the molecular mechanisms of pigment biosynthesis in coloured cotton, which may provide new insights into the development of new cotton colour types by genetic manipulation.

11.8.8 Forage Crop Proteomics

The improvement of forage crops translates into the improvement of livestock. These crops are mainly used in food supply for grazing livestock such as cattle and sheep. Few examples of these include members of the Poaceae family, such as *Lolium*, *Poa*, *Cynodon* and *Bromus*. Herbaceous legumes such as *Medicago* are also included in forage crops. Sorghum and maize are also included in forage crops in some countries, through their use as silage for livestock feeds. Being used as fodder, these crops are of importance and their improvement adds to the food security issue through healthy livestock. The focus on studies with forage crops centres on the improvement of stress tolerance in different climates. A study on the effects of cold and frost on the proteome of *Lolium perenne* showed differences in the leaf proteomes of high and low frost tolerance (Bociana et al. 2011). Forty-two differentially abundant proteins were identified of which 35 were found to be chloroplast proteins, suggesting resistance to photo-inhibition and survival during cold acclimatization through continued food manufacture. Cold acclimatization was shown to be effective in increasing freezing tolerance in velvet bentgrass (*Agrostis canina*) by Espevig et al. (2012). Allowing the plants to experience a more tolerable cold climate enhanced their cold tolerance and increased survival under more stringent cold conditions. Additionally, when the proteomes of these acclimatized plants were observed, proteins involved in amino acid metabolism, energy production, stress defence and secondary metabolism were differentially abundant. Xu et al. (2011b) also studied heat-shock proteins (HSPs) and their relation to heat tolerance in grasses, focusing on HSP role in heat tolerance, allowing essential cellular processes such as amino acid and protein metabolism, photosynthesis and carbon allocation to continue, thus allowing the survival of the plant.

11.9 Plant Proteomics Update: 2009–2013

Proteomics is a relatively young science considering the term per se was only coined by Marc Wilkins in 1994. However, in almost two decades, tremendous headway has been made both in plants and animals. There has been appreciable progress in plant proteomics because of the following reasons: (1) the completion of the genomic sequence of model plants such as *Arabidopsis* and rice, (2) the swift dynamic growth of mass spectrometry having provided immense opportunities for high-throughput proteomic studies that have gone from simple protein identification to analysing functional proteomics, (3) database construction and mining through bioinformatics to deal with large and complex set of proteome data and (4) the innovative and diverse ways in which the methodologies in protein and peptide

analysis have been applied. This has led to modern proteomics which required a multidisciplinary expertise from various fields such as software engineering, statistics, protein chemistry, cell biology, biophysics and mass spectrometry (Have et al. 2011).

The updates in plant proteomics made in the year 2004–2006 (Rossignol et al. 2006) surveyed about 200 original articles in plant proteomics. By the time the next review on the plant proteomics was published in the year 2008 (Jorrín-Novo et al. 2009), 50 more papers had been published. However, by December 2013 there were 3,806 plant proteomics research articles. Rapid advancement in and availability of MS instrumentation tools and techniques, and databases mining software, has made it possible to identify thousands of proteins within a single day (Cox and Mann 2007).

In terms of plant species being studied, thale cress (*Arabidopsis thaliana*) and rice (*Oryza sativa*) are still the most popular models. However, by 2009 an advent into studying the proteome of other major crops such as maize, soybeans, sorghum, citrus fruits, cotton, grasses, oilseeds, etc., had already commenced. Most of the published papers have focused on profiling organs, tissues, subcellular proteomes, developmental processes and responses to biotic and abiotic stresses using differential expression strategy (Jorrín-Novo et al. 2009). If the strength of proteomics studies in the past was more on targeted and focused analyses of proteomes, nowadays, a shift on the global functional approaches is starting to roll on. Many studies have engaged in the subcellular localizations of protein, molecular interaction networks and large signalling complexes such as PTMs with the aim of a full understanding of the functions of protein in relation to biological functions.

The technological platform whereon plant proteomics is based depends still on the classical 2DE for protein separation and enhanced with differential staining via DIGE. The use of gel-free and second-generation quantitative proteomics techniques [i.e. shotgun proteomics (MudPiT, ICAT, ITRAQ, LC-MS/MS, MALDI-TOF, etc.)] to quantify and analyse differential abundance of protein has increased. In addition, proteomic data is also verified and enhanced using other omics complements such as transcriptomics, metabolomics, ionomics, etc. Moreover, proteomics nowadays is pushing the boundaries of computation, database generation, curation and mining, with advanced statistics for true positives to handle large and complex data sets efficiently.

Advanced and sophisticated proteomic studies have been undertaken in rice and *Arabidopsis* due to the availability of data on most of the subcellular localizations of proteins in different tissues. Other crops, particularly citrus fruits and oilseed, manifest rapid progress as well. One major highlight in fruit proteomics is the discovery of two new successful protein extraction methods which for some time had been a problem (Marquez et al. 2013). Another highlight is the novel discovery of a software tool in oilseed which can now access large-scale predictions of both general and specific kinase and phosphorylation sites (Gao et al. 2010). However, the progress made in roots, tubers, forage and condiment type of crops is slow. Efforts have been put to initiate and successfully accomplish complete genome sequencing of other species such as *M. truncatula* and *Brachypodium distachyon*. With even more pace gathering in the coming years on proteome characterization, a wider understanding on the biological processes in plants may be rapidly achieved.

11.10 Gaps in General Proteomics Versus Plant Proteomics

Plant proteomics in general is lagging as compared to mammalian and microbial proteomics. Others have described plant proteomics as in its infancy (Agrawal et al. 2006; Annan, http://www.thecatalyst.org/proteomics/). Such disparity may be attributed to several factors. Firstly the microarray facilities for mammalian and microbes have already been well established and accessible for postgenomic activities such as the proteomics and other "omics" technology as compared to plants, where there is lack of access in most plant laboratories. Another is the lack of information, of close cooperation among proteomic researchers and most importantly of suitable platform to globally discuss updates, successes and challenges

encountered in plant proteomics (Agrawal et al. 2009). The organization that is supposed to serve as globally recognized platform with user-friendly and centralized data base called the International Plant Proteomics Organization (INPPO) is new. The 1st INPPO World Congress was held in Germany in 2014 and brought together leading experts in plant proteomics from all over the world. This should provide an ideal venue for researchers in proteomics technologies and applications on all aspects of plant proteomics to share and discuss their new findings and ideas. On the contrary, the Human Proteome Organization (HUPO) organized its 13th Annual World Congress in Madrid in October 2014. It possesses an established database for a single species (human) and encourages the spread of proteomics technologies. Plant proteomics will have a long mile to tread before the proteome databases of important crops are gathered from among other nations to facilitate exchange of resources and information from among plant proteomic researchers. This scenario would be somewhat like an insurmountable challenge due to variations in individual plants (genus and species) being so vast. Moreover, the influence and role of industries who serve as sponsors or users of the technology play an important role. This is evident by the fact that research effort on disease treatment, drug discovery and medical diagnostics and forensics are more appealing to private sectors and/or investors who exploit much of the results of various proteomic initiatives (Kenyon 2002). On the contrary the issue of food security is a public or government thrust with limited support from the private sector. Nonetheless, the impact of plant proteomics will be substantial if proteome databases can be brought to bear upon agriculture per se (Agrawal et al. 2009).

11.11 Pitfalls, Challenges and Future Perspectives

Proteomics techniques have unique strengths but also have several challenges and considerable limitations. Proteomics is very much a technology-driven field. The enormous range of exciting new technology for separation and quantification of proteins has been applied to various intriguing biological queries. However, the stringency to validate proteomic data generation and analysis has somewhat been lenient, and as a result, numerous published findings with questionable quality and reproducibility are now available (Wilkins 2006). The challenge now is to establish standardized protocols and regulations to validate methods, statistical designs, computations and results before publications are made. At present, there is no common gel matrix that would enable researchers to reproducibly align protein patterns. No matter how much 2DE has been improved, the technology is still tedious and difficult. It did not develop and mature with the same breathtaking speed as that of the other downstream MS technologies (Huber 2003). Nevertheless the enthusiasm to perform proteomic studies has no signs of slowing down; that is why voluminous proteomic data are swamped incredibly fast in a database which mainly contains abundant and common proteins, whereas the rare regulatory proteins of low abundance (e.g. GTPases, kinases and phosphatases) or "difficult" proteins remained dawdling. As a challenge, technological improvements are considered and are needed to enhance quantitative and comparative large-scale proteomic studies (Zhang and Riechers 2008). The proteome is in a state of flux and is thus extremely complex. The analysis of the proteome is exacerbated by the huge dynamic concentration range of proteins in the cellular environment. The challenge to map plant proteomes is quite formidable due to the myriad post-translational modifications of proteins in each subcellular location and tissue. The future development of more high-throughput tools for fractionation and suitable MS/MS approach, the discovery of more biomarkers and the link with "omics" and bioinformatics will surely make a difference (http://www.thecatalyst.org/proteomics/). Furthermore, if the collection of proteome data becomes systematic, it can help build a baseline information or databank that can be accessed globally and can be utilized in finding patterns and trends across multiple experiments. The sophisticated, highly sensitive throughput technologies in plant proteomics need to shed light in the multifaceted, complex

nature of global proteins. This will provide a clear, fast dimensional analytics, as well as a clear understanding of how a plant biological system works (Have et al. 2011). This may sound a wishful thinking at present, but were not the sequencing of human genome, *Arabidopsis* and the like, once in this formidable situation?

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Epigenetic Mechanisms in Plants: An Overview

12

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Abstract

Plant epigenetics has become one of the hottest topics of research not only as a subject of basic research but also as a possible new source of beneficial traits for plant breeding. In addition, epigenetic mechanisms are also crucial to appropriate plant reactions to stress. Given the sessile lifestyle and the late differentiation of the germ line, plants can perceive stresses during vegetative growth and also memorize them, possibly by epigenetic mechanisms. Plants use three systems to initiate and regulate epigenetic gene regulation, like other higher organisms, which include DNA methylation, histone modifications, and RNA interference. New concepts are being evolved to show how these epigenetic components interact and stabilize each other. The role of epigenetic mechanisms in hybrid vigor and epigenetic transgene silencing is also being explored. In this chapter, we have tried to highlight the epigenetic mechanisms that play key roles in plants.

Keywords

Epigenetics • DNA methylation • Histone modification • RNA interference • Transgene

12.1 Introduction

The term epigenetics was first introduced by Conrad Waddington (1942) which is referred to as the intermediate factor between the genotype and phenotype. The redefined term "epigenetics"

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includes all mitotically and meiotically heritable changes in gene expression which are not coded in the DNA sequence itself (Tsaftaris et al. 2005). Epigenetics now refers to the heritable changes which are not related to the DNA sequence but can be reliably inherited from one generation to another (Chen et al. 2010). However, this definition is still under debate among epigeneticists and biologists (Bird 2007). Over the last decade, epigenetic studies have focused mainly on mammals, and plants have received much less attention. At present, the epigenetic research is at forefront of plant biology and molecular genetics. Plants are excellent models for studying epigenetics since epigenetics has been suggested to play a role in both plant development and epigenetic response. There is a fair amount of epigenetic information on certain model plants such as *Arabidopsis*, *Oryza sativa*, and *Zea mays*. Several mechanisms such as DNA methylation, histone modifications, siRNAs, paramutations, nucleosome arrangements, and others have been identified. Various enzymes and homologous factors participating in the regulation of plant epigenetics have been identified. Depending upon analytical resolution and precise data interpretation, epigenetic code of the DNA sequence in plants might be determined in the near future. The three main hallmarks of the process of epigenetics in plants as well as in higher organisms are DNA methylation, histone modification, and RNA interference. These three molecular epigenetic mechanisms, their effects on plant development, and particularly their multiple implications have been discussed in this chapter.

12.2 DNA Methylation

DNA methylation was the established epigenetic mark to be studied. Methylation is a heritable enzymatic modification which results from the addition of a methyl group in the cyclic carbon-5 of cytosine. In plants, DNA methylation is species, tissue, organelle, and age specific and is controlled by phytohormones and changes such as seed germination and flowering (Vanyushin 2005). It is also influenced by various pathogens (viral, bacterial, fungal). In mammals, it is restricted to symmetrical CG sequences (Bird 2002). However, in plants, DNA methylation is found at CG sites, CHG sites (H indicates A, C, or T), and CHH sites (asymmetrical site). DNA methylation is catalyzed by a family of conserved enzymes, DNA methyltransferases (MTases). Different types of DNA MTases include (a) maintenance methylases, which maintain stable cytosine methylation patterns through successive cell generations; (b) de novo methylases, which are able to transfer methyl groups to cytosines of unmethylated DNA; and (c) domain-rearranged methylases (DRMs) that are directed from short RNAs and specifically methylate homologous genes in a process termed as RNA-directed DNA methylation (RdDM).

Arabidopsis has been found to possess ten different members of MTases, and mutations of each have been reported to affect normal development or other features of the plant (Tariq and Paszkowski 2004). Methylation can be removed from DNA by either passive or active mechanisms. Replacement of methylated cytosine with non-modified cytosine results in passive demethylation. The participation of specific proteins that demethylate DNA sequences cause active demethylation. Passive demethylation can occur when methylated cytosines are replaced with non-modified cytosines during DNA replication (Vairapandi and Duker 1993; Jost et al. 1995; Tsaftaris and Polidoros 2000). Two *Arabidopsis* mutations, one interfering with maternal expression of an imprinted gene (Choi et al. 2002) and the other inducing transcriptional gene silencing (TGS) of transgenes and endogenous homologous loci (Gong et al. 2002), were mapped to the genes DEMETER (DME1) and ROS1, respectively, which encode DNA glycosylases that remove 5mC from DNA. The prominent roles proposed for methylation are (i) to provide a heritable epigenetic mark directing the developmental program of the organism (Holliday and Pugh 1975; Regev et al. 1998; Wolffe and Matzke 1999), (ii) to provide genome defense against the activity of parasitic mobile elements (Yoder et al. 1997), (iii) to reduce background transcriptional

noise in organisms possessing a large number of genes (Bird 1995), and (iv) to "memorize" pattern activity of the gene by stabilizing gene silencing brought about by other mechanisms (Bird 2002).

DNA methylation has been found to exert its effects through repression of gene expression. Methylation inhibitor studies have been reported to induce developmental abnormalities in several plant species including *Oryza sativa*, *Triticale*, *Linum usitatissimum*, and *Nicotiana tabacum* (Richards 1997). A clear correlation between methylation status and gene activity has been demonstrated by numerous studies including tissue-specific genes, using different techniques. In fact, abundant evidence supporting a role of methylation in suppression of gene expression comes from studies on regulation of transposable elements (Fedoroff 1996; Martienssen 1996) and transgene silencing in genetically engineered plants (Morel et al. 2000; Paszkowski and Whitham 2001; Fojtova et al. 2003; Matzke et al. 2004).

12.2.1 DNA Methylation and Stress Response

Studies have shown that under stress, DNA methylation and RNA interference (RNAi) rapidly and reversibly modify plant genomic DNA, which further avoids the need for excessive genetic recombination and population diversity (Goll and Bestor 2005; Lukens and Zhan 2007; Boyko and Kovalchuk 2008). The plant somatic cells can "memorize" the experience of stress by epigenetic mechanisms (Bruce et al. 2007). Studies have shown that methylation of GC-rich sequences acts as a major coordinating point for many epigenetic mechanisms and ensures that the "memory" is transferred faithfully to the progeny (Mathieu et al. 2007). A large number of the genes modified by methylation possess a close relationship with the stress response in plants. It has been reported that the silencing of type I DNA methyltransferase NtMET1 in tobacco (*Nicotiana tabacum*) plant using an antisense RNA technique resulted in the upregulated expression of approximately 30 genes, 62.5 % of which are related to responses to biotic or abiotic stress (Wada et al. 2004). Another example is of rice (*Oryza sativa*) variety Wase Aikoku 3, which is not resistant to the blight pathogen *Xanthomonas oryzae* pv. *oryzae* in the seedling stage but develops resistance in the adult plant. After artificial inoculation of seedlings and adult plants, methylation-sensitive amplified polymorphism (MSAP) analysis showed that most polymorphic loci possessed a higher level of methylation in adult plants than in seedlings (Sha et al. 2005). This may contribute to the development of adult plant resistance (APR) in rice plants. One of the demethylating reagent 5-azadeoxycytidine can also induce resistance to the blight pathogen in Line-2 rice (Akimoto et al. 2007). The MSAP technique was used to screen DNA fragments that were methylated differently between Line-2 and wild-type rice, and one clone, Xa21G (similar to the rice blight resistance gene Xa21), was selected. The promoter of Xa21G was not methylated at any cytosine residue in Line-2 rice but was fully methylated in the wild type, which was consistent with the high expression of Xa21G in Line-2 (Akimoto et al. 2007). Therefore, demethylation of the Xa21G promoter enhances the expression of this gene and contributes to the resistance of the plants to pathogen. It was suggested that as a rule, rice blight pathogen resistance was related to genomic hypermethylation and hypomethylation of resistance-related genes (Akimoto et al. 2007). This rule is explained in more detail via TMV-infected tobacco. After infection with the virus, an overall increase in genomic methylation was accompanied by the hypomethylation of resistance-related leucine-rich repeat (LRR)-containing loci, and this was found to be consistent with the increased amount of recombination observed within the LLR-containing resistance gene (N-gene) (Kovalchuk et al. 2003; Boyko et al. 2007). It was found that the overall increase in methylation might favor genomic stability when the plant is attacked by the virus, whereas reduced methylation of the resistance genes may accelerate genetic recombination and thus result in the development of novel resistance genes (Engler et al. 1993). Therefore, both phenomena

are crucial for plant resistance. TMV resistance can be transferred to the progeny, and blight pathogen resistance and the related methylation status are inherited for at least nine generations (Boyko et al. 2007; Akimoto et al. 2007). Classical Mendelian genetics cannot explain the inheritance of stress-derived phenotypic variations, which reminds us of the "acquired" genetics theory that was proposed by Lamarck two centuries ago.

12.2.2 Metal Ions and Plant Genomic DNA Methylation

High levels of methylation protect DNA against endonuclease cutting and multi-copy transposition favoring resistance to heavy metals. For example, cadmium (CdCl2) has been reported to change the methylation status of both oilseed rape and radish (*Raphanus sativus* L.) DNA (Yang et al. 2007; Filek et al. 2008). MSAP ratio of radish genomic DNA increased in proportion to the intensity of the stress (Yang et al. 2007). The methylation level of rape genomic DNA has been suggested to be altered by chromium. Using MSAP and immuno-labeling techniques, the analysis revealed that the level of hypermethylation in the rape genome was correlated positively with the stress dosage of chromium, which suggested de novo synthesis of methylated cytosine (Labra et al. 2004). However, in some reports, a contrary trend was observed. For instance, nickel, cadmium, and chromium stress can reduce cytosine methylation levels in clover and hemp by 20–40 %, and the decrease is proportional to the stress dosage of the heavy-metal ions (Aina et al. 2004). Chromium stress can therefore promote methylation in rape but induces a decrease in methylation in clover and hemp, suggesting that different methylation mechanisms for heavy-metal resistance exist in different plant species (Labra et al. 2004). MSAP has revealed that the methylation patterns of different individual plants within CCGG sites were similar, before and after heavy-metal stress, which indicates that the changes in methylation that were induced by stress were not distributed randomly (Aina et al. 2004).

Except for a few genes such as NtGPDL, we still know very little about methylation modification at specific genomic loci under heavy-metal stress. Bisulfite mapping has revealed that CG sites in the NtGPDL coding region were demethylated rapidly in detached leaves, within 1 h, after aluminum stress. However, the methylation pattern of the promoter did not change. NtGPDL transcripts appeared within 6 h which is in agreement with the theory that DNA methylation levels are inversely related to the levels of gene expression (Choi and Sano 2007). The study also shows that the regulation of gene expression under stress is not limited to the promoter region, but can be achieved by changing the methylation status of the coding region (body methylation) as well. Zhang et al. (2006) also reported that genes that are methylated in their coding and promoter regions are generally expressed at high and low levels, respectively (Zhang et al. 2006). In addition to aluminum, both salt and low temperature can induce demethylation of NtGPDL leading to upregulation of its expression (Choi and Sano 2007). Furthermore, both aluminum and herbicides cause overproduction of reactive oxygen species (ROS), suggesting that oxidative damage as a result of stress might cause variations in DNA methylation patterns (Cerda and Weitzman 1997; Boscolo et al. 2003; Apel and Hirt 2004). Cadmium stress activates superoxide dismutase and catalase, altering the methylation status of genomic DNA. Selenium prevents the change in methylation levels and reduces the amount of ROS, suggesting that this is one of the causes of the change in methylation status (Filek et al. 2008). It is interesting that pathogens do not affect the levels of NtGPDL methylation and expression (Choi and Sano 2007). Hence, it is apparent that DNA methylation is affected differently by biotic and abiotic stresses.

12.2.3 Epigenetics and Plant Genetic Engineering

Silencing of the introduced transgenes in plants constitutes a major commercial risk which hampers the general economic exploitation of

transgenic plants. The transgenes are silenced at both transcriptional and translational level (Matzke and Matzke 2004). Transcriptional gene silencing is achieved by methylation of transgene promoter, whereas the posttranscriptional gene silencing is associated with the methylation of the coding sequence. There is a lot of commercial interest to avoid the epigenetically imposed transgene silencing. Of course, the most efficient way to avoid the silencing of transgenes is to carefully construct and analyze the transformants thoroughly at the molecular level. Studies of silenced transgenes and of silenced endogenous sequences have shown that repeated sequence arrays, particularly inverted repeats, are most prone to silencing. Selection of transgenic plants with single copy transgene insertions is the first line of defense against silencing. Even with this safeguard, the transgene might still be silenced if it inserts near heterochromatin or if it expresses an RNA trigger for silencing. But with an increased understanding of epigenetic patterning across the plant genome, and the identification of novel gene products that control gene silencing, the tools are now in place to manipulate more effectively plant transgene expression with the goal of improving agriculture.

12.2.4 DNA Methylation and Somaclonal Variation

Somaclonal variation is generally attributed to tissue culture-induced heritable changes manifested as cytological abnormalities, frequent qualitative and quantitative phenotypic mutations, sequence change, and gene activation and silencing. Quiescent transposable elements and retrotransposons may also activate, indicating that epigenetic changes occur through the culture process. It has been observed that DNA methylation patterns are highly variable among regenerated plants and their progeny. DNA modifications are less stable in culture than in seed-grown plants.

A somatic cell or small group of cells, bearing a unique genetic constitution or epigenetic modification, may reproduce preferentially in culture. Any plant eventually regenerated from these cells would probably be labeled as a variant acquired during the culture period. No doubt in reality it comprised a minuscule, possibly even undetectable, proportion of the cells in the original explant (Matzke and Matzke 1995). As it is becoming more apparent that somatically acquired epigenetic modifications in plants can be mitotically stable and meiotically heritable, more emphasis is given to variation in DNA methylation as a source of somaclonal variation. The first such evidence came from studies of regenerants from crown-gall tumor lines where changes in thymidine methylation as well as from demethylation were found to be associated with phenotypic variation (John and Amasino 1989), showing reactivation of silent Ac elements following tissue culture (Brettell and Dennis 1991; Peschke et al. 1991). Neuhuber et al. (1994) demonstrated that variation in antibiotic resistance markers in regenerated double transformants was due to the presence of the transgene complex in the original line as an epigenetic mosaic resulting from partial methylation.

Sun et al. (2013) studied the phenotypic variation in pollen viability of regenerants of *Torenia* after subculturing for one to nine generations (Sun et al. 2013). They found that pollen viability of regenerants continuously decreased with increasing subculture time. High concentrations of plant growth regulators applied to the Murashige and Skoog (MS) medium also resulted in diminished pollen viability. Furthermore, antibiotic application during gene transformation also decreased pollen viability of the transformants. However, the process of long-term culture did not significantly change pollen viability. The mean methylation level of regenerants showed a 0.28–3.95 % decrease in seedlings subcultured in vitro for nine generations. Moreover, when the ninth subcultured regenerants with reduced pollen viability were recovered in soil to get seeds, the pollen viability of seed-derived plants was similar to that of the wild type. They concluded that plant growth regulators, antibiotics, and the number of subculture generations influence somaclonal variations in *Torenia* sp., and these variations in *Torenia* might have resulted from epigenetic changes.

12.2.5 DNA Methylation, Heterosis, and Hybrid Breeding

Heterosis refers to the increased vigor of crosses between inbreds, species, or distantly related variants within a species in comparison with the parents. Although heterosis, or hybrid vigor, has been recognized for well over a century, its molecular basis is not been understood clearly (Birchler et al. 2010). However, recent progress has pointed that epigenetics has a role to play in heterosis (Groszmann et al. 2011). Shen et al. (2012) have provided valuable information about the underpinnings of heterosis by comparing global DNA methylation, small RNA (sRNA) expression, and gene expression patterns in hybrids in comparison with their parents.

Variability in gene expression can be assessed through polymorphism of individual RNA amount (RAP) as well as the polymorphism of individual protein amount (PAP). Significant correlations between PAP indices and hybrid vigor for agronomic traits have been found (Leonardi et al. 1988, 1991). Studies have shown that genes controlling protein amounts and enzyme activities, and particularly those with multiple effects, directly affect the expression of hybrid vigor. Examining the deviation in quantity of each RNA of every one of the two hybrids at the same mRNA in the two parental hybrids at the same developmental stage, the heterotic hybrid was found to have significant number of genes expressed over the quantity of the better parent at three stages. The work by Song and Messing (2003) provides evidence for altered regulatory effects in hybrids. DNA methylation could be considered as a genome-wide general regulatory mechanism that affects the expression of many genes playing an important role in the manifestation of heterosis (Tsaftaris and Kafka 1998). Tsaftaris and Polidoros (1993) found a significant negative correlation between genome activity and total DNA methylation. Studies have also been carried out to monitor the pattern of DNA methylation in maize parental inbreds and their hybrids using different techniques. The results indicated that (1) hybrids in general are less methylated than their parental inbreds; (2) heterotic hybrids are less methylated than related non-heterotic hybrids; and (3) old, low-yielding inbreds are highly methylated. Shen et al. (2012) established reciprocal crosses between *Arabidopsis thaliana* C24 and *Landsberg erecta* ecotypes. The F1 hybrids exhibited hybrid vigor, with increased growth and more siliques than either parent. Then, methyl *Cseq* was used to sequence the entire cytosine methylome of both parents and the reciprocal hybrids. It was found that both hybrids had increased cytosine methylation compared with the parents. In addition, the growth of both hybrids was more sensitive to a chemical inhibitor of methylation in comparison with the parents, supporting a role for DNA methylation in growth vigor.

Does methylation have a role in a hybrid's genomic DNA, especially in tolerating stress, leading to more stable yield? Studies have shown that F1 hybrids are in general less methylated than their parental inbreds. The possible role of methylation in the expression of maize genes and performance of hybrids under different growth conditions has been examined in experiments with maize inbreds and hybrids grown under different plant densities (Tsaftaris and Kafka 1998; Tani et al. 2005). It has been concluded that the genotype, the developmental stage, and conditions of growth do affect the methylation status of genomic DNA. Stressful growth conditions result in more methylated DNA (less expressed), and in general, vigorous hybrids are more resistant to such density-induced methylation and suppression of genome activity in their genomic DNA. The resistance of the hybrid genome to methylation understress and, consequently, avoidance of suppression of many of its genes could be the reason underlying the high F1 yield and, more importantly, F_1 stable yield. Shen et al. (2012) examined the transcriptomes of the parents as well as hybrids. They found that more genes were downregulated in the hybrids than were upregulated. Integration of the DNA methylome, the sRNAome, and the transcriptome data supports the idea that increased methylation of the circadian clock genes CIRCADIAN CLOCK ASSOCIATED1 and LATE ELONGATED HYPOCOTYL, which

have been reported to be involved in heterosis, leads to their downregulated expression in the hybrids. Further, the authors found DNA methylation and repression of flavonoid biosynthesis genes, with concomitantly increased auxin transport and signaling activity in the hybrids (Shen et al. 2012).

12.3 Histone Modifications

Histone modifications have emerged as vital epigenetic modifiers that regulate the genetic information encoded in DNA. The basic unit of chromatin, the nucleosome, consists of 147 bp of DNA wrapped nearly twice around the octamer, containing two copies of each of core histones H2A, H2B, H3, and H4. Each core histone within the nucleosome contains a globular domain which mediates histone-histone interactions and also bears a highly dynamic amino acid terminal tail around 20–35 residues in length and is rich in basic amino acids. These tails extend from the surface of the nucleosome. Histone H2A has an additional ~37 amino acid carboxy-terminal domain protruding from the nucleosome. All histone proteins are modified inside the nucleus of the cell, but so far only few modifications have been studied. Nature uses several modifications in proteins to complement and expand its chemical repertoire. In fact, more than 200 distinct posttranslational modifications (PTMs) have been identified to date, and the number and variety of modifications continue to grow. Histones are subject to enormous number of posttranslational modifications including acetylation and methylation of lysines (k) and arginines (R), phosphorylation of serine (S) and threonines (T), ubiquitination, sumoylation, and biotinylation of lysines as well as ADP ribosylation.

Generally, the acetylation of histones marks active, transcriptionally competent regions, whereas hypoacetylated histones are seen in transcriptionally inactive euchromatic or heterochromatic regions. Histone methylation can be a marker for both active and inactive regions of chromatin. Two important targets for epigenetic modifications in plants are histone H3 lysine 9 (H3K9) and H3 lysine 27 (H3K27). H3K9 methylation has been associated with heterochromatin formation or gene repression, while H3K27 methylation profile is related to gene expression required for developmental decisions (Zhou 2009). Methylation of lysine 4 of histone H3 (H3K4) leads to activity and has been found predominantly at promoters of active genes (Lachner et al. 2003). Methylation H3K9 marks silent DNA and is found throughout heterochromatic regions including centromeres and telomeres. Lysine methylation can be monomeric, dimeric, or trimeric. These observations have led to the idea of a "histone code," although the degree of specificity of these codes may vary as particular combinations of histone marks do not always dictate the same biological function (Strahl and Allis 2000). Important links between histone modifications on one hand and DNA methylation on the other have been found in H3K9 methylation, a prerequisite for DNA methylation (Tamaru and Selker 2001; Jackson et al. 2002; Malagnac et al. 2002), while DNA methylation can also trigger H3K9 methylation (Johnson et al. 2002; Soppe et al. 2002; Lehnertz et al. 2003; Tariq et al. 2003). The histone deacetylases (HDACs), histone methyltransferases, and methylcytosine-binding proteins (Nan et al. 1998; Fuks et al. 2003) have been reported to lead to the recruitment of DNA methyltransferases (Fuks et al. 2000), although it is not yet clear what initiates the recruitment of the different epigenetic modifiers to their specific target sequences.

Multimeric protein complexes have been reported to function as "cellular memory keys" that "lock" expression of genes and enable their inheritance over many cell division cycles both mitotic and meiotic (Narlikar et al. 2002). The multiprotein complexes involved in the regulation of developmental stages have been identified and studied in *Drosophila*. Two such main complexes, the polycomb (PcG) and the trithorax groups (trxG), were originally described in *Drosophila* (Mahmoudi and Verrijzer 2001). Both of these are known for their role in controlling the expression of homeotic genes but with opposite effect: Polycomb group maintains them in an inactive state, whereas trithorax group

maintains them in active state. Similar multiprotein complexes have recently been identified to execute conserved functions in plants. The PcG protein complex is the most studied in plants. This complex is involved in the control of the expression of homeotic genes that are essential transcriptional factors for the proper development of the plants . Although there is no structural relevance between the PcG target genes in *Drosophila* and *Arabidopsis*, their functions are similar in terms of controlling the developmental processes. A main component of the PcG complex in plants is, again, methyltransferases like the MEDEA protein of *Arabidopsis* that specifically methylates H3K27 leading to a silent state of chromatin (Reyes and Grossniklaus 2003). The regulatory mechanism of histone H3K9 and H3K27 modification provides important clues to unravel the molecular basis of establishment, inheritance, and erasure of epigenetic information regarding plant cell division and differentiation.

Chromatin modifications and remodeling in plants regulate abiotic stress responses (Luo et al. 2012). Histone modifications may also have a decisive function in regulating plant responses to abiotic stresses. Hypoxia in rice seedlings leads to the acetylation of histone H3 and to the conversion of dimethyl H3K4 to trimethyl H3K4 in stress-responsive genes ADH1 and PDC1 (Tsuji et al. 2006). These modifications associated with active transcription were reversible after the removal of the stress. Tobacco and *Arabidopsis* cells have been observed to show dynamic changes in histone modifications in response to high salinity and cold stress, manifested by transient upregulation of H3 phosphoacetylation and histone H4 acetylation (Sokol et al. 2007). Enrichment of histone acetylation at H3K23 and H3K27 has been reported to occur in response to drought stress on the coding regions of drought stress-responsive genes, RD29B, RD20, and At2g20880 (Kim et al. 2008). Histone H3K4 methylation patterns in response to dehydration stress in *Arabidopsis* has also been observed (van Dijk et al. 2010). Abscisic acid (ABA) and salt stress induce histone H3K9K14 acetylation and H3K4 trimethylation but decrease H3K9 dimethylation of some ABA and abiotic stress-responsive genes. This suggests that functionally related gene groups are regulated coordinately through histone modifications in response to abiotic stress in plant cells. All these studies show that epigenetic modifications of chromatin are implicated in plant stress responses.

12.4 RNA Interference (RNAi)

The discovery that double-stranded RNAs (dsRNAs) can robustly silence genes in *Caenorhabditis elegans* and plants, RNA interference (RNAi) has become a new paradigm for understanding the regulation of gene expression. RNAi is a gene downregulation mechanism in which small RNAs (of about 21–24 nucleotides) function to direct specific effector proteins (members of the Argonaute protein family) to a target nucleotide sequence by complementary base pairing. Then, the effector protein complex downregulates the expression of the targeted RNA or DNA. Small RNA-directed gene regulation systems have been discovered and named in plants, fungi, worms, flies, and mammalian cells (Lindbo 2012). Depending on the protein composition of the effector complex and the nature of the target sequence, the outcome can be either degradation of mRNA, translational repression leading to PTGS (posttranscriptional gene silencing) and mRNA alternative splicing, or genome modification leading to TGS, all of which result in silencing the gene expression. In most of the organisms, it has been observed that nuclear RNAi predominantly operates at heterochromatic loci, where it facilitates sequence-specific silencing through the direction of histone H3K9 methylation and/or cytosine methylation. However, differences are found in small RNA biogenesis, particularly in the subcellular localization of small RNA processing and loading of Argonaute proteins, and this could represent alternative approaches to regulating nuclear RNAi. As far as the cotranscriptional model is concerned, it is mechanistically unclear as to how the RNAi complexes regulate transcriptional machinery.

Outside constitutive heterochromatin, RNAi co-transcriptionally regulates some genes, and research is being carried out to determine whether this is a widespread phenomenon across organisms.

TGS was observed when two different transgene complexes were introduced in sequential steps into the tobacco genome (Matzke and Matzke 2004). Each complex encoded different proteins but had identical gene regulatory regions. The first transgene complex, which was stably active on its own, often became silenced in the presence of the second. The silenced transgenes acquired the most common genome modification associated with silencing, the DNA methylation. The silencing and methylation were abolished when the transgene complexes segregated from each other in progeny (Matzke et al. 1989; Park et al. 1996). As far as PTGS is concerned, it was discovered in two ways. One involved experiments to evaluate antisense suppression, a promising approach at the time for selectively silencing plant gene expression. Antisense RNA encoded by a transgene should base pair to the complementary mRNA of a plant gene, preventing its translation into protein. Although the control "sense" transgene RNAs are unable to base pair to mRNA and therefore should not induce silencing, they often inexplicably did (Smith et al. 1990). In another experiment, efforts to enhance floral coloration in petunia by overexpressing a transgene encoding a protein involved in pigment synthesis led to partial or complete loss of color. This resulted from coordinate silencing ("co-suppression") of both the transgene and the homologous plant gene (Napoli et al. 1990; Van der Krol et al. 1990) which was later shown to occur at the posttranscriptional level (de Carvalho et al. 1992; van Blokland et al. 1994). PTGS has also been associated with DNA methylation of transgene sequences (Ingelbrecht et al. 1994). RNA-mediated gene silencing pathways have been reported to play essential roles in plant development, chromosome structure, and virus resistance.

RNA has found its application in biotechnology, especially in the engineering of food plants which produce lower levels of natural plant toxins. RNAi has been used for the reduction of the cyanogenic natural product linamarin in cassava plants (Siritunga and Sayre 2003). Another example is of cotton seeds which are rich in dietary protein but naturally contain the toxic terpenoid product gossypol which is known to make them unsuitable for human consumption. Cotton stocks have been produced using RNAi with seeds containing lower levels of delta-cadinene synthase, a key enzyme in gossypol production, without affecting the enzyme's production in other parts of the plant, where it is important in preventing damage from pests (Kumar et al. 2006). The levels of allergens in tomato plants have successfully been reduced, and the precursors decreased of likely carcinogens in tobacco plants (Le et al. 2006; Gavilano et al. 2006). Other engineered plant traits include the production of non-narcotic natural products by the opium poppy resistance to common plant viruses and fortification of plants such as tomatoes with dietary antioxidants (Allen et al. 2004; Zadeh and Foster 2004). RNAi/PTGS provides protection from viral infections, prevents transposon mobilization, and regulates endogenous genes. No doubt the errors are on; no plant products using RNAi-based genetic engineering have yet passed the experimental stage.

12.5 Genomic Imprinting

Genomic imprinting is an epigenetic mechanism that produces functional differences between the paternal and maternal genomes and plays an essential role in the development and growth of organisms. There are approximately 156 genes in human genome that are subject to genomic imprinting where one parent copy of the gene is expressed while the other is silent (Luedi et al. 2007). There is a rapid and possibly active genome-wide demethylation in both male and female primordial germ cells resulting in the erasure of imprints (Mann 2001). The same mechanism also erases any aberrant epigenetic modification, preventing the inheritance of epimutations (Tam et al. 1994). The precise mechanisms responsible for epigenetic erasure and demethylation in primordial germ cells are not yet clear. The initiation of new

imprints occurs subsequently during gametogenesis and primarily during oogenesis (Murphy and Jirtle 2003). Imprinting is controlled by methylation of stretches of regulatory DNA, the imprinting control regions (ICRs) (Feil and Berger 2007). Interestingly, in order to get ready for the establishment of imprints, methyl marks on histones need to be removed (Ciccone et al. 2009).

It has been evolved in flowering plants and mammals (Feil and Berger 2007; Meaney and Ferguson-Smith 2010) . In case of flowering plants, a double fertilization event in reproduction takes place in which one sperm fertilizes the egg cell to form an embryo and a second sperm fuse with the central cell to give rise to endosperm. It is the endosperm tissue, where imprinting occurs in plants (Gehring et al. 2009). The first plant gene shown to be imprinted in endosperm was A SET domain polycomb group of genes MEDEA (MEA) . Its maternal expression is controlled by DNA methylation and demethylation. Recently, there has been significant progress in identifying imprinted genes as well as understanding molecular mechanisms of imprinting in plants. Up to date, approximately 350 genes have been reported to have differential parent-of-origin expression in the plant endosperm (*Arabidopsis*, *Zea*, and *Oryza*). In *Arabidopsis*, many imprinted genes are regulated by the DNA *METHYLTRANSFERASE1* (MET1) and the DNA-demethylating glycosylase *DEMETER* (DME) and/or their chromatin states regulated by polycomb group proteins (PRC2). There have been also some maternally expressed genes regulated by unknown mechanisms in the endosperm.

Many researchers are currently interested in manipulating the development of endosperm through the epigenetic mechanisms that underlie the parental imprinting. Studies in *Arabidopsis* have shown that removal of the parental imprinting leads to over proliferation of the endosperm, which is a desirable trait for seed crops (Berger 2003). Exploring the epigenetic mechanisms of seed development will eventually reveal the mysteries behind apomixis in which the fertile plant progeny is produced without double fertilization (Koltunow and Grossniklaus 2003). If this mechanism can be applied to commercial crops, hybrids can be regenerated indefinitely, therefore overcoming the current limitations of plant breeding to maintain hybrid vigor for more than one single generation.

12.6 Epigenetic Inheritance and Plant Breeding

Phenotypic variation is directed by an interaction between genetic and environmental variation. However, the line between these two components is currently blurred by inherited epigenetic variation, which is quite sensitive to environmental inputs. Careful study of epigenetic plant phenotypes directed plant geneticists to recognize the role of epigenetic inheritance systems in plant evolution (Kalisz and Purugganan 2004), as well as domestication and breeding (Tsaftaris and Polidoros 2000). The significance of epigenetic inheritance in plants has been gradually recognized over the past 50 years, and the researchers have found that the epigenetic phenotypes can result from (1) activation, excision, and translocation of transposon elements (McClintock 1951); (2) allelic interactions known as paramutations (Brink 1956); (3) transgene silencing in plants (concurrently observed from several groups working with transgenic plants in the late 1980s (Matzke and Matzke 2004); and (4) epialleles of endogenous plant genes that control floral induction and morphogenesis, seed development, and parental imprinting (Jacobsen and Meyerowitz 1997; Luff et al. 1999; Melquist et al. 1999).

Epigenetic states in plants can be inherited through the transmission of epigenetic alleles (epialleles) over many generations (Kakutani 2002). These heritable epigenetic alleles can be considered as a new source of variation that may produce novel phenotypes. One of the examples of the stability and heritability of alleles and epigenetic variation is found in the morphological variant of toad flax *Linaria vulgaris* described 250 years ago by Linnaeus. This variant has played such a significant role in the history of botany. But ironically, it turned out to

be neither a new species (as Linnaeus thought) nor a mutation (as de Vries and others thought) but a fairly stable epimutation due to an alteration in DNA methylation of the Lcyc gene. What caused the methylation change in the first place is unclear, but once formed, it seems to have been transmitted, more or less steadily (although there is residual instability), for many generations . Such epigenetic variants might have significant implications in plant breeding because the selection and breeding in these cases are based on heritable phenotypic variation within populations.

12.7 Conclusions and Future Perspectives

As it is evident from the above discussion that epigenetics has a lot of role to play in plant biology, however, this has not been fully appreciated. In contrast to the ease with which the DNA sequence can be analyzed, studying the complex patterns inherent in epigenetics poses many difficulties. Greater knowledge of patterns of epigenetic variation is expected to be highly informative in taxonomy and systematics, as well as population biology and conservation.

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Bioinformatics: Application to Genomics

13

S. Parthasarathy

Abstract

In this chapter, the basic concepts of bioinformatics and some of the important tasks in biological sequence and structure analysis are described. In biological sequence analysis, (i) pairwise and multiple sequence alignments, (ii) database search against a query sequence and (iii) phylogenetic analysis are fundamental tasks. Similarly, in the structure analysis of biological molecules, the most important tasks are (i) visualisation of 3D structures, (ii) prediction of three-dimensional structures of proteins, (iii) docking and (iv) molecular dynamics simulation. Further, the recent developments in bioinformatics, namely, 'omes' and 'omics' concepts along with their integration in systems biology, which are emerging areas in the postgenomic era, are also briefly described. Finally, some of the important applications of bioinformatics in various areas like agricultural, pharmaceutical and medical sciences are provided.

Keywords

Bioinformatics • Database • Multiple sequence alignment • Genomics • Functional genomics • Transcriptomics • Proteomics • Systems biology

13.1 Introduction

During the past two decades or so, biological data explosion has occurred, and the accumulation of biological data has got accelerated at a faster rate. In the field of molecular biology, high-throughput deoxyribonucleic acid (DNA) sequencing methods generate the genome sequence data of different organisms continuously in an exponential rate resulting in biological sequence databases of unimaginable sizes. These sequence databases along with the structure databases play an important role in life sciences, as they are needed for understanding more about the biological functions of an organism. They also have a lot of potential applications in biotechnology, agriculture and pharmaceutical industries. These

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developments really triggered off a new field in biological sciences, namely, bioinformatics.

Bioinformatics is an offshoot of biotechnology, and it is the application of computational methods to analyse biological data to obtain useful information (Lesk 2005; Baxevanis and Ouellette 2005; Mount 2003; Pevsner 2009). There are several bioinformatics tasks and applications on sequence and structural analysis of biological data. In biological sequence analysis (Durbin et al. 1998; Gromiha 2010), (i) pairwise and multiple sequence alignments, (ii) database search against a query sequence and (iii) phylogenetic analysis are fundamental tasks. Similarly, in structure analysis of biological sequences (Higgins and Taylor 2000; Orengo et al. 2003), the most important tasks are (i) visualisation of 3D structures, (ii) prediction of three-dimensional structures of proteins, (iii) docking and (iv) molecular dynamics simulation. There are many popular and standard tools available for the above-mentioned biological sequence and structural analysis.

In the postgenomic era, the high-throughput studies like 'omes' and 'omics' concepts along with their integration in systems biology have emerged as the recent developments in bioinformatics. For example, the complete DNA content of an organism is called the genome, and the study of genomes becomes genomics. Similarly, there are many 'omes' such as transcriptome, proteome and metabolome, and their corresponding 'omics' studies are known as transcriptomics, proteomics and metabolomics, respectively. These high-throughput studies are possible because of the technological advancements that provide high-precision equipments for making the biotechnological measurements and analysis leading to the large amount of data of 'omes' and their analysis 'omics'. The integration of the entire high-throughput 'omes' and 'omics' data provides a system-oriented approach to understand biological systems, which forms the subject matter of the new field called 'systems biology'.

Bioinformatics has a lot of potential applications in various fields including agricultural, pharmaceutical, clinical and medical sciences. It has been used directly/indirectly for computer-aided drug/inhibitor designing, genetically modified plants and animals, gene therapy, molecular evolution and so on.

In this chapter, we describe about the basic concepts of bioinformatics and some of the important tasks in biological sequence and structural analysis. Further, we describe briefly about the recent developments in bioinformatics, especially the high-throughput 'omes' and 'omics' concepts along with their integration in systems biology, which are emerging in the postgenomic era. Finally, we provide a few important applications of bioinformatics.

13.2 Bioinformatics: Definition and Basic Concepts

It is interesting to note that Krishnamurthy (2003) in his book entitled *An Advanced Textbook on Biodiversity* has already provided a general definition to informatics. Krishnamurthy had quoted the following three lines from the poem entitled 'Choruses from the rock' written by the famous poet T.S. Eliot in 1934, which reads as

> Where is the Life we have lost in living?
> Where is the Wisdom we have lost in knowledge?
> Where is the Knowledge we have lost in information?

To these lines, Krishnamurthy had added two more lines as

> Where is the Information we have lost in data?
> Where is the Data we have lost in database?

And here, I wish to add one more line as

> Where is the Database we have lost in the Internet?

The above lines clearly indicate the definition of informatics of any kind, whether it is bioinformatics, cheminformatics, pharmacoinformatics, immunoinformatics, medical informatics and so on. In any subject, informatics begins its role when the amount of data becomes large and the size of the data grows in a rapid manner. In informatics, the first step is to classify the data and store them in organised and integrated databases for easy retrieval and use with the help of computers. Next step is to analyse the data using any

suitable computational methods to obtain useful and pertinent information. From the information, one has to derive knowledge, and from the knowledge, one has to attain wisdom by taking wise and proper decisions resulting in efficient actions. So, the definition of informatics is not only to classify, store and retrieve the data but also to analyse the data to get useful information, and from that, the knowledge has to be derived and also to attain the wisdom.

13.2.1 Need for Bioinformatics

Before we define bioinformatics, let us know about the major reasons behind the need for bioinformatics. The first and foremost reason for any kind of informatics is the existence of large amount of data and its rapid growth. In biological sciences, there has been a data explosion that occurred due to the high-throughput DNA sequencing projects that generate the genome sequence data of different organisms, which has started around the year 1990. The draft genome of humans was completed in the millennium year 2000 and was announced (Lesk 2005) on 26 June 2000. The importance of this discovery becomes evident from the fact that the top journals like *Nature* and *Science* had published a special issue on 'The human genome' in their 15th February 2001 and 16th February 2001 issues, respectively. However, many other genome sequences data of microbes, plants and animals are also available for analysis to date (Lesk 2005; Baxevanis and Ouellette 2005; Gregory 2005; Pevsner 2009). This is reflected in the size of the GenBank database, which is one of the popular DNA sequence databases maintained by the National Centre for Biotechnology Information (NCBI), USA. There is an exponential growth of the size of the GenBank database, which is evident from its statistics (http://www.ncbi.nlm.nih.gov/genbank/statistics) from 1990 to date. The current release #201 of GenBank database contains 159,813,411,760 (i.e. approximately 159 billion) bases corresponding to 171,744,486 (i.e. approximately 171 million) sequences, as of April 2014.

On the other hand, the experimentally determined three-dimensional (3D) structures of biological macromolecules available in structure databases are very less in number compared to the sequence databases. For example, Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do), which is one of the most popular 3D structure databases of biological macromolecules including proteins, nucleic acids and complexes, contains 99,293 structures only as of April 2014. This clearly indicates that the sizes of the sequence databases are huge compared to structure databases. The reason for this disparity is mainly because the experimental determination of 3D structures of biological macromolecules (Higgins and Taylor 2000; Gautham 2006) is tedious and time consuming compared to that of the experimental high-throughput sequencing techniques available at present. Hence, the biological data explosion and the disparity in the size of the sequence and structure databases may be regarded as the two major reasons behind the need for bioinformatics.

13.2.2 What Is Bioinformatics?

From the broad definition of informatics and the reasons for the need of bioinformatics, one can easily define bioinformatics (Lesk 2005; Mount 2003). Bioinformatics is an interdisciplinary subject, which requires the knowledge/expertise from diverse backgrounds, including all branches of biological sciences, computer science, mathematics, statistics, physics and chemistry. In a popular way, Arthur Lesk (2005) in his book on *Bioinformatics* provided a simple definition as 'The marriage of biology and computer science has created a new field called bioinformatics'.

In general, bioinformatics is the application of various computational methods from mathematical, statistical and computer sciences to analyse biological data to obtain useful information. We use computer programmes to make inference from the biological data, to make connections among them and to derive useful predictions. From these predictions, one has to derive knowledge and attain wisdom by taking wise and proper decisions.

13.2.3 Understanding Gene Function: Importance of 3D Structures

From bioinformatics' point of view, we look at an organism in the form of data. An organism must have one genome, fixed number of chromosomes in the nucleus of each cell, the total number of nucleotide bases in all the chromosomes and so on. For example, in the case of humans (*Homo sapiens*), which is the complex eukaryotic organism, there exists one genome, 24 haploid chromosomes (22 autosomes, X and Y sex chromosomes) of nuclear DNA along with the mitochondrial organelle DNA in each cell, a total of approximately 3.3×10^9 base pairs and approximately 21,000 genes predicted by gene prediction methods. In the case of plants, in addition to nuclear and mitochondrial DNA, genome includes plastid DNA, which is responsible for photosynthesis. For example, in the case of rice (*Oryza sativa*) genome, there are 12 chromosomes, plastid and mitochondrial DNAs. In fact, immediately after the experimental determination of the genome sequence of an organism, the very first result predicted is the possible number of coding genes, which is obtained only from the gene prediction algorithms of bioinformatics.

It is also interesting to note that most of the mapping and sequencing technologies were developed from studies of simpler organisms such as microbes, plants and animals. Some of the representative model organisms of each major type along with their genome sizes are given in Table 13.1. It is interesting to note from Table 13.1 that the genome size of the mouse and human is of the same order of 2.8 Gbp and 3.3 Gbp, respectively. This clearly indicates that for any organism, the organism size does not have any relation to its genome size. For any organism, the total amount of DNA of the haploid genome measured in picograms (pg) is called its 'C-value' (Gregory 2005). The lack of a consistent relationship between the C-value and the complexity of an organism is called the 'C-value paradox'. Further, we know that the mouse has been used as a model organism for humans in carrying out many in vivo experiments for several decades without knowing that they do have the same order of genome size.

In order to understand the various functions of an organism, we need to understand the functions of their coding genes. In order to find the function of a gene, first, we have to obtain the corresponding protein expressed by the gene, as coded by it, using the appropriate standard genetic code. The function of the protein depends on the 3D or folded structure of it, assumed in typical biological environments surrounding it. Hence, understanding protein structure will be essential in determining gene function. Once the function of the protein is known, then it is assigned as the function of the associated gene.

This is the reason, why we have compared the size of PDB, the 3D structure database, with that of the GenBank, the DNA sequence database in Sect. 13.2.1. Since the size of the 3D structure database is small and also the experimental

Table 13.1 Genome of sizes of non-human model organisms

Sl. no.	Organism type	Organism name	Chromosomes[§] (haploid)	Genome size*
1.	Bacterium	*Escherichia coli*	–	4.6 Mbp[a]
2.	Yeast	*Saccharomyces cerevisiae*	16 (1–16)	12.4 Mbp
3.	Fruit Fly	*Drosophila melanogaster*	5 (2,3,4,X,Y)	122.6 Mbp
4.	Roundworm	*Caenorhabditis elegans*	6 (1–5,X)	100.2 Mbp
5.	Plant	*Arabidopsis thaliana*	5 (1–5)	135.0 Mbp
6.	Animal	*Mus musculus* (mouse)	21 (1–19,X,Y)	2.8 Gbp[b]

Source: [§]http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/Chromosomes.html
*http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/G/GenomeSizes.html
[a]1 Mbp (Mega base pairs) = 10^6 base pairs
[b]1 Gbp (Giga base pairs) = 10^9 base pairs

determination of 3D structures of biological macromolecules is tedious and time consuming, computational algorithms for 3D structure prediction play an important role in predicting 3D structures from the sequences of proteins for which experimentally determined 3D structures are not available.

13.3 Bioinformatics Tasks: Biological Sequence and Structure Analysis

In this section, some of the basic bioinformatics tasks in sequence and structure analysis of biological data are described. The important tools used for various tasks related to biological sequence and structural analysis are also discussed. Before that, it is necessary to know about how to download DNA and protein sequences, structures and scientific articles from the respective databases, required for any analysis. DNA sequences can be downloaded either directly from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), EMBL (http://www.embl.de/) and DDBJ (http://www.ddbj.nig.ac.jp/) databases or from the genome databases. Protein sequences can also be downloaded from the Protein-NCBI (http://www.ncbi.nlm.nih.gov/protein), PIR (http://pir.georgetown.edu/) and UniProt (http://www.uniprot.org/). Scientific Literature can be accessed from PubMed (http://www.ncbi.nlm.nih.gov/pubmed), PubMed Central – PMC (http://www.ncbi.nlm.nih.gov/pmc/) and also directly from the home pages of many journals publishing articles with open access.

13.3.1 Sequence Analysis Tasks

In biological sequence analysis, the two basic tasks are sequence alignment and database search (Lesk 2005; Pevsner 2009). Suppose we get a DNA or protein sequence for our analysis, either the new sequence may be isolated, purified and extracted from a source organism in the lab or may be downloaded from a database. We know that *in nature*, new sequences are adapted from the pre-existing sequences rather than invented de novo (Durbin et al. 1998). There exists a significant similarity between a new sequence and already known sequences. This is very fortunate for computational sequence analysis, as any new sequence can be studied by comparing it with the existing sequences.

Now, if we have some clues about the new sequence, then the basic question is whether this DNA or protein sequence is related to any other DNA or protein sequence of our choice. The relatedness of a pair of sequences suggests that they may be homologous (Durbin et al. 1998; Pevsner 2009). By the homology of sequences, we mean that the two sequences and the organisms in which they occur are descended from a common ancestor, with the implication that they do have shared ancestral characteristics. If two related sequences are homologous, then we can transfer information about structure and may also function through homology. It is important to note that the 'similarity' of two sequences simply means that the measurement of resemblance and difference is independent of the source (i.e. organism) of resemblance. Homology implies similarity and the converse is not true always. Further, if two amino acid residues in the same position of both the sequences are the same (i.e. D vs D), then it is known as 'identity' and have maximum score in the scoring matrices, such as BLOcks SUBstitution Matrix (BLOSUM), used for sequence alignment. For example, the score for D vs D in BLOSUM50 scoring matrix is 8. Suppose if two amino acid residues in the same position of both the sequences are conserved (i.e. D vs E), then they possess 'similarity' and have positive score in the scoring matrices. In the case of D vs E, the score in BLOSUM50 scoring matrix is 2. Again, identity implies similarity and converse is not true.

The question now becomes how to find the relatedness of two sequences, and the answer is simply by aligning the sequences, which is known as pairwise sequence alignment (Mount 2003; Pevsner 2009). Since genome sequences of many organisms become available, the task of finding out how DNA/proteins are related within an organism and between organisms becomes

increasingly fundamental to our understanding of life. Comparison of DNA (or nucleotide) sequences is important in (i) confirming the identity of a DNA sequence in a database, (ii) searching for polymorphisms (SNPs) and (iii) analysing the identity of a cloned cDNA fragment. More often, protein sequence alignment is more informative than DNA alignment. Protein sequence comparisons can identify homologous sequences from organisms that last shared a common ancestor over 1 billion years ago (BYA). However, DNA sequence comparisons typically allow look-back time up to about 600 million years ago (MYA) only. Further, with the whole genome sequences of many organisms, it becomes the fundamental task to find out how proteins are related within an organism (known as paralogues) and between organisms (known as orthologues) for our understanding of life.

Some of the important types of sequence comparisons used for biological sequence analysis (Lesk 2005) are given below:

(i) Pairwise sequence comparison is the assignment of character by character match between two sequences. Two types of pairwise alignments are useful. (a) Global alignment aligns end to end of two sequences as a whole, and (b) local alignment aligns one or more portions or subsequences of two sequences.

(ii) Motif search finds matches of a short sequence in one or more regions internal to a long sequence.

(iii) In multiple sequence alignment, the algorithm tries to align all the sequences (more than two in number) that are given as query. This type of alignment is useful to find out conserved regions among proteins. For example, the multiple sequence alignment of the insulin protein sequences of various organisms may provide the regions of residues in the given set of query sequences which are conserved, while the other residues are not. Finding these regions is helpful to determine the function of the proteins for which sequence similarity is not significant.

Apart from these, it may also be interesting to search for similar sequences through databases for any given new sequence, which is known as database search. But comparing the sequences with other sequences or through sequence databases is not a simple process that can be done manually, as the size of the sequence databases is very huge and hence computational algorithms are used to perform the analysis. Biological sequence analysis algorithms are used in aligning a new sequence with the millions of available sequences, and by using similarity between the sequences, one can predict the structure and function. Let us describe few sequence analysis tasks mentioned above with suitable examples.

13.3.1.1 Pairwise Sequence Alignment

The most fundamental sequence analysis task is aligning two sequences (either DNA or protein) in a pairwise manner and to find whether the two sequences are related or not. If there is any similarity, we can be sure of the structure and function of the new protein. The key issues involved in pairwise sequence alignments are:

(i) *Types of alignment needed and gap penalties*

Global alignment: It is an end-to-end matching of two entire sequences using the Needleman–Wunsch algorithm (Needleman and Wunsch 1970; Durbin et al. 1998). This kind of alignment is used when the sequence sizes are close to each other and the algorithm tries to align all the residues (letters) end to end. Gaps are introduced to make the alignment better and are denoted by hyphens.

Local alignment: It is the matching of portions or subsequences from two sequences using the Smith–Waterman algorithm (Smith and Waterman 1981; Durbin et al. 1998). This is used when the sequences are distantly related or partial sequence matching is required for analysis.

Gap penalties: While aligning, if a gap is to be introduced in any one of the sequences for optimal alignment, a penalty is given (Durbin et al. 1998). The standard penalty associated with a gap of length g is given either by a linear score $r(g) = -gd$ or an affine score $a(g) = -d - (g-1)e$, where d is the gap open penalty (gap is introduced) and

e is the gap extension penalty (gap is extended).

(ii) *Scoring System used to rank alignments*
A score is needed as a measure of similarity when a residue from the first sequence is aligned with a residue in the second sequence. The residues may be identical (same amino acids), similar (amino acids with similar properties) or different (amino acids with entirely different properties), and the score is given accordingly using appropriate substitution matrices. The values in these matrices are derived using the probability of occurrence derived from the statistics based on known sequences. Point accepted mutation (PAM) and BLOcks SUBstitution Matrix (BLOSUM) are two popularly used scoring matrices. They are given different numbers to denote the different levels of similarity or relatedness. For example, PAM40 and BLOSUM90 are equivalent which are obtained from closely related/similar sequences. PAM500 and BLOSUM30 are obtained from distantly related sequences. When we have no idea of similarity beforehand, PAM250 and BLOSUM62 are used as default substitution matrices.

(iii) *Algorithms used to find optimal* (*or good*) *scoring alignment*
Dynamic programming algorithms are used for pairwise alignment, in which the alignment score is dynamically given as an additive score based on the previous alignment. These types of algorithms are guaranteed to find the optimal scoring alignment or set of alignments.

(iv) *Statistical methods used to evaluate the significance of an alignment score*
The statistical quantity, namely, E-value, is used commonly to evaluate the significance of the scores obtained.

Let us consider the sequence of myoglobin of humans as query, and the question is to find how much similar is the query sequence with haemoglobin subunit beta sequence of human. One has to perform pairwise alignment of these two sequences using any pairwise sequence alignment tools such as EMBOSS Needle, EMBOSS Water and LALIGN available at http://www.ebi.ac.uk/Tools/psa/, BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and so on. First, the two sequences have to be downloaded from the relevant databases and are given below:

>gi|4885477|ref|NP_005359.1| myoglobin [*Homo sapiens*]
MGLSDGEWQLVLNVWGKVEADIPGHG
QEVLIRLFKGHPETLEKFDKFKHLKSED
EMKASEDLKKHGATVLTALGGILKK
K G H H E A E I K P L A Q S H A T K H K
IPVKYLEFISECIIQVLQSKHPGDFGAD
A Q G A M N K A L E L F R K D M A S N
YKELGFQG

>gi|4504349|ref|NP_000509.1| haemoglobin subunit beta [*Homo sapiens*]
M V H L T P E E K S A V T A L W G K V N V D E V G
G E A L G R L L V V Y P W T Q R F F E S F
G D L S T P D A V M G N P K V K A H G K K
V L G A F S D G L A H L D N L K G T F A T L
S E L H C D K L H V D P E N F R L L G N V L
V C V L A H H F G K E F T P P V Q A A Y Q K V
VAGVANALAHKYH

The NCBI BLAST input and output of the pairwise alignment of the above two sequences are given in Figs. 13.1 and 13.2, respectively. The following steps have to be carried out in giving the BLAST input shown in Fig. 13.1:

(i) Go to BLAST website at http://blast.ncbi.nlm.nih.gov/Blast.cgi and choose 'protein blast'.
(ii) Copy the 'myoglobin' sequence into the 'Enter Query Sequence' box.
(iii) Check the 'Align two or more sequences' box.
(iv) Copy the 'hemoglobin subunit beta' sequence into the 'Enter Subject Sequence' box.
(v) Choose 'BLOSUM50' matrix in 'Scoring parameters' box in the 'Algorithm parameters' section.
(vi) Click 'BLAST' button to run BLAST.

In Fig. 13.2, the graphic summary and alignments are shown as BLAST output.

13.3.1.2 Database Search

The database search for the above-mentioned human myoglobin sequence as query sequence is carried out using BLAST, and the screenshots of both input and output are given in Figs. 13.3 and 13.4, respectively. The following steps have to be carried out in giving the BLAST input as shown in Fig. 13.3:

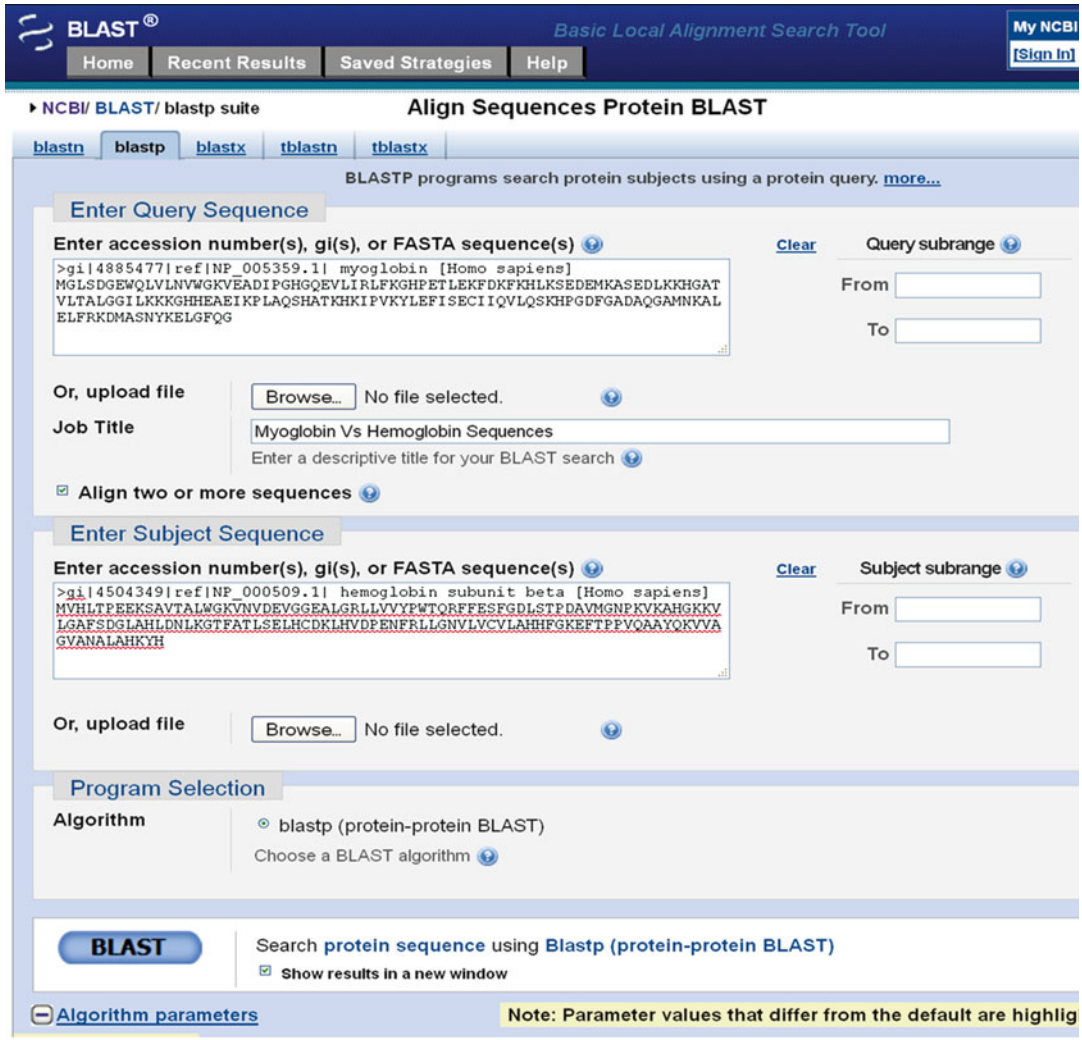


Fig. 13.1 Pairwise sequence alignment using BLAST – Input

(i) Go to BLAST website at http://blast.ncbi.nlm.nih.gov/Blast.cgi and choose 'protein blast'.
(ii) The human myoglobin sequence is copied into the 'Enter Query Sequence' box.
(iii) Choose 'No Adjustment' in 'Compositional Adjustments' option in 'Scoring parameters' box in the 'Algorithm parameters' section.
(iv) Click 'BLAST' button to run blast.

In Fig. 13.4, the graphic summary and descriptions are shown as BLAST output.

13.3.1.3 Multiple Sequence Alignment

The insulin sequences from five different organisms are downloaded from the NCBI database and are given below:

>Hamster gi|69316|pir||INHY insulin – hamster
F V N Q H L C G S H L V E A L Y L V C G E R G
FFYTPKSGIVDQCCTSICSLYQLENYCN
>Monkey gi|538566|pir||INMKSQ insulin – common squirrel monkey
FVNQHLCGPHLVEALYLVCGERGFFY
APKTGVVDQCCTSICSLYQLQNYCN

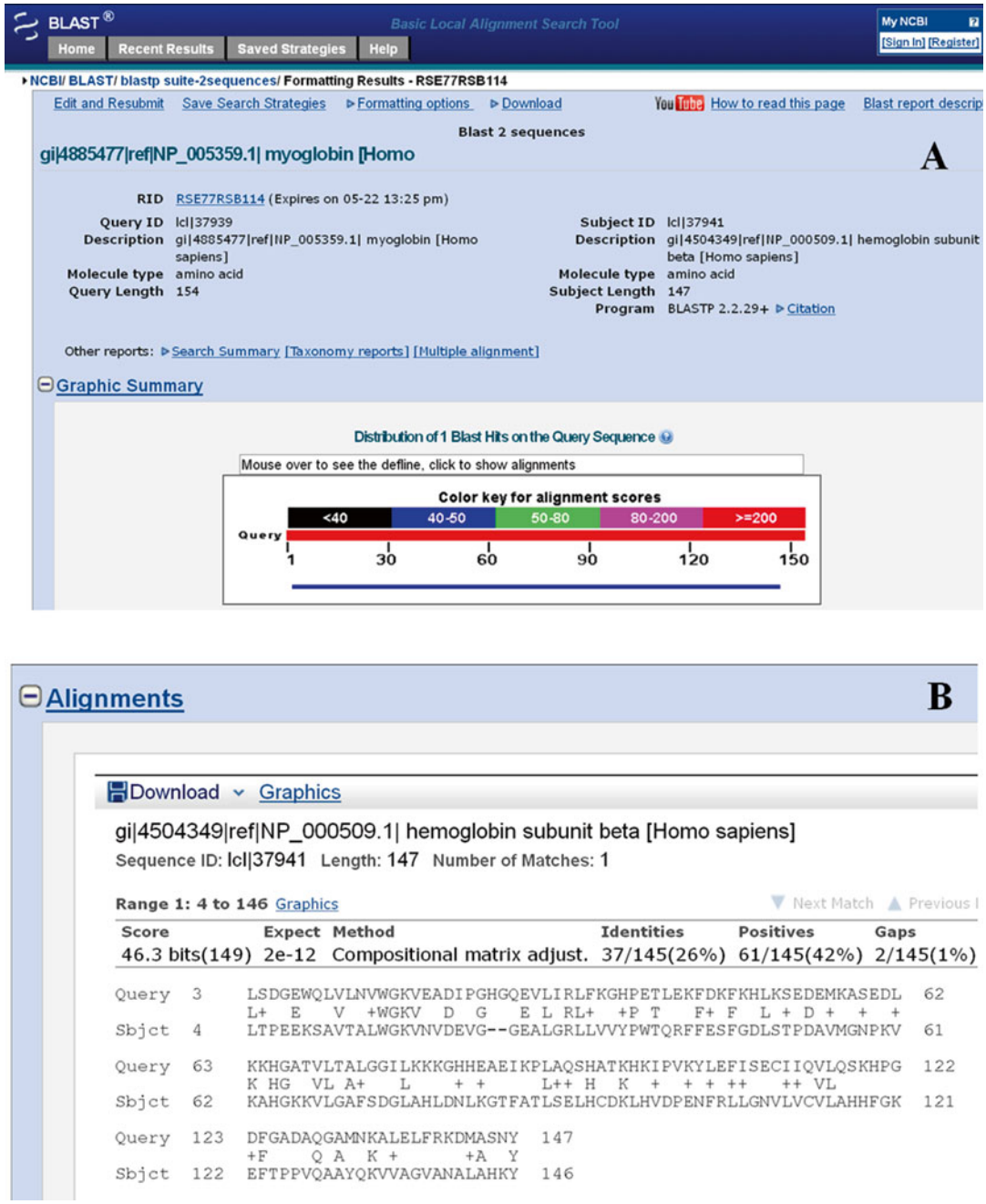


Fig. 13.2 Pairwise sequence alignment using BLAST – Output. (**a**) Graphic summary and (**b**) Alignments

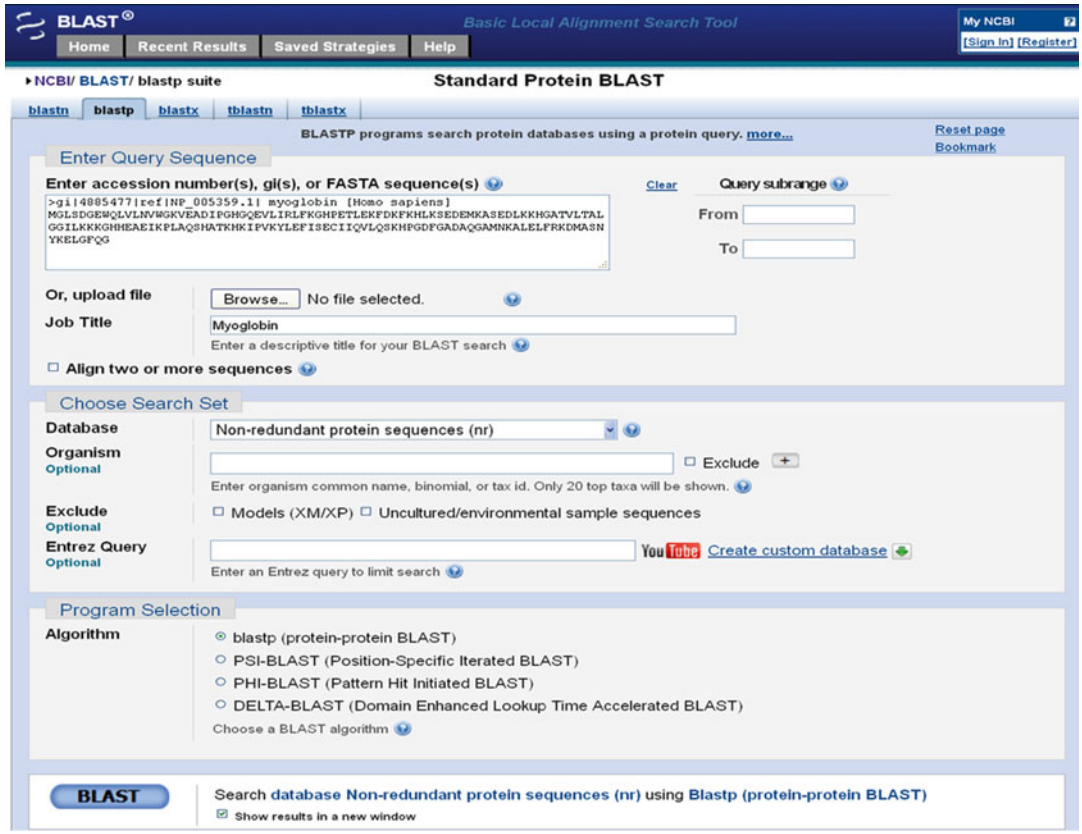


Fig. 13.3 Database search of a query using BLAST – Input

>Ostrich gi|69327|pir||INOS insulin – ostrich
AANQHLCGSHLVEALYLVCGERGFFYSPK
AGIVEQCCHNTCSLYQLENYCN
>Elephant gi|69307|pir||INEL insulin – elephant
FVNQHLCGSHLVEALYLVCGERGFFYTPKT
GIVEQCCTGVCSLYQLENYCN
>Turkey gi|69326|pir||INTK insulin – turkey (tentative sequence)
AANQHLCGSHLVEALYLVCGERGFFY
SPKAGIVEQCCHNTCSLYQLENYCN

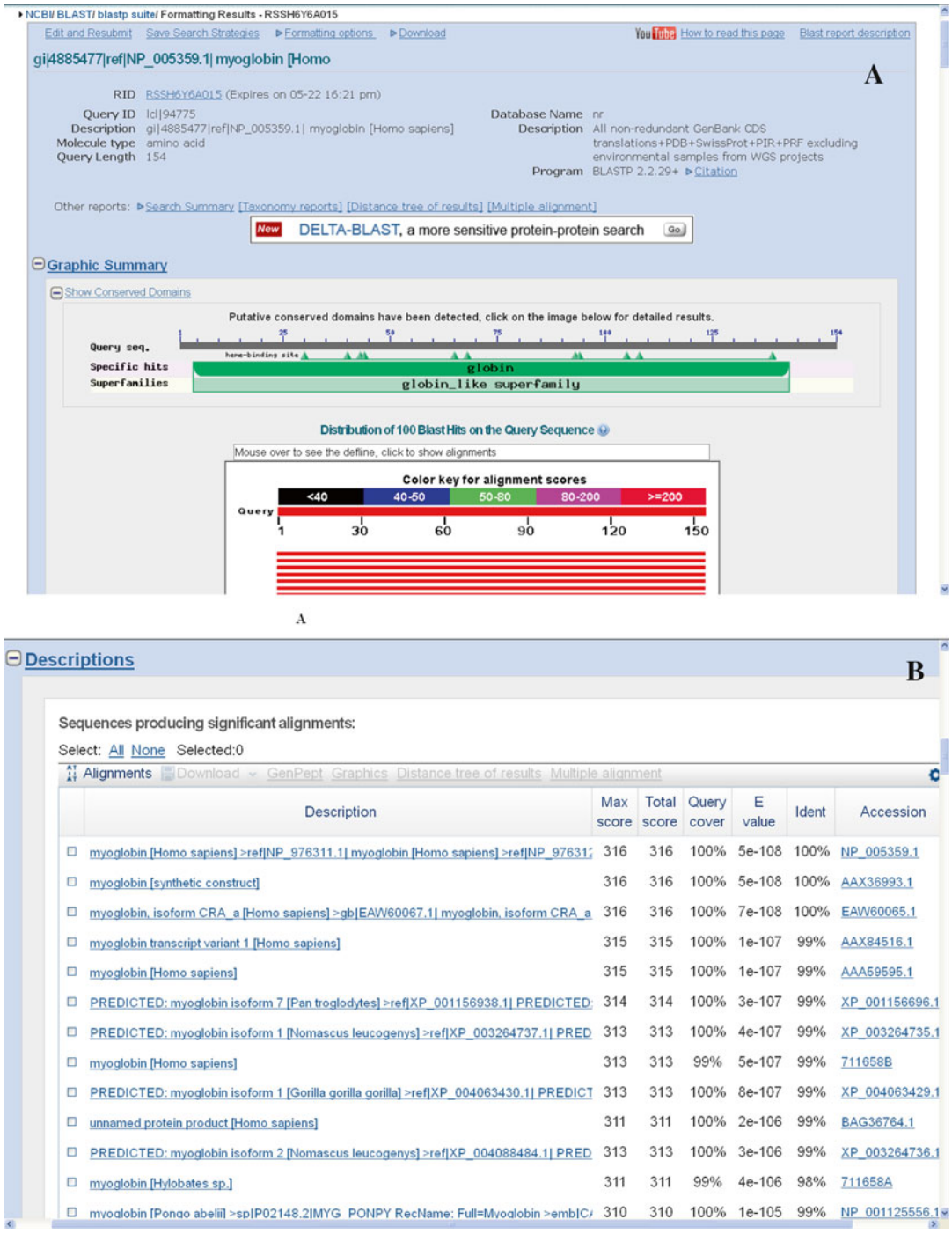
The multiple sequence alignment of the above insulin sequences of five different organisms are carried out using Clustal Omega tool available at http://www.ebi.ac.uk/Tools/msa/clustalo/. The screenshots of both input and output are given in Fig. 13.5. The following steps have to be carried out in giving the 'Clustal Omega' input as shown in Fig. 13.5:

(i) Go to 'Clustal omega' website at http://www.ebi.ac.uk/Tools/msa/clustalo/.
(ii) Copy all the above five 'insulin' sequences into the 'STEP 1 Enter into input sequences' box.
(iii) Click 'Submit' button to run 'Clustal Omega'.

The multiple sequence alignment alone is shown as output.

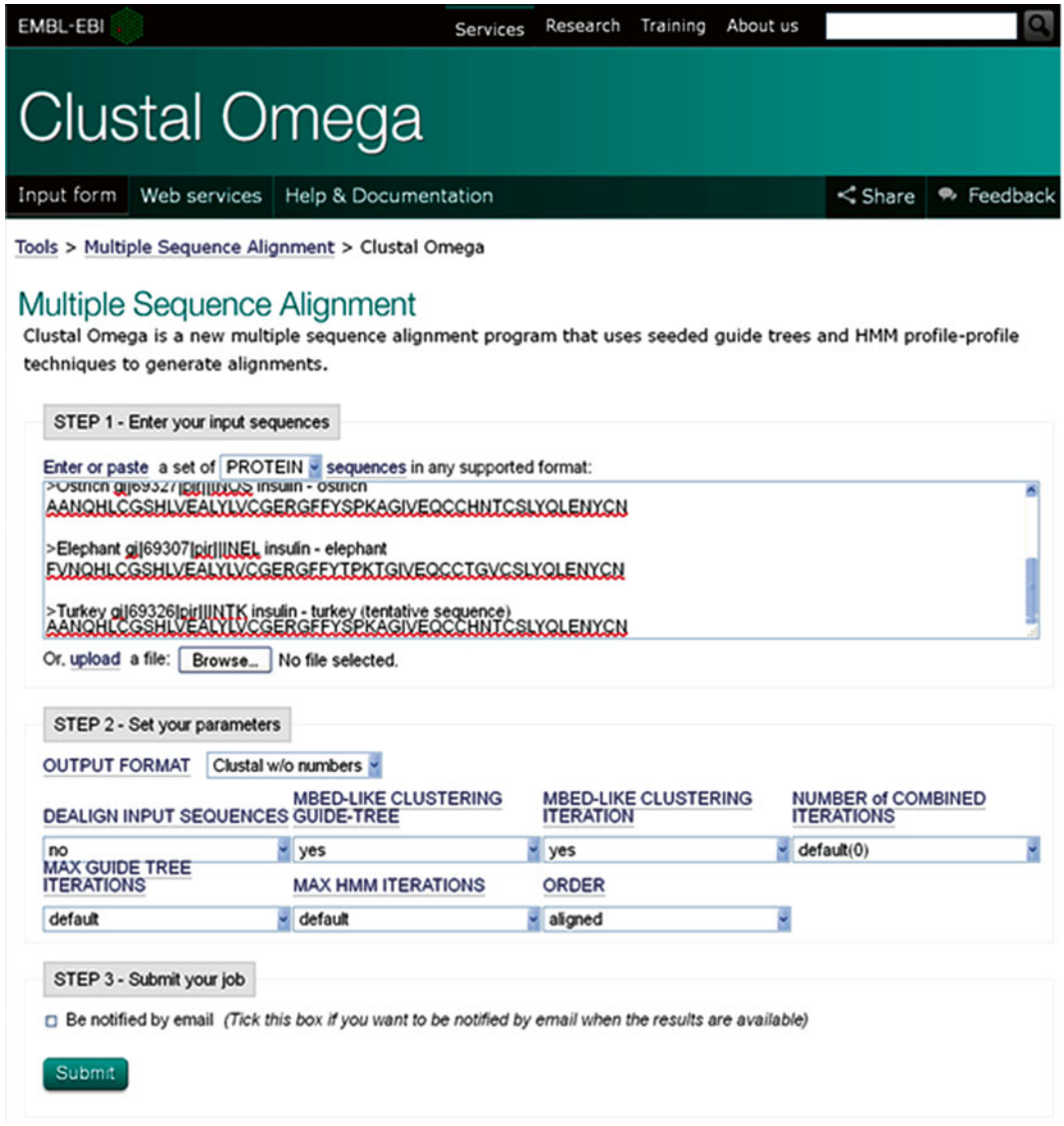
13.3.1.4 Other Sequence Analysis Tasks

Apart from the above-mentioned basic sequence analysis tasks, other computational sequence analysis tools are also available to predict structural and functional features of proteins from the sequence information alone. Secondary structure prediction is an important structural feature prediction where the computational tools can predict which portion of the sequence may form helix, sheet and coil. Similarly, protein fold recognition tools may predict the possible fold the sequence



A

Fig. 13.4 Database search of a query using BLAST – Output. (**a**) Graphic summary and (**b**) Descriptions



Output:

```
CLUSTAL O(1.2.1) multiple sequence alignment


Ostrich         AANQHLCGSHLVEALYLVCGERGFFYSPKAGIVEQCCHNTCSLYQLENYCN
Turkey          AANQHLCGSHLVEALYLVCGERGFFYSPKAGIVEQCCHNTCSLYQLENYCN
Monkey          FVNQHLCGPHLVEALYLVCGERGFFYAPKTGVVDQCCTSICSLYQLQNYCN
Hamster         FVNQHLCGSHLVEALYLVCGERGFFYTPKSGIVDQCCTSICSLYQLENYCN
Elephant        FVNQHLCGSHLVEALYLVCGERGFFYTPKTGIVEQCCTGVCSLYQLENYCN
                 .****** ****************:**:*:*:***    ******:****
```

Fig. 13.5 Multiple sequence alignment of insulin protein sequences using Clustal Omega – Input & Output

Table 13.2 List of sequence analysis tools

Sl. no.	Task(s)	Free online tools
1.	Pair-wise sequence alignment	EMBOSS Needle, EMBOSS Water, LALIGN, BLAST
2.	Multiple sequence alignment and phylogenetic analysis	ClustalW & Tree View, Clustal Omega, PHYLIP, PAUP, MEGA
3.	Database search	BLAST, FASTA
4.	Secondary structure prediction	GOR, PHD, DSSP, SOPMA, NPS@
5.	Protein fold recognition	3D-PSSM/PHYRE, nFOLD3, PredicFold PSS-3D1D
6.	Amyloidogenic regions prediction	AGGRESCAN, FoldAmyloid

can adopt by using the structural features of known folds. Further, functional features like amyloidogenic regions of proteins may also be predicted. Some of the important sequence analysis tools for the above-mentioned tasks are provided in Table 13.2.

13.3.2 Structure Analysis Tasks

In structure analysis, some of the important bioinformatics tasks are (i) visualisation of three-dimensional (3D) structures of biological macromolecules; (ii) computing bond lengths, bond angles, torsion angles and other structural parameters; (iii) superposition of protein structures and to compute root–mean–square deviation (RMSD) between them; (iv) 3D structure prediction of proteins; (v) molecular docking and (vi) molecular dynamic simulations (Higgins and Taylor 2000; Orengo et al. 2003; Bourne and Weissig 2003). As we have mentioned in Sect. 13.2.3, structure is responsible for the function of a protein; structural analysis tools are very important in understanding the functions of biological molecules.

The major experimental techniques available for the determination of 3D structure of biological macromolecules (Higgins and Taylor 2000; Gautham 2006) are (i) X-ray diffraction and (ii) NMR methods. However, both these methods are tedious and time consuming. Even the latest high-throughput crystallographic structure determination method using high-flux synchrotron radiation sources for data collection and employing multiple anomalous diffraction methods for data interpretation is not keeping the pace at which DNA sequences are determined using the modern high-throughput sequencing methods. Hence, computational 3D structure prediction algorithms of bioinformatics become useful in structural analysis of biological macromolecules.

The experimentally determined 3D structures of biological macromolecules are made available in the structure databases like (i) Protein Data Bank (PDB) maintained by Research Collaboratory for Structural Bioinformatics (RCSB), USA (http://www.rcsb.org/pdb/home/home.do); (ii) Molecular Modelling Database (MMDB) of NCBI, USA (http://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml); and (iii) Nucleic Acid Database (NDB) (http://ndbserver.rutgers.edu/). There are many tools available for 3D structure visualisation such as (i) RasMol (http://rasmol.org/), (ii) Swiss-PDBViewer Deep View (http://spdbv.vital-it.ch/), (iii) VMD – visual molecular dynamics (http://www.ks.uiuc.edu/Research/vmd/) and (iv) PyMOL (http://www.pymol.org/).

Further, three types of methods are available for 3D structure prediction of proteins, namely, (i) homology modelling, (ii) threading and (iii) 'ab initio' methods. In order to perform homology modelling, a template 3D structure is required whose sequence identity must be more than 40 % with the query sequence for which the 3D structure has to be predicted. Some of the free online tools, which can perform homology modelling, are Modeller (https://salilab.org/modeller/) and SWISS-MODEL (http://swissmodel.expasy.org/). If such a template structure is not available, then one may have to use threading method performed by typical tool such as I-TASSER (http://zhanglab.ccmb.med.umich.

edu/I-TASSER/). There exists another method, namely, 'ab initio' method, which can predict the three-dimensional structure from the first principles using the sequence information alone, and hence, it is computationally intensive. The Robetta server (http://robetta.bakerlab.org/) is used to predict protein structure maintained by the Baker laboratory for noncommercial ab initio and comparative modelling.

Once it is possible to predict three-dimensional structure of proteins and visualise it, one can also perform many other structure analysis tasks, namely, docking of small molecules with target protein structures, protein–protein docking and protein–DNA interactions. Further, one can also perform computational protein engineering experiments like (i) mutation experiments where one alters any particular amino acid or base for a desired effect and (ii) site-directed mutagenesis experiments in which one identifies potential sites where one can do alterations. All these computational experiments have a lot of potential applications in various fields including agriculture, pharmaceutical and medical sciences. Let us describe few structure analysis tasks mentioned above with suitable examples.

13.3.2.1 Visualisation of 3D Structure of Proteins Using RasMol

The 3D structure of haemoglobin molecule of humans (PDB ID: 1A3N) is downloaded from PDB. Visualisation of this molecule using RasMol is shown in Fig. 13.6. Using the visualisation tool, we can identify the chains A, B, C and D and indicate them with different colours. In addition, the heme molecules in each chain are also shown in ball and stick display in purple colour.

13.3.2.2 Structure Function Relationship Using Swiss-PDBViewer Deep View

In order to exhibit the structure–function relationship, let us consider the single point mutation in haemoglobin gene that has resulted in sickle-cell anaemia in human population. It is evident that a single nucleotide change has led to the substitution of glutamic acid (E) in the 6th position of normal to valine (V) of mutant, which resulted in the structural change in the protein which in turn has altered the cell shape. So, let us compare the structure of the B chain of haemoglobin of normal (PDB ID: 1A3N_B) and mutant (PDB ID: 2HBS_B) forms of humans after

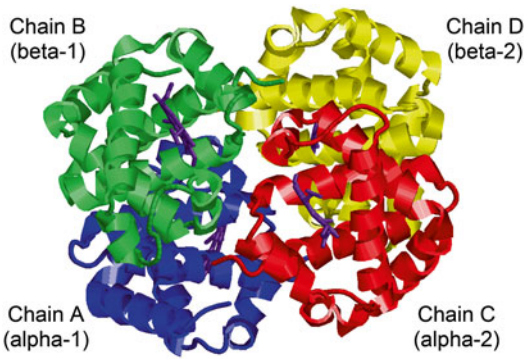


Fig. 13.6 Visualisation of 3D structure of human hemoglobin molecule using RasMol (PDB ID: 1A3N) with indivudal chains A, B, C and D are depicted in different colours with heme molecule of each chain in *purple* colour

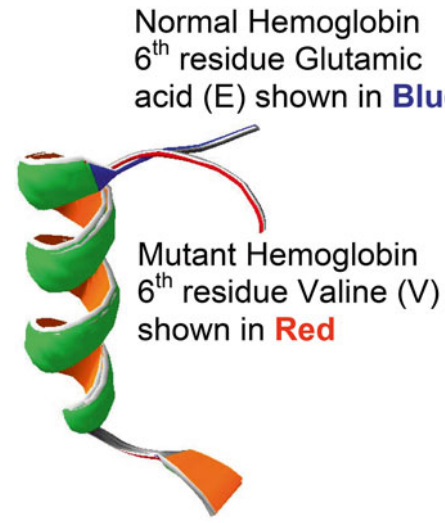


Fig.13.7 Structure function relationship – Superimposing the first 20 amino acids of the 3D structures of chain B of normal (1A3N_B) and mutant (2HBS_B) hemoglobin molecule of human using Swiss-PDB Viewer Deep View

superimposing their structures using Swiss-PDBViewer Deep View tool and is shown in Fig. 13.7. In order to recognise the structural change in the 6th position (E to V) of normal and mutant haemoglobin structures, the first 20 amino acids are shown in Fig. 13.7.

13.3.2.3 Structural Classification of Proteins

Proteins are classified based on their three-dimensional structures. The following are the important databases on structural classification:

i. SCOP – Structural Classification of Proteins (New SCOP2 database is available at http://scop2.mrc-lmb.cam.ac.uk/.)
ii. CATH – Class, Architecture, Topology, Homologous superfamily (CATH database is available at http://www.cathdb.info/.)
iii. FSSP – Fold classification based on Structure–Structure alignment of Proteins – obtained by Distance-matrix alignment (DALI) (FSSP database is available at http://protein.hbu.cn/fssp/.)

13.3.2.4 Other Structural Analysis Tasks

One of the major important structural bioinformatics tasks is docking. Docking is the process of predicting (or checking) the fitness of a small molecule (drug or ligand) into the active site (or a binding pocket) of a target protein molecule. After docking, the resulting docked structure is a protein–ligand complex. Computational docking algorithms are used to compute the binding of a drug to the target protein and their interactions in a computer-simulated environment using specialised software. The other structural bioinformatics tasks are (i) active site prediction, (ii) molecular dynamics simulation, (iii) quantitative structure activity relationship (QSAR), (iv) virtual screening, (v) pharmacophore modelling and so on. Some of the important structural analysis tools for the above-mentioned tasks are provided in Table 13.3.

13.4 Recent Developments in Bioinformatics

The tasks and tools mentioned above are used to analyse single sequence or structure only. In the postgenomic era, the availability of genome sequences of many organisms and the data available through the modern high-throughput experiments require more comprehensive tools to analyse them. Recent developments in bioinformatics include (i) genomics, (ii) transcriptomics, (iii) proteomics, (iv) metabolomics and (v) systems biology. Let us briefly explain about these recent concepts.

13.4.1 Genomics

As we know, the total DNA content (i.e. nuclear, organelle and plastid DNA) is known as genome. Genomics is the study of the structure, content, evolution and functions of genes in genomes (Lesk 2007). The major aims of genomics are to (i) establish an integrated web-based database and research interface; (ii) assemble physical, genetic and cytological maps of the genome; (iii) identify and annotate the complete set of genes encoded within a genome and (iv) provide the resources for comparison with other genomes.

There are many computational tools available to analyse the genomes. Genomes of many

Table 13.3 List of structure analysis tools

Sl. no.	Task	Free online tools
1.	3D structure visualization	RasMol, Swiss-PDBViewer, VMD, PyMOL
2.	3D structure prediction	Modeller, SwissModel, I-TASSER, Robetta
3.	Docking	AutoDock, Argus Lab, Hex
4.	Active site prediction	Q-SiteFinder, CASTp
5.	Molecular dynamics simulations	AMBER, GROMACS

organisms and plants are available in NCBI genome database (http://www.ncbi.nlm.nih.gov/genome). Further, NCBI genome Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/) is a very useful tool in viewing genomes and also to download the sequence of any particular gene or set of genes. A typical screenshot of NCBI Map Viewer of the *Homo sapiens* (human) genome is shown in Fig. 13.8. All the 22 autosomes, X and Y chromosomes and mitochondrial DNA are depicted in Fig. 13.8. Similarly, the screenshot of NCBI Map Viewer showing the genes on sequence of *Homo sapiens* (human) chromosome 1 is shown in Fig. 13.9. The other popular genome browsers are (i) UCSC genome browser (http://genome.ucsc.edu/) and (ii) Ensembl human genome browser (http://www.ensembl.org/index.html).

One of the important applications of genomics is the prediction of coding genes using the gene finding algorithms from the genomes of prokaryotes and eukaryotes. Once the genome sequence is obtained through experimental high-throughput sequencing projects, the coding genes are predicted using computational gene prediction tools such as (i) GeneMark (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/genemark.cgi), (ii) GENSCAN (http://genes.mit.edu/GENSCAN.html), (iii) GLIMMER (http://www.ncbi.nlmnih.gov/genomes/MICROBES/glimmer_3.cgi) for prokaryotic and eukaryotic genomes, (iv) GRAIL (http://www.broadinstitute.org/mpg/grail/) and (v) ORF Finder (http://www.bioinformatics.org/sms2/orf_find.html) for eukaryotic genomes.

Single nucleotide polymorphism (SNP) analysis is another application of genomics. SNP is a genetic variation between individuals, limited to a single base pair, which can be substituted, inserted or deleted. The dbSNP is the database of SNPs available at http://www.ncbi.nlm.nih.gov/SNP/. SNPs are useful in genetic disorders and identification of evolutionary traits of an organism or a plant across geographic variations. One of the examples of SNPs is a genetic disorder, namely, sickle-cell anaemia disease, which is caused by a specific SNP, an adenine to thymine mutation in the β-globin gene that changes a glutamic acid to valine in the 6th residue (c.f. Fig. 13.7), creating a sticky surface on the haemoglobin molecule that leads to the polymerisation of the deoxy form.

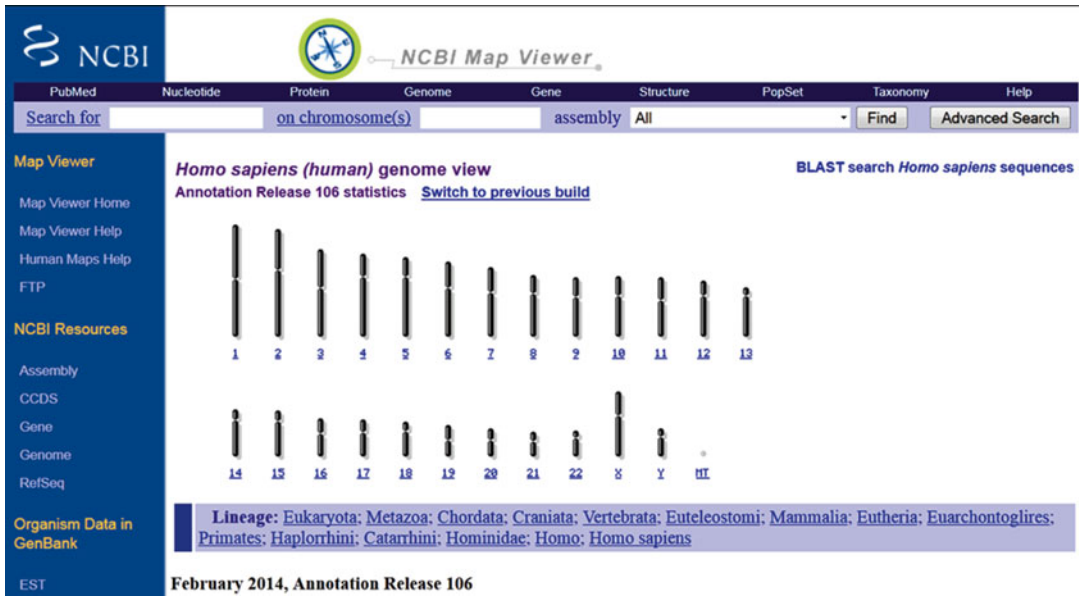


Fig. 13.8 Screen shot of NCBI Map Viewer of the *Homo sapiens* (human) genome view

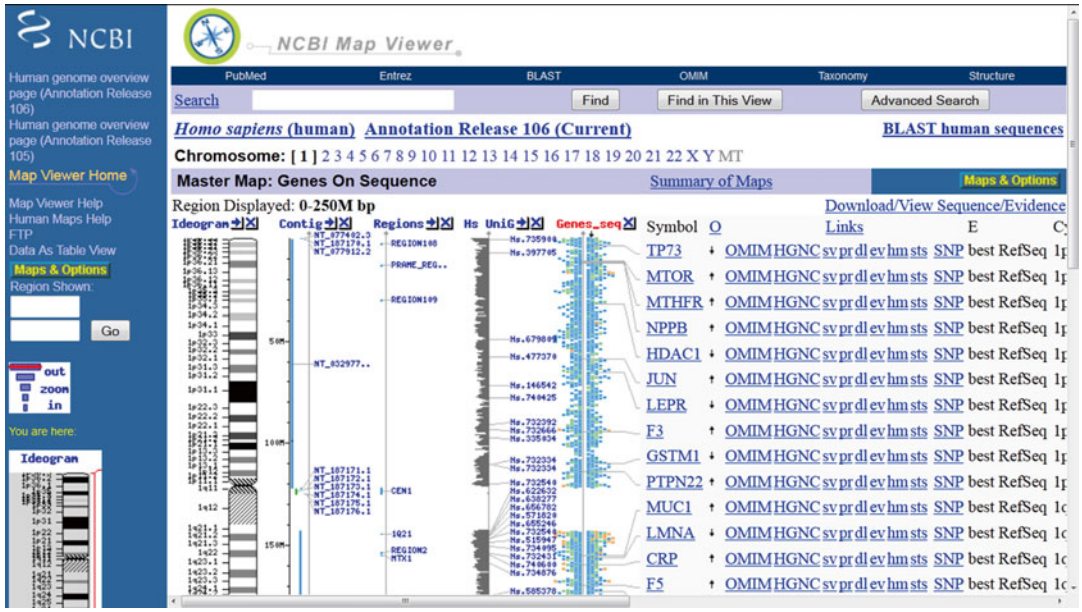


Fig. 13.9 Screen shot of NCBI Map Viewer showing the genes on sequence of *Homo sapiens* (human) chromosome 1

Comparative genomics is another important application of genomics, and its tools like TaxPlot (http://www.ncbi.nlm.nih.gov/sutils/taxik2.cgi) and MUMmer (http://mummer.sourceforge.net/) are useful in comparing genomes of related organisms. Further, genomics has applications in many other areas including genetically modified plants and animals, treating genetic disorder diseases, personalised medicine and so on.

13.4.2 Transcriptomics

Transcriptome is the complete set of mRNA trasncripts of an organism. Transcriptomics performs global analyses of gene expression with the help of high-throughput techniques, such as DNA microarrays.

Some of the important techniques used in transcriptomics are (i) cDNA arrays, (ii) DNA microarrays, (iii) photolithography – 250,000 oligonucleotide spots per sq.cm. and (iv) serial analysis of gene expression (SAGE). The ExPASy transcriptomics server (http://www.expasy.org/transcriptomics) is one of the major servers containing many transcriptomics tools.

13.4.3 Proteomics

Cellular activities like metabolic processes are mediated by proteins of the 'proteome' and not by the genes of the 'genome' or mRNA of the 'transcriptome'. Proteome is the complete collection of proteins in a cell/tissue/organism at a particular time. Unlike genomes which are stable over the lifetime of the organism, proteomes change rapidly as each cell responds to its changing environment and produces new proteins at different quantities.

It is interesting to note that genome is a more stable entity. An organism has only one genome but many proteomes. In the case of human, there may be (i) one body-wide proteome, (ii) about 200 tissue proteomes and about a trillion ($\sim 10^{12}$) individual cell proteomes.

The study of proteomes that includes determining the 3D shapes of proteins, their roles inside cells and the molecules with which they interact and defining which proteins are present and how much of each is present at a given time. The important techniques used in proteomics are (i) two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) used for separation by charge and mass, (ii) matrix-assisted laser desorption/ionisation–time-of-flight (MALDI-TOF) mass spectrometry and (iii) protein microarrays. The ExPASy Proteomics Server (http://www.expasy.org/proteomics) is one of the major servers containing many proteomics tools.

Some of the important applications of proteomics are (i) to correlate proteins on the basis of their expression profiles, (ii) to observe patterns in protein synthesis and this observed pattern changes can be used as an indicator of the state of cell and its gene expression, (iii) to characterise bacterial pathogens and to develop novel antimicrobials, (v) to identify regions of the bacterial genome that encode pathogenic determinants and (vi) to develop drugs. Further, proteomics can also be used as a tool for plant genetics and breeding.

13.4.4 Metabolomics

Metabolites are the small molecules, which are the intermediates and products of metabolism. The total metabolite pool of a cell is called the metabolome of an organism. The research area dealing with metabolic profiling is termed metabolomics. Cells can be profiled with mass spectroscopic techniques to quantify cellular metabolites. In the Human Metabolome Database (http://www.hmdb.ca/), approximately 3,000 known metabolites are deposited.

13.4.5 Systems Biology

Systems biology (Kitano 2001, 2002; Szallasi et al. 2006) is a new perspective and emerging field for research in the postgenomic era. It aims at system level understanding of biological systems. Systems biology is an approach to quantitative study of biological systems that attempts to embrace the complexity of life as a fact of life. Systems biology studies whole cells/tissues/organisms not by a traditional reductionist's approach but by holistic means in a reiterative attempt to model quantitatively the complete cell/tissue/organism. It is an integrated and interacting network of genes, proteins and biochemical reactions, which give rise to life.

The latest high-throughput procedures, namely, genomics, transcriptomics, proteomics, metabolomics and phenomics, have become important technologies that facilitate the functional determination of gene products. The integration of all available data from all these high-throughput 'omics' procedures and analyse them together improves the reliability and the generation of more reliable hypotheses. Systems biology is the new emerging research field that focuses on the integration of various high-throughput 'omics' data because it analyses an entire biological system.

Systems biology aims to produce as accurate a picture as possible of all the regulatory processes within a cell or organism by analysing the interactions between component parts of the biological system, e.g. metabolic pathways, organelles, cells and tissues. Further, the ambitious aim of systems biology is to develop computer models that simulate biological systems and predict consequences upon changing parameters (e.g. changing the concentration of a specific metabolite).

Some of the important software tools and projects in systems biology are (i) Systems Biology Markup Language (SBML), (ii) Systems Biology Workbench (SBW), (iii) E-Cell, (iv) Virtual Cell, (v) Cytoscape and (vi) Virtual Rice Project. Let us briefly explain about them.

13.4.5.1 Systems Biology Markup Language (SBML)

Systems Biology Markup Language (SBML) is an Extensible Markup Language (XML)-based format for representing computational models in a way that can be used by more than 80 software tools worldwide to communicate and exchange those models. Therefore, each calculated model

can be tested in different software environments without additional effort. The home page of SBML is http://sbml.org/Main_Page. The BioModels Database contains several biological models, and it is available at http://www.ebi.ac.uk/biomodels-main/.

13.4.5.2 Systems Biology Workbench

Systems Biology Workbench (SBW) is the software that allows communications between diverse software modules using SBML. SBW 2.7.9 is available at http://sys-bio.org/. Some of the important SBW components are (i) Jarnac, which is a programme for interactive systems biology and (ii) JDesigner 2 is used for modelling biochemical networks.

13.4.5.3 E-Cell

E-Cell project aims to make precise computational whole-cell simulation at the molecular level possible; E-Cell System is an object-oriented software suite for modelling, simulation and analysis of large-scale complex systems such as biological cells, architected by Koichi Takahashi team. The core part of the system, E-Cell Simulation Environment version 3, allows many components driven by multiple algorithms with different timescales to coexist. E-Cell System consists of the four major parts, namely, (i) E-Cell Console, (ii) E-Cell Model Editor, (iii) E-Cell Sessions Monitor and (iv) E-Cell Tool launcher. E-Cell is available at http://www.e-cell.org/ecell.

13.4.5.4 Virtual Cell

The Natural Resource for Cell Analysis and Modelling has developed the Virtual Cell Modelling and Simulation Software to model cell biological processes. The VCell 4.7 Server is available at http://vcell.org/. This new technology associates biochemical and electrophysiological data describing individual reactions with experimental microscopic image data describing their subcellular locations. Cell physiological events can then be simulated within the empirically derived geometries, thus facilitating the direct comparison of model predictions with experiment. The simulations can be run over the Internet. Models developed by VCell can be reused, updated and published so that they are available to the scientific community.

13.4.5.5 Cytoscape

Cytoscape is an open-source bioinformatics software platform for visualising molecular interaction networks and integrating these interactions with gene expression profiles and other state data. Cytoscape 2.6.3 is available at http://cytoscape.org/.

13.4.5.6 Virtual Rice Project

Japan had set up the Rice Simulator Project (Harris 2002) to create an in silico rice plant. This is an experimental scheme funded by MAFF, Japan, to the tune of 1.2 billion Yen. The two main aims of the project are to (i) create a network of databases from sequence, expression and microarray data and (ii) create in silico models of the plant at the level of single cells, tissues and the whole plant. Mathematical models for use in prediction studies will be developed in four areas, namely, (i) knockout simulation, (ii) stress response simulation, (iii) growth simulation and (iv) pollination or breeding simulation. The simulation software being developed under the project will also allow researchers to set up a model for viewing, for example, the effects of a drop in temperature on a plant at various stages of growth such as sprouting, flowering or heading. The Rice Simulator Project will create the technological engine to drive future development of crop varieties.

13.4.5.7 Research in Systems Biology

Research in systems biology requires the collaboration of researchers from diverse backgrounds, including biology, computer science, mathematics, statistics, physics and biochemistry. These collaborations are necessary because of the enormous breadth of background needed for research in this field. These collaborations can be hindered by differing understandings of the limitations and applicability of techniques and concerns from different disciplines.

Systems biology will necessarily be a leading academic and industrial thrust in the years to

come. Its impact on agriculture, medicine, biological energy production and many other areas will make biotechnology a powerful driving force in the near future. Systems biology requires scientists able to cross boundaries between many disciplines. So far, biology has traditionally been an observational science rather than a predictive science. Systems biology is the only hope of understanding biological systems at the predictive level required for disease detection, prevention or cure.

13.4.6 The ENCODE Project

The draft human genome was published in 2000 and the finished human genome was published in 2003. Human genome consists of approximately three billion base pairs of genetic sequence, and it codes for around 20,000 genes that amount to little more than 1 % of the genome only. More than 98 % of the genetic sequence may not be simply 'junk' DNA. The ENCODE project known as Encyclopedia of DNA Elements, which started in 2003, is a massive data collection effort designed to identify the information responsible for the 'complexity' of humans lay somewhere in the 'deserts' between the genes. The aims of ENCODE project are to (i) catalogue the functional DNA sequences in the regions between the genes, (ii) learn when and in which cells they are active and (iii) trace their effects on how the genome is packaged, regulated and read.

After an initial pilot phase, the scientists of ENCODE project consortium started applying their methods to the entire genome in 2007, and the results of that phase were completed in 2012. The ENCODE project consortium (2012) had recently published these results in 30 papers in three major journals, namely, (i) *Nature* (6 papers), (ii) *Genome Biology* (6 papers) and (iii) *Genome Research* (18 papers). The consortium had assigned some sort of function to roughly 80 % of the genome, including (i) more than 70,000 'promoter' regions, which are the sites, just upstream of genes, where proteins bind to control gene expression, and (ii) nearly 400,000 'enhancer' regions that regulate expression of distant genes. These results of the present and future phases of the ENCODE project will prove useful in many areas of science including personalised medicine, in the decades to come.

13.5 Applications of Bioinformatics

There are many applications of bioinformatics in various branches of science including agriculture, pharmaceutical, clinical and biomedical sciences. The major application of bioinformatics is in the computer-aided drug discovery process in the pharmaceutical sciences. The tasks include molecular modelling; molecular dynamic simulation; molecular docking; virtual screening; pharmacophore modelling; absorption, distribution, metabolism, excretion and toxicity (ADMET) properties and so on involved in structure-based and ligand-based drug/inhibitor designing processes.

Bioinformatics is also used in the development and analysis of genetically modified plants and animals. It is useful in identifying the location of genes to be modified, computing various possible gene modifications, the computational analysis on the structure and functions of the modified genes and so on. In addition to computer-aided drug designing, bioinformatics tools can be used in clinical and biomedical sciences especially in the computational investigations of genetic disorders, gene therapy, personalised medicine and so on.

More recently, 'DNA Barcode of Life' (Hebert et al. 2003; Janzen and CBOL Plant Working Group 2009) is an application of phylogenetic analysis of bioinformatics for taxonomical classification of biodiversity. Microgenomic identification systems use a small segment of the genome for taxonomic classification of organisms such as viruses, bacteria and Protista, which are unable to be classified using morphological features. Such molecular phylogeny has been extended from microorganisms to all organisms. Recently, DNA-based identification systems have been applied not only to microorganisms but also to higher organisms. DNA barcoding is to provide a

standardised approach to identify animals and plants by using small fragment of DNA sequences from the same segment of all the genomes, called DNA barcodes. The major goal of DNA barcoding is that anyone, anywhere and anytime will be able to identify quickly and accurately the species of a specimen, whatever its condition and stage of life.

Recently, Hebert et al. (2003) had established that the mitochondrial cytochrome *c* oxidase I (COI or COX I) gene can serve as a core of a global bio-identification system for animals. However, for plants, mitochondrial genes do not differ sufficiently to distinguish among closely related species. In 2005, at the first international barcode meeting, Kress and his colleagues nominated chloroplast genes of about 450 bases, part of a 'spacer' sequence between two genes as barcode for the plants, trnH–psbA. More recently, the consortium for the Barcode of Life (CBOL) plant working group (Janzen and CBOL Plant Working Group 2009) had identified that seven leading plastid DNA regions (atpF–atpH spacer, matK gene, rbcL gene, rpoB gene, rpoC1 gene, psbK–psbI spacer and trnH–psbA spacer) can be used as barcodes for plants. Once fully developed, DNA barcoding has the potential to completely revolutionise our knowledge of biodiversity and our relationship to nature. There are many other applications of bioinformatics including the new areas, namely, genomics, proteomics and systems biology in various branches of science.

13.6 Conclusions

Biological data is growing rapidly and becomes so voluminous. Because of its size, it requires lots of information technology skills to develop more efficient algorithms to analyse the biological data. In this chapter, we explained the need for bioinformatics and also the basic concepts of bioinformatics. Some of the fundamental biological sequence and structure analysis tasks and the bioinformatics tools used are described. The recent developments through the high-throughput experiments, the 'omes' and 'omics' concepts, namely, genomics, transcriptomics, proteomics, metabolomics and systems biology, are also briefly explained. Finally, some of the important applications of bioinformatics are mentioned.

Bioinformatics requires novel methods and algorithms to handle genome sequences as such and also to compare genomes of related organisms. It also requires lots of IT skills not only to develop many new bioinformatics software tools but also to use the existing tools more efficiently. The molecular modelling and docking of drug molecules need tremendous computing skills for designing novel drugs required to control or cure various diseases, which results in huge cost cuts in pharmaceutical industries. Many of the biotechnology and pharmaceutical industries have a separate bioinformatics division. Hence, bioinformatics including biotechnology (BT) have already taken on information technology (IT) for its genomics, proteomics and systems biology projects. Many prominent IT companies like IBM, WIPRO and TCS had already started a separate division for either life sciences or health care. Information technology professionals with some basic knowledge in any branch of life sciences will find bioinformatics as a quite interesting and challenging job. In general, bioinformatics provides lot of job and research opportunities.

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Systems Biology: A New Frontier in Science

14

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Abstract

Systems biology is an emerging field with the potential of making significant contribution to human life and other living organisms. It helps to understand the entirety of life and elucidates basic principles behind the biological life. It captures the complexity of complex biological system and explains the relationship between every gene, transcript, protein, and phenotype. Advancements in functional and structural genomics have enabled the molecular biologists to come a long way toward understanding molecular constituents of the cell. Yet, we fail to understand how organisms function. To date, we face lot of problems in complete understating of several diseases caused to plants, animals, and other productive organisms. To understand underlying biological processes and to find out potential new drug targets, we need to understand complete molecular network systems. Systems biology is an approach based on interdisciplinary fields which focuses on systematic study of complex biological systems using new perspective of holism instead of conventional reductionism. Systems biology follows a holistic approach to understand life by using interactomics, genomics, transcriptomics, metabolomics, proteomics, and informational science. In this chapter, we explained different components of systems biology and its connections with other disciplines. In the future, this integrated science can answer several devastating diseases and mysteries in biological systems. They can be readily tested by computer based models.

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Systems biology • Bioinformatics • Transcriptomics • Proteomics • Metabolomics • Interactomics • Genetic networks • Metabolic networks • Cellular networks

14.1 Introduction

Although systems biology is known for years, it is still relatively new compared to other fields of science. The developments in the different fields of biological sciences have made possible shift from combined molecular and system-level approaches to biological research under the name of *systems biology*. It integrates molecular and structural knowledge, which can best be achieved by the combinatorial use of models and experimental data. In fact, systems biology has several definitions: Systems biology has been described as a comprehensive quantitative analysis of the manner in which all the components of a biological system interact functionally over time (Aderem 2005); this area of work can integrate behavior of complex biological organization and process in terms of molecular constituents (Kirschner 2005); and "systems biology" aims at a quantitative understanding of biological systems to the extent that one is able to predict systemic features (Bork and Serrano 2005). Different types of modeling approaches are useful depending on the amount and quality of the molecular data available and the purpose of the model. Two main approaches of systems biology can be distinguished as *top-down* and *bottom-up*. In top-down systems biology method, characterization of cells can be achieved by combinative model of system-wide data that is obtained from genomics and metabolomics. Those models are often phenomenal but serve to discover new insights into the molecular networks. In bottom-up systems biology method, the study does not start with data, but detailed model of a molecular network on the basis of its molecular properties. In this approach, molecular networks can be quantitatively studied leading to predictive models; they can be applied in drug design and optimization of product formation in bioengineering (Bruggeman et al. 2007; Bruggeman and Westerhoff 2007) as shown in Fig. 14.1. Based on these two approaches in systems biology, we have solved

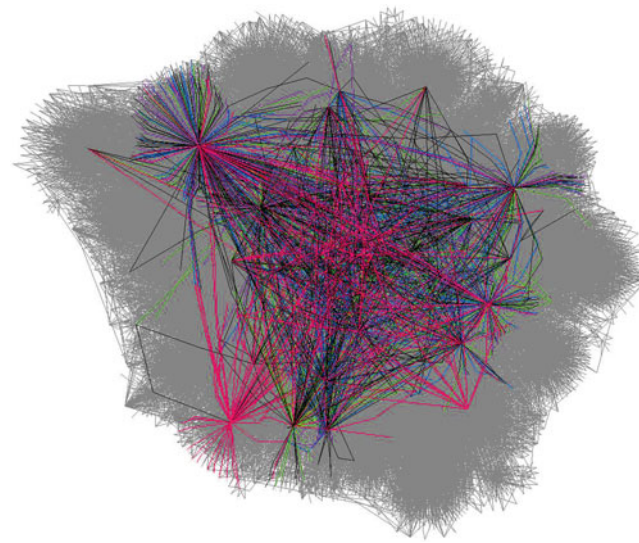

Fig. 14.1 Causal model of cancer: union of all three networks activated by epidermal growth factor receptor (EGFR) drugs – (*blue*) drug1, (*yellow*) drug2, (*green*) drug3, (*black*) two of the above, and (*pink*) all of the three

attained number of successes. The most important consequence of Human Genome Project (HGP) and structural genomics is that these are pushing scientists toward new view of biology. Systems biology investigates neither individual gene nor protein one at a time, rather investigates in a network manner. This data can be integrated, graphically displayed, and used for computations. In this respect, complete genome sequence is crucial in systems biology; the complete genome sequences of human (International Human Genome Sequencing 2004; Lander 2011; Schmutz et al. 2004), nematode (Harris et al. 2014; van der Velde et al. 2014), fly (Levine and Malik 2011), *Arabidopsis* (Arabidopsis Genome 2000; Meinke et al. 1998), yeast (Bassett et al. 1996; Levy 1994; Zagulski et al. 1998), and several microbes and parasites are in the pipeline. HGP has improved the ability of the cells systematically; it is also facilitated new technologies for systematically characterizing the cellular response: DNA sequences, microarrays, and high-throughput proteomics. Alternatively, gene-expression data could be combined with either information on protein-protein interactions and protein phylogenetic profiles (Chen and Xu 2003; Rao et al. 2014) or with the genomic location of each gene to find shared regulatory elements (Barrera and Ren 2006; Bischof et al. 2013). Finally, it is difficult to decide significant changes in expression level and is also an active area of research. Gene-expression clustering and its extensions, method of expression data analysis, have been reviewed elsewhere (Brazma and Vilo 2000; Claverie 1999; Heyer et al. 1999; Sherlock 2000). Although this analysis has been certainly informative, global data sets undoubtedly provide additional information that remains untapped. Ultimately, it would be highly desirable to analyze expression levels and other global measurements in a way that it validates our current knowledge of a cellular process and isolates discrepancies between expected and observed levels. To achieve this level of analysis, the model of biological process of interest is well defined with comparative and integrated global data.

14.2 The Increasing Importance of Computer Models

Conventionally, a biological model begins in the mind of the individual researcher as a proposed mechanism to account for some experimental observations. Often, the researcher represents his/her ideas by sketching a diagram using pen and paper. This diagram is an incredible aid in thinking evidently about the center of the idea by framing a model to predict possible experimental outcomes. To convey this model, not surprisingly, diagrammatic representation of models is discussed in journals, textbooks, presentations, and lectures.

Despite the use of these conventional models is simple, advances in systems biology are prompting some biologists to forego mental models or pen-and-paper diagrams to more sophisticated computer representations. The notion of modeling a biological process computationally is almost as old as the computer; such models are becoming popular for several reasons. First, it is apparent that the comprehensive interactions in a cell are too complicated and unpredictable to process and organize the interactions (Bhalla 2003; Eungdamrong and Iyengar 2004; Gianchandani et al. 2006; Weng et al. 1999). Second, the tradition of whole genome sequencing, high-throughput microarray data, and state-of-the art technology begin to generate a vast amount of biological data; now, the idea of descriptive science is shifting toward predictive science (Bhalla et al. 2005; Gilbert 1991; Sakaki 2005; Weatherall 1999; Yu et al. 2008). Computer systems play an important role in storing, sorting, and condensing the accumulating mass of data rapidly; in addition, automated tools are assimilating these data into a network model; it can predict network behaviors and outcomes that may be tested experimentally. It is encouraging that recent computer simulations of partial or whole genetic networks have demonstrated network behaviors, commonly called systems properties or emergent properties, that were not apparent from examination of a few isolated interactions alone (Cleary et al. 2014; Dall'Olio et al. 2014; Weckwerth 2011).

The molecular biosciences deliver lots of data that require systems approaches to understand their implications for cell function. The more systems-oriented sciences avail of several systems approaches that have worked well for inanimate systems. Not all molecular bioscientists are familiar with current mathematical methods, nor are all mathematicians aware of acceptable biological mechanisms. Of course, implementation of mathematics to large data sets will deliver results that might look remarkable enough to be published. However, unless systems biology is carried out correctly, it might not deliver an understanding of cell function that is based on molecular interactions.

14.3 Genetic Networks

To understand biology at the system level, we must examine the structure and dynamics of cellular and organismal function, rather than the characteristics of isolated parts of a cell or organism (Kitano 2002). Cell is not just an assembly of genes and proteins. Systems biology complements molecular biology. Genomics has provided vast amount of information, which links gene activity with diseases. However, there are a number of reasons why gene sequence information does not provide a complete and all properties of proteins and their function. Because of piecewise nature, the current approach of predicting each gene function based on sequence similarity searches often fails to reconstruct cellular functions.

14.3.1 Complete Pathway Simulations in the Cell-E-Cell

The complete software environment for simulation of activities of a whole cell (Fig. 14.2) is called an E-cell (electronic cell). In general, scientific work on biochemical and genetic simulations has helped in understanding individual pathways, but methods for building integrative models of gene regulation, metabolism, signaling, and phenotypic expression have not been established. Hence, scientists have evolved a new approach, involving the development of software environment for building such integrative models based on gene sets and coordinated network of genes to conduct experiments in silico. E-cell is hence an approach for modeling and simulating environment for understanding biochemical and genetic processes. This system allows a user to define functions of proteins, protein-nucleic acid, and protein-protein interactions. The E-cell is

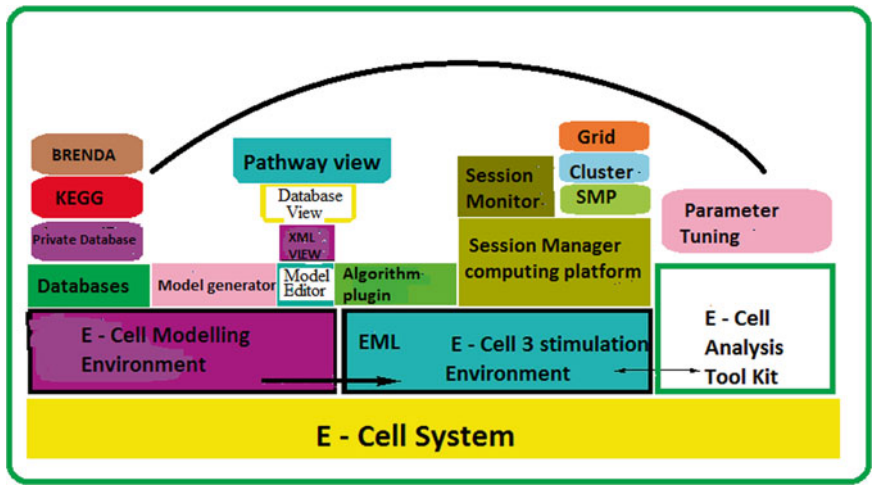

Fig. 14.2 Diagrammatic representation of E-cell

majorly modeled using three classes of objects: genes, products, and reaction rules.

This new field requires a strong combination of computational approach with biological data. E-cell modeling is an art of converting biology into numbers and entities. A model is an optimal mix of hypothesis, evidence, and abstraction to explain a phenomenon.

The following biological activities would be important to model a complete virtual cell:

1. Metabolic pathways
2. Cell membrane dynamic
3. Intracellular molecular trafficking
4. Signaling pathways
5. Membrane transport and ion channels
6. Cell division
7. DNA replication and repair
8. Transcription
9. Translation
10. Aging

A cell represents a dynamic environment of interactions among nucleic acids, proteins, carbohydrates, ions, pH, temperature, stress, and environmental signals. Three elements are necessary to make a good model: (a) precise knowledge of biological phenomenon, (b) an accurate and appropriate conversion of biological data into mathematical data, and (c) good simulation tools. Mapping cellular networks is always a challenging job, especially in the presence of a large number of cross-link pathways.

(a) Linear chains: represent a unidirectional flow of information or energy.
(b) Branched chains: one enzyme acts on one substrate and results on two products.
(c) Loops: two or three branches unite, giving rise to inherent dependencies between them.
(d) Cycles: larger loops composed of many intermediates, having one overall entry and one exit point in the system.

The resultant model structure will ultimately be custom built to the user's needs and not according to the software abilities. The goal is that by combining model building with algorithm implementation, the modeling of any system within the E-cell simulation environment should be easy and open ended. Large-scale complex models can be best created and composed in arbitrary algorithms.

14.3.2 Applications of E-Cell

(a) *A model library of bacterial chemotaxis*: Bacterial organisms like *Escherichia coli* have developed stress signaling mechanism to detect and direct cell movement toward the substrate when stared or exposed to unfavorable conditions. Such behavior is known as chemotaxis. This behaviour of chemotaxis is demonstrated in http://www.e-cell.org initial amount of substances, kinetic parameters are taken from the original models and published papers (Bray et al. 1993; Mello and Tu 2003; Morton-Firth et al. 1999). Both deterministic and stochastic models of chemotaxis have been successfully implemented.
(b) *Electrophysiological simulations on cardiomyocytes*: The cardiac membrane contains various ionic channels and pumps that allow specific ions to travel as well as exchanged through the membrane. Models from the action potential simulation at different developmental stages were constructed on the basis of Kyoto and Luo-Rudy models. The latest version of the Luo-Rudy model was implemented in the E-cell version 3.1.
(c) *Model and simulations of human erythrocyte metabolism*: The simulation studies enabled us to see the rational design of metabolic pathways in human erythrocytes as an evolutionarily optimized system. Mathematical and logical models can be applied not only for straightforward simulations, but also for the nature of explanations of the nature of network properties complying nonlinear dynamics in the cell. The metabolic model of human erythrocyte on E-cell system, which consists of glycolysis, pentose phosphate pathways, nucleotide metabolism, simple membrane transport systems, and the ATP-dependent Na^+/K^+ pump, carried out simulation analysis of G6PDH. This basic model was developed by Joshi and Palsson (1990).

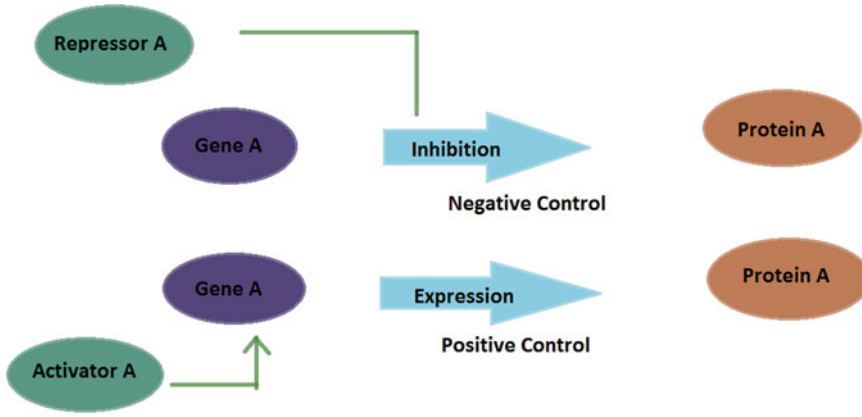

Fig. 14.3 Diagrammatic representation of negative and positive control in feedback-dependent signaling system

(d) *Modeling of mitochondrial energy metabolism*: This model includes 58 enzymatic reactions and 117 metabolites to represent the respiratory chain, TCA cycle, fatty acid β-oxidation, and inner membrane metabolite transporters. In this model specially inner membrane metabolite transporters were included to allow simulation of metabolite insertion from outside mitochondrion (Yugi and Tomita 2004). This model was based on published kinetic parameters. With this model, it is possible to compare mitochondrial ATP production in vivo and in silico and also comprehensive profiling of intracellular metabolite concentrations.

14.3.3 Genetic Network Analysis

Living cells can be viewed as signal processors also. They will have metabolic and genetic network systems. The purpose of the network is to control the life processes so that the organism can grow, survive, and multiply in a constantly changing, unfavorable, and challenging environment. The cells receive signals from the environment in the form of hormones, nod factors, nutrient levels, and/or stresses (cold, heat drought, toxins). The cells have intra- and intercell signaling, e.g., events such as DNA damage, cell division, and chromosomal duplication. These cell signals may start repair or begin apoptosis.

An essential mechanism in cell regulation is feedback (Fig. 14.3). Negative feedback is a system where the product or output downregulates the process or system. This is very common in metabolic reactions and amino acid synthesis pathways. Positive feedback is a signaling loop where the product reinforces the process. Cyclical processes are processes that never reach a steady state but move between stages and states in an ordered fashion. The cell cycle is a prime example and another one circadian clock in flowering plants.

14.4 Metabolic Networks

Metabolism is the totality of all the chemical reactions that operate in a living organism. It performs two types of reactions: (a) catabolic reactions where breakdown of larger molecules produces energy and (b) anabolic reactions which involve the use of energy and build up essential cell components. The current approach of predicting each gene function based on sequence similarity searches often fails to reconstruct cellular functions with all necessary components. Metabolic pathway data is used in systems biology to predict the biological function of a partic-

ular genome sequence either by activation or interference of pathway intermediates. For instance, the metabolic pathway diagram from KEGG (Kyoto Encyclopedia of Genes and Genomes) database (Fig. 14.4) organizes and digitalizes all current knowledge of molecular and genetic pathways from experimental observations. It maintains the catalog of every organism that has been sequenced and the mapping of each gene product onto a component in the pathway. The main aim of KEGG database is to describe, predict, and possibly design systems behavior of living organisms. The KEGG database consists of two sections: (a) metabolic pathway section and (b) regulatory section. The first one is well organized in KEGG database, whereas regulatory pathways are still rudimentary. KEGG is based on binary relations, which help in drawing the pathway diagrams based on successive conversion of various states. The substrate-product binary relations have been utilized in computing possible reaction paths, and the enzyme-enzyme binary relations have been correlated with other types of molecular relations. In metabolic networking systems, three types of links are used in path calculations:

1. Factual link – cross-references in databases – links between genes and functions
2. Similarity link – sequence homology (orthologous/paralogous) – 3D similarity and complementarity
3. Biological link – substrate–product relations in enzymatic reactions – interacting molecules in a cell neighboring in the genome

The WIT (what is there) (http://wit.mcs.anl.gov/WITs2) system has been designed to support comparative analysis of sequenced genomes and to generate metabolic reconstructions. WIT works based on modules from EMP/MPW family of databases. The Enzymes and Metabolic Pathways (EMP) database is an encoding of the contents of more than 10,000 original research publications on the topics of enzymology and metabolism; a project, which was started November 2006, uses the model bacterium *Bacillus subtilis* (*B. subtilis*) to gain insight into the global structure of the regulatory networks that control bacterial metabolism.

14.4.1 Applications of Metabolic Network Models

1. Predict drug targets for metabolic disorders
2. Dispensability of metabolic genes
3. Robustness and evolution of metabolic networks
4. Bioengineering and overproduction of desired compounds
5. Knockout lethality
6. Nutrient uptake/secretion rates

14.5 Transcriptional Networks

Transcriptional control is embedded into a hierarchical flow of information from gene to phenotype, in which many regulatory steps occur (Fig. 14.5). Data need to be generated about the network components at all the levels of the information flow, in order to understand, at the system level, the global regulation of gene transcription in bacteria. The modeling experimental strategy developed in *B. subtilis* model, when validated, can lead to an understanding of regulatory networks controlling pathogenesis in disease-causing bacteria. The BaSysBio has started on November 2006 and has been developing and implementing high-throughput technologies for the quantitative determination of the cellular transcriptional responses. To discover and monitor the relevance of each protein to a biological process in the cellular networks, it is important to identify where, when, and to what extent a protein is expressed. Two of the tools which are extensively used for protein characterization are (a) microarrays and (b) mass spectrometry approaches. A microarray is a set of short expressed sequence tags (EST) made from a cDNA library of a set of known gene loci or partial known loci. The ESTs are spotted onto a cover-slip-sized glass plate. A complete set of mRNA transcripts is prepared from the tissue of an experimental treatment or an individual with breast cancer. Complementary DNA reverse transcripts are prepared and labeled with red fluorescent. A control library is prepared from an untreated source or noncancerous breast tissues;

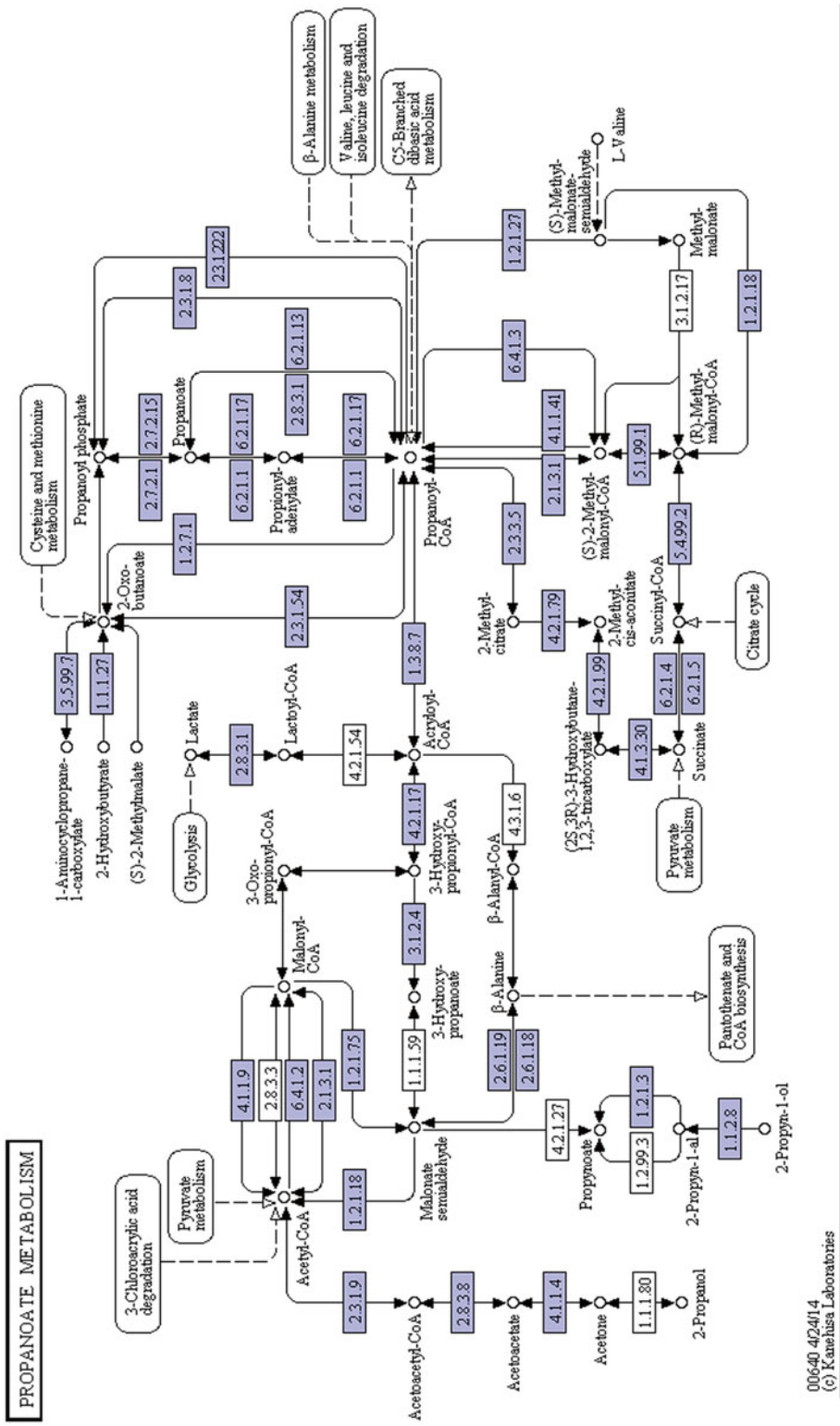

Fig. 14.4 Pathway of propanoate metabolism, obtained from KEGG database

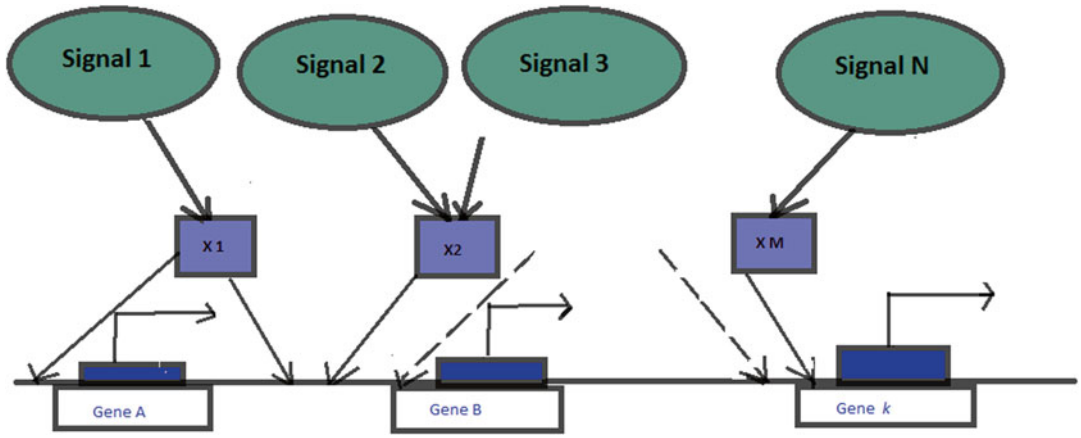

Fig. 14.5 Schematic representation of transcriptional signaling

this library is labeled with different fluorescents. The experimental and control libraries are hybridized to the microarray. Relative gene expression is measured as the ratio of the two fluorescence wavelengths.

(a) *Upregulation*: Genes in the experimental transcriptome relative to the control will be visualized as a hotter red.
(b) *Downregulation*: Genes in the experimental transcriptome show less green fluorescence than control.
(c) *Constituent expression*: Genes in the experimental transcriptome exhibit equal expression to that of control set, genes to control ratio is 1:1; neutral black fluorescence will be observed.

DNA microarray technology can accomplish this because mRNA and protein concentration are often correlated. In mass spectroscopy approach method, mixture of proteins in cellular extract is resolved, and individual proteins are identified using MALDI-TOF and MS peptide finger printing. Although in theory mass spectroscopic approaches have the potential to characterize the entire protein complement of a cell, in practice it has proved difficult to identify proteins of low abundance. Systems biology plays a major role in providing connecting links in whole cell protein networks.

14.6 Cellular Signaling Networks

Cell signaling is a complex system of communication that governs the basic cellular activities and actions. The cell converts one signal to another in a network fashion. In these networks, proteins are nodes and edges between them are directed. Building complex models of signaling networks is best accomplished in a stepwise manner (Fig. 14.6). Systems biology plays a major role in understanding complex networks with limited experimental data also. In this case it may help in identifying the types of data that are still unknown and are needed for better understanding the system. For example, when modeling growth factor activation of MAP kinase, one may use input of activated RAS. Thus, the output may be dually phosphorylated MAPK. Several modeling environments are available that can be used to develop kinetic simulation out of the three computer-based programs, which help model the cell signaling networks with ease.

(a) GENESIS/Kinetikit: It makes cellular networks based on assumption that "well-stirred" reactions where every molecule has equal access to any other molecule. It runs with large-scale General Neural Simulation System (GENESIS).
(b) Gepasi 3: Gepasi 3 is a modeling platform that allows the simulation of signaling pathways. It runs on Windows operating system

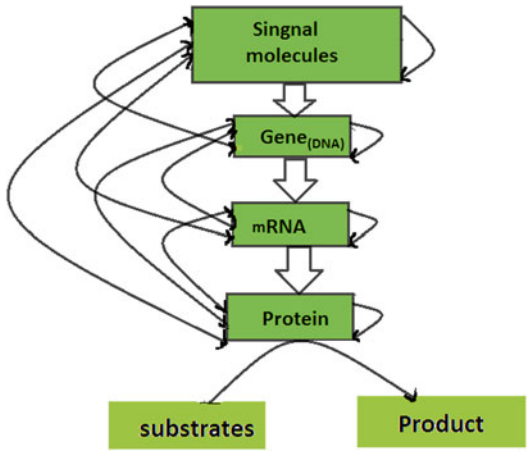

Fig. 14.6 Depiction of wiring diagram for the cell signaling information network

and it performs steady state and time-course simulations. It has parameter scan capability. The user can select parameters that will be varied, and the range and extent of variation can be compared with experimentally observed data. This feature can be used to optimize unknown parameters.

(c) Virtual Cell: The model is built by using a graphical interface that allows the user to determine the geometry of the model. This usually involves a collection of molecules, with their respective parameters and subcellular localizations. It gives the output in different formats: spreadsheets, pseudocolor images, and QuickTime movies.

14.7 Systems Biology and Personalized Medicine in the New Era

Starting from the last century, advances in high-throughput biological data capturing instruments such as DNA sequencers, microarrays, mass spectroscopy, electron microscopy, X-ray crystallography, and NMR made it possible to routinely perform structural and functional genomics on complex biological samples. In isolation the study of genes, transcripts, proteins, and metabolites in the context of human physiology and pathology has been challenging, because these molecular entities must ensemble into molecular machinery and coordinate in molecular pathways to play their role inside the cells. Systems biology studies can help interpret molecular functions and bridge molecular understanding of human diseases. Electronic databases play a central role in storing and analyzing clinical data, which has been useful for conventional clinical decision-making. The establishment of connections between clinical data and molecular data requires integrative models to help enhance medical decision-making. In the near future, development of systems biology may help physicians to prescribe a drug to their patients based on "proteomics profiling" and "genomics profiling." This may help in determining correct subtype of the disease. The "genome-wide genotyping" test of patients would help to look for possible genetic variability that helps in determining the right dosage. Personalized medicine has been adapting to a new economic reality (Osnabrugge et al. 2014). Given that ~60 % of the prescriptions written produced desired benefits, 30–40 % of prescribed medicine do not help and up to 7 % have hard or negative impact on patients. Now recent advances in systems biology and ensemble of omics (genomics, proteomics, transcriptomics, and metabolomics) made physicians to predict accurate drug targets which can virtually guarantee patient that the drug they were prescribed would help them without causing any side effects.

14.8 Reverse Vaccinology and Pan-genomics

Classical approaches to vaccine development are time consuming and economically costly, biased toward the identification of several antigens which may not lead to immunity. It is also dependent on the ability to cultivate the pathogen under laboratory conditions. The recent advances of systems biology have led us into a new era of vaccinology. The "omics" field has provided us with a continuous flow of data to look at host-pathogen interactions in a holistic way. Genome sequences of many pathogens including viruses are available. In the future, many vaccines previously thought impossible can be designed by

using reverse vaccinology (based on pathogen genome information) method (Fig. 14.7). In addition, the comparison of the genomes of multiple strains of a single pathogen, known as "pan-genomics," has expanded our understanding of virulence (Tettelin 2009). In the coming years, systems biology will play a major role in solving deadly diseases by integrating information from other disciplines.

14.9 Single Nucleotide Polymorphism (SNP) and HapMap Applications in Mapping Disease Loci

SNP is a genetic variation in a person's DNA sequence that occurs when a single nucleotide is replaced by any one of the other three nucleotides. Identification of genes that confer disease susceptibility can be found by studying DNA markers such as SNP associated with a disease trait. Genome-wide association study (GWAS) offers a systematic analysis of the linkage of thousands of SNPs with a quantitative complex trait. The methodology was successfully applied to a wide variety of common diseases and traits and has generated valuable findings that have improved the understanding of the genetic basis of many complex disease traits. For example, in recent studies on lipid metabolism by genome wide analysis, found 19 loci are being associated with LDL/HDL or triglyceride levels.

The HapMap is a catalog of common genetic variants that occur in human beings. The elucidation of the entire human genome has made possible our current efforts to develop a haplotype map (Fig. 14.8). The "HapMap" is a tool that allows researcher to find genes and genetic variations that affect human health. The HapMap is valuable for it reduced the number of SNPs required to examine the entire genome for association with a phenotype from the ten million SNPs that exist to roughly 500,000 tag SNPs. This allows the development of test to predict which drugs or vaccines would be most effective in individuals with particular genotypes.

14.10 Conclusions

Computational biology and experimental biology for many decades have been separate disciplines. The dramatic progress of molecular biology and completion of the sequences of many genomes have provided us with a lot of data to be interpreted. This required an integration of theoretical and experimental approaches at the highest levels. This integrated field rapidly evolved into systems biology. The present dominant research paradigm of one laboratory-one gene-one function-one pathogen has reached its limits in many areas, as disease arises from a combination of several factors. A systems approach to life sciences is often necessary for better qualitative and quantitative understanding of biological systems. Systematic understanding of complex biological processes will allow taking many real-world problems. This is not achieved by better data alone. Rather, the way the model is built and software used makes the calculations critical. Systems biology might therefore be one of the key approaches of the twenty-first century in providing solutions to cellular biology, physiology, and medicine and also for pharmaceutical and biotechnology industries.

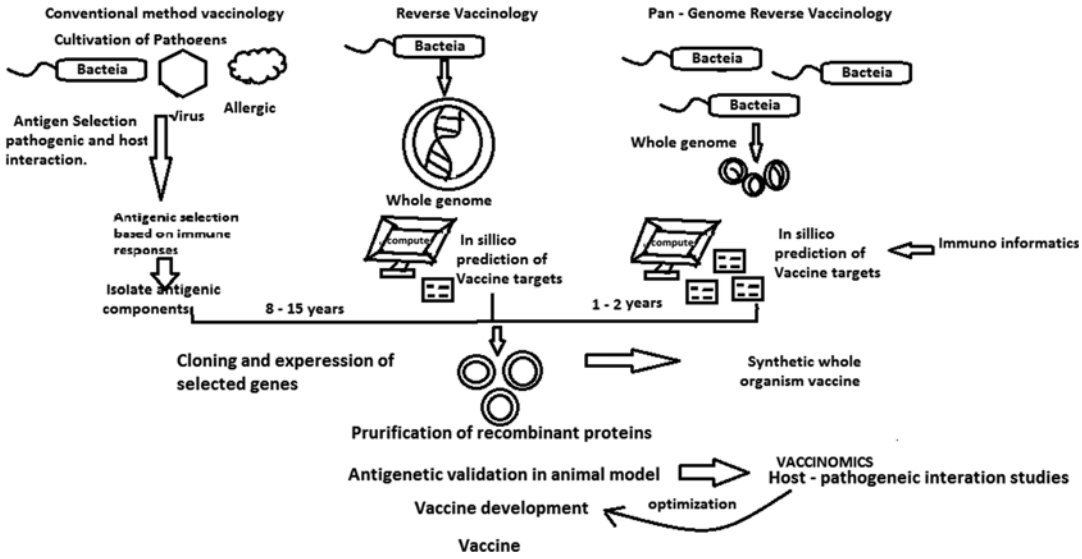

Fig. 14.7 Diagrammatic representation of conventional, reverse, and pan-genome reverse vaccinology

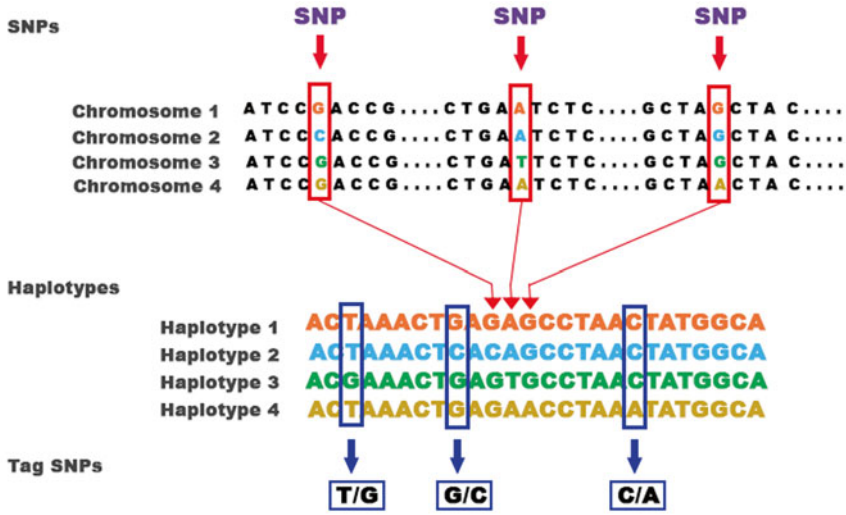

Fig. 14.8 Diagrammatic representation of HapMap and tagSNPs

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Somatic Embryogenesis

15

Leela Sahijram and Bir Bahadur

Abstract

In addition to natural embryogenesis termed "apomixis" in some species, plant cells can also be induced to form embryos in plant tissue culture. Such embryos are called *somatic embryos* and can be used to generate plants from single cells. Plants possess cellular totipotency which makes it feasible for individual somatic cells to regenerate into a whole plant in vitro, a process termed "somatic embryogenesis" (SE). Development of somatic embryos closely resembles that of zygotic embryos, both morphologically and physiologically. Since the first reports of this phenomenon in 1958 in carrot, SE has been reported in innumerable plant species. SE with a low frequency of chimeras, high efficiency of regenerates, and limited occurrence of somaclonal variation is more attractive than organogenesis as a plant regeneration system. This can be successfully applied to plant transformation, in vitro mutagenesis, and selection. Several factors, however, are known to influence initiation and progression of somatic embryogenesis in plants.

Keywords

Somatic embryo • Embryogenesis • Organogenesis • Regeneration systems

15.1 Introduction

Somatic embryogenesis (SE) is the process by which somatic cells, under conditions suitable for induction, generate embryogenic cells, which go through a series of morphological and biochemical changes that result in the production of a bipolar structure without vascular connection with the original tissue (Solis Ramos et al. 2012). SE with a low frequency of chimeras, a high number of

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regenerants, and a limited level of somaclonal variation is more attractive than organogenesis as a plant regeneration system (e.g., in genetic transformation, in vitro mutagenesis, and selection). However, several factors influence initiation of SE in plants. Most of the important crops and grasses are recalcitrant to in vitro culture, which hampers the development of reliable regeneration techniques in these plant groups. This phenomenon was first reported in carrot (Steward et al. 1958), and ever since, SE has been reported in innumerable plant species. Somatic embryogenesis poses even greater challenges in recalcitrant plants; e.g., two plant species, habanero chili (*Capsicum chinense* Jacq.) and coconut palm (*Cocos nucifera* L.), have been studied in some detail. Several factors, however, are known to influence initiation and progression of somatic embryogenesis in plants (Yalcin Elidemir et al. 2007; Perrin et al. 2001).

Somatic embryos are formed from plant cells that are not normally involved in the development of (zygotic) embryos, i.e., ordinary plant tissue. No endosperm or seed coat is formed around a somatic embryo. Applications of this process include clonal propagation of genetically uniform plant material; elimination of viruses; provision of source tissue for genetic transformation; generation of whole plants from single cells called protoplasts; development of synthetic seed technology, etc.

Cells derived from competent source tissue are cultured to form an undifferentiated mass of cells, called a callus. Plant growth regulators in tissue culture media can be manipulated to induce callus formation and, subsequently, changed to induce embryos to form from the callus. Ratio of various plant growth regulators required for induction of callus or embryo formation varies with type of the plant. Asymmetrical cell division also appears to be important in developing somatic embryos; and, while failure to form the suspensor cell is lethal to zygotic embryos, it is not lethal to the somatic embryos.

15.2 Plant Regeneration: Somatic Embryogenesis Versus Organogenesis

Organogenesis and somatic embryogenesis, two patterns of in vitro differentiation, are distinctly different processes that also occur in nature. Adventive embryony within ovules of polyembryonic plant species, including many fruit and forest trees, is identical with in vitro somatic embryogenesis. Adventitious shoot meristems and adventitious roots that develop in vivo from many different tissues are identical with in vitro organogenesis. Since somatic embryogenesis and organogenesis reflect different developmental events, Ammirato (1985) suggested that it is likely that the two processes are mutually exclusive, so that cells can be committed to only a single pathway.

15.2.1 Somatic Embryogenesis

Somatic embryo is an embryo formed in vitro from somatic cells or embryogenic cells by mitotic cell division. "Mature somatic embryo," however, is a fully developed embryo with evidence of root and shoot apices and exhibits a bipolar structure. Preferred mature somatic embryos are those with well-defined cotyledons. Rangaswamy (1986) and Ammirato (1989) have reviewed the phenomenon of somatic embryogenesis.

Development of somatic embryos directly from intact explants (without an intervening callus phase) is known as primary direct somatic embryogenesis (Ammirato 1989). According to Quiroz-Figueroa et al. (2006), somatic embryos originate by two pathways: (i) unicellular and (ii) multicellular. When somatic embryos have unicellular origin, cell division is seen to be coordinated, and the somatic embryo is sometimes connected to the maternal tissue by a suspensor-like structure. In contrast, somatic embryos of multicellular origin

are observable initially as a protuberance, showing no coordinated cell division, and those somatic embryos here that are in contact with the basal area are typically fused to the maternal tissue. Srinivasan and Mullins (1980) demonstrated SE from unfertilized ovules of seedless grapes, while Salunkhe et al. (1997) developed SE and subsequent plantlet formation from tendrils of grape plants. Carimi et al. (2005) could induce somatic embryogenesis in stigmas and styles of grapevine. Ontogenesis, differentiation, and precocious germination in anther-derived somatic embryos of grapevine through proembryogenesis have also been studied (Faure et al. 1996).

15.2.2 Organogenesis

Cersosimo et al. (1990) reported organogenesis, embryogenesis, and plant regeneration from cultures of *Vitis*. Organogenesis has also been reported by Mezzetti et al. (2002) wherein they used the technique for genetic transformation of grape.

Two different cultivars and two rootstocks were used in the study of Bayir et al. (2007) where they studied the effect of genotype on callus formation and organogenesis. MS medium supplemented with BAP and 2, 4-D was found to be the best for callus formation for all grape cultivars/rootstocks used.

Venkatachalam et al. (2006) induced direct shoot and cormlet regeneration from leaf explants of "silk" banana (AAB), while Novak et al. (1989) induced indirect somatic embryogenesis and plant regeneration in suspension cultures of dessert (AA and AAA) and cooking (ABB) bananas (*Musa* spp.).

A procedure for the regeneration of *Vitis* plantlets by organogenesis (Kwon et al. 2000) and in rootstocks (Clog et al. 1990) from foliar tissues was described earlier. Leaves from mature plants grown in growth chambers, or from plantlets grown in tubes, were wounded with a scalpel and cultured on modified Murashige and Skoog liquid medium containing different concentrations of benzylaminopurine. Presence of benzylaminopurine was required for shoot formation. Age of the source explant, composition of the culture medium, and culture incubation temperature were found to be important parameters for regeneration.

Kwon et al. (2000) optimized medium composition and culture conditions for plant regeneration through organogenesis from leaf explant in "Kyoho" grape (*Vitis labrusca* × *V. vinifera*). Adventitious shoot formation from in vitro-cultured leaf segments was affected by basal medium, plant growth regulator, and light condition. Within the first 7 days of culture on shoot regeneration medium, the petiolar stub of explants was swollen and formed callus. Adventitious structures formed on the petiolar stub after 14 days. Among plant growth regulator treatment, 2.0 mg/L BA and 0.01 mg/L IBA produced the highest shoot regeneration frequency. Adventitious shoots were regenerated effectively on the modified MS medium such as 1/2 TMS and 1/2 MMS. Among three gelling agents, agar showed higher shoot regeneration than agarose and Phytagel. The dark incubation for 3–4 weeks at the initial culture periods was effective for shoot regeneration. In conclusion, the most excellent shoot regeneration from leaf explants was obtained with dark incubation of initial 3 weeks on 1/2 TMS medium with 2.0 mg/L BA, 0.01 mg/L IBA, and 8 g/L agar, in which the regeneration frequency reached up to 80 %. The regenerated shoots rooted successfully in MS medium with 0.1 mg/L IAA, and plantlets were acclimated.

In addition to the above, the "embryonic condition" is considered to also occur in buds that form on stems. The buds have tissue that has differentiated but not grown into complete structures. They can be in a resting state and lying dormant over the winter or when conditions are dry and, then, commence growth when conditions turn favorable. Before they start growing into stem, leaves, or flowers, the buds are said to be in an "embryonic" state.

15.2.3 Factors Governing Somatic Embryogenesis

Several factors are known to influence induction of somatic embryogenesis (Gaj 2004) and maturation of the so-formed somatic embryos. Chief among these are:

1. Genotype of plant

2. Type of plant (plant species)
3. Plant/explant age
4. Developmental stage of the explant
5. Physiological status of the explant donor plant/explant (including endogenous levels of plant growth regulators)
6. Environment external to the explant, viz., composition of culture medium (chemical), and physical culture conditions (incubation temperature, quantity/quality/duration of light)

Polar transport of auxin in early globular embryos is essential to establish bilateral symmetry during plant embryogenesis. Interference with auxin transport causes failure of transition from the axial to bilateral symmetry, thereby resulting in embryos with fused cotyledons (Liu et al. 1993).

Details of factors governing SE are elaborated later in the chapter under grape, wherein the crop has been treated as a case study.

15.2.4 Genes Involved in Somatic Embryogenesis

Some genes have been shown to be related to expression of somatic embryogenesis, such as *WUSCHEL* (WUS), *Baby Boom* (BBM), and *SERK* (somatic embryogenesis receptor kinase). The last of these three, *SERK*, has been detected in early stages of the process of somatic embryogenesis and forms a subgroup in the leucine-rich repeat receptor-like kinases (LRR-RLKs) comprising the largest subfamily of RLKs in plants. These genes are also related to key processes in plant growth (Sharma et al. 2008).

15.3 Grape: A Case Study

Somatic embryogenesis is the most common regeneration system for grapevine (*Vitis* spp.) (Gribaudo et al. 2007). After rice, the second food crop to be fully sequenced genomically is the grape (var. Pinot Noir). This is viticulture's equivalent of the first moonwalk. The genetic blueprint will make it possible to develop new, more resistant varieties (www.biotech-weblog.com). Thus, grape is the first fruit crop genome to be mapped. The far-reaching consequences of having sound regeneration systems in grape to plumb the wealth of genetic information, therefore, cannot be undermined. However, implementation of these strategies for grape improvement requires the ability to manipulate and achieve regeneration from cells and tissues in vitro. Somatic embryogenesis being central to such manipulations encompasses culture initiation, maintenance, manipulation of somatic embryos, and plant development and, finally, incorporates specific uses of embryogenic culture systems. For perennial embryonic culture system (e.g., grape), several methods have proven effective for regeneration by somatic embryogenesis.

15.3.1 Regeneration System in Grapes

Somatic embryogenesis of grapevine has been described in several instances. The first description of regeneration of plantlets by the route of somatic embryogenesis was reported for an ancient clone of grapevine *(Vitis vinifera* L. cv. Cabernet Sauvignon) in liquid culture conditions (Coutos-Thevenot et al. 1992). Other cultivars have been shown to be competent for somatic embryo production, and different explants like leaves, anthers, zygotic embryos, and ovaries were used for somatic embryogenesis induction (Rajasekaran and Mullins 1983). Somatic embryogenesis was observed by Salunkhe et al. (1997) in callus initiated from tendril explants of *Vitis vinifera* L. cvs. Thompson, Sonaka, and Tas-e-Ganesh on Emershad and Ramming medium supplemented with 1 μM 6-benzylaminopurine.

Current methods for improving grapevines are time-consuming and labor intensive. For example, genetic improvement in grapes through conventional breeding is severely limited by a number of factors such as long prebearing age and varying ploidy levels. Cultivated grapes are also highly heterozygous and do not generally breed true from seeds. Moreover, grape breeding programs are expensive, long-term projects. Although plant biotechnology is an attractive alternative for

genetic improvement of grapes, in vitro genetic manipulation can be addressed only if there is an effective regeneration system. Accordingly, methods that reduce any of these problems would represent a significant advancement in the art.

Methods for growing perennial grape embryogenic cultures, and for growing large quantities of somatic grape embryos from such perennial embryogenic cultures, in a relatively short period using liquid suspension culture are available. Several advantages are provided by the present methods. These approaches, for example, facilitate an extraordinarily high frequency of somatic embryo formation and plant regeneration. Such frequencies have not been previously reported for grapevine regeneration of any known cultivar and render the method useful for large-scale production of clonal planting stock of grape plants. In addition, the methods produce embryos free of such common abnormalities as fusion and fasciations of somatic embryos. The methods of the invention also result in enhanced embryogenic culture initiation frequency, allowing for the production of highly embryogenic cultures that can then be successfully carried through the subsequent stages of the regeneration process to the whole plant level. Because of these advantages, the methods of the invention are especially useful in the application of biotechnology for the genetic improvement of this crop.

Production of an embryogenic perennial culture of *Vitis vinifera* cv. Thompson Seedless: mother plant-derived cultures were obtained from a leaf that was surface disinfected and inoculated onto culture initiation medium.

Subculture of embryogenic cultures typically results in the formation of the somatic embryos. Embryogenic cultures and early-stage somatic embryos obtained from these cultures are then further cultured in a suitable liquid plant growth medium, for example, a plant tissue culture nutrient medium consisting of B5 medium devised by Gamborg et al. in 1968 (Sigma Chemicals, St. Louis, MO) that has been modified as described by DeWald and coworkers (*J Amer Soc Hort Sci* 114: 712–716, 1989) and Litz et al. 1995 ("Somatic embryogenesis in mango," 1995, supra). This modified medium consisted of B5 major salts, MS minor salts and vitamins, glutamine (about 400 mg/L), and sucrose or commercial table sugar (about 60 g/L).

In the experiments of Emershad and Ramming, somatic embryo formation was shown to occur from immature zygotic embryos within ovules of stenospermoearpic seedless grapes *(Vitis vinifera* L.) when cultured for 2 months on liquid Emershad/Ramming medium. Somatic embryos continued to proliferate after excision and transfer to Emershad/Ramming medium supplemented with 1 μM benzylaminopurine and 0.65 % TC agar. Plant development from somatic embryos was influenced by genotype, medium, phase (liquid, agar), stage (torpedo, mature), and their interactions. Optimal plant development occurred on Woody Plant Medium supplemented with 1.5 % sucrose + 1 μM benzylaminopurine + 0.3 % activated charcoal and 0.65 % TC agar.

Cultures are scanned weekly for the presence of emerging embryogenic cells or embryogenic cell masses. Embryogenic cells or cell masses are identified based on morphology. Embryogenic cell masses, in general, tend to be white to pale yellow in color and are often hyaline. They may be recognized from a very early, small stage (10–20 cell aggregates), based on color/friable, granular appearance. Embryogenic cultures are also identified by their compact nature, containing cells rich in cytoplasm, as seen under the microscope. A multitude of factors affect somatic embryogenesis including genotype, nature and type of explant, medium composition, and season of harvest. Cultures can be prompted to develop for enhanced embryogenesis or diminished embryogenesis, or more unorganized embryogenic cell growth, by repeated manipulation of the culture, which entails careful selection of embryogenic cells and cell masses during transfer.

Ever since Mullins and Srinivasan (1980) and, later, Gray and Mortensen (1987) demonstrated initiation and long-term maintenance of somatic embryogenesis in grape, several success stories have been reported. Petioles, tendrils, leaf and nodal explants, anthers, ovaries, and unfertilized ovules have all been used for inducing somatic embryogenesis (Croce et al. 2005; Perrin et al. 2004). Suspension cultures too have been successfully

used to obtain high-efficiency somatic embryogenesis and plantlet production (Jayasankar et al. 1999).

Lopez-Perez et al. (2006) achieved plant regeneration from somatic embryogenesis in cvs. Sugraone, Crimson Seedless, and Don Mariano. Conversion rate to plantlets was higher in medium with IAA and GA_3. Medium-dependent response (e.g., use of activated charcoal, silver nitrate, etc.) has also been reported by other workers. Indirect somatic embryogenesis using stem nodal explants was obtained for six clones of *Vitis vinifera* cultivars, including rootstocks (Maillot et al. 2006). Gribaudo et al. (2007) identified the best development stage to initiate anther culture and improve somatic embryo germination by some treatments and optimized two key steps of grape somatic embryogenesis. Goussard et al. (1991) elucidated the role of in vitro somatic embryogenesis in eliminating fan leaf virus and leaf roll–associated viruses from grapevines.

Grapevine somatic embryos have also been used for the selection of toxin-resistant strains (Jayasankar et al. 2000). Das et al. (2002) developed an efficient leaf-disc culture method for transforming grape via somatic embryogenesis. Very often, patents are sought from researchers successfully developing protocols for somatic embryogenesis in crops/plants, particularly in recalcitrant/woody/difficult-to-manipulate systems, for example, walnut.

15.3.2 Regeneration System for Grape and Uses Thereof

Method of producing a mature somatic embryo: The method includes the steps of (a) providing a liquid culture that includes an embryogenic cell, (b) recovering embryogenic cell from the culture, (c) transferring the embryogenic cell to a second culture, and (d) growing a mature somatic grape embryo from the embryogenic cell.

Culture Initiation: Embryogenic cultures were initiated from anthers and ovaries of the cultivar "Chardonnay" (clones CH 01 and CH 02) and from the leaves of the cultivar "Thompson Seedless" according to standard methods, e.g., those described herein.

Preferably, the embryogenic cell is obtained from anthers, ovaries, ovules, floral tissue, vegetative tissue, tendrils, leaves, roots, nucellar tissue, stems, seeds, protoplasts, pericycle, apical meristem tissue, embryogenic tissue, somatic embryos, or zygotic embryos. In still other preferred embodiments, the liquid culture medium of step (a) includes a plant growth regulator (e.g., an auxin, NOA (naphthoxyacetic acid), indole-3-acetic acid (IAA), dicamba, picloram, naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), a cytokinin, benzyladenine (BA), thidiazuron, zeatin, abscisic acid (ABA), or gibberellic acid (GA)). In other preferred embodiments, the initiation culture medium includes at least 0.01 mg/L of a cytokinin, at least 0.1 mg/L of a carbohydrate, and at least 0.1 mg/L of a nitrogenous compound. Exemplary carbohydrates include sucrose, glucose, maltose, and glycerol. Exemplary nitrogenous compounds include potassium nitrate, calcium nitrate, ammonium nitrate, and ammonium sulfate. If desired, the embryogenic cell or the somatic embryo can be transformed with DNA (e.g., DNA encoding a gene which is capable of conferring disease resistance). The embryogenic cell can be recovered from the first culture by filtration, sedimentation, or selection. In addition, the first culture can be sieved to synchronize the formation of differentiated somatic grape embryos. Preferably, the embryogenic cell of step (a) is obtained from a source selected from the group consisting of an embryogenic cell produced according to steps (a) and (b) of the first aspect; a somatic grape embryo produced according to steps (a) to (d) of the first aspect; a mature somatic embryo produced according to method of the first aspect; a plantlet according to the fifth aspect; and anthers, ovaries, ovules, floral tissue, vegetative tissue, tendrils, leaves, roots, nucellar tissue, stems, seeds, protoplasts, pericycle, apical meristem tissue, embryogenic tissue, somatic embryos, or zygotic embryos from a plant produced from a mature somatic embryo produced according to the method of the first aspect. In preferred embodiments, the method further includes transferring the somatic grape embryo to a germination medium to grow a grape plantlet.

15.3.3 Method for Producing Somatic Grape Embryos

The method includes the general steps of (a) providing a liquid culture comprising an embryogenic cell, (b) selection of embryogenic cell from the culture, (c) transferring the embryogenic cell to a second culture, and (d) growing a somatic grape embryo from the embryogenic cell.

In a third aspect, the invention features a method of producing a somatic grape embryo. The method includes the general steps of (a) providing a liquid culture that includes an embryogenic cell and medium consisting of B-5 major salts and MS minor salts, (b) recovering the embryogenic cell from the culture, (c) transferring the embryogenic cell to a second culture, and (d) growing a somatic grape embryo from the embryogenic cell.

15.3.4 Plantlet Germinated from the Mature Somatic Grape Embryo

A method for long-term storage of a mature somatic grape embryo: The method includes the general step of drying the embryo and storing the embryo at a temperature less than 8 °C. Preferably, the mature somatic grape embryo is produced by the method of the first aspect. In a seventh aspect, the invention features a method for direct seeding of a somatic grape embryo. The method includes the general step of placing the said somatic grape embryo in potting medium comprising of sand and potting mixture. In an eighth aspect, the invention features a method of producing a somatic grape embryo using a liquid culture medium from a perennial grape embryogenic culture. The method includes the steps of (a) culturing a perennial grape embryogenic culture in a first liquid culture medium to grow a cellular suspension culture, the first liquid culture medium including a plant growth regulator; (b) recovering an embryogenic cell or embryogenic cell mass from the cellular suspension culture; and (c) culturing the embryogenic cell or embryogenic cell mass from the cellular suspension culture of step (b) in a second liquid culture medium to produce a somatic grape embryo. In preferred embodiments, the method further includes transferring the somatic grape embryo to a germination medium to grow a grape plantlet. In other preferred embodiments of the eighth aspect, the plant growth regulator is an auxin, NOA, BA, zeatin, dicamba, picloram, NAA, IAA, 2, 4-D, a cytokinin, thidiazuron, ABA, or GA. In yet other preferred embodiments, the liquid cell culture of step (a) is subcultured, and the recovery of the grape cell or grape cell cluster from the cellular suspension culture includes filtration, sedimentation, or selection. In other preferred embodiments, the second liquid culture medium further includes a plant growth regulator (e.g., a cytokinin). The liquid cell culture of step (c) can be sieved to synchronize the formation of differentiated somatic grape embryos. If desired, the somatic grape embryo or embryogenic cell of step (a) can each be transformed with DNA. The embryogenic cell or embryogenic cell mass recovered in step (b) may also be transformed with DNA. Similarly, the recovered somatic embryo may be transformed with DNA.

In about 6–8 weeks, a fine cell suspension culture is produced, which consists of highly vacuolated elongated cells (nonembryogenic cells), and also a lesser number of small, cytoplasm-rich, isodiametric cells (embryogenic cells). Repeated transfer of embryogenic cells or cell masses has not only been found to enrich the growth of embryogenic tissue but also to facilitate the process of somatic embryogenesis. The cultures are perennial in that they typically persist for over 2 years.

Embryogenic suspensions of grapevine (*Vitis vinifera* L.) were initiated from somatic embryos of “Thompson Seedless” and “Chardonnay” by Jayasankar et al. (1999). Suspension cultures consisted of proembryonic masses (PEM) that proliferated without differentiation in a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D). “Chardonnay” somatic embryos developed fully from PEMs following subculture in medium without 2,4-D; however, somatic embryo development did not advance beyond the heart stage in “Thompson Seedless” suspension cultures.

Highly synchronized development of somatic embryos was obtained by inoculating <960-μm PEMs into liquid medium without 2,4-D. Somatic embryos were also produced in large numbers from suspension-derived PEMs of both cultivars on semisolid medium lacking 2,4-D. Somatic embryos matured and regenerated into plants in MS basal medium containing 3 % sucrose. Using this method, over 60 % somatic embryos regenerated plants, of which more than 90 % plants were successfully transferred to the greenhouse.

Somatic embryogenesis and subsequent diploid plants were obtained from anthers of *Vitis vinifera* Cabernet Sauvignon, a cultivar considered recalcitrant to in vitro regeneration (Mauro et al. 1986). Anthers enclosing microspores near the first pollen mitosis were found to be the most responsive. However, from a practical point of view, anther length proved to be an easier criterion to determine the optimal physiological stage of the anther. Calluses derived from somatic tissues of the anther produced embryoids only when cultured on medium supplemented with casein hydrolysate. Glutamine and adenine were found to stimulate embryoid production. Early removal of cotyledons increases the frequency of development of embryoids into normal plantlets. Immature inflorescences of coconut belonging to three different genotypes were cultured by Jean-Luc et al. (1994) on a solid medium supplemented with activated charcoal (2 %) and 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations ranging from 1.5 to 3.5×10^{-4} M. Globular white callus is formed from immature floral meristems, depending on inflorescence age and 2,4-D concentration. Acquisition of embryogenic competence was confirmed histologically. Somatic embryos presented a functional bipolar organization with a completely differentiated shoot meristem, reported for the first time in coconut tissue culture. Embryo maturation allowed reliable plant regeneration in this in vitro recalcitrant species. Details of exogenous hormonal requirements were worked out for acquisition of embryogenic competence and embryo maturation.

15.4 Other Plant Species

A new protocol was developed by Jiahe et al. (2004) for a highly efficient somatic embryogenesis and plant regeneration of ten recalcitrant Chinese cotton cultivars. Calluses and embryogenic calluses were induced on MSB1 medium containing optimal combination of indolebutyric acid (IBA; 2.46 μM) and kinetin (KT; 2.32 μM). Up to 86.7 % of embryogenic calluses differentiated into globular somatic embryos 2 months after culture on MSB2 medium containing double the KNO_3 and free of growth regulators. Up to 38.3 % of the somatic embryos converted into complete plants in 8 weeks on MSB3 medium with L-asparagine (Asn)/L-glutamine (Gln) (7.6/13.6 mM). The plants were successfully transferred to soil and grew to maturity. With this protocol, they obtained hundreds of regenerating plantlets from ten recalcitrant cultivars, which is important for the application of tissue culture to cotton breeding and biotechnology.

An improved protocol was also developed for somatic embryogenesis and plant regeneration in recalcitrant cotton cultivars. High callus frequency and embryogenic tissues were developed on MSB medium supplemented with gradient concentrations of KT and 2,4-D, with concentration decreasing from 0.1 to 0.01 mg/L. Somatic embryos were successfully incubated in one-half macronutrient MSB suspension supplemented with 0.5 g L^{-1} glutamine and 0.5 g L^{-1} asparagine. Decrease in macronutrient concentration in MSB significantly alleviated browning and was beneficial to suspension cells. Transformation of somatic embryos into plants was induced on MSB medium supplemented with 3 % sucrose, 0.5 g L^{-1} glutamine, 0.5 g L^{-1} asparagine, and 6.0 g L^{-1} agar. Effect of sucrose as the carbohydrate source was better than glucose for plant germination. Regenerated plantlets from CCRI521 and Zhongzhi86-6 could be recovered at the rate of as much as 19.6 and 18.5 % somatic embryos, respectively.

Rao and Bahadur (1990) induced somatic embryogenesis and subsequent plant formation in distylous self-incompatible *Oldenlandia umbellata* (Rubiaceae), a vegetable crop valued for its red pigment, from primary cultures of leaf, axillary bud, stem, and immature fruit explants. This protocol is useful for self-incompatible species that fail to produce seeds.

Sahijram and Doreswamy (1988, 1991) induced morphogenesis and nucellar embryogeny in monoembryonate mango cultivars and somatic embryogenesis in embryo cultures. Sahijram et al. (1996) studied factors influencing somatic embryogenesis in this crop. Ravindra et al. (2000) demonstrated somatic embryogenesis in nucellus cultures of polyembryonic mango cv. "Vellaikolumban." Sahijram et al. (2011) and Mirajkar et al. (2010) gave a protocol for plant regeneration from petal cultures in pomegranate. Sahijram (2014) also observed differential response to MS and Gamborg's B5 media of petal-derived embryogenic calluses of pomegranate cv. Bhagwa with/without cocultivation with *Agrobacterium*, while Sahijram and Rao (2012) induced high-frequency regeneration from segmented root explant cultures in this fruit crop (*Punica granatum* L.). They also regenerated plantlets from petals of pomegranate for *Agrobacterium*-mediated transformation. Madhusudhana Rao and Sahijram (2013) studied the comparative response of epicotyl, hypocotyl, and cotyledonary explants for the induction of somatic embryogenesis in pomegranate. Somatic embryogenesis is also reviewed by Sahijram (2011) in grape. Regeneration systems developed in horticultural crops are reviewed by Vageeshbabu et al. (2007).

Litz et al. (1995) opined that in mango, it was preferable to remove the somatic embryos from liquid medium as soon as they germinated precociously and transfer these to semisolid medium. For somatic embryo maturation, germination, and plant regeneration studies, three maturation media – mango maturation medium, mango maturation medium solidified with agar (7 g/L), and MS basal medium with 3 % sucrose – were evaluated for their ability to promote somatic embryo germination and plant regeneration. MS basal medium containing 3 % sucrose was the most effective at promoting embryo maturation, germination, and plant regeneration, for both embryos derived from semisolid medium and for the precociously germinated embryos obtained from liquid medium cultures. Although good germination was obtained by the authors on mango maturation medium with agar, quality of the regenerants was not as good as with MS salts containing 3 % sucrose in the medium.

Genetic engineering of corn, and other cereals, is currently limited because of the difficulty of regenerating plants via cell culture. Hodges et al. (1986) reported commercially important inbred lines and hybrids from several different maize groups to be capable of regeneration via somatic embryos. Inbred line A188 had a high frequency of plant regeneration, and, when crossed to recalcitrant inbreds, the resulting F1 hybrids too were found to be "regenerable." These results demonstrated that nuclear genes exhibiting dominance were important information of somatic embryos and regeneration of corn plants.

Hakman and von Arnold (1988) regenerated plantlets from long-term embryogenic cultures of *Picea glauca* (Moench) Voss. (white spruce). Embryogenic calluses, initiated from immature zygotic embryos and maintained by monthly subculture for 16 months, were used to establish suspension cultures. Small somatic embryos were continuously produced in liquid culture medium containing auxin and cytokinin, and the cultures showed sustained regeneration capacity for >6 months. Somatic embryos propagated in suspension cultures developed further into embryos bearing cotyledons about 1 month after transfer to solidified medium containing abscisic acid. Electron microscopic examination revealed that storage nutrients, lipids, proteins, and carbohydrates accumulated in the somatic embryos during treatment with abscisic acid (ABA). Upon subculture to medium lacking plant growth regulators, such embryos could develop into small, green plantlets.

Maheshwaran and Williams (1984) reported direct somatic embryoid formation in immature embryos of *Trifolium repens, T. pretense*, and *Medicago sativa*. They also demonstrated the

effect of cytokinins on somatic embryo initiation/maturation especially cotyledon development and subsequent plantlet formation in several plant species. Bhattacharya and Sen (1980) successfully produced plantlets through somatic embryogenesis in *Brassica campestris*.

An efficient procedure was developed by Gill and Saxena (1992) for inducing direct somatic embryogenesis, organogenesis, and regeneration of plants from tissue cultures of peanut (*Arachis hypogaea* L.). Thin transverse sections of cotyledons and juvenile leaves were cultured on Murashige and Skoog (1962) medium supplemented with N^6-benzylaminopurine (BAP) or a substituted phenylurea, thidiazuron (TDZ). Somatic embryos or shoot buds differentiated from cut surfaces of the cotyledons and midrib region of the leaves. Application of BAP induced differentiation of shoot buds, whereas treatment with TDZ resulted in the production of somatic embryos. Somatic embryos developed into plants upon subculture on basal medium. Agar-solidified medium was found to be superior over liquid medium for the development of embryos and shoot buds. TDZ-induced somatic embryogenesis and plant regeneration were successfully applied to three genotypes. A distinct feature of their study was induction of morphogenic competence in cultures of seedling explant origin in peanut – until then known to be recalcitrant to somatic embryogenesis in vitro.

15.5 Molecular Markers for Assessing Genetic Fidelity of Regenerants

Gesteira et al. (2008) used random amplified polymorphic DNA (RAPD) markers for evaluating genetic stability of regenerants (plants) in soybean obtained through somatic embryogenesis using 180 μM 2,4-dichlorophenoxyacetic acid. Twenty primers were used for screening 44 regenerants in cv "Spring" and 28 in cv "CAC-1." Three primers were polymorphic for two of the "Spring"-derived regenerants, showing somaclonal frequency of 4.5 %. Four primers were polymorphic for "CAC"-l-derived regenerants, with somaclonal frequency of 3.57 %. Thus, usefulness of RAPD markers in detecting genetic instability in soybean primary regenerant plants derived from somatic embryogenesis was demonstrated; this could also be used as a certification tool for monitoring genetic stability during the regeneration process.

15.6 In Vitro Conservation

Several methods of in vitro conservation in grapes have been developed. Cryopreservation of embryogenic cell suspensions by encapsulation-vitrification; influence of pretreatment, freezing and thawing, and postculture conditions and freezing in liquid nitrogen of alginate-coated shoot tips are some of the aspects investigated. Genetic stability over long periods and over variable temperatures imposed during preservation is a critical component of in vitro conservation methodologies. This has been studied using molecular markers.

Tissue culture-based conservation strategies for use in in vitro gene banks have been worked out for grape germplasm by Ganeshan. In his study, meristem culture and differentiating, ovary explant–derived callus showed amenability to conservation under reduced input culture conditions.

Synthetic seed technology in grape, which would allow clonal germplasm of grape to be conserved in seed repositories, is relatively well advanced. Cotyledonary-stage somatic embryos originating from leaf explants have been encapsulated in alginate gel in Murashige and Skoog (1962) medium by Das et al. (2006). The percentage germination in encapsulated somatic embryos was found to be higher than in nonencapsulated embryos. The same workers encapsulated somatic embryos as an efficient method for storing and propagating pathogen-free planting material. Patented technologies are available for a number of cultivars for achieving successful somatic embryogenesis.

15.7 Conclusions

Biotechnology promises to dramatically decrease the time needed to develop new grape varieties in view of the exciting strides made recently, especially in the sequencing of the genome and somatic embryogenesis promises to deliver nonchimeric, solid transgenics. These could be further multiplied, in turn, using somatic embryogenesis protocols. Already patented technologies have been developed for generating disease-resistant grape cultivars. Patented technologies are available for regeneration systems in grape (US Patent No. WO/1999/059398) for exploitation by the biotechnologist for incorporation of genes of choice into cultivars of choice.

Improved methods of germplasm conservation and vegetative propagation should arise through synthetic seed technology. In vitro selection for desirable traits in established cultivars is an attractive approach and has been successful recently. Haploids and dihaploids have not been developed, stifling the evaluation of their use in grape improvement.

However, ongoing research in these areas is encouraging and the rapid progress displayed to date demonstrates that we are on the verge of utilizing an array of biotechnological procedures.

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Micropropagation of Plants

16

Aneesha Singh

Abstract

Micropropagation is a rapid multiplication of a selected plant using in vitro culture techniques. In this chapter various aspects of micropropagation have been discussed. The propagation of selected plant through micropropagation would be useful for raising plantation using apical and nodal segment. They are best for micropropagation and mostly result in true to type plants. These segments upon the subsequent subcultures result in a number of multiple shoots. These multiple shoots on elongation allowed to root in vitro. After rooting, they are in vitro hardened and transferred to field. The potential of plant tissue culture is well recognized, as it increases agricultural production and generates rural employment. But the high cost of production on micropropagation is a major bottleneck. Low-cost protocol development can popularize this method. High multiplication rate, use of low-cost chemicals, high rooting and survival percentage, and use of ex vitro rooting method can minimize the expense of any protocol. Presently, both the developing and the developed countries require low-cost technologies to progressively reduce the cost of production. The performance of tissue plants generally better than cutting and seedling. Initially, some morphological changes occur in micropropagated plant, but in the course of time, they minimize. Somaclonal variation caused during callus cultures can be used to generate variants for hybridization. Variation in phenotype of plants produced in tissue culture can be characterized as either temporary or permanent. Temporary variation includes increased branching, greater susceptibility to disease, and lack of uniform response. Micropropagated plants need to be monitored to get long-term data for its

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growth, survival, yield, and disease attach under field condition as very limited information is available for many plants. In this chapter micropropagation of commercially important plants like *Jatropha curcas*, *Eucalyptus*, banana, and bamboo has been discussed.

Keywords
Plant tissue culture • Regeneration • Micropropagation • Shoot proliferation • Clonal plants • Tissue hardening • Genetic fidelity • Somaclonal variation • *Jatropha curcas* • Banana • Bamboo

16.1 Introduction

In vitro clonal propagation provides true to type plants of a selected genotype using in vitro culture techniques (Thorpe 2007). Endangered, threatened, and rare species have successfully propagated by micropropagation because of its high multiplication rate with minimal number of starting material/plants used for initiation of cultures. Most often micropropagation is coupled with mass production at a viable price. Gottlieb Haberlandt (1902) is the pioneer of plant tissue culture and was the first person to isolate plant cell. With course of time, micropropagation has become an essential tool for the propagation of plants that are otherwise difficult to propagate conventionally by seed and/or vegetative means. It has many distinctive advantages over conventional propagation methods such as rapid multiplication of valuable genotypes, expeditious release of improved varieties, production of disease-free plants, production round the year, germplasm conservation, and facilitating their easy exchange. Micropropagation has raised high expectation for large-scale propagation, and lot of research has been focused on the development of protocols for large-scale propagation of many plants. This biotechnological dream was fulfilled by a large number of publications; although there are a number of publications, only a few of the published protocols could be converted into commercially viable propagation systems, and problems were either technological or associated with marketing. Most of the published work lacked crucial issue of selecting seeds or adult plants as a starting material, and the choice of the propagation method is crucial. The advantage of using seedlings is that the technology is easier but with the disadvantages of unknown genetic background. Using adult plants as a starting material, it has the problem of endogenous contamination, poor response, instability of multiplication rates, and rooting problems. However, the use of adult plant as the starting material is desirable as its pedigree is known. It is more useful to propagate the elite plant material than unknown. Most of the conventional techniques for vegetative propagation are useful for the production on a smaller scale, but for large-scale propagation (lakhs and millions of the plants per year) requires highly efficient techniques. For large-scale propagation, conventional methods are insufficient and ineffective, and tissue culture is the only viable method. Nobody would think of propagating a weedy fig tree via tissue culture as can be propagated easily via cuttings in the tropical countries. More than fifty lakh plants of *Ficus* plants are generated in Europe through tissue culture. In some plants, micropropagation has completely replaced propagation via cuttings.

Plant can be micropropagated in three different ways: (1) axillary bud, where axillary bud gives rise to many new shoot buds; (2) direct regeneration of shoot buds, further gives rise to many buds or through somatic embryos and gives rise to plantlet as they are diploid with shoot and root connected by vascular system; and (3) indirect regeneration of shoot buds or somatic

embryos from callus. Numerous examples of its application have been reported such as culturing of gametes, meristematic culture for virus elimination, zygotic embryo culture, embryo rescue (ER), and germplasm conservation; ER technique nurtures the immature or weak embryo, and this way it helps to germinate. Recently, this technique has been proven useful for breeding seedless grape; thus, remarkable progress in micropropagation technologies is occurring as their applications are diverse and significant.

Genetic transformation is the most recent aspect of micropropagation that provides the mean of transfer of genes with desirable trait into host plants. Plant can be genetically modified by direct gene transfer or indirect gene transfer method. In the indirect gene transfer method, *Agrobacterium*-mediated genetic transformation is most commonly used for the expression of foreign genes in plant tissue. Successful introduction of agronomic characters in plants was achieved by using root explants for the genetic transformation. Virus-based vectors offer an alternate for stable and quick ephemeral protein expression, thus providing an efficient mean of recombinant protein production.

16.2 Micropropagation

In vegetative propagation use of micropropagation has become very popular due to its application in agriculture, forestry, and horticulture. It has been possible to propagate forest trees, crop plants, vegetables, ornamentals, medicinal plants, and other commercial useful plants using this technique. Micropropagation is performed in following steps: (1) generations of aseptic cultures, (2) multiple shoot induction, (3) shoot elongation and proliferation, and (4) rooting, hardening, and field trials (Fig. 16.1). We will be discussing in this present chapter about micropropagation of commercially important plants like *Jatropha curcas*, *Eucalyptus*, banana, and bamboo.

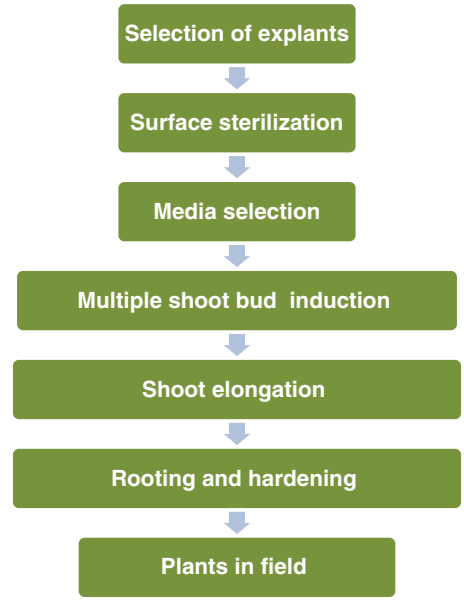


Fig. 16.1 Flow chart summarizing micropropagation method

16.2.1 Case Study 1 Micropropagation of *Jatropha curcas* L.

16.2.1.1 Generation of Aseptic Cultures

Seedling, nodal and apical shoot buds, cotyledonary leaf, petiole, stem, epicotyls, and hypocotyls were used for culture generation. Axillary, apical, stem, and leaf were used for the generation of large-scale cultures using explants from Council of Scientific and Industrial Research – Central Salt and Marine Chemicals Research Institute (CSIR-CSMCRI) field station, Bhavnagar, Gujarat, India. The establishment success of shoot cultures was >90 % when explants were taken from actively growing stems, while explants from the dormant plant were difficult to establish, as lot of infection occurred. Young shoots of new branches or priory pruned branch was cut, and petiole was removed by

keeping 1 cm petiole base attached to stem. Shoots were then cut keeping single bud in each segment. Explants were surface sterilized for 8 min in 0.1 % mercuric chloride and washed thrice with sterile distilled water. Trim the remaining part of petiole along with the lower and upper end of segment and cultured on bud initiation medium (Singh et al. 2010; Reddy et al. 2008, 2009). While most of the reports have used systemic fungicide, Bavistin, two to four liquid soap solution or two to five drops Teepol for 10–20 min prior to surface sterilization and followed by 0.1 % $HgCl_2$ (w/v) for surface sterilization.

16.2.1.2 Multiple Shoot Bud Induction

Nodal segment, leaves, and stem were used for shoot bud induction and cultured on MS medium supplemented with different concentrations and combinations of auxin and cytokinins. Multiple shoot bud induction is the most important stage in micropropagation. Success of any protocol depends upon the rate and number of multiple shoots. It mainly depends upon the formulation of medium used and combinations of plant growth regulators (PGRs). Cytokinins play key role in multiple shoot induction in this plant. Large-scale cultures were generated by CSIR-CSMCRI (Fig. 16.2), during the study cytokinin alone resulted 3–6 multiple shoots in primary cultures, and upon subculture on MS medium supplemented with cytokinin and auxin, 6–12 multiple shoots were achieved (Reddy et al. 2009; Fig. 16.3a, b). MS medium with 6-benzylaminopurine (BAP) alone induced shoot buds with two multiple shoots per explants. Activated charcoal when added to the culture medium was found to have promoted high frequency of multiple shoots in shoot tip cultures of *J. curcas*. While, BAP and kinetin (KN) in combination with indole-3-acetic acid (IAA) induced 45 multiple shoots. BAP in combination with indole-3-butyric acid (IBA) adenine sulfate + glutamine + L-arginine + citric acid induced shoots bud (Shrivastava and Banerjee 2008). Axillary shoot bud proliferation was best (six shoots per nodal explants) initiated on BAP with adenine sulfate. The multiplication rate (30 multiple shoot buds per culture) was significantly enhanced upon transfer to MS medium with KN, IBA, and adenine sulfate (Datta et al. 2007) and more than 20 shoot buds from leaf explants (Reddy et al. 2008) and 10–15 buds from stem explants were achieved (Singh et al. 2010).

In vitro culture studies on *Jatropha* have been conducted on somatic embryogenesis, zygotic embryogenesis, and direct and indirect organogenesis. Demissie and Lele (2010) reported phorbol ester from solid and liquid culture

Fig. 16.2 Large-scale culture generation at CSMCRI-CSIR

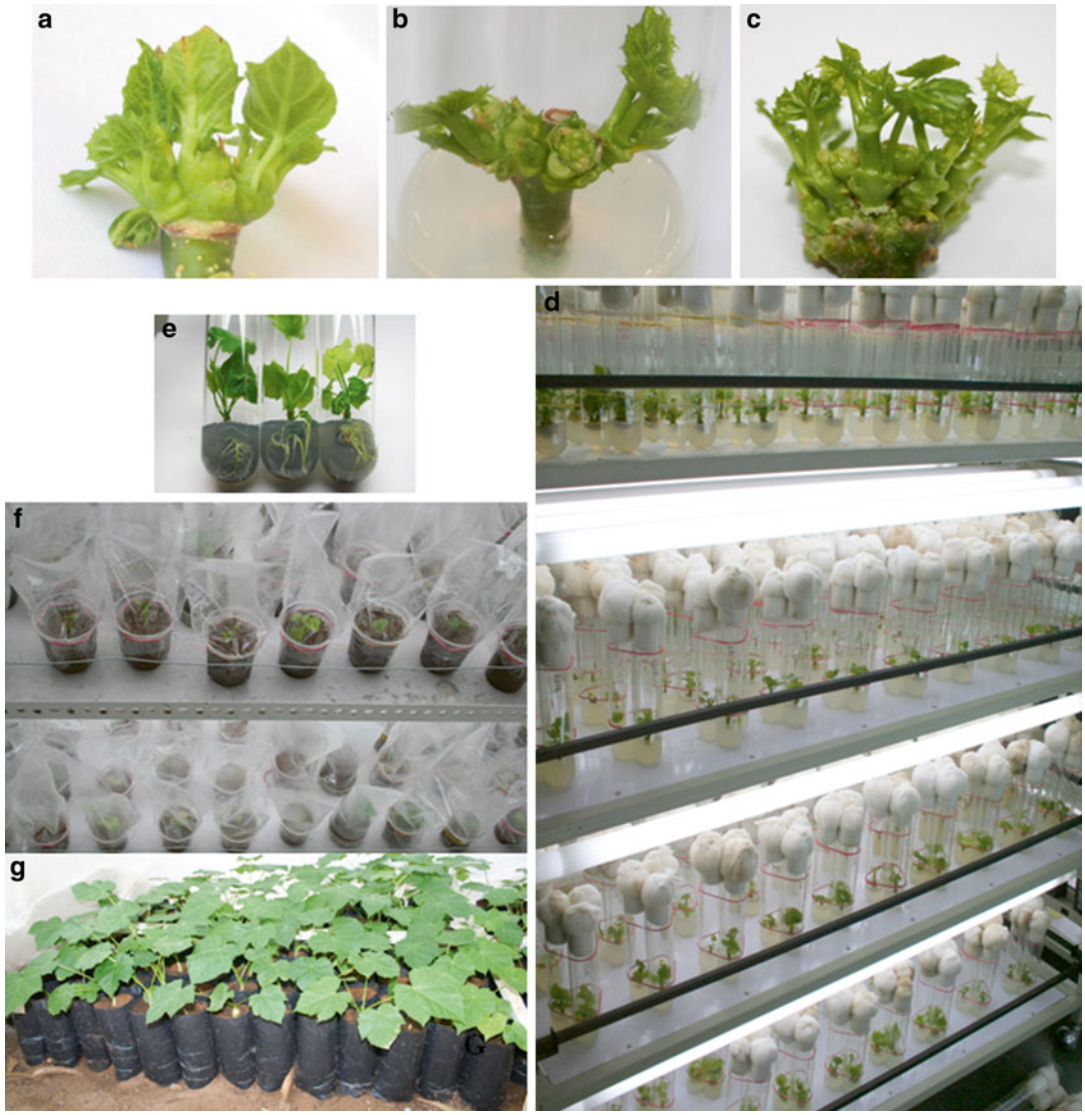


Fig. 16.3 Large-scale micropropagation of *J. curcas* using axillary shoot bud (**a**, **b**) multiple shoot induction upon subculture of explant, (**c**) large-scale cultures generation of *J. curcas* (**d**), in vitro rooting of shoots (**e**), ex vitro rooted shoots (**f**), well-rooted plants in nursery (**g**)

medium of *Jatropha*; phorbol ester content was high in liquid medium as compared to solid medium. Callus cultures were initiated from leaf and hypocotyl explants of *J. curcas*; hypocotyl explants were proved to be best in inducing large-scale callus in the short time (Soomro and Memon 2007). Attempts were also made for in vitro maturation and germination of *J. curcas* microspores. The rate of germination was 71.6 % and pollen matured in vitro exhibited a rate of germination of 13.7 %, when it was transferred into the optimal medium (Li et al. 2010). However, the use of cyanobacterial filtrate instead of synthetic PGRs was found to be better for in vitro germination of *J. curcas* embryo. In medium containing cyanobacterial filtrate, shoot and root lengths were comparatively higher than the other treatments with synthetic plant growth regulators. The same

trend was found for chlorophylls a and b (Shrivastava and Banerjee 2009). Somatic embryos were generated using different explants like cotyledonary leaf and leaf callus with different regeneration efficiencies. In recent years, plant regeneration in *J. curcas* has been accomplished through organogenesis from various explants, including mature leaf, petiole, hypocotyls, and stem.

16.2.1.3 Shoot Elongation and Proliferation

Shoot elongation and proliferation is an important stage for the successful plantlet development in tissue culture. Generally, reduction in the PGRs in the subculture medium is favorable for elongation and proliferation, but sometimes subculturing to the same composition also found to be equally good. In an experiment MS medium supplemented with BAP and IAA induced 6–12 multiple shoots upon subsequent subculture on the same medium (Fig. 16.3c, d). Subculturing from alone BAP to BAP in combination with IAA and other additives was found optimal for shoot proliferation. While subculturing on the same medium used for primary culture, BAP/KN in combination with IAA was also found equally good for shoot elongation and proliferation. Similar results were reported of subsequent subculturing onto the same medium found to be best for shoot proliferation and elongation with 100 shoot buds per explants (Shrivastava and Banerjee 2008). MS medium with KN, IBA, and adenine sulfate was found best for shoot elongation and proliferation as both shoot number and shoot length were found to increase significantly.

16.2.1.4 Rooting, Hardening, and Field Trials

Rooting of shoot not only depends upon the medium composition and PGRs but also depends upon other factors like shoot length, light, and age of culture. The full-strength MS medium was found to best for rooting as compared to half MS medium. As only 33 % rooting could be achieved in half MS medium and 88 % rooting in PGR-free full MS medium. The shoots rooted efficiently on half-strength MS medium (Purkayastha et al. 2010). Healthy elongated shoots were rooted on half-strength MS medium supplemented with IBA. The addition of phloroglucinol (200 μM) to the full MS medium enhanced the frequency of rooting to 76.7 % (Kumar and Reddy 2010). While, IAA (1.0 mg/l) was found to be more suitable than IBA for root induction. More than 50 % rooting could be achieved with the use of combinations of auxins IBA, IAA, and NAA (Reddy et al. 2009). Addition of activated charcoal (AC) to the medium does not have any significant role to play on rooting percentage (Fig. 16.3e) The role of AC on plant growth is still unclear, but it is believed that AC may slowly release certain adsorbed products, such as nutrients and growth regulators, and become available for growth and rooting of the plant. Long mature shoots could ex vitro root while tender shoots dried. Only few protocols are available to minimize the cost of the protocol via ex vitro rooting (Singh et al. 2010; Reddy et al. 2009; Fig. 16.3f). Hardening of the plant involves in vitro hardening by placing plants transferred into sterile polythene bags under in vitro conditions for a month, then shifted to the greenhouse and slowly humidity minimized. During ex vitro rooting, time minimizes in this whole process and so expense minimizes. Sapling shifted to nursery for further acclimatization and then shifted to field (Figs. 16.3g and 16.4).

More than 85 % of saplings of particular accession well established in different wasteland sites of CSMCRI, Bhavnagar, Gujarat. It was observed that tissue culture–raised plants were superior to cuttings and seed-raised plants in the field in terms of consistent yield.

Morphologically saplings were similar and-verified by molecular marker (Rathore et al. 2014), and suggesting plants -were true to type. The survival rate of 60–70 % was achieved in field (Rajore and Batra 2005). Most of the workers limited their workup to pot experiments. Most of the work has been done using explants of unknown pedigree or used seedlings. Available protocols do not have information regarding their survival in the field, which is a very crucial issue in tissue culture. Though plant is susceptible to

Fig. 16.4 Micropropagated plant growing in amended wasteland

abiotic and biotic stress, only few attempts have been made for genetic transformation in the plant. Although many methods have been published, implementation of this technology on a commercial scale is a constraint, due to the high cost involved. Some Indian units are currently producing *J. curcas* at the commercial level (Table 16.1). CSIR-CSMCRI in collaboration with MNRE (Ministry of New and Renewable Energy) worked on project large-scale micropropagation of *Jatropha curcas* and generated cultures on a large scale and developed plants were well established in the field. Micropropagated plants were found genetically stable (Rathore et al. 2014).

Table 16.1 *J. curcas* being currently micropropagated by commercial tissue culture units in India

1	Labland Biotech Private Limited, Mysore	*J. curcas*
2	Reliance Life Sciences Pvt. Ltd. Bombay	*J. curcas*
3	Nandan Biomatrix Limited, Hyderabad	*J. curcas*
4	Global Flora Biotech, Coimbatore	*J. curcas*

16.2.2 Case Study 2 Micropropagation of *Eucalyptus*

16.2.2.1 Generation of Aseptic Cultures

Seedling, lignotubers, and nodal and apical coppice explants from mature plants were used for culture generation. Pretreatment of fungicide was found essential for mature plants by many workers, but not found essential by Girijashankar (2012). Surface sterilization of nodal segments carried out using 0.1 % mercuric chloride ($HgCl_2$) for 10–17 min and 1 % sodium hypochlorite ($NaOCl_2$) for 7–15 min; 70 % ethanol and few drops of Tween 20 were added to the treatment solution. Regeneration through callus is difficult; however. callus induction has been reported in more than 30 species of *Eucalyptus*, and successful regeneration was achieved in a few species only.

Regeneration efficiency depends on the type of explants (plant part used for culture generation), time of subculture, nutrients, and PGR formulation. Malt extract was used in callus cultures of *E. viminalis* and *E. coccifera* (lshii 1982) and coconut milk for callus cultures of *E. polybractea*. Callus generated from lignotuber, stem, and

cotyledons could regenerate shoot buds. These shoot buds could elongate on medium with auxin in combination with cytokinin. Shoots regenerated from stamen callus of *E. marginatu* showed low rooting percentage. Explants source plays an important role in regeneration efficiency and rooting percentage.

16.2.2.2 Multiple Shoot Bud Induction

The best micropropagation is achieved by axillary shoot bud proliferation using nodal segments of mature plant. For multiple shoot bud induction, explants were cultured onto the medium supplemented with cytokinin and auxin (Roux and Staden 1991; Hartney and Barker 1980). Multiplication rate depends upon factors like species, clone, explant source, and juvenility. Highest multiplication was achieved by Hartney (1982) with a potential production of millions shoots from a single shoot within a year. However, seedling performed even better in *E. marginatu*. Cultures showed problem like unwanted callus and vitrification of shoot buds. Vitrified shoot is difficult to multiply and impossible to root. This problem could be overcome by the use of activated charcoal into the medium in this and other plants (Boulay 1983; Singh et al. 2008). Callusing retards shoot bud growth; it adversely affects on multiplication rate and rooting of the shoot. *Eucalyptus* cultures of *E. marginatu* showed callusing. Storage of cultures has been reported in *E. camaldulensis* and *E. grandis* on a simple medium for over 8 months and *E. gunnii* and *E. dalrympleana* for 6 months. Embryonic cultures of *E. citriodora* could be stored for more than 9 months without subculture and without loss of embryogenic potential.

16.2.2.3 Shoot Proliferation and Elongation

Multiple shoot buds need to be separated so that they could elongate sufficient enough to get rooted. By deceasing cytokinin shoot bud elongation was achieved in *E. torelliana* shoots. Whereas a transfer from liquid to solid medium achieved a similar effect with *E. tereticornis* shoots (Mascarenhas et al. 1982). Gibberellic acid in different concentrations ranging from 0.1 to 15 mg/L has been added to media to obtain shoot elongation with and without activated charcoal (Furze and Cresswell 1985). The use of different medium has been reported for culture generation of *Eucalyptus*. MS medium was found suitable for culture generation *E. camaldulensis* and *Eucalyptus spp*.

16.2.2.4 Rooting, Hardening, and Field Trials

Root initiation using different media, e.g., White's (1963) medium, Knop's (1865) medium, and half-strength Murashige and Skoog's (1962) medium, has been reported. Rooting efficiency also gets affected by source explant. The range of rooting percentages for seedling shoots was 5–80 %, whereas the range for an adult shoot was 2–20 %. Burger (1987) achieved 4 % rooting with adult explants of *E. sideroxylon* and 100 % rooting from coppice explants shoots. Gupta et al. (1983) observed 50 and 70 % rooting of shoots from mature trees of *E. torelliana* and *E. camaldulensis*, respectively. The rooting percentage of shoots varies upon the number of subcultures. Mature shoots root better than tender shoot. As the number of subculture increases, rooting percentage increased from 20 to 100 % from the third to the sixth subculture (Badia 1982a). Mc Comb and Bennett (1982) found similar results with mature explants of *E. marginata*. The use of half-strength MS supplemented with activated charcoal favored rooting. The use of auxins like NAA (naphthaleneacetic acid) and IAA (indole acetic acid) promoted rooting by minimizing callus formation. The addition of riboflavin into the rooting medium changed rooting pattern. Vitamin E, enhanced shoot growth, was reported by Badia(1982b), while Boulay (1983) found it noneffective. Ex vitro rooting is a promising method for cost minimization of the protocol (Singh et al. 2010). Shoots of *E. camaldulensis* were ex vitro rooted directly in the sand and in nonsterile conditions.

Adaptation of plants to the new environment is acclimatization, and the process of adjusting

the in vitro grown plantlet to the outside or surrounding climate is hardening. Rooted shoots are subjected to hardening so that they can be transferred to field conditions. During hardening plant is slowly acclimatized to the outside environment. Hardening material and humidity play an important role in plant establishment. Transferring plantlets to container containing a mixture of 70 % pine bark, 25 % sphagnum peat, 5 % brown coal ash, and 4 g dmW3 Osmocote fertilizer resulted in a good establishment of the plant. As plantlets come from complete sterile conditions, they are sensitive to diseases; also frequent sprays with fungicides and insecticides are recommended. Establishment of plant varies species to species. Bell et al. (1993) studied the behavior of micropropagated plants and seedling. They found some variation in the initial phase of growth, but soon those variations were lost after 40 days. Goodger and Woodrow (2010) studied *E. polybractea* and found somaclonal variants with increased foliar oil concentrations. The study on micropropagated *E. grandis* × *nitens* showed that, despite of root morphological differences, micropropagated plants did not significantly reduce the efficiency of roots to take up and transport water to the leaves in a field trial. Greater uniformity was found in micropropagated plants from the normal microcutting. Uniformity is desirable for the practice of clonal forestry. There is a need to monitor micropropagated plant and study its behavior in field condition for years, as many time elite features of the plant disappear with time, for example, increased branching of ornamental plants, but the effect is lost in successive generations when cuttings are generated from the tissue cultured plants.

List of commercial unit's micropropagating *Eucalyptus* (Table 16.2). Center of Agriculture and Forestry Research in Italy together with Celulosas de Asturias SA worked on a collaborative project Biogenie and successfully applied molecular technique for clonal identity on a commercial basis. Mondi forest, a division Paper Company Ltd. in South Africa, uses tissue culture technique to establish microcuttings of cold-tolerant clones.

Table 16.2 *Eucalyptus* being currently micropropagated by commercial tissue culture units

Eucalyptus sp.	Green Valley of India, Aligarh, India	Rs. 37
	Revolving Earth Agro Pvt. Ltd, Mumbai, India	Rs. 30
	Merar Limited, London	–

The cost of production of micropropagated plants depends mainly on labor cost in the country/place/region of origin, energy cost, number of cultures per culture vessel/unit area of production, rate of multiplication and number of weeks between subculture, percent of culture contamination, and saleable plants after the production process.

16.2.3 Case Study 3 Micropropagation of Banana (*Musa* sp.)

In 1972 tissue culture of banana (*Musa*) was first time reported using shoot apex; later on meristem tissue combined with thermotherapy was used for generation of virus-free banana plants. Since then people are working on large-scale propagation of good varieties of banana. The leading producer of banana is Maharashtra and then followed by Tamil Nadu, Gujarat, Karnataka, and Andhra Pradesh. They contribute more than 85 % of total banana production in the country.

16.2.3.1 Generation of Aseptic Cultures

Cultures are initiated using different explant sources of banana plant. The most commonly used explant sources are shoot apex, suckers, lateral buds, peepers, and terminal inflorescence. For culture initiation, explant must be disinfected; various methods of surface sterilization have been proposed by researchers. Sodium hypochloride is most commonly used for surface sterilization of banana and also use of mercury chloride found to be effective by many researchers. Pretreatment of antibiotic, fungicide, and ethanol minimized infection rate. However, cultures could be initiated without any disinfection reported. For in vitro culture, banana seeds were treated with 1.4 % sodium hypochloride and washed with ethanol.

16.2.3.2 Multiple Shoot Bud Induction

Shoot tip and shoot tip meristem are commonly used explants for multiple shoot bud induction; use of male flower for embryogenesis is also reported. Shoot tips are cultured on the medium intact, wounded or fragmented for shoot bud induction. During fragmentation, shoot apex was cut longitudinally into two or more halves and cultured onto the medium. Different media supplemented with PGRs (plant growth regulators) are used for culture initiation. Some researchers used the same medium for initiation and multiplication, while others have changed the concentration of PGRs in initiation and multiplication medium. Assani et al. (2003) initiated cultures on MS medium supplemented with casein hydrolysate and adenine sulfate (Nandwani et al. 2000). Diploid and triploid cultures could be generated using male flower explants. Anthers were also used as explant for generation of haploid plants. Lee et al. (1997) used sliced rhizome tissue for the induction of somatic embryos.

16.2.3.3 Shoot Proliferation and Elongation

PGRs are the essential components of the medium; their concentration and ratio generally determines the growth and development of the explant. The response varies explant to explant; Mendes et al. 1996 reported that shoot tips of different rhizomes behave differently as some of them were highly productive and others were less productive. A high concentration of 5–20 mg/L BAP in combination with 10–40 % sucrose and 5–20 mg/L ancymidol (AVC) is found best for shoot proliferation and elongation, medium supplemented with high concentration of BAP and coconut milk enhanced regeneration efficiency of shoot buds. BAP in combination with auxin enhanced shoot proliferation and elongation, BAP with IBA is found to be best for three cultivars (Cavendish, Bluggoe, and Silk) of banana, and BAP with IAA enhanced shoot multiplication of leaf regenerants. TDZ is frequently used in tissue culture; its combination with IAA is found to be best for shoot elongation as compared to BAP in combination with IAA. Also more than one cytokinin, with or without auxin, were used together to enhance the shoot proliferation and elongation of banana cultures. Kinetin and BAP were used in combination, and their combination with 2iP was used for shoot multiplication of cv. Sabri. However, kinetin and BAP combination produced the highest number of shoot buds as compared with 2iP. The addition of ANC (growth retardants) induced significant difference in plant height during hardening. Also, the use of ANC and paclobutrazol in liquid medium decreased the excessive growth of leaf and stem. Browning of the medium during multiplication is observed due to the oxidation of phenols. It interferes in the plant growth and leads to necrosis of the tissue. It could be minimized by the addition of 50–100 mg/L of calcium chloride in the medium. Washing with 0.125 % potassium citrate before culturing was beneficial to control necrosis of the explant. Agar medium is expensive as compared to liquid medium, and also liquid medium is easy to handle. The highest multiplication rate was observed in the explant cultured in temporary immersion as compared to other methods using cellulose as substrate and partial immersion. Though there are many reports using liquid medium and bioreactors, but commercially propagation is carried by solid culture medium.

16.2.3.4 Rooting, Hardening, and Field Trials

Generally for successful rooting, concentration of auxins, MS, and sucrose is mainly responsible. However, auxin with cytokinin is also reported for rooting in many plants. High auxin and low cytokinin concentration are favorable for root induction. The most frequently used auxins for rooting of banana shoots are NAA, IAA, and IBA; however, rooting without PGRs has been also reported in banana. Shoots regenerated many roots when cultured on MS medium supplemented with 1.0 g/L activated charcoal (AC); use of 0.025 % AC was also good for rooting. Activated charcoal (AC) was initially added to tissue culture media in an attempt to simulate soil conditions: now a day it is usually added in many tissue culture media

formulations. The promotary effects of AC on rooting may be due to its irreversible adsorption of inhibitory compounds in the culture medium and substantially decreasing phenolic exudation and brown exudate accumulation. In addition to this, activated charcoal is also involved in a number of stimulatory and inhibitory activities which promote growth. IBA was effective for root induction of banana shoots and frequently used for this purpose in solid and semisolid medium. Half MS medium supplemented with IBA produced a good number of roots. Pulse treatment of IBA and NAA combination was used to produce in vitro rooted plants. Alone NAA could root in vitro shoots of *Musa textilis* and AAB bananas. The establishment success of in vitro plant in a field depends upon the method used for hardening. In vitro plants which have poor cuticle and open stomatas, therefore, lose water rapidly under field condition and also prone to different diseases. Shoots cultured on medium supplemented with triazoles established better as compared to control. In vitro triazole treatment acts as a conditioner and obviated the need for hardening. To minimize the cost, plantlets were hardened without a greenhouse. The humidity was maintained by spraying water after 2 h. On an average 92 % of the plantlets survived.

Hardened plants are shifted to the field, and their establishment in the field is found to be good by many workers. The yield performance of plant depends upon the spacing between the plants (Nokoe and Ortiz 1998). Plant number varies according to variety as the growth of the plant varies plant to plant. The leaf and pseudostem were bigger in micropropagated plant as compared to original suckers. Higher yield in micropropagated plant is due to large bunch and shorter cycle to harvest. However, in some cases phenotypical variations were seen, and the plants did not show any higher yield. To evaluate, regenerants were compared with mother clone, and significant variation was observed in fruit maturity and leaf size. Among all the variants, three of four variants were inferior to mother clone from which they were derived. Only one regenerant resembled to mother clone with reference to yield.

Among the banana growers, popular banana varieties are Grand Naine (G9), Yelakki, Dwarf Cavendish, and Nendran in India. Grand Naine (G9) is high-yielding variety and resistant to Panama wilt. Yelakki is good in taste and is preferred in religious ceremony but prone to Panama wilt, and sometimes complete devastation of the crop occurs by the disease. The selling price of them is different; Yelakki market value is higher than G9, but as G9 is resistant Panama wilt is more popular among growers. Desai Fruits and Vegetables (DFV) is an agribusiness and India's leading exporter company of premium banana. Company works with the farmers in Gujarat, Maharashtra, Tamil Nadu, and Andhra Pradesh and also exports banana in Middle East (Dubai, Iran, Iraq, Kuwait), Saudi Arabia, Oman, Qatar, and Eastern Europe (Ukraine). In the year 2013–2014, the production of banana tissue culture was 250,000 MT and is expected to reach 300,000 MT in 2014–2015 (www.desaifv.com). Cadila produces eight millions of plants per annum of Grand Naine (G9), and SECON Agriventure produces G9, Yelakki, and Nendran bananas.

16.2.4 Case Study 4 Micropropagation of Bamboo (*Bambusa* sp.)

Bamboos are natural polyploids and variability occurs within the seedling of tropical and temperate bamboos. In the seedling, variability occurs at the genetic and the phenotypic level. Micropropagation via tissue culture has attracted lots of interest, and it was thought that this method could crack most or at least some problems in propagation of bamboo. Woody bamboos have been used for different applications, but recently interest from paper and wood industries has increased, for both tropical and temperate woody bamboos. Bamboo can be cultivated by different ways like seeds, clump division, and rhizome cuttings. But these methods are insufficient for small- or large-scale propagation, especially when the target is in lakhs. Bamboo has huge market round the world; temperate bamboos are used for agroforestry in China and as

ornamental plants in Europe and the USA. The magnitude of the demand for bamboo planting materials indicates that micropropagation will be necessary for large-scale propagation. The total world production of all tissue cultured plants in 1995 was estimated in millions. Through micropropagation within a very short time, lakhs and millions of true to type plants can be made from one single elite clone. For large-scale production, efficiency of the propagation methods is important, but perhaps even more crucial is the genetic stability. Gielis and Oprins (1998) practiced commercially feasible micropropagation for large-scale propagation for species and varieties of temperate and tropical bamboos, and the technology was capable to propagate any bamboo. The main emphasis was on the propagation of more than 60 different species, cultivars of bamboo, ornamental bamboos, and tropical bamboos. The approach for successful method involves three major components: (1) research on bamboo physiology and genetics, (2) the development of propagation method using axillary branching into a universal technique with a high clonal fidelity and very high efficiency, and (3) evaluation of in vitro to in vivo performance. The developed method could possibly micropropagate almost any selected bamboo at mass scale in a short time frame. Huge numbers of bamboo can be transported from laboratory to site or anywhere in the world. The quality of young plants derived through micropropagation was excellent. The quality of plants from tissue culture was considerably better than those propagated via conventional methods of propagation. The technology developed ensures the best possible quality, which is also reflected in the bamboo tissue culture standards in India. Some of these practices are:

1. Micropropagation cultures are only initiated from selected mother plants kept in greenhouses with known phenotypes. Only adult plants were used as explant.
2. Cultures for large-scale production need to be changed yearly; high-production genotypes are multiplied maximum of 16 subcultures using axillary branching.
3. During dispatch detailed labeling of the kind and variety of the plants.
4. Propagation rates (multiplication rates) are kept below five. Overproducing cultures are not used in production.
5. During the transfer of cultures, only the qualitatively best cultures are maintained; the others are simply discarded.

16.3 Somaclonal Variation

In vitro-regenerated plants show variations; it can be genetic (permanent) or epigenetic (reversible and temporary). This phenomenon is known as "somaclonal variation," and the frequency of somaclonal variation (SV) is usually elevated far beyond that expected in nature. Bird (2007) defined epigenetic events as the structural adaptation of chromosomal regions so as to register, signal, or responsible for altered activity. Epigenetic variation in vitro reflects the adaptation process of cells in a different environment which includes the response to signals that may trigger button in the developmental program. However, such adaptive alterations may compromise the genetic makeup for which the plant cells or tissues were cultured. Therefore, a detailed study is necessary of the epigenetic occurrence and its consequences likely to occur in the longer term. Chromatin structure may change by epigenetic mechanisms such as DNA methylation, histone modifications (histone methylation, histone deacetylation, phosphorylation, and carbonylation), RNA interference (RNAi), and ultimately lead to phenotypic variation.

DNA methylation affects genomic imprinting, chromosome inactivation, silencing of transposons, and other repetitive DNA sequences. DNA methylation can be divided into three different types in the reference sequence of cytosine, high methylation at CG sites, medium methylation at CHG sites, and lowest methylation at CHH sites (H =A, C, or T) (Feng et al. 2010). In comparison to eukaryotic organisms, plants and well-developed multicellular organisms show high DNA methylation, may be due to a complex

development system requiring additional epigenetic regulation to manage the development of many different types of cells. Histone deacetylases (HDACs) are involved in eukaryotic gene regulation by catalyzing the acetyl groups and deletion from lysine residues on histone and this way transcriptionally repress gene expression. The changes in chromatin structure influence gene transcription in response to diverse exogenous and endogenous stimuli like pathogen attack, water, heat, cold, light, and hormones (Anzola et al. 2010). Hyperacetylation of histones is involved with active gene expression and hypoacetylation with gene repression. It was found that H3K27me3 regulates many genes that act independently of other epigenetic pathways like DNA methylation and smRNAs. SmRNAs not only influence at the posttranscriptional level but also affect RNA-directed DNA methylation. During grafting of *Arabidopsis*, it was known that small interfering RNA (siRNA) and microRNA (miRNA) can be responsible for epigenetic changes in the genome of recipient cells (Carlsbecker et al. 2010). Let us understand the process by the schematic diagram as variation occurs at different developmental stages of the regeneration, at each stage different genes involve in the process (Fig. 16.5).

16.3.1 Dedifferentiation

The regeneration of plants through differentiation is a clear example of the plasticity of plant cells. Cells regenerate in response to certain environmental signals, and cells acquire capability for undergoing dedifferentiation process followed by new developmental pathway. Histone methylation activity is essential for the generation of the dedifferentiated state into the cell cycle. During the cell cycle, change in chromatin structure and histone modifications occurs, and some of them are plant specific. Berdasco et al. (2008) treated undifferentiated cultures of *Arabidopsis* cell with a demethylating drug and found out many hypermethylated genes in callus and cell suspensions. Promoters of the MAPK12, GSTU10, and BXL1 genes were closely hypermethylated in callus and cell suspensions, at the same time as the TTG1, GSTF5, and SUVH8 genes occasionally become hypermethylated only in cell suspensions

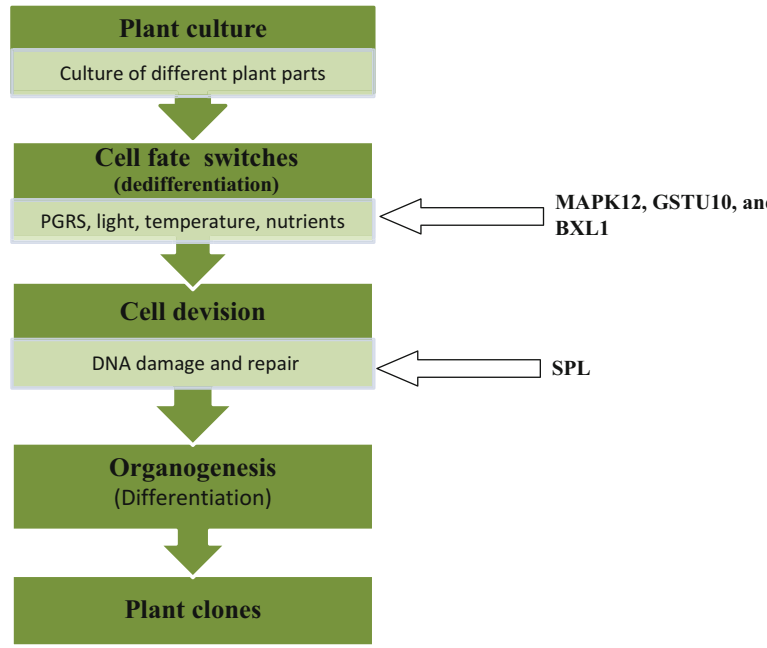


Fig. 16.5 In vitro plant regeneration and epigenetic variation at dedifferentiation and differentiation

(Fig. 16.5). In long-term suspension cultures, it was seen that euchromatin was hypermethylated, and heterochromatin was DNA hypomethylated resulting in activation of specific transposable elements, and its reprogramming of proliferating cells causes DNA methylation, histone modifications, and RNAi.

16.3.2 Differentiation

During organogenesis the proliferating cells differentiate when growth regulators (specific changes) are added to the culture medium and the process of plant restructuring takes place. Polycomb-group (Pc-G) proteins are known as epigenetic regulators and are key regulators for differentiation pathways. The miRNAs facilitated embryogenesis in *Arabidopsis* by preventing advance expression of differentiation promoting transcription factors Squamosa Promoter Binding Protein-Like (SPL) (Fig. 16.5). During in vitro regeneration, reset of epigenetic reprogramming may be needed to erase the effects of epigenetic marks caused by external stimuli. An accumulation of epigenetic changes during successive cell divisions may increase the risk responsible for dangerous epigenetic alleles. Ex vitro clonally propagated plants are also prone to these changes.

16.3.3 Methodologies for Detecting Epigenetic Variation

The most common tools to analyze somaclonal variations are random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), simple sequence repeat (SSR), and DNA methylation markers; however, RAPD has a problem with reproducibility. Variation in the plants cultured in vitro has been analyzed mainly by AFLP (amplified fragment length polymorphism)-based techniques (Vos et al. 1995). MSAP has found to be the best method for finding changes in cytosine; however, it has limitations like it can only detect a small proportion of the methylated cytosines and the changes between unmethylated sites and internally methylated sites. Another method of quantification of 5mC can be carried out by HPCE (high performance capillary electrophoresis) and HPLC (high performance liquid chromatography) techniques (Fraga and Esteller 2002); here genomic DNA is digested into nucleotides, nucleosides, or bases. Through HPCE method, epigenetic variation has been detected in some plant species. These methods can map genomic DNA methylation and histone modifications at unmatched levels. The tools like chromatin immunoprecipitation (ChIP)-chip and ChIP-seq can be used to map DNA protein relations (Miguel and Marum 2011).

The developmental state of the explant is affecting the ability to regenerate and unable to regenerate shoots, roots, or embryos from somatic tissues. Needle maturation in *Pinus radiata* caused depletion in organogenic capability was due to DNA methylation and low acetylated histone. Under in vitro conditions, DNA methylation, histone modification, and other regulatory proteins have important roles to play in plant development. Postculture behavior and genetic, epigenetic effects, and their related problems need to be studied. Phonotypical variations like dwarfs, color changes or mosaic patterns (chlorosis), growth pattern changes with the inconsistent multiplication rate, change in leaf shape, growth vigor, and changes in productivity or prolonged juvenility are observed in micropropagated plants. Large numbers of plants are produced in vitro; each year and phonotypical variations like dwarfs, color changes or mosaic patterns (chlorosis), growth pattern changes with the inconsistent multiplication rate, change in leaf shape, growth vigor, and changes in productivity or prolonged juvenility are observed in micropropagated plants. Recent advances in molecular genetics, as well as more detailed physiological and cytological study of the effect of tissue culture on genotypic stability and plant performance, have allowed a clearer understanding of the observed variation. By understanding epigenetic regulation, it may be possible to avoid the negative consequences of variation as it's the only way to avoid it and there

no single recipe to control it. However, epigenetic changes are reversible and the plant regains its original makeup, but the duration and time is not fixed. In due course, it will be possible to deviate/change the regenerant outcomes by selecting conditions leading to varied epigenetic landscapes. This controlled change opens the way to epigenetic engineering in plants.

16.3.4 Advantages of Somaclonal Variations

- Useful for crop improvement by generating variability
- Creation of additional genetic variation like biotic stress resistance
- Increased and improved production of secondary metabolites
- Suitable variants for breeding of tree species of monoculture

16.4 Problems in Micropropagation

16.4.1 Plant Status

The use of mature tissue and older age explants for micropropagation is still a very difficult task. Generation of cultures using seedling is comparatively easy as compared to multiply elite germplasm growing in an open-field condition. In perennial plants, selection (required character, e.g., yield) is made after they gain maturity as fruiting starts after 3–5 years (optimum yield). Older age mother plants are difficult to multiply as aging causes poor response. Tree species/perennial plants growing in an open-field condition may be diseased (virus infection in latent form) and may cause poor or erratic culture behavior. Plants growing in high humid area are difficult to multiply under in vitro condition, as they are prone to different infections caused by bacteria or fungus. After a prolong number of culture passages, culture growth/multiplication rate declines.

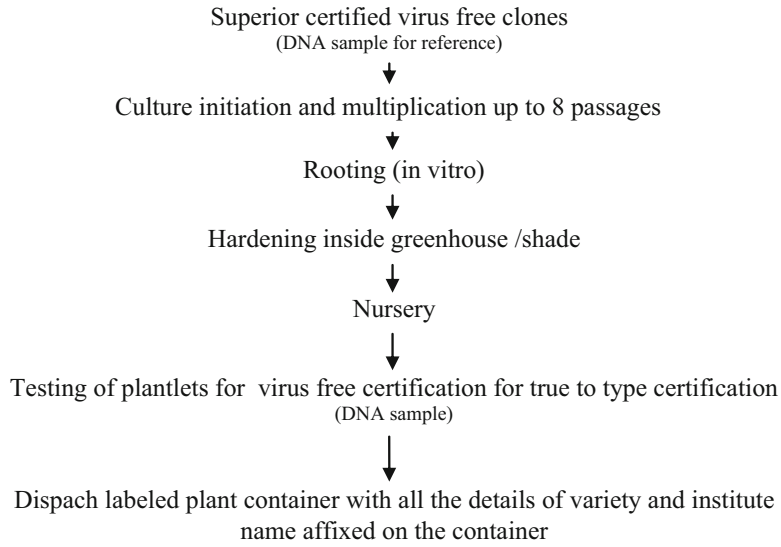
16.4.2 Contamination

Contamination is caused by poor inoculation technique, unhygienic laboratory condition, or by faulty autoclaving. The use of jars, boxes, and test tubes with lids/caps is safer as compared to cotton plugs for long-term culture maintenance. The poor explant sterilization technique can cause culture contamination. Contamination after numerous passages may be due to hidden microbes in groves of explants. The use of antibiotics can inhibit the growth of microbes, and its use in the medium can save cultures. Contamination can damage cultures in large number and lead to poor production.

16.5 Role of Government to Encourage Plant Tissue Culture Industry

In the year 2002–2003, the consumption of TCP was 44 million with 41 % of banana share. Looking the growth rate, the demand for tissue culture plants is expected to increase at a high rate of 25–30 %. It may increase to approximately 72 million for the year 2003–2004 and may reach 144 million in the next 5 years (survey report). To support the tissue culture industry, various central and state government departments have several schemes. Agriculture and Processed Food Products Export Development Authority (APEDA) under the Ministry of Commerce and Industry provides airfreight subsidy up to 25 % to tissue culture plants and 50 % subsidy for the development of infrastructure (Singh and Shetty 2011). In addition, 50 % subsidy is given to the farmers for purchase of tissue culture fruit crops by the Andhra Pradesh State Agriculture Department under the Macro Management Scheme. The rate of subsidy for export to Europe other than CIS countries, North America, and Far East, at the rate of Rs. 25 per kg or 25 % of the airfreight rate approved by IATA or one third of the FOB value whichever is the least (survey report). The Government of India has set up a national facility for virus diagnosis and quality control of tissue culture plants at New Delhi.

Procedures and standard parameters by government of India for production of banana by tissue culture



16.6 Conclusions and Future Prospective

Plant tissue culture techniques have an immense potential to produce plants of superior quality, but this potential has to be fully exploited by developing cost-effective protocols for micropropagation. In *J. curcas* compared to vegetative propagation through cuttings, high multiplication can be possible through micropropagation. It offers a much quicker way of propagation with a consistent supply of plantlets throughout the year. Limited number of shoot buds and slow growth of culture lead to high-cost propagation and can be one of the major hindrances to the direct use of micropropagation. There is a need to use low-cost options like bioreactors and liquid culture; they are meant for large-scale culture generation in liquid media. The advantages of bioreactors include high multiplication rate, reduction in medium cost and low power consumption, reduction in labor, and laboratory space. Due to the use of liquid medium in the bioreactor, the medium cost reduces; the estimated cost of 1-l solid MS medium is three times greater than for the liquid MS medium. There is the need to generate cultures using liquid medium as no attempt has been by anyone in *J. curcas*. There are, however, some disadvantages with the use of bioreactors. Enormous scope exists for genetic improvement of *J. curcas* through in vitro techniques, adopting biotechnological tools, including development of transgenic crops to synthesize high oil content in conventional oil seeds (Mazumdar et al. 2010). Somaclonal variation can be a useful tool to generate variability in the plant for hybridization. Micropropagated plants (are usually with fibrous root system) can be generated with a taproot system using grafting. There is a need to monitor micropropagated plant for its growth, survival, yield, and disease attach under field condition as very limited information is available. Long-term data will be useful for commercial growers/farmers and will define their future use in large-scale propagation/plantation generation.

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Efficacy of Biotechnological Approaches to Raise Wide Sexual Hybrids

17

K.R. Shivanna and Bir Bahadur

Abstract

Present-day cultivars of crop species have become susceptible to a range of biotic and abiotic stresses because of their narrow genetic base. The genes imparting resistance to these stresses are no more available within the cultivated species but are present in many wild and weedy relatives. To transfer desirable genes to the cultivars, the production of wide hybrids is an important pre-breeding requirement. One of the major limitations of wide hybridization is the presence of strong crossability barriers that operate before as well as after fertilization. During the last several decades, many biotechnological methods have become available, and their integration into the traditional methods of wide hybridization greatly increases the efficacy and reduces the time and efforts needed. Some of these include pollen storage, application of growth substances, stump pollination, placental pollination, in vitro fertilization and embryo rescue in the form of ovary, ovule and embryo culture. This review highlights the efficacy of these techniques in realizing wide hybrids and emphasizes the use of combination of techniques to make these approaches more successful. The interest on production and use of sexual wide hybrids has greatly reduced after the development of the techniques of somatic hybridization and genetic transformation. Although they provide powerful technologies to achieve some specific objectives of the breeding program, they cannot replace sexual hybridization for gene transfer. It is important for the plant breeders to refine and exploit fully some of the underexploited techniques described in this review in raising wide sexual hybrids.

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Brassica hybrids • Crossability barriers • Embryo rescue • *In vitro* fertilization • Ovary and ovule cultures • Placental pollination • Pollen storage • Wide hybrids

17.1 Introduction

The transfer of desirable genes to crop species from other accessions or species through sexual hybridization has been one of the most effective crop improvement programs (Goodman et al. 1987). Until recently, the major objectives of crop improvement programs have been on increasing the yield and improving the quality and genetic uniformity of the cultivars. Breeders have been able to achieve remarkable success on these lines in almost all crop species. Intensive breeding and selection for economic traits over the years have greatly reduced genetic variability in crop species. Because of their narrow genetic base, present-day cultivars have become susceptible to a range of biotic and abiotic stresses. Although most of the cultivars have the genetic potential for high yield under optimal agronomic conditions, their increased susceptibility to the stresses brings down the yield substantially (Boyer 1982). Broadening the genetic base of cultivated species, therefore, has become an important pre-breeding requirement to safeguard crop yield in the coming years.

In recent years, the objectives of the breeding program, apart from improvement in yield and quality, have become more diverse. Some of these additional breeding objectives are:

1. To breed varieties tolerant/resistant to biotic and abiotic stresses.
2. To breed varieties that have lower requirement for environmentally unfriendly chemicals particularly pesticides and herbicides. Excessive use of agrochemicals has been a major environmental problem of modern agriculture. The trend is to grow crops 'organically' without the use of environmentally unfriendly agrochemicals.
3. To breed varieties that can be grown on marginal lands. A large area of agricultural land is being degraded, particularly in developing countries, due to water logging, soil erosion, salinity, alkalinity and contamination with industrial chemicals. The recovery of such vast areas of land is not feasible because of the lack of suitable technologies and/or the high cost involved. Breeding crops that can be grown on marginal lands has become an alternate strategy.

Because of the constant erosion of genetic diversity in our crop species, genes imparting the required traits are no longer available within the cultivated species. A large number of wild and weedy relatives of crop species, however, form a good repository of such desirable genes (Harlan 1976). The plant breeders, therefore, often have to extend the breeding program across the species limits to tap genes of the wild and weedy species (wide hybridization). A good number of examples of successful gene transfer through wide hybridization are already available (Hawkes 1977; Hadley and Openshaw 1980; Stalker 1980; Goodman et al. 1987; Hermsen 1992; Kalloo 1992; Chopra et al. 1996; Sareen et al. 1992; Wang et al. 2005; Prakash et al. 2011). The primary requirement for transferring genes from wild species to the cultivars is the production of wide hybrids (hybrids from distantly related species).

In addition to the transfer of desirable genes from wild species to the cultivars, wide hybridization is also one of the standard approaches to develop new cytoplasmic male sterile lines (through cytoplasmic substitution), which are important for crop species (Labana et al. 1992; Shivanna and Sawhney 1997; Shivanna 2003; Prakash et al. 2011). Another application of wide

hybrids is to develop new alloploid crops such as triticale (*Triticum* × *Secale*) and *Raphanobrassica* (*Raphanus* × *Brassica*) (Sareen et al. 1992). Wide hybrids are also useful to study a range of problems of traditional and molecular cytogenetics, particularly for elucidating homology (Prakash et al. 2011).

The presence of strong crossability barriers between the species is the major constraint in any wide hybridization program. Early plant breeders used to resort to mass pollination, generally in thousands, to realize a few wide hybrids. Now, a number of biotechnological approaches have become available to overcome crossability barriers. Integration of these techniques with conventional breeding program greatly improves the efficiency of wide hybridization and reduces markedly the time and cost of breeding. This review briefly discusses some of the important biotechnological approaches that have been used to produce wide hybrids through sexual pathway and highlights their importance in the coming decades.

The following are a few general guidelines to be followed to achieve success in any wide hybridization program:

1. It is desirable to try different accessions of both the parents in crossing experiments since genetic variability within the species may affect the intensity of the barriers. Some of the accessions/genotypes may turn out to be more compatible when compared to others.
2. It is also necessary to check the viability of the pollen sample used for crosses and the receptivity of the stigma at the time of pollination (Shivanna and Rangaswamy 1992). If possible, it is better to use pollen grains collected from freshly dehisced anthers.
3. Many of these crosses show unilateral incompatibility (de Nettancourt 2001); it is, therefore, better to make crosses in both the directions.
4. Recent studies have shown that knowledge on the phylogenetic relationships of the cultivar with its wild relatives would help in selecting the wild parent which is likely to be more successful (Kubota et al. 2011). For example, in *Hydrangea*, interspecific crosses were found to be successful up to an average genetic distance of 0.01065. The crosses are likely to fail when the average genetic distance is 0.01385 and higher (Mendoza et al. 2013a, b). Similarly, in wheat-rye hybrids, significant differences in the frequency of normal embryo development were found when different lines of rye were used in rye × wheat crosses (Taira et al. 2011). It would therefore be helpful to check the available information on phylogenetic relationship of the selected species and use closely related parents for making crosses.

17.2 Crossability Barriers

Crossability barriers operate at different levels before and after fertilization (Sastri 1985; Shivanna 1997; de Nettancourt 2001). One of the common barriers particularly in distantly related species is the physical barrier imposed by geographical and/or temporal isolation of the parent species. The male and female parents do not grow at the same place and/or their flowering may not be synchronous, thus preventing effective pollination. Pre-fertilization barriers inhibit pollen germination or pollen tube entry into the stigma or subsequent growth of the pollen tubes before reaching the ovule. More often these barriers operate in a cumulative way; the proportion of pollen grains that complete germination or post-germination growth of pollen tubes at different levels may get reduced at each step, resulting in the failure of fertilization. In multiovulate systems, fertilization may occur in a few ovules which may not be sufficient to activate the development of the fruit and thus no seeds are realized.

Post-fertilization barriers operate at various levels: abortion of hybrid embryos at preglobular/globular/later stages, failure of hybrid seeds to germinate or the seedling to grow up to the flowering, hybrid sterility and lack of recombination. Post-fertilization barriers also operate in a cumulative way with the result that no usable hybrid is realized. A number of biotechnological approaches are now available to overcome such

barriers. Identification of the barrier(s) would help in the selection of effective method(s) to circumvent the barrier(s).

17.3 Methods to Overcome Physical Barriers

17.3.1 Pollen Storage

Pollen storage is one of the simple and effective methods to overcome physical barriers imposed by temporal and spatial isolation of the parent species. A number of techniques are now available to store pollen grains for extended periods in viable condition. When once a suitable technique is standardized for a given species, pollen grains of the male parent can be stored and used routinely for pollination when the female parent flowers. Apart from its application to overcome physical barriers, successful pollen storage eliminates the need to grow pollen parent continuously in the breeding program. Another important application of pollen storage relevant to the breeding program is that it provides a convenient and simple means of pollen exchange amongst breeders within and between countries. Many of the horticultural societies such as the American Rhododendron Society maintain their own pollen banks which are accessible to their members (Mayer 1983). Pollen grains are generally free from pathogens even when the parent plant is infected. Except for some viral diseases, there are no authentic reports of systemic transmission of fungal and bacterial diseases through pollen (Mink 1993). Therefore, the quarantine restrictions for the exchange of germplasm through pollen are much less when compared to seeds and vegetative parts of the plant. The utility of 'pollen banks', which would ensure the availability of pollen of the desired species/variety at any time of the year and at any place, has been emphasized since long. Pollen banks would greatly facilitate the breeding program, particularly of tree species which have to complete their juvenile phase, often lasting for several years, before flowering. Extensive literature, available on pollen storage, has been reviewed regularly (Johri and Vasil 1961; King 1965; Stanley and Linskens 1974; Shivanna and Johri 1985; Towill 1991; Hanna and Towill 1995; Barnabas and Kovacs 1997; Ganeshan and Rajasekaran 2000; Shivanna 2003). Many of these reviews list the pollen species stored for various periods under different storage conditions. The following is a brief description of the important methods used to store pollen grains of various species:

Storage of pollen under low temperature (+4 to −20 °C) and humidity (<10 % RH) conditions is one of the simple and commonly used methods particularly for horticultural species. Pollen grains are kept in small unsealed vials and stored in a desiccator or a suitable airtight vial containing an appropriate dehydrating agent such as dry silica to maintain low RH (Shivanna and Johri 1985; Shivanna and Rangaswamy 1992). The sealed desiccators are then kept in a refrigerator or a deep freeze. This method is very convenient and effective for short-term storage (for a few weeks/months) and has been used extensively by amateur horticulturists.

Storage under subfreezing temperatures (ca −20 °C) is effective for storing pollen grains of several species for more than a year. Frozen pollen of *Rhododendron* in home freezer has been reported to be viable for 3 years (Mayer 1983). Pollen grains of cereals in general cannot withstand desiccation and need to be stored under high RH in the refrigerator. Even under these conditions, viability of cereal pollen lasts only for a few days (Shivanna and Heslop-Harrison 1981).

Storage of freeze-dried/vacuum-dried pollen is an effective method for long-term storage. Freeze-drying involves rapid freezing of pollen (−60 to −80 °C) and gradual removal of water under sublimation. In vacuum-drying, the pollen is subjected to simultaneous cooling and vacuum-drying. Freeze-dried and vacuum-dried pollen grains generally do not show any differences in their responses to storage. The freeze-dried pollen is usually stored at sub-zero temperatures. For effective use of this method, optimum pollen water content, duration of drying and subsequent

rehydration have to be optimized (Barnabas and Kovacs 1997). Freeze-drying method has been effective for long-term storage of pollen grains of a number of species (King 1965; Towill 1991). However, in recent years, this method has not been very popular probably because of the success achieved by using a simpler technique of cryopreservation.

Cryopreservation of pollen is another effective method for long-term pollen storage and has become more popular than freeze-drying method in recent years. In this method, pollen grains are dried to bring their water content below a threshold level and stored in liquid nitrogen (Towill 1991; Barnabas and Kovacs 1997). Initial attempts to cryopreserve pollen grains of cereals were not successful largely because of their susceptibility to desiccation (which is critical for cryopreservation). Unlike the earlier methods used for drying pollen grains over a desiccant, Barnabas and her associates used a 'pollen drier' in which the air of 20 °C and 20–40 % humidity is blown through pollen, facilitating a rapid but gentle and uniform drying (Barnabas and Kovacs 1997). Through this drying method, it was possible to successfully cryopreserve the pollen grains of many cereals for several years.

17.4 Methods to Overcome Pre-fertilization Barriers

A number of techniques have been developed over the years to overcome pre-fertilization barriers that operate after pollination but before fertilization. Some of these methods along with a few examples are presented in Table 17.1. Additional examples are found in Van Tuyl and De Jeu (2003) and Shivanna (2003). According to Bates and Deyoe (1973), the inhibition reaction of pollen in the pistil is analogous to immunochemical reaction found in animals. On the basis of this hypothesis, the flowers were treated with some immunosuppressors such as E-amino caproic acid, salicylic acid or acriflavin (using similar methods used for treatment with growth substances), and a few wide

Table 17.1 Details of in situ pollination methods used to produce hybrids with some examples

Effective methods	Some examples
Application of growth substances	
Growth substances (auxins, cytokinins and gibberlins) applied around the pedicel of the flower or to the wound caused by removing one of the sepals/petals, at the time of pollination or soon after pollination. This is one of the oldest methods tried and has been successful in several crosses. Growth substances delay floral abscission and facilitate slow-growing pollen tubes to effect fertilization	Interspecific crosses of *Vigna* (Chen et al. 1978), *Agropyrum* (Alonso and Kimber 1980), *Triticum* (Mujeeb-Kazi 1981), *Arachis* (Sastri 1985; Sastri and Moss 1982; Sastri et al. 1983), *Corchorus* (Park and Walton 1990) and *Hordeum* (Subramanyam 1999)
Mentor pollen	
Pollinated with a mixture of mentor pollen (compatible pollen made ineffective to effect fertilization, generally prepared by exposing pollen to high doses of irradiation) and viable pollen of the male parent	Very effective in several crosses of *Populus* (Knox et al. 1972; Stettler 1968; *see* Villar and Gaget-Faurobert 1997). In wide crosses of *Sesamum*, it was effective in overcoming the inhibition of pollen germination but not of pollen tube growth in the style (Sastri and Shivanna 1976). Not effective for *Lilium* (Van Tuyl and de Jeu 2003)
Stump pollination	
The stigma and a part or the whole of the style are excised, and pollen grains are deposited at the cut end of the style or the tip of the ovary. This reduces the distance through which pollen tubes have to grow to reach the ovules. Application of suitable medium on the stump before pollination may facilitate pollen germination	Interspecific crosses of corn (Heslop-Harrison et al. 1985), *Lilium* (van Tuyl et al. 1991; Janson et al. 1993), *Lathyrus* (Herrick et al. 1993), *Fritillaria* (Wietsma et al. 1994)

(continued)

Table 17.1 (continued)

Effective methods	Some examples
Style grafts	
Pollination is carried out on compatible stigma, and after pollen grains have germinated and pollen tubes have grown through a part of the style, the style is excised in front of the growing pollen tubes and grafted onto incompatible pistil. This is a delicate operation and can be done only in species with robust pistils. In *Lilium*, grafting was carried out using a straw filled with the exudates of compatible stigma	Interspecific crosses of *Lilium* (Van Tuyl et al. 1991; Van Tuyl and De Jeu 2003)
Bud pollination	
Incompatible pollination carried out in the bud stage when the factors that induce crossability barriers are not likely to be fully developed. This approach has not been used frequently; as it is one of the simplest techniques, it may be worthwhile trying	Interspecific crosses of *Nicotiana* (Kuboyama et al. 1994)
Intraovarian pollination	
Injection of pollen grains suspended in a suitable medium directly into the ovary. This is another simple technique which has not been tested with additional systems. Lack of ovarian cavity in many species is a limitation	Interspecific crosses of *Argemone* (Maheshwari and Kanta 1961)

hybrids were realized in some cereals and legumes (Van Tuyl and De Jeu 2003; Shivanna 2003). However, so far, there are no evidences to indicate the involvement of immunochemical reactions in crossability barriers, and there have been no additional reports on the success of immunosuppressors in realizing wide hybrids.

17.4.1 *In Vitro* Pollination of Cultured Ovules

In this method, instead of carrying out pollination on the stigma, pollen grains are deposited directly on the ovules. In one of these methods, a group of ovules are excised from the ovary and cultured on a nutrient medium (Kanta et al. 1962; Kameya and Hinata 1970); the ovule mass is dusted with pollen grains. In another method termed placental pollination, the entire mass of ovules intact on the placenta(e) together with a short length of pedicel is excised and cultured by inserting only the pedicel in the medium (Rangaswamy and Shivanna 1967). Pollen grains are dusted on cultured ovule mass (Shivanna and Rangaswamy 1992). As this technique eliminates pollen-pistil interaction altogether and brings pollen grains in direct contact with the ovules, it is likely to be more effective than other methods to overcome pre-fertilization barriers. In successful pollinations, pollen grains germinated on the ovule mass; pollen tubes entered the ovules and effected fertilization. The fertilized ovules developed into seeds. Ovule pollination has been used successfully to produce interspecific as well as intergeneric hybrids particularly by Zenkteler and his associates (Zenkteler 1980, 1990; Zenkteler and Bagniewska-Zadworna 2001). Some of the successful crosses include *Melandrium album* × *M. rubrum*, *M. album* × *Silene schafta* (Zenkteler 1980, 1990) and several interspecific crosses of *Nicotiana* (Reed and Collins 1978; DeVerna et al. 1987; Zenkteler 1990). Attempts were also made to obtain interspecific/intergeneric hybrids in Brassicaceae (Kameya and Hinata 1970; Zenkteler 1990; Zenkteler et al. 1987). Hybrids were realized in a few combinations, but in some others, although there was normal fertilization, hybrid embryos degenerated. Isolation and culture of embryos on a suitable nutrient medium would probably enable the production of hybrids in such crosses also. In an interesting treatment,

placental cultures of some flowering plants were pollinated with pollen grains of a few gymnosperms; pollen grains of *Pinus* and *Ephedra* could germinate on placental cultures of several flowering plants (Zenkteler and Bagniewska-Zadworna 2001). Although some pollen tubes were occasionally seen entering the ovules, no hybrid embryos were realized.

17.4.2 *In Vitro* Fertilization

Fertilization in flowering plants takes place deep inside the ovule, and this is one of the limitations to conduct experimental studies on fertilization. Following the dramatic progress of protoplast technology of somatic cells, a number of investigators started work on isolation of male and female gametes of flowering plants in the 1980s. Success was soon achieved in isolation of protoplasts of embryo sacs and sperm cells of a number of species and to keep them in viable condition for considerable length of time (Cass 1997; Mathys-Rochon et al. 1997). In three-celled pollen, sperm cells were isolated by incubating pollen grains in hypotonic medium that results in bursting of pollen grains or mechanically rupturing the pollen grains by gentle grinding in an isolation medium. The released sperm cells were purified and washed suitably. In two-celled pollen, a semi-vitro method (Shivanna and Rangaswamy 1992) was used to allow pollen tubes to grow partly inside the style and then emerge into a nutrient medium. This was followed by the treatment of pollen tubes with cell wall-degrading enzymes or subjecting pollen tubes to osmotic shock to the release of sperm cells from the tip of pollen tubes (Shivanna et al. 1987). Subsequently it was possible to isolate the components of the embryo sac (egg, synergids, and central cell). By 1990s, all the basic requirements needed to try in vitro fertilization in flowering plants were available.

Kranz and his associates were the first to achieve in vitro fertilization in maize by bringing isolated egg and sperm cells together under the microscope in microdroplets of the fusion medium (Kranz and Lorz 1993; Kranz 1997; Okamoto and Kranz 2005). In vitro formed zygotes were grown successfully by using nurse culture method (by culturing zygotes on a semi-permeable membrane placed on fast-growing non-morphogenetic cell suspension cultures derived from maize embryos or microspores) into plantlets and eventually into adult fertile plants. Subsequently the sperm cell was also fused with the central cell; the fusion product did not give rise to the embryo but gave rise to an unorganized tissue comparable to the endosperm in vivo. Subsequently several modifications in the protocols to improve the efficacy of fusion and embryo development have been reported (Kranz 1997; Wang et al. 2006; Kranz et al. 2008).

Apart from its application in tackling a range of problems fundamental to fertilization (*see* Okamoto and Kranz 2005; Wang et al. 2006), one of the most obvious practical applications of in vitro fertilization is in realizing wide hybrids. Although the success has so far been confined in achieving fertilization and embryo development between gametes of compatible species, studies of Kranz and his associates (Kranz 1997, 2008; Scholten and Kranz 2001) have shown that there is no technical difficulty in achieving in vitro fertilization between isolated egg and sperm cells in interspecific and intergeneric combinations. However, the development of zygotes is restricted to crosses between closely related species (Kranz and Dresselhaus 1996). Hybrid zygotes between maize (egg donor) and sperm cell of many other members of Poaceae gave rise to multicellular structures, while those resulting from the egg of maize and sperm of a distant species, *Brassica*, failed to divide. Further studies are needed to extend this technology to other species and to realize useful interspecific and intergeneric hybrids from in vitro fused zygotes.

17.5 Methods to Overcome Post-fertilization Barriers

Post-fertilization barriers operate after fertilization. Depending on the extent of reproductive isolation between the parent species, the embryo abortion may initiate at a very early stage of development or after the growth of embryo to different stages.

17.5.1 Embryo Rescue

Embryo rescue has become the most effective and routinely used technique to overcome post-fertilization barriers. When the abortion occurs at a very early stage, it is difficult to excise the embryo and also to culture it successfully as its nutrient requirements are more complex and precise. Embryo excision is a problem even at later stages when the developing seeds are very small. In such instances, it is more convenient to culture the whole ovule or even the ovary. Ovule and ovary culture facilitates embryo growth in situ without exposure to in vitro disturbances. In crosses where the embryo aborts at later stages, embryo culture is the most ideal. There are a large number of successful hybrids realized through embryo rescue (*see* also Chap. 18 of this volume). For details of the technique and comprehensive examples, the reader may refer to Maheshwari and Rangaswamy (1965), Raghavan (1977, 1986, 1999), Rangan (1982), Williams et al. (1987), Sharma et al. (1996) and Van Tuyl and De Jeu (2003). A modified technique of embryo rescue termed 'sequential culture' has been reported to be more effective than either ovary or ovule culture alone in realizing many wide hybrids (Nanda Kumar et al. 1988; Agnihotri 1993; Shivanna 2000). In sequential culture, ovaries are cultured 4–8 days after pollination; cultured ovaries are taken out 7–10 days after culture, dissected under aseptic conditions, and enlarged ovules (young seeds) are then re-cultured on a fresh medium. In successful crosses, cultured ovules grow further and germinate in vitro. In some crosses, it was necessary to dissect the embryo from cultured ovules and re-culture the embryo. Some examples of wide hybrids realized through ovary, ovule, embryo and sequential cultures are presented in Table 17.2. In a few crosses of legumes, hybrid embryos have been transplanted into the developing endosperm of compatible seeds

Table 17.2 Some wide crosses produced through embryo rescue

Effective methods	Some examples
Ovary culture	
Pollinated ovaries are excised before the onset of embryo abortion and cultured on a suitable nutrient medium	*Phaseolus* (Sabja et al. 1990), *Lilium* (Van Tuyl et al. 1991), *Eruca* × *Brassica* hybrids (Agnihotri et al. 1990a), *Brassica* (Gundimeda et al. 1992), *Brassica napus* × *Raphanobrassica* (Agnihotri et al. 1990b)
Ovary slice culture	
Pollinated ovary, a few days after pollination, is transversely cut into slices and the slices along with attached ovules are cultured	So far confined to *Lilium* (Kanoh et al. 1988; Van Tuyl et al. 1991; Janson et al. 1993; Obata et al. 2000)
Ovule culture	
Ovules are excised from pollinated ovaries before the initiation of embryo degeneration and cultured. In some crosses, cultured ovules are re-cultured on a different medium	*Alstroemeria* (Bridges et al. 1989; De Jeu et al. 1992), *Cyclamen* (Ishizaka and Uematsu 1992), *Nicotiana* (Reed and Collins 1978; Iwai et al. 1986), *Lilium* (Ikeda et al. 2003), *Gentiana* (Morgan 2004), *Hylocereus* (Cisneros and Tel-Zur 2010), Asteraceae species (Wang et al. 2014)
Embryo culture	
Embryo is dissected from fertilized ovules and cultured	*Allium* (Nomura and Oosawa 1990), *Lilium* (Van Tuyl et al. 1991), *Solanum* (Singsit and Hannaeman 1991), *Gossypium* (Mehetre and Aher 2004), *Hylocereus* (Sage et al. 2010)
Sequential culture	
Culture of pollinated ovary, followed by culture of ovules from cultured ovaries and finally culture of embryos from cultured ovules. The time gap between each varies. In some species, culture of ovary and then ovule was enough to recover hybrids, while in some other species, culture of ovary, ovule and then embryo was successful in recovering hybrids	*Brassica* (Nanda Kumar et al. 1988; Agnihotri et al. 1990a; Gundimeda et al. 1992; Nanda Kumar and Shivanna 1993; Vyas et al. 1995), *Medicago* (McCoy and Echt 1993), *Lilium* (Van Tuyl and De Jeu 2003)

(after removing compatible embryo), and such transplanted embryo-endosperm complex has been successfully cultured (Williams and Latour 1980).

17.5.2 Wide Hybrids in *Brassica*

Brassica is an important oilseed crop in several countries. All the three major cultivated species, *B. rapa*, *B. juncea and B. napus*, are susceptible to a number of diseases, pests and abiotic stresses and result in low productivity. The genes imparting resistance to these stresses are not available in the cultivated species, but many of their wild relatives possess such genes (*see* Prakash et al. 2011). A large number of wide hybrids have been produced in crop brassicas with many of their wild relatives through embryo rescue, especially from the University of Delhi in collaboration with the Indian Agricultural Research Institute, New Delhi (Shivanna 1995, 2003; Prakash et al. 2011). The details of the crossability barriers between all the cultivated species and 12–20 wild relatives have been documented by Singh et al. (2007). Of the 100 cross combinations tested, 73 crosses showed pre-fertilization barriers and 27 crosses showed post-fertilization barriers. All the wide hybrids produced by this group through embryo rescue are listed in Table 17.3.

Table 17.3 Wide (interspecific and intergeneric) hybrids of *Brassica* realized through embryo rescue

Sr no.	Hybrids	Reference
1	*Brassica rapa* × *Erucastrum gallicum*	Batra (1991)
2	*B. rapa* × *E. abyssinicum*	Rao (1995)
3	*B. juncea* × *Sinapis pubescens*	Gundimeda et al. (1992)
4	*B. juncea* × *B. gravinae*	Nanda Kumar et al. (1989)
5	*B. juncea* × *Enarthrocarpus lyratus*	Gundimeda et al. (1992)
6	*B. napus* × *B. gravinae*	Nanda Kumar et al. (1989)
7	*Eruca sativa* × *B. rapa*	Agnihotri et al. (1990a)
8	*Brassica napus* × *Raphanobrassica*	Agnihotri et al. (1990b)
9	*B. cossoneana* × *B. rapa*	Verma (1993)
10	*B. cossoneana* × *B. carinata*	Verma (1993)
11	*B. cossoneana* × *B. juncea*	Verma (1993)
12	*B. cossoneana* × *B. napus*	Verma (1993)
13	*B. cossoneana* × *B. oleracea*	Verma (1993)
14	*B. fruticulosa* × *B. rapa*	Nanda Kumar et al. (1988)
15	*B. maurorum* × *B. rapa*	Chrungu et al. (1999)
16	*B. maurorum* × *B. nigra*	Chrungu et al. (1999)
17	*B. maurorum* × *B. juncea*	Chrungu et al. (1999)
18	*B. maurorum* × *B. oleracea*	Chrungu et al. (1999)
19	*B. maurorum* × *B. carinata*	Chrungu et al. (1999)
20	*Diplotaxis assurgens* × *B. rapa*	Vyas (1993)
21	*D. berthautii* × *B. rapa*	Vyas (1993)
22	*D. catholica* × *B. rapa*	Mohanty (1996)
23	*D. catholica* × *B. juncea*	Mohanty (1996)
24	*D. erucoides* × *B. rapa*	Vyas et al. (1995)
25	*D. erucoides* × *B. juncea*	Vyas et al. (1995)
26	*D. erucoides* × *B. napus*	Vyas et al. (1995)
27	*D. erucoides* × *B. oleracea*	Vyas et al. (1995)
28	*D. siifolia* × *B. juncea*	Batra et al. (1990)
29	*D. siettiana* × *B. rapa*	Nanda Kumar and Shivanna (1993)
30	*D. siifolia* × *B. napus*	Batra et al. (1990)

(continued)

Table 17.3 (continued)

Sr no.	Hybrids	Reference
31	*D. tenuisiliqua* × *B. rapa*	Vyas (1993)
32	*D. tenuisiliqua* × *B. juncea*	Vyas (1993)
33	*D. tenuisiliqua* × *B. napus*	Vyas (1993)
34	*D. tenuisiliqua* × *B. oleracea*	Vyas (1993)
35	*D. viminea* × *B. carinata*	Mohanty (1996)
36	*D. viminea* × *B. napus*	Mohanty (1996)
37	*Enarthrocarpus lyratus* × *B. campestris*	Gundimeda et al. (1992)
38	*E. lyratus* × *B. carinata*	Gundimeda et al. (1992)
39	*E. lyratus* × *B. napus*	Gundimeda et al. (1992)
40	*E. lyratus* × *B. oleracea*	Gundimeda et al. (1992)
41	*Erucastrum abyssinicum* × *B. rapa*	Rao et al. (1996)
42	*E. abyssinicum* × *B. carinata*	Rao et al. (1996)
43	*E. abyssinicum* × *B. juncea*	Rao et al. (1996)
44	*E. abyssinicum* × *B. oleracea*	Rao et al. (1996)
45	*E. cardaminoides* × *B. oleracea*	Mohanty et al. (2009)
46	*E. gallicum* × *B. juncea*	Batra et al. (1989)
47	*E. gallicum* × *B. napus*	Batra et al. (1989)
48	*E. varium* × *B. rapa*	Das (1993)
49	*E. varium* × *B. juncea*	Das (1993)
50	*E. varium* × *B. oleracea*	Das (1993)

17.6 Bridge Cross Hybrids

As pointed out earlier, the strength of the cross-ability barriers of the cultivar may vary with different wild relatives. The barriers may not be so strong with some of the wild relatives, but such species may not have desirable traits; those that have desirable traits may show strong barriers with the cultivar. Another simple method, referred to as bridge cross method, has been used by the breeders to circumvent such barriers. In this method, the hybrid is produced between the cultivar and one of the wild species (which does not show strong barriers with the cultivar), and this hybrid or its amphidiploid is used as the bridge species to raise hybrids with another wild species (which shows strong barriers with the cultivar but has desirable trait). This approach has been very effective in a number of crop plants – wheat, tobacco, potato, lettuce, sugarcane and *Brassica* (*see* Shivanna 2003). In some combinations, it was possible to realize bridge cross hybrids through field pollinations without resorting to application of any special technique. Bridge cross technique is simple and very useful particularly to transfer the cytoplasm from one species to the other (Shivanna 1995, 2000), although the transfer of nuclear genes through this method involves more elaborate breeding program (Hadley and Openshaw 1980). The bridge cross hybrids raised in *Brassica* by Delhi group are listed in Table 17.4.

17.7 The Development of New Cytoplasmic Male Sterile (CMS) Lines in *Brassica*

Male sterile lines are very important to exploit hybrid vigor in crop plants. By using male sterile line as the female parent, intra-line pollinations are prevented completely in hybrid seed production plots. Male sterile lines developed through different approaches (genic male sterile lines, cytoplasmic male sterile lines, male sterility induced through application of chemicals and also through recombinant DNA technology) are

Table 17.4 Wide hybrids in *Brassica* realized through bridge cross method

Sr. no.	Hybrid	Reference
1	(*Diplotaxis siettiana* × *Brassica rapa*) × *B. juncea*	Nanda Kumar and Shivanna (1993)
2	(*D. siettiana* × *B. rapa*) × *B. napus*	Nanda Kumar and Shivanna (1993)
3	(*D. berthautii* × *B. rapa*) × *B. juncea*	Vyas (1993)
4	(*D. tenuisiliqua* × *B. rapa*) × *B. napus*	Vyas (1993)
5	(*D. erucoides* × *B. rapa*) × *B. juncea*	Vyas et al. (1995)
6	(*Erucastrum abyssinicum* × *B. juncea*) × *B. napus*	G.U. Rao (unpublished)
7	(*E. abyssinicum* × *B. juncea*) × *B. nigra*	G.U. Rao (unpublished)
8	(*E. abyssinicum* × *B. juncea*) × *B. rapa*	G.U. Rao (unpublished)
	(*E. cardaminoides* × *B. oleracea*) × *B. napus*	Mohanty et al. (2009)
	(*E. cardaminoides* × *B. oleracea*) × *B. carinata*	Mohanty et al. (2009)

available. Of these, cytoplasmic male sterile (CMS) lines have several advantages over others in producing hybrid seeds (*see* Shivanna and Sawhney 1997; Shivanna 2003). One of the standard approaches to produce CMS lines is through the development of alloplasmic lines (containing the cytoplasm of one species and the nuclear genes of another species) as many of them result in cytoplasmic male sterility. This involves production of wide hybrids (wild species as the female and cultivar as the male parent). As the cytoplasm is inherited only through the egg in a majority of species, the hybrids possess the cytoplasm of the wild species and the nuclear genome of both the parents. The hybrid or its amphiploid is repeatedly backcrossed with the cultivar (as male parent) to eliminate the nuclear genome of the wild species. Although many CMS lines were available in crop *Brassica* (Banga 1992; Shivanna 2000; Prakash et al. 2011), they show many limitations such as chlorophyll deficiency, thermosensitivity of male sterility and presence of female sterility. Also, many of them do not have suitable restorers needed for hybrid seed production. There has been a need to produce more

Table 17.5 Cytoplasmic male sterile (CMS) lines of *Brassica* produced through sexual alloplasmics

Cytoplasmic donor	Recipient species	Reference
Diplotaxis siifolia	*Brassica juncea*	Rao et al. (1994)
D. siifolia	*B. napus*	Rao and Shivanna (1996)
D. erucoides	*B. juncea*	Malik et al. (1999)
D. berthautii	*B. juncea*	Malik et al. (1999)
D. catholica	*B. juncea*	Mohanty (1996)
Erucastrum lyratus	*B. rapa*	Gundimeda et al. (1992), Deol et al. (2003)

CMS and restorer lines. The Delhi University group has been able to produce several new CMS lines in the background of the cytoplasm of different wild species. These CMS lines are listed in Table 17.5.

Restorer lines have been developed subsequently for some of these alloplasmic lines: *B. juncea* carrying *Diplotaxis catholica* cytoplasm (Pathania et al. 2003); *B. rapa* (Deol et al. 2003), *B. juncea* (Banga et al. 2003) and *B. napus* (Janeja et al. 2003) carrying *Enarthrocarpus lyratus* cytoplasm; and *B. juncea* carrying *D. erucoides* cytoplasm (Bhat et al. 2005).

17.8 Multiplication of Hybrids

The number of hybrids realized in wide crosses even after using biotechnological methods is rather limited. A large number of hybrids are needed for morphological and cytological studies, induction of amphiploidy to restore fertility and to raise backcross progeny. Hybrids can easily be multiplied through the use of in vitro culture technique (Nanda Kumar and Shivanna 1991; Agnihotri et al. 1990a, b; Shivanna 2000). Culture of shoot tips or single node segments on a medium containing one of the cytokinins is effective in inducing multiple shoots. The shoots are isolated and cultured on an auxin-containing medium to induce rooting. The plantlets are then hardened and transferred to the soil. Hybrids can also be multiplied through the induction of callus from hybrid embryos or hypocotyl segments and

subsequent regeneration of plantlets through shoot and root regeneration pathway or somatic embryogenesis pathway (Nanda Kumar and Shivanna 1991; Agnihotri et al. 1990a, b).

17.9 Concluding Remarks

The production of wide hybrids is an important pre-breeding step in transferring genes across species limits. Early breeders, in spite of low rate of success, were able to realize a number of wide hybrids in many crop species and to transfer important traits from the wild species to the cultivars. Integration of some of the tools of biotechnology to the breeding program has made the production of wide hybrids more effective, and a large number of wide hybrids have been produced through this approach. Pollen storage is probably the simplest and effective technique to overcome nonsynchronous flowering and geographical isolation of the parent species. Although several pollen banks of many horticultural species have come up to supply pollen for the breeding programs, pollen banks for crop species are yet to be established as a routine facility at the international level. Establishment of effective pollen banks, similar to seed banks, at global level would greatly facilitate the breeding programs of crop species.

There is a scope for further refinement of many of the available techniques to overcome pre-fertilization barriers. For example, by carrying out stump pollination or stylar grafting on cultured pistils (instead of flowers retained on plants), the operations can be performed and controlled more efficiently. Cultured pistils would provide a more convenient system to conduct additional treatments such as irradiation or high-temperature treatment to the pistil, which have not yet been tried seriously to raise wide hybrids. Cultured pistils are also ideal to perform intrastylar pollinations (in hollow-styled systems) and mixed/mentor pollinations. These treatments can be combined with manipulation of the medium and other conditions to improve the growth of cultured pistils. Also, the full potentials of other in vitro techniques particularly in vitro pollination of ovules and in vitro fertilization are yet to be fully exploited.

More importantly, a combination of techniques integrating the methods used to overcome pre- and post-fertilization barriers is likely to give better results. In the absence of such integration, even when a particular method is effective in circumventing pre-fertilization barriers, it may not yield hybrids because of the operation of post-fertilization barriers. For example, in crosses between *B. rapa* and *B. juncea*, showing pre-fertilization barriers, bud pollination and stump pollination increased the frequency of ovules that were fertilized. However, hybrids could be recovered only when bud pollination and stump pollination were combined with ovule culture (Bhat and Sarla 2004). Combination of placental pollination with embryo rescue would be particularly effective in providing suitable conditions for hybrid embryos to continue their growth. Thus, plant breeders in the coming decades should try combination of techniques rather than a single technique to raise wide hybrids.

Although a large number of wide hybrids have been realized in a number of crop species, introgression of desirable traits from wild species to the cultivars is limited due to lack of intergenomic chromosome homoeology. This is one of the major problems to achieve alien gene transfer even after getting wide hybrids. Interspecific hybridization and polyploidization in ornamental plants are more simple and straightforward, as it aims to produce novel cultivars combining the traits of both the parents rather than to transfer specific traits from one parent to the other (Kato and Mii 2012). Even the fertility of the hybrid may not be a serious problem in ornamental plants as many of them can be propagated through vegetative multiplication or through micropropagation.

The interest on wide hybridization through sexual pathway has greatly reduced after the development of the techniques of somatic hybridization and genetic transformation. There are not many reports on the production of wide sexual hybrids in recent years. Although both somatic hybridization and genetic transformation have great potential in specific areas of crop

improvement program, they cannot replace sexual hybrids; they can only supplement them. Thus, there is a continuous need for the production of sexual wide hybrids in the coming years. It is necessary for the breeders to re-establish interest in sexual hybrids, particularly wide hybrids for transferring desirable traits from wild species/accessions to the cultivars.

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Hybrid Embryo Rescue in Crop Improvement

18

Leela Sahijram and B. Madhusudhana Rao

Abstract

Failure to produce a hybrid may be due to pre- or post-fertilization incompatibility barriers. The hybrid embryo, if produced, may abort before maturation. In such cases, the embryo resulting from interspecific or intergeneric crosses can be potentially rescued and cultured to produce a whole plant, thus facilitating transfer of desirable genes from wild relatives into cultivated species. This approach is especially warranted in horticultural crops in situations where one or both parents may be seedless, initial fruitlet drop post-fertilization may be very high, the endosperm may be inimical, etc. Protocols have been developed for hybrid embryo rescue in several top-of-the-line fruit crops suffering from an inability to breed naturally in wide crosses. Techniques of hybrid embryo rescue figure among the very important commercially applicable breeding strategies under non-GM biotechnologies. It is a nonconventional, tissue culture-based technique used for creating new genotypes, often used to rescue plant embryos from aborting progeny ovules/seeds. Distant crosses are often sought to transfer genetic traits from secondary and tertiary gene pools to the cultivated, primary gene pool of crop plants.

Keywords

Hybrid embryo rescue • *In vitro* culture • Post-zygotic incompatibility • Interspecific • Intergeneric horticultural crops • Nonconventional breeding

18.1 Introduction

Successful sexual hybridization involves a series of events including pollen germination, pollen-tube growth, fertilization, embryo and endosperm development and seed maturity, leading to germination (Debbarama et al. 2013). These events can

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be hampered by somatoplastic sterility, cytoplasmic-genic male sterility and structural differences in chromosomes. A very important and useful biotechnological tool for raising hybrid progeny in intractable crosses is embryo rescue. A given cross may fail for any of a number of reasons: seedlessness in either or both parents, inimical influence of endosperm resulting in inhibited embryo development, poor fruit set/heavy fruit drop at initial stages of the cross, distant crossing (interspecific/intergeneric/intervarietal or 'intraspecific' breeding resulting in embryo abortion), genetic incongruity of parental genomes, mismatch in ploidy levels, etc. Embryo culture is one of the earliest forms of *in vitro* culture applied to practical problems and is probably the tissue culture technique that has proven of greatest value to breeders (Dunwell 1986). Technologies using excised hybrid embryo/sequential embryo culture have been developed in a number of horticultural crops (Leela Sahijram 2004, 2011; Leela Sahijram et al. 2013). The underlying principle of embryo rescue technique is aseptic isolation of the embryo and its transfer to a suitable medium for facilitating its development under optimal conditions. The potential these techniques hold is a viable alternative to parasexual hybridization and somatic embryogenesis (Stewart 1981). Post-zygotic barriers such as endosperm abortion and, at later stages, embryo degeneration are of common occurrence, leading to low fertility; but these have been overcome through the use of embryo rescue, and several hybrids have been developed. Genetic variation necessary for crop improvement is conventionally generated through hybridization, mutagenesis and, occasionally, polyploidy. More recently, biotechnological approaches have become available for creating genetic variability.

In embryo rescue, however, the embryo is removed before seed abortion occurs and is grown outside the parent plant to produce a new plant to enable crosses to be made between species not normally sexually compatible. Plant breeders usually rescue inherently weak, immature or hybrid embryos to prevent degeneration. Successful production of plants from cultured embryos largely depends upon maturation stage and composition of the medium (Raghavan, 2003). Abortion of embryos at one or the other stage of development is a characteristic feature of distant hybridization. Several refinements have been made in embryo culture/embryo rescue techniques which have been a popular approach for raising hybrids from a number of incompatible crosses. It has even allowed synthesis of triticale, a new hybrid species, resulting from a cross between rye and wheat. Related applications like ovule/ovary/placental cultures through sequential embryo culture have also been developed. Embryo culture applied to practical problems is a tissue culture technique that has proven to be of greatest value to breeders.

There are several situations where it may not be possible to obtain hybrids from parent plants. Distantly related plant species in a breeding programme ('wide hybridization') may result in no/aborted hybrid embryos. Seedless parents may be unable to produce seeds in a cross. Alternately, breeding may be hindered owing to heavy fruit drop in the early stages of fruit development. In all such cases, hybrid embryo rescue is a very powerful and useful tool and an indispensable technique. The procedure involves dissection of the developing embryo out from its environment and culturing it on a synthetic medium to produce hybrid plants. In certain instances, ovule culture may be needed preceding excised embryo culture. Using this method, we have raised hybrids in seedless grape and lime, in difficult crosses of papaya, mango, banana, rose, etc.

Currently, embryo rescue holds great promise not only for effecting wide crosses but also for obtaining plants from poorly developed embryos, for obtaining haploid plants as well as for shortening the breeding cycle (Sharma et al. 1996).

18.2 The Technique of Embryo Culture

Following fertilization, the zygote undergoes an asymmetrical cell division that gives rise to a small apical cell, which becomes the embryo and

a large basal cell (called the suspensor), which functions to provide nutrients from the endosperm to the growing embryo. From the eight-cell stage (octant stage) onwards, the zygotic embryo shows clear embryo patterning, which forms the main axis of polarity, and the linear formation of future structures. These structures include the shoot meristem, cotyledons, hypocotyl and root and root meristem: they arise from specific groups of cells as the young embryo divides, and their formation has been shown to be position dependent. In the globular stage, the embryo develops radial patterning through a series of cell divisions, with the outer layer of cells differentiating into the 'protoderm'. The globular embryo can be thought of as two layers of inner cells with distinct developmental fates; the apical layer will go on to produce cotyledons and shoot meristem, while the lower layer produces the hypocotyl and root meristem. Bilateral symmetry is apparent from the heart stage; provascular cells will also differentiate at this stage. In the subsequent torpedo and cotyledonary stages of embryogenesis, the embryo completes its growth by elongating and enlarging.

Embryo culture is the sterile isolation and growth of an immature or mature embryo *in vitro*, with the goal of obtaining a viable plant. The first attempt to grow embryos of angiosperms was made by Hannig in 1904 who obtained viable plants from *in vitro* isolated embryos of two crucifers *Cochlearia* and *Raphanus*. In 1924, Dietrich grew embryos of different plant species and established that mature embryos grew normally, but those excised from immature seeds failed to achieve the organization of a mature embryo. They grew into seedlings, skipping the stages of normal embryogenesis and without completion of the dormancy period. Laibach (1925, 1929) demonstrated the practical application of this technique by isolating and growing the interspecific cross *Linum perenne* × *L. austriacum* that otherwise aborted *in vivo*. This led Laibach to suggest that in all those crosses where viable seeds are not formed, it may be appropriate to excise their embryos out and grow them in an artificial nutrient medium. Embryo culture is now a well-established and useful branch of plant tissue culture.

18.2.1 Types of Embryo Cultures

There are broadly two types of embryo cultures:

1. *Mature embryo* culture is the culture of mature embryos derived from ripe seeds. This type of culture is done when embryos do not survive in vivo or become dormant for long periods of time or is done to eliminate inhibition of seed germination. Seed dormancy of many species is due to chemical inhibitors or mechanical resistance present in the structures covering the embryo, rather than dormancy of the embryonic tissue. Excision of the embryos from the testa and culturing them in nutrient media may bypass such seed dormancy. Some species produce sterile seeds (which may be due to incomplete embryo development). Embryo culture procedures may yield viable seedlings. Embryos excised from the developing seed at or near the mature stage are autotrophic and are grown on a simple inorganic medium with a supplemental energy source.
2. *Immature embryo* culture, also known as *embryo rescue*, is the culture of immature embryos to rescue embryos from wide crosses, crosses involving seedless parent(s), or where fruit fall is heavy in early stages of embryo development. This is mainly used to avoid embryo abortion, with the purpose of producing a viable (hybrid) plant. Wide hybridization, where individuals from two different species of the same genus or different genera are crossed, often leads to failure of the cross. There are several barriers which operate at pre- and post-fertilization levels to prevent successful gene transfer from wild into cultivated species. Pre-fertilization barriers include all factors that hinder effective fertilization, which is usually due to inhibition of pollen-tube growth by the stigma or upper style. Post-fertilization barriers hinder or retard development of the zygote after fertilization and inhibit normal development of the seed.

This frequently results from failure of the hybrid endosperm to develop properly, leading to starvation of the hybrid embryo, or results from embryo-endosperm incompatibility where the endosperm produces toxins that kill the embryo.

Evidence suggests that embryos of inviable hybrids possess the potential for initiating development, but are inhibited from reaching adult size with normal differentiation. Endosperm development precedes and supports embryo development nutritionally, and endosperm failure has been implicated in several cases of embryo abortion. Endosperm failure generally results in abnormal embryo development and eventual starvation. Thus, isolation and culture of hybrid embryos prior to abortion may circumvent these strong post-zygotic barriers. Production of interspecific and intergeneric hybrids is the most impressive and conspicuous application of embryo rescue and culture technique, particularly for subsequent valuable gene transfer from wild species. The underlying principle of embryo rescue technique is the aseptic isolation of the embryo and its transfer to a suitable medium for development under optimal conditions. There are normally no problems of disinfection of embryos in such cultures. Florets are removed at the proper time, and either the florets or the ovaries are sterilized. Ovules can then be removed from the ovaries. The tissue within the ovule in which the ovule is embedded is already sterile. For mature embryo culture, either single, mature seeds are disinfected, or if the seeds are still unripe, then the still-closed fruit is disinfected. The embryos can then be aseptically removed from the ovules. Using embryo culture to overcome seed dormancy requires a different procedure. Seeds having hard seed coats are sterilized and soaked in water for a few hours to a few days. Sterile seeds are then split open and the embryos excised out.

The most important aspect of embryo culture work is the selection of the medium necessary to sustain continued growth of the embryo. In most cases, a standard basal plant growth medium with major salts and trace elements may suffice. Mature embryos can be grown in a basal salt medium with a carbon energy source, such as sucrose. Young embryos, in addition, require various vitamins, amino acids and growth regulators and, in some cases, natural endosperm extracts. Young embryos should be transferred to a medium with high sucrose concentration (8–12 %) which approximated the high osmotic potential of the intracellular environment of the young embryo sac and a combination of hormones that supports growth of the heart stage embryo (i.e. a moderate level of auxin and a low level of cytokinin). Reduced organic nitrogen in the form of asparagine, glutamine or casein hydrolysate is always beneficial in embryo culture. Malic acid is often added to the embryo culture medium. After 1–2 weeks (when the embryo ceases to grow), it must be transferred to a second medium with normal sucrose concentration, low levels of auxin and a moderate level of cytokinin, which allows for renewed embryo growth, with direct shoot growth in many cases. Where the embryo does not show direct shoot formation, it can be transferred to a medium for callus induction, followed by shoot induction. When the embryos have grown into plantlets *in vitro*, these are generally transferred to sterile soil and grown to maturity. Hybrids have been successfully developed in mango, banana, papaya, seedless grape and citrus, onion, capsicum and hot peppers using intergeneric/interspecific/intervarietal crosses.

18.2.2 General Applications of Embryo Culture

Obtaining rare hybrids: In plant breeding programmes, not all desirable crosses are successful. Where a hybrid embryo capable of normal development aborts due to inadequate supply of nutrition inside the seed, or due to embryo-endosperm incompatibility, it is possible to raise complete hybrid plants through embryo rescue. Success has been achieved in prevention of abortion in wide crosses, and interspecific crosses in cotton, barley, tomato, rice, legumes, flax, etc., have been generated.

18.2.2.1 Development of Intergeneric Hybrids

Prevention of abortion in wide crosses has been shown to be possible. Success has been achieved in inter*specific* crosses in cotton, barley, tomato, rice, legumes, flax, etc. In several crop plants, even inter*generic* hybrids have been successfully developed through hybrid embryo rescue. Examples include wheat × barley, wheat × rye, barley × rye, maize × *Tripsacum*, *Raphanus sativus* × *Brassica napus*, *Actinidia deliciosa* × *A. eriantha* and *Actinidia de*liciosa × *A. arguta*. In *Carica* and *Citrus* also distant hybrids have been produced from their relatives using embryo rescue. In peach, a cross between an early Japanese cultivar Sunago Wase and a Chinese cultivar Yuhualu resulted in 'Zaoxialu' – an extra early maturing hybrid (55 days to maturity, in Hangzhou, China). Some hybrids obtained through embryo rescue have recombined desirable genes like earliness and disease and pest resistance (see Table 18.1). Embryo rescue is also used to recover crosses between diploids and tetraploids.

18.2.2.2 Production of Haploids

Embryo culture can be used for the production of haploids or monoploids. In 1970, a technique was developed to produce barley monoploids. Interspecific crosses are made with *Hordeum bulbosum* as the pollen parent, and the resulting hybrid embryos are cultured. But, these exhibit *H. bulbosum* chromosome elimination, resulting in monoploids of the female parent *H. vulgare*.

18.2.2.3 Overcoming Seed Dormancy

Seed dormancy can be caused by numerous factors, including endogenous inhibitors, specific light requirements, low temperature, dry storage requirements and embryo immaturity. Seed dormancy in *Iris* is due to the presence of a stable chemical inhibitor in the endosperm. In American basswood (*Tilia americana*), the seed is born within a tough, indehiscent pericarp where the resistance is mechanical. Naturally vegetatively propagated plants like bananas and *Colocasia* produce seeds that do not germinate in nature, probably due to recalcitrant dormancy. These stand to benefit from embryo culture.

18.2.2.4 Shortening the Breeding Cycle

Many species exhibit seed dormancy which is localized in the seed coat and/or in the pericarp. Removal of these inhibitions promotes seed germination, e.g. Brussels sprout, rose, apple, oil palm and *Iris*. Hollies (*Ilex*) embryos remain in the immature heart stage even though the fruits may have reached maturity. It takes 3 years for *Ilex opaca* seeds from mature berries to complete their embryonic development and to begin germination. Through embryo rescue, hybrid plants from this can be obtained in 2–3 weeks. *Rosa* normally takes a year to come to flowering; through embryo culture, it was shown that it was possible to produce two generations in a year.

Prevention of embryo abortion with early ripening stone fruits: Early ripening varieties of peach, cherry, apricot and plum produce sterile seeds which do not germinate under natural

Table 18.1 Resistance traits transferred to crop species using hybrid embryo rescue

S. no.	Species used for crossing	Resistance trait(s)
1	*Lycopersicon esculentum* × *L. peruvianum*	Virus, fungi, nematodes
2	*Solanum melongena* × *S. khasianum*	Brinjal fruit and shoot borer (*Leucinodes orbonalis*)
3	*Solanum tuberosum* × *S. etuberosum*	Potato leaf roll virus
4	*Brassica oleracea* × *B. napus*	Triazine resistance
5	*Brassica napus* × *B. oleracea* (kale)	Cabbage aphid
6	*Triticum aestivum* × *Thinopyrum scirpeum*	Salt tolerance
7	*Hordeum sativum* × *H. vulgare*	Powdery mildew and spot blotch
8	*Hordeum vulgare* × *H. bulbosum*	Powdery mildew
9	*Oryza sativa* × *O. minuta*	Blast (*Pyricularia grisea*), bacterial blight (*Xanthomonas oryzae*)

conditions and, eventually, decay in the soil. Seed sterility here may be due to incomplete embryo development. In early ripening stone fruits, transport of water and nutrients to the immature embryo is cut off too soon sometimes, resulting in embryo abortion.

18.2.2.5 Propagation of Rare Plants

'Macapuno' coconuts are prized for their characteristic soft endosperm which fills the whole nut. These nuts fail completely to germinate because the endosperm invariably rots before the germinating embryo can emerge out of the shell.

Embryo culture has been practised as a general method in horticultural crops including avocado, peach, nectarine and plum. Two cultivars, 'Goldcrest peach' and 'Mayfire nectarine', have resulted from embryo culture and are now commercially grown.

18.2.2.6 Rapid Seed Viability Test

Breaking of seed dormancy is possible through embryo culture. Therefore, it also allows the use of this technique as a rapid test for seed viability. Germination of excised embryos is reliable and is capable of more exact interpretation of seed viability than the commonly used stain tests.

18.2.2.7 Haploid Formation

Haploid production with the aid of embryo culture following distant hybridization: In such crosses, double fertilization may occur normally, but selective elimination of the entire complement of chromosomes of one parent may occur, leading to haploid embryo formation. Such embryos do not survive unless rescued by culturing them.

18.2.2.8 Embryos for Propagation/Conservation/Germplasm Exchange

Embryos are excellent material for in vitro propagation, especially in conifers and in Gramineae family. As per IPGRI, Rome, it is compulsory to exchange germplasm of coconut internationally using embryo cultures (otherwise, shipping of whole nuts is cumbersome, besides requiring huge cargo space on flights or ships).

18.2.2.9 Germination of Seeds of Obligate Parasites

Parasitic plants produce seeds that can be germinated to produce plantlets only on their chosen host plant. Germination of seeds of obligate parasites *in vivo* without the host plant is impossible, but is achievable with embryo culture. Insectivorous plants have also been shown to propagate successfully on a synthetic medium using these techniques.

18.3 Application of Embryo Rescue in Fruit Crops

Embryo rescue techniques in fruit crops play an important role in breeding new early maturing seedless triploid types as well as obtaining distant hybrids preventing embryo degeneration at early development stage and shortening the breeding cycle (Yi Hualin et al. 2001). Application and progress of embryo rescue in fruit crop breeding was reviewed, and several problems, e.g. suitable culture period nutrient and environment conditions, were briefly introduced as well in this paper. With interspecific crosses, intergeneric crosses and crosses between diploids and tetraploids, the endosperm often develops poorly or not at all. A synthetic medium can compensate for lack of support from the endosperm.

18.3.1 Seedless Grapes

The second food crop to be fully sequenced genomically after rice is the grape. Improvement in grape is slow and time-consuming since the crop is heterozygous and exhibits pronounced inbreeding depression (Leela Sahijram 2011). Thompson Seedless is the ruling variety of grape in India and other parts of the world, but is susceptible to downy mildew disease caused by a deuteromycete fungal pathogen, *Plasmopara viticola*. Obtaining a downy mildew-resistant Thompson Seedless would be a boon to the global viticulture industry, resulting in massive savings in pesticide use while providing a cleaner, safer environment. Seedless grapes are

stenospermocarpic, in that these form embryos successfully, but the embryo does not develop and aborts due to the inability of the ovular wall to expand. These embryos can, however, be rescued before they abort by initially culturing the whole ovule; after about 8 weeks, the developing embryo can be dissected out from the ovule and grown on a different medium. This is termed 'sequential embryo culture'. Ramming (1990) pioneered hybrid embryo rescue in seedless grape breeding. Sequential hybrid embryo rescue was carried out using 'Thompson Seedless' as the maternal parent and 15 different pollen donor parents (Tables 18.2 and 18.3) variously resistant to downy mildew disease (Leela Sahijram and Raghavendra 2005). The authors used a 2-step, sequential embryo rescue: *in ovulo* embryo culture followed by *ex ovulo* embryo culture (Kanamadi et al. 1999a, b, c; Leela Sahijram and Kanamadi 2004; Leela Sahijram et al. 2005a, b).

Seedless grape cultivars (*Vitis vinifera* L.) are widely grown in Europe, America and Asia. Fungal diseases are a great threat of these cultivars. Several wild Chinese *Vitis* species show high resistance to several fungal diseases. Therefore, an investigation was conducted by Lili Tian et al. (2008) to assess the potential of incorporating these species into a breeding project for the development of disease-resistant seedless cultivars. Hybridization was accomplished using *V. vinifera* as the female parent and wild, Chinese *Vitis* spp. as male parents. *In ovulo* embryo rescue was used for developing hybrid plants from seedless females. An efficient protocol was reported from them for in vitro embryo rescue and plant development using the cross Emerald Seedless × Beichun. Ovules were excised from immature fruits 7 weeks after pollination (WAP) and cultured in the double-phase ER medium supplemented with 6.0 % sucrose and 0.3 % activated charcoal (AC). Following 8–12 weeks of culture, embryos were removed from the ovules and transferred onto WPM supplemented with 1.0 mM 6-benzyladenine (6-BA), 2.0 % sucrose and 0.2 % AC and solidified with 0.6 % agar. After 8 weeks of culture, the embryos germinated and subsequently grew into whole plantlets. With optimized parameters developed in this study, about 34.0, 91.2 and 77.4 % of embryo formation, embryo germination and plant development, respectively, were obtained. When this protocol was applied to 11 other cross combinations,

Table 18.2 Data on crossing 'Thompson Seedless' (female parent) with 14 downy mildew-resistant pollen donor parents and open-pollinated 'Thompson Seedless'

Sl. no.	Pollen donor parent used	Total no. of ovules cultured from the cross	No. of fully developed hybrid plantlets recovered	% hybrid plants recovered
1	*Vitis tiliifolia*	165	28	16.97
2	'Madresfield Court'	134	57	42.54
3	*Fruehort veltliner*	110	48	43.64
4	*Vitis candicans*	39	3	7.69
5	'SV 23501'	5	0	0.00
6	'SV 18315'	172	14	8.14
7	'SV 12309'	58	33	56.90
8	'Catawba'	60	23	38.33
9	'James'	117	19	16.24
10	*Riparia* × *Rupestris*	134	25	18.66
11	'SV 12364'	189	64	33.86
12	*Vitis assamica*	111	26	23.42
13	'SV 18402'	51	11	21.57
14	*Vitis lanata*	8	0	0.00
15	Open-pollinated Thompson Seedless	37	13	35.14
	Total	**1,390**	**364**	**26.19**

Table 18.3 Comprehensive data on crossing 'Thompson Seedless' (female parent) and 15 pollen donor parents resistant to downy mildew

Sl. no.	Male parent	No. of panicles used	Total no. of buds used	No. of ovules obtained 6–10 weeks post-pollination					Total no. of berries dissected	Total no. of ovules recovered in a cross	Total no. of apparently viable embryos	No. of fully developed plants regenerated
				6	7	8	9	10				
1	*V. tiliifolia*	5	700	31	53	–	–	81	289	165	28	24
2	Madras field Court	4	1,070	21	41	–	24	48	303	134	38[a]	54
3	*F. veltliner*	4	630	8	26	–	76	0	276	110	36[a]	39
4	*V. candicans*	6	1,230	0	0	–	29	10	446	39	2[a]	3
5	SV 12375	1	80	–	–	–	–	–	0	0	0	0
6	SV 23501	2	230	0	5	–	–	–	123	5	0	2
7	SV 18315	4	460	55	103	–	–	14	247	172	20	11
8	SV 12309	3	360	10	30	18	–	–	146	58	21[a]	27
9	Catawba	3	360	12	1	–	–	47	192	60	7[a]	18
10	James	3	210	23	51	43	–	–	195	117	16	15
11	*Riparia* × *Rupestris*	3	180	–	114	–	20	–	161	134	21	20
12	SV 12364	6	415	41	44	104	–	–	269	189	35[a]	48
13	*V. assamica*	3	210	19	–	92	–	–	191	111	20[a]	26
14	SV 18402	3	220	17	–	34	–	–	225	51	8	7
15	*V. lanata*	1	100	–	–	–	–	8	44	8	3	0
	Total	**51**	**6,455**						**3,107**	**1,353**	**255[a]**	**294**
								OpTS[b]	52	37	11	8

Source: Leela Sahijram et al. (2015)
Total no. of crosses = 15
Average no. of buds used per panicle = 127
Ovules harvested at an age ranging from 6 to 11 weeks post-pollination
[a]No. of apparently viable embryos, but some of the apparently non-viable embryos also developed into plantlets
[b]Open-pollinated Thompson Seedless

genotype was found to significantly influence embryo formation, embryo germination and plant development, with different frequencies of hybrid plants obtained successfully in all the crosses.

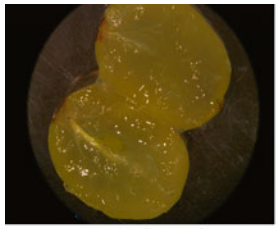
A highly efficient embryo rescue technique using *in ovulo* culture is critical for maximizing success when using stenospermocarpic female parents to breed new seedless grape varieties. The effects of medium composition, pollen parent and year of cross on embryo development and recovery in cultured ovules were investigated by Liu et al. (2008) to improve *in ovulo* embryo rescue from stenospermocarpic parents commonly used in breeding seedless grapes for the Australian table and dried grape industries. Increasing CaCl2 concentrations in the culture medium improved embryo recovery, but embryo emergence from ovules and germination rates were unaffected by varying Fe-EDTA concentrations. Casein hydrolysate supplementation in the culture medium improved embryo recovery, emergence and germination. Embryo recovery varied with parental genotype and ranged from 5 to 14 % in four self-pollinated genotypes. Mean embryo recoveries from ovules cultured 50 days after controlled cross-pollination from berries of the pollen-sterile, stenospermocarpic variety 'Carina' were 36 and 26 % where the pollen parents were seeded (11 crosses) and seedless (6 crosses), respectively. Embryo recovery and germination were affected by the year in which the cross was made; there was also a significant cross × year interaction. Results indicated that *in ovulo* embryo rescue can be improved by increasing CaCl2 concentration and incorporating casein hydrolysate supplement in the basal medium, by exploiting genotypic differences using seedless parents yielding higher proportions of rescued hybrids and, possibly, by understanding environmental effects on female parents to maximize the number of hybrids produced.

Pommer et al. (1995) used 18 seedless grape genotypes differing in ripening season (early, mid and late) and in seed trace size (small, medium and large) and harvested at 6, 10, 14, 18 and 22 weeks post-bloom (wpb). Using embryo rescue techniques, it was studied if embryos aborted as the fruit matured and what percentage of embryos remained viable at later stages. Size of the seed trace was also investigated to determine its influence on embryo viability during maturation. Genotype was found to have a great influence on embryo culture. Late-maturing genotypes showed fewer (1) embryos that could be rescued, (2) germinated embryos and (3) transplantable plants, than early and midseason ones. The best time for grape embryo rescue/culture was 6 and 10 weeks post-bloom when the largest number of germinated embryos and transplantable plants was obtained. Genotypes with the largest ratio for seed trace weight/seed trace length (i.e. largest density) showed the greatest tendency to have the largest number of ovules with embryos, more germinated embryos and more transplantable plants. The study also showed that it was possible to recover plants from mature fruits harvested late, although the rate of success was much reduced.

Somatic embryo formation was shown from immature zygotic embryos within ovules of stenospermocarpic seedless grapes *(Vitis vinifera* L.), when cultured for 2 months on liquid medium (Emershad and Ramming 1994). Somatic embryos continued to proliferate after excision and transfer to Emershad/Ramming medium supplemented with 1 #M benzylaminopurine and 0.65 % TC agar. Plant development from somatic embryos was influenced by genotype, medium, phase (liquid, agar), stage (torpedo, mature) and their interactions. Optimal plant development occurred on Woody Plant Medium supplemented with 1.5 % sucrose + 1/~M benzylaminopurine + 0.3 % activated charcoal and 0.65 % TC agar.

In grape, pollen donor parent overwhelmingly determined the outcome of crossing and success rate thereof. From the directed crosses, of the 1,500 hybrids generated and grown in the glasshouse, about 700 were transferred to the field (Fig. 18.1), established to maturity and evaluated for downy mildew resistance while retaining desirable pomological qualities of the seed parent. In some crosses involving seedless × seedless parents, the authors have demonstrated through histological studies that the zygote does not develop beyond the first cell division (i.e. 2-cell stage).

Bharathy et al. (2003, 2005) in their study on 'Thompson Seedless', 'Flame Seedless' and eight other varieties of grape found embryo

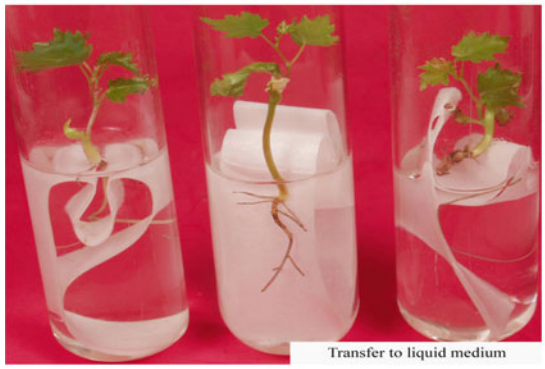


Immature berry in longitudinal section showing seed traces

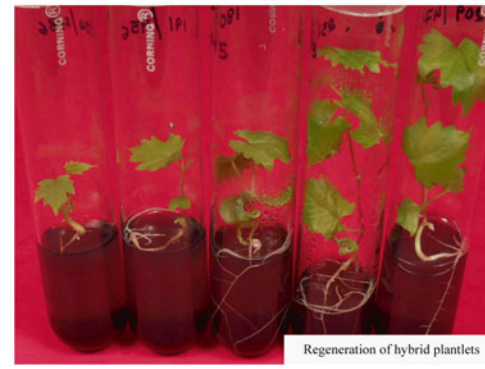
Stereozoom micrograph of ovule containing hybrid embryo

Excised embryo under high magnification

Microscopic embryos germinating on synthetic culture medium



Transfer to liquid medium



Regeneration of hybrid plantlets

Transfer of vitroplants to soilrite

Polybagged vitroplants

Acclimatization in glasshouse

Field-transfer for evaluation

Fig. 18.1 Sequential hybrid embryo rescue/culture in controlled crosses of grape with 'Thompson Seedless' as female parent and downy mildew-resistant pollen donors (Source: Leela Sahijram et al. 2013)

recovery to increase significantly with application of benzyladenine – a cytokinin – at pre-flowering and flowering stages. Maximum embryo recovery (47.57 %) was obtained with 'Thompson Seedless' (TS) × 'Concord', followed by TS × SV18402 (29.75 %). Murthy et al. (2006) achieved field establishment and screened embryo-rescued hybrid seedlings of grape with special reference to downy mildew.

18.3.2 Mango

Mango (*Mangifera indica* L.) is the leading fruit crop of India. It has long been known to be a difficult system in traditional breeding programmes because of inherent characteristics such as:

1. Long juvenile phase
2. High level of heterozygosity resulting in unpredictable outcome in hybridization
3. Heavy fruit drop leading to low retention of crossed fruits
4. One seed per fruit
5. Polyembryony in some cultivars
6. Large acreage required for meaningful assessment of hybrids

Classically, barring a few hybrid varieties resulting from planned hybridization programmes, almost all known cultivars have resulted from selection of chance seedlings resulting from natural cross-pollination. In Florida and South Africa, for example, none of the cultivars developed have come from a breeding programme. Modern horticultural and industrial requirements in mango breeding (Iyer and Degani 1997) emphasize:

1. Precocious bearing
2. Dwarf tree habit
3. Heavy and regular bearing
4. Freedom from physiological disorders
5. Resistance to major pests and diseases
6. Good shipping qualities and shelf life
7. Ideal tree architecture

In controlled crosses in this crop, fruit set in relation to the original number of flowers pollinated is very low (Table 18.4), sometimes as low as 0.01 % (Iyer 1991). Therefore, embryo rescue-based technology was applied for improving breeding success.

Table 18.4 Controlled intraspecific cross in mango (cv. Alphonso × Kerala Dwarf)

Total no. of panicles used for crossing	487
Total no. of flowers used as female parent for pollination	2,646
Fruit set (no.)	208
% Fruit set	7.86 %
No. of fruit/fruitlets lost to leaf-hopper infestation/sooty mould	193 (92.8 %)
No. of excisable hybrid embryos	15
Ratio of panicle to fruit set	1:0.42
Ratio of flowers crossed to fruit set	176.4:1

Source: Leela Sahijram et al. (2013)

18.3.2.1 Floral Biology and Pollination in Mango

The total number of flowers in a panicle may vary from 1,000 to 6,000 (Mukherjee 1953). Initial fruit set in mango is directly related to the proportion of perfect (hermaphrodite) flowers to staminate flowers, although the final fruit set does not necessarily depend on this ratio. This proportion becomes critical for optimum fruit set in a cultivar when the proportion drops to 1 %.

18.3.2.2 Breeding Objectives in Mango

Developing cultivars with:

1. Regular bearing
2. Dwarf tree habit with precocity in bearing
3. Attractive, good-sized (300–500 g), good shape, good quality fruits (taste, flavour and firm flesh without fibre), high pulp-stone ratio

With regard to improvement of rootstocks by breeding, the main desirable features are:

1. Polyembryony (a recessive trait)
2. Dwarfing influence on the scion
3. Tolerance to adverse soil conditions (pH, calcareous soil, etc.)
4. Good scion compatibility

18.3.2.3 Breeding for Special Objectives

18.3.2.3.1 Dwarfness: For Orchard Management and Fruit Quality

Owing to the obvious benefits of comparatively dwarf trees for orchard management and fruit quality, attempts were focused on obtaining hybrids with a dwarf tree framework. Breeding

for dwarfness is important in mango, since a consistent dwarfing effect of any rootstock has not been established to date. Indian cultivars found to be useful as a source of imparting dwarfness include 'Kerala Dwarf', 'Amrapali', 'Janardhan Pasand' and 'Nileshwar Dwarf' (Singh 1990).

Controlled crosses were made using the following mango varieties as parents, based on breeding priority (Leela Sahijram et al. 2013) 'Kerala Dwarf' as the male parent to introgress dwarfness. Factors detrimental to initial embryo growth and development were found to be fungal/bacterial contamination, excessive exudation of phenolics into the medium and poor response of the embryo.

S. no.	Cross combination
1.	'Amrapali' × 'Kerala Dwarf' – (AKD)
2.	'Alphonso' × 'Kerala Dwarf' – (ALK)
3.	'Arka Anmol' × 'Kerala Dwarf' – (AnKD)
4.	Open-pollinated Totapuri (OpTp)

Young fruitlets containing immature embryos from these monoembryonic cultivars were harvested at 4–6 weeks post-pollination and excised embryos cultured *in vitro*. In AKD, 69.23 % and in OpTp 82.68 % of the embryos cultured on defined media developed into normal plantlets *in vitro*. In AKD, cultures responded, with shoot and root induction. Upon normal plantlet development, hybrid vitroplants were transplanted *extra vitrum* in a phased manner into mini pots. Eight weeks later, these hybrid plants were transferred to larger pots and, finally, into the field. In OpTp plantlet survival at final field establishment was just 3.81 %. Field-established plants showed overall survival percentage of 34.4 % in AKD. In the controlled cross ALK and in OpTp, rooting was achieved in 70.9 and 72.4 % cultures, respectively. Performance of ALK and OpTp was comparable (77.8 and 77.5 %, respectively) on a medium containing 1,500 mg/l casein hydrolysate (CH) + 6 % sucrose (w/v). OpTp responded best (87.5 %) on a medium containing 1,000 mg/l CH + 3 % sucrose (w/v).

In ALK, 88 hybrid embryos out of a total of 109 from the controlled cross formed plantlets *in vitro* (80.73 %), of which only 2 hybrids survived field establishment (2.27 % survival rate). In AnKD, 244 hybrid embryos were excised and cultured *in vitro* of which 177 formed normal plantlets (72.5 %). In the subsequent year, in OpTp, out of 232 embryos, 166 formed plantlets *in vitro* (79.55 %).

Cultures showing both shoot and root inductions were transplanted *extra vitrum* into large, polyvinyl pots. In all, 80.7 % cultures of ALK (Fig. 18.2) and 82.7 % of OpTp were found suitable for transfer to soil. Finally, overall survival percentage of 4.54 and 3.81 % was obtained in ALK and OpTp hybrids, respectively. 84.77 % of AnKD embryos developed into plantlets *in vitro*. Overall percentage field survival in AnKD was 4.11.

18.3.2.4 Ex Vitro Shoot-Tip Grafting (Ex Vitro STG)

A protocol earlier also worked out for the cross 'Mulgoa' × 'Neelum'. Using immature zygotic embryos as starter explants, Leela Sahijram et al. (1996) could also induce somatic embryogenesis in mango embryos/nucellus and elucidate factors influencing the same. These plantlets did not survive beyond 4 months of ex vitro transfer, resulting in very high mortality despite their healthy status at transplanting. Therefore, an alternative strategy was devised to rescue the hybrid vitroplants. Hence, a novel technique of ex vitro shoot-tip grafting (ex vitro STG) was devised by the authors (Leela Sahijram and Bhaskara Reddy 2009) using 'Totapuri' as the rootstock. The hybrids were grown to maturity and evaluated. These were further multiplied on various rootstocks.

'Totapuri' (rootstock) stones were germinated in the traditional manner in seed pans on a sand medium. Shoot apices of the hybrid plantlets were excised and grafted ex vitro onto the rootstock using the epicotyl grafting technique. The diameter of the scion shoot apex at the cut surface was 2–4 mm. Graft union established successfully in 3 weeks, and at the end of 8 weeks, the scions showed vigorous growth and development. Overall graft success rate was 79.54 %. Ex vitro STG was carried out using vitroplantlets of Alphonso × Kerala Dwarf, open-pollinated (1) Alphonso, (2) Ratnagiri Alphonso and (3) Totapuri, with a success rate of 66.7, 100, 72.2 and 92.31 %, respectively. These grafts were transferred to polybags of size 9″ ht × 7″ dia containing sand, soil and vermicompost at 1:1:1, at 10–12 weeks. The grafts were further transferred to larger pots containing sand,

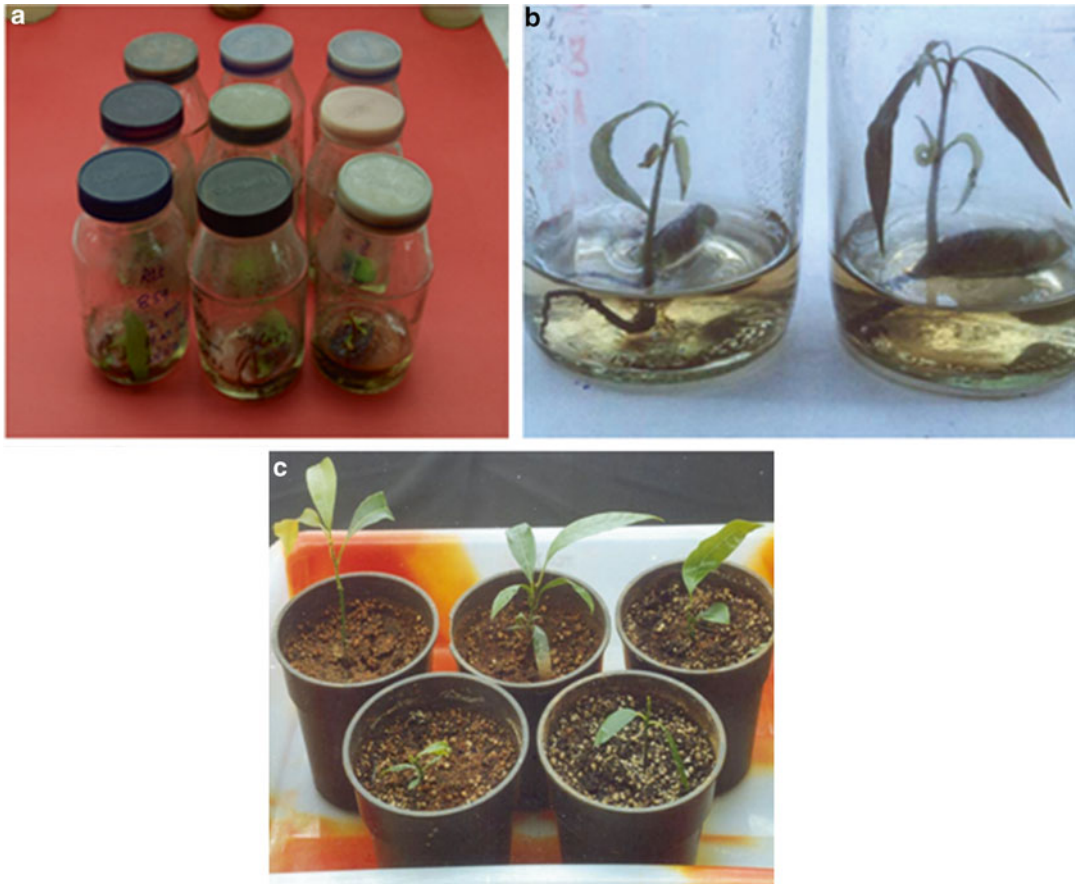


Fig. 18.2 Hybrid embryo rescue in the mango cross 'Alphonso' × 'Kerala Dwarf': vitroplants derived through embryo rescue (**a**) on semisolid medium in culture vessels and (**b**) on ex vitro transfer to non-sterile tap water and (**c**) transplanted into soil mix in polyvinyl mini pots (Source: Leela Sahijram et al. 2013)

soil and farmyard manure at 1:1:1, at 6 months, and, subsequently, field-planted and hybrids evaluated for desirable traits.

18.3.3 Papaya

Leela Sahijram and Doreswamy (1993) tested the efficacy of using placental cultures for rescuing hybrid embryos from controlled crosses involving *Carica papaya* and *Vasconcellea cauliflora*. They were able to induce a transient expression of anthocyanin gene (inky blue in colour) in placental cultures of hybrid papaya. Intergeneric and interspecific crosses were used for hybrid embryo rescue, and hybrid plants were recovered. Vitroplantlets were transplanted into soil ex vitro.

18.3.4 Seedless Lime

Prasad et al. (1996) emphasized the importance of embryo rescue in improving seedless lime quality. Controlled crossing between seedless lime and acid lime resulted in immature zygotic embryos that were rescued *in vitro* and grown to maturity in the field. The objective here was to develop a hybrid of acid lime with resistance to citrus canker.

The role of embryo culture techniques in the improvement of fruit quality in seedless lime was elucidated by Prasad et al. (1996). Rao et al. (2011) highlighted the importance of genetic resources in citrus fruits and their exploitation in citrus improvement programmes using various in vitro techniques such as embryo rescue, nucellus culture, etc.

18.3.5 Banana

Commercially popular varieties are seedless owing to their triploid status. Protocols have been developed for rescuing excised hybrid embryos from crosses involving *Musa acuminata* and *M. balbisiana*. Hybrid plants were raised to maturity in the field and evaluated (Doreswamy and Leela Sahijram 1991, 1993; Chadha and Leela Sahijram 2000a, b). The hybrids were added to the germplasm pool in the Institute for further use in breeding.

18.3.6 Olive

Acebedo et al. (1997) found the growth of plantlets derived from *in vitro* germinated embryos in the greenhouse to be normal. Thus, embryo culture can increase the efficiency and shorten the time for starting initial progeny evaluation, thereby speeding up seedling development in olive breeding programmes.

18.4 Application of Embryo Rescue in Floricultural Crops

Several crops in floriculture have benefitted from *in vitro* interventions in breeding programmes. Van Tuyl and Lim (2003) reviewed interspecific hybridization and polyploidization as tools in ornamental plant breeding. Some of the successes are reported hereunder.

18.4.1 Rose

Marchant et al. (1994) used embryo culture for the production of F1 hybrids in English rose. Rout et al. (1999) reviewed advances made in the biotechnology of rose and Holeman (2009) outlined a simplified method of embryo culture in rose. Hybrid/zygotic embryos were rescued from controlled crosses and open pollination, respectively, in rose cultivars. Mohapatra and Rout (2005) in their study on embryo rescue in floribunda roses rescued immature embryos from 'Arunima' and 'Shocking Blue' roses. The time required for breeding roses through conventional breeding, for example, is very long. It is occasionally hampered by premature abortion of the developing embryo, resulting in few or no viable seeds (Rout et al. 1999). Rose is highly heterozygous. Lack of germination is due to mechanical restriction of embryo expansion by the presence of a thick, hard pericarp or due to dormancy regulated by growth inhibitors present within the achene. There are also a few reports of culture of mature embryos *in vitro*. Embryo rescue in *Rosa hybrida* and English roses is also reported from elsewhere. Gudin (1994) also demonstrated the usefulness of embryo rescue in *Rosa hybrida* L.

18.4.2 Lilies/Liliums

In order to introduce new traits such as disease resistances, flower shape and colour from wild species into the cultivar assortment of lily, it is necessary to overcome interspecific crossing barriers. Several techniques like the cut-style method, the grafted-style method and the *in vitro* isolated ovule pollination technique have been developed to overcome pre-fertilization barriers. Post-fertilization barriers can be circumvented by *in vitro* pollination and/or rescue methods as embryo, ovary-slice and ovule culture. Using these techniques, wide interspecific lily crosses with species and cultivars from the different sections of the genus *Lilium* (*L. longiflorum*, *L. henryi*, *L. canadense*, *L. pardalinum*, *L. concolor*, *L. dauricum*, *L. candidum*, *L. rubellum*, *L. martagon*, Asiatic and Oriental hybrids) could be made (Van Tuyl et al. 2000, 2002) and breakthroughs achieved.

Interspecific hybrids have been developed in liliums using early stage ovule culture *in vitro* (Wang et al. 2009). Wang et al. (2009) successfully generated interspecific hybrids between *Lilium longiflorum* and *L. lophophorum* var. *linearifolium* culturing whole ovules at early stages of their development. Low seed set following self-pollination is known to be caused by reduced ovule availability resulting from embryo sac degeneration. Tammy et al. (1999) hypothesized this to be due to the absence of a required stimulus for normal ovule development. If this is

correct, current concepts of SI may need to be broadened to include a wider range of pollen-pistil interactions.

Crosses between the diploids *Hylocereus polyrhizus* and *H. undatus* in both directions were performed by Aroldo and Noemi (2010). The aim of their study was to develop an efficient methodology to rescue embryos following interspecific crosses in the genus *Hylocereus*.

Fertilized ovules carrying embryos at very early pro-embryonic stages were excised from ovaries 5 days after pollination (DAP) and placed on half-strength basal MS medium containing 680 μM glutamine, 0.55 μM α-naphthaleneacetic acid (NAA), 0.45 μM thidiazuron (TDZ) and various concentrations of sucrose. After 30 days in culture, ovules were isolated from the surrounding tissue and transferred to the same fresh medium. Significant differences were found between the main effects (cross and sucrose concentration) in ovule response, i.e. increased ovule size and callus formation. The best responses were obtained in the cross, *H. polyrhizus* × *H. undatus*, and sucrose concentration of 0.09 M. In terms of embryo conversion, polyembryony and the number of regenerated plants, the highest responses were observed on a culture medium supplemented with 0.17 M sucrose in both the interspecific crosses. All the plants tested by flow cytometric ff were found to be diploid. Fluorescent amplified fragment length polymorphism (fAFLP) confirmed hybrid origin of the regenerated plants. This study reported the success of a three-step embryo rescue procedure for *Hylocereus* species. The procedure developed provides means for producing plants from very early embryo stage, thus expanding the prospects for vine cactus breeding programmes. Yuan et al. (2003) demonstrated shortening of breeding cycle in spider lily (*Lycoris* spp.) through embryo culture.

Differential ovule development following self- and cross-pollination and the basis of self-sterility in *Narcissus triandrus* (Amaryllidaceae) were shown by Sage et al. 1999. Earlier, Hayashi et al. (1986) demonstrated ovary-slice culture in *Lilium formosanum*. Wallace and Ikeda et al. in 2003 produced seedlings from ovules excised at a time when they contained zygote stage of the hybrid product and cultured them in *Lilium* spp.

18.4.3 Pelargonium

As a means of integration of *in vitro* techniques in ornamental plant breeding, interspecific crosses were made in *Pelargonium* by applying embryo rescue methods. Some reports of artificial hybrids are available, but no evidence of natural hybridization is seen.

18.4.4 *Alstroemeria*

Interspecific hybridization in the genus *Alstroemeria* is hindered by post-fertilization barriers (Buitendijk et al. 1995). Histological analysis revealed poor endosperm development from 18 days after pollination onwards, followed by malformation and abortion of embryos. To create interspecific hybrids between *Alstroemeria aurea*, *A. pelegrina*, *A. magnifica*, *A. inodora* and *A. psittacina* in diallelic combinations, an ovule culture technique was developed. Influence of age of ovules, sucrose concentration of medium and temperature and light during culture were tested. Harvesting ovules before the onset of endosperm degeneration, i.e. at 14 days after pollination, cutting them into halves and culturing the micropylar halves in a rotating liquid culture medium containing 6 % sucrose at 21 °C in the dark, led to successful embryo rescue. Germinated embryos were subcultured in vitro until rhizomes were formed, a prerequisite for successful transfer to the greenhouse. Full-grown plants all showed interspecific morphological traits, and an analysis of chromosome complement confirmed their hybrid nature. Diploid hybrid plants were obtained in all the 20 interspecific 2*x*-2*x* combinations. A total of 260 interspecific hybrid plants were produced. Half-ovule culture of 2*x*-4*x* and 4*x*-2*x* crosses resulted in 43 triploid hybrid plants. Because interspecific hybrids were obtained in 100 % of the interspecific combinations, it is expected that the described technique can be applied to overcome post-fertilization barriers in most crosses within the genus *Alstroemeria*. Chunsheng and Bridgen (1996) studied the effect of genotype, culture medium and developmental stage of the embryo on *in vitro* responses of ovule cultures in the interspecific hybrids of *Alstroemeria*.

18.4.5 Cacti

Embryo rescue and plant regeneration have been successfully demonstrated in cacti following interspecific crosses in the genus *Hylocereus* (Aroldo and Noemi 2010). Gynogenesis has also been shown to be possible in the vine cacti *Hylocereus* and *Selenicereus*.

18.4.6 Other Ornamental Crops

In primulas, Kato et al. (2001) obtained different genomic combinations in intersection hybrids from crosses in primulas through embryo rescue. Two types of triploids with different genome combinations were generated. Interspecific hybridization in rhododendron has been shown to be possible using embryo rescue in the genus *Rhododendron* (Eeckhaut et al. 2007).

Other attempts at embryo rescue include that in *Narcissus* and *Zinnia* (Miyajima 2006) and *Ornithogalum* (Neiderweiser 1990). Kasten and Kunert (1991) devised a method for culture of isolated immature embryos of various lupins (*Lupinus* species); Custers (1995) overcame interspecific crossing barriers in tulips using embryo rescue by successful direct transfer of *Tulipa kaufmanniana* Regel germplasm into that of *T. gesneriana* L.

18.5 Application of Embryo Rescue in Vegetable Crops

Wide hybridization to a vegetable breeder and cytogeneticist is the first step in transferring genes from wild species into cultivated ones. Embryo rescue technique has been successfully demonstrated in tomato, capsicum, hot peppers, okra and radish. In India, embryo culture work has been initiated in okra for rescuing interspecific hybrids. The following is by no means an exhaustive account of work reported in literature thus far.

18.5.1 Tomato

Smith (1944) and Demirel and Seniz (1997) made observations on the possibility of utilizing embryo culture for improvement of tomato. For *in vitro* breeding in tomato, Aragao et al. (2002) used three culture media in combination with distinct accessions, crossing generations and periods of time after artificial pollination and evaluated them to identify more efficient protocols to recover interspecific hybrids between *Lycopersicon esculentum* and *L. esculentum*. Bhattarai et al. (2009) found germination of immature seeds to be a better alternative to culture of excised immature embryos in a bid to hasten tomato breeding programmes.

18.5.2 Capsicum/Hot Peppers

In the state of Meghalaya in India, chilli (hot peppers) is the third most important spice crop after ginger and turmeric. However, diseases, namely, tobacco mosaic virus, root rot, tomato spotted wilt virus, etc., lead to considerable decline in yield. *Capsicum chinense*, *C. annuum* and *C. frutescens* were crossed with each other and embryos rescued between 27 and 33 days after pollination. The highest percentage of embryo growth was observed on MS medium with 0.5 mg/l GA_3 and 0.05 mg/l NAA. Hybrid plants were obtained and their hybridity confirmed using morphological as well as RAPD markers (Debbarama et al. 2013).

18.5.3 Radish

Intergeneric herbicide resistance transfer to radish has been recently accomplished using embryo rescue (Mithila and Hall 2012). For introgressing auxinic herbicide (Dicamba) resistance from wild mustard (*Sinapis arvensis*) into radish (*Raphanus sativus*; $2n = 18$), embryo regeneration and hybrid plant production was achieved involving several hundred reciprocal crosses performed between these two species. Upon altering

cultural conditions and media composition, a high frequency of embryo regeneration and hybrid plant establishment was achieved.

18.5.4 Okra (Lady's Finger)

As resistance is not available in cultivated species of okra (*Abelmoschus esculentus* Moench), interspecific crosses were made between *A. esculentus* and *A. moschatus* (resistant wild species) to develop resistant varieties. Post-zygotic incompatibility was found to operate between the species. Crossed seeds were shrivelled and non-viable. *In vitro* embryo rescue to overcome the incompatibility revealed that culturing 12- and 15-day-old embryos of *A. esculentus* var. Kiran (a high-yielding line) × *A. moschatus* and *A. esculentus* var. Anakomban (a landrace) × *A. moschatus* on MS medium supplemented with BA 0.5 mg l^{-1} and CW 150 ml l^{-1} yielded transplantable hybrids. Embryos of crosses *A. moschatus* × *A. esculentus* (i.e. reciprocal cross) turned brown by the 11th day of pollination and could not be cultured *in vitro* (Rajamony et al. 2006).

In another study (Fatokun 1987), crosses were made between members of two West African okra types, 'Soudanien' and 'Guineen'. All crosses succeeded in both directions, but F_1 plants that showed hybrid vigour for plant stature were partially sterile. Cytological observations of these F_1 plants revealed abnormal meiosis which resulted in the production of microspores of variable size. The frequency of viable pollen (indicated by acetocarmine staining) was low in the hybrids: 35.80 % (U.I.92 × U.I.313) and 39.41 % (1bk-1 × U.I.215). The number of seeds produced per fruit was also low in the hybrids and only a few of these seeds were viable. The authors' laboratory has initiated work on *in ovulo* and *ex ovulo* culture of okra embryos with a view to generating interspecific hybrids in the long run.

18.5.5 Artichoke

Globe artichoke breeders have two important problems: lengthy seed-to-seed cycle and seed-borne diseases caused by fungal pathogens. Both problems can be solved by embryo rescue. Embryos were collected from five cultivars in different post-pollination stages (Cravero and Crointy 2007). Root induction was poor, but shoot development per explant was better. Twenty days were required as the optimal time for embryo rescue.

18.5.6 Tuber Crops

Cassava is one of the most cultivated tuber crops in the tropical world. The importance of cassava is growing as a food security crop in sub-Saharan Africa, where malnutrition is a menace (Akinbo et al. 2010). However, a major hindrance in rapid improvement is the long generational cycle of cassava, poor germination of seeds and a low multiplication rate of stem cuttings. *In vitro* germination of 495 seeds from backcross population was done. Each genotype was multiplied for sufficient planting material, hardened in the greenhouse and transplanted to the field. Percentage germination of the seeds in embryo culture was high (66 %). Raising plantlets in the greenhouse was found to be useful to select healthy plants and thus obtain a uniform stand in the field. The genotypes were planted in a single row trial and harvested 8 months after planting. Transplanted plantlets gave 98.89 % establishment. Yield-related traits were significantly high compared to results from past experiments. The high percentage of plant recovery from seed to the field is a means of overcoming some problems associated with the traditional method of cassava breeding through direct seed planting to generate planting materials.

18.6 Molecular Studies on Putative Hybrids Obtained Through Embryo Culture

Morphological parameters of the two parents in a cross and their hybrid progeny can be compared to look for clear-cut differences. Morphological traits, however, are subject to environmental influences and can be misleading. Besides, these lack power of resolution to identify hybrids at juvenile stages, for which it becomes necessary to wait until plant maturity (Debbarama et al. 2013). Distant crosses rescued through embryo rescue can be confirmed for hybridity using molecular markers such as RAPD, AFLP, etc. Therefore, testing of hybridity using molecular markers is advantageous, as it obviates these hindrances.

18.6.1 Use of Molecular Markers in Mango Embryo Rescue

Mini- and microsatellite DNA markers are highly polymorphic in many species due to a variable number of tandem repeats. DNA fingerprints obtained by hybridization of mini- and microsatellite probes with genomic DNA were shown to be useful for cultivar identification. DNA fingerprint information for identification of nucellar and zygotic seedlings was attempted. For this purpose, DNA was isolated using CTAB method from fully mature leaves of 6-month-old seedlings (stones with single plants, as well as polyembryonic seedlings) and from field-grown mother trees. DNA samples were quantified using spectrophotometer and confirmed for the presence of DNA using agarose gel electrophoresis.

To multiply Jeffrey's minisatellite probe (33.6 plasmid DNA was used as the probe), the plasmid was incorporated into DH5α *E. coli* competent cells and cultured on LB Agar medium with ampicillin at 50 µg/ml. Single colonies from the transformed ones were isolated and inoculated onto the same medium. After adequate growth of the culture, the plasmid was isolated and checked for the presence of minisatellite 33.6 (700 bp DNA) on 0.8 % agarose gel, using restriction enzymes *Bam H1* and *EcoR1*. Further development of the nonradioactive labelled probe (33.6 minisatellite), restriction digestion of the plant genomic DNA and Southern transfer/hybridization were worked out.

18.6.1.1 PCR Amplification Using ISSR Primers

ISSR primers UBC 814, 835, 841, 844, 848, 868, 873, 881, 898 and 901 were used. The reaction mixture (20 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM mixed dNTP, 1 mM primer, 0.5 unit Taq polymerase and 25 ng/µl of genomic DNA. DNA amplification was obtained through 40 cycles (92 °C for 1 min, 42 °C for 1 min 30 s and 72 °C for 1 min 30 s), followed by extension at 72 °C for 8 min. Of the ten ISSR primers used, only six (UBC 835, 841, 848, 868, 873, 898) showed amplification. Bands amplified in the hybrids were similar to that in the parents suggesting that these were true hybrids. Forward and reverse primers, viz. 1F1R, 5F5R, 6F6R and 9F9R, were also used, of which 1F1R, 6F6R and 9F9R showed amplification.

18.6.2 Use of Molecular Markers in Grape Embryo Rescue

Leela Sahijram and Raghavendra (2005), Raghavendra et al. (2006) and Leela Sahijram and Reddy (2009) carried out molecular studies using ISSR markers in seedless grape hybrids and confirmed the hybridity of rescued progeny.

18.7 Basic Studies Carried Out Using Embryo Cultures

In addition to applied uses of embryo culture, the procedure is useful in basic studies. Growing embryos outside the ovule (*ex ovulo*) is an excellent way to study nutrition and metabolism of the embryo at various stages of its development.

The technique can also be used to examine growth requirements of embryos, effects of phytohormones and environmental conditions on zygotic embryogenesis and the regeneration potential of whole embryos or their segments. Embryo culture can be used to localize sites of germination promoters and inhibitors, for studies on embryogenesis and for cryopreservation. Embryo culture is useful in understanding precocious germination. Studies have shown that seed tissues play an important role in regulating the development of embryos in situ. Cotyledon growth stops almost immediately upon excision of immature embryo, indicating probable cessation of cell division, as seen in cotton. An embryo is programmed to germinate even when it is very small.

In situ, the embryo may not germinate because it lacks the germination-specific mRNA. But, removal of the seed from its environment activates the required machinery of embryo cells to synthesize the information necessary for germination. It has been shown in cotton that germination-specific mRNA is normally transcribed when cotyledons are about three-fifths their final size. However, to check precocious germination that may lead to vivipary (a lethal development), translation of mRNA is prevented by simultaneous appearance of abscisic acid (ABA) in the seed. Embryo excised at this stage undergoes precocious germination which suggests that ABA is contained in tissues surrounding the embryo rather than in the embryo itself. This conclusion is also supported by the fact that exogenous application of ABA to excised embryos also prevents their precocious germination. Accumulation of ABA during late stages of seed development has been shown to occur in a number of plants.

18.8 Conclusions

A very important and valuable biotechnological tool for raising hybrid progeny in intractable crosses is embryo rescue. It is most often used to rescue embryos from interspecific and intergeneric crosses and from embryos that do not fully develop naturally (as in early ripening and seedless fruit crops where the embryo aborts). Distantly related plant species in a breeding programme ('wide hybridization') may result in no/aborted hybrid embryos. Seedless parents may be unable to produce seeds in a cross. Alternately, breeding may be hindered owing to heavy fruit drop in the early stages of fruit development. In all such cases, hybrid embryo rescue is a very powerful and useful tool and an indispensable technique. The method also can be used to rescue seedless triploid embryos, produce haploids, overcome seed dormancy or determine seed viability. It is useful in understanding embryo morphogenesis and precocious germination. As research continues with this technique, new and valuable uses will be developed to assist biotechnological breeding of plants.

Embryo rescue/culture as a technique has found wide acceptance and utilization. Many interspecific and intergeneric hybrids have been successfully produced by culturing immature embryos that normally do not survive to maturity in situ. Embryo culture techniques are also used to rescue embryos from early maturing fruit varieties, to hasten maturation in some species and to overcome dormancy requirements in others. *In ovulo* embryo culture facilitates embryo development from the zygote stage to maturity when the ovule is cultured. A few interspecific and intergeneric hybrids have been made by first accomplishing fertilization *in vitro* and then culturing the ovules to maturity. Self-incompatibility in some species can be circumvented with these techniques. The potential of these may be a viable alternative to parasexual hybridization and somatic embryogenesis.

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Applications of Triploids in Agriculture

19

Ashwani Kumar and Nidhi Gupta

Abstract

Triploid hybrids have one of the most important traits, seedlessness, which is the characteristic for the fresh-fruit market. Triploid embryos are found in small seeds that do not germinate. Hybridization-based extensive breeding programmes require very efficient methodologies for embryo rescue and evaluation of ploidy. Biotechnology provides powerful tools for plant breeding. Triploid plants raised from endosperm are generally sterile. Endosperm-ploidy levels and its applications in plant breeding have been discussed here. Endosperm-raised triploid plants are of commercial value, e.g. timber-yielding plants, edible fruit plants or ornamentals propagated vegetatively and multiplied mainly through micropropagation. Illustration cases of many successful endosperm cultures are described here.

Keywords

Triploids • Embryo rescue • Plant tissue culture • Biotechnology • Polyploidy breeding

19.1 Introduction

In a fertilization process, the egg fuses with one of the male gametes to form a zygote, which afterward forms the embryo. The other male gamete fuses with the central cell containing two haploid nuclei. This second fusion is actually a double fertilization and triple fusion which often results in a triploid structure, the endosperm, and found to be present in all angiosperm families except Orchidaceae, Trapaceae and Podostemaceae. Such endosperm-raised triploid plants are generally sterile, but this seedlessness does not affect commercial utility of such plants, e.g. edible fruit plants, timber-yielding plants or ornamentals which are multiplied mainly through micropropagation or propagated vegetatively. The growth of triploids is generally higher than

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respective diploids (Thomas and Chaturvedi 2008). Also, triploids are more vigorous than diploids (Morinaga and Fukushima 1935).

Rather than the typical pair of chromosomes, a cell having three complete sets of chromosomes is called triploid. To produce viable offspring, chromosomes need to occur in pairs. But due to chromosomal number three, the triploid plants are sterile as the odd numbers of chromosomes are unable to pair up properly. Such plants do flowering and bear fruits, but flowers cannot be fertilized and fruit is sterile. Some of the examples of triploid crops are:

- Seedless watermelons (*Citrullus vulgaris*) produced due to cross between tetraploid females and diploid males. These are commercially cultivated in Japan.
- Triploid sugar beets (*Beta vulgaris*) produce larger roots with more sugar content.
- TV29 of tea produced by Tea Research Association of India is cultivated in North India. It produces larger shoots and leaves and is tolerant to drought.
- Cultivated banana (*Musa paradisiacal*) produces larger and seedless fruits.

19.2 Endosperm and Origin of Triploids

Endosperm is a natural and unique triploid tissue in its origin, ploidy level and nature of growth. It is the triploid stage of the flowering plant which is produced by fusion of three haploid nuclei; two from the female gametophyte and one from the male gametophyte (Thomas et al. 2000). It lacks histological differentiation. Lampe and Mills' (1933) first report on endosperm culture was on maize, whereas La Rue (1949) first reported the establishment of tissue cultures in maize from immature endosperm. Since then, mature and immature endosperm of various species has been shown to form continuously growing calli (Bhojwani and Razdan 1996). Johri and Bhojwani (1965) demonstrated totipotency of endosperm for the first time. They also demonstrated direct shoot formation from cultured mature endosperm of cherry ballart (*Exocarpos cupressiformis*). By the time, embryo/shoot/plantlet regeneration from endosperm has been reached to dozen of species (Bhojwani and Razdan 1996).

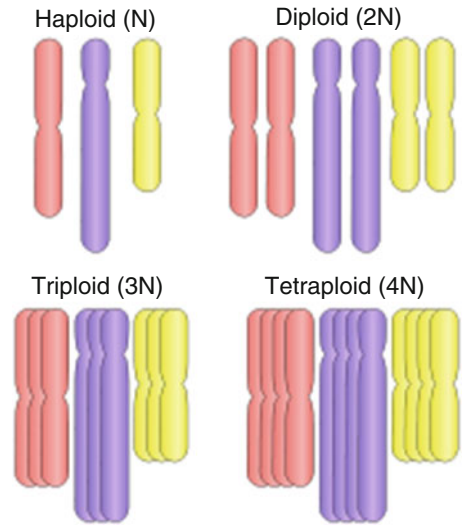

Fig. 19.1 Haploid (*single*), diploid (*double*), triploid (*triple*) and tetraploid (*quadruple*) sets of chromosomes

In tissue culture, endosperm tissues provide natural material for regenerating plants with triploid chromosome number, and thus, regeneration of plants from this tissue offers a direct method to produce triploids. A number of successful regeneration reports of organogenesis and somatic embryogenesis are available. Endosperm culture (Johri and Bhojawani 1977), reviews on endosperm (Cheema and Mehra 1982; Bhatnagar and Sawhney 1981), micropropagation (Driver and Kuniyuki 1984), walnut tissue culture (Mc Granahan et al. 1987), embryo rescue (Mc Granahan et al. 1986), somatic embryogenesis (Tulecke and McGranahan 1985), triploids in woody perennials (Lakshmi Sita 1987), *Hordeum vulgare* (Sehgal 1974; Sun and Chu 1981), *Triticum aestivum* (Sehgal 1974) and *Oryza sativa* (Bajaj et al. 1980; Nakano et al. 1975) are already in records (Fig. 19.1).

19.3 Production of Triploids

Triploids can be produced by crossing an induced tetraploid plant with normal diploid plant. Tetraploids can be produced by treating the terminal buds of diploid plants with chemicals such

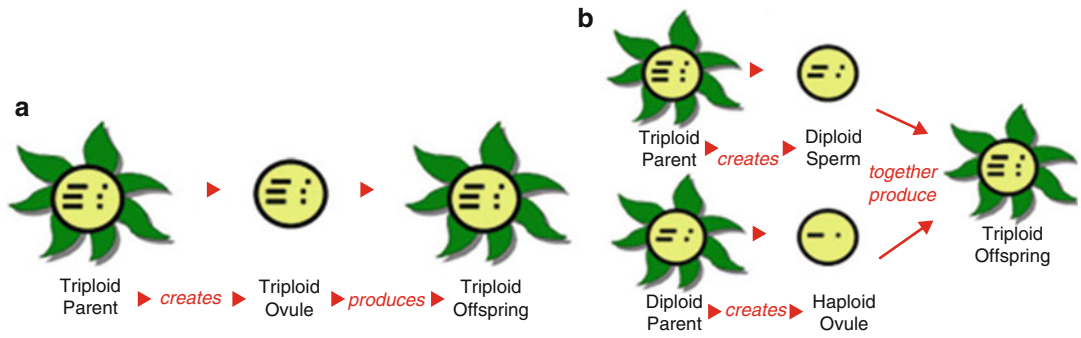

Fig. 19.2 (**a**) Asexual triploid reproduction via parthenogenesis. (**b**) Triploid-diploid sexual reproduction

as colchicine, oryzalin, pronamide, amiprophos methyl and trifluralin (Wan et al. 1991). However, such crosses are not always fortunate as it results in reduced seed setting compared to cross between two diploids (Sikdar and Jolly 1995). Moreover, seedling survival and seed germination are also very low. Still, triploids play an important role in biomass and soil conservation and thus represent a significant importance in shrubs and trees. They help in preserving vast amounts of photosynthetic energy and thus promote vegetative growth. Similarly, seedlessness is used to increase the quality of several fruits, like banana, papaya, grapes, apple, etc. In some plants, like *Miscanthus sinensis*, seed-sterile triploids have been grown to prevent seed dispersal in the environment (Petersen et al. 2002) (Fig. 19.2).

19.4 Examples of Triploid Plants

Triploid seedless trait has been described in many crops, especially in fruits. Artificially, triploid fruits are produced by first developing tetraploids using above-mentioned chemicals, which are then crossed with respective diploids. Such fruits are then commercially used.

19.4.1 Watermelon (*Citrullus vulgaris*)

When tetraploid females are crossed with diploid males, seedless watermelons (*Citrullus vulgaris*) are produced. Native African vine *Citrullus lanatus* (syn. *C. vulgaris*) derived modern varieties of the watermelon that are unable to produce viable gametes during meiosis, and thus, their ripened melons are seedless. Wild populations of *C. lanatus* var. citroides are common in Central Africa and give rise to domesticated watermelons var. lanatus (Robinson and Decker-Walters 1997). Wild, ancestral watermelons (var. citroides) have a spherical, striped fruit and white, slightly bitter or bland flesh and are commonly known as the citron or citron melon (Fig. 19.3).

Japan commercially grows seedless watermelons which are produced by crossing tetraploid female with diploid male lines. Reciprocal cross was also tried but was not successful. Seeds produced by triploid plants are not true seeds; they are small in size having white rudimentary structures like that of cucumber (*Cucumis sativus*) seeds. However, a few normal sized seeds may occur, but they are generally empty. It is also to be noted that all cultivated triploid watermelons do not have red pulpy flesh. They may have seedless yellow, sweet flesh (Fig. 19.4).

19.4.2 Little Gourd (*Coccinia grandis*)

Babu and Rajan (2001) developed a triploid variety of *Coccinia grandis*, fruit of which is used as a vegetable. It was also produced by crossing a normal diploid parent with colchicine-induced tetraploid. 2.4 % of seeds per fruit were observed. Morphologically, the triploid plants were somewhat resembled to the diploid, but the substantial

Fig. 19.3 Citron melon

features were its vigorous growth, increased fruit size, lower astringency and higher yield. However, these triploid fruits were tastier with good amount of vitamin A, vitamin C and iron and had less polyphenols; hence, they could be used as a salad crop. This plant also has many medicinal properties against diabetics, skin infections and bronchitis (Table 19.1 and Fig. 19.5).

19.4.3 Citrus

Citrus fruits are the most extensively and primarily produced fruit tree crop in the world (FAO 2009) for the fresh-fruit market, especially in the Mediterranean area. Area-wise, Spain is the main producer which covers a surface of 330,000 ha and produces about 6.3 million tons of citrus.

Diploids are the available genetic resources for citrus fruit, and their naturally produce seeds include polyploid individuals. These natural polyploid plants can give rise to interesting characteristics in citrus fruit; thus, they are very useful for genetic breeding projects. CIRAD (French Agricultural Research Centre for International Development) has developed genetic breeding programmes for citrus fruit in the Mediterranean Basin to create triploid varieties of sterile and seedless fruit and tetraploid rootstocks resistant

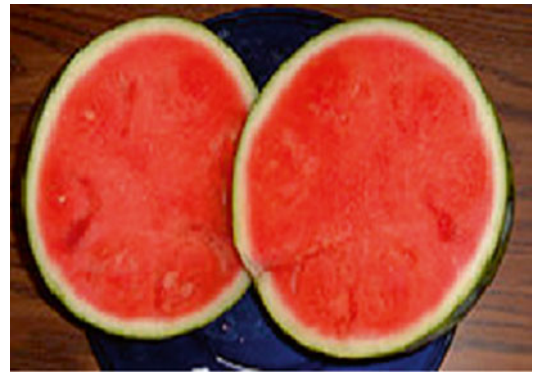

Fig. 19.4 Triploid watermelon having red flesh

to abiotic constraints, such as water deficiency or salinity, both from predominantly diploid genetic resources that would meet agronomic constraints, market expectations and consumer demand (Fig. 19.6).

19.4.4 Mandarin

As per increasing consumer demand, seedless citrus fruits are the basic requirement for the fresh market. Mandarin triploid hybrids have this seedlessness trait as its one of the most important characteristics. The availability of a number of high-quality seedless varieties in mandarins is very low; thus the production and recovery of new seedless triploid hybrids of mandarin varieties have a high priority for many citrus industries worldwide (Fig. 19.7).

Citrus triploid hybrids can be recovered by $2x \times 4x$ (Esen and Soost 1971b; Oiyama et al. 1981; Starrantino and Recupero 1981), $2x \times 2x$ (Cameron and Frost 1968; Esen and Soost 1971a; Geraci et al. 1975) and $4x \times 2x$ (Cameron and Burnett 1978; Esen et al. 1978; Aleza et al. 2009) sexual hybridizations as a consequence of the formation of unreduced gametes at low frequency (Aleza et al. 2010).

For the first time, Esen and Soost (1971a) indicated that triploid embryos were mainly found in between one third and one sixth smaller seeds than normal seeds that do not germinate in conventional greenhouse conditions. However, still at relatively low germination percentages,

Table 19.1 Comparative evaluation of diploid, tetraploid and triploid of *Coccinia grandis* (Source: Babu and Rajan 2001)

Characters	Diploid	Tetraploid	Triploid
Days to flowering	40	42	38
Flower size	Medium	Large	Medium
Fruit size	Medium	Small	Large
Fruit length (cm)	6.59	4.49	7.50
Fruit girth (cm)	7.40	8.20	11.60
Fruit weight (g)	15.20	14.50	44.20
Polyphenol per gram of fruit (fig)	0.300	0.311	0.090
Fruit colour	Green white strips	Green with white strips	Green with white
Leaf size	Medium	Large	Medium
Leaf thickness	Medium	Very thick	Medium
Fruit yield/plant/year (kg)	10.32	9.34	15.25

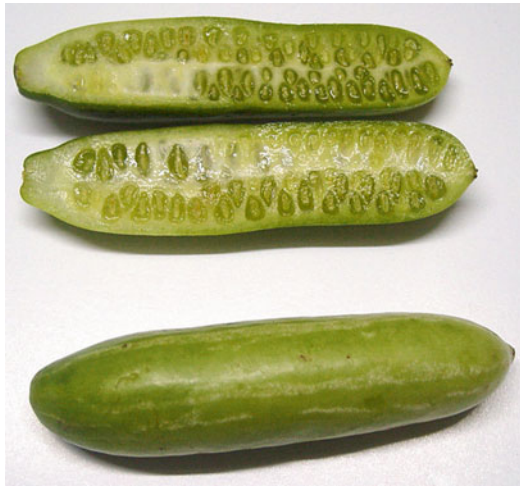

Fig. 19.5 *Coccinia grandis*

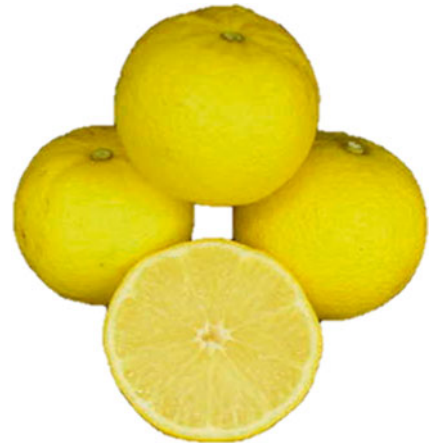

Fig. 19.6 Seedless lemon (*Citrus limon*)

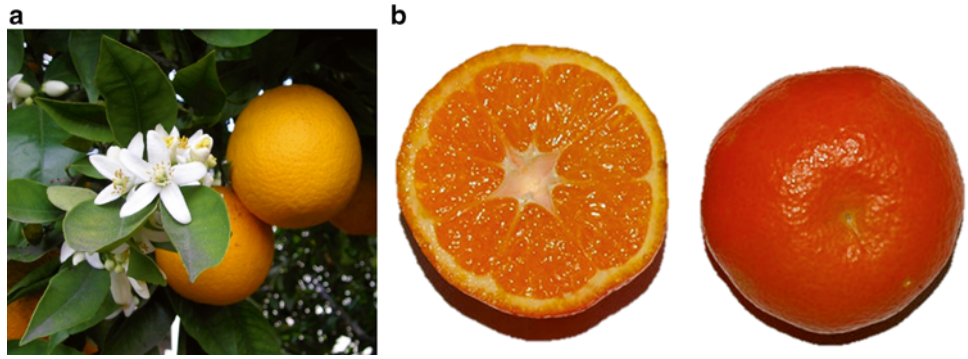

Fig. 19.7 (**a**) Mandarin plant having flowers and fruits. (**b**) Seedless mandarin

the in vitro culture of whole seeds with their integuments can improve germination rates (Ollitrault et al. 1996). In rare cases, triploid hybrids can be found in conventional greenhouse seedlings, as in 'A-12' mandarin (Bono et al. 2004) and 'Winola' mandarin (Vardi et al. 1991).

19.4.5 Neem (*Azadirachta indica*)

Because of the arising use of neem and its products in medicine, agriculture, cosmetics and animal health care, it is an important and economic tree of India. Triploid plants of neem were obtained from immature endosperm culture (Chaturvedi et al. 2003). Over 66 % of the plants were triploid with chromosome number 36. A characteristic feature of the shoots of endosperm origin is the presence of a large number of multicellular glands. The selected triploids, expected to be sexually sterile, can be bulked up by micropropagation (Fig. 19.8).

19.4.6 Acacia (*Acacia nilotica*)

Garg et al. (1996) describe somatic embryogenesis and triploid plant regeneration from immature endosperm cultures of *Acacia nilotica*, an important leguminous tree species suitable for afforestation of arid and marginal lands (Fig. 19.9).

19.4.7 Shanin (*Petunia violacea*)

Gupta (1983) reported the formation of haploid, diploid and triploid plants by direct pollen embryogenesis in *Petunia violacea*. In certain species, especially in *Petunia*, an almost exclusive production of androgenic triploids has been reported which is useful in ornamental plants for the introduction of vigorous foliage and flowers (Fig. 19.10).

19.4.8 Triticale

Triticale, first bred in laboratories during the late nineteenth century, is one of the most successful synthetic allopolyploids produced by crossing tetraploid wheat or hexaploid wheat with rye. The grain was originally bred in Sweden and Scotland; however, now it is being grown commercially in many parts of the world, e.g. Germany, Canada, France and Poland (the largest area), covering an area of around 2.6 million hectares with an annual production of 8 million tons. Triticale high-yielding ability and grain qualities of wheat combined with tolerance ability for adverse environment of rye provide its important and desirable features. In more than 15 years, the yielding ability of triticales has been increased to about 90 %. However, in Sweden, the raw triticales yielded about 50 % of the standard varieties of wheat.

Fig. 19.8 Neem

Fig. 19.9 Acacia plant having flower and fruit

Fig. 19.10 Shanin flower

Fig. 19.11 Triticale

India have released three varieties of triticales: TL419, DT46 (amber colour grains) and TL1210. Although TL1210 grain yield is comparable to that of the best wheat varieties, its deep grain colour represents its chief drawback, thus mainly grown as a fodder crop in Punjab. To overcome the problem, Indian breeders have developed amber-coloured triticales by using white-seeded rye as one of the parents of the triticales (Fig. 19.11).

Some other examples of allopolyploids are *Raphanobrassica*, the triploid (AAC) produced by crossing *B. campestris* (AA) with *B. napus* (AACC), *Festuca-Lolium* hybrids, allopolyploid clovers and some species hybrids in *Rubus* and jute (*Corchorus* sp.).

19.4.9 Sugar Beet (*Beta vulgaris* L.)

The triploid varieties of sugar beet are mixtures of diploid, triploid and other ploidy level plants. As compared to diploids, triploid sugar beets produce more sugar and larger roots and 10–15 % higher yields per unit area, while tetraploids produce smaller roots and lower yields. Commercially, Japan and Europe produce triploid varieties of sugar beet, but their popularity is declining rapidly. As the beet flower is small in size, triploid sugar beet seed production is quite difficult (Fig. 19.12).

Triploid sugar beet seed may be produced by using any of the following two ways: (1) using 4*x* plant as male and 2*x* as female or (2) using 4*x* plants as female and 2*x* as male. The first cross provides higher seed yield but a lower proportion of triploids, while the second gives lower seed yield but a higher proportion of triploids. Commercially, interplanting 4*x* and 2*x* lines in the ratio 3:1 is used for producing triploid sugar beet seed, and finally, seeds from both 4*x* and 2*x* plants are harvested. These harvested seeds consist of about 75 % triploid (3x) seeds.

19.4.10 Cassava (*Manihot esculenta*)

Cassava, commonly known as poor man's crop, is an important root crop to be cultivated in tropical countries and propagated by stem cuttings. It has become a subsidiary food in many countries. It is also used as cattle feed and its raw material for starch-based industries. Cultivated cassava is highly heterozygous and cross-pollinated, having a diploid number of chromosomes ($2n = 36$). Among artificially produced polyploids, cassava triploids have higher starch potential and a higher yield (Jos et al. 1987; Sreekumari and Jos 1996).

The first triploid variety of cassava named Sree Harsha was released in 1996 (Sreekumari et al. 1999) and was produced by crossing induced tetraploid plants with natural diploid. The use of a 2*x* female plant yielded better results than reciprocal crosses. Many features of triploid

Fig. 19.12 Sugar beet: (**a**) seed, (**b**) flower, (**c**) root

cassava make it superior than its diploid. These include higher harvest index, rapid bulking, higher yield, early harvestability, increased dry matter and starch content in the roots, shade tolerance and tolerance to cassava mosaic virus (Fig. 19.13).

19.4.11 Tea (*Camellia sinensis*)

Tea Research Association, India, has recently released a triploid clone of tea (*Camellia sinensis* var. assamica) for its commercial cultivation in northern parts of the country. This triploid cultivar, TV29, produces larger shoots and, thereby, biomass yields more cured leaf per unit area and is more tolerant to drought than the available diploid cultivars. The quality of the triploid clone is comparable to that of diploid cultivars used for making CTC (curl-tear-cut) tea (Fig. 19.14).

19.4.12 Mulberry (*Morus alba* L.)

Being an exclusive source of feed for silkworms, mulberry is an indispensable crop for the sericulture industry. Both natural and in-vivo-induced mulberry triploids have been reported (Das et al. 1970; Katagiri et al. 1982; Dwivedi et al. 1989). Many of the triploid lines are superior to its diploids (Thomas et al. 2000), in cold and disease resistance (Hamada 1963) and in yield and nutritive qualities of leaves (Seki and Oshikane 1959). The endosperm callus differentiated shoots, which could be rooted, and full triploid plants have already been established in soil (Fig. 19.15).

19.4.13 Ornamental

Excised cultures of endosperm from immature fruits having zygotic embryo have been used at

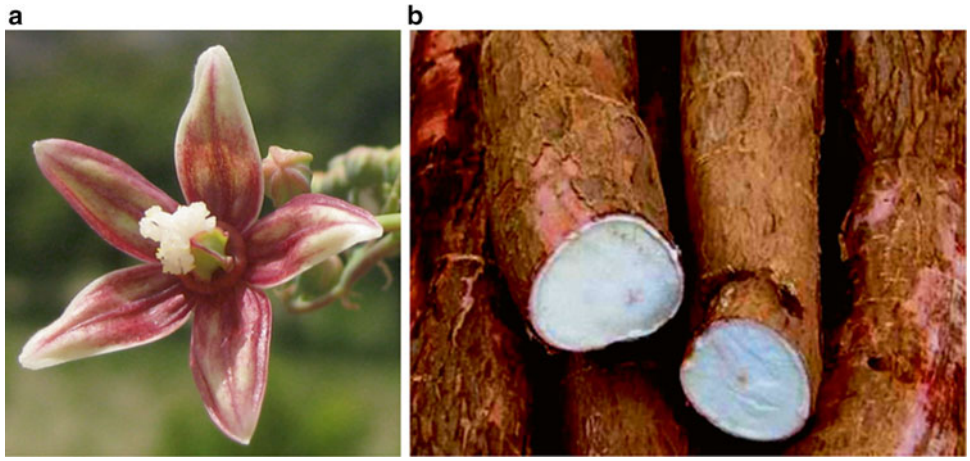

Fig. 19.13 Cassava: (**a**) flower and (**b**) root

Fig. 19.14 Tea leaves

Fig. 19.15 Mulberry plant with fruit

early dicotyledonous stage to produce triploid annual phlox or Drummond's phlox (*Phlox drummondii* Hook.) ornamental plants (Razdan et al. 2013). It was reported that over 70 % of annual phlox plants were triploid with a chromosome number of $2n = 3x = 21$. The growth of triploids is generally higher than respective diploids (Thomas and Chaturvedi 2008). These triploid plants have greater size of leaves, stem, flowers and/or foliage with higher number of pollen and larger stomata as compared to naturally occurring diploid plants (Miyashita et al. 2009). Moreover, triploid plant flowers showed enlarged central eye and bright colour, adding to their ornamental value (Razdan et al. 2008) (Fig. 19.16).

19.4.14 Pomegranate (*Punica granatum* L.)

Pomegranate is one of the oldest known fruit trees of the tropics and subtropics, cultivated for its delicious edible fruits. In addition, the tree is also valued for its pharmaceutical properties.

Fig. 19.16 *Phlox drummondii* flowers

19.5 Discussion

Endosperm is a unique tissue in its origin, ploidy level and nature of growth. It is mostly formed by the fusion product of three haploid nuclei, one from the male gametophyte and two from the female gametophyte, and is, therefore, triploid. Traditionally, triploids are produced by crossing induced superior tetraploids and diploids. This approach is not only tedious and lengthy (especially for tree species), but in many cases, it may not be possible due to high sterility of autotetraploids. In contrast, regeneration of plants from endosperm, a naturally occurring triploid tissue, offers a direct, single-step approach to triploid production (Bhojwani and Razdan 1996; Kumar 2010; Kumar and Roy 2006, 2011; Kumar and Shekhawat 2009; Neumann et al. 2009).

19.6 Conclusion

In conclusion, gametic embryogenesis hold great promise for making a significant, low-cost and sustainable contribution to plant breeding, aimed at increasing farm productivity and food quality, particularly in developing countries and in an environmentally friendly way, helping to reduce the proportion of people suffering from chronic hunger and from diseases due to malnutrition.

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Improving Secondary Metabolite Production in Tissue Cultures

20

Ashwani Kumar

Abstract

Plant cell and tissue culture has been suggested as an alternative means for year-round production of secondary metabolites with an added potential of increasing yields by culture selection and manipulation, genetic transformation, hairy root cultures, and use of bioreactors for mass production. Secondary metabolite pathways and genes involved in those pathways have been identified, and regulation of transcription and transcription factors has been determined by studying functional genomics in conjunction with data-mining tools of bioinformatics. Besides this, advances in metabolic engineering enable researchers to confer new secondary metabolic pathways to crops by transferring three to five, or more, heterologous genes taken from various other species. As an alternative, the metabolic pathways of useful secondary metabolites have been modified to improve their productivity via genetic transformation. However, there is a need to understand metabolic pathways of secondary metabolism at the molecular level. Plant hairy roots offer a novel and sustainable tissue-based system that preserves multiple specialized cell types believed to be important in maintaining a better consistency in synthesis of bioactive secondary molecules. This paper will review state-of-the-art reports on improving production of secondary metabolites in tissue cultures in various plant species.

Keywords

Secondary metabolites • Alkaloids • Saponins • Terpenoids • Nicotine

20.1 Introduction

Plant cell and tissue culture technology has been known as a possible tool for studying both the biosynthesis and the production of plant secondary metabolites (Kong et al. 2004, 2013, 2014).

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It has been used for production of large number of secondary metabolites. The degree of cellular differentiation and organization of the tissue, which is implied in a culture of this nature, favors the accumulation of these secondary compounds (Flores 1992; Wu et al. 2003, 2005; Kim et al. 2004; Kong et al. 2004; Thwe et al. 2012). An increased secondary metabolite production is correlated with a slow cell division rate in cell suspension cultures (Lindsey and Yeoman 1983; Sharma et al. 2011). Similarly, secondary metabolite production at the stationary phase of growth has been related with tissue organization (Tabata et al. 1972), morphological differentiation (Ramawat et al. 1985; Sharma et al. 2009), and low growth rates (Lindsey and Yeoman 1983).

Controlled transcription of biosynthetic genes achieved by specific transcription factors is one major mechanism regulating secondary metabolite production in plant cells (Bopana and Saxena 2010). Transcription factors are sequence-specific DNA-binding proteins that interact with the promoter regions of target genes and modulate the initiation of mRNA synthesis by RNA polymerase II. These proteins regulate gene transcription depending on tissue type and/or in response to internal signals. The two well-studied secondary pathways are the phenylpropanoid pathways and its flavonoid branch and the terpenoid indole alkaloid biosynthetic pathway (Neumann et al. 2009).

Hairy root cultures provide novel opportunities for production of valuable phytochemicals that are synthesized in roots. Hairy roots are developed by infecting plant leaf or stem tissue with *Agrobacterium rhizogenes* that transfers genes that encode hormone biosynthesis enzymes into the plants. Hairy root cultures have several advantages over undifferentiated plant suspension cell cultures. Hairy roots are genetically stable and grow in hormone-free culture media. Hairy roots show rapid growth and promote the synthesis of phytochemicals whose biosynthesis requires differentiated cell types. Hairy root lines producing valuable phytochemicals have been developed from various plant species (Dehghan et al. 2012; Cardillo et al. 2013). Recently, have been used to hairy root cultures to improve secondary metabolism compounds in *Hyoscyamus niger* (Zhang et al. 2004) and p-hydroxybenzoic acid (pHBA) glucose ester production in hairy roots of *Beta vulgaris* (Rahman et al. 2009), express foreign proteins or vaccine in tobacco (Shadwick and Doran 2007). Several TIAs' biosynthesis genes have also been overexpressed in *C. roseus* hairy root cultures (Zhao et al. 2012a, b). Kochkin et al. (2013) demonstrated for the first time the presence of large amounts of ginsenosides malonyl-Rb1, malonyl-Rc, malonyl-Rb2, and malonyl-Rd in a suspension culture of *Panax japonicus* var. *repens* cell.

Due to an increased appeal of natural products for medicinal purposes, metabolic engineering can have a significant impact on the production of pharmaceuticals and help in the design of new therapies (Bender and Kumar 2001; Kumar and Roy 2006, 2011; Kumar and Sopory 2008, 2010; Neumann et al. 2009; Kumar and Shekhawat 2009; Kumar 2010; Fernandez et al. 2010; Sharma et al. 2011; Kumar 2014). According to Bailey (1991), metabolic engineering is "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology." Application of recombinant DNA methods can improve production of metabolite and protein products by altering pathways and regulate release process in downstream processing. In many cases, this approach relies on the identification of limiting enzyme activities after successful pathway elucidation and metabolite mapping (metabolomics) (Neumann et al. 2009). Some of the important metabolites being produced in tissue culture and some technologies to improve their production are presented in this review paper.

20.2 Hairy Root Cultures

20.2.1 Azadirachtin from *Azadirachta indica* A. Juss. (Neem) Cultures

Azadirachtin (C35H44O16), obtained from *Azadirachta indica* (neem), is a high-value secondary metabolite commercially used as a broad-spectrum biopesticide. Neem (*Azadirachta*

indica A. Juss.) plant tissue and cell culture have been used to obtain year-round production of azadirachtin and other neem metabolites with the added potential of increasing yields by culture selection and manipulation. Allan et al. (2002) established hairy root cultures from stem and leaf explants of *Azadirachta indica* A. Juss (neem) following infection with *Agrobacterium rhizogenes*. Transformation was confirmed using polymerase chain reaction analysis. Srivastava and Srivastava (2013) reported batch cultivation of *Azadirachta indica* hairy roots in different liquid-phase bioreactor configurations (stirred tank, bubble column, bubble column with polypropylene basket, and polyurethane foam disk as root supports) to investigate possible scale-up of the *A. indica* hairy root culture for in vitro production of the biopesticide, azadirachtin. The hairy roots failed to grow in the conventional bioreactor designs (stirred tank and bubble column). They reported batch cultivation of *A. indica* hairy roots in modified bubble column reactor (with polypropylene mesh support). The incorporation of a PUF disk as a support for the hairy roots inoculated inside the bubble column reactor facilitated increased biomass production and azadirachtin accumulation in hairy roots (Srivastava and Srivastava 2013).

20.2.2 Tropane Alkaloids from *Nicotiana tabacum*

Hairy root cultures of *Nicotiana tabacum* are a better alternative for tropane alkaloid production than cell suspension cultures, mainly because they are stable, both genetically and in alkaloid production during long subculture periods (Maldonado-Mendoza et al. 1993). The utility of hairy root cultures to produce valuable phytochemicals could be improved by repartitioning more of the desired phytochemical into the spent culture media, thereby simplifying the bioprocess engineering associated with the purification of the desired phytochemical. The majority of nicotine produced by tobacco hairy root cultures is retained within roots, with lesser amounts exuded into the spent culture media. Reduced expression of the tobacco nicotine uptake permease (NUP1), a plasma membrane bound transporter, results in significantly higher nicotine accumulation in the media. Thus, NUP1-reduced expression lines provide a genetic means to repartition more nicotine into the culture media (Dewey and Xie 2013; Murthy et al. 2014).

20.2.3 Diosgenin from *Trigonella foenum-graecum* L. (Fenugreek)

Plant tissue cultures (in vitro techniques) offer an opportunity to improve the plant properties via genetic engineering, and recently it has been used as a tool for genetic transformations. *Trigonella foenum-graecum* L. (in Arabic, Hulabah) is also employed as a herbal medicine in many parts of the world. Diosgenin provides about 50 % of the raw material for the manufacture of cortisone, progesterone, and many other steroid hormones and is a multibillion-dollar industry. However, the supply of diosgenin cannot currently satisfy the demands of the ever-growing steroid industry, and therefore new plant species and new production methods, including biotechnological approaches, are being researched (Verpoorte 2000; Neumann et al. 2009).

20.2.4 Terpenoid Indole Alkaloids (TIAs)

Terpenoid indole alkaloids (TIAs) are very important pharmaceutical compounds. The monomeric alkaloids serpentine and ajmalicine are used as a tranquilizer and to reduce hypertension, respectively. The dimeric alkaloids vincristine and vinblastine are potent antitumor drugs. The biosynthesis of TIAs is highly regulated and depends on tissue-specific factors as well as environmental signals. Low productivity is the main hindrance toward commercial application of the production of secondary metabolites by plant cells in suspension culture. Different strategies have been developed to overcome this problem (Kumar and Roy 2006;

Kumar and Sopory 2008, 2010; Kumar and Shekhawat 2009; Kumar 2010; Bopana and Saxena 2010; Sharma et al. 2011).

20.2.5 Ginsenoside Saponin

Han et al. (2013) established dammarenediol-II production via a cell suspension culture of transgenic tobacco overexpressing PgDDS. Dammarenediol-II is a biologically active tetracyclic triterpenoid, which is a basic compound of ginsenoside saponin and is a useful candidate with potentially biologically active triterpenes. Transgenic tobacco plants overexpressing PgDDS (AB122080) under the control of the CaMV35 promoter were constructed.

20.2.6 Benzylisoquinoline Alkaloids (BIAs)

This group of alkaloids is derived from aromatic amino acid tyrosine. BIAs include the narcotic analgesic morphine, the cough suppressant codeine, the muscle relaxants papaverine and (+)-tubocurarine, the antimicrobial compound sanguinarine, and the cholesterol-lowering drug berberine (Kong et al. 2004; Kong and von Aderkas 2007). Recently, efforts have been made to assemble BIA biosynthetic pathways in microorganisms through the heterologous expression of multiple alkaloid biosynthetic genes (see Kumar and Sopory 2010; Nakagawa et al. 2011). Farrow et al. (2012) suggested that species-specific metabolite accumulation is influenced by the presence or absence of key enzymes and perhaps by the substrate range of these enzymes. They provided a valuable functional genomics platform to test these hypotheses through the continued discovery of BIA biosynthetic enzymes.

BIA noscapine is cough suppressant and promising anticancer agent (Dumontet and Jordan 2010), BIA biosynthetic enzymes from a number of related plant species have been characterized using EST (Farrow et al. 2012). The integration of transcript and metabolite profiles predicts the occurrence of both functionally redundant and novel enzymes.

20.2.7 Hypericin from *Hypericum perforatum* L.

Hypericin is a traditional medicinal plant for the treatment of depression and wound healing, and hypericin is one of the main effective active substances. To optimize the culture system for producing hypericin in adventitious root, Wu et al. (2014) reported the use of balloon-type airlift bioreactors. They investigated the effect of air volume, inoculation density, indole-3-butyric acid (IBA) concentration and methyl jasmonate (MeJA) concentration on hypericin content, and productivity during adventitious root culture. MeJA efficiently elicited the hypericin synthesis of *H. perforatum* adventitious roots (Wu et al. 2014).

20.2.8 Anthraquinones

Baque et al. (2013) reported improve root growth and production of bioactive compounds such as anthraquinones (AQ), phenolics, and flavonoids by adventitious root cultures of *Morinda citrifolia*. They studied effects of aeration rate, inoculum density, and Murashige and Skoog (MS) medium salt strengths using a balloon-type bubble bioreactor.

20.2.9 Isoflavone Production

Isoflavones have an affinity to estrogen-β receptors in humans and are reported to exhibit numerous health-promoting effects, including the allevia-

tion of menopausal symptoms, the prevention of osteoporosis and cardiovascular diseases, and the lowering of risk of breast cancer (Patisaul and Jefferson 2010). The growing demand for isoflavonoid derivatives resulted in numerous research projects focused on the in vitro cultures of selected plant species of Fabaceae which is rich in isoflavones. Some of the best known phytoestrogens are genistein, genistin, daidzein, and puerarin (Patisaul and Jefferson 2010). Biotechno-logical production of isoflavones, mainly from the family Fabaceae, is based on suspension cultures of *Pueraria* sp. (Goyal and Ramawat 2008a, b; Sharma et al. 2009; He et al. 2011), *Psoralea* sp. (Shinde et al. 2009a, b), and *Glycine max* (Federici et al. 2003; Terrier et al. 2007). Calycosin, formononetin, and pseudobaptigenin are also present in the more widespread legume *Trifolium pratense* (Kokotkiewicz et al. 2013).

20.3 Adventitious Root Culture Versus Hairy Root Culture

Culture of adventitious roots in bioreactors offers several advantages such as faster growth rates, tremendous quantities of metabolite accumulation, and stable production year-round. This can also reduce production costs and time and final product quality can be more easily controlled (Lee et al. 2011). Baque et al. (2013), using large-scale bioreactors, raised adventitious root culture as an efficient and attractive alternative to cell, hairy root, or whole-plant cultivation for biomass and metabolite production.

20.4 Bioreactors for Secondary Metabolite and Plant Propagules

Recent advances with large-scale production have successfully produced ginseng roots in a 10,000 l bioreactor establishing the feasibility of the root system to accommodate industrial processes (Sivakumar et al. 2006). A suitable air supply inside a bioreactor is an important factor. Air volume promoted the hypericin production of adventitious roots (Wu et al. 2011). Higher efficiency of genetic transformation resulted not only from greater target tissue yield, but there was also evidence of improved transgenic event production with the tissue produced with airlift bioreactors than tissue produced in shaken flasks (Kong et al. 2013).

Numerous studies have applied bioreactors in plant cell (Huang and McDonald 2012) and organ (Srivastava and Srivastava 2012) culture to obtain specific metabolites. Modern bioreactor culture systems provide a more advanced technology to produce higher secondary metabolites from plant cell, tissue, or organ using artificial nutrients with MeJA. Yu et al. (2002) found that the ginsenoside content was obviously enhanced by the addition of 100 μM MeJA during adventitious root culture of *Panax ginseng*; Donnez et al. (2011) examined that 0.2 mM MeJA was optimal for the efficient production and high accumulation of resveratrol in grape cell.

20.5 Propagules in Bioreactors

Besides this, a considerable number of researchers have cultured plant propagules in bioreactors to produce high-quality seedling (Zhao et al. 2012a, b). It is clear from these studies that temporary immersion bioreactor culture systems are appropriate for shoot multiplication and regeneration and the continuous immersion system is suitable for the proliferation of propagules (without leaves) such as bulblets (Kim et al. 2004), PLBs (Yang et al. 2010), and rhizomes (see Gao et al. 2014). Kong et al. (2013) constructed and tested airlift bioreactors (ALBs) for their potential to enhance chestnut embryogenic tissue proliferation for genetic transformation and mass propagation.

20.5.1 Ginsenoside Production

Panax ginseng roots have been widely used in Chinese traditional medicine since ancient times owing to their stimulating and tonic properties.

The pharmacological activities of ginseng or its crude extracts are based on the presence of a mixture of triterpenic saponins referred to as ginsenosides. The two major groups of ginsenosides are the Rb and Rg groups, which have proto-panaxadiol and protopanaxatriol, respectively, as the sapogenins (Mallol et al. 2001). Rb group includes the ginsenosides Rb1, Rb2, Rc, and Rd, while Rg group includes the Re, Rf, and Rg1 ones as the main compounds. Among all these ginsenosides, Rb1 and Rg1 are the most effective compounds (Tanaka and Kasai 1984; Mallol et al. 2001). Ginsenosides accumulate in the root of the plant, but the agricultural production of roots is expensive. Therefore, the production of ginsenosides, by means of different biotechnological alternatives, has been extensively studied by a number of researchers, using callus tissues (Mallol et al. 2001), cell suspensions (Mathur et al. 1994; Kochkin et al. 2013), and root cultures (Yoshikawa and Furuya 1987); nevertheless, the productivity obtained so far has been low because of the low growth rates of cultures. The induction and establishment of hairy roots after the infection of *Panax ginseng* rhizomes with *Agrobacterium rhizogenes* has been successfully performed (Washida et al. 1998). These roots grow more rapidly and produce higher levels of saponins than the ordinary cultured roots obtained by hormonal control. In the case of agropine-type strains (such as *A. rhizogenes* A4), two T-DNA fragments (TL-DNA and TR-DNA) are separately transferred into the plant material (Jouanin 1984). The integration of the TL-DNA into the plant genome is essential for developing transformed roots; three genes of this fragment, known as rolA, rolB, and rolC, are responsible for the full hairy root syndrome (Palazon et al. 1998). Although the TR-DNA is not essential for hairy root formation, it has been shown that the aux1 genes harbored in this T-DNA segment provide to the transformed cells with an additional source of auxin. Aux genes play a significant role in the morphology and alkaloid production of transformed roots of *Datura metel* and *Duboisia* hybrid (Mallol et al. 2001).

20.5.2 Resveratrol 1

These plant polyphenols have received considerable interest based upon a number of associated health benefits (Baur and Sinclair 2006; Delmas et al. 2006). Most notably, the significant levels of resveratrol 1 in red wine have been credited to the phenomenon known as "the French Paradox," wherein low incidence of heart disease is observed among a population with a relatively high-saturated-fat diet and moderate wine consumption (Frankel et al. 1993). Over the past two decades, numerous health benefits impacting cardiovascular disease, various cancers, atherosclerosis, and aging have been linked with resveratrol 1 (reviewed; Baur and Sinclair 2006; Roupe et al. 2006). The majority of resveratrol-containing dietary supplements are composed of unknown/unidentified botanical components wherein resveratrol 1 and resveratrol derivatives only make up a small fraction of the product. While chemically synthesized resveratrol 1 may address this issue, natural sources often contain derivatives, cofactors, and other phytonutrients that provide added or synergistic benefits to the nutraceutical product and are often preferred by the consumer (Wallace 1998). Recent studies showing antiaging benefits of resveratrol 1 (Baur et al. 2006) further accelerate interest in a natural, food-grade source of enriched resveratrol/resveratrol derivatives that delivers a more defined and consistent product composition and ensures a stable supply chain, and several biotic production strategies targeting recombinant plants, yeast, and bacteria have been advanced (Watts et al. 2006).

20.5.3 Camptothecin (CPT)

Camptothecin (CPT), a monoterpene indole alkaloid, has been found in several plant species including *Camptotheca acuminata*, *Nothapodytes foetida*, and *Ophiorrhiza pumila* (Saito et al. 2001; Lorence and Nessler 2004). Since it possesses topoisomerase I poisoning properties, its semi-

synthetic derivatives, topotecan and irinotecan, have been developed to be clinically used as anticancer drugs. Previously, we have established a hairy root culture of *O. pumila* which has already been shown to be a desirable experimental system to study the biosynthesis of camptothecin, since the culture produces a high level of CPT and excretes it into the culture medium (Sirikantaramas et al. 2007).

20.5.4 *Catharanthus roseus* (Madagascar Periwinkle)

As an important medicinal plant, *Catharanthus roseus* (Madagascar periwinkle) produces a large amount of terpenoid indole alkaloids (TIAs). Among them, vinblastine and vincristine are important antitumor bisindole alkaloids. However, these two anticancer compounds are produced at a very low level in *C. roseus* leaves, about 5.8 μg/g for vinblastine and 0.9 μg/g (fresh weight) for vincristine, leading to their high price in the market (Favretto et al. 2001). The lack of vinblastine and vincristine in *C. roseus* hairy roots has been ascribed to an absence of vindoline (Bhadra et al. 1998). This may be due to the undetectable expression of the D4H and DAT genes in the transgenic hairy roots. Very recently the DAT gene, which is responsible for the terminal step of vindoline biosynthesis in *C. roseus*, was overexpressed in *C. roseus* hairy roots. Interestingly, overexpression of DAT did not increase vindoline production but improved the accumulation of another monoterpenoid indole alkaloid, horhammericine (Magnotta et al. 2007). Biotechnological methods may provide an efficient alternative for producing natural products since a number of genes involved in the TIAs' biosynthetic pathway have been cloned (Pasquali et al. 2006; Wang et al. 2010; Zhou et al. 2011). Deacetylvindoline-4-O-acetyltransferase (DAT) is a key enzyme for the terminal step of vindoline biosynthesis. In this research, the DAT gene promoter was cloned, sequenced, and analyzed.

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Somaclonal Variation in Micropropagated Plants

21

Leela Sahijram

Abstract

Plants generally exhibit cytogenetic and genetic variations that are helpful to plant breeders for crop improvement. When such variants arise through the cell and tissue culture process, using any plant portion as an explant material, these are termed 'somaclonal variations' (SV). Variants obtained using callus cultures are referred to as 'calliclones', while variants obtained using protoplast cultures are known as 'protoclones'. On the other hand, 'gametoclonal variation' refers to variations arising in cell cultures of gametic origin, as in pollen and microspore cultures, to distinguish them from somatic cell-derived regenerants. Somaclonal variation is a double-edged sword whereby its presence in micropropagation programmes is inimical, while it can be gainfully exploited to create stable variations, e.g. disease resistance, where other methods fail or are cumbersome.

Keywords

Somaclonal variation • Genetic stability • Gametoclonal • Detection of variants • Useful variants

21.1 Introduction

Somaclonal variation (SV) is a phenotypic variation either genetic or epigenetic in origin displayed among somaclones (soma=vegetative; clone=identical copy) and occurs among plants regenerated from tissue culture. A general term 'somaclonal variation' was proposed to describe genetic variation in plants regenerated from any form of cell cultures. Accordingly, plants derived from cell and tissue cultures are termed '*somaclones*'. Somaclonal variation has come to represent genetic variability present among all kinds of cells/plants obtained from cultures in vitro. SV can be problematic during micropropagation and in vitro conservation and in genetic transformation of crop plants, although it may be put to good use as a tool in plant breeding. Plants regenerated from tissue and cell cultures show heritable variation for both qualitative and quantitative

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traits. Several useful somaclonal variants too have been obtained in a large number of plant species such as potato, sugarcane, banana, tomato, etc. Somaclonal variation is well documented in the widely commercialized tissue culture-raised fruit crop, banana (*Musa* spp.).

Variants obtained using callus cultures are referred as *calliclones*, while variants obtained using protoplast cultures are known as *protoclones*. Larkin and Scowcroft (1981) proposed a general term 'somaclonal variation' to describe genetic variation in plants regenerated from any form of cell cultures. Accordingly, the plants derived from cell and tissue cultures are termed as *somaclones*, and the plants displaying variation as 'somaclonal variants'. However, generally the term somaclonal variation is used for genetic variability present among all kinds of cell/plants obtained from cell cultures in vitro. Plants regenerated from tissue and cell cultures show heritable variation for both qualitative and quantitative traits. Several useful somaclonal variants have been obtained in large number of plant species such as potato, sugarcane, banana, tomato etc. Chaleff (1981) labelled plants regenerated from tissue cultures as R_0 generation and their successive sexual generations as R_1, R_2, etc.

SV can be problematic during micropropagation and in vitro conservation and in genetic transformation of crop plants, although it may be put to good use as a tool in plant breeding. These changes are heritable. Early detection of SV is, therefore, very useful. Shoot-tip culture preserves genetic stability much better than callus or cell suspension cultures, yet somaclonal variation appears to be widespread among plants regenerated from banana shoot-tip cultures. Off-type frequencies are reported to vary from 1 to 74 %.

In banana, a globally important fruit crop that is extensively micropropagated, it is even more pertinent to study SV as the crop is especially prone to this phenomenon. To date, somaclonal variation affecting in vitro propagated banana is not well understood, suggesting a complex genetic cause of this phenomenon. A molecular biology-based approach of analysis would help throw light on causes and detection of variants to cure this scourge of the banana micropropagation industry. In vitro conditions can induce mitotic instability. Labile portions are known to exist in the genome rendering it susceptible. These portions get modulated when cells undergo 'stress' in tissue culture, resulting in higher rearrangement and mutation rates than other portions of the genome. Occurrence of hotspots of mutation and recurring menus of alternative alleles is consistent with this response being limited to a sub-fraction of the genome. In banana, where production of somaclonal variants is substantial, only those plants that show side shoots as well with the same type of variation are considered as 'variants'.

21.2 Gametoclonal Variation

Another term for variations arising due to the tissue culture process is *gametoclonal variation* for variations arising in cell cultures of gametic origin, like in pollen and microspore cultures, to distinguish them from somatic cell-derived regenerants.

When gametic cells are cultured under in vitro conditions and variations observed in such cultures, these are called *gametoclonal variations*. Products obtained from gametoclonal variations are termed *gametoclones*. In gametoclonal variation, gametes (being products of meiotic division) possess only half the number of parent chromosomes. Gametoclones can be developed by culturing male or female gametic cells. Anthers or isolated microspores are widely used for developing gametoclones. A large number of plants have been regenerated from gametoclonal variations like *Oryza sativa*, *Nicotiana tabacum*, *Brassica napus* and *Hordeum vulgare*. Improvements have been made in several plant species through gametoclonal variation, e.g. rice, wheat and tobacco. There are three major reasons that can cause genetic variations in gametoclones:

- The technique used in cell culture may induce genetic variation(s).
- Doubling of haploid chromosomes may generate variation(s).
- Heterozygosity in diploids may induce genetic variation(s).
- Variations may result from segregation and independent assortment.

Gametoclones differ from somaclones in three distinct ways: (1) Gametoclones regenerate into

haploid plants in comparison to somaclones which develop into diploid plants. (2) The recombination process occurs by meiotic crossing over in gametoclonal variation. (3) Gametoclones can be stabilized by doubling their chromosome number.

21.3 Factors Contributing to Occurrence of Somaclonal Variation

- *Genotype*
  Obvious chromosome breakages or aberrant number of chromosomes are found even in conventional sucker-grown plants. However, these defects get magnified in plants grown in tissue cultures.
- *Ploidy level*
  In general, more incidence of SV is observed with increase in ploidy.
- *Number of subculture cycles*
  Restricting the number of subculture cycles to five to eight is considered safe. SV is known to increase to 2.9 and 3.8 % at 9th and 11th subcultures, respectively, i.e. mutation rate is higher in prolonged culture. Almost all somaclonal variants produce poor quality bunches.
- *Overdosing with hormones in vitro*
- *Starter material*: *sword suckers vs. water suckers*

The basic cause of these variations may be attributed to changes in karyotype (chromosome number and structure), chromosome rearrangements, somatic crossing over, sister chromatid exchange, DNA amplification and deletion, transposable elements and DNA methylation. Somaclonal variation can be characterized based on morphological, biochemical (isozymes) and DNA markers such as random amplified polymorphic DNA (RAPDs), restriction fragment length polymorphism (RFLPs) and inter-simple sequence repeats (ISSR).

The variations could also arise in tissue culture due to physiological changes induced by the culture conditions. Such variations are temporary and are caused by *epigenetic changes*. These are non-heritable variations and disappear when the culture conditions are removed. Changes in DNA methylation pattern have been implicated.

There are different approaches (steps) to create somaclonal variations, which include:

1. Growth of callus or cell suspension cultures for several cycles
2. Regeneration of a large number of plants from such long-term cultures
3. Screening for desirable traits in the regenerated plants and their progenies. For example, in vitro selection to select agronomically desirable somaclones for tolerance to various biotic and abiotic stresses, herbicides, high salt concentration and extremes of temperature
4. Testing of selected variants in subsequent generations for desirable traits
5. Multiplication of stable variants to develop new breeding lines

To be of commercial use, a somaclonal variant must fulfil certain basic requirements:

1. It must involve useful characters.
2. It should be superior to the parents in the character(s) in which improvement is sought.
3. The improved character(s) must be combined with all other desirable characters of the parent.
4. The variations must be inherited stably through successive generations by chosen means of propagation.

21.3.1 Identification of Variants

There are identifiable and predictable DNA markers for early diagnosis of SV. DNA methylation has been recognized to cause SV. Representational difference analysis (RDA) has been employed to isolate unique fragments (‘difference products’) between visible off-types and ‘normal’ tissue culture (TC)-derived plants. Various other molecular techniques are available to detect sequence variation between closely related genomes such as those of source plants and somaclones, viz. RAPD, AFLP (including MSAP – methylation-sensitive amplification polymorphism), microsatellites, etc.

Somaclonal variants have also been shown to have gibberellic acid profiles different from those of normal TC plants. Overdosing with cytokinins and culture frequency or number (or both) have all been shown to cause SV. However, genome is

the predominant predisposing factor for occurrence of somaclonal variants.

21.3.2 Exploiting Somaclonal Variations to Advantage

Plants generally exhibit cytogenetic and genetic variations that are helpful to plant breeders in crop improvement. When such variants arise through the cell and tissue culture process using any plant portion as an explant material, variations arising are termed as somaclonal variations. Hwang and Ko (2004) in Taiwan successfully developed 'Cavendish' banana cultivars *resistant to Fusarium wilt* acquired through somaclonal variation. There is no known source of natural resistance within the banana group to this deadly deuteromycetan pathogen of the bananas.

21.3.3 Disadvantages of Tissue Culture-Propagated (TCP) Plants

The primary disadvantage is somaclonal variation (SV). Technically, somaclonal variation is a phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones. In the context of TC industry, it can be loosely defined as a genetic/epigenetic change in the plant system inimical (harmful) to commercial production, because the primary objective of micropropagation is to produce true-to-type planting material of a desired clone. Micropropagated bananas have shown a high propensity for variation. Considerable research on multiplication schedules, phytohormone concentrations and relative proportions in vitro has been performed to minimize/eliminate the problem. Additional issues affecting production success and marketing strength are (1) selection and quality of explant donor (source) plants and (2) recognition and control of endogenous (nonpathogenic) bacteria/contaminants, with adherence to acceptable commercial principles.

Micropropagated banana plants can also be more susceptible to various pests and diseases. Infection can be overcome by inoculation of the rhizosphere or the root system of these micropropagated plants with fungi or mycorrhizae and improved growth with beneficial bacteria.

In short, major disadvantages are:

1. Inability to lower high frequency of off-types in certain cultivars
2. Inability to deal with endogenous bacteria or bacterium-like contaminants contributing to significant losses in, and immediately after, culture

Thus, micropropagation of bananas has not been without obstacles. SV has been widespread in both bananas and plantains. The result is an increase in the number of off-types and this is genotype dependent (Sahijram et al. 2003a, b, c). Several distinct off-types have been described, including dwarfism, in the Cavendish group. Inflorescence changes have been reported in plantain. Low-vigour and low-production off-types are also very common. Apart from obvious visual characteristics such as distorted/mosaic/variegated plants, off-types cannot usually be identified at the in vitro stage. Some undesirable somaclones in commercial banana micropropagation are illustrated below in Figs. 21.1 and 21.2.

21.3.3.1 Factors to Consider for Dealing with Off-Type Production

- Off-types can arise at any time in culture.
- They may arise from stimulation of adventitious buds.
- Growth regulators do not *directly* induce mutagenesis (their effect is indirect).
- Nursery screening procedures are available for rouging off-types.
- Cavendish types can be identified by morphological characters.
- Molecular markers can be used.

Fig. 21.1 Dwarf off-type somaclonal variant banana plant showing abnormal, spindle-shaped leaf lamina (**a**) and leaf texture/colour variants (**b**) (Source: R. DoreSwamy, formerly at IIHR, Bangalore)

21.3.3.2 Methods for Minimizing Variants

- Correct mother-plant selection
- Low-cytokinin medium for multicycles
- Correct dissection protocols
- Minimal subculture cycles
- Correct subculture cycle time
- Clonal conformity during multicycle
- Culling out variants during primary and secondary hardening cycles
- Grading and removal of variants at planting

21.3.4 Molecular Basis of Somaclonal Variation

Molecular basis of somaclonal variation is not precisely known, but both genetic and epigenetic mechanisms have been proposed (Cullis et al. 2007). Banana is a crop where in vitro culture is widely practised on a commercial scale. Available evidence points toward the existence of labile portions of the genome that can be modulated when cells undergo the stress of tissue culture.

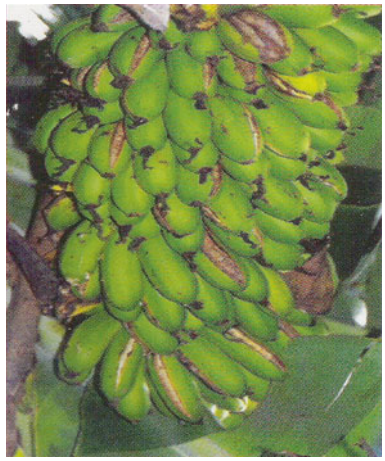

Fig. 21.2 Variant: bunch morphology – an abnormal banana bunch showing split fingers

Therefore, the hypothesis that there are identifiable and predictable DNA markers for the early diagnosis of somaclonal variation has been tested. Representational difference analysis (RDA) was used to isolate unique fragments of DNA (difference products) between visible culture-induced off-type and normal banana plants. Markers generated from six difference products differentiated between some of the off-type and normal pairs. The genomic region around one of these difference products has been extensively characterized and has a high degree of polymorphism, with variation in up to 10 % of the nucleotides sequenced in the region. This same region has been shown to vary in other pairs of off-type and normal banana plants derived from tissue culture as well as in plants propagated commercially in vitro. The data are consistent with the hypothesis that there is at least one particularly labile portion of the genome that is especially susceptible to the stress imposed during tissue culture and that is associated with higher rearrangement and mutation rates than other portions of the genome. Consequently, the regions that are reported here have the potential to be used as early detection tools for identifying somaclonal variants.

Lakshmanan Venkatachalam et al. (2007), in their study, used two PCR-based techniques, RAPD and ISSR, for identification of genetic variations in tissue culture-derived banana plantlets because of their simplicity and cost-effectiveness. The use of the two types of markers that amplify different regions of the genome allows better chances for identification of genetic variation in the plantlets. Although this study did not detect any genetic change, it is possible that some changes may have occurred that went undetected as there is a possibility of point mutations occurring outside the priming sites. This study screened a large number of random primers common for higher plants, some of which are present in monocots. Since no changes were seen in the banding pattern of tissue culture plants compared to that of the mother plant, they concluded that micropropagation protocol developed by them for banana, var. Nanjanagudu Rasabale, may be applicable for a considerable length of time without any significant risk of generating genetic instability.

The extent of DNA methylation polymorphisms was evaluated by Peraza-Echeverria et al. (2001) in micropropagated banana (*Musa* AAA cv. 'Grand naine') derived from either the vegetative apex of the sucker or the floral apex of the male inflorescence using the methylation-sensitive amplification polymorphism (MSAP) technique. In all, 465 fragments, each representing a recognition site cleaved by either or both of the isoschizomers, were amplified using eight combinations of primers. A total of 107 sites (23 %) were found to be methylated at cytosine in the genome of micropropagated banana plants. In plants micropropagated from the male inflorescence explant, 14 (3 %) DNA methylation events were polymorphic, while plants micropropagated from the sucker explant produced 8 (1.7 %) polymorphisms. No DNA methylation polymorphisms were detected in conventionally propagated banana plants. These results demonstrated the use of MSAP to detect DNA methylation events in micropropagated banana plants and indicate that DNA methylation polymorphisms are associated with micropropagation.

Biochemical, isozyme activities and molecular marker, DNA fingerprinting were used to analyse somaclonal variations in tissue culture-derived banana plants BBTV and CMV tested. Variation

was found in glutamate oxaloacetic and polyphenol oxidase isozyme (DISC-PAGE) activities as well as peroxidase activity among in vitro regenerated banana plants. Fifteen arbitrary-base primers were successfully used to amplify DNA extracted from banana plants propagative in vivo and in vitro of these, four primers revealed characteristic. DNA fingerprinting revealed genetic variations and 25 % polymorphisms. The frequency of somaclonal variations was found to be dependent upon number of subcultures (times of micropropagation cycles). The genetic variations were only detected in 7-month-old cultures. It was observed also that morphologically abnormal shoots and change in chlorophyll showed genetic variations at the molecular level.

Tissue culture propagation of banana has gained attention due to its potential to provide genetically uniform, pest-free and disease-free planting materials. A large number of varieties of banana are multiplied in vitro the world over for commercial cultivation.

Yuval Cohen and co-workers (2007) analyzed somaclonal variants in date palm (*Phoenix dactylifera*). Propagation of date palms using tissue culture techniques is very advantageous. However, among normal trees, many off-type trees are generated. These abnormal phenotypes include variegation, 'low level fruit setting' and dwarfism. The last two tend to occur in mass numbers mainly in plantlets of specific tissue culture laboratories. While some off-types are easily detected in young plantlets, others are commonly detected in the field only years after planting. Therefore, both preventing generation of and developing methods for early detection of these off-types are necessary. Their study applied molecular techniques to characterize abnormal phenotypes where they applied amplified fragment length polymorphism (AFLP) to characterize all three, common date-palm off-type phenotypes. In the variegated trees, multiple mutations seemed to occur. In contrast, only relatively few mutations were detected among 'low level fruit setting' and dwarf trees. No single specific mutation was found to be associated with these phenotypes. Differences in DNA methylation patterns were seen among the off-type trees. Reduction in overall DNA methylation levels appeared to be associated with 'low fruit setting' phenotype. Changes in DNA methylation patterns have been previously suggested to be involved in the generation of somaclonal variation. It seems that, similarly, changes in DNA methylation patterns occurring during the tissue culture process may generate a developmentally altered scheme. This may result in off-types which can be detected only long after planting of the trees. Specific sequence variations and/or DNA methylation assays can be useful in detecting off-type plantlets before they are field planted.

Other studies on the use of molecular techniques for analyzing somaclonal variants in plant species of commercial importance are also available (Sahijram et al. 2010). Goto et al. as early as in 1998 determined genetic stability of long-term micropropagated shoots of *Pinus thunbergii* Parl., using RAPD markers. RAPD assessment was also carried out by Carvalho et al. (2004) for the identification of clonal identity and genetic stability of in vitro propagated chestnut hybrids; Rahman and Rajora (2001) used microsatellite DNA for detecting somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*). Palombi and Damiano (2002) successfully used and made comparison between RAPD and SSR molecular markers for detecting genetic variation in kiwifruit (*Actinidia deliciosa*).

21.3.5 Genetic Stability and Molecular Profiling

ISSR technique is also very simple, fast, cost-effective, highly discriminative and reliable. At present, RAPD and ISSR markers have been successfully applied to detect the genetic similarities or dissimilarities in micropropagated material in various plants. Molecular profiling has recently been done to assess genetic stability of three economically important banana cultivars of the lower Indo-Gangetic plains by Ray et al. (2006) using RAPD and ISSR markers. Venkatachalam et al. (2007) carried out genetic analysis of micropropagated and regenerated banana plantlets as assessed by RAPD and ISSR markers.

Lakshmanan et al. (2007) earlier studied banana cv. Nanjanagudu Rasabale (NR) (silk group, AAB) to assess the impact of protocol and long-term in vitro effects on induction of somaclonal variation. In this study, they adopted two PCR-based techniques, RAPD and ISSR, for the identification of somaclonal variants because of their simplicity and cost-effectiveness. The use of two types of markers, which amplify different regions of the genome, allows better chances for identification of genetic variation in the plantlets.

A large number of micropropagated plantlets of NR developed from axillary shoot bud explants over 10 years ago were screened for genetic variation, if any, using RAPD (random amplified polymorphic DNA) and ISSR (inter-simple sequence repeats) markers. Of the 4,000 in vitro plantlets, 11 were used for screening that involved shoot cultures with distinct variation in morphological characteristics (morphotypes). Similarly, the mother maintained in the field was also subjected to genetic analysis. Out of the 50 RAPD and 25 ISSR primers screened, 30 RAPD and 5 ISSR primers produced a total of 424 clear, distinct and reproducible band classes resulting in a total of 5,088 bands where the banding pattern for each primer was highly uniform and comparable to that of field-grown mother clone from which these cultures had been established. This is the first report on the use of genetic markers to establish genetic fidelity of long-term micropropagated banana using RAPD and ISSR. Since there were no changes in the banding pattern observed in tissue culture plants as compared with that of mother plant, they concluded that that their micropropagation protocol for banana var. Nanjanagudu Rasabale can be carried out for long without much risk of genetic instability.

21.3.6 International Scenario

Two crops of major commercial importance have suffered in the past from susceptibility to somaclonal variation. Both of these are monocots, viz. banana/plantain group and oil palm. Appearance of off-types during the in vitro multiplication process is an important drawback for mass propagation of bananas. Visual screening at prehardening and hardening phases in the greenhouse helps detect putative off-types which can then be eliminated. In any micropropagation programme, 3–5 % somaclonal variation is permissible, but in bananas, up to 10 % variation is permitted owing to the flexible genetic make-up of the crop.

There is an increasing demand for quality and uniformity in developing dessert banana TC products for international trade (Altman and Loberant 2000). Many commercial banana-producing countries use TC plants in an annual or biennial crop cycle to improve yield and to reduce disease pressure. Greenhouse production using TC plants in Canary Islands (Spain) resulted in an increase in yield from 47.3 t/ha/year under conventional production systems to 83.7 t/ha/year.

Large-scale commercial production has developed particularly in Taiwan, France, South Africa, Israel, etc. In vitro plantlets are sent to their destination where they are acclimatized, placed in shade nurseries and planted in prepared fields. By the 1990s, companies were providing millions of plantlets annually for commercial plantations in South Africa, in the continent of Africa and in Southeast Asia. In S. America alone, nearly 100 million micropropagated banana plants were planted in a decade since the 1980s. Israel produces micropropagated banana for domestic consumption and export. With a highly developed micropropagation industry, coupled with an academic infrastructure and experienced nursery/field agronomists, Israeli companies have been in the forefront of providing disease-free, selected clones of in vitro dessert banana plantlets to the international market.

21.3.7 Government Initiatives in India

Like in any other trade, quality assurance and suppliers' guarantee are sought by the buyer of micropropagated plants. One of the chief constraints in TC product exports is lack of the concept of quality. To begin with, selection of

'mother stock' requires great care. Genetic instability should be accorded high importance while selecting a particular genotype. Thus, quality control of the raw material becomes paramount.

The Department of Biotechnology (DBT), Ministry of Science and Technology (GOI), New Delhi, India, set up a Consortium on Micropropagation Research and Technology Development. To prevent risks, it suggests effective testing (indexing) procedures *prior to* bulking up of cultures and adoption of standard guidelines as follows:

- Careful selection of mother plants
- Ensure establishment of virus-free culture through indexing of 100 % plants
- Proper package and practices to be followed such as limiting the number of subculture cycles (multiplication cycles), grading cultures as well as plantlets, insect/pest monitoring in the hardening area, etc.

Based on the needs of the TC industry, DBT has set up national facilities for virus diagnosis and quality control of TC plants at many institutions in the country. All these measures have contributed immensely to the TC industry. The crop-wise share of micropropagated plants in India pegs the sales of TC banana at 45 % of the total volume (i.e. quantity in terms of numbers), with crop-wise estimated sales value of TC banana in the domestic market at 52 %. A number of progressive farmers and nurserymen in Andhra Pradesh, Maharashtra, West Bengal, Karnataka, Tamil Nadu, etc. are major consumers (customers) of TC plants. Banana is a priority TC crop for several state agriculture departments (SADs) and for Kerala AEZ (agri-economic zone).

In India, several plant tissue culture laboratories and commercial facilities have been set up recently, and they have been generating a large number of tissue culture-raised plants of commercial crops. However, the country lacks organized testing of the quality of regenerants besides their freedom from viruses. The most deleterious variants in tissue culture-raised plants are those that affect yield and quality and carry infection of viruses difficult to diagnose. To bridge the gap, the Department of Biotechnology, Govt. of India, has established recognized test centeres for testing tissue culture-raised plants for quality and freedom from viruses.

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In Vitro Conservation of Plant Germplasm

22

P.E. Rajasekharan and Leela Sahijram

Abstract

Biotechnology has brought about a revolution in the way that plant genetic resources can be utilized. Clonal crops cover a wide range of species from the root and tuber crops, such as potato, cassava, yam, taro and sweet potato, to fruits, such as apple, pear, citrus, banana and the cooking banana (plantain). Other miscellaneous crops including vanilla, ginger, turmeric, hops and sugarcane are also clonally propagated. In some of these cases, seed production is impossible due to sterility. In others, it is undesirable to produce seeds for conservation as this would break up highly heterozygous clonal genotypes. The foundation technologies that make up an in vitro conservation system are collection, disease eradication and indexing, culture initiation, multiplication, storage and distribution. There are two basic options for in vitro storage, slow growth for the short to medium term and cryopreservation for the long term. (Since the definition of storage time-span and the concepts of active or base storage derive strictly from motivation rather than methodology, there is no reason why, in fact, cryopreservation should not have applications in short- to medium-term conservation.) Intensive conservation efforts are needed for clonally propagated crops, constituting about 1,000 species, and for difficult-to-store seeds, constituting about 88,250 species throughout the world. In vitro approaches, including tissue culture maintenance and cryopreservation,

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are recognized as useful tools for medium- to long-term conservation of these groups of species. The in vitro techniques for conserving plant biodiversity include shoot apical or *axillary-meristem-based micropropagation*, somatic embryogenesis, cell culture technologies and embryo rescue techniques, and a range of in vitro cold storage will be discussed in this chapter.

Keywords
In vitro conservation • Clonal • Slow growth • Tissue culture

22.1 Introduction

Seed storage is a preferred method of conservation, but it is not feasible for germplasm from crops that are either clonally propagated or that do not produce seeds. For some genotypes, elite genetic combinations are only preserved through clonal means as their conservation is dictated by breeding strategy; this is because heterozygosity does not permit the maintenance of desired characteristics. Clonally propagated plants thus require special conservation approaches. Options include maintenance in field gene banks and the conservation in cold stores of dormant vegetative propagules (Reed 2001); however, these methods have limitations regarding efficiency, costs, security and long-term maintenance. In vitro conservation is preferentially applied to clonal crop germplasm as it also supports safe germplasm transfers under regulated phytosanitary control (IBPGR 1988).

Conservation in IVGBs combines tissue culture and cryopreservation for medium-term (MTS) and long-term (LTS) storage, respectively. For MTS, subculture intervals are extended, reducing processing costs by arresting growth using reduced temperature treatments and/or growth retardants. For LTS, germplasm (usually shoot tip meristems) from in vitro propagated plants is cryobanked for long-term storage in liquid nitrogen (LN) to a minimal temperature of −196 °C in the liquid phase.

22.1.1 Need for In Vitro Conservation

There are a number of crops which are normally propagated vegetatively, such as potato, sweet potato, yams, cassava, several fruit tree species and many others. In this category, the clonal material carries variable gene combinations which have been maintained by the avoidance of sexual reproduction. When these clones are maintained in field gene banks, the traditional procedures tend to be expensive due to (1) high labour costs, (2) vulnerability to environmental hazards and (3) requirement for large amount of space. An even more serious problem is the vulnerability of such clones to pests and pathogens or natural disasters to which they are almost continuously exposed. This can lead to sudden loss of valuable germplasm or accumulation of systemic pathogens, especially viruses. In such cases, in vitro conservation is complementary to field gene banks, seed gene banks and pollen/DNA preservation which along with in situ conservation measures provide an integrated conservation strategy. In vitro gene banks, where plant material is stored in nutrient medium under artificial conditions, are being increasingly used as alternatives to conserve vegetatively propagated species and threatened plant species (Fay 1994; Bhat et al. 1995; Sharma and Chandel 1996).

Much of the world's germplasm is currently maintained as breeders' collections in gene banks, plantations, orchards or even in evolution

gardens primarily raised from seeds, such as rubber and coconut, and vegetatively propagated plant species such as citrus, cocoa, banana and many other fruits. These also include clonal collections of important staple food crops, such as cassava, sweet potato and yams, and aroids such as *Colocasia* and *Xanthosoma*. These field gene banks do not represent the entire range of genetic variability within the respective crop gene pool, and most of them represent only a fraction of the variability which should be conserved (Withers and Williams 1985). The IBPGR programme initiated a move to include fruits, vegetables and forages. Any strategy for collection and conservation of samples of crops that are normally propagated vegetatively or that produce seeds which cannot be stored using normal procedure of storage may require alternative methods. This led to the consideration of in vitro techniques and cryopreservation of seeds for germplasm conservation (Withers and Alderson 1986). Problems of in vitro storage of such material, when solved, should also relate to cycling of the material through multiplication schemes, distribution of germplasm and also its characterization and evaluation. Hence, the development of the full potential of in vitro culture storage and associated biochemical techniques could revolutionize the handling of germplasm.

A range of in vitro techniques have been developed in the last few decades. The organized culture systems have a high degree of genetic stability and are more likely to be of importance for germplasm storage, especially the 'shoot tips' or meristem cultures. In vitro techniques are employed to eliminate diseases and pests. However, some viroids and viruses particularly are not necessarily eliminated or even detected and can readily multiply in tissue culture. These can be eliminated by meristem or shoot tip cultures possibly in combination with both heat and cold therapy. The International Potato Research Center (CIP) maintains the pathogen-tested potato germplasm in the form of in vitro plantlets or tuberlets. Potato germplasm is being preserved at CIP through in vitro techniques using meristem and shoot tip culture for international exchange of germplasm.

With in vitro techniques, it is now possible to provide a germplasm storage procedure which uniquely combines the possibilities of disease elimination and rapid clonal propagation (Henshaw and Grout 1977). Further, the virus-tested cultures could provide ideal material for international exchange and distribution of germplasm as they will be acceptable to plant quarantine authorities (Paroda et al. 1987) and comply with international quarantine regulations.

In vitro conservation strategy offers an appropriate alternative and would be discussed in detail in the present chapter. With in vitro techniques, it is now possible to provide a germplasm storage procedure which uniquely combines the possibilities of disease elimination and rapid clonal propagation (Henshaw and Grout 1977). However, field gene banks have the potential risk of germplasm being lost due to disease, stress or disaster and are labour intensive. Cryogenic preservation of seeds or vegetative material is another potential mode of ex situ conservation which is still at experimental stages.

Various aspects of in vitro conservation and cryopreservation were reviewed by Normah et al. (1996), Ashmore (1997), Engelmann and Takagi (2000), Reed et al. (2004), Sarasan et al. (2006) and Krishnan et al. (2011). Research requirements identified by Reed et al. (2004) were:

1. *Germplasm health*: virus surveys, indexing techniques, development of effective virus testing in vitro and whether viruses can be transmitted in vitro and development of indexing techniques for latent endogenous bacteria
2. *Slow growth*: research into the effects of plant growth regulators and growth retardants, light and light-temperature interactions, propagule type, size, growth stage (microtubers, bulbs, rooted plantlets, unrooted shoots), statistical

rigour in experimental design and minimizing the use of growth retardants
3. *Cryopreservation*: widening its applicability to more crops and genotypes, methods developed for several localities, and use of cryotherapy
4. *Genetic stability*: selection pressure of in vitro maintenance, genetic variation in field compared to in vitro, field evaluations on material with known instabilities and development of markers to monitor genetic stability

22.2 In Vitro Conservation: An Overview

In vitro storage of germplasm first reported two decades ago (Henshaw 1975) offers promise for conservation of threatened species of known and/or potential medicinal and aromatic value and for species clonally propagated. The material for such species could be available once the true potential of the species is realized. The fundamental objectives of in vitro conservation technology are the maintenance and exchange of germplasm in disease-free and genetically stable state through tissue culture. The essential prerequisites for an in vitro conservation programme are:

1. Creation of special facilities including tissue culture facility, green-/glasshouse facilities, storage facility, computer facility and facility for monitoring genetic stability
2. Presence of trained scientists and technicians
3. Linkage with farmers' fields

Information on the in vitro multiplication and/or conservation of the plant species is also desirable. Many laboratories and institutes in India have been engaged mainly in developing protocol for micropropagation of various threatened endemic species. The Department of Biotechnology established India's first national facility of plant tissue culture repository in 1986 at National Bureau of Plant Genetic Resources (NBPGR), New Delhi, which has made concerted efforts towards developing in vitro technology for conservation of several vegetatively propagated agri-horticultural and several threatened/rare species, especially of medicinal and aromatic value. The number of species being worked upon has increased appreciably with the initiation of DBT funded G-15 project operative at three centres, namely, NBPGR, Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, and Tropical Botanic Garden and Research Institute (TBGRI), Thiruvananthapuram.

Any in vitro conservation programme mainly comprises of two stages:

1. In vitro multiplication to build up a large number of plants
2. In vitro storage/preservation

The cultures may be conserved for either short, medium or long term, depending on the requirement as well as the technique applied and infrastructure availability. For short-term maintenance of cultures, regular subculture (4–8 weeks' interval) may suffice. To conserve cultures for a longer period of time, two strategies normally adopted include slow growth and cryopreservation. The use of artificial seeds in combination with the above two is a more recent approach in the conservation programmes.

22.3 Type of Culture Systems

In vitro techniques rely on the concept of 'totipotency' of plant cells. Cultures could be initiated from two types of explants: first, explants that retain developmental integrity such as meristem shoot tips and axillary buds and, second, explants that differentiates to a more or less organized state such as somatic embryos and adventitious buds through a disorganized callus phase. One of the important requirements of in vitro conservation is to get high-frequency regeneration of plantlets from organized explants such as meristem/shoot tips, embryos, embryonic axes and plantlets as they offer the lowest frequency of genetic variation during conservation (Karp 1989). In contrast, callus, cell suspension and protoplast culture are preferred systems only when endowed with special attributes or required for biotechnological applications.

Germplasm of threatened plants is collected from difficult areas and may be available in the form of either seeds or cuttings or vegetative propagules such as bulb, corm or tubers in limited number. In such cases, the 'less preferred' culture system may be the only choice for conservation.

22.4 In Vitro Propagation

Development of efficient plant regeneration protocols for clonally propagated species and threatened plants of medicinal and aromatic value is a recent phenomenon. The methods for micropropagation include stimulation of axillary bud, proliferation from shoot tip and nodal explants, induction of somatic embryogenesis using explants from juvenile or mature plants depending on the availability of material and inducing adventitious bud directly from explants or through intervening callus. Protocol for rapid multiplication involves five stages:

1. Section and preparation of stock plants
2. Establishment of aseptic cultures
3. Multiplication of propagules
4. Preparation for re-establishment in soil
5. Transfer to greenhouse and acclimatization

Since multiplication is carried out under artificial conditions on a nutrient medium, plants can be produced round the year if photoperiod and temperature are properly maintained. As plants are produced under aseptic conditions, they are free from pest and pathogen. Even virus can be eliminated by meristem culture technique, that is, regeneration of plants from 0.1 to 0.2 mm shoot meristem.

Various techniques have been used for micropropagation of plants. From conservation, the most useful is the propagation from existing meristem as by this method plants with desired traits are obtained. Micropropagation protocols have been developed in an increasingly large number of species. However, certain species are recalcitrant to tissue culture (*Coptis*), and this is a major obstacle in using tissue culture for germplasm conservation. The rate of shoot multiplication varies from 3.5-fold per 3 weeks in *Saussurea lappa* (Arora and Bhojwani 1989; Bhojwani et al. 1989) to as high as 150 shoots every 4 months in *Coleus* (Sen and Sharma 1991). High multiplication rate has major advantages for raising plans for nurseries and commercial plantings; however, for conservation programmes, very high multiplication rate is not desirable. The mode of regeneration has been either direct or through callus, in the form of shoots or somatic embryos. It is evident that in most of the cases, the propagation is through axillary branching.

It is worthwhile to mention here that tissue culture methods also provide potential means of multiplying threatened species and clonally propagated with possible reintroduction into their original habitats for throated species. However, it is too early to predict the survival and fate of reintroduced tissue culture-raised endangered plants in their natural/native habitats. Sustainable utilization of this germplasm depends on the development of appropriate in vitro conservation procedure to ensure its availability for future utilization.

22.5 Conservation of Tissues

Six major steps defined in the conservation use cycle are collection, quarantine, propagation, characterization, evaluation, monitoring, storage and distribution. The role of in vitro conservation techniques in the overall conservation strategies should be indicative of the fact that it should complement other conservation strategies within the total programme of a given species or population. The methods chosen should be carefully considered taking into account the feasibility, practicality, economy and security.

Generally, field conservation of plants requires more space and is labour intensive and expensive. They also run the risk of being damaged by natural calamities and biotic stress factors. Techniques to conserve such species in vitro have recently been developed. For some species, while in situ conservation is the only option available, tissue culture systems offer advantages, which are listed below:

1. Very high multiplication rates
2. Aseptic system

   - Free from fungi, bacteria, viruses and insect pests
   - Production of pathogen-free stocks
3. Reduction of space requirements
4. Genetic erosion reduced to zero under optimal storage conditions
5. Reduction of the expenses in labour costs

In vitro collections of species could be maintained at the same or separate site, but should have clear linkages with field gene banks. The properties required for a successful in vitro conservation system as defined by Grout are:

The ability of the biological system to:

1. Minimize growth and development in vitro
2. Maintain viability of stored material at the highest possible level along with minimum risk of genetic stability
3. Maintain full developmental and functional potential of the stored material when it is returned to physiological temperatures
4. Make significant savings in labour input, materials and commitment of specialized facilities

Some of the advantages favouring this conservation strategy are:

1. Collection may occur at anytime, independent of flowering periods for each species.
2. There is potential for virus elimination from contaminated tissue through meristem culture.
3. Clonal material may be produced.
4. Rapid multiplication.
5. Germination of 'difficult' immature seed/ embryo rescue may be facilitated for breeding.
6. Distribution across borders may be safer.

Issues of concern that could limit potential application of in vitro techniques for conservation of plants include:

1. The whole programme can be initially expensive, but with low recurring cost. Technology inputs as adopted by the developed nations will have to play a major role for successful implementation of this conservation strategy.
2. In vitro storage techniques, particularly cryopreservation procedures, are not yet well optimized for routine application across a wide range of species or genotypes. Although cryopreservation techniques are currently being tested and protocols optimized for gene pool components of several plant species, the rate of success is limited to only a few, where the conditions need to be carefully monitored to ensure viability, minimize genetic damage and prevent contamination by diseases and pests.
3. Somaclonal variation can be a major limitation among tissue culture regenerated plants; some of the methodological basis for variation is explant source, age of culture, hormone used, genotype, ploidy status, etc.
4. The problems of genetic stability manifested by loss of cellular integrity among most tissue culture systems pose a major obstacle in using this technique as a conservation strategy. Variation can be observed at different levels, such as morphological, karyotypic or biochemical. The use of axillary or apical meristem for micropropagation reduces the probability of genetic variation among plant tissue culture systems. The genetic stability at all stages of an in vitro conservation programme should be monitored. No well-defined techniques are available for conservation of endangered medicinal plants. Therefore, special attention is required in this regard, since it is a question of species extinction, and it is essential to retain the quality and quantities of secondary metabolites contained in the species
5. There is a need to establish the basic tissue culture competence of the plant species in question; difficulties can be encountered during culture initiation, micropropagation, rooting and establishment of plants *extra vitrum*. All these stages for any given medicinal plant species must be optimized. Some species show recalcitrance in culture system for which may require special attention.

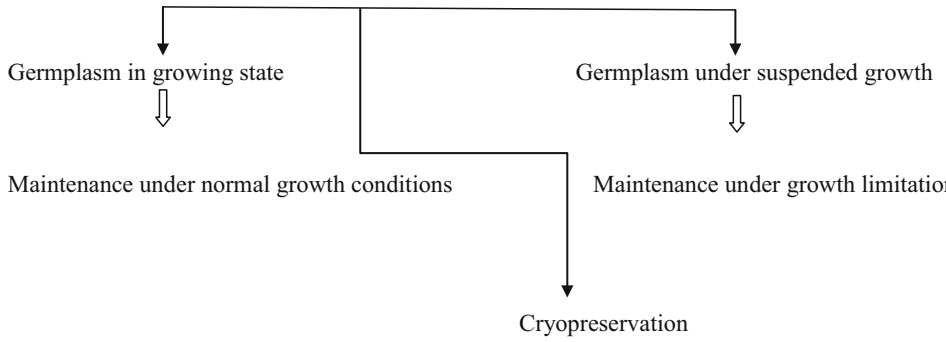
22.6 In Vitro Collection

In vitro collection involves initial disinfestations and placement of plant explants in sterile culture medium, before transport to a tissue

culture laboratory for further in vitro procedures. In vitro collection is particularly useful for species that are vegetatively propagated and for those with recalcitrant seeds or embryos, which deteriorate rapidly. The technique has much potential to facilitate the collection of germplasm of tropical and subtropical fruit species, as has already been demonstrated with cassava and coconut. Recently, 300 *Musa* accessions were collected in Papua New Guinea using this technique; before being transported to a collection in Australia, an added advantage of this exercise is that it complied with quarantine regulations that are in place to stop the spread of *Fusarium* and other diseases.

22.7 In Vitro Storage

As with short-term storage, there have been very few attempts to apply cryopreservation techniques to tropical and subtropical fruit species, with the exception of *Musa* spp. (Panis 1995) and *Citrus* spp. (Pérez-Molphe-Balch and Ochoa-Alejo 1997). Withers (1992), in a review article, reported successful cryopreservation of the recalcitrant tropical species, *Theobroma cacao* (cocoa), *Artocarpus heterophyllus* (jackfruit), *Cocos nucifera* (coconut) and *Nephelium lappaceum* (rambutan), but provided no details. Very low survival rates have been reported when excised embryos from seeds of jackfruit, rambutan and coconut were cryopreserved (Chin 1988). No survival was achieved when excised embryos from partially dehydrated seeds of rambutan, durian and cempedak (*Artocarpus integer*) were cryopreserved (Hor et al. 1990). However, before cryopreservation can be universally applied to woody perennial fruit species, there is still much research, development and field testing that need to be done. For example, the issue of genetic stability is rarely mentioned. Because growth is suspended, the potential to store material for long periods without genetic variation is assumed. However, any system based on cell suspension or callus (including embryogenesis) is prone to somaclonal variation and should be field-tested before being accepted unreservedly. Field testing of tropical and subtropical fruits should be continued through to the fruiting stage, as fruit production is the primary reason for their collection and use. Unfortunately, this requires long-term projects for many species. Nevertheless, a research effort into cryopreservation of tropical and subtropical fruit species should be encouraged because of its potential for long-term preservation of germplasm. At the current rate of development, it is reasonable to assume that routine protocols for cryopreservation and subsequent regeneration of explants will eventually become available for most plant species. However, protocols must be repeatable and result in high percentages of preserved tissue being viable after thawing, before they can be used routinely for storage of germplasm.



Technical approaches to *in vitro* storage

22.8 In Vitro Conservation: Strategies

Once cultures have been established and multiplied in sufficient number, an effective method for conservation is required. Conservation can partly be achieved by regular subculture on fresh media. However, it may not be practical due to the danger of microbial contamination and equipment failure and may be uneconomical in terms of labour, physical resources and time requirement. Additionally a few systems may have constraints such as loss of morphogenic capacity and occurrence of somaclonal variation.

The main aim of in vitro conservation programmes is to reduce frequent demand for subculture, which can be accomplished in two ways: by maintaining cultures under normal growth (SCC) or by subjecting them to growth limiting strategies (for detailed reviews see Grout 1995). The latter includes slow growth and suspended growth (cryopreservation). Normal growing cultures along with those in slow growth comprise the active collections whereas those cryopreserved constitute the in vitro base collection.

The expectations are high about tissue culture methods providing sound strategy for both clonal propagation and medium-term storage. Literature survey revealed that till date there is very limited documented information on in vitro conservation.

22.8.1 Normal Growth

It is possible to maintain cultures virtually indefinitely under normal growth conditions provided nutrients are supplied and accidents avoided. This method is preferred for inherently slow-growing, stable systems and for cultures for which there is no other method though it is laborious and abounds with risks of genetic alterations with time, contamination or loss through human errors; in specific cases such as tropical germplasm, it can be useful because of the following advantages:

1. It minimizes requirement of low temperature facility (particularly for developing countries' saves inputs).
2. It promises avoidance of stress-induced variability.
3. It helps in getting cultures for instant multiplication and exchange.

22.8.2 Slow Growth

The aim of this method is to reduce requirement for subculture without causing any damage to the tissue. It is the most direct way of restricting growth and development of in vitro materials and is usually applied to differentiated plantlets or shoot cultures. Slow growth involves one or a combination of the following techniques:

1. Type of enclosure
2. Temperature and/or light reduction
3. Use of minimal media and osmotic
4. Use of growth retardants
5. Other approaches:
   (i) Reduction of oxygen pressure
   (ii) Mineral oil overlay
   (iii) Encapsulation
   (iv) Desiccation

22.8.2.1 Type of Enclosure

Type of enclosure seems to have direct influence on subculture requirement of growing cultures. One of the simplest and cost-effective approaches for slowing growth rate of cultures has been replacement of the commonly used cotton plugs with polypropylene caps as culture tube enclosures (Balachandran et al. 1990; Sharma and Chandel 1992). The increase in storage time is attributed to the reduction in evaporation of water from the medium in culture tubes.

Shoot cultures of *Coleus forskohlii*, *Rauvolfia serpentina* and *Tylophora indica* have been conserved for 12–20 months at 25 °C without requiring any intermittent subculture (Chandel and Sharma 1992; Sharma and Chandel 1992; Sharma and Chandel 1996). In *Allium tuberosum* and *Dioscorea* too, the shelf life of shoot cultures was extended for up to 9 months at 25 °C. Encouraging results have been obtained in other species also in our laboratory. This technique seems to work well with species belonging to subtropical or tropical region probably due to their inherent property of growing at higher temperature. In most of the

species of temperate region, optimum subculture period could be extended only to 5 months. The added advantage of this approach is that mostly the cultures can be visibly assessed for viability and can readily be brought back to fresh culture medium to produce plants on demand.

Excised roots to *Rauvolfia serpentina* could be conserved for 16 years, and these retained the regenerative capacity to produce plants (Chaturvedi et al. 1991). This approach needs to be tested for other important threatened species.

22.8.2.2 Reduction in Temperature and/or Light

The most commonly used method for reducing growth of tissues in cultures is by decreasing the temperature in which cultures are maintained. This may also be accompanied with reduction of light intensity. The basic principle in this method is that incubation at temperature lower than that required for optimum growth would reduce or decrease the metabolic activities, thereby restricting the growth of the plants. However, care must be taken to avoid temperature below freezing or where chilling injury could occur (Lyons et al. 1979). In most cases, the storage temperature is species specific. Usually, the most suitable storage temperature for temperate species is between 1 and 10 °C, whereas tropical species are stored in the range of 10–22 °C. The light intensity can be reduced by 60 % from standard requirement, i.e. 1,000 1× and 16th photoperiod. This method is very simple and easy to use and may be applicable to a wide range of genotypes. However, uninterrupted maintenance of reduced temperature, required for the purpose, may be difficult and uneconomical particularly for tropical and developing countries.

Low temperature incubation of in vitro cultures appears highly promising. This may also be accompanied by reduction in light intensity. Cultures are kept at ambient culture conditions for 1 week and subjected to screening for detection and elimination of contamination prior to low temperature incubation. Cultures exhibit reduction in growth when transferred to low temperature.

Shoot culture/nodal cultures of *Rauvolfia serpentina* have been successfully conserved for over 15 months at 15 °C, while 10 °C and 5 °C were found deleterious to growth of cultures (Sharma and Chandel 1992). These in vitro conserved cultures showed shoot multiplication comparable to the cultures maintained at 25 °C. The plantlets obtained from these conserved cultures could be established in soil and no morphological differences were observed in these plants.

Tylophora indica have been successfully conserved from more than 12 months tested so far at 10 °C (Sharma and Chandel 1996). These in vitro conserved cultures exhibited normal growth and multiplication on rejuvenation.

Reduction of light along with temperature has extended shelf life of cultures in *Saussurea lappa*. According to Arora and Bhojwani (1989), shoot cultures of *Saussurea* could be successfully stored at 4 °C in the dark for 12 months, with 100 % viability. Shoot tip cultures of the same species have been conserved for more than 15 months at 4 °C in the dark (Sharma et al. 1995). Further experiments have indicated that cultures can be maintained for 22 months at 10 °C under reduced light. However, the result has to be repeated for confirmation. An interesting observation has been that some of the cultures that were subjected to reduced temperature/light regime exhibited a better rate of multiplication following storage as compared to cultures maintained at normal temperatures and light.

In *Picrorhiza kurroa*, shoot cultures have been successfully conserved for 6 months at 25 °C and over 9 months at 10 °C. Shoot cultures of this species are reported to have been stored for 10 months at 5 °C in the dark, with 70 % survival. Cold-stored shoots, on transfer to 25 °C, multiplied at rates comparable to cultures maintained under normal conditions (Upadhyay 1989).

Embryogenic calli of *Podophyllum hexandrum* cold stored for 7 months at 5 °C exhibited 95 % viability and differentiated globular embryos (Bhojwani et al. 1989). However, similar studies carried out in our laboratories indicated that cold-stored calli failed to regenerate beyond the globular stage.

Gentiana kurroo, another threatened medicinally important species, has been conserved for 11 months at 4 °C (Sharma et al. 1993; Sharma and Chandel 1996). In *Allium tuberosum*, shoot

cultures stored at 10 °C survived for 18 months without subculture.

22.8.2.3 Minimal Media/Osmotica

Inclusion of osmotica with or without low temperature incubation has also proved to be an important technique to prolong subculture period. Change of carbon source may have a very remarkable effect on growth rate. Use of either increased or decreased doses of sucrose yielded interesting results in some species. Inclusion of non-metabolizable, inert sugar alcohol, particularly mannitol and sorbitol, in the range of 3–6 % (w/v) has been quite effective in restricting the growth of many plant species. The major limitation of this method is that different genotypes may react differently under these conditions. However, when used in combination with reduced temperature incubation, it becomes the most realistic and cost-effective method because the combinations of these two treatments show synergistic effects on in vitro conservation of many species. Inclusion of osmotica such as sucrose and mannitol, with or without low temperature incubation, has also proved to be an important technique to prolong subculture period. There is no documented information regarding its use in case of threatened plant species. Effect of addition of mannitol in the medium to cultures of *Rauvolfia*, *Picrorhiza*, *Gentiana* and *Saussurea* has been studied in extending shelf life. Addition of mannitol led to maintenance of these species for more than 9–12 months at 25 °C. Reduction of temperature further resulted in prolongation of shelf life of the same cultures up to 16 months. Experiments are underway to study the effect of mannitol and other osmotica, in other species too. Incorporation of high concentration of sucrose has also been beneficial in extending the storage time of cultures of *Allium tuberosum*. In *Rauvolfia serpentina*, half-strength medium and full-strength medium without hormones were used to test their effect on survival of cultures at 25 °C (Sharma and Chandel 1992). None of these were effective in improving the shelf life of cultures in comparison to low temperature storage, with survival rates of only 33–50 % after 6 months of storage. An important observation made in author's lab is that rooted cultures survive longer probably due to roots being capable of absorbing water and nutrients from the medium more efficiently than shoot cultures especially at later stages.

22.8.2.4 Growth Retardants

The basic principle of using these compounds is simply to reduce the overall growth rate of the in vitro plantlets and thereby enhance the subculture interval. The choice of inhibitory growth regulator necessarily depends upon the circumstances and species. Abscisic acid, N-dimethylaminosuccinamic acid, maleic hydrazide (MH), trans-cinnamic acid (TCA), chlorocholine chloride (phosphon-D), daminozide (B 995) and Cycocel (CCC) at a concentration range 2–50 mg/l have been reported to extend subculture period for 6–12 months.

This method is simple, efficient and cheaper. Using these chemicals, cultures can be stored at normal culture room, thereby eliminating the requirement of low temperature facility. However, its use may pose serious problems in the germplasm conservation:

1. The plant may become stunted and show abnormal growth, thereby posing problems in regenerating normal plants.
2. The presence of these retardants may lead to selection of lines with resistance or tolerance to growth retardants.
3. Chances of induction of genetic alterations increase because some of the growth retardants have mutagenic properties.

22.8.2.5 Other Approaches

Growth rates of in vitro culture are influenced by composition and volume of atmosphere inside the culture vessel (Gould and Murashige 1985). Mineral oil overlay and reduction of oxygen pressure in the culture vessel may extend shelf life cultures from a few weeks to months. The main problem with the mineral oil overlay is the recovery growth of shoot tips. The limitation of modification of gaseous environment is the diffi-

culty of maintaining gas atmosphere of individual culture vessel. However, the use of gas permeable culture vessel such as star packs (Reed and Abdelnour 1991) offers the possibility of controlling the gas atmosphere of the entire incubation chamber which may be more practical. This technique therefore needs further investigation. Storage of encapsulated buds and somatic embryos at room temperature for 8 months when water content was reduced down to 15 %. Though the result of these methods is encouraging and may be useful in specific cases, the methods need further standardization for their acceptability and wider use. Thus, these may be viewed as experimental techniques with future application. It is worthwhile to mention here that the technique of applying slow growth to reduce subculture frequency is not without drawbacks, mainly of somaclonal variation. Moreover, reduction of temperature has to be used with caution for tropical species which require higher temperatures for growth. Besides cost-effectiveness of low temperature storage, maintenance of constant low temperature for desired long periods may pose a practical constraint, especially in subtropical/tropical regions.

22.9 Principles of Medium-Term Storage

Standard culture conditions can only be used for medium-term storage of slow-growing species. However, in most cases, environmental conditions and culture medium have to be modified to induce growth reduction. This is most frequently achieved by temperature reduction, often in association with a decrease in light intensity or even its complete suppression. Temperatures in the range of 0–5 °C are employed with cold-tolerant species, but higher temperatures are required in the case of tropical species, which are generally cold sensitive. It is also possible to limit growth by modifying the culture medium, mainly by reducing the sugar and/or mineral element concentration. Finally, the type of culture vessel, its volume as well as the type of enclosure influence survival of cultures. Notably, it is important to limit the evaporation of the culture medium by using a type of enclosure, which is tight enough.

Medium-term conservation techniques have been developed for a wide range of plant species, but they are used routinely for the conservation of only a limited number of species such as *Musa*, potato or cassava (Engelmann 1997).

New medium-term conservation techniques include reduction of the oxygen level available to cultures (achieved by covering explants with a layer of liquid medium or mineral oil or by placing them in controlled atmosphere), desiccation and encapsulation of explants in alginate beads (Engelmann 1997). However, these techniques are still at the experimental stage.

The objective of slow growth (or minimal growth) is to reduce subculture intervals to a critical level that does not impose a long-term deleterious effect on germplasm or put at risk the stability of regenerated/regrown plants. However, slow growth treatments incur some level of stress, and it is essential to optimize MTS with respect to the timing of subculture regimes and regeneration. When this is achieved, slow growth is a successful method of securing plant germplasm in MTS (Cha-um and Kirdmanne 2007). Minimal growth storage is useful for genotypes that cannot be cryopreserved, and it is a key part of in vitro gene banks that clonally propagate crops for distribution services. Several MTS treatments are applied, either singularly or in combination to retard growth:

- *Physical growth limitation:*
  - Low temperature
  - Low light/restricted photoperiod
  - Minimal containment
  - Minimal O_2
  - Osmotic (water) stress
- *Chemical growth limitation:*
  - Growth regulator retardation
  - Growth inhibitors
- *Nutrient limitation:*
  - Low macronutrient levels
  - Low micronutrients levels

Ashmore (1997) listed key issues and actions for future consideration.

22.10 In Vitro Conservation: Practical Considerations

The real utilization of any conservation programme lies in carefully selecting the species, designing work plan and sincerely executing it. The first and foremost and essential step, once the infrastructure is available, is prioritization of species. In deciding the priority, the main points to be considered include threat of genetic erosion, importance of the species and potential demand for distribution/exchange for commercial utilization, besides size of collection and cost of field maintenance. It is equally important to select a strategy after assessing its economy and safety.

An ideal strategy should meet the following requirements:

1. Wide and easy applicability of protocols and techniques over a range of species
2. Economy (establishment and running cost) in operation/management
3. Ensure high viability after storage
4. Low risk of genetic alteration
5. Technically less demanding techniques

The material needs to be well documented and conserved under safe condition and duplicated in at least one other location/country to minimize accidental loss and to ensure its availability. Before embarking on in vitro conservation, the potential approach should be balanced against other conservation strategies. Experience with in vitro maintenance is limited; thus, time and cost compliment other conservation strategies for the same crop species (seed, field gene banks, etc.) including in situ conservation. Issues of concern in potential application of in vitro techniques for germplasm conservation of medicinal and aromatic plants include:

1. Genetic stability
2. Basic tissue culture competence

22.11 Genetic Stability

Maintenance of specific gene combination (genotypes) is vital for any germplasm conservation programme. The phenomenon of somaclonal variation associated with certain culture systems is perceived as major obstacle in using plant tissue culture for germplasm conservation. The instability has been documented to occur at the morphological, karyotypic and biochemical levels. The variations primarily result from the change in ploidy level, structural changes in chromosome morphology and mitotic aberrations. It has been reported that these changes may occur during culture, though genetic variation occurs spontaneously in nature also. Long preservation in culture media composition can also give rise to variations. Somaclonal variation can be enhanced, particularly where regeneration is adventitious. It has also been observed that certain in vitro storage conditions can increase the risk of integrity because of directional change in response to selection. The use of axillary bud for multiplication reduces the probability of genetic variation. Therefore, formation of callus and adventitious shoots should be avoided (Roca et al. 1989).

Monitoring of genetic stability is an important aspect of in vitro conservation. Technique of monitoring of stability will depend on the need, type and nature of plant species and its economic value. The characterization may be for morphological, cytological, histological and/or biochemical traits. Some of the techniques like field testing for morphological traits cannot be avoided. Other techniques have to be chosen judiciously depending on the crop, the economic product, the life cycle of the plant and the resources available. Use of sophisticated high-technology monitoring (molecular markers/DNA polymorphism) has value when there is sufficient basis to assume instability, either intrinsic or due to the culture system and storage conditions. In the case of medicinal and aromatic plants, however, it is more relevant to monitor the potentially useful metabolites of plant species.

Greater understanding of the causes and nature of somaclonal variation (SCV) is needed, particularly after prolonged storage in culture and cryopreservation and in establishing safe storage procedures, developing improved markers and methods for characterizing SCV and monitoring genetic instability. Encouragement of

research aimed at understanding causal factors of SCV and comparative assessments of genetic stability in germplasm conserved in the field and IVGBs is recommended.

22.11.1 Reproducible and Wider Use of Slow-Growth and Cryopreservation Methods

Improved, reproducible and robust protocols required for slow growth and cryopreservation optimized across genotypes held in gene banks, greater provision for species that have received limited attention, application of in vitro methods for safe movement of germplasm and prioritizing problem species.

Developing methods using simple facilities, with general application, optimized, and tested in IVGBs; more information on in vitro distribution and transportation of in vitro material.

22.11.2 Management Issues

Database of in vitro collections and their activities and operations are required including routine methods and guidelines, particularly addressing what are acceptable ranges for survival and amount of material to be stored per accession for in vitro storage techniques.

22.12 Performance Indicators for Slow Growth

Performance indicators include (1) plant health, (2) extension of subculture interval, (3) contamination frequency and (4) capacity [viability, vigour, health status] to recover from stress treatments. Reed et al. (1998b, 2003) use descriptive scales of 0 and 1–5 to rate the performance of in vitro *Pyrus* and *Humulus* cultures maintained under minimal, low temperature growth:

0 = all of the plantlet is brown and no visible indication of growth.
1 = very poor, questionable viability, brown, necrotic shoots, only extreme shoot visibly green, plantlet mostly brown.
2 = poor, much browning, most shoot tips necrotic, shoot tip green, leaves and stems mostly brown, base may be brown.
3 = fair, some browning, some shoot tips necrotic, shoot tips and upper leaves green, etiolation present, base green.
4 = good, elongated shoots, shoot tips generally healthy, green leaves, stem and limited etiolation.
5 = excellent condition, dark green leaves and shoots, no etiolation.

A widely used method for conservation is seed storage. Several categories of crop present problems with regard to seed storage. At present, the most common method to conserve the genetic resources of these problem species is as whole plants in the field (field gene banks – FGBs). However, several serious problems such as labour-intensive field maintenance, shrinking land resources, etc. are associated with field gene banks. The conservation of rare and endangered plant species has also become an issue of concern.

During the last 20 years, in vitro culture techniques have been extensively developed and applied for more than 1,000 different species (George 1996). Tissue culture techniques are of great use for the collection, multiplication and storage of plant germplasm. Storage techniques, which allow to reduce the procedures and to preserve the genetic integrity of the plant material, are urgently needed. Different in vitro conservation methods are employed depending on the storage need (Engelmann 1991). For short- and medium-term storage, the aim is to reduce growth and to increase the interval between subcultures. For long-term storage, cryopreservation in liquid nitrogen is the only available method.

Tissue culture techniques are of great interest for collection, multiplication and storage of plant germplasm (Engelmann 1991). Tissue culture systems allow propagating plant material with high multiplication rates in an aseptic environment. Virus-free plants can be obtained

through meristem culture in combination with thermotherapy, thus ensuring the production of disease-free stocks and simplifying quarantine procedures for the international exchange of germplasm. The miniaturization of explants allows reducing space requirements and consequently labouring costs for the maintenance of germplasm collections.

The methods employed vary according to the storage duration required (Engelmann 1991). For medium-term conservation, the aim is to reduce growth, thus increasing intervals between subcultures. For long-term conservation, cryopreservation, i.e. conservation at ultralow temperature, usually that of liquid nitrogen (−196 °C), is the only method currently available. Indeed, all metabolic processes as well as cell divisions are stopped at this temperature. Plant material can thus be conserved without modification or alteration for extended periods, under a reduced volume and with reduced maintenance.

22.13 Stability: Optimal Storage

In vitro gene bank practices need to ensure their biological resources maintain their special characteristics and are not changed because of storage and associated tissue culture practices. Genetic instability includes the risks of in vitro generated instability termed somaclonal variation (SCV), defined by Scowcroft (1984) as heritable genetic variability in plants generated through tissue culture. Genetic changes can also arise because of epigenetic processes, stress and selection pressure. The consequences of SCV are significant for genetic resource conservation as it is manifest in the regenerated plant; therefore, reducing the potential risk of SCV is necessary. Scowcroft (1984) suggested that in vitro storage protocols should avoid practices that increase the risks of genetic variation occurring. These are (1) avoiding germplasm propagation via dedifferentiated (callus) and adventitious routes, (2) limiting the use of plant growth regulators that increase the possibility of dedifferentiation and adventitious development and (3) selecting germplasm from young cultures as SCV increases and totipotency decreases during prolonged culture.

Genetic instability arising from tissue culture is particularly significant for clonally propagated crops as compared to sexually propagated species in which chromosomal abnormalities are eliminated by gametogenesis and fertilization (Cassels and Curry 2001). Ideally, germplasm with a higher risk of manifesting genetic instability should be monitored at the genetic level, as recessive genetic changes occurring during the tissue culture of asexually propagated species will have no phenotypic expression. In this context (Scowcroft 1984), clonal crops may thus be expected to display a potentially higher frequency of SCV than those propagated by seed; however, as off-types can arise in field-grown, clonally propagated plants, some variation may be unrelated to culture conditions. In vitro conservation can help safeguard against the genetic changes that occur in field-propagated materials that have a predilection to producing off-types.

22.13.1 General Status of In Vitro Conservation

22.13.1.1 Guidelines

Pence et al. (2002) described in vitro collecting techniques for *Musa*, *Citrus*, avocado, taro, coconut, tropical rainforest trees and wild and endangered species. Standards for in vitro culture and slow growth storage are developed by FAO. Reed et al. (2004) developed technical guidelines for management of field and in vitro germplasm collection (Tables 22.1 and 22.2).

Table 22.1 Examples of in vitro conservation in representative species of different categories of crops

Species	Storage regime	Conditions	Storage period	Reference
Forest species				
Castanea sativa (chestnut)	8 °C and 23 °C	Slow growth storage has been achieved for *Castanea sativa* (cv. 'Montemarano') shoot cultures over a duration of 48 months at a temperature of 8 °C, where 82 % of explants survived and were able to resume normal growth after transfer to standard culture conditions at 23 °C	48 months	Capuana and Di Lonardo (2013)
Cedrela odorata L., *Guazuma crinita Mart. and Jacaranda mimosaefolia*	12 °C, 20 °C, 25 °C	Shoot tips excised from in vitro plantlets were encapsulated in calcium alginate beads and stored on different substrates at 12, 20 and 25 °C. Percent viability when encapsulated shoot tips were stored on substrate containing only water solidified with 1 % (wt/vol) agar was 80 % after 12 months at 12 °C for *C. odorata*, 90 % after 12 months at 25 °C for *G. crinita* and 70 % after 6 months at 20 °C for *J. mimosaefolia*	6 and 12 months	Maruyama et al. (1997)
Coffea arabica L. (coffee zygotic embryos)	1 °C	Immature zygotic embryos of *Coffea arabica* L. can be preserved with a little loss of viability in the presence of ABA even at the normal room temperature (25 + 1 °C) up to 2 years without any transfer	2 years	Naidu and Sreenath (1999)
Drosophyllum lusitanicum (L.) Link	25 °C	Double-node cuttings were maintained for 4, 8 and 12 months at 5 or 25 °C in the dark. The cultures could effectively be conserved under minimum growth at 5 °C for 8 months on Murashige and Skoog's medium supplemented with 60 g dm-3 sucrose, 20 g dm-3 mannitol and 0.91 PM zeatin	4, 8 and 12 months	Gonçalves and Romano (2007)
Elettaria cardamomum Maton. (cardamom)	25±2 °C	In vitro conservation by slow growth method was achieved on 1/2 MS (major salts) +5 μM BAP +0.7 % agar (conservation medium); about 70 % of the cultures survived up to 18 months at 25±2 °C. Some 96 % of the plants survived the hardening treatment and grew normally in a greenhouse	18 months	Tyagi et al. (2009)
Eucalyptus grandis	24–28 °C	Slow growth storage, for up to 10 months, has been achieved for *Eucalyptus grandis* shoot cultures by either the addition of 10 mg l^{-1} abscisic acid to the growth medium or by the halving of nutrient supply (half MS) and removal of exogenous plant growth regulators. Reduction of light intensity or addition of mannitol to the media was less effective in reducing growth rate. Isolated in vitro axillary buds encapsulated in calcium alginate and stored under low temperature and low light intensities survived for up to 3 months without loss in viability	10 months	Watt et al. (2000)

(continued)

Table 22.1 (continued)

Species	Storage regime	Conditions	Storage period	Reference
Jacaranda mimosaefolia D. Don.	20 °C or 12 °C	When encapsulated shoot tips were stored on substrate containing only water solidified with 1 % (wt/vol), agar was 70 %	6 months	Watt et al. (2000)
Medusagyne oppositifolia	22±2 °C	Long-term in vitro cultures of *M. oppositifolia* were slow growing and eventually lost the ability to multiply and root. Because of the lack of new seed and vegetative materials, efforts were made to rejuvenate the existing cultures	6 months	Marriott and Sarasan (2010)
Olea europaea L. (olive)		Italian local cultivar showed a notable difficulty for the in vitro establishment due to heavy pathogen contamination. Mercury chloride and sodium hypochloride in the sterilization step and antibiotics in culture media allowed overcoming the problem. Proliferation of shoot apical bud on olive culture medium with 36 g dm^{-3} mannitol and 4.56 μM zeatin appeared very satisfactory	1 month	Zacchini and De Agazio (2004)
Pyrus L. (pear)		Pear (*Pyrus* L.) germplasm may now be stored as seeds (species), dormant buds or pollen from field-grown trees or shoot tips from in vitro grown plants (cultivars). Pear germplasm may now be cryopreserved and stored for long periods (>100 year) utilizing slow freezing or vitrification of in vitro grown shoot tips. Dormant bud freezing, pollen and seed cryopreservation of other lines are being developed to complete the base collection for *Pyrus*	More than 100 years	Reed et al. (1998a)
Quercus suber L. (cork oak shoot)	1–5 °C	Cultures were stored in vitro on multiplication medium at 5± 1 °C without an intervening subculture for 2 years	6, 12 or 24 months	Romano and Martins-Loução (1999)
Ornamental crops				
Ipsea malabarica	2 °C	Half-strength MS medium with 3 % sugar and 1.5 mg l kinetin in jam bottles facilitated storage of shoots up to 14 months without subculture which developed 25 shoots. Medium facilitated storage up to 20 months. Half-strength MS growth regulator and sugar-free (photoautotrophic) medium were best for in vitro conservation, and the storage period on this medium was 27 months	20–27 months	Martin and Pradeep (2003)

(continued)

Table 22.1 (continued)

Species	Storage regime	Conditions	Storage period	Reference
Lilium L. (lily)	−2 °C and 25 °C	In vitro regenerated bulblets of 10 lily (*Lilium* L.) genotypes (Asiatic hybrids, Oriental hybrids, *L. longiflorum* and *L. henryi*) were stored for 28 months at −2 °C and 25 °C on four different media: 1/4 or full-strength Murashige and Skoog nutrients with 9 % (w/v) or 6 % sucrose. Sprout growth, bulb growth and viability were determined. The combination of 1/4 strength MS nutrients and 9 % sucrose gave the highest reduction in sprout and bulb growth, the highest viability and the highest percentage of regrowth after 28 months of storage. At 25 °C, all lily genotypes survived 28 months of storage under these conditions. At −2 °C, Asiatic and Oriental hybrids survived 28 months of storage, whereas genotypes of *L. longiflorum* and *L. henryi* survived 6 months of storage, but died during prolonged storage	28 months	Bonnier and Van Tuyl (1997)
Spiranthes brevilabris (Orchidaceae)	SCC	It maintains a high degree of mycobiont specificity under in vitro symbiotic seed germination conditions	12 months	Stewart and Kane (2007)
Vegetable crops				
Cassava germplasm	24 °C–25 °C	Plant regrowth by encapsulated nodal cuttings and shoot tips was significantly affected by the duration of storage period as shoot recovery decreased from almost 100–73.3 % for encapsulated nodal cuttings and 94.4–60 % for shoot tips after 28 days of storage		Danso and Ford-Lloyd (2003)
Cucumis sativus L. (Haploid cucumber)	25 °C	During the first year of storage, between 30 and 80 %, the clones were lost, due to disturbances in plant development, increased levels of endogenous bacteria and physiological changes resulting in continuous flowering. After 2 years of storage, haploids showed reduced vigour. Therefore, plants were regenerated directly from primordial leaf microexplants	16 h photoperiod for 3–5 weeks	Niemirowicz-Szczytt et al. (2000)
Potato microplants	8 °C	At 8 °C, culture in the presence of either acetylsalicylic acid or 4 % mannitol retarded microplant stem growth and required intervals between subcultures ranged from 8 to over 12 months, depending on the genotype. Prolonged culture on acetylsalicylic acid had no adverse effects on the yield of minitubers on plantlets transferred to glasshouse cultivation. At 18 °C also, intervals between subcultures could be prolonged to 5.5–6 months by culture on either mannitol or acetylsalicylic acid media	8 to over 12 months	Lopez-Delgado et al. (1998)

(continued)

Table 22.1 (continued)

Species	Storage regime	Conditions	Storage period	Reference
Solanum tuberosum (potato)	24/22 °C	Culture growers can successfully use their existing facilities, small refrigerators and coolers with low light intensity, set at 4 °C, for short-term storage of potato, choke cherry and Saskatoon berry cultures	6–12 weeks	Pruski (2001)
Solanum tuberosum L. (potato)	4 °C and 10 °C	'Regrowth' of 93–100 % was recorded for non-stored encapsulated shoot tips, directly transferred on soil in the greenhouse after 2 weeks pre-culture on MS solid medium with an added fungicide (Carbendazim) in the encapsulating gel. The 'regrown' shoot tips produced plants showing normal development	Up to 390 days	Nyende et al. (2003)
Solanum tuberosum L. (potato germplasm)	6±1 °C	Microtubers produced on media devoid of ABA and containing high sucrose concentrations and N6-benzyladenine (44.38 mM) could be stored for 12 months under diffused light at 6–18 °C	12 months	Gopal et al. (2004)
Solanum tuberosum (potato)	SCC	Poor microplant quality at standard Ca [ext] over prolonged storage under minimal growth was due to limiting Ca nutrition, and this could be improved by using Ca [ext]-enriched (5.0–7.0 mM) minimal growth medium for conservation of potato microplants	Not given	Sarkar et al. (2005)
Solanum stenotomum (potato)	–	In both in vitro and greenhouse conditions, potato and the somatic hybrids produced few bigger tubers, while many small tubers were obtained from the relative. The hybrid tubers were morphologically intermediate. The starch content of hybrid tubers was much lower than that of potato, but similar to that of the relative species. Interestingly, the level of bacterial resistance, introgressed from *S. stenotomum* into potato, was shown to be very stable and remained as high as that of the relative after a long-term period of in vitro conservation	More than 5 years	Fock et al. (2007)
Fruit crops				
Apple genotypes	4 °C	Microvessels appeared to be suitable for storing single node cuttings under slow growth conditions up to a year at least	6, 8,12 and 18 months	Negri et al. (2000)
Phoenix dactylifera (Date palm)	5 °C in the dark	High percent of cultures remained viable without serious signs of senescence. The growth rate decreased as storage period increased. The role of sorbitol as osmotic agent in storage was examined. Healthy shoot bud cultures were obtained after 6 months of storage on medium containing 40 g dm^{-3} sorbitol	3, 6, 9 and 12 months	Bekheet et al. (2002)

(continued)

Table 22.1 (continued)

Species	Storage regime	Conditions	Storage period	Reference
Ensete	15 °C and 18 °C	In vitro cultures can be effectively maintained for 6 months at 15 °C and 18 °C on MS medium supplemented with 10 µM BAP, in the presence of mannitol at concentrations of 0 %, 1 % or 2 % as a growth retardant. Shoots were subsequently recovered and multiplied on MS medium supplemented with 10 and 20 µM BAP at 25 °C, and rooted shoots were successfully transferred to the greenhouse	6 months	Negash et al. (2001)
Grapevine (*Vitis vinifera* L.)	4 °C	After 42 months of dehydrated storage, 90 % of the somatic embryos regenerated into plants. To test utility of this storage method, dehydrated embryos stored for 12 and 26 months cool temperature storage of dehydrated somatic embryos is a simple and inexpensive method of clonal germplasm preservation when compared to alternatives such as cryopreservation	42 months	Jayasankar et al. (2005)
Mulberry (*Morus* spp.)	15–20 °C	A low temperature of 0–5 °C is used for cold-tolerant species, but plants from tropical and subtropical regions may not survive such low temperatures, in which case they are stored at 15–20 °C	Not mentioned	Vijayan et al. (2011)
Raspberry	4 °C	In the first, subculture after storage was lower than that of non-stored cultures. In the second, subculture was lower, and in the third, subculture was similar to that in non-stored cultures	3 and 6 months	Lisek and Orlikowska (2004)
Medicinal and spice crops/species				
Acorus calamus	10 °C and SCC	MS medium devoid of growth regulators for medium-term storage (under standard culture conditions). These cultures were incubated at 26±2 °C with 31.55 µm-2s-2 light intensity. In vitro established cultures of *Acorus calamus* were relocated to chambers having reduced light and temperature conditions (reduced temperature of 10 °C and reduced light of intensity of 2.97 µm-2s-1) to study the effect of storage	6 months	Rajasekharan et al. (2010)
Artocarpus heterophyllus Lam.	10 °C and SCC	Protocols have been optimized for IVAG for this species	4 years	Ganeshan et al. (2010)
Bacopa monnieri (L.)	2 °C	A slow growth protocol was developed for medium-term conservation using mineral oil (MO) overlay. Nodal segments of *B. monnieri* (two genotypes; IC249250, IC468878) were conserved using MO for 24 months. Normal plants regenerated from conserved cultures were successfully established in soil	3, 6 and 24 months	Sharma and Anwar (2012)

(continued)

Table 22.1 (continued)

Species	Storage regime	Conditions	Storage period	Reference
Coleus forskohlii		In vitro plants could be successfully conserved in full MS under SCC for 6 months without any subculture	6 months	Rajasekharan et al. (2004)
Coleus sp.	10 °C and SCC	Vitro plants could be successfully conserved under SCC for 6 months without subculture and full potential to regenerate	6 months	Rajasekharan et al. (2010)
Curcuma longa L. (turmeric)	SCC	Genetic stability of 12-month-old in vitro conserved plants was assessed using 25 random amplified polymorphic DNA (RAPD) primers; no significant variation was observed in RAPD profiles of mother plants and in vitro conserved plantlets on CM and low-cost media	Up to 12 months	Tyagi et al. (2007)
C. malabarica and *C. aromatica* (*Curcuma* sp.)		Plantlet regeneration and medium-term genotype conservation phenomena were genotype dependent and influenced significantly by type and concentration of cytokinins used. The number of shoots per culture ranged from 1.3 to 7.2 and conservation period from 264 to 379 days. In 30-day-old cultures, highest frequency of shoot regeneration could be obtained in *C. malabarica* (7.2 shoots per culture) on MS + 11.4 µM zeatin. *Curcuma* sp. (unidentified wild species) could be conserved for maximum period (379 days) on MS + 24.6 µM 2iP followed by *C. aromatica* (363 days) on MS + 22.8 µM zeatin	264–279 days (*C. malabarica*) 379 days (*C. aromatica*	Tyagi et al. (2004)
Decalepis hamiltonii	2 °C	Microshoots, recovered from encapsulated nodal segments (capsule), were best rooted on half-strength MS medium containing 2.5 µM a-naphthalene acetic acid (NAA). Complete plantlets (with shoot and root) were successfully acclimatized and established in field where they grew well without any detectable variation	6 weeks	Sharma and Anwar (2012)
Eleutherococcus senticosus Maxim.	2 °C	An average of 115,370 germinated somatic embryos (SEs) developed from an initial 400 mg of embryogenic callus, and 64.7 % of germinated SEs converted into plantlets after a 4-week culture on agar medium. During the bioreactor culture process, secondary SEs were induced directly from SEs at various stages, a phenomenon that rarely occurred in suspension culture. These secondary SEs developed quickly and germinated during the bioreactor culture process	4 weeks	Yang et al. (2012)

(continued)

Table 22.1 (continued)

Species	Storage regime	Conditions	Storage period	Reference
Kaempferia galanga	10 °C and RCC	Reduced culture conditions (RCC) of light, temperature and media showed that vitro plants retained the capacity to regrow after storage for a duration of 6 months without intervening subculture	6 months	Rajasekharan et al. (2009)
Medicinal plants	10 °C and SCC	Protocols for conserving plant diversity for 22 species of medicinal plants have been optimized. SCC (standard culture conditions) and RCC (reduced culture conditions: light and temperature)	Different periods	Rajasekharan et al. (2010)
Momordica sahyadrica	10 °C and SCC	In vitro multiplication and conservation of wild *Momordica sahyadrica*	6 months	Rajasekharan and Ganeshan (2010)
Nothapodytes nimmoniana	10 °C and SCC	Under reduced temperature and light, in vitro plants grew well (without any subculture) even after 6 months of incubation. Cultures in MS basal medium showed shoot elongation of 5–10 mm compared to MS with TDZ (3–50 mm)	6 months	Rajasekharan et al. (2010)
Pelargonium sidoides DC. (Geraniaceae)	2 °C	Based on the pharmacological data generated in this study, aerial parts can be used to substitute the commonly preferred underground tubers for the preparation of herbal formulations and antioxidant properties	6 weeks and 12 months	Moyo et al. (2013)
Piper longum	SCC	Only shoots that indexed negative for endogenous bacteria were used for proliferation and in vitro conservation studies. The shoot cultures could be maintained without subculturing for as long as 8 weeks in MS medium supplemented with 1 mg/l paclobutrazol (PBZ) and 40 mg/l ascorbic acid	4–8 weeks	Rani and Dantu (2012)
Rauvolfia serpentina	10 °C and RCC	Lengthening the time between subcultures up to 1 year and consequently reducing maintenance requirements could be useful for in vitro germplasm collections	1 year	Rajasekharan et al. (2007)
Splachnum ampullaceum (moss buds)	1 °C in darkness	Moss regeneration was evaluated at different periods of time to examine the efficiency of the technique for moss germplasm conservation	Long term (2 and 2.5 years)	
Taxus media	5 °C	Paclitaxel levels increased in the cells after conservation during the first recovery subculture cycle and then decreased during the subsequent recovery subculture cycle. The results of high-performance liquid chromatography indicated that DNA methylation increased during the course of repeated subculture. A decrease in DNA methylation level caused by treatment with 5-Aza- 2′ -deoxycytidine coincided with an increase in paclitaxel levels	180 days	Li et al. (2013)

(continued)

Table 22.1 (continued)

Species	Storage regime	Conditions	Storage period	Reference
Tylophora indica	10 °C and RCC	Vitroplants could be successfully conserved in full-strength MS medium (FMS) under SCC for 6 months without subculture with full potential to regenerate, producing viable shoots and nodes	6 months	Rajasekharan et al. (2009)
Vanilla (*Vanilla planifolia*)	2 °C	The conserved material could be retrieved and multiplied normally in MS medium with 1.0 mg l^{-1} BA and 0.5 mg l^{-1} IBA	10 months to 7 years	Divakaran et al. (2006)
Zingiber sp.	–	A total of 33 genotypes of cultivated and wild species of *Zingiber* were subsequently tested for conservation through in vitro rhizome formation on CM under 16 h light condition. All genotypes produced rhizomes of varying size with numbers ranging from 3 to 15 per culture and were conserved for at least 12 months; some genotypes could be conserved even up to 16–20 months	2–3 weeks	Tyagi et al. (2006)
Pulse crops				
Wild *Arachis*	10 °C and 45 % relative humidity	*Arachis* seeds display a sub-orthodox behaviour due to their high lipid content and the fragile tegument, which may lead to oxidative damages. It leads to loss of germinative potential, which occurs even under optimal storage conditions. Plant multiplication under field conditions can be limited by specific soil and environmental requirements or by low seed yielding	15 years	Pacheco et al. (2009)
Commercial crops				
C. malabarica (*Curcuma* sp.)		Plantlet regeneration and medium-term conservation phenomena were genotype dependent and were influenced significantly by type and concentration of cytokinins used. The tissue culture-raised plantlets were morphologically similar to their parents. The in vitro conserved plants multiplied rapidly in tissue cultures and produced normal rhizomes upon transfer to soil in net house	264–379 days	Tyagi et al. (2006)
Corymbia torelliana x C. *citriodora* and *Khaya senegalensis*	14 °C and zero irradiance	Encapsulated explants of both trees were preserved most effectively on high-nutrient (half-strength MS medium containing 1 % sucrose), which provided very high frequencies of shoot regrowth (92–100 % for *Corymbia* and 71–98 % for *Khaya*) and excellent shoot development after 12 months of storage. This technique provides an extremely efficient means for storage and exchange of eucalypts and mahoganies, ideally suited for incorporation into plant breeding and germplasm conservation programmes	0, 3, 6 and 12 months	Hung and Trueman (2012)

(continued)

Table 22.1 (continued)

Species	Storage regime	Conditions	Storage period	Reference
Hibiscus moscheutos	5 °C calcium alginate microencapsulation	80 % of encapsulated hardy hibiscus nodal segments survived refrigerated storage for 1.5 years (78 weeks). After 3 months on proliferation medium, the nodal segments produced nearly the same length axillary shoots with the same number of axillary nodes per shoot as compared to encapsulated segments either not stored at 5 °C or stored for 24 weeks at 5 °C		West et al. (2006)
Olive (*Olea europaea* L.)	–	Italian local cultivar showed a notable difficulty for the in vitro establishment due to heavy pathogen contamination. Mercury chloride and sodium hypochloride in the sterilization step and antibiotics in culture media allowed overcoming the problem. Proliferation of shoot apical bud on olive culture medium with 36 g dm^{-3} mannitol and 4.56 μM zeatin appeared very satisfactory	1 month	Zacchini and De Agazio (2004)
Saccharum spp.	2 °C	For storage of whole plantlets, the highest survival rates and shoot regrowth after 8 months were observed at 18 °C on half-strength MS and 10 g l^{-1} sucrose, with or without 30 g l^{-1} sorbitol	2 weeks	Watt et al. (2009)
Saccharum spp. hybrid (genotype 88H0019)	2–18 °C	For storage of whole plantlets, the highest survival rates and shoot regrowth after 8 months were observed at 18 °C on half-strength MS and 10 g l^{-1} sucrose, with or without 30 g l^{-1} sorbitol	8 months	Watt et al. (2009)
Sequoia sempervirens (D. Don.) Endl	4 °C encapsulated and nonencapsulated shoots	Survival and regrowth of encapsulated shoots declined within 3 months, regardless of the storage medium composition. No significant decrease in survival and regrowth was noted with nonencapsulated shoots after 12 months of storage on Quoirin and Lepoivre medium supplemented, or not, with 1 mgl^{-1} benzyladenine. Regrowth dropped to 60–61 % after 15 months of storage on the same media	15 months	Ozudogru et al. (2011)
Strawberry beads	4 °C	In the first, subculture after storage was lower than that of non-stored cultures. In the second, subculture was generally higher than in non-stored cultures, and in the third, subculture was similar to that in non-stored cultures	9 months	Lisek and Orlikowska (2004)

SCC standard culture conditions, *RCC* reduced culture conditions

Table 22.2 Tissue culture and cryopreservation at National Gene Bank of India (NBPGR, New Delhi) as on 28 February 2014

Crop/crop group	Present status (no.)
Tropical fruits	420
Temperate and minor tropical fruits	305
Tuber crops	619
Bulbous crops	171
Medicinal and aromatic plants	153
Spices and industrial crops	225
Total	**1,893**
Recalcitrant	0
Intermediate	6,113
Orthodox	3,253
Dormant bud (mulberry)	387
Pollen	446
Total	**1,0199**

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Gene Banking for *Ex Situ* Conservation of Plant Genetic Resources

23

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Abstract

Seed storage is one of the most widespread and valuable *ex situ* approaches to conservation. Extensive expertise has been developed in this field by agencies and institutions involved with plant genetic resources over the past 30 years. Seed banking has considerable advantages over other methods of *ex situ* conservation such as ease of storage, economy of space, relatively low labour demands and, consequently, the capacity to maintain large samples at an economically viable cost. Depending on the species, seeds are dried to suitably low moisture content according to an appropriate protocol. Typically this will be less than 5 %. The seeds then are stored at −18 °C or below. Because seed DNA degrades with time, the seeds need to be periodically replanted and fresh seeds collected for another round of long-term storage. There are about six million accessions, or samples of a particular population, stored as seeds in about 1,300 gene banks throughout the world as of 2006. The procedure for seed storage along with the latest development on gene banking is discussed in this chapter.

Keywords

Gene bank, accessions seeds community seed bank, permafrost

23.1 Introduction

The former Soviet Union gained an early lead in collecting and conserving plant genetic resources as a result of the work of Vavilov, who was responsible for the extensive and valuable collections assembled at the All-Union Institute of Plant Industry at St. Petersburg (formerly Leningrad). The Vavilov All-Union Institute of Plant Industry (VIR), as it was to be known later, became the central nationwide institution responsible for collecting and conserving global plant diversity and studying it for the purposes of plant breeding and crop improvement in the Soviet Union (Vavilov 1997) In the United States, collection and evaluation of germplasm has been

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performed since the 1900s, but the first repository of crop seeds was not established until 1947 when the Regional Plant Introduction Station in Ames, IA, began operation. In 1958, the first national facility for storage of germplasm at low temperatures in cold rooms was constructed at Fort Collins, CO. These facilities were recently upgraded (Qualset and Shands 2005). An IBPGR survey in 1975 revealed that there were only eight long-term genetic resources' conservation centres globally (almost all of them in the industrialised countries). But only 7 years later, the total had jumped to 33, and today there are over 1,000 major germplasm collections in gene banks all over the world (Qualset and Shands 2005). Crop genetic resources grown from true seed are stored in three main types of gene banks. In long-term gene banks, whose aim is to store material for 50–100 years, samples are kept at −10 °C to −20 °C in airtight containers. Usually before such storage, the seed samples are dried to a moisture content of 5–7 %. In medium-term facilities, as most working collections are stored, temperatures of 0–5 °C are maintained and seeds may last up to 10–15 years. The short-term collections or breeder's collections are usually kept in paper envelopes or cloth bags or tin cans at 5–15 °C without any seed drying. Samples are constantly withdrawn for evaluation. Much of the germplasm of major crops and their wild and weedy relatives already resides in gene banks. The effect of the vast increase in accessions since the 1970s on germplasm utilised for crop production has thus far been modest (Damania 2008). More than a decade ago, a US survey found around three-quarters of soy and wheat breeders and around three-fifths of cotton and sorghum breeders, but only 45 % of corn breeders used gene banks more than 'rarely'.

Since the 1970s, work on the conservation of crop genetic resources has increasingly become a large-scale independent activity detached from crop improvement efforts. A substantial germplasm collecting effort was launched in the 1970s in response to concerns about genetic erosion and crop vulnerability. Over 1,000 gene banks have been established, holding about six million accessions (FAO 1998). The germplasm holdings in major international agricultural research centres are provided in Table 23.1. Seed banks of a global network of international agricultural research institutions, coordinated by the Consultative Group on International Agricultural Research (CGIAR), Washington, are focused on crops and have extensive seed collections for such crops as rice, maize, wheat, barley, millets, pulses, oil seeds, tuber crops, banana, tropical forage and fruits. The collections in these seed banks are well documented, and the institutions are networked among themselves and with several other institutions. The Millennium Seed Bank Project (MSBP) at the Royal Botanic Gardens, Kew, England, is one of the largest conservation projects. MSBP's 47 partner organisations in 17 countries intend to store 25 % of the world's plant species by 2020. The Seed Information Database (SID) at Kew is an ongoing compilation of seed characteristics and traits worldwide, targeted at >24,000 species.

What makes seed banks such an effective *ex situ* conservation technique is that the methodology can be applied to a wide range of species in a universal and straightforward way and that large amounts of intraspecific diversity can be conserved and for long periods of time without intervention. Additionally, germinating seeds to obtain fully grown plants is relatively simple compared with obtaining plants from *in vitro-stored* material. Plants recovered from banked seeds can also be compared with natural populations from which the material was harvested

Table 23.1 PGR holdings at IARCs (Source: SINGER)

Name of IARC	Number of accessions
AVRDC	52,845
Bioversity International	1,208
CIAT	72,246
CIMMYT	120,527
CIP	15,092
ICARDA	140,189
ICRAF	1,785
ICRISAT	114,865
IITA	27,596
ILRI	20,177
IRRI	108,272
WARDA	21,752

years before and which may have been subjected subsequently to environmental change (e.g. as a result of global warming).

23.2 Current Status of Seed Banks for Food and Agriculture

Just under half of the six million accessions are held in 12 national collections. To some degree, this is a function of the early establishment of their genetic resource collections. The collections include those in Russia and the United States (noted earlier), Japan (established in 1966), Germany (1970), Canada (1970) and Brazil (1974). Of the 1,308 national or regional collections currently noted by FAO (1996), only 397 within 75 countries are held in long- or medium-term seed banks. Medium-term storage might be assumed to be in the order of 10 or more years.

23.3 Scientific Principles Underlying Seed Banking

Viable seeds of many species when maintained in a dry and cold state are capable of being germinated many years later. This capability means that the long-term *ex situ* conservation of many higher plants is a realistic possibility.

23.3.1 Seed Storage Conditions

The science of seed storage is not a new one and dates back, at least, to China in the sixth century. Advances in the quantification of seed longevity under different storage conditions were made in the second half of the twentieth century through the work of Harrington in the United States and Ellis and Roberts in the United Kingdom (see review in Hong, Jenkins et al. 1998). Critical factors that determine seed longevity are the seed's moisture content, temperature, and gaseous environment; its initial viability; and its genetic background. With respect to the latter, differences between species would appear to be much greater than those within. Genetics particularly influences the relationship between seed longevity and seed moisture content. Most species produce seeds that can be dried to low moisture contents (e.g. where less than 5 % of the seeds' fresh weight is water) without loss of viability. The seeds of such species are termed 'orthodox'.

23.3.2 Genetic Considerations

The genetics of seed bank storage is an important issue. A criticism occasionally levelled at seed banks is that there is selection in storage. Selection can occur during collection through a biassed sample (e.g. for early- or late-flowering genotypes). It can also occur when samples are grown out under conditions that differ dramatically from those where the seeds were harvested. There is, however, little evidence to show that, compared with, say, room conditions, the seed bank environment does other than slow down the (normally distributed) times for individuals to die. In other words, there is no greater risk of selection out of any individual by the conditions applied than under natural conditions.

23.4 Other Forms of Gene Banks

23.4.1 Conservation of Pollen

Conservation of nuclear genetic diversity (NGD) using pollen is desirable in horticultural species for a variety of reasons. Cryopreserved pollen can be a major access point for pre-breeding germplasm lines, hybrid seed production and biotechnological and other basic studies. In the case of tree species, germplasm can be easily received and exchanged through pollen, eliminating a long juvenile phase. The objective of a useful pollen cryostorage protocol is to collect mature pollen from plant and treat it so as to retain its normal function, ultimately assessed by its ability to germinate in vivo and effect normal fertilisation (Hanna and Towill 1995). Alexander and Ganeshan (1993) reviewed the work on pollen storage in fruit crops. Hoekstra (1995) has

assessed the merits and demerits of pollen as genetic resource. Ganeshan and Rajasekharan (1995) reviewed work on ornamental crop pollen storage. Grout and Roberts (1995) detailed the methodology for pollen cryopreservation. Recently, Barnabas and Kovacs (1997) and Berthoud (1997) stressed the importance and need for pollen conservation. Response to cryopreservation of pollen of 45 species belonging to 15 families is presented in Table 23.1. In some of the recently cryostored pollen, only feasibility tests were carried out. In most species, protocols are optimised for establishing pollen cryobanks.

Besides the already existing role of pollen banks in breeding, there are many promising applications which have come to focus with the recent advances in allied bioscientific areas. Some of the practical utilities are discussed below:

23.4.2 Advantages to the Use of Pollen

- Gene-banked pollen can be made available to breeders upon request. For tree species, this obviates the need for growing the male parents in the breeding orchards. It allows for wide hybridisation across seasonal and geographical limitations and reduces the coordination required to synchronise flowering and pollen availability for use in crosses. With adequate pollen available, one can also load additional pollen onto stigmas to increase pollination and yield.
- Pollen is available for research programmes. As single cells, pollen provides a simple model system for research on conservation. Storage of pollen within gene banks also ensures its availability year-round for basic biology and allergy research programmes (Shivanna 2003).
- Pollen captures diversity within small sample sizes, and documentation is available for long-term survival of pollen from many diverse species. Pollen also serves as a source of genetic diversity in collections where it is hard to maintain diversity with seeds (species of low fecundity, large seeds, or seeds that require an investment of labour to store).
- Pollen can also be shipped internationally, often without threat of disease transfer (Hoekstra 1995) (http://cropgenebank.sgrp.cgiar.org/).

Detailed protocols for pollen cryopreservation have been given by Ganeshan et al. (2008) and Rajasekharan et al. (2013).

23.4.3 Conservation of DNA

Advances in genomics have provided technologies for high-throughput analysis of plant genomes with potential for use in gene discovery in germplasm collections. Biotechnology has made possible DNA conservation of plant species, in the form of extracted DNA or as genomic DNA libraries. However, this technique needs advanced technological inputs to match with the importance and requirement for the species in question, to justify its need as a practical conservation strategy. For any given plant species, DNA conservation could be advantageous for:

1. The study of molecular phylogenetics and systematics of extant and extinct taxa
2. Production of previously characterised secondary compounds in recombinant DNA-mediated transgenic cell cultures
3. Production of transgenic plant using genes from gene families
4. *In vitro* expression and study of enzyme structure and function, synthesising genomic probes for research laboratories

The establishment of DNA banks facilitates this screening by making DNA from large numbers of plant accessions widely available. DNA banks require the development of appropriate policies for access and benefit sharing. Tools for automating sample and data handling are essential. Standard molecular methods for fingerprinting DNA accessions for international comparisons need to be determined. New screening technologies are required to take advantage of the emerging availability of large DNA collections.

The Australian Plant DNA Bank aims to collect DNA from all Australian plant species and to sample the diversity within each species. DNA from all individuals of the species is being stored for rare species. Domesticated or economically important species from all countries are also being collected and stored. The international networking of DNA banks will be a key step in linking genomics tools to global plant diversity (Rice et al. 2006).

23.4.3.1 The Use of DNA Banks

Molecular techniques are becoming increasingly important in the study and management of genetic resources. DNA has been routinely extracted and stored from the nuclei, mitochondria and chloroplasts of many plant species, together with derivatives such as RNA, cDNA and genes. Technologies are available to allow all these to be stored quickly and at low cost in DNA banks as an insurance policy against loss of crop diversity. DNA storage has so far been undertaken with objectives other than conservation in mind, usually to allow genetic material to be made readily available for molecular applications, for distribution or for training (http://cropgenebank.sgrp.cgiar.org/).

23.4.3.2 Advantages

DNA banking is an efficient, simple and long-term method to conserve the genetic information.

23.4.3.3 Disadvantages

There are problems with subsequent gene isolation, cloning and transfer of DNA back to a plant, and it currently does not allow the regeneration of the same genotype as the original sample.

23.4.3.4 Storage

There is little information on the long-term stability of extracted DNA during frozen storage, but most repositories consider several years to decades as realistic. Information on the stability of purified DNA dissolved in buffer suggests that the overall fragment size decreases with storage time and that the usefulness of the specimen for PCR-based assays may be 1–2 years when stored at 4 °C, 4–7 years when stored at −18 °C and greater than 4 years when stored at −80 °C (Madisen et al. 1987; Visvikis et al. 1998). The choice of temperature usually depends on the moisture level within the sample.

23.5 Community Seed Banks (CSBs)

Community seed banks fulfil diverse purposes of sustainable agriculture for small and marginal farmers. These seed banks serve as focal point in maintaining indigenous genetic diversity on farms involving farmer community. CSBs serve local farmers to form an informal seed distribution system prevailing in villages since ancient time at no or very low cost. Community participation in maintaining local genetic diversity provides pride to farmers and sense of belonging for local landraces. This system is run, maintained and promoted by farmers to facilitate good quality seeds and input (Malik et al. 2013). There are no set guidelines available to establish and manage community seed banks as they form an important part of informal seed distribution system in villages since ancient time. The farming community as per their convenience has developed this system and the same is being continued by the farmers.

23.6 Soil Seed Bank

The soil seed bank is the natural storage of seeds, often dormant, within the soil of most ecosystems. The study of soil seed banks started in 1859 when Charles Darwin observed the emergence of seedlings using soil samples from the bottom of a lake. The first scientific paper on the subject was published in 1882 and reported on the occurrence of seeds at different soil depths. Weed seed banks have been studied intensely in agricultural science because of their important economic impacts; other fields interested in soil seed banks include forest regeneration and restoration ecology.

23.7 Seed Bank Management

23.7.1 Procedure

The basic elements of the seed banking procedure (more or less in order) are as follows (Linington et al. 2001):

- Collection planning and permission seeking
- Seed (and pressed specimen) collecting and field data recording
- Shipment of seeds
- Creation of a data record about the accession
- Seed cleaning (sometimes preceded by initial drying and sometimes accompanied by X-ray analysis and quantity determination)
- Main drying
- Seed moisture determination
- Initial germination test (sometimes left until after banking)
- Packaging and banking and security duplication
- Characterisation (including verification of identity in the case of wild species) and evaluation (where appropriate)
- Distribution of stocks to users (through time)
- Germination retests (through time)
- Regeneration/multiplication (as required)

23.7.2 Seed Collection

Set against the background of the CBD and the International Undertaking, collecting should only be carried out with the permission of the national and local authorities. In India as per the Biodiversity Act, one has to take permission from local bodies for collection. Foreign collectors should work collaboratively with local scientists, and clear agreements on benefit sharing should be in place. One immediately tangible benefit is the sharing of collections and the information relating to them. Other international regulations need to be adhered to. These include the Convention on International Trade in Endangered Species (CITES) and national quarantine laws. Seed collecting methodology and genetic resource exploration have been thoroughly covered by Guarino et al. (1995). In most instances, random and even sampling of wild plant or crop populations is recommended, including careful note taking of the sample method (in often less than perfect conditions). Objective data recording is essential as is accurate recording of location. This latter aspect is now facilitated by the use of Global Positioning System receivers that help fix latitude, longitude and even altitude using satellites.

23.7.3 Seed Cleaning

Seed cleaning is the removal of debris, inert material, damaged and infested or infected seeds and seeds of different species (e.g. weeds) to achieve clean and pure samples of seeds of high physiological quality for storage.

Seed cleaning is necessary to:

- Reduce bulk during transportation.
- Improve purity of the sample.
- Optimise storage space and reduce costs.
- Prevent seed from going mouldy and help reduce ‘damping off’ or fungal contamination after germination.
- Allow precise regulation of seed moisture content during storage. Seeds should be cleaned immediately after harvest or soon after they arrive at the gene bank. Cleaning methods vary according to the type of seed.

Cleaning should not cause damage to samples or lead to waste. It can be done manually or by machines, but gene banks are strongly advised to clean accessions by hand for the following reasons:

- Mechanical cleaning could result in selection within genetically heterogeneous accessions (due to exclusion of very small and very large seeds passing through mechanical apertures).
- Equipment requires rigorous cleaning and often careful adjustment between accessions.

23.7.4 Seed Bank Storage Standards

Seeds can be classified into the following types according to seed storage behaviour:

1. *Orthodox* – seeds that can withstand low seed moisture content and can be stored at low temperature without losing viability
2. *Recalcitrant* – seeds that lose viability if moisture content is less than 12–13 %
3. *Intermediate* – seeds that exhibit storage characteristics between orthodox and recalcitrant seeds

For orthodox seeds, the storage potential is influenced by inherent and external factors; for example, some legumes that are hard-seeded are long-lived, while seeds with high oil content are short-lived. There is also observed variation at the eco-geographic races (e.g. *indica* vs. *japonica* rice) and cultivar levels. Seeds harvested at their physiological maturity generally store better. The environment (temperature, moisture, nutrition and light) under which the crop is planted can also affect storability. The longevity of seeds depends on the initial seed quality, moisture content and temperature during storage. In general, low moisture content and low temperature reduce the loss of seed viability. Harrington's rule of thumb can be applied as rough estimate of length of storage in reference to temperature and seed mc. The rule states that beginning at 14 % mc, for every 1 % decrease, the lifespan of the seeds doubles, and beginning at 50 °C, for every 5 °C decrease in storage temperature, the lifespan of the seeds doubles.

No two banks are the same. Traditionally, seed banks have been classified into the following categories:

- *Base* collections that are for the long-term storage of seed lots and from which seeds are not normally sent to users (though this is not always the case).
- *Active* collections from which seeds are made available to users. Often such stores are maintained under less optimal storage conditions compared to those holding base collections though this need not be the case. FAO/IPGRI (1994) recommends that storage lives of 10–20 years might be appropriate.

23.7.5 Seed Drying

Seed drying is the reduction of seed moisture content to the recommended levels (which should not be lower than the critical seed moisture content) for storage using techniques which are not detrimental to seed viability. Dry seeds retain viability for longer periods during storage. Seeds are hygroscopic and absorb or desorb moisture depending on the relative humidity of the surrounding air or the gradient in water potential between the seed and surrounding air. If the water vapour pressure of the seed is greater than the surrounding air, the seed will lose moisture and becomes drier (desorption). If the water vapour pressure of the seed is lower than the surrounding air, the seed will gain moisture (absorption). Absorption or desorption occurs until the water vapour pressure in the seed and the surrounding air is balanced. The water content of seeds at equilibrium with the RH of surrounding air is referred to as equilibrium moisture content. Understanding the relation between equilibrium seed moisture content and relative humidity is important for the gene bank technician to decide on the drying regime for seeds. Several methods are available for drying seeds. Methods that minimise loss of viability during drying should be used. The most common and safe methods used for drying are dehumidified drying and silica gel drying. Drying rate depends on seed size, shape, structure, composition, initial seed moisture content, amount of seeds and layers, air movement, temperature and relative humidity.

23.7.6 Packaging

Seed packaging is the placing of a counted or weighed sample of seeds of an accession into a container which is then hermetically sealed for subsequent storage.

Seeds are packaged to:

- Prevent absorption of water from the atmosphere after drying.
- Keep each accession separate and avoid the mixing of accessions.
- Prevent contamination of the seeds from insects and diseases.

The best time to package seeds is immediately after the moisture content has been determined and found to be within the required limits for safe storage. Dry seeds will reabsorb moisture from ambient air. Therefore, seeds should be packaged into containers and hermetically sealed without delay, soon after removal from the drying room or cabinet. Different types of containers are available for packaging. The choice depends on storage conditions and species. The most important thing is that the packing material should be completely impermeable to water and suitable for long-term use. Some frequently used containers in gene banks are glass bottles, aluminium cans, aluminium foil packets and plastic bottles. These different types of containers all have advantages and disadvantages. Glass bottles are good but fragile and can easily break. Aluminium cans are difficult to reseal once they are opened. Aluminium foil can be resealed and occupy less space in storage room. However, seeds with sharp projection can pierce the packets and moisture can leak inside. Plastic bottles are moisture resistant but not moisture proof. They should be used with caution if relative humidity of the storage room is not controlled. Packaging is best carried out in an air-conditioned room where the relative humidity is controlled. It is important to ensure that seeds taken from the drying room are exposed to the ambient air for the shortest possible time so that they do not reabsorb water.

23.7.7 Storage Temperature

Many long-term seed banks store seed under deep-freeze conditions using either purpose-built prefabricated cold rooms or domestic deep freezers. To reduce staff time at subzero temperatures, a few seed bank cold rooms, such as one at the National Institute of Agrobiological Resources (NIAR), Japan, have mechanised banking/retrieval systems. The uses of such systems have implications to energy consumption by the bank. Use of permafrost has been considered for long-term duplicate storage of seed in places such as Svalbard. Stores are usually unable to match the lowering of temperatures possible in conventional base storage conditions. In 1997, the Japanese-based Biological and Environmental Specimen Time (BEST) Capsule 2001 Project discussed the possibilities of long-term storage of flagship samples under Antarctic ice at 58 °C (which incidentally is not sufficiently low for animal tissue preservation) or even on the dark side of the moon at 230 °C.

23.7.8 Monitoring Seed Lot Viability

Perhaps one of the most important parameters of seed bank effectiveness is the result of germination monitoring. Germination is the preferred test for seed lot viability. Providing such information to those using the seed is helpful. Additionally, other viability tests such as vital staining using tetrazolium solution have a greater element of subjectivity about them. This staining test is, however, sometimes used to help distinguish between dead and dormant seeds among those that did not germinate under a given test regime. Two problems relate to the germination monitoring of seed bank accessions: First, because seeds are tested soon after arrival at the bank and then at regular intervals (often every 5–10 years) during their storage life, the tests need to be repeatable and operator independent. Second, in order to recover as many genotypes as possible represented within a seed lot, it is necessary to break seed dormancy. This can be a particular problem in the seed of wild species and where the seed is freshly harvested. Key techniques include scarification of hard seed coats to facilitate water or oxygen permeation, imbibed chilling at 5–10 °C and later incubation at diurnal alternating temperatures with fluorescent light (i.e. rich in red light) provided only in the higher temperature phase. Tests have to be seed lot specific as most seed dormancy is not strongly genetically inherited and the form it takes depends on the conditions under which the seed matured. Once determined, the same treatments can be used during the monitoring of that seed lot through time.

23.7.9 Duplication

One of the main advantages of seed banks is that they centralise collections of genetic material making them more easily accessed and studied. Indeed, some seed banks might be seen as some of the world's greatest plant diversity hot spots with more individuals and, in some banks, more species per square metre than anywhere else on the planet. This centralisation poses a risk to all but the most carefully located and constructed facilities. Potential catastrophic loss, which of course threatens plants, conserved both *ex situ* and in situ, means that duplication of collections and their associated data is an important element of seed bank safety. The FAO report (1996) indicates that the level of security duplication of plant genetic resources for food and agriculture still needs to be improved and is at best uncertain.

23.7.10 Characterisation and Evaluation

Characterisation can vary from accurate naming of the species or subspecies represented by the collection through to more detailed recording of characters governed by genes that are little modified by environmental factors (major genes). Such information, published in the form of descriptor lists, is of great value to plant breeders wishing to narrow their choice of material from, often, vast collections. Similarly, the concept of core collections has been established to facilitate use by breeders. A core collection genetically represents a limited set of accessions of a crop gene pool with the minimum repetition. Increasingly, characterisation is taking the form of more detailed molecular techniques such as screening by amplified fragment length polymorphism. By contrast to characterisation, evaluation records data on traits such as yield that are strongly influenced by the environment in which the plants are grown. Such data are thus site and year specific and are perhaps of less use to breeders.

23.7.11 Distribution to Users

A very important element of seed bank work is to make the seed available wherever possible. In evidence of the scale of such dispatch, Kerry Ten Kates and Laird (1999) quote an annual distribution of nearly 120,000 samples by the US National Plant Germplasm System, of which 65 % are sent abroad, many requested by reference to the Germplasm Resources Information Network (GRIN) available on the Internet. Furthermore, the usage rate of crop banks by plant breeders is probably less than the rate of request from banks holding broader plant diversity collections where uses include a wide array of pure and applied research in addition to field trials. During 1994–1996, there was a 50 % request rate for seeds offered through an extensive list offered by the Kew Seed Bank.

23.7.12 Regeneration

Seed bank accessions are grown out for the purposes of regeneration of seed stock (either when seed numbers are low or when viability has reduced), for characterisation and for evaluation. Many banks have a regeneration standard below which the germination of a seed lot should not fall. This is usually set at 85 %. This high value limits the risk of accumulated genetic damage that is associated with seed ageing. Even though falling levels of seed germination are correlated with falling levels of field establishment, many banks have adopted lower standards. This may in part be due to the backlog of regeneration work that in some national facilities highlighted by FAO (1996) is nearly 100 % of the collection. By collecting high-quality seed lots in good quantity, other banks have reduced the necessity for regeneration that can be time- and labour-consuming and that can have adverse effects on the genetics of the collection. Samples regenerated under conditions different from where they originated can experience selection. If too small a sample is regenerated, genetic drift may occur in which

rarer alleles are lost through chance. Under some circumstances, recollection, if possible, may be the more desirable option.

23.8 Seed Bank Design

Having considered the aspects of seed bank management, a brief consideration of seed bank design is appropriate (also see Cromarty et al. 1985). The location of the bank is important from political, practical and security aspects. Potential risks have to be considered be they earthquake, flooding or radiation fallout. Some facilities are placed underground such as the seed bank at Krasnodar in Russia and the Millennium Seed Bank in the United Kingdom. Others such as the NSSL are located on the first floor to limit possible impact from structures above resultant from seismic activity. The size of most banks should be dictated by peak annual intake (seed drying and cleaning facilities), projected capacity before a rebuild which is practical (seed storage) and annual collection maintenance (germination, field and greenhouse facilities). Cold storage facilities vary from a few domestic deep freezers up to large rooms such as one of 140 m^2 (with capacity for 150,000 samples) at NIAR in Japan.

23.9 Gene Bank Standards

The *Genebank Standards for Plant Genetic Resources for Food and Agriculture* is intended as a guideline for gene banks conserving plant collections (seeds, live plants and explants). They were developed based on a series of consultations with a large number of experts in seed conservation, cryopreservation, *in vitro* conservation and field gene banks worldwide. The standards are voluntary and nonbinding and have not been developed through standard-setting procedure. They should be viewed more as targets for developing efficient, effective and rational *ex situ* conservation in gene banks that provides optimal maintenance of seed viability and genetic integrity, thereby ensuring access to and use of high-quality seeds of conserved plant genetic resources (FAO 2014).

The standards, as described in this document, define the level of performance of a routine gene bank operation below which there is a high risk of losing genetic integrity. Each section is divided into:

A. Standards
B. Context
C. Technical aspects
D. Contingencies

Specific standards have been developed for eight key areas and include the following sections:

- Acquisition and initial handling
- Testing for nonorthodox behaviour and assessment of water content, vigour and viability
- Hydrated storage for recalcitrant seeds
- *In vitro* culture and slow growth storage
- Cryopreservation
- Documentation
- Distribution and exchange
- Security and safety duplication

An operation manual is also available for gene bank (Rao et al. 2006) (Tables 23.2, 23.3, 23.4 and 23.5).

Promoting genetic resources' utilisation, precise evaluation and documentation of plant genetic resources is a prerequisite for their utilisation. The following areas of research need to be paid more attention for promoting effective utilisation of PGR gene resources:

Table 23.2 PGR holdings in the National Gene Bank, India

Crop group	No. of accessions
Cereals	145,765
Minor millets	53,466
Pseudo-cereals	6,619
Grain legumes	56,870
Oilseeds	54,994
Fibre crops	11,483
Vegetables	24,112
Fruits	382
Medicinal and aromatic crops	6,304
Spices and condiments	2,708
Agroforestry	2,433
Safety duplicates	10,235
Total	*375,371*

Source: NBPGR website

Table 23.3 Details of various *ex situ* conservation sites for PGRFA in India

Type of conservation and nodal ministry	Number of department facilities
Seed gene bank (long-term collections, −18 °C), Ministry of Agriculture	ICAR 1
Seed gene bank (medium-term collections, 4 °C), Ministry of Agriculture	ICAR 28
Seed gene bank (short-term collections at around 10 °C), Ministry of Agriculture	ICAR 13
Botanical gardens, Ministry of Environment	BSI 150
In vitro conservation (4–25 °C), Ministry of Agriculture	ICAR 5
Field gene bank, Ministry of Agriculture	ICAR, SAU 25
Cryopreservation (using liquid nitrogen), Ministry of Agriculture	ICAR 2

Table 23.4 Number of samples of PGR distributed over the last 10 years

Year	No. of samples
1996	20,775
1997	27,022
1998	23,313
1999	11,064
2000	9,714
2001	10,771
2002	12,274
2003	15,487
2004	15,543
2005	9,366
2006	9,537
Total	*164,866*

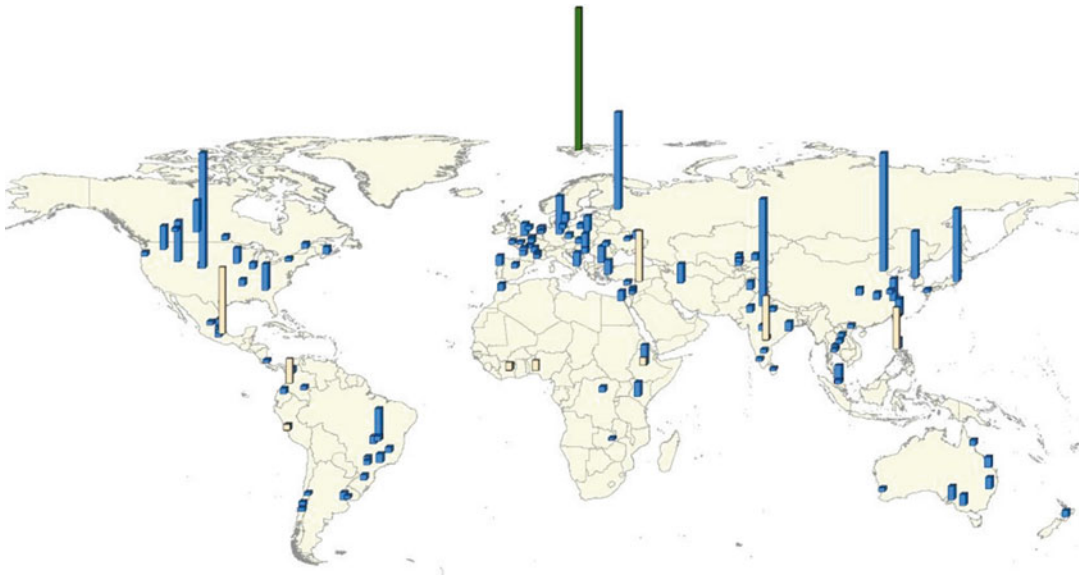
- Development of core collections
- Focused Identification of Germplasm Strategy (FIGS)
- Pre-breeding
- Gene prospecting and allele mining for a trait of interest from genetic resources (Tables 23.6 and 23.7)

On 18 April 2013, the Commission on Genetic Resources for Food and Agriculture (CGRFA) of the United Nations Food and Agriculture Organization (FAO) endorsed and adopted the revision of the *Genebank Standards*, last published in 1994. The revised *Genebank Standards* take into account the changes in *ex situ* conservation conditions, diversity in storage requirements and purpose and period of germplasm conservation, ranging from temperate to tropical provenances. Field gene banking is the most commonly used method for nonorthodox seed-producing plants, for plants that produce very few.

Seeds are vegetatively propagated and/or have a long life cycle, and their standards have been defined accordingly. The standards for *in vitro* culture and cryopreservation are broad and generic in nature, due to the marked variation among nonorthodox seeds and vegetatively propagated plants (Tyagi and Agrawal 2013).

23.10 Economic Considerations

The annual cost (in year 2000 US$) of conserving and distributing the genetic material presently held in all 11 CGIAR gene banks is estimated to be 5.7 million US$ (m US$), which could be maintained for all future generations by setting aside a fund of 149 m US$ (invested at a real rate of interest of 4 % per annum) (Virchow 2003). This would be sufficient to underwrite the costs for the CGIAR's current conservation activities in perpetuity (estimated to be 61 m US$), as well as the cost of maintaining the distribution activities (88 m US$) that provide germplasm to breeders, scientists, farmers and others worldwide (Koo et al. 2003).



Gene banks of the world

Table 23.5 Total accessions conserved *ex situ* and the number of safety duplicates for various categories of crop species (1996–2005)

Sl. no.	Status	No. of crop spp.	No. of accessions	Safety duplicates as active collections	Safety duplicates (%)
1.	Traditional cultivar/landrace	280	121,274	84,931	70
2.	Wild	314	15,881	4,745	30
3.	Weedy	70	267	11	4
4.	Breeders line	37	14,661	2,272	15
5.	Mutant/genetic stock	26	7,898	4,880	62
6.	Advanced/improved cultivar	59	9,080	4,867	54
7.	Others	73	27,662	2,378	9

Table 23.6 Genetic diversity collection and utilisation

Commodity	Landraces	% in collections	Wild species	In situ collections	*Ex situ* collections (acc.)	Utilisation distribution
Rice	140,000	90	20	Few	420,000	High
Maize	65,000	90	–	Few	277,000	High
Sorghum	45,000	80	20	Few	169,000	Low
Millets	30,000	80	–	None	90,000	Low
Soybean	30,000	60	–	None	174,000	Low-medium
Chickpea	22,230[a]	90	19	None	33,782[a]	High
Pigeon pea	8,220[a]	80	57	Few	13,628[a]	Medium-high
Groundnut	6,374[a]	90	45	None	15,419	Medium-high
Potato	30,000	95	65	Few	31,000	High

Modified after Evenson et al. (1998)
[a]ICRISAT and ICARDA holdings

Table 23.7 Status of base collections in the National Gene Bank of India [NBPGR, New Delhi (−18 °C)] (as of 28 February 2014; Source: NBPGR website)

Crop/crop group	Number of accessions conserved
Paddy	97,314
Wheat	40,340
Maize	9,639
Others	12,276
Cereals	*159,569*
Sorghum	20,542
Pearl millet	8,930
Minor millet	22,614
Others	5,437
Millets and forages	*57,523*
Amaranth	5,579
Buckwheat	886
Others	389
Pseudo-cereals	*6,854*
Chickpea	17,373
Pigeon pea	11,432
Mung bean	3,705
Others	26,246
Grain legumes	*58,756*
Groundnut	14,937
Brassica	10,759
Safflower	8,081
Others	24,700
Oilseeds	*58,477*
Cotton	7,329
Jute	2,915
Others	2,214
Fibre crops	*12,458*
Brinjal (eggplant)	4,119
Chilli	2,012
Others	19,199
Vegetables	*25,330*
Custard apple	59
Papaya	23
Others	448
Fruits	*530*
Opium poppy	350
Ocimum	473
Tobacco	1,490
Others	4,559
Medicinal and aromatic plants and narcotics	*6,872*
Coriander	911
Sowa (dill)	91
Others	2,845
Spices and condiments	*3,847*
Pongamia (oil tree)	395
Others	2,048
Agroforestry	*2,443*
Lentil	7,712
Pigeon pea	2,523
Duplicate safety samples	*10,235*
Total	*402,894*[a]

No. of crop species conserved: 1,586

[a]The figure includes 4,305 released varieties and 2,300 genetic stocks; regenerated accession not included

23.11 Svalbard

Svalbard Global Seed Vault: On 26 February 2008, the Svalbard Global Seed Vault (SGSV) opened near Longyearbyen (Norway), 600 miles from the North Pole. SGSV is designed to hold 4.5 billion batches of seeds of the world's main crops. The SGSV is a glazed cave-like structure, drilled 500 ft below permafrost, in the middle of a frozen Arctic mountain topped with snow, with the goal to store and protect samples from every seed collection in the world, which will stay frozen. An automated digital monitoring system controls temperature and humidity and provides high security. The SGSV is an insurance against natural disasters, such as earthquakes and tsunamis, or deliberate attacks like bomb blasts or human errors such as nuclear disasters or failure of refrigeration that may erase the seeds of any important species in the other seed banks or in the wild, in the other countries. Such seed can be re-established using seeds from SGSV. NBPGR facilitated safe transfer of about 40,000 accessions of ICRISAT mandate crops to SSGV during the past 2 years.

The Svalbard Global Seed Vault was established with the 'objective to provide a safety net for the international conservation system of plant genetic resources, and to contribute to the securing of the maximum amount of plant genetic diversity of importance to humanity for the long term in accordance with the latest scientific knowledge and most appropriate techniques'.

Ensuring that the genetic diversity of the world's food crops is preserved for future generations is an important contribution towards the reduction of hunger and poverty in developing countries. This is where the greatest plant diversity originates and where the need for food security and the further development of agriculture is most urgent. The Svalbard Global Seed Vault, which is established in the permafrost in the mountains of Svalbard, is designed to store duplicates of seeds from seed collections around the globe. Many of these collections are in developing countries. If seeds are lost, e.g. as a result of natural disasters, war or simply a lack of resources, the seed collections may be re-established using seeds from Svalbard. The loss of biological diversity is currently one of the greatest challenges facing the environment and sustainable development. The diversity of food crops is under constant pressure. The consequence could be an irreversible loss of the opportunity to grow crops adapted to climate change and new plant diseases, and the seeds of an expanding population of more than 200,000 crop varieties from Asia, Africa, Latin America and the Middle East—drawn from vast seed collections maintained by the Consultative Group on International Agricultural Research (CGIAR)—will be shipped to a remote island near the Arctic Circle, where they will be stored in the Svalbard Global Seed Vault (SGSV), a facility capable of preserving their vitality for thousands of years.

23.12 Conservation Benefits

The value of genetically coded information can never be determined a priori but rather only from a posterior observation as a result of their success on the market. To assign monetary value to the benefits generated by PGR conserved in ex situ collections is exceedingly difficult because they have multiple dimensions. The use of germplasm in plant breeding leads to changes in crop output and breeder's requests represent the demand for germplasm in terms of crop in production and trade. Little information is available on the germplasm movement. Only some gene banks and national programmes have drawn up some statistics. Over the past 3 years, for instance, the CGIAR centres have distributed an annual average of over 50,000 accessions to national programmes all over the world (SGRP 1996). Similarly between 1992 and 1994, the Unites States distributed over 100,000 samples each year (FAO 1996). In developing countries, 34 % of all accessions are stored in public gene banks, 49 % in developed countries, 1 % private companies and 0.2 % local conservators (Iwanaga 1993). In India, Ethiopia and China, the expected value for PGRFA conservation is assessed as being very high (Virchow 1999). The benefits of conservation must be analysed more in depth to guide the conservation investment on national and international levels. It is necessary to define the conservation objectives for adjusting these to some cost-efficiency criteria. The conservation investments must follow the criteria of cost efficiency. Objectives must be quantified. In the accessibility of germplasm for present and future use, the link between conservation and utilisation and distribution and characterisation has to be strengthened (http://cropgenebank.sgrp.cgiar.org/).

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Conservation and Management of Endemic and Threatened Plant Species in India: An Overview

24

Radhamani Jalli, J. Aravind, and Anjula Pandey

Abstract

Endemic and threatened plant species are a vital component of plant biodiversity which require immediate human intervention to ensure their long-term survival. Natural and anthropogenic factors are putting in jeopardy such vulnerable plant groups in India, which on the one hand is one of the mega-diverse nations in terms of plant diversity while on the other hand is one of the most populous nations of the world with a rapidly growing economy. Strategies for conservation of plant diversity include *in situ* approaches in the form of protected areas such as national parks, biosphere reserves and gene sanctuaries and *ex situ* approaches such as seed genebanks, field genebanks and *in vitro* conservation. A judicious integration of diverse conservation approaches can not only help in rescuing plant species in danger but also in widening our knowledge base of these species which can in turn pave way for their sustainable use for the benefit of mankind. The present chapter reviews the current status of endemic and threatened plant species in India, followed by the various conservation strategies which are being or which can be employed for the conservation of such species in India along with the future perspectives in this field.

Keywords

Biodiversity • Indian flora • Endemic species • Genebanks • *In vitro* conservation • *Ex situ* conservation • Field genebanks • Botanical gardens • Cryopreservation • DNA banks

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24.1 Introduction

The single most important factor that makes our planet unique is perhaps the existence of life. The sum total of the multitudes of forms in which life exists is known as biodiversity. From the point of its origin, around three to four billion years ago, life has been shaped by the dynamic process of evolution to form the diversity in living organisms we see today (Dawkins 2005). The balance between speciation and extinction dictates this diversity. Planet earth has undergone five periods of mass extinctions due to a variety of reasons. It is suggested that today, we are in the middle of the sixth period which is predominantly driven by anthropogenic reasons including climate change, unlike the previous five (Barnosky et al. 2011; Root et al. 2003). High extinction rates coupled with the fact that only 86–91 % of the estimated 8.7 million species on earth have been described lead to the conclusion that many species will go extinct before we have a chance to describe them (Mora et al. 2011). With a goal of stemming this biodiversity loss through conservation and policy interventions, the United Nations has declared 2010–2020 as the decade on biodiversity (CBD c2014a).

Conservation in any form involves considerable costs, so prioritization of conservation efforts is of prime importance. The identification of priority areas and species for conservation is undertaken on the basis of extent of biodiversity, level of past, present of future threat, species richness, percentage endemism and potential end use (Plucknett et al. 1983; Wilson et al. 2006). Among the 298,000 estimated plant species on earth (Mora et al. 2011), around 7,000 have been grown for human consumption in human history, and presently, we depend on only 30 species for 95 % of food energy needs (FAO c2014). Considerable success has been achieved in the conservation of diversity of such species of immediate utility particularly under *ex situ* conditions. Most of the accessions held in the genebanks around the world such as the National Genebank (NGB) in National Bureau of Plant Genetic Resources (NBPGR), New Delhi, are of such species. The conservation of the vast majority of other plant species has been largely limited to *in situ* conservation in protected areas, even though specialized genebanks such as Kew's Millennium Seed Bank have achieved considerable success in *ex situ* conservation.

In the present chapter, the status and management efforts for plant species of prime concern in India, one of the richest nations in the world with respect to floral diversity, are discussed with emphasis on management of endemic and threatened taxa.

24.1.1 India: A Hub of Biodiversity

India, the seventh largest country in the world, is considered as one of the most biodiverse regions of the planet. The multifarious combinations of topography, geology and climate have led to the evolution of highly diverse life forms in the country. This rich biological heritage has led to the recognition of India as one of the elite megadiverse nations of the world, a group of 17 countries estimated to hold ~70 % of the Earth's biodiversity (Mittermeier et al. 1997). Occupying only 2.4 % of world's land area, India harbours 7–8 % of recorded species of the world which includes 7.43 and 11 % of global faunal and floral diversity, respectively (Goyal and Arora 2009).

24.1.2 Floristic Diversity of India

India is bestowed with a huge diversity has made in the plant species. Hooker and Thompson (1855) in one of the earliest attempts and to inventorize the Indian floral diversity had observed the presence of representatives of almost every family of the world. In terms of plant diversity, India ranks tenth in the world and fourth in Asia. There are over 45,500 recorded floral species in India, including 17,527 angiosperms, 67 gymnosperms, 1,200 pteridophytes, 2,500 bryophytes, 2,223 lichens, 14,500 fungi, 7,175 algae and 850 virus/bacteria. 7 % of world's documented flowering plants are from India with the major families being Poaceae, Orchidaceae,

Leguminosae, Asteraceae, Rubiaceae, Cyperaceae, Euphorbiaceae and Acanthaceae (Goyal and Arora 2009). Four of the 234 global centres of plant diversity exist in India (UNEP–WCMC 2013). Furthermore, India is one of the Vavilovian centres of origin and diversity of cultivated plants possessing 811 cultivated crop species and 902 crop wild relatives (Singh et al. 2013).

24.1.3 Endemism and Threat Status of Indian Flora

A taxonomic category entirely restricted to a given biogeographical unit is referred to as an endemic. Such a restricted range is enforced through a combination of geographical factors such as unique edaphic, geological, ecological and climatic conditions and biological factors such as reduced seed production, reduced pollination, limited dispersal and interspecies interactions (Hobohm et al. 2014).

The migration of the Indian plate northward from Gondwanaland to Eurasia has left their mark, and the present-day Indian flora is an amalgamation of several global genera (Axelrod 1971). Notwithstanding the unique geological history, the relatively recent isolation of Indian flora owing to the Himalayas in the north, hot and arid desert in the west and the Arabian Sea, Bay of Bengal and the Indian Ocean in the south has aided the evolution of several endemic taxa (Nayar 1996). The diverse ecosystems and habitats of India have made it a home of several endemic plant species. Species endemism is one of the criteria for defining biodiversity hotspots for setting conservation priorities. The geographical boundaries of India overlap with 4 of the 34 such biodiversity hotspots identified around the world, viz., the Himalaya, Indo-Burma, the Western Ghats and Sri Lanka and Sundaland (Mittermeier et al. 2004). Further, Nayar (1996) has identified three mega-centres of endemism and 27 micro-endemic centres in India (Fig. 24.1).

Around 33.5 % of Indian flora have been identified as endemic and are distributed predominantly in the Indian Himalaya, Peninsular India and the Andaman and Nicobar Islands (Nayar 1996). Even though several of the 20,074 India angiosperm species are endemic, only 49 endemic genera and no angiosperm family strictly endemic to India have been identified (Irwin and Narasimhan 2011). Among the 101 species of gymnosperms existing within India, seven are endemic (Srivastava 2006). Even though not sufficiently surveyed in comparison to higher plants, 49 pteridophyte taxa out of 900–1,000 and 340 bryophyte taxa out of 2,489 are endemic to India (Chadha et al. 2008; Dandotiya et al. 2011).

This rich and unique floral diversity of India is extensively under threat as India is also one of the most populous countries in the world with a fast-growing economy. Furthermore, the very nature of endemics makes them unusually vulnerable to extinction (Myers 2003). Global assessment of species threat status to highlight species threatened with extinction and prioritize conservation efforts is carried out globally by the International Union for Conservation of Nature (IUCN) since 1966 in the form of red lists (The IUCN Red List of Threatened Species, Version 2013.2 2013). The IUCN Red List 2013.2 lists 5,512 Indian angiosperm species. Most of the assessed species (4,511 species) are of least concern status (LC) unlike the world status of assessed plants (Fig. 24.2). However, 137 are not threatened (NT), 242 are vulnerable (VU), 232 are endangered (EN) and 77 are critically endangered (CR). Six species have been declared extinct (EX) and one species is extinct in the wild (EW) (Fig. 24.2). The state-wise distribution of these assessed angiosperm taxa shows their non-uniform distribution according to the threat status across India (Fig. 24.3).

In India, in addition, national level assessments have been undertaken by the Botanical Survey of India (BSI) leading to the publishing of three volumes of the *Red Data Book of Indian Plants* following the pre-1994 IUCN Red data book threat status categories. Assessment was undertaken for 940 taxa, of which 99 are indeterminate (I), 6 are indeterminate/potentially extinct, 397 are rare (R), 178 are vulnerable (V), 32 are endangered or potentially extinct (E/PEx), 175 are endangered (E) and 47 are extinct or poten-

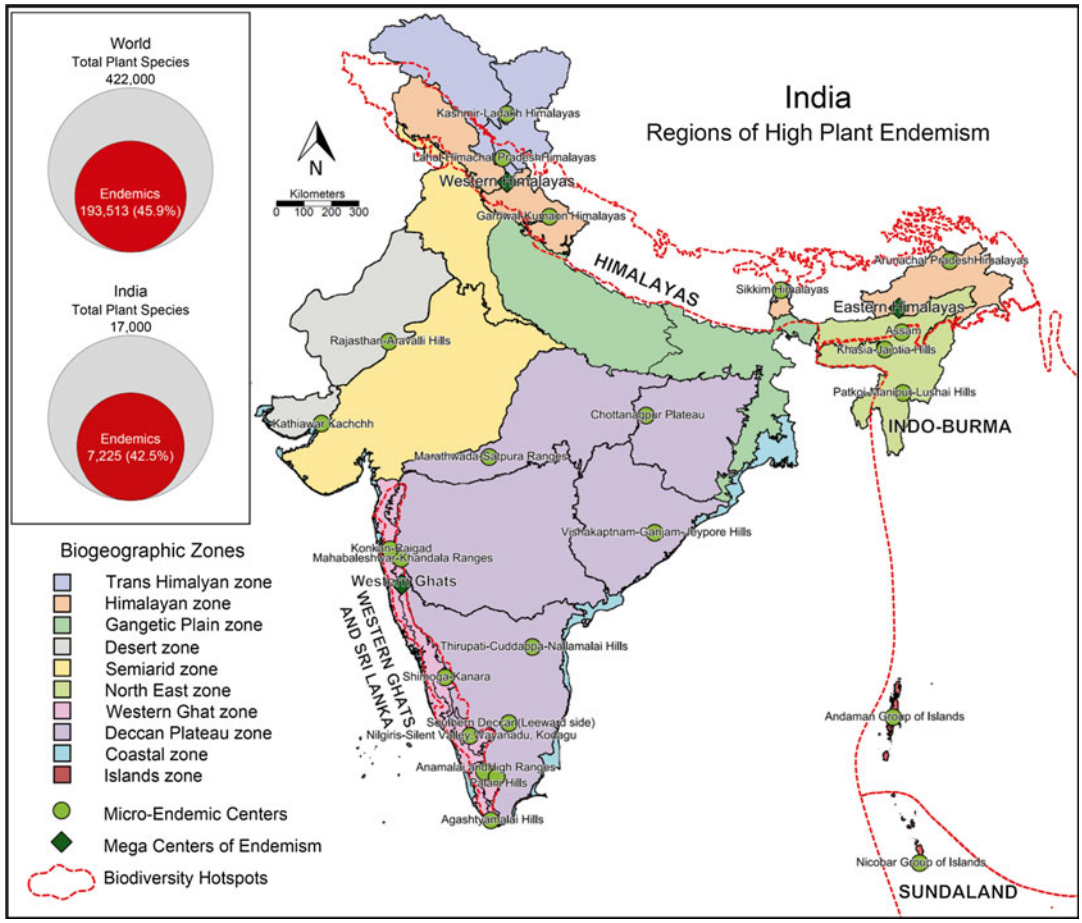

Fig. 24.1 Regions of high plant endemism in India – the biodiversity hotspots (Mittermeier et al. 2004; Conservation International 2004), mega-centres of endemism and micro-endemic zones (Nayar 1996), across biogeographic zones of India (Rodgers et al. 2000). Comparison of status of endemic plants of India with the world is given in the inset (Pitman and Jørgensen 2002)

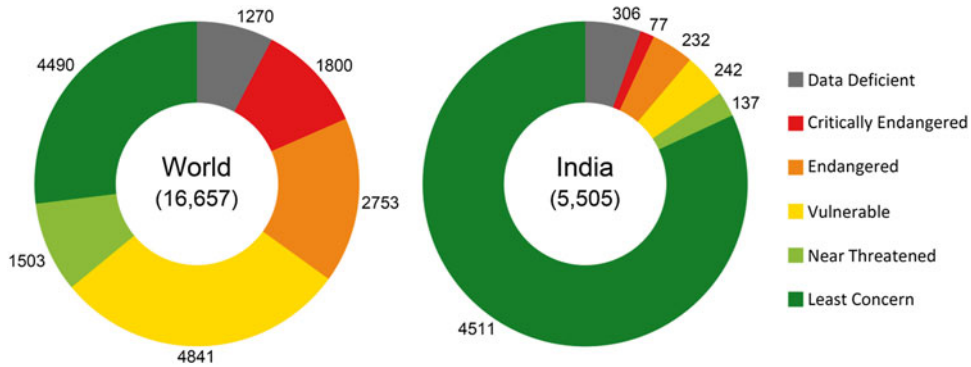

Fig. 24.2 Proportion of living plant species from India and the world under different threat categories (The IUCN Red List of Threatened Species, Version 2013.2 2013)

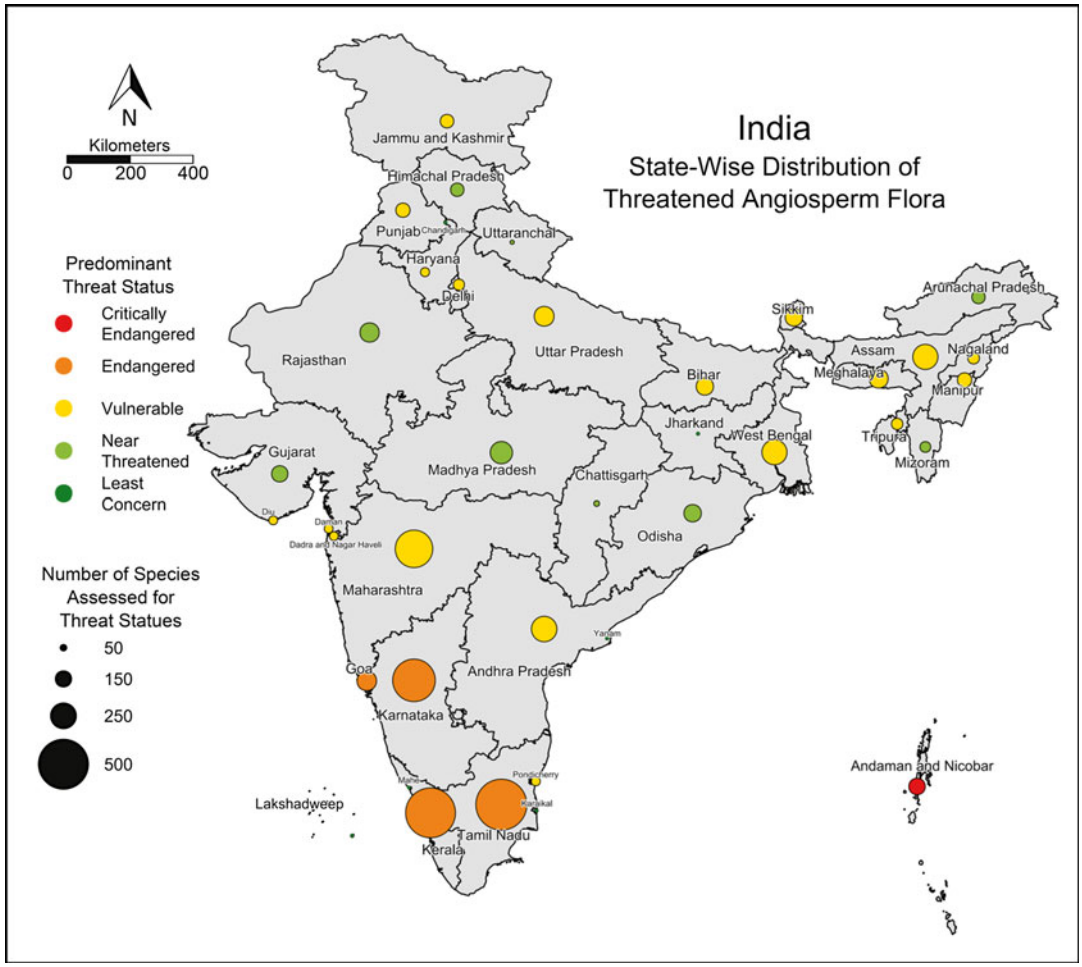

Fig. 24.3 State-wise distribution of threatened angiosperm flora in India (The IUCN Red List of Threatened Species, Version 2013.2 2013)

tially extinct (Ex/PEx) (Nayar and Sastry 1987, 1988, 1990).

However, these assessments of endemism and threat are far from adequate, as only a tiny fraction of Indian flora have been systematically investigated for threat status. Furthermore, the state-wise distributions of threatened angiosperm flora in Fig. 24.3 may partially be a reflection of the regional differences in botanical research intensity. Similar differences are also revealed when the number of assessed and threatened plant species in different angiosperm families are compared (Fig. 24.4) as several major Indian families are underrepresented. Other plant groups such as pteridophytes and bryophytes are also grossly underrepresented in such assessments.

For the current chapter, we have largely followed the assessments of *Red Data Book of Indian Plants* (Nayar and Sastry 1987, 1988, 1990) and The IUCN Red List of Threatened Species, Version 2013.2 (2013) in describing the threat status of species.

24.2 Conservation Strategies

Germplasm conservation is defined as the management and use of biosphere resources which may yield the sustainable benefit to present generations while maintaining its potential to meet the needs of future generations (IUCN/UNEP/WWF 1980). Anthropogenic forces have been

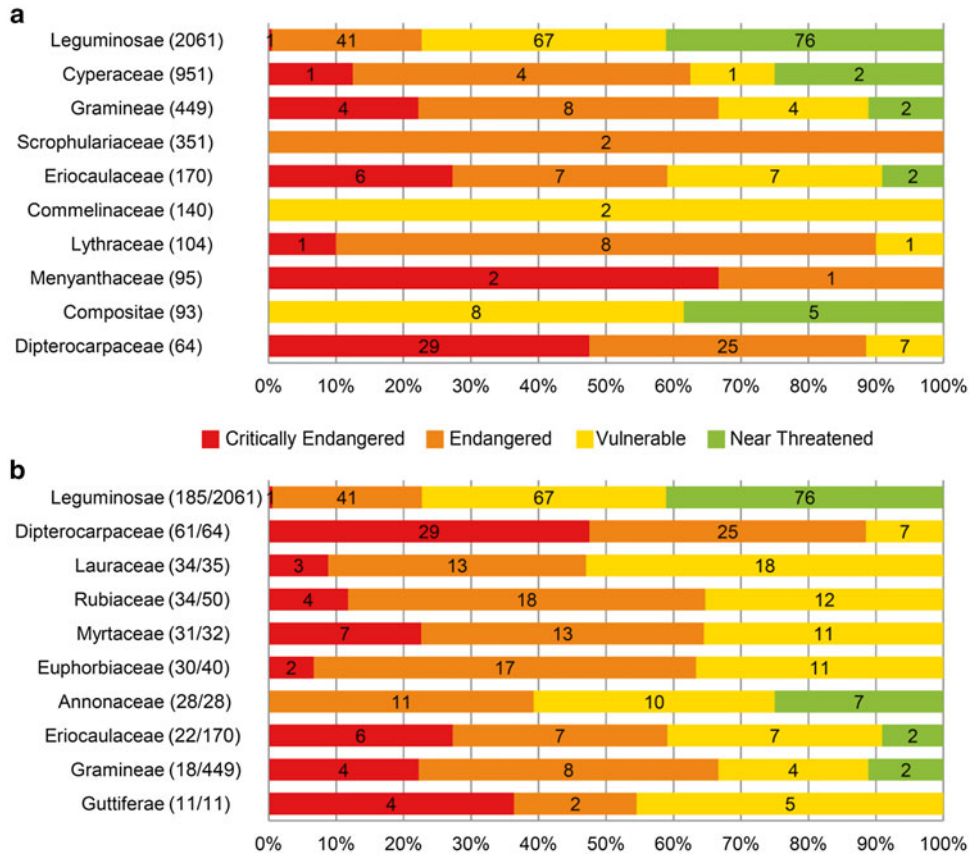

Fig. 24.4 The top ten (**a**) assessed and (**b**) threatened Indian angiosperm families according to the number of species (The IUCN Red List of Threatened Species, Version 2013.2 2013); numbers in parentheses indicate (**a**) the number of assessed species or (**b**) the number of threatened species/number of assessed species

largely detrimental to the survival of a great number of plant species including several unique and important taxa. The conservation of these plant species on priority is the need of the hour. The conservation of plant genetic resources has long been realized as an integral part of biodiversity conservation, and the systematic efforts towards its comprehensive execution are gathering pace albeit under several limitations such as limited information and resources and the lack of adequate guidelines for implementing the new and evolving policy requirements under the framework of the Convention on Biological Diversity (CBD). CBD is the umbrella multilateral treaty under the aegis of the United Nations for ensuring conservation, sustainable use and equitable sharing of benefits of biodiversity around the world (UNEP 1992).

Conservation strategies broadly involve two approaches – *in situ* and *ex situ* based on the site of implementation. They are complementary to each other and can be judiciously integrated based on the species to be conserved and whether medium- or long-term preservation is required. Conservation methods to be employed depend on the objective of conservation, the breeding system of species, its threat status, seed physiology and storage behaviour and the available resources for conservation. *In situ* conservation involves maintenance of genetic resources within the existing natural habitat of the species, which is highly susceptible to natural calamities, whereas *ex situ* conservation involves conservation outside the natural environment of the species and is used to safeguard populations of species which

are in immediate danger of destruction or as a safety duplicate of the existing *in situ* collection.

24.2.1 *In Situ* Conservation

In situ conservation is the management of species within their natural ecosystems and habitats. Viable populations of the target species are maintained in their natural surroundings with minimal human intervention (UNEP 1992). Generally to achieve this, whole forest ecosystems and the containing species are preserved in protected areas through managed use (Iwanaga 1995). This is particularly preferred for those taxa which are less amenable to external disturbances. Moreover, for several species with highly specialized adaptations, it is the only viable conservation strategy available. Apart from being the most convenient and economical conservation approach, it allows the dynamic forces of evolution to continue to act on the natural populations enabling them to continue to adapt to the prevailing conditions (Saunier and Meganck 1995). Furthermore, as habitats and ecosystems as a whole are protected, aside from the target species, several associated species are simultaneously conserved in the natural environment.

In situ conservation is generally practised for forestry species, wild relatives of crop species, medicinal and aromatic plants and the species that are under various levels of threat of extinction, because they cannot be grown outside their habitat or they are the members of complex ecosystems, like tropical forest trees, have high dormancy or possess highly specialized breeding system.

Depending on the ecological hierarchy targeted for conservation, *in situ* conservation approaches are of species, habitat or ecosystem kind.

24.2.1.1 Species Approach

The conservation efforts are concentrated towards a single species in this approach with the intention that along with it, the associated species in the ecosystem are conserved. These may be 'keystone' species, may be those which have a crucial role in the ecosystem and may be whose disappearance may lead to the collapse of the same. They can also be 'umbrella species' with large home ranges for survival of a breeding population, whose protection ensures that a huge area with all the concomitant species is protected. Moreover, to engage the society in conservation efforts, certain threatened 'flagship species' with features that appeal to the general public are used as rallying points (Caro 2010).

24.2.1.2 Habitat Approach

Conservation efforts in this approach are focused towards the maintenance of the quality of strategic habitats on a landscape scale in an ecosystem which harbour multiple species. As the habitats and the dynamic ecological processes existing within are conserved as a whole, thriving populations of multiple interacting species are conserved. Such protected areas include managed forests, sacred groves, nature reserves, preservation plots, wildlife sanctuaries and national parks (Griffiths and Vogiatzakis 2011).

24.2.1.3 Ecosystem Approach

This approach has become the foundation of conservation efforts under CBD. It is a strategy for the integrated management of land, water and living resources that promotes conservation and sustainable use in an equitable way. In addition to the different processes, functions and interactions among different organisms and environment and the integral role of humans in ecosystems are acknowledged under this. Biosphere reserves of the United Nations Educational, Scientific and Cultural Organization-World Network of Biosphere Reserves (UNESCO-WNBR) come under this approach. Initiated under UNESCO's Man and Biosphere (MAB) programme, a biosphere reserve involves the conservation of various representative ecosystems of earth which are large enough to ensure self-perpetuation and evolution of organisms. It also envisages national and international cooperation for harmonious interaction between people and environment (Arora and Paroda 1991).

24.2.1.4 Protected Areas (PAs) in India

The term 'protected area' is defined in the Convention on Biological Diversity (Article 2) as 'a geographically defined area, which is designated or regulated and managed to achieve specific conservation objectives'. Protected areas (PAs) are the important strength of biodiversity conservation as they maintain key habitats, provide refugia, allow for species migration and movement and ensure the maintenance of natural processes across the landscape. They not only ensure biodiversity conservation but also secure the well-being of humanity itself. PAs provide livelihoods for nearly 1.1 billion people, are the primary source of drinking water for over a third of the world's largest cities and are a major factor in ensuring global food security. PAs, when suitably managed, are recognized as the key to both mitigation and adaptation responses to climate change. They are set aside for the protection of biological diversity along with the natural and associated cultural resources and are managed through legal or other effective means (CBD c2014b). They include national parks and nature reserves, sustainable use reserves, wilderness areas and heritage sites (Chape et al. 2008). For the effective conservation in PAs, IUCN has categorized them according to management objectives.

In India, a network of 668 PAs has been established comprising of 102 national parks, 515 wildlife sanctuaries, 47 conservation reserves and 4 community reserves (MoEF c2014a) (Fig. 24.5). Generally, PAs are constituted and governed under the provisions of the Wild Life (Protection) Act, 1972, enforced through the Ministry of Environment and Forests. The act has been amended several times according to the changing requirement of PAs management.

Moreover, in India, two important land mammals, the tiger and the Asiatic elephant, have been used as flagship species for conservation, and today, there are 43 tiger reserves and 26 elephant reserves with a cumulative protecting area of over 60,000 km^2 for each species. In addition, sanctuaries in Tura range in Garo Hills of Meghalaya for the conservation of *Citrus* and *Musa* species and for *Rhododendrons* and orchids in Sikkim have been established.

With an objective of an ecosystem-level conservation, the government of India has established 18 biosphere reserves (MoEF c2014b), which protect large land and/or coastal environments and often include several protected areas within. People being an integral component of the system, their rights are also protected to ensure sustainable economic use to a limited extent. Nine of these are part of UNESCO-WNBR international network of biosphere reserves.

Being party to several major international conventions such as the Convention on International Trade in Endangered Species of wild fauna and flora (CITES), the Convention on Biodiversity (CBD), the International Union for Conservation of Nature (IUCN), the International Convention for the Regulation of Whaling, the UNESCO World Heritage Convention, the Ramsar Convention on Wetlands, the United Nations Framework Convention on Climate Change (UNFCCC), the United Nations Convention to Combat Desertification (UNCCD), the United Nations Commission on Sustainable Development (UNCSD), the International Treaty on Plant Genetic Resources (ITPGR), the United Nations Convention on the Law of the Sea (UNCLOS) and the Convention on Migratory Species (CMS), the biodiversity protection efforts in India are in tune with the global goals of conservation.

In addition to these official efforts, there are several non-governmental organizations (NGOs) involved in the conservation in India (WWF India 2008), for example, *in situ* conservation of medicinal plants by the NGO Foundation for Revitalisation of Local Health Traditions (FRLHT) in medicinal plant conservation areas established in the South Indian states (FRLHT 2006).

24.2.2 *Ex Situ* Conservation

Efforts for *ex situ* conservation of plant biodiversity have so far mainly focused on cultivated crop species. Such activities for wild, rare and endan-

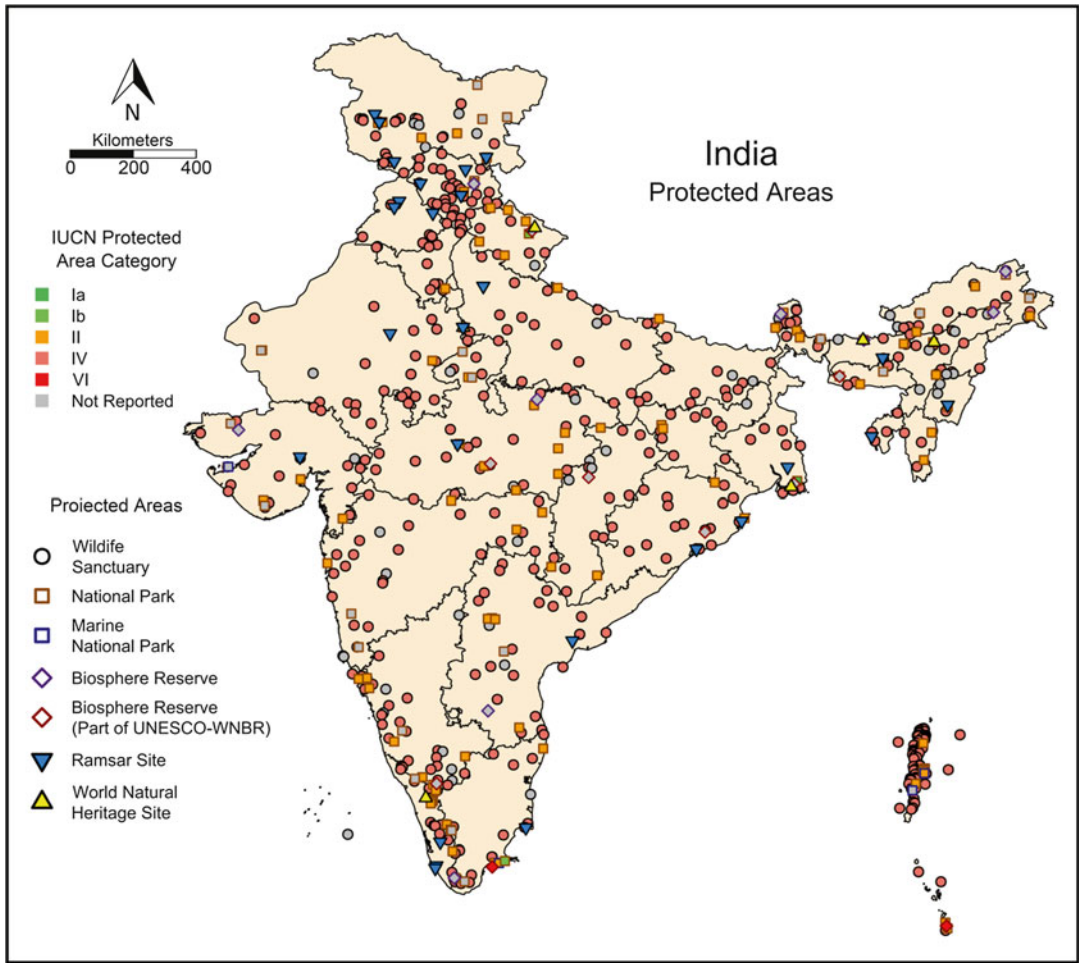

Fig. 24.5 Protected areas in India (Data sourced from The World Database on Protected Areas (WDPA) (2010))

gered species have largely been modest in comparison which is a key issue of concern today. After inclusion in Article 9 of CBD, *ex situ* conservation has gained greater focus globally (Glowka et al. 1994). At the same time, the world's biodiversity is declining at an alarming rate (Sarasan et al. 2006). During the period 1996–2004, a total of 8,321 plant species have been added to the Red List of Threatened Species (Baillie et al. 2004), and the number of plants recorded as critically endangered has increased by 60 %. In the case of wild species, the traditional conservation approach is *in situ*; however, it is now recognized that *ex situ* techniques can be efficiently used to complement *in situ* methods, and they may represent the only option for conserving certain highly endangered and rare species (Ramsay et al. 2000). The different *ex situ* conservation approaches are:

- Field Genebank
- Seed Genebank
- *In Vitro* Genebank
- Cryo-Genebank
- Pollen bank
- DNA Bank

24.2.2.1 Field Genebank

Field genebanks provide an easy and ready access to the plant genetic resources for characterization, evaluation or utilization, while the same material conserved in seed *in vitro* or cryo-genebanks must be germinated or regenerated

and grown before it can be used. Field genebanks, however, are generally more expensive to maintain, requiring more labour, more inputs and more space (land) than other methods of conservation. Moreover, they also have higher levels of risk from natural disasters and adverse environmental conditions like drought, floods or attacks from pests and diseases. They include botanical gardens, arboreta, herbal gardens and clonal repositories.

24.2.2.1.1 Botanical Gardens

Botanical gardens hold adequate and accurately identified samples of plants as living collections, including collections from arboreta, other field genebanks, seed genebanks and *in vitro* propagated material. In addition, they possess both propagation facilities and the scientific and technical skills for species recovery and establishment. UNEP (1995) has estimated that botanical gardens conserve more than one third of the world's flowering plants. Such botanical gardens present worldwide have been classified based on their roles by Wyse Jackson and Sutherland (2000) (Table 24.1).

Botanical gardens play a vital role in *ex situ* conservation and protection of endemic and threatened plants. Bringing plants into cultivation is the primary initiative towards saving them from extinction followed by ensuring their survival in suitable protected sites in the wild (Heywood and Jackson 1991). The International Union for Conservation of Nature and Natural Resources (IUCN) has greatly emphasized the need of developing botanical gardens into a global site for *ex situ* conservation of plants (Bramwell et al. 1987). The network of botanical gardens in every country plays a significant role in *ex situ* conservation of plant genetic resources (Nayar 1987). In India, there are over 140 botanical gardens, including those attached to university botany departments, public parks and agri-horticultural gardens (Chakraverty and Mukhopadhyay 1990). Over 38 botanical gardens conserve around 246 rare and threatened plants of India as living plant collections, and among these, 32 species are catalogued in the Indian Red Data Books (Chakraverty et al. 2003). A few such examples are given in Table 24.2. Furthermore, there are several plants species of botanical interest such as *Rafflesia*, several orchids, Bromeliads, *Nepenthes* and other insectivorous taxa and aquatic species including *Victoria amazonica*, *Nymphaea gigantea*, *Nelumbo nucifera* and *Euryale ferox* which can only be protected in botanical gardens under *ex situ* conditions.

24.2.2.1.2 Arboreta

Arboreta are special spaces set apart for the cultivation and display of a wide variety of different kinds of trees and shrubs. Many tree collections have been established as arboreta. Arboreta differ from pieces of woodland or plantations because they are botanically significant collections with a variety of species. For example, in India, the Ooty arboretum has both indigenous and exotic tree species such as *Hypericum hookerianum* and *Alnus nepalensis*. In addition, the Regional Plant Resource Centre (RPRC), Bhubaneswar, Odisha, has an arboretum with 1,430 species of trees.

24.2.2.1.3 Herbal Gardens

There are about 71 herbal gardens with 4,024 species in India which have been established with the objective of conservation of local biodiversity in medicinal and aromatic plants (Rao and Das 2011). These are basically maintained to strengthen *ex situ* conservation of medicinal and aromatic plants and provide access to quality planting material for utilization.

24.2.2.1.4 Clonal Repositories

Plant genetic resources of different fruit plants/tree species and many ornamentals are maintained by vegetative propagation in order to keep their genetic makeup true to the type. In the case of fruit germplasm, usually, conservation is done in such field genebanks which are also known as varietal collection or living collection. A clonal repository is a facility where clonal materials are conserved as living collections in a field, orchard or plantation. Here, the plant genetic resources are kept as live plants that undergo continuous growth and require continuous maintenance. They are often used when the germplasm is either

Table 24.1 Types of botanical gardens (Wyse Jackson and Sutherland 2000)

Type	Description
'Classic' multipurpose gardens	These institutions have a broad range of activities in horticulture and related training; research, particularly in taxonomy with associated herbaria and laboratories and public education and amenity
Ornamental gardens	These are establishments with diverse plant collections that are documented They may or may not have any role in research, education or conservation
Historical gardens	They are earliest gardens developed for the teaching of medicine and for religious purpose. A number of these gardens are still active in medicinal plant conservation and research and today are primarily concerned with the collection and cultivation of medicinal plants and increasing public awareness about them
Conservation gardens	They are developed in response to local needs for plant conservation. Some have associated areas of natural vegetation in addition to their cultivated collections. Included in this category are native plant gardens. Most conservation gardens play a role in public education
University gardens	Many universities maintain botanical gardens for teaching and research. Many are open to the public
Combined botanical and zoological gardens	These are currently reassessing the roles of their botanical collections. Plants collections are being researched and developed that provide habitats for the displayed fauna, and interpretation of these habitats to the general public is an important element
Agro-botanical and germplasm gardens	These function as an *ex situ* collection of plants of economic value or potential for conservation, research, plant breeding and agriculture. Several are experimental stations associated with agricultural or forestry institutes and contain associated laboratories, plant breeding and seed testing facilities, but many are not open to the public
Alpine or mountain gardens	These are most frequently in mountain regions of Europe and some tropical countries. They are specifically designed for the cultivation of mountain and alpine flora or in the case of tropical countries for the cultivation of subtropical or temperate flora. Some alpine and mountain gardens are satellite gardens of larger lowland botanical gardens
Natural or wild gardens	Contain an area or natural or seminatural vegetation, which is protected and managed. Most are established to play conservation and public education roles and include areas where native plants are grown
Horticultural gardens	These are often owned and maintained by horticultural societies and open to public. They exist primarily to foster the development of horticulture through the training of professional gardeners and are involved in plant breeding, registration and conservation of garden plant varieties
Thematic gardens	These specialize in growing a limited range of related or morphologically similar plants or plants grown to illustrate a particular theme generally in support of education, science, conservation and public display. These include orchids, rose, *Rhododendron*, bamboo and succulent gardens or gardens established on such themes as ethnobotany, medicine, bonsai, butterfly gardens, carnivorous plants and aquatic plants
Community gardens	These are generally small gardens with limited resources, developed by a local community to fulfil its particular needs, such as recreation, education, conservation, horticultural training and the growth of medicinal and other economic plants

difficult or impossible to conserve as seeds (i.e. when no seeds are formed, seeds are recalcitrant or seed production takes many years, as for many tree species) or the plant reproduces vegetatively.

24.2.2.2 Seed Genebank

A large number of plant species particularly crop species have seeds with orthodox seed storage behaviour which can be dried down to low moisture content (3–7 %) and thus can be stored at

Table 24.2 Some important rare and endangered species maintained at botanical gardens in India (BGCI c2014)

Sl. no.	Botanical/Latin name	Family	Conservation status	Method of conservation (remarks)
1.	*Adhatoda beddomei*	Acanthaceae	Rare	Seedlings established in botanical garden
2.	*Bentinckia condapanna*	Arecaceae	Rare	Seedlings transplanted and establishment in botanical garden
3.	*Cycas beddomei*	Cycadaceae	Critically endangered	Saplings acclimatized and established as seedlings in botanical garden
4.	*Eremostachys superba*	Lamiaceae	Critically endangered	Seedlings from its origin transplanted in botanical garden (poor seed viability was observed)
5.	*Frerea indica*	Asclepiadaceae	Critically endangered	Seedlings from its origin multiplied by *in vitro* propagation and established as seedlings in botanical garden
6.	*Peucedanum dehradunensis*	Apiaceae	Critically endangered	Saplings established from seeds collected from natural habitat in botanical garden (poor seed germination was observed)
7.	*Sophora mollis*	Fabaceae	Endangered	Seedlings established in botanical garden
8.	*Trachycarpus takil*	Arecaceae	Critically endangered	Saplings established from seeds collected from natural habitat in botanical garden (good seed germination was observed)

subzero temperature for a long term (Roberts 1973). Since, handling of dry seeds is much easier over other plant materials and they can remain viable for long periods under controlled conditions, the conservation of orthodox seeds is considered to be the cheapest and safest method for *ex situ* conservation. Today, there are about 7.4 million accessions, or samples of a particular population, stored as seeds in about 1,750 genebanks throughout the world (FAO 2010).

The seeds of intermediate and recalcitrant seeds cannot be conventionally stored for a longer time. Seeds of intermediate category such as papaya, gooseberry, oil palm, etc. can be dehydrated to low moisture levels but are sensitive to freezing, so can be desiccated and stored only for a medium term (Ellis 1991), whereas recalcitrant seeds which are intolerant to both desiccation and freezing can be stored only for a short term, few days to few weeks. Cryopreservation is the only option wherever the standardized protocols are available in recalcitrant seeds.

Seeds can be stored under different storage conditions depending on seed types and time period to be stored. Seeds can be shade dried and stored up to 2 years in sealed plastic containers, paper bags or muslin cloth bags at 18–20 °C and 45 % RH for short term, while for medium term, the seeds can be shade dried and stored up to 5 years in cloth bags, metal cans and plastic jars at 4–10 °C and 35 % RH, and for long term, seeds can be dried and stored in sealed aluminium foil pouches at −18 °C with 6–8 % moisture content on fresh weight basis. At NBPGR, New Delhi, active collections are kept at medium-term storage which are immediately available for distribution, utilization and multiplication, while base collections are stored under long-term conditions for posterity. The minimum standards followed for long-term seed storage in NGB are untreated seeds, seeds with maximum viability ≥85 % minimum 2,000 seeds in self-pollinated and 4,000 in cross-pollinated species, seed moisture content 3–7 %, seed storage temperature −18 °C for long-term and +4 °C for medium-term storage and monitoring of viability – after 10 years in long-term and 5 years in medium-term storage.

The operational sequence to integrate an accession into the genebank involves cleaning, seed health testing, determination of moisture content, drying, viability testing and packaging. The management of seed collections requires that

germplasm accessions be maintained with a high proportion of viable seeds, and this involves storage under appropriate conditions, periodic monitoring of seeds for viability and quantity and their regeneration as and when required.

24.2.2.2.1 Storage of Orthodox Seeds

The methodology followed for processing of orthodox seeds for long-term conservation in the NGB at NBPGR, New Delhi, is as follows. In case of species with known seed storage behaviour, the corresponding standard protocols for viability testing are followed, while for species of unknown category, the seed storage behaviour is predicted based on the seed size, family it belongs to, reproductive biology, desiccation and freezing sensitivity, etc. (Hong and Ellis 1996, Fig. 24.6). Similarly, the germination protocols for viability testing are worked out based on the seed size and ISTA (2003) rules.

Receipt, verification of samples and seed health testing Seed cleaning is done by removal of debris, inert material, damaged and infested or infected seeds and seeds of other species (e.g. weeds) to obtain clean and pure samples of seeds of high physiological quality for storage. To achieve the pest-free status of the germplasm, visual examination and X-ray radiography are done to detect infection and/or infestation, and those samples which may be salvaged without compromising the seed health status are passed on for conservation in NGB wherever possible.

Moisture testing and accessioning For estimation of moisture content, the low constant oven drying method for oily seeds and high constant oven drying method for the other species are used. The moisture content is expressed as a percentage of the weight of the original sample. Qualified seed samples are given a national unique identity – exotic code (EC) numbers or indigenous code (IC) numbers.

Drying and viability testing Seeds are dried in specially designed walk-in seed dryers maintained at 15 % RH and 15 °C temperature. The initial seed viability test is conducted after drying

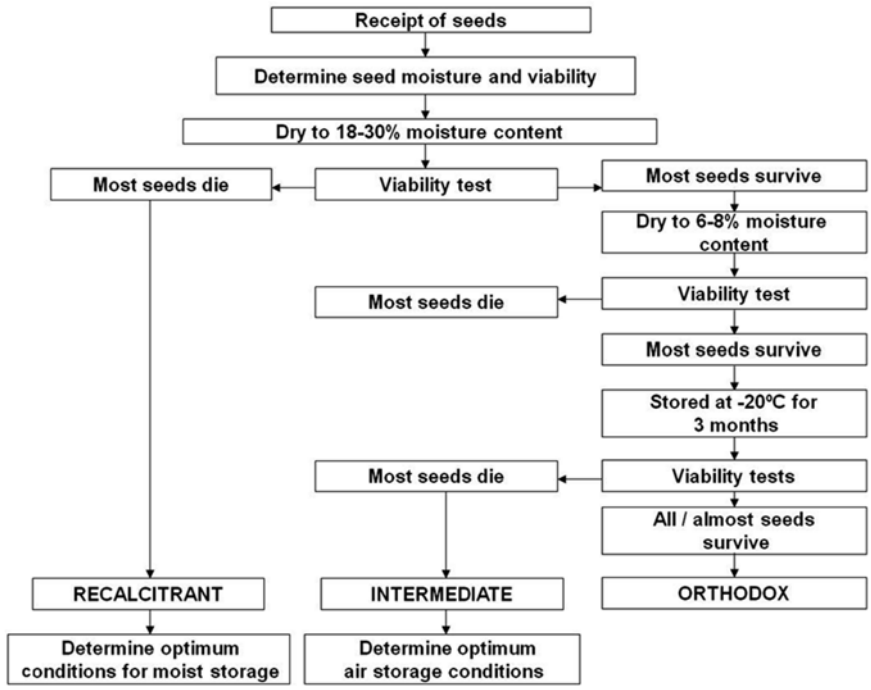

Fig. 24.6 Protocol followed to determine the seed storage behaviour (Hong and Ellis 1996)

of the seed sample and before packaging (ISTA 2003). The initial germination value should exceed 85 % for most seeds of cultivated field crop species. Exceptions may be granted for seed of endemic and threatened plant species.

Seed packaging and storage After drying (desired moisture content of 3–7 %) and viability testing (≥85 %), the seed samples are packed in tri-layered aluminium foil packets (outer polyester layer, 12 µm; middle aluminium foil layer, 12 µm; inner polythene layer, 250 gauge), which have been found most effective for long-term conservation of all types of seeds. Packaging is done in an air-conditioned room with controlled humidity (preferably 15 % RH). The passport data along with the national identity is documented. The minimum data is printed on the labels which are pasted on the respective seed packets. The processed accessions are arranged in plastic baskets serially and placed in cold storage vault at −18 °C at a defined location in the genebank.

Germplasm documentation Correct and reliable recording of data, its documentation and information transfer are as important as proper handling of germplasm itself. Correct and proper documentation allows us to retrieve and utilize the information effectively. Being the most important component of NGB, it is accomplished efficiently through a well-managed computer network.

Today, the National Genebank holds a total of 4,00,098 accessions of different agri-horticultural crops of various crop groups such as cereals, grain legumes, millets, oilseeds, vegetables, fibre and forages, medicinal and aromatic, spices, etc. There are ten modules running at a temperature of −18 °C for a long-term storage of seeds and two modules maintained at medium-term storage conditions for storage of active collections (Fig. 24.6). About 60 accessions belonging to 32 families with orthodox seed storage behaviour holding the status under endemic and threatened category are available in the National Genebank which are listed in Table 24.3. All these species are regularly monitored at an interval of 10 years for seed quality, health and quantity. NBPGR is strengthened with ten regional stations in different agro-climatic regions of India and 59 crop-based National Active Germplasm (NAG) sites for timely regeneration of the required germplasm and its utilization.

24.2.2.2.2 Ultra-Desiccation: A Cost-Effective Conservation Strategy

Ultradry storage technique is a 'low-input' alternative method to the conventional cold storage of seed and allows preservation at room temperature. It is considered a useful low-cost option where no adequate refrigeration is required (Theilade et al. 2004). Research on various aspects of ultradry seed storage, including drying techniques such as sun/shade drying (Probert and Hay 2000) or vacuum/freeze drying (Corbineau and Come 1988) and their applicability to a broader number of species, is under investigation to assess its feasibility in various species.

24.2.2.2.3 Storage of Nonorthodox Seeds

Seeds under this category cannot be maintained under conventional storage conditions. The processing of seeds under this category needs special attention due to the limitation in the availability of specific species and the seed quantity. So seed samples are acceptable even if quantity is limited. These seeds can be only maintained for short to medium term depending on its seed physiology. All seeds are hygroscopic in nature, and they tend to absorb and desorbs moisture depending on the surrounding humidity of the air. So depending on the seed moisture content, the seeds are equilibrated over the saturated salts to a safe and critical moisture content at a safe temperature of 12–15 °C which extends its storability to about 24 weeks (Radhamani et al. 2003). Similarly, the storability could be extended for a period of 3 months by sub-imbibed storage in high osmoticum salts of polyethylene glycol 6000 (PEG 6000) alone or in combination with abscisic acid (Fig. 24.7). A few successful storage methods for nonorthodox seeds are as follows:

Table 24.3 Endemic and threatened species stored in NGB showing orthodox seed storage behaviour (NBPGR 2012)

Sl. no.	Family	Scientific name
1	Acanthaceae	*Barleria prionitis*
2	Alliaceae	*Allium stracheyi*
3	Amaranthaceae	*Achyranthes aspera*
4	Apiaceae	*Pinda concanensis*
5	Apocynaceae	*Tabernaemontana heyneana*
6	Araceae	*Arisaema caudatum*
7	Asparagaceae	*Chlorophytum borivilianum*
8	Asteraceae	*Inula racemosa*, *Saussurea costus*
9	Burseraceae	*Boswellia ovalifoliolata*, *Canarium strictum*, *Commiphora wightii*
10	Caesalpiniaceae	*Hardwickia binata*
11	Calophyllaceae	*Calophyllum inophyllum*
12	Celastraceae	*Lophopetalum wightianum*
13	Colchicaceae	*Iphigenia magnifica*
14	Cycadaceae	*Cycas beddomei*
15	Dioscoreaceae	*Dioscorea deltoidea*
16	Dipterocarpaceae	*Shorea robusta*
17	Fabaceae	*Caesalpinia sappan*, *Gymnocladus assamicus*, *Mucuna pruriens*, *Pterocarpus indicus*, *Pterocarpus santalinus*, *Tephrosia purpurea*, *Vigna khandalensis*, *Vigna trilobata*, *Atylosia cajanifolia*, *Alysicarpus vaginalis*, *Dalbergia latifolia*, *Kingiodendron pinnatum*
18	Flacourtiaceae	*Flacourtia indica*, *Hydnocarpus pentandra*
19	Liliaceae	*Chlorophytum glaucoides*
20	Lythraceae	*Woodfordia fruticosa*
21	Myrtaceae	*Knema attenuata*, *Syzygium stocksii*, *Syzygium zeylanicum*
22	Papaveraceae	*Meconopsis robusta*
23	Poaceae	*Andropogon pumilus*, *Brachiaria reptans*, *Cenchrus biflorus*, *Cenchrus prieurii*, *Digitaria pennata*, *Oryza rufipogon*
24	Ranunculaceae	*Aconitum ferox*
25	Rhizophoraceae	*Rhizophora apiculata*, *Rhizophora mucronata*
26	Rosaceae	*Prunus cornuta*
27	Rubiaceae	*Canthium dicoccum*
28	Santalaceae	*Santalum album*
29	Scrophulariaceae	*Picrorhiza scrophulariiflora*
30	Solanaceae	*Solanum vagum*
31	Vitaceae	*Cayratia pedata*
32	Zingiberaceae	*Hitchenia caulina*

Short- to medium-term storage:

Successful storage methods of recalcitrant seeds

- Short-term storage has been found successful by moist/sub-imbibed storage in polyethylene glycol 6000.
- Partial drying with the application of fungicide (0.2–05 % captan/thiram) and storage in perforated polyethylene bags.
- Equilibration over saturated salt of potassium nitrate ≅94 % RH before storage.
- Alternate drying and wetting of seeds every 15 days during storage.

Successful storage methods of intermediate seeds

- Medium-term storage at 10–12 °C in screw cap plastic bottles
- Medium-term storage at 10–12 °C in paper bags

Seedling conservation. Under this technique, the growth of the young seedlings is arrested at low temperature (12–15 °C) in dark conditions with minimum water-soaked blotting sheets in petri plates (Fig. 24.8). Such seedlings at requirement are transferred to the pots, and further growth is continued at normal temperature

Fig. 24.7 A view of the National Genebank (NGB) at NBPGR showing storage modules on both sides (Pandey et al. 2010)

and light conditions. This technique has been attempted in several tree species such as *Symphonia globulifera*, *Dryobalanops aromatica*, *Syzygium cuminii*, *Caryota urens*, *Saraca indica*, etc. (Marzalina et al. 1992; Radhamani et al. 2003).

Long-term storage:

- *Cryopreservation.* With the advancement of new and innovative technologies in the field of cryobiology, several attempts in different crops have been made to successfully cryopreserve the zygotic embryos and embryonic axes of intermediate and recalcitrant seed species. The embryonic axes are preferred as explants in most cases as they are highly meristematic well-organized small, independent structures. Further details are discussed in Sect. 24.2.2.3.

24.2.2.3 *In Vitro* Conservation

In vitro techniques are useful tools for the conservation of plant biodiversity especially the genetic resources of nonorthodox seeds (where seeds are desiccation/freezing sensitive), vegetatively propagated crop species and rare and endangered plant species. In vitro culture techniques ensure the production and rapid multiplication of disease-free material. Medium-term conservation of *in vitro* cultures is achieved by reducing growth (slow growth) of plant cultures by altering the mineral concentration of the media, adding high osmotic solutions, changing light and

Fig. 24.8 Conventional short-term storage methods in some recalcitrant seed species

temperature conditions of growing cultures thus increasing intervals between subcultures (Fig. 24.9). For long-term conservation, cryopreservation (storage in liquid nitrogen at −196 °C) is the best option which allows the storage of germplasm by ceasing the complete metabolic activity, for extended periods, protected from contamination and with limited maintenance. In recalcitrant seeds, only limited success has been achieved due to their structural complexity and heterogeneity in seed characteristics and seeds being shed at high water content. Excised embryos/embryonic axes, somatic embryos, buds and apical meristems which can be desiccated easily to low moisture content are the alternative systems for low temperature storage. Under this technique, the protocol for the development of the whole plant from the embryos and subsequently their hardening and successful transfer to the field is an essential prerequisite (Radhamani and Chandel 1992; Engelmann 1991).

24.2.2.3.1 Slow Growth Storage

Slow growth in tissue culture is a technique used for the preservation of *in vitro* grown plants (Engelmann 1997). However, the use is limited to few crop species. Minimal growth storage is

Fig. 24.9 A view of the national tissue culture repository at NBPGR (Pandey et al. 2010)

usually applied to differentiated plantlets and developing meristem cultures. The *in vitro* storage programme is influenced by various parameters such as the type of explants used, its physiological maturity, the type of storage material and post-collection storage conditions. These techniques are being developed and practised in several rare and endangered crop species as listed in Table 24.4 (Fay 1992; Sarasan et al. 2006).

24.2.2.3.2 Cryopreservation

Cryopreservation is the storage of biological material at low temperature of −196 °C in the liquid phase of liquid nitrogen for the maximal phenotypic and genotypic stability (Steponkus 1985). This method being relatively convenient and economical, a large number of genotypes and variants could be conserved and thus maximize the potential for storage of genetically desirable material (Engelmann and Takagi 2000). Seeds of some orthodox species, embryos/embryonic axes and dormant buds display natural dehydration processes and do not require any pretreatment for cryopreservation. However, other systems such as cell suspensions, calluses, shoot tips, embryos, etc. contain high amounts of cellular water thereby making the system sensitive and prone to freezing injury. Under such conditions, cells have to be dehydrated artificially to protect them from the damages caused by crystallization of intracellular water (Mazur 1984).

The techniques used are of two types – traditional/classical and new (Withers and Engelmann 1998). Under traditional techniques, system is slowly cooled down to a prefreezing temperature, followed by the rapid immersion in liquid nitrogen. With temperature reduction during slow cooling, the cells and the external medium initially supercool, followed by ice formation in the medium (Mazur 1984). Traditional freezing procedures include pregrowth of samples, cryoprotection, slow cooling to a determined prefreezing temperature and quick immersion of samples in liquid nitrogen, storage, rapid thawing and recovery, whereas the new advance novel cryopreservation techniques involve vitrification-based procedures. Cell dehydration is performed prior to freezing by exposing the samples to concen-

Table 24.4 *In vitro* techniques available in endemic and threatened plant species (Fay 1992; Sarasan et al. 2006)

Sl. no.	Explant used	Botanical name
1	Axillary branching	*Feronia limonia*
2	Axillary bud	*Ceropegia candelabrum*, *Gerbera aurantiaca*, *Rumex acetosella*, *Trichopus zeylanicus*, *Valeriana wallichii*, *Wrightia tomentosa*
3	Bulb scale	*Sternbergia fischeriana*
4	Chlorophyllous root, leaf, internode	*Holostemma ada-kodien*
5	Corm	*Gloriosa superba*
6	Hypocotyl segment	*Pimpinella tirupatiensis*, *Coptis teeta*
7	Hypocotyl, cotyledon	*Meconopsis simplicifolia*
8	Leaf	*Arnebia euchroma*
9	Mature leaves and stem, petiole and roots of young seedlings	*Psoralea corylifolia*
10	Nodal segment (seedling explant)	*Coleus forskohlii*, *Anogeissus sericea*
11	Nodal shoot	*Aconitum napellus*, *Aconitum noveboracense*
12	Nodal segment, hypocotyl, cotyledon and leaf	*Gymnema sylvestre*
13	Node	*Decalepis hamiltonii*, *Pittosporum napaulensis*, *Nepenthes khasiana*, *Syzygium travancoricum*, *Adhatoda beddomei*
14	Node, internode, leaf	*Rotula aquatica*
15	Petiole	*Nardostachys jatamansi*
16	Rhizome	*Geodorum densiflorum*, *Ipsea malabarica*, *Kaempferia galanga*
17	Root	*Decalepis arayalpathra*
18	Root, hypocotyl, cotyledon, leaf	*Saussurea obvallata*
19	Seedling	*Oncidium catharinensis*
20	Seedlings and shoots	*Pterocarpus marsupium*
21	Shoot bases and immature floral buds	*Chlorophytum borivilianum*
22	Shoot segments	*Caralluma edulis*, *Commiphora wightii*
23	Shoot tip	*Swertia chirata*
24	Shoot tips, axillary buds	*Aconitum carmichaeli*, *Rheum emodi*
25	Shoot tips, axillary buds and segment of young leaves	*Renanthera imschootiana*
26	Shoot tips, lateral buds, node, leaf calli, internode	*Rauvolfia serpentina*
27	Shoot tips, leaf base, foliar leaves	*Vanda coerulea*
28	Shoot tips, nodes	*Gentiana kurroo*
29	Shoot tips, nodes, internodes, hypocotyl, embryos, leaf	*Withania somnifera*
30	Stem	*Celastrus paniculatus*, *Dendrobium moschatum*
31	Terminal and nodal segment axillary bud	*Picrorhiza kurroa*
32	Zygotic embryos	*Ocotea catharinensis*, *Podophyllum hexandrum*, *Santalum album*
33	Nodal segment/axillary bud	*Ceropegia fantastica*, *Daphne cneorum*, *Coronopus navastii*, *Frerea indica*, *Huernia hystrix*

trated cryoprotective media and/or air desiccation followed by rapid cooling. Several different vitrification-based procedures are being developed: (a) encapsulation-dehydration, (b) vitrification, (c) encapsulation-vitrification, (d) dehydration, (e) pregrowth, (f) pregrowth-dehydration and (g) droplet-vitrification. Similarly, the seeds/embryos of endemic and threatened species can be cryopreserved using different explants, where the simple known protocols are possible to be implemented.

Although methodology and freezing protocols have been developed for various higher plants (Bunn et al. 2007) including orchids (Hirano et al. 2006) and also bryophytes and fern spores (Pence 2008) using different explants, limited literature is available for cryopreservation of rare and endangered species. Touchell and Dixon (1993) successfully cryopreserved 68 native Western Australian species with pretreatment of seeds with DMSO. Desiccation and freezing were undertaken in seeds of rare temperate orchids by direct immersion in liquid nitrogen (Nikishina et al. 2007). Studies were also conducted with endangered, rare, ancient and wild *Citrus* species (Malik and Chaudhury 2006; Lambardi et al. 2007; Hamilton et al. 2009). Protocorms of *Cleisostoma arietinum*, a rare Thai orchid species, have been cryopreserved following the method of encapsulation-dehydration (Maneerattanarungroj et al. 2007). Similarly, shoot tips of the endemic endangered plant *Centaurium rigualii* were cryopreserved (Gonzalez-Benito and Perez 1997). Turner et al. (2001) and Tanaka et al. (2008) reported successful cryopreservation of shoot tips of endangered Australian and Japanese species, respectively, using the vitrification technique. The droplet-vitrification technique has been used for freezing shoot tips of wild potatoes (Yoon et al. 2007) and wild relatives of *Diospyros* (Niu et al. 2009).

Presently, cryopreservation is employed on a large scale for different types of materials where conventional seed storage methods are not feasible, and established protocols are available. Cryopreservation is used mainly for storing seeds with limited longevity and of rare or endangered species at several genebanks such as the National Center for Genetic Resources Preservation (NCGRP; Fort Collins, CO), the National Bureau for Plant Genetic Resources (NBPGR) New Delhi, India (Fig. 24.10), Kings Park and Botanic Garden in Perth, Australia, and the Center for Conservation and Research of Endangered Wildlife at the Cincinnati Zoo in the USA.

24.2.2.4 Pollen Banks

Cryopreservation of pollen is easy and cost-effective in supplementing the usual germplasm preservation techniques by seed and clonal storage particularly for enriching haploid gene pools. Pollen banks are efficient, economical and space saving, compared to the maintenance of live field collections. Pollen storage was developed as a tool for controlled pollination of asynchronous flowering genotypes, especially in fruit trees and agroforestry species (Alexander and Ganeshan 1988) and is an emerging technology for genetic conservation (Roberts 1975; Omura and Akhima 1980; Withers 1991). Pollen can easily be collected and cryopreserved in large quantities in a relatively small space. In addition, exchange of germplasm through pollen poses less quarantine problems compared with seed or other plant propagules.

24.2.2.5 DNA Banks

DNA from the nucleus, mitochondria and chloroplasts can be routinely extracted and immobilized into the nitrocellulose sheets where it can be probed with numerous cloned genes. Since these techniques are simple, efficient and the samples occupy limited space, they are considered advantageous and may be considered as a practical method of conserving germplasm in the future. Such DNA banks for endemic and threatened species have been established at Kew Botanical Gardens, the UK, and Missouri Botanical Garden, the USA. In India, NBPGR, New Delhi has initiated a DNA banking facility for the conservation of genomic resources of India.

Fig. 24.10 A view of the cryobank repository at NBPGR (Pandey et al. 2010)

24.2.3 Bridging *In Situ* and *Ex Situ* Conservation

Unlike crop species where conservation purpose of a collection is to fuel crop improvement, for endemic and threatened wild plants, the objective is to ensure the survival of self-perpetuating viable populations in the wild. The *ex situ* collections can act as backup and facilitate *in situ* conservation of wild populations (Husband and Campbell 2004). Several strategies to combine the strengths of the two basic approaches to conservation while minimizing the weaknesses have been proposed.

The basic method of such an *inter situ* approach envisages establishment and maintenance of an artificially managed off-site collection maintained within the natural habitat of the species (Given 1987). Such sites may be within the current range of the distribution of the species (Guerrant and Pavlik 1998), in recent past range (Burney and Burney 2007) or completely new areas possessing similar environmental space as the original habitat (Maschinski et al. 2012), where a population can be reintroduced. Uma Shaanker and Ganeshaiah (1997) have proposed a modified methodology for conservation of threatened plant species, wherein by mapping the genetic diversity and geographical distribution of the target species, donor and recipient sites can be identified, and such recipient sites termed as 'forest genebanks' act as sinks for the import of gene pool from outside sites as pollen. A similar strategy termed *quasi in situ* approach has been suggested by Volis et al. (2009). Such strategies have been successfully tested for the conservation of endemic and threatened flora in different parts of the world (Volis et al. 2009; Cochrane et al. 2010) and can be adapted to the Indian scenario.

24.2.4 Geospatial Technology

Geospatial techniques available today are key and powerful tools for assessing geographic information for monitoring land use and land cover changes, changes in landscape, mapping potential, species distributions and for monitoring biodiversity losses. Such techniques have fur-

ther proven useful for conservation and management of natural resources worldwide. The geospatial techniques are a combination of several integrating technologies such as geographic information system (GIS), remote sensing (RS), global positioning system (GPS) and modern information and communication technologies (ICT).

With respect to conservation biology, geospatial techniques have fuelled studies on biodiversity fragmentations, species distributions and levels of species richness and how they operate. With the ongoing simplification of native ecosystems, declining population and rising loss of biodiversity, such techniques take on an even greater role in biodiversity conservation efforts. Increasing number of earth observation satellites with better repetitive information, improvement in spectral bands, spatial resolution from 50 cm to 1 km and also unprecedented number of remote sensing tools have facilitated addressing various biodiversity conservation challenges. The availability of advanced sensors with increased spatial and spectral resolution has enabled conservation biologists for making the direct use of remote sensing for distinguishing species assemblages or even identifying species of individual trees. In India, remote sensing techniques have been used for land use classification mapping particularly for improved management of biodiversity-rich areas and wildlife through precise biodiversity assessment on a larger spatial scale. These techniques can be further exploited to conserve and maintain the forest tree species, wild relatives and medicinal plants particularly for the endemic and threatened species to ensure their efficient and effective conservation (Yadav et al. 2013).

24.3 Future Perspectives

The past 50 years have witnessed a major revolution in the area of biodiversity conservation yet utilization of the same is a recent attempt. The development of new techniques for germplasm conservation and its implementation offers new opportunities for conservation of biodiversity in the form of seeds, pollen, vegetative propagules and DNA. NBPGR, New Delhi, equipped and enriched with all the facilities *such as* seed genebank, *in vitro* repository, cryobank and field genebank widens the scope for the conservation and management of endemic and threatened plant species in India.

The IUCN Red List categories have been widely used throughout the world which was in place for last 30 years. As knowledge about the existing species and threat classification progressed, some countries have developed improved versions at national levels. The threat status kept changing with changing environmental conditions. The species which was at the verge of extinction might have been rescued, regenerated and recovered the status of which is not updated. The lack of available basic biological data led to the failure in species recovery programme. So sequential improvement in indentifying the species and its exact status needs to be identified regularly and updated in the database especially in the areas which are prone to various catastrophes, such as cyclone, landslide, floods and drought.

Limited availability and narrowness of species diversity are the indicators to find out the status of species extinction. Climate change is one of the major factors responsible for species extinction. The conservation of threatened species is challenging such as their ability to move with changing climatic conditions, and often, it may not be feasible due to restricted habitats and interdependence on other species (Preston et al. 2008). The endemic species have evolved in geographic isolation which limits their dispersal to new areas, so the conservation of biodiversity under such conditions demands special challenges for threatened and endemic species, which needs to be designed to fit into the present scenario.

The selection procedure for conservation of threatened species depends on the biological nature of the targeted species, practicality and feasibility of particular method, its cost-effectiveness and security afforded by that application. The development of suitable complementary technique for the species needs to be identified for successful conservation of

gene pool which requires multidisciplinary approach. The future challenges for the conservation are scientific, technical, socio-economical, legal and political including public awareness. Although several strategies suggest creating space for the protection of the natural habitats, but in the developing countries, population explosion may be a major limiting factor for the availability of space. During the last few years, progresses have been made with the development of various biotechnological tools and their implementation in new conservation strategies which should be modified accordingly to accommodate in the changing trends. A coordinated effort of researchers, research institutions, NGOs and communities is the need of the hour that can jointly make an effort for the best suitable conservation depending on the available resources.

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Biotechnological Approaches in Improvement of Spices: A Review

25

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Abstract

Biotechnological approaches like micropropagation, somaclonal variation, *in vitro* conservation, synseed technology, protoplast fusion, production of flavour and colouring components and development of novel transgenics have great potential in conservation, utilization and increasing the production of spices. Efficient micropropagation systems are available for many spices which are being used for propagation, conservation, safe movement and exchange of germplasm, crop improvement through somaclonal variation and transgenic pathways. Studies on the production of metabolites, flavour and colouring compounds using immobilized and transformed cell cultures are being attempted. Molecular markers and maps are being generated for crop profiling, fingerprinting, identification of duplicates, and marker-assisted breeding. Transcriptome sequencing is becoming an important tool for identification, isolation and cloning of useful genes. Bio-technological approaches involving microbials (antogonists/hyper parasites and PGPRs) with broad spectrum of disease suppression and growth promotion have been found effective in crop health management in spice crops.

Keywords

Fingerprinting • Ginger • *In vitro* conservation • Micropropagation • Molecular markers • Somaclonal variation • Spices • Synseed technology • Transgenics • Tree spices • Turmeric • Vanilla

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25.1 Introduction

Spices and herbs are aromatic plants – fresh or dried plant parts of which are mainly used to flavour our food and confectionery and also in medicine and perfumery industry. Spices and herbs are grown throughout the world, and it has been estimated that these crops are grown on an area of 8 million ha globally contributing to 31.6 million tons of spices annually. India's share to world spices production is 6 million tons. The global spice industry amounts to 1.1 million metric tons, accounting to US$3.475 billion in value. India's share at the global level is 0.575 million metric tons, accounting to US$2.037 billion, i.e., 52 % in volume and 58.6 % in value (Source: Spices Board of India 2014).

Black pepper, cardamom, ginger, turmeric, vanilla, cinnamon, clove, nutmeg, tamarind, etc. constitute the major spices, while coriander, cumin, fennel and fenugreek are important seed spices followed by saffron, lavender, thyme, oregano, celery, anise and sage are important herbal spices. The productivity of many of these crops is low due to the lack of high-yielding, pest and disease-resistant varieties. The past few years have witnessed a quantum jump in utilization of biotechnological tools to achieve the above through commercial propagation, development of novel varieties and marker-assisted breeding (Nirmal Babu et al. 2011d).

25.2 Black Pepper

25.2.1 Micropropagation and Plant Regeneration

Micropropagation has been employed for large-scale production of disease-free planting materials and germplasm conservation. High rate of multiplication coupled with the additional advantage of obtaining disease-free planting material makes micropropagation a viable alternative to conventional propagation (Nirmal Babu and Minoo 2003). Black pepper, *Piper nigrum* L., is native to India and is the most important spice in the world. Conserving the genetic diversity and development of *Phytophthora* foot rot resistance are the immediate priorities for all breeding programmes. Technologies for micropropagation of black pepper using various explants were reported (Nirmal Babu 1997; Nirmal Babu et al. 2012b). Multiple shoots can be induced using BA in the culture medium (MS or SH Medium) either alone or in combination with auxins (Fig. 25.1a). Endogenous contamination severely hampers establishment of black pepper cultures to over come this constrains. A commercially viable protocol for large-scale *in vitro* multiplication of black pepper these problems was reported by Nazeem et al. (2004). Protocols were standardized for micropropagation of other endangered and medicinally important species of *Piper* like *P. longum* and *P. chaba*, *P. betle*, *P. barberi* and *P. colubrinum* (Nirmal Babu 1997; Nirmal Babu et al. 2012b). Joseph et al. (1996) and Yamuna (2007) reported the somatic embryogenesis from zygotic embryos, while Nair and Gupta (2003, 2006) reported the cyclic somatic embryogenesis from the maternal tissues, which have tremendous potential for automated micropropagation. Nirmal Babu et al. (2005a) reported the somatic embryogenesis from mature leaf tissues (Fig. 25.1b, c). These protocols are useful in transgenic experiments. Plant regeneration was reported in other *Piper* species like *P. longum*, *P. betle, P. chaba, P. attenuatum* and *P. colubrinum* through direct and indirect organogenesis (Nirmal Babu et al. 2012b). Attempts on induction of variability on somaclones for tolerance to *Phytophthora* foot rot resistance by Shylala et al. (1996) resulted in identification of tolerant somaclones through *in vitro* selection of calli as well as somaclones using crude culture filtrate and toxic metabolite isolated from *Phytophthora capsici.*

25.2.2 Molecular Characterization

Recent advances in molecular biology led to more emphasis on molecular markers for characterization of the genotypes, genetic fingerprinting, identification and cloning of important genes and marker-assisted selection and in understanding interrelationships at the molecular level. Menezes et al. (2009) and Joy et al. (2007, 2011) developed and characterized a total of 16 microsatellite markers from black pepper and used them to

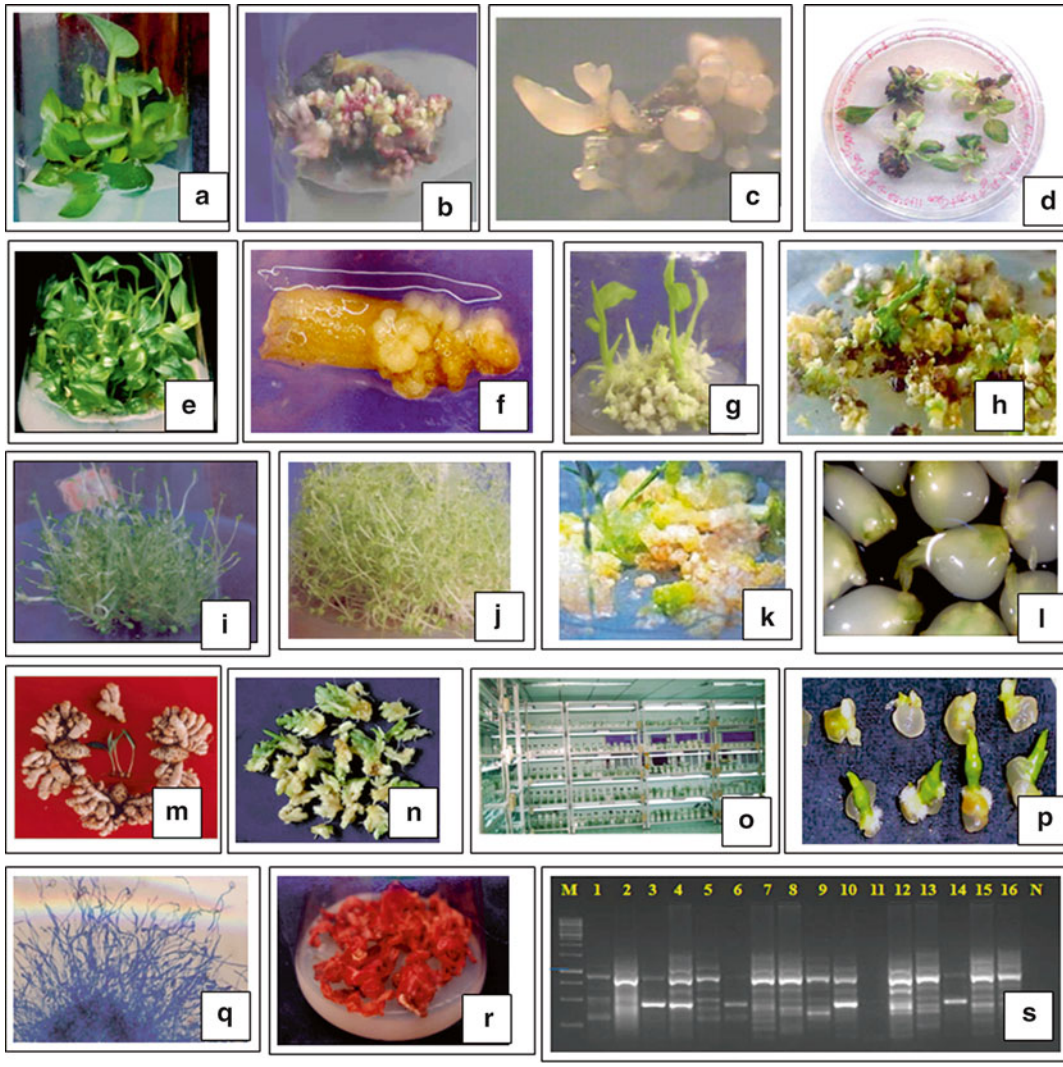

Fig. 25.1 (**a**) Biotechnological approaches for spices. Micropropagation in black pepper; (**b**) plant regeneration in black pepper; (**c**) somatic embryogenesis in black pepper; (**d**) regeneration of transformants in black pepper; (**e**) micropropagation in cardamom; (**f**) regeneration of embryoids from cardamom anthers; (**g**) plant regeneration in ginger; (**h**) somatic embryogenesis in cinnamon; (**i**) micropropagation in thyme; (**j**) micropropagation in mint; (**k**) plant regeneration in coriander; (**l**) synthetic seeds in cardamom; (**m**) microrhizomes in turmeric; (**n**) microrhizomes in ginger; (**o**) *in vitro* gene bank of spices; (**p**) germinating cryopreserved encapsulated buds of vanilla; (**q**) germinating cryopreserved pollen of vanilla; (**r**) *in vitro* multiplication of nutmeg mace; (**s**) ISSR profiles of black pepper varieties

study the genetic diversity of 20 varieties from Brazilian collections and 40 popular genotypes and 4 different species of black pepper from India, respectively. Liao et al. (2009) reported the isolation and characterization of 11 and 9 polymorphic microsatellites loci from a *Piper polysyphonum* and *Piper solmsianum*, respectively. These microsatellite markers provide a reliable means to understand the population structure and interrelationships in the genus *Piper*. Jaramillo and Manos (2001) used phylogenetic analysis of sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA based on a worldwide sample of the genus *Piper*. Nirmal Babu et al. (2011a) reported the molecular interrelationships between 24 *Piper* species using RAPD profiles. Chaveerach

et al. (2002) studied interrelationships between three *Piper* species, viz., *P. kadsura, Piper retrofractum* and *P. chaba* using morphological characters and RAPD profiles to demonstrate a closer relation between *P. retrofractum* and *P. kadsura* than between *P. chaba* and *P. retrofractum*. Genetic diversity of *Piper* species using RAPD and ISSR (Fig. 25.1s) was also reported recently by many workers (Sen et al. 2010; Nirmal Babu et al. 2012b). Jiang and Liu (2011) applied RAPD and SRAP markers to analyze genetic diversity in 74 individual plants of *Piper* spp. in Hainan Island. The SRAP technique clearly distinguished all *Piper* spp. from each other.

Molecular markers like RAPD, AFLP, ISSR, SSR and ITS polymorphism were used for assessment of genetic variability in black pepper and to characterize important cultivars, varieties and related species of black pepper to develop fingerprints and to study the interrelationships (Pradeep Kumar et al. 2003; Nazeem et al. 2005; Nirmal Babu et al. 2011a). A mapping population was developed for the preparation of genetic map of black pepper (Nirmal Babu et al. 2011a), and DNA markers were also used to study the genetic fidelity among micropropagated black pepper and long pepper plants (Nirmal Babu et al. 2011a).

Johnson et al. (2005) used male parent specific RAPD markers for the identification of hybrids in black pepper (*Piper nigrum* L.). Sex-specific markers were developed in *Piper longum* L. using RAPD (Banerjee et al. 1999) and differential display (Manoj et al. 2008) *Piper betle* L. using ISSR (Khadke et al. 2012) and RAPD markers (Samantaray et al. 2012b). Sheji et al. (2006) developed a SCAR marker for identifying *Phytophthora*-resistant lines of black pepper. RAPD technique was also employed for the authentication of dried black pepper from its adulterant *Carica papaya* L (Dhanya et al. 2009).

25.2.3 Protoplast Culture

The 'protoplast' is devoid of cell wall and this makes the protoplast technology suitable for genetic transformation by introduction of transgene DNA and somatic hybridization by protoplast fusion of species or subspecies resistant to traditional cross-breeding, or isolation of subcellular organelles etc. Reliable procedures are available for isolation, culture and fusion of protoplasts from a range of spices. Successful isolation and culture of protoplasts were reported in leaf tissues in black pepper (Shaji et al. 1998), and these protoplasts could be successfully developed up to microcalli stage.

25.2.4 Genetic Transformation

Reports are available on *Agrobacterium*-mediated gene transfer system (Fig. 25.1d) in *P. nigrum* (Nirmal Babu et al. 2005a, 2011d, 2013). Maju and Soniya (2012) reported an efficient protocol for genetic transformation using *Agrobacterium tumefaciens* strain EHA 105 and multiple shoot production in *Piper nigrum* var. Panniyur-1 to understand the mechanisms that control the regeneration process in the species. Mani and Manjula (2011) reported the *Agrobacterium-mediated* transformation and endogenous silencing in *Piper colubrinum* by vacuum infiltration. The *in planta* transformation via pollen tube pathway was done by Asha and Rajendran (2010) in black pepper variety Panniyur-2, using the total exogenous DNA of *Piper colubrinum*, a species resistant to *Phytophthora capsici*.

25.2.5 Cloning and Isolation of Candidate Genes

Spices are sources of many important genes with antibiotic and pharmaceutical properties. Efforts are being made to identify and isolate genes of interest both for crop improvement and of industrial value. Molecular cloning of a cDNA fragment encoding the defence-related protein β-1,3-glucanase in black pepper (*P. nigrum* L.) and methyl-glutaryl-CoA reductase in *Piper colubrinum* was reported (Girija et al. 2005a, b). PCR-based SSH technique was used to generate a leaf-specific subtracted cDNA library for *Piper nigrum* L. for the identification of more number

of tissue-specific genes (Alex et al. 2008). Bhat et al. (2005) reported the isolation and sequencing of CMV coat protein gene infecting black pepper and its possible deployment in black pepper to induce virus resistance. Dicto and Manjusha (2005) used PCR-based SSH to identify *P. colubrinum* resistance genes which are differentially expressed in response to salicylic acid. Mani and Manjula (2011) reported the cloning and characterization of two isoforms of osmotin, an antifungal PR-5 gene homologue, from a salicylic acid-induced subtracted cDNA library in *P. colubrinum*. Cloned isoforms of osmotin from resistant species could be used for molecular breeding of black pepper. Bioprospecting of novel genes from black pepper was attempted by Sujatha et al. (2005). Heterologous probes were used to identify the presence of pea lectin genes and tomato protease inhibitor genes in black pepper. Resistance gene analogues (RGAs) have been isolated in *Piper nigrum* and *Piper colubrinum* by Tiing et al. (2012).

25.3 Cardamom

25.3.1 Micropropagation and Plant Regeneration

Cardamom, considered the 'Queen of Spices', is also native to India. The productivity of cardamom is hampered by various diseases of viral aetiology. Utilization of virus-free planting material is an important input into disease management strategy. Cardamom is one of the first crops where commercialization of micropropagation has been achieved. Efficient *in vitro* methods for rapid clonal propagation of cardamom (Fig. 25.1e) are available (Nadgauda et al. 1983; Nirmal Babu et al. 1997; Nirmal Babu and Minoo 2003, 2011b, 2011e). Successful regeneration of plantlets from callus of seedling explants and anthers (Fig. 25.1f) of cardamom was reported (Nirmal Babu 1997; Nirmal Babu et al. 2011b, Rao et al. 1982). Manohari et al. (2008) also reported an efficient protocol for the induction of somatic embryogenesis and plant regeneration in small cardamom. Identification of a few Katte tolerant somaclones was reported by Peter et al. (2001).

25.3.2 Molecular Characterization

Molecular profiling of 11 species representing 5 major tribes, viz., *Amomum, Aframomum, Alpinia, Hedychium* and *Elettaria*, and 96 collections of cardamom germplasm using RAPD, PCR, RFLP and ISSR markers to elucidate their interrelationships, identification of duplicates and geographical origins of Indian cardamom was reported by Nirmal Babu et al. (2011a). Molecular characterization of selected cardamom genotypes using AFLP markers was also done in Columbia by Tamayo 2007. Molecular profiling of Indian, Sri Lankan and Guatemalan exported cardamoms using RAPD/ISSR primers indicated that though there is a lack of genetic polymorphism among them, they show the variation in quality parameters (Thomas et al. 2006).

25.3.3 Protoplast Culture

Successful isolation and culture of protoplasts were reported from cell suspensions and leaf tissues in cardamom (Geetha et al. 2000), and these protoplasts could be successfully developed up to microcalli stage.

25.3.4 Genetic Transformation

A preliminary study on the transformation of cardamom was attempted using the biolistic process to study the optimum conditions for gene delivery and the efficiency of the plasmid vector pAHC 25 and promoter Ubi-1 (maize ubiquitin) for transformation and gene expression in cardamom embryogenic callus. Transient expression of GUS gene was noticed in the bombarded callus tissue (Nirmal Babu et al. 1998).

25.4 Ginger

25.4.1 Micropropagation and Plant Regeneration

Ginger is the third most important spice that originated in South Asia. There is no seed set in ginger

leading to limited variability, and this hampers crop improvement programme. Rhizome rot caused by *Pythium aphanidermatum* and bacterial wilt caused by *Ralstonia solanacearum* are the major diseases affecting ginger. Diseases of ginger are often spread through infected seed rhizomes. Tissue culture will help in the production of pathogen-free planting material. Clonal multiplication of ginger from vegetative buds (Nadgauda et al. 1980; Nirmal Babu et al. 1997, 1998; Sharma and Singh 1997); optimization of media composition for micropropagation of ginger (Nirmal Babu et al. 2011c); regeneration of plantlets *via* callus phase from leaf (Nirmal Babu et al. 1992a), vegetative bud (Fig. 25.1g), ovary and anther explants (Kacker et al. 1993; Nirmal Babu 1997; Nirmal Babu et al. 2005a, b; Lincy et al. 2009; Ramachandran and Chandrashekaran 1992) and plant regeneration from ginger immature anthers (Nirmal Babu et al. 1992b) and anther have been reported (Samsudeen et al. 2000). This system was used for inducing somaclonal variability in ginger where lack of seed set hampers conventional breeding. A few promising high-yielding rhizome rot-tolerant somaclones also have been identified (Nirmal Babu et al. 1996; Sumathi 2007).

In nature, ginger fails to set fruit. However, by supplying required nutrients to young flowers and by *in vitro* pollination, 'fruit' development and subsequently plants could be recovered. *In vitro* pollination attempts were successfully made by Nazeem et al. (1996) to overcome the pre-fertilization barriers like spiny stigma, long style and coiling of pollen tube that interfered with natural seed set in ginger successful seed set was obtained. Induction of tetraploid ginger through *in vitro* colchicine treatment and tetraploid somaclone with extra bold rhizomes was also reported (Adaniya and Shirai 2001; Smith et al. 2004; Nirmal Babu et al. 1996, 2005b; Wang et al. 2010).

25.4.2 Molecular Characterization

RAPD profiling of 90 accessions of ginger indicated moderate to low level of polymorphism (Sasikumar and Zachariah 2003; Nirmal Babu et al. 2005a, b; Parthasarathy and Nirmal Babu et al. 2011c). Various markers like AFLP (Wahyuni et al. 2003; Sajeev et al. 2011) and ISSR (Kizhakkayil and Sasikumar 2010) were used to study the genetic diversity in ginger. Jiang et al. (2006) used metabolic profiling and phylogenetic analysis to investigate the diversity of plant material within the ginger species and between ginger and closely related species in the genus *Zingiber* and also for authentication of ginger. Chavan et al. (2008) developed SCAR markers as complementary tools for distinguishing *Z. officinale* from the other *Zingiber* species.

25.4.3 Protoplast Culture

Successful isolation and culture of protoplasts were reported from cell suspensions and leaf tissues in ginger (Nirmal Babu 1997; Geetha et al. 2000), and these protoplasts could be successfully developed up to microcalli stage. Somatic hybridization of ginger through chemical fusion (PEG mediated) and its regeneration was reported by Guan et al. (2010). RAPD technique was used for the identification of hybrids, and flow cytometry analysis revealed the diploid nature of all regenerated progenies.

25.4.4 Genetic Transformation

Transient expression of GUS was successfully induced in ginger embryogenic callus bombarded with plasmid vector pAHC 25 and promoter Ubi-1 (maize ubiquitin) callus tissue (Nirmal Babu 1997). *Agrobacterium tumefaciens* strain EHA105/p35SGUSInt, effective in expressing β-glucuronidase activity, was used to transform ginger by Suma et al. (2008).

25.4.5 Cloning and Isolation of Candidate Genes

Nair and Thomas (2007) identified the three primer pairs designed from the conserved motifs of NBS domain of NBS-LRR gene class as most successful in isolating RGCs in ginger, and these

provide a base for isolation of RGC mining in ginger. They also reported the efficiency and sensitivity of SSCP (single-strand conformation polymorphism) analysis in discriminating *Pythium* susceptible and resistant *Zingiber* accessions (Nair and Thomas 2012; Nair et al. 2010) and the isolation of resistant gene designated ZzR1 from *Z. zerumbet* and its correlation between ZzR1 expression and resistance of the wild taxa to *Pythium aphanidermatum* infection (Nair and Thomas 2013). Swetha Priya and Subramanian (2008) reported the presence of R gene of CC-NBS-LRR class of plant resistant gene in ginger varieties against *Fusarium oxysporum* f. sp. *zingiberi.* Fujisawa et al. (2010) cloned a novel gene that could encode s (beta) bisabolene synthetase from ginger. Prasath et al. (2011, 2013) reported the isolation of PR5 protein genes CaPR5 and ZoPR5 which code for the precursor proteins of 227 and 224 amino acids. They used a PCR-based suppression subtractive hybridization (SSH) method to identify *C. amada* (a potential donor for bacterial wilt resistance to *Zingiber officinale*) genes that are differentially and early expressed in response to the *R. solanacearum* infection compared to *Z. officinale*. The study highlighted the expression of LRR, GST and XTG much higher in resistant species (*C. amada*) than in susceptible species (*Z. officinale*).

25.5 Turmeric

25.5.1 Micropropagation and Plant Regeneration

Turmeric of commerce is the dried rhizomes of *Curcuma longa* L. which belongs to the family Zingiberaceae. India is the major producer and exporter of this spice. Curcumin is the important colouring material from turmeric, and development of varieties with high recovery of curcumin is the need of the hour. Successful micropropagation of turmeric has been reported (Nadgauda et al. 1978; Nirmal Babu et al. 1997; Sunitibala et al. 2001; Salvi et al. 2002; Panda et al. 2007; Ghosh et al. 2013). This technique is used for the production of disease-free planting material. Organogenesis and plantlet formation were achieved via callus cultures of turmeric (Nirmal Babu et al. 1997; Sunitibala et al. 2001; Salvi et al. 2000). Variants with high curcumin content were isolated from tissue-cultured plantlets (Nadgauda et al. 1982). Root rot disease-tolerant clones of turmeric cv. Suguna were isolated using continuous *in vitro* selection technique against pure culture filtrate of *Pythium graminicolum* (Gayatri et al. 2005).

Renjith et al. (2001) reported the *in vitro* pollination and hybridization between two short duration types VK-70 and VK-76 and reported the seed set and seed development. This reduces the breeding time and helps in recombination breeding which was so far not attempted in turmeric. Protocols for micropropagation of many economically and medicinally important zingiberaceous species like *Amomum subulatum* (large cardamom), *Curcuma aromatica* (kasturi turmeric), *C. amada* (mango ginger), *C. zedoaria, Kaempferia galanga, K. rotunda* and *Alpinia* spp. were developed (Vincent et al. 1992; Chang and Criley 1993; Ravindran et al. 1996; Geetha et al. 1997; Chan and Thong 2004; Chithra et al. 2005; Raju and Anita-D 2005. Islam et al. (2004) described an efficient protocol for microrhizome production, *in vitro*, in turmeric. Rahman et al. (2004) reported the efficient plant regeneration through somatic embryogenesis from leaf base-derived callus of *Kaempferia galanga* L.

25.5.2 Molecular Characterization

Kress et al. (2001) proposed a new phylogenetic analysis of Zingiberaceae based on DNA sequences of the nuclear internal transcribed spacer (ITS) and plastid *mat K* regions. The results suggest that *Curcuma* is paraphyletic with *Hitchenia, Stahlianthus* and *Smithatris.* Sasaki et al. (2004) applied single-nucleotide polymorphism analysis of the trnK gene for the identification of *Curcuma* plants. Genetic diversity evaluation among *C. longa* and other 14 *Curcuma* species was done using ISSR and RAPD markers (Syamkumar and Sasikumar 2007) placing them into seven groups which is somewhat congruent with classification based on morphological

characters proposed by the earlier workers. Genetic diversity in turmeric was assessed by using molecular markers like RAPD (Jan et al. 2011), both RAPD and ISSR (Singh et al. 2012) and PCR-based markers, viz., RAPD, ISSR and AFLP (Das et al. 2011; Nirmal Babu et al. 2007, 2011d). Development and characterization of EST-derived and genomic microsatellites in *Curcuma longa* were reported by Joshi et al. (2010), Siju et al. (2010) and Senan et al. (2013) which could be used for diversity analysis. Nayak and Naik (2006) carried out four C nuclear DNA content and RAPD analysis of 17 promising cultivars of turmeric from India.

Salvi et al. (2003) and Praveen (2005) using RAPD analyzed turmeric somaclones and concluded that plants regenerated using shoot tips showed genetic stability, while the callus-derived and inflorescence-derived plants showed variations. Tyagi et al. (2007) confirmed the genetic stability of 12-month-old *in vitro* conserved turmeric by RAPD profiling. Sinus et al. (2010) developed and amplified microsatellite markers from ESTs of turmeric. Sasaki et al. (2002) used sequence analysis of Chinese and Japanese *Curcuma* drugs on the 18S rRNA gene and *trnK* gene and its application based on amplification-refractory mutation system analysis for their identification and authentication. Sasaki et al. (2004) used SNP analysis based on the differences in the *nucleotide* positions in the 177, 645, 724 and a 4 base indel on the *trnK* gene obtained using three different lengths of 26 mer, 30 mer and 34 mer reverse primers for the identification of four *Curcuma* sp. studied by Xia et al. (2005) used 5S rRNA spacer and chemical fingerprints for quality control and authentication of Rhizoma *Curcumae*, a Chinese medicine used for the removal of blood stasis and alleviating pain.

25.5.3 Protoplast Culture

Successful isolation and culture of protoplasts were reported from cell suspensions and leaf tissues in turmeric (Geetha et al. 2000), and these protoplasts could be successfully developed up to microcalli stage.

25.5.4 Genetic Transformation

An efficient method for stable transformation was developed in turmeric using particle bombardment on callus cultures (Shirgurkar et al. 2006). Transgenic shoots regenerated were multiplied, and stably transformed plantlets were produced. Polymerase chain reaction (PCR) and histochemical GUS assay confirmed the stable transformation. Transformed plantlets were resistant to glufosinate. A protocol for genetic transformation and regeneration was established in *C. alismatifolia* using shoot explants through *Agrobacterium* strain AGLO harbouring binary vector pBI121 or pBI121-Ca-ACSI. Transformation was confirmed by PCR, GUS assay and Southern blotting of regenerated plants (Mahadtanapuk et al. 2006).

25.5.5 Cloning and Isolation of Candidate Genes

Isolation, cloning and characterization of a mannose-binding lectin from cDNA (Chen et al. 2005), a stress-responsive CDPK gene (ZoCDPK1) from ginger rhizome, using rapid amplification of cDNA end (RLM-RACE) technique (Vivek et al. 2013) and violaxanthin deepoxidase (GVDE) (Huang et al. 2007) have been reported. Over-expression of ginger CDPK1 gene in tobacco conferred tolerance to salinity and drought stress. ZoCDPK1 functions in the positive regulation of the signalling pathways that are involved in the response to salinity and drought stress in ginger, and it is likely operating in a DRE/CRT independent manner. Joshi et al. (2010) used degenerate primers designed based on known R gene in combination to elucidate resistance gene analogues from *Curcuma longa* cultivar Suvarna. Kar et al. (2013) used a previously isolated resistance gene candidate Czp11 from *C. zedoaria* resistant to *Pythium aphanidermatum* as a template to characterize a major resistance gene CzR1 through candidate gene approach in combination with RACE-PCR strategy.

25.6 Vanilla

25.6.1 Micropropagation and Plant Regeneration

Vanilla planifolia, native to Mexico and Central America, now cultivated in other parts of the tropics, is the source of natural vanillin. Micropropagation of vanilla using apical meristem was standardized for large-scale multiplication of disease-free and genetically stable plants (Kononowicz and Janick 1984; Minoo 2002; Minoo et al. 2006; Minoo and Nirmal Babu 2009). Successful plant regeneration from shoot- and seed-derived callus was reported in vanilla (Nirmal Babu et al. 1997; Minoo 2002).

25.6.2 Molecular Characterization

Markers like RAPD and AFLP coupled with morphological characters were utilized to assess the variability and hybrid nature of genotypes and of successful interspecific hybridization and production of hybrids between *V. planifolia* and *V. aphylla* (Minoo et al. 2006). RAPD marker was used to estimate the level of genetic diversity and interrelationships among different clones of *V. planifolia* and related species (Minoo et al. 2010). The data showed very limited variation within accessions of *V. planifolia* indicative of its narrow genetic base and its close relationship with *V. tahitensis* (Besse et al. 2004; Schluter et al. 2007; Minoo et al. 2008b; Minoo and Nirmal Babu 2009, Minoo et al. 2010). A comparative study of RAPD and ISSR was reported to analyze the interrelationships among nine cultivated, wild and hybrid *Vanilla* species (Verma et al. 2009). Fourteen microsatellite loci developed from *V. planifolia* shown to be monomorphic within the cultivated accessions and 11 markers out of 14 were polymorphic when transferable to *V. tahitensis* (Bory et al. 2008).

25.6.3 Protoplast Culture

Minoo et al (2008a) reported the isolation of viable protoplasts in *Vanilla* species, i.e. in *V. andamanica*, and that of PEG-mediated protoplast fusion between *V. planifolia* and *V. andamanica.* The protoplast fusion technology can be useful in gene transfer of useful traits to *V. planifolia* especially the natural seed set and disease tolerance observed in *V. andamanica.*

25.6.4 Genetic Transformation

Protocols are available for genetic transformation of *Vanilla planifolia* using indirect procedure, viz*., Agrobacterium tumefaciens* using shoot tip sections (Malabadi and Nataraj 2007) and protocorm-like bodies from shoot tips as explants (Ratheesh and Ishwara Bhat 2011), providing a very useful basis for further genetic improvement of the orchid.

25.6.5 Cloning and Isolation of Candidate Genes

Sequencing of neutral genes has been used for reconstructing the evolutionary history of vanilloid orchids, including a few vanilla species (Cameron et al. 1999; Cameron and Chase 2000; Cameron 2004, 2009; Cameron and Molina 2006). Nuclear and plastid sequences were also used for unravelling the origin of the Tahitian vanilla (Lubinsky et al. 2008). Recently, the length polymorphism of the nonneutral caffeic acid O-methyl transferase gene was also used to analyze 20 vanilla species and confirmed the strong differentiation of Old World versus New World species in the genus (Besse et al. 2009).

25.7 Tree Spices

25.7.1 Micropropagation and Plant Regeneration

Cinnamon, clove, nutmeg, curry leaf, pomegranate, tamarind, allspice and garcinia are some of the important tree spices. In these perennial tree crops, identification and clonal multiplication of high-yielding 'elite' genotypes become a priority due to long pre-bearing period. Micropropagation

of cinnamon, Chinese cassia and camphor was reported from seedlings and mature tree explants (Mini et al. 1997; Nirmal Babu et al. 1997; Huang et al. 1998). Multiple shoots were induced from shoot tips and nodal segments of *Cinnamomum camphora* on Woody Plant Medium (WPM) (Huang et al. 1998 and from a cotyledonary node on MS medium (Azad et al. 2005). Successful micropropagation of Chinese cassia was reported by Inomoto and Kitani (1989) using nodal explants from seedlings on MS medium. Micropropagation protocols for *C. camphora* were developed by Nirmal Babu et al. 2003.

In vitro multiple shoot induction was worked out in *G. indica* (Kulkarni and Deodhar 2002). Murashige and Skoog's medium supplemented with BAP gave optimal response in different genotypes investigated. Micropropagation of three species of garcinia was reported by Huang et al. (2000), Malik et al. (2005) and Mohan et al. (2012). *In vitro* shoot initiation from explants of field-grown trees of nutmeg was reported by Mallika et al. (1997). Micropropagation of clove from seedling explants have been reported (Mathew and Hariharan 1990; Suparman and Blake 1990). MS medium supplemented with IBA or activated charcoal induced root formation. However, there are no reports on successful micropropagation of clove from mature shoot explants.

Reports on micropropagation of curry leaf, pomegranate, camboge and tamarind are also available (Mascarenhas et al. 1987; Hazarika et al. 1995; Rao et al. 1997; Bhuyan et al. 1997; Mathew et al. 1999; Nirmal Babu et al. 2000; Mehta et al. 2000). High-frequency direct shoot proliferation was induced in intact seedlings of *M. koenigii* (Bhuyan et al. 1997). Shoot proliferation is also reported from different explants like nodal cuttings (Nirmal Babu et al. 2000), leaves (Mathew and Prasad 2007) and immature seeds (Rani et al. 2012). Efficient micropropagation protocols for Pomegranate were reported (Bin and Jiang 2003; El-Agamy et al. 2009; Patil et al. 2011). The plantlets grown on WPM were found to be significantly better in average survival, plantlet height and average leaf number per shoot when compared to MS and NN media (El-Agamy et al. 2009; Kaji et al. 2013). *In vitro* regeneration and high-frequency regeneration of tamarind were achieved in different media compositions (Hussain et al. 2004; Pattepur et al. 2010). Thidiazuron can play a major role to induce germination in tamarind seedlings Mehta et al. (2004). Reports on successful callus induction and plant regeneration in nutmeg, cinnamon (Fig. 25.1h), camphor, pomegranate and curry leaf are available (Bhansali 1990; Iyer et al. 2000; Kong et al. 2009; Shi et.al. 2009, 2010; Paul et al. 2011).

25.7.2 Molecular Characterization

Genetic identification among four cinnamon species (*Cinnamomum cassia*, *C. zeylanicum*, *C. burmannii* and *C. sieboldii*) using nucleotide sequences of chloroplast DNA was studied by Kojoma et al. (2002). The nucleotide variation at one site in the *trnL-trnF* IGS and at three sites in the *trnL* intron was used for the correct identification of *Cinnamomum* species. Furthermore, single-strand conformation polymorphism (SSCP) analysis of PCR products from the *trnL-trnF* IGS and the *trnL* intron resulted in different SSCP band patterns among *C. cassia*, *C. zeylanicum* and *C. burmannii*. RAPD technology was applied for the analysis of normal and excellent types of *Cinnamomum camphora* L. and genetic interrelationship of nine *Cinnamomum* species (Hui and Linshui 2003 and Joy and Maridass 2008), respectively. Sheeja et al. (2013) reported the diversity analysis of nutmeg (Myristica) and related genera using both RAPD and ISSR markers. Species-specific bands could be identified from all the accessions, which can be converted into SCAR markers for genotype identification and authentication.

Yapwattanaphun et al. (2004) used ITS sequence data to elucidate phylogenetic relationship of mangosteen (*Garcinia mangostana*) and several wild relatives (*Garcinia* spp.). The ITS sequence analysis showed that *G. atroviridis, G. cowa, G. dulcis, G. malaccensis, G. mangostana, G. rostrata* and *G. vilersiana* have nucleotide additivity (two different nucleotides at the same nucleotide position) at several sites in the ITS region. The occurrence of these species might be related to hybridization with ancestors, but the

genomic compositions, even chromosome numbers, of these species are still unknown. Sulassih and Santosa (2013) worked on the diversity analysis of mangosteen and its relatives based on morphological and ISSR markers, revealing a close relationship between *G. celebica, G. malaccensis* and *G. mangostana*. It was determined that *G. malaccensis* and *G. celebica* were ancestors based on morphological and ISSR markers. Thatte and Deodhar (2012) identified male- and female-specific molecular markers in *Garcinia indica*.

Genetic variability and relationship studies of curry leaf were done using RAPD, DAMD and ISSR by Verma and Rana (2013). Genetic diversity studies of pomegranate using RAPD, SSR, AFLP, RAMP, nuclear rRNA and internal transcribed spacer were reported by many workers (Durgaç et al. 2008; Jbir et al. 2008; Awamleh et al. 2009; Zamani et al. 2010; Hasnaoui et al. 2010; Pirseyedi et al. 2010; Ebrahimi et al. 2010; Soriano et al. 2011; Zhao et al. 2013; Singh et al. 2013). Genetic diversity studies of tamarind using RAPD and AFLP markers were done by Diallo et al. (2007) and Algabal et al. (2011) and in allspice by Wadt et al. (2004).

25.7.3 Cloning and Isolation of Candidate Genes

Genes encoding cinnamomin (a type II RIP), which has three isoforms, were isolated from camphor seeds by Yang et al. (2002). A geraniol-synthase gene is also isolated from *Cinnamomum tenuipilum* by Yang et al. 2005. Expression of CtGES was exclusively observed in the geraniol chemotype of *C. tenuipilum*. Furthermore, *in situ* hybridization analysis demonstrated that CtGES mRNA was localized in the oil cells of the leaves.

25.8 Seed and Herbal Spices

25.8.1 Micropropagation and Plant Regeneration

Seed spices and herbs constitute a large group of widely different aromatic plants which are used as spices, culinary herbs and medicinal herbs and those which are used in aroma therapy. Micropropagation protocols for many seed and herbal spices are available. They include coriander, anise, thyme (Fig. 25.1i), peppermint (Fig. 25.1j), spearmint, celery, lavender, savory, *Ocimum*, oregano, basil, sage, fennel, parsley, dill and garlic, saffron and *Eryngium foetidum* capsicum (Bhojwani 1980; Venkataraman and Ravishankar 1987; Cellarova 1992; Furmanowa and Olszowska 1992; Panizza and Tognoni 1992; Patnaik and Chand 1996; Vandemoortele et al. 1996; Sajina et al. 1997a; Ochoa-Alejo and Ramirez-Malagon 2001; Gupta and Bhargava 2001; Sharma et al. 2004; Aflatuni et al. 2005; Karaoglu et al. 2006; Majourhat et al. 2007; Minas 2009; Song et al. 2009; Ascough et al. 2009; Falk et al. 2009; Kothari et al. 2010; Fadel et al. 2010; Irikova et al. 2011; Samantaray et al. 2012a; Nhung and Quynh 2012; Kara and Baydar 2012; Navroski et al. 2012; Zeybek et al. 2012; Rodeva et al. 2013; Santoro et al. 2013; Dixit and Chaudhary 2013; Keller and Senula 2013). Clonal propagation of chemically uniform fennel plants through somatic embryoids was reported by Miura et al. (1987). Shoot regeneration protocols for fenugreek, cumin and coriander (Fig. 25.1k) were reported (Nirmal Babu et al. 1997; Tawfik and Noga 2001; Ebrahimie et al. 2003; Aasim et al. 2009). Jakhar et al. (2003) reported the *in vitro* flowering and seed formation in cumin.

Somatic embryogenesis has been established in saffron, garlic (Keles et al. 2010) and chilli (Blazquez et al. 2003; Sheibani et al. 2006; Munyon et al. 1989). An efficient protocol for organogenesis from root protoplasts (Xu et al. 1982) and adventitious shoot formation in fennel was developed by investigating the effect of plant growth regulators by Jakhar and Choudhary (2012). Profuse callus differentiation was observed when medium was supplemented with 1.0 mg/l BAP followed by 1.0 mg/l BAP + 0.5 mg/l IBA. The shoot morphogenesis was observed in callus proliferated from the shoot apex explants incubated at 1.0 mg/l BAP + 0.5 mg/l IBA, upon subculture on the same levels of plant growth regulator. In order to create variability, organogenesis followed by mutagenesis

has also been identified as a potential *in vitro* technique. In this process, stock organogenetic callus is treated with physical or chemical mutagens. The studies have shown positive indications to isolate promising mutants in cumin (Raje et al. 2004). Similar type of efforts can be made for the creation of variability in cumin for resistance to *Alternaria* blight, root rot in fenugreek, and for many other stresses.

In vitro methods of screening could prove highly useful in screening a large germplasm collections or cell lines for resistance to prevalent fungal diseases and tolerance to drought and salt stress. The reports are available on *in vitro* selection for salt tolerance in fenugreek on media containing 0.025–1.5 % NaCl (Settu et al. 1997) and drought-tolerant cell lines cultured on media containing 0.25–1.50 % PEG in coriander (Stephen and Jayabalan 2000) through tissue culture. Selection of somaclonal variants resistant to *Septoria apiicola* by callus culturing in the presence of fungal culture filtrate in celery (Evenor et al. 1994), *Fusarium* yellow-resistant celery line, a somaclonal variant (Lacy et al. 1996), and resistant to *Alternaria* blight in cumin (Shukla et al. 1997b) has been reported by different workers.

25.8.2 Molecular Characterization

Genetic diversity study of fennel, fenugreek, cumin, coriander, thyme, dill, garlic and saffron using RAPD, ISSR, AFLP and SSR was reported by many workers (Paran et al. 1998; Lopez et al. 2008; Rubio-Moraga et al. 2009; Imran et al. 2010; Zahid et al. 2009; Abdoli et al. 2009; Singh et al. 2011; Jana and Shekhawat 2012; Jo et al. 2012; Keify and Beiki 2012; Khalil et al. 2012; Suresh et al. 2013; Siracusa et al. 2013; Singh et al. 2013). Molecular marker systems based on SCAR, CAPS, RAPD, STS, SSR. TRAP and SRAP are available for identification and fingerprinting of fennel varieties by Agbiotech (2009). Genetic diversity and identification of variety-specific AFLP markers were developed in fenugreek by Vinay Kumar et al. (2012). Twelve novel polymorphic microsatellite loci were developed and characterized from a repeat-enriched genomic library of *Crocus sativus* to study population and conservation genetics of this economically and medicinally important species Nemati et al. (2012). Fenwick and Ward (2001) studied the RAPD-based cultivar identification in mint. RAPD- and AFLP-based hybrid identification in Mentha was reported by Shasany et al. (2005).

25.8.3 Protoplast Culture

Shekhawat and Galston (1983) reported the isolation, culture and shoot regeneration from mesophyll protoplasts of fenugreek. Successful regeneration of whole plants from tissue-cultured shoot primordial of garlic was reported by Ayabe et al. (1995).

25.8.4 Genetic Transformation

Wang and Kumar (2004) reported the heterologous expression of *Arabidopsis* ERS1 which causes delayed senescence in coriander. *Agrobacterium rhizogenes* which mediate transformed root cultures were analyzed for the production of essential oils in dill (Santos et al. 2002). The development of efficient genetic transformation protocol using glucuronidase gene in caraway was studied, and gene transfer was more efficient when cotyledonary node explants were used (Krens et al. 1997). Preliminary experiments on *Agrobacterium*-mediated transformation of uidA (GUS) gene into cotyledon and hypocotyls explants in fenugreek were reported by Khawar et al. (2004). Stable transformation in cumin through particle bombardment was reported by Singh et al. (2010) and through *Agrobacterium*-mediated transformation by Pandey et al. (2013).

25.8.5 Cloning and Isolation of Candidate Genes

Molecular cloning of mannose-6-phosphate reductase and its developmental expression in celery was studied by Everard et al. (1997). Wang and Kumar (2004) reported the heterologous

expression of *Arabidopsis* ERS1 that causes delayed senescence in coriander.

25.9 Development of Synthetic Seeds

Synthetic seeds or artificial seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates or any other tissue which can be used for sowing as a seed and those that posses the ability to convert into a plant under *in vitro* or ex vitro conditions and that retain its potential after storage also. Artificial or synthetic seeds can be an ideal system for low-cost plant movement, propagation, conservation and exchange of germplasm. Synthetic seeds were developed by encapsulation of *in vitro* developed small shoot buds in 3–5 % calcium alginate in black pepper, shoot buds in cardamom (Fig. 25.1i), somatic embryos and *in vitro* regenerated shoot buds in ginger and turmeric, *in vitro* regenerated shoot buds, protocorms in vanilla (Sharma et al. 1994; Sajina et al. 1997b), somatic embryos in cinnamon and curry leaf and nodal segments in pomegranate leaf (Sundararaj et al. 2010; Minoo 2002; Gayatri et al. 2005; Naik and Chand 2006; Nikhil and Shukla 2013). Synseeds have been reported in cumin (Tawfik and Noga 2002a, b), coriander (Kim et al. 1996; Stephen and Jayabalan 2000), fennel (Sajina et al. 1997b), celery (Pratap 1992), dill (Ratnamba and Chopra 1974; Sehgal 1978) and nigella (Hamid Elhag and Olemy 2004), and regeneration of these plants has been successfully obtained.

25.10 Microrhizome

Microrhizome technology is useful for developing disease-free planting material and hence is an ideal source of planting material suitable for germplasm exchange, transportation and conservation. *In vitro* induction of microrhizomes in ginger (Fig. 25.1m) was reported by many workers (Bhat et al. 1994; Sharma and Singh 1995; Nirmal Babu 1997; Nirmal Babu et al. 2003, 2005b; Tyagi et al. 2007; Sumathi 2007; Zheng et al. 2008). The microrhizome-derived plants have more tillers but were shorter. They gave fresh rhizome yield ranging from 100 to 800 g per plant with an estimated yield of 10 kg per 3 m^2 bed. Many reports are available on *in vitro* microrhizome formation in turmeric (Fig. 25.1n) (Nirmal Babu et al. 2003; Cousins and Alderberg 2008). Low sucrose is reported to decrease the size of microrhizome, but optimum microrhizome production at 6–9 % sucrose was also reported. Sucrose (6–9 %) was most effective in rhizome formation.

25.11 Conservation of Genetic Resources

25.11.1 *In Vitro* Conservation of Germplasm

The genetic resources of spices are conserved either in seed gene banks and/or in field repositories. Conservation of the germplasm in *in vitro* (Fig. 25.1o) and cryobank is a viable and a safe augment to conventional conservation strategies (Nirmal Babu et al. 2012c). Conservation of pepper, cardamom, herbal spices, vanilla and ginger germplasm in *in vitro* gene bank by slow growth was reported (Nirmal Babu et al. 1999; Tyagi et al. 2007). Protocols for *in vitro* conservation by slow growth of black pepper and its related species, viz., *P. barberi*, *P. colubrinum, P. betle* and *P. longum*, were standardized by maintaining cultures at reduced temperatures, in the presence of osmotic inhibitors and at reduced nutrient levels or by minimizing evaporation loss by using closed containers. The technology for *in vitro* conservation of zingiberaceous crops like ginger, turmeric, *Kaempferia*, cardamom and their related species was standardized by Geetha (2002) and in vanilla by Minoo (2002), Minoo and Nirmal Babu (2009). Slow growth techniques are being used for medium-term conservation of spices in *in vitro* repository at NBPGR (Mandal et al. 2000). Suspensions of embryogenic cell lines of fennel, conserved at 4 °C for up to 12 weeks, produced normal plants upon transfer to laboratory conditions (Umetsu et al. 1995).

25.11.2 Cryopreservation

Cryopreservation of black pepper and cardamom seeds in liquid nitrogen (LN_2) was reported by Choudhary and Chandel 1994, 1995. The technology for cryopreservation of black pepper, cardamom, ginger, turmeric and vanilla germplasm (Fig. 25.1p) using vitrification, encapsulation and encapsulation-vitrification methods is available (Minoo 2002; Yamuna 2007; Minoo et al. 2012; Nirmal Babu et al. 2012). Cryopreservation of encapsulated shoot buds of endangered *Piper barberi* has achieved (Nirmal Babu et al. 2012c). Efficient cryopreservation technique for *in vitro* grown shoots of ginger based on encapsulation-dehydration, encapsulation-vitrification and vitrification procedures was reported by Yamuna (2007). Minoo (2002) reported the cryopreservation of vanilla pollen (Fig. 25.1q) for the conservation of haploid genome as well as assisted pollination between species that flower at different seasons and successful fertilization using cryopreserved pollen. Cryopreservation of coriander (*Coriandrum sativum* L.) somatic embryos using sucrose preculture and air desiccation was reported by Popova et al. 2010. Cryopreservation of celery using LN_2 was reported by González-Benito and Iriondo 2002.

25.12 Production of Secondary Metabolites

Biotechnology can be utilized to exploit the potential of spices for bio-production of useful plant metabolites. The use of tissue culture for the biosynthesis of secondary metabolites particularly in plants of pharmaceutical significance holds an interesting alternative to control production of plant constituents. This technique is all the more relevant in recent years due to the ruthless exploitation of plants in the field leading to reduced availability. *In vitro* proliferation of nutmeg mace (Fig. 25.1r) and synthesis of flavour components in culture were reported by Nirmal Babu et al. (1992a). Since mace is the source of anticarcinogenic compound myristicin, this technique with improvement can be used for the production of myristicin. Most of the reports in saffron were on the *in vitro* proliferation of stigma and *in vitro* synthesis of colour components and metabolites. The proliferation of stigma of saffron *in vitro* and chemical analysis of metabolites produced through tissue cultures of *Crocus sativus* were reported (Sano and Himeno 1987; Himeno et al. 1988; Sarma et al. 1991).

Plant cells cultured *in vitro* produce wide range of primary and secondary metabolites of economic value. Production of phytochemicals from plant cell cultures has been presently used for pharmaceutical products. Production of flavour components and secondary metabolites *in vitro* using immobilized cells is an ideal system for spices crops. Ahmad et al. (2013) concluded that regenerated tissues of *P. nigrum* are a good source of biologically active metabolites for antimicrobial activities, and callus culture presented itself as a good source for such activities. Production of saffron and capsaicin was reported using such system (Ravishankar et al. 1993, 1995; Johnson et al. 1996; Venkataraman and Ravishankar 1997). Johnson et al. (1996) reported the biotransformation of ferulic acid vanillylamide to capsaicin and vanillin in immobilized cell cultures of *Capsicum frutescens*. Reports on the *in vitro* synthesis of crocin, picrocrocin and safranal from saffron stigma (Himeno and Sano 1995) and colour components from cells derived from pistils (Hori et al. 1988) are available for further scaling up. Callus and cell cultures were established in nutmeg, clove, camphor, ginger, lavender, mint, thyme (Furmanowa and Olszowska 1992), celery, etc. Cell immobilization techniques have been standardized in ginger, sage, anise and lavender (Ilahi and Jabeen 1992). The production of essential oils from cell cultures (Ernst 1989) and accumulation of essential oils by *Agrobacterium tumefaciens-transformed* shoot cultures of *Pimpinella anisum* were reported (Salem and Charlwood 1995). The regulation of the shikimate pathway in suspension culture cells of parsley (Conn and McCue 1994) and production of anethole from cell cultures of *Foeniculum vulgare* (Hunault and Du Manoir 1992) were reported. Growth and production of monoterpene by transformed shoot

cultures of *Mentha citrata* and *Mentha piperita* in flasks and fermenters was reported by Hilton et al. 1995. The production of rosmarinic acid in suspension cultures of *Salvia officinalis* has been discussed by Hippolyte et al. (1992). Reports on the production of phenolic flavour compounds using cultured cells and tissues of vanilla are also available (Dorenburg and Knorr 1996). *In vitro* production of petroselinic acid was reported from cell suspension cultures of coriander (Kim et al. 1996a). Kintzios et al. (2004) reported the scaling up of micropropagation of *Ocimum basilicum* L. in an airlift bioreactor and accumulation of rosmarinic acid. Though the feasibility of *in vitro* production of spice principles has been demonstrated, methodology for scaling up and reproducibility need to be developed before it can reach commercial levels. Once standardized, this technology has tremendous potential in industrial production of important compounds like capsaicin, vanillin, crocin, picrocrocin, safranal, myristicin, anethole, menthol and curcumin.

25.13 Biotechnological Approaches on Disease Management in Spice Crops

Crop losses in spice crops due to biotic stress are serious. Particularly, soilborne nature of the plant pathogens, poor amenability for effective disease management and lack of durable host resistance are the real constraints of production (Sarma et al. 2014). Foot rot and slow decline of black pepper (Sarma 2010), clump and capsule rot of small cardamom rhizome rot, *Fusarium* yellows and bacterial wilt in ginger and turmeric and root rots and wilts in cumin, coriander and fenugreek still remained as major threats in these crops. Though high degree of host resistance was identified for *Phytophthora capsici*, *Radopholus similis* and *Meloidogyne incognita* (root/foot rot and slow decline diseases), in *Piper colubrinum*, a native of Brazil, efforts to develop resistant transgenics are yet unsuccessful. Pending successful incorporation of R genes in susceptible cultivars, it is imperative to explore the available natural resources to combat these biotic stresses in these crops. Microbial biocontrol technology as a part of integrated disease management (IPM) is an ideal option. This technology is ecofriendly and would reduce pesticide load into the ecosystem. Hence, it received considerable attention during the last four decades. In this well-proven microbial biotechnology, biological control (Cook 1985) was found effective in reducing the crop losses in soilborne diseases of several crops, including spice crops (Sarma et al. 2014). Antagonism/antibiosis, hyperparasitism, growth promotion and induced systemic resistance (ISR) are the basic principles that govern this technology. Hyperparasitic fungi like *Trichoderma harzianum* and *T. viride*, *Pochonia chlamydosporia* and *Paecilomyces lilacinus* and PGPRs (plant growth-promoting rhizobacteria) like *Pseudomonas fluorescens, Pseudomonas cepacia* (Fridlender et al. 1999), *Bacillus subtilis* and Arbuscular mycorrhiza (AM) were found effective in reducing crop losses caused by soilborne plant pathogens in the spice crops. The large-scale production of these microbes adopting both solid and liquid fermentation technologies is in place. The delivery systems/formulations of these microbials both in solid and liquid (Chet 1987; Batta 2004) are now available in the market for the farming community to utilize them for the seed treatment and soil application. Application of these along with organic inputs that would serve as food base for their multiplication is suggested. The seed treatment is particularly important for seed spices, ginger and turmeric, where contaminated seed is the primary source of infection (Singh et al. 1972; Sarma and Anandaraj 1998).

Either fungus or bacterium to be successful biocontrol agents in a given ecosystem, rhizosphere competence (Ahmed and Baker 1987; Harman 1992) and competitive saprophytic ability (CSA) are the important traits that are of paramount importance (Sarma 2006).

25.13.1 *Trichoderma*

Intensive investigations carried out during the last four decades clearly established the potential

of *Trichoderma* as an effective biocontrol agent in suppression of several soilborne plant pathogens (Papvizas 1985; Papvizas and Chet 1987). These biocontrol agents received considerable attention in the management of spice crop diseases, viz., black pepper (Rajan et al. 2002; Saju 2004; Sarma and Saju 2004; Sarma 2010; Sarma et al. 2013), small cardamom (Suseela Bhai 1998; Suseela Bhai et al. 1993), ginger (Sharma and Jain 1979; Usman et al. 1996; Usman 1997), turmeric, cumin (Vyas and Mathur 2002; Hagag and Abosedera 2005), coriander (Gopal Lal et al. 2010) and fenugreek (Kakani et al. 2009). *In vitro* selection based on the inhibition of the target pathogen, the inhibitory effects of both volatile and nonvolatile antibiotics on suppression of the target pathogen, disease suppression and growth promotion of the target crop both in pot culture and field evaluation are the criteria adopted to evaluate their bio-efficacy. The mode of action of this hyperparasite includes production of the lytic enzyme like cellulases, glucanases and chitinase, and antibiotics that are necessary for the degradation of the cell wall of pathogen were studied in these pathogens of spice crops. The disease suppressive effect of *T. harzianum* was established through soil application that reduced the capsule rot of cardamom and was on par with treatment with copper fungicides and potassium phosphonate (Suseela Bhai 1998). Suppression of root rot in cardamom nurseries was demonstrated with soil application of *T. harzianum* (Sarma 2006). Similar results were obtained in rhizome rot of ginger caused by *Pythium aphanidermatum* both as seed treatment and soil application along with neem cake (Usman et al. 1996). In cumin, seed contamination with *F. oxysporum f. sp. cumini* and also wilt suppression were accomplished with seed treatment and soil application (Vyas and Mathur 2002). Root rot caused by *Rhizoctonia solani* in fenugreek was successfully controlled with *T. viride* application both as soil treatment and seed coating (Kakani et al. 2009). Economic viability of *Trichoderma* technology in black pepper was reported by Madan et al. (2006).

25.13.2 Plant Growth-Promoting Rhizobacteria (PGPRs)

Rhizosphere of any crop plant is associated with abundant microbial load that would impact the crop health and productivity. This is an established fact and has been researched extensively. Microbes associated with rhizoplane, rhizosphere and endophytes (Hallmann 2001) received greater attention in plant microbe interaction to focus on growth promotion and disease suppression in crop plants (Kloepper et al. 1980, 1993). In general, the parameters for selection and studies on mode of action remained the same as that were adopted in fungal biocontrol agents. There is a spurt of research activity on this group of organisms taking advantage of their potential both for growth promotion and disease suppression in several plant pathosystem (Lopper 1988). Kumar et al. (2013) have characterized *Pseudomonas aeruginosa* for their genetic and functional properties. Extensive studies carried out on black pepper – *P. capsici* – pathosystem revealed that *Pseudomonas fluorescens* increased growth in black pepper through production of growth promoters (Diby et al. 2001). Similarly, the induced systemic resistance (ISR) was achieved through the enhancement of defence-related enzymes like PAL, peroxidase, polyphenol oxidase (Diby and Sarma 2005; Diby et al. 2001) and mycolytic enzymes in black pepper (Diby et al. 2005). The enhancement of nutrient uptake in black pepper through this microbial association was found promising (Diby et al. 2001). In the case of ginger, the bio-efficacy of a bacterial strain mixture (*Bacillus subtilis -S2BC1* and *Burkholderia cepacia)* in suppressing rhizome rot and *Fusarium* yellows (Shanmugam et al. 2012, 2013) has been reported. The efficacy of microbial mixtures in root disease suppression has been reported (Raupach and Kloepper 1978). Similar results were reported in *P. capsici*-Black pepper pathosystem (Jisha et al. 2002). There is a need for field evaluation of these microbial mixtures to exploit their commercial potential. Harman and Stasz (1991) have utilized protoplast

fusion technique, for the production of superior biocontrol fungi.

25.13.3 Arbuscular/Vesicular Arbuscular Mycorrhiza (AM/VAM)

The importance of AM/VAM was extensively studied for their role in enhanced nutrient uptake and the protection of root system from soilborne plant pathogens (Atkinson et al. 1994; Schenk 1981). Increased nutrient uptake, altered host metabolism through enhanced production of phenolics, growth regulators and defence mechanisms are the factors implicated in root protection from soilborne plant pathogens. *Glomus fasciculatum* inoculum incorporated in black pepper nursery mixture was found effective in reducing root infection caused by *P. capsici, R. similis* and *M. incognita* in black pepper (Anandaraj et al. 1996).

25.13.4 Production of Healthy Nursery Stock of Spice Crop

The production of root rot-free rooted cuttings of black pepper received considerable attention. Root rot caused by *Phytophthora* and plant parasitic nematodes at nursery stage go unnoticed without any visible foliar symptoms. Hence, fortifying nursery mixture with biocontrol agents (AM, fungi and PGPRs) is an accepted technology which is becoming popular in all pepper-growing countries (Sarma 2010; Sarma et al. 2013, 2014). Similar methodology is also practiced in cardamom nurseries.

25.13.5 Biosafety Regulations

The formulations of these microbials both solid and liquid (Batta 2004) with ideal population of these biocontrol agents are now available in the market for seed treatment and soil application. In general, the use of these formulations along with organic inputs is suggested to ensure their multiplication and colonization. However, biosafety of microbials, to be used for crop protection, is an essential prerequisite. The regulatory norms for many of these are now in place (Kulakshetra 2004) and are further being improved. Alarmingly *Pseudomonas aeruginosa* an endophyte in black pepper highly effective in suppressing all the three major pathogens of the crop could not be used in crop protection, since *P. aeruginosa* is a human and animal pathogen (Kumar et al. 2012). The use of *P. aeruginosa* has been banned in Europe for crop protection as a policy. Similar biosafety regulatory mechanisms are needed in India and elsewhere to make this technology farmer friendly. However, large-scale field demonstrations and correct appraisal among the farming community is called for to popularize this technology. Critical ecological studies of these microbes, their quantification in relation to protection through molecular techniques (Aravind et al. 2011), identification of organisms with multiple mode of action to address the disease complexes, strainal improvement and the field demonstrations are needed to popularize this microbial biotechnology for crop protection in spice crops (Sarma et al. 2014).

25.14 Conclusions

Biotechnology has the potential to be a key tool to achieve sustainable agriculture and agri-based industry, through improvement of food production in terms of quantity, quality and safety while preserving the environment. Significant progress has been made in the field of biotechnology for micropropagation, conservation and management of genetic resources, disease and pest management and molecular characterization. Identifying markers linked to important agronomic characters will help in marker-assisted selection to shorten breeding time. Application of recombinant DNA technology for the production of resistant types to biotic and abiotic stress has to go a long way before they can be effectively

used in spices improvement. Though programmes have been initiated in many laboratories for *in vitro* secondary metabolite production, these techniques are to be refined and scaled up for possible industrial production of the products. Owing to their commercial potential, intensification and application of biotechnology in spices is important and indispensable in the coming decade. Microbial intervention through *T.harzianum, T.viride, P.florescens* and AM fungi has been found effective in disease suppression and growth promotion. Induced systemic resistance (ISR) as reflected in defense response appears to be one of the modes of action in disease suppression in black pepper and ginger. This microbial biocontrol technology at present is being practiced by the farming community for effective disease management in spice crops.

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Metabolic Engineering in Plants

26

Ashwani Kumar

Abstract

Metabolic engineering considers metabolic and cellular system as an entirety and accordingly allows manipulation of the system with consideration of the efficiency of overall bioprocess, which distinguishes itself from simple genetic engineering. Metabolic engineering in plants involves the modification of endogenous pathways to redirection of one or more enzymatic reactions to produce new compounds or improve/retard production of known compounds. Metabolic engineering is the redirection of one of more enzymatic reaction to produce new compounds in an organism, improve production of existing compounds or mediate the degradation of compounds. It reviews the current status of metabolic engineering.

Keywords

Metabolic engineering • Terpenoids • Alkaloids • Cellulose • Gene • Transcription regulation

26.1 Introduction

Significant advances have been made in metabolic engineering through the application of genomics and proteomics technologies to elucidate and characterise metabolic pathways and their regulation at all levels like genes, enzymes, compartmentation, transport and accumulation. Recently, the focus has been on metabolic pathways in the context of the entire cell rather than at the single pathway level. The various interactions between proteins, DNA and metabolites, called the interactome, are the key determinant of any cellular process. Proteins are the functional entities that carry out the actual metabolic process by interconverting metabolites (metabolome). It is necessary for the metabolic engineer to understand these relationships in order to accurately design and control biological systems (Vemuri and Aristidou 2005).

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26.2 Aims of Metabolic Engineering

The goal of metabolite engineering is to increase the production of certain compounds in the normal producing plant species or to transfer a pathway to other plant species or production of novel compounds not yet produced in nature by plants by gene manipulation. To increase the production of compounds, two general approaches have been followed. (1) Methods have been employed to change the expression of one or a few genes, thereby overcoming specific rate-limiting steps in the pathway, or to shut down competitive pathway and to decrease catabolism of the product of interest. (2) Attempts have been made to change the expression of regulatory genes.

26.3 Metabolic Engineering

Neumann et al. (2009) and Kumar (2015) (this volume) reviewed in detail the production of secondary metabolites, pharmaceutical and other biologically important compounds in plant cell and cultures. Ramawat et al. (2009) and Kumar and Sopory (2010) reviewed metabolic engineering in plants with respect to primary and secondary metabolites including terpenoids, carotenoids, phenolics and flavonoids. Plant molecular breeding and use of plant biotechnology for production of novel compounds have been described by Kumar et al. (2014). Several antitumor compounds have been isolated from plants. Kumar (2010), Kumar and Roy (2011) and Dar et al. (2013) reviewed breeding methods for plant tissue culture and under field conditions for medicinally important plants. Sujatha and Kumar (2008) and Tang and Newton (2010) described different plant tissue culture systems with improved efficiency using *Agrobacterium*- mediated genetic transformation. Various natural products like tropane alkaloids, atropine, hyoscyamine, scopolamine and steroidal precursors are mainly produced by roots (Ramawat and Merillon 2000; Kumar and Roy 2006; Kumar and Sopory 2008, 2010; Kumar and Shekhawat 2009). *Eclipta alba* (L.) Hassk. was transformed by *Agrobacterium rhizogenes* strain MTCC 532 (Bhansali and Kumar 2014). Plants of family Valerianaceae, e.g. *Nardostachys jatamansi*, have been induced in tissue culture to produce higher levels of compounds than in intact plants. Studies on medicinal uses of ferns and their biotechnology have been extensively covered by Fernadez et al. (2011).

26.3.1 Biofuels

The world's fossil fuel reserves are running out. Governments around the world are looking to diversify their energy sources with nuclear, hydroelectric, wind and biofuel energy. Recent advances in synthetic biology and metabolic engineering can engineer microorganisms to produce new fossil fuel replacements. Such products might include short-chain, branched-chain and cyclic alcohols as well as alkanes, alkenes, esters and aromatics.

Biofuels include bioethanol, biodiesel (methyl and ethyl esters), biohydrogen, alkanes and various other hydrocarbon mixtures (such as wood gas and syngas). Today, however, ethanol is by far the most widely available; in the United States, for example, it constitutes 99 % of all biofuel produced (a total of over four billion gallons in 2005 from the fermentation of sugars derived from corn). Ethanol is biodegradable and produces slightly less greenhouse emissions than fossil fuel (carbon dioxide is recycled from the atmosphere to produce biomass); it can replace harmful fuel additives (e.g. methyl tertiary butyl ether, MTBE).

Ethanol is derived from corn or wheat for bioethanol and soya bean for biodiesel. However, recently biotech innovations and metabolic engineering are making it possible to use cellulosic biomass (wood chips, corn stalks, willow trees and switchgrass) for biofuel production.

Metabolic engineering for the production of biofuels has been reviewed by Kumar (2013). Bioethanol from bamboo is shown to be both technically and economically feasible, as well as competitive with petrol in China. Some measures may include improving sugar release with more

effective pretreatments and reduced enzyme usage, accessing low-cost bamboo feedstock or selecting feedstocks with higher/more accessible cellulose.

26.3.2 Lignocellulosic Biomass to Ethanol

Lignocellulosic biomass, such as agricultural residues and forestry wastes, has been widely recognised as a sustainable source for biofuel production (Gong et al. 2014). Roy and Kumar (2013) reviewed methods for lignocellulosic materials. However, cellulose and hemicellulose, two major sugar polymers of lignocelluloses, have to be depolymerised by hydrolysis to enable more efficient microbial utilisation (Acker et al. 2013b). Biomass hydrolysates usually contain monosaccharides as well as various amounts of oligosaccharides. Oligosac-charides are more challenging substrates than monosaccharides for microorganisms, because assimilation of oligo-saccharides may require additional hydrolytic enzymes and transportation systems.

26.4 Metabolic Engineering of Oilseed Crops

Recently, the demand for bioenergy produced from plant materials has increased. Transcription factors that control the biosynthesis of lignocellulose and lipids should be particularly investigated. Increase of the lipid content and modification of fatty acids in oil seeds have been required by the oleo-chemical industry.

The metabolic engineering of seed oils is attractive as the modification of lipid metabolism to change the quantity and quality of fatty acids in plants has important applications in the food industry, as well as in the production of detergents, fuels, lubricants, paints and plastics (Drexler et al. 2003).

It is possible to genetically manipulate the composition of long-chain and medium-chain fatty acid in some of common oil crops, such as *Glycine max* (soybean), *Brassica napus* (oilseed rape), *Helianthus annuus* (sunflower) and *Zea mays* (maize) (Drexler et al. 2003).

Recent notable studies include experiments in which oilseed rape (*Brassica napus*) was transformed with multiple cDNAs encoding enzymes for the desaturation and functional modification of long-chain fatty acids. Liu et al. (2003) expressed 18:1 D12 desaturase either alone or in combination with 18:2 D6 desaturase. With the 18:1 D12 desaturase alone, the seeds produced up to 46 % linoleic acid, whereas with both enzymes, the seeds produced up to 43 % linolenic acid. Recently, the a-linolenic acid content of rice grains has been increased tenfold by expressing the soybean FAD3 gene encoding O3 fatty acid desaturase (Anai et al. 2003).

26.5 Metabolic Engineering of Secondary Metabolites

26.5.1 Terpenoids

Terpenoids, also known as isoprenoids, are the largest family of natural compounds, consisting of more than 40,000 different molecules. All terpenoids are synthesised from the common precursors, dimethylallyl pyrophosphate and isopentenyl pyrophosphate. This occurs through two distinct pathways, the mevalonate-independent pathway and the mevalonate pathway, both of which have been targets for metabolic engineering (Broun 2004). Transgenic plants with modified terpenoid production help in fundamental studies of the biosynthesis and regulation of these compounds. Monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20) and tritrepenoids (30) are considered to be secondary metabolite. Following the recent discovery of the role of the 2-C-methyl-D- erythritol-4-phosphate (MEP) pathway in the biosynthesis of plastidial terpenoid, such as the carotenoids and monoterpenes and diterpenes, several genes of this pathway have been cloned (Broun 2004; Broun and Somerville 2001). Luecker et al. (2004) have reported the transformation of tobacco plants with three different monoterpene synthases from lemon. When the three transgenes

were stacked in one transgenic line, the tobacco plants produced more terpenoids and displayed a different terpenoid profile to wild-type plants.

26.5.2 Ornamentals

The global value of the ornamental sector of the horticulture industry is likely to be 250–400 billion USD (Chandler 2013). Value addition to ornamentals used in the floristry, gardening, landscaping and the environmental amenity industries can be done by metabolic engineering. The molecular breeding approaches represent vast opportunities as alternatives to chemical treatments. Genetic engineering has potential to create ornamental plants suitable for environmentally friendly production. Metabolic engineering approaches targeting elements of the ethylene signal transduction pathway and its perception (e.g. etr1-1 and IPT) serve as possible alternatives to avoid the use of chemical ethylene inhibitors. Over-expression of GA2ox genes and reduction of expression of GA20ox genes as well as modulation of regulatory genes involved in GA perception and signal transduction (GAI, SHI) and overall hormone homeostasis (KNOX) have successfully led to compact plants (see for details Lütken et al. 2012).

Lilies, *Lilium* spp., are of importance in floriculture. New lily cultivars with improved qualities such as resistance to insects and diseases, longevity and novel flower colour are desirable for both producers and consumers. Genetic modification has been proven to be an alternative to conventional breeding for the introduction of resistance to insects and disease in ornamental crops (Chandler and Brugliera 2011). Wang et al. (2012) provided information on factors affecting lily regeneration in a broad range of cultivars and also on gene transfer for further studying the transformation in the commercially most interesting lily hybrid cultivars, i.e. Orientals and OTs.

26.5.3 Beta-carotene

'Golden rice' contains up to 200 mg β-carotene per 100 g grain. Engineering high levels of β-carotene in the endosperm of 'golden rice' is a major breakthrough. Rice endosperm accumulates geranylgeranyl diphosphate. Therefore, four novel enzymes are required for β-carotene synthesis in this tissue: phytoene synthase, phytoene desaturase and z-carotene desaturase and lycopene b-cyclase. The researchers found that phytoene synthase and crtI alone were sufficient not only for the synthesis of lycopene but also for that of β-carotene and zeaxanthin.

Brassica napus seeds and oil contain negligible amounts of β-carotene. In this case, the simple over-expression of a bacterial phytoene synthase, crtB, fused to a plastidic transit peptide and under the control of a seed-specific napin promoter, is sufficient to boost β-carotene 300-fold, to a level similar to that found in oil palm. The β-carotene (provitamin A) levels have been increased in rice and canola seeds and in tomato fruits (Roemer et al. 2002). Astaxanthin, a non-plant carotenoid, has been produced in tobacco flowers (Mann et al. 2000). For several reasons, the carotenoid biosynthetic pathway is a very interesting target for genetic engineering. The carotenoids are important colour compounds in flowers, fruits and food products, and they are also good antioxidants. Vitamin A is formed from β-carotene. Vitamin A deficiency is a widespread problem in many areas like Asian countries. The introduction of β-carotene biosynthesis into the major staple food rice, by over-expression of phytoene synthase, phytoene desaturase and lycopene-cyclase, is thus an important achievement.

26.5.4 Insect Repellents

In one study, petunia was transformed with the (S)-limonene synthase gene from *Clarkia breweri* resulting in the production of linalool, which repels aphids (Lucker et al. 2001).

26.5.5 Flavonoids

Flavonoids have a wide range of colours from pale yellow to red, purple and blue. The flavonoid

biosynthetic pathway has been the choice to genetically engineer flower colours to obtain novel colours. Because of their antioxidant activity, higher levels of anthocyanin and flavonoids in food are an interesting objective. Much work has focused on tomato. Chalcone isomerase (CHI), an early enzyme of the flavonoid pathway, was found to be the key to increase flavonol production (Muir et al. 2001). Over-expression of petunia CHI gene led to a 78-fold increase of flavonoid levels in the tomato peel. Upon processing such tomatoes, a 21-fold increase of flavonols in tomato paste was achieved, if compared with non-transgenic controls.

26.5.6 Alkaloids

The terpenoid indole alkaloid pathway has been the target of numerous genetic engineering attempts, owing to the fact that about 15 terpenoid indole alkaloids are industrially important, including the antitumour alkaloids vinblastine, vincristine and camptothecin. Most efforts have been concentrated on the early part of the pathway and on over-expression of early genes, aiming to increase the metabolite flux into the alkaloid pathway. In particular, the genes encoding tryptophan decarboxylase (TDC) and strictosidine synthase (STR) have been studied extensively in *Catharanthus roseus* cell cultures (Verpoorte et al. 1994; Verpoorte et al. 2000; Verpoorte and Memelink 2002).

26.6 Alkaloid Production in Tryptophan Decarboxylase Silenced Lines

Tryptophan decarboxylase, a pyridoxal phosphate–dependent enzyme that produces tryptamine **1** from tryptophan **2** (Facchini et al. 2000), was targeted for gene silencing (Helliwell and Waterhouse 2005). The plasmid designed to suppress tryptophan decarboxylase (pTDCi) was transformed into *Agrobacterium rhizogenes*, which was then used to infect *C. roseus* seedlings to generate hairy root culture as described (Hughes et al. 2002; Runguphan and O'Connor 2009). Hairy root lines harbouring the pTDCi silencing plasmid were cultured in liquid media, and alkaloids were extracted from the plant tissue according to standard protocols. Gratifyingly, the production of all major tryptamine-derived alkaloids ajmalicine **3**, serpentine **4**, catharanthine **5** and tabersonine **6** was substantially decreased in the five silenced lines that were examined. Suppression of alkaloid production appears to be stable, with little change in alkaloid production levels after seven subcultures performed thus far.

26.7 C3 and C4 Metabolism and Plant Tissue Culture Studies

The majority of terrestrial plants, including many important crops such as rice, wheat, soybean and potato, are classified as C3 plants that assimilate atmospheric CO2 directly through the C3 photosynthetic pathway. C4 plants such as maize and sugarcane evolved from C3 plants, acquiring the C4 photosynthetic pathway to achieve high photosynthetic performance and high water- and nitrogen-use efficiencies. So, there is great scope to study the metabolic pathway and try to engineer this in another plant, which is having less photosynthetic performance and high water and nitrogen efficiencies. The good news is that technology continues to hold great promise for the future of plant metabolite engineering.

26.8 Conversion of Cellulose into Microbial Lipids by *Cryptococcus curvatus*

The oleaginous yeast *C. curvatus* can produce microbial lipids using a mixture of glucose and xylose as well as cellulosic biomass as feedstocks. Gong et al. (2014) has developed the simultaneous saccharification and lipid production (SSLP) process for direct conversion of cellulose into lipids by oleaginous species in the presence of cellulase and β-glucosidase.

26.9 Tocopherols

Higher vitamin E doses of 100–1,000 IU have been associated with cancer reduction, improved immune response and cardiovascular benefits. Such high doses are currently realised only through supplemental tocopherol intake. Tocopherols are also used in the pharmaceutical and cosmetics industry and as animal feed additives to improve the quality and shelf life of meat (Bramley et al. 2000). Natural vitamin E originates almost exclusively from soybean oil and is used predominantly for human applications due to its premium price and limited availability.

26.10 Role of Plant Tissue Culture Studies on Secondary Metabolite Production and Metabolic Engineering

Plant cell walls determine the shape and architecture of the plant body and play major roles during cell differentiation, defence against pathogens and cellular growth. Cellulose, the main polysaccharide of plant cell walls, is made of β-1,4-linked glucan chains, which form crystalline microfibrils and act as a framework for the deposition of other wall components. The matrix polymers, such as hemicelluloses and pectins, are structurally diverse polysaccharides, and most of them harbour heavily substituted side chains. Second-generation biofuels are generally produced from the polysaccharides in the lignocellulosic plant biomass, mainly cellulose. However, because cellulose is embedded in a matrix of other polysaccharides and lignin, its hydrolysis into the fermentable glucose is hampered.

26.11 Cell Wall Polysaccharides Are Synthesised in Different Cellular Compartments

Cell wall polysaccharides are synthesised in different cellular compartments. Cellulose is synthesised at the plasma membrane by hexameric cellulose synthase (CESA) complexes, which are believed to hold 36 individual CESA proteins (Somerville et al. 2004). In contrast to cellulose, the synthesis of the matrix cell wall polymers, that is, hemicelluloses and pectins, is carried out in the Golgi apparatus (see Liepman et al. 2005; Sterling et al. 2006; Jensen et al. 2008). These polymers are synthesised by a plethora of glycosyltransferases and are subsequently modified by different hydrolases, esterases and lyases (Henrissat et al. 2001; Somerville et al. 2004). Modification and secretion of cell wall components can depend on the demand for cell wall biosynthetic events that may be transduced by cell surface signalling devices needs to be transformed into cellular responses. These may include post-translational modifications of enzymes, modulation of the rate of secretion and transcriptional activations or repressions.

Although the lignin content had the main effect on saccharification, also other cell wall factors could be engineered to potentially increase the cell wall processability, such as the galactose content. A better understanding of the effect of lignin perturbations on plant cell wall composition and its influence on saccharification yield provide new potential targets for genetic improvement (Acker et al. 2013a).

In contrast to common perception, a reduction in lignin was not compensated for by an increase in cellulose, but rather by an increase in matrix polysaccharides.

Cell wall components to the cell surface are dependent on the secretory shuttling system (Taylor 2008). The endomembrane system also contains V-ATPase complexes which acidify the vesicle lumen, a process required for certain aspects of membrane trafficking. The vesicles containing the CESAs are transported to the plasma membrane with the help of actin cables via interactions with actin-associated proteins, possibly KAM1/MUR3 (Tamura et al. 2005; Geisler et al. 2008). After the delivery of the CESA complexes to the plasma membrane, vesicle components are recycled back to the endomembrane system. Microfibrils are produced by CESA complexes and are co-aligned with the

microtubules beneath the plasma membrane via microtubule-associated proteins (see review Geisler et al. 2008).

This system encompasses protein synthesis and folding in the ER, glycan modification and synthesis in the Golgi apparatus and endosomal vesicle loading and shuttling to different cellular locations, including the plasma membrane and the vacuole. Brux et al. (2008) reported that the cellulose content is dependent on V-ATPase activity. The pH difference across the vesicle membrane is established by endosomal-localised vacuola ATPases (V-ATPases). There is also evidence that these differently located isoforms may carry out different biological functions in the cell. RNA interference of the TGN-localised, but not of the tonoplast-located, VHA-a isoform leads to reduced cell expansion (Brux et al. 2008). The VHA-a1 protein is located at the trans-Golgi network (TGN), whereas two other isoforms, VHA-a2 and VHAa3, are specific for the tonoplast (Dettmer et al. 2006). This is consistent with phenotypes observed in other mutants for V-ATPase subunits, which also display altered morphology of Golgi stacks (Strompen et al. 2005; Dettmer et al. 2010).

It is therefore possible that the defects in TGN trafficking caused by blocking V-ATPase activity result in retention of the CESAs in the TGN. Thus, the activity of TGN-localised V-ATPases appears crucial for vesicle trafficking during cellulose synthesis and cell expansion in plant cells. As in other eukaryotic cells, small GTPases and guanine nucleotide exchange factors (GEFs) are involved in the regulation of vesicle formation and trafficking in plants and also in the definition of the membrane identity of endomembrane compartments (Strompen et al. 2005; Hurtado-Lorenzo et al. 2006). This suggests that the V-ATPase could act both as a pH modulator and sensor that governs the recruitment of proteins from the cytosol to the endosomal membrane (Paredez et al. 2006; Dettmer et al. 2005, 2006). At least one component that is directly involved in the synthesis of cell wall polymers also affects vesicle trafficking (Madson et al. 2003; Tamura et al. 2005). The importance of the actin filaments for cell wall deposition was further underscored in a recent study performed in xylem cells (Wightman and Turner 2008). They suggested that not only microtubules, which may guide the CESAs (Paredez et al. 2006), but also actin cables may be crucial for cellulose deposition (Wightman and Turner 2008).

26.12 Compounds of Pharmaceutical Importance

In the 1950s, Charles Pfizer Co. (USA) tried unsuccessfully to produce pharmaceutical compounds through the generation of large-scale cultures of plant cells. Such an attempt was however well received abroad, i.e. Germany and Japan, and resulted in the development of feasible industrial applications of cultured cells in the late 1970s (see Kumar and Roy 2006, 2011; Thorpe 2012; Neumann et al. 2009; Kumar and Sopory 2010). By using radioimmunoassay techniques and enzyme-linked immunosorbent assays, as well as cell cloning and repeat selection of high-yielding strains from heterologous cell populations, improvements in production have been attempted.

Cell immobilisation techniques have also been used. The utilisation of bioreactors allowing the large-scale growth of cells has also contributed to the field. Stirred tank reactors and several types of air-driven reactors have been employed. Such methods involve a two-step process: biomass accumulation through active cell growth followed by product synthesis coupled with reduced cell proliferation (Sharma et al. 2011).

Enhancement of secondary product formation has also been achieved by selecting mutant lines overproducing the desired product and by using abiotic factors, including ultraviolet treatments and exposure to heat or to cold and heavy metals (Neumann et al. 2009; Kumar and Sopory 2010; Kumar and Roy 2011).

26.13 Conclusions

Metabolite engineering is generally defined as the redirection of one or more enzymatic reaction to produce new compounds in an organism,

improve the production of existing compounds or mediate the degradation of compounds. As presented above, several points in a given metabolic pathway can be controlled simultaneously either by over-expressing and/or suppressing several enzymes or through the use of transcriptional regulators to control several endogenous genes. Over the past few years, there has been a growing realisation that metabolic pathways must be studied in the context of the whole cell rather than at the single pathway level and that even the simplest modifications can send ripples throughout the entire system. Metabolic engineering in plants involves the modification of endogenous pathways to increase flux towards particular desirable molecules or redirection of one or more enzymatic reaction to produce new compounds in an organism or mediate the degradation of compounds. Recently, significant advances have been made in metabolic engineering through the application of genomics and proteomics technologies to elucidate and characterise metabolic pathways. The major challenge for the coming years is to obtain more information about regulation at all levels like genes, enzymes, compartmentation, transport and accumulation. This will open the way for successful strategies for altering the accumulation of certain compounds. In this review, efforts are made to look at recent examples of single- and multiple-gene metabolic engineering in primary and secondary metabolism groups.

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Genetically Modified Crops

27

S.B. Nandeshwar

Abstract

Genetic engineering is the alteration of the genome of an organism by introduction of one or a few specific foreign gene mainly with the help of *Agrobacterium* or particle gun. The modified organism is described as 'transgenic organism'. If gene is inserted in plants, they are called as transgenic plants. In this highly sophisticated method of genetic modification of plants, the genetic material or DNA sequence coding for a desirable trait is located in the donor organism by a variety of molecular technique and then cut out from the parental DNA using the 'molecular scissors' known as restriction endonucleases. Genetic engineering makes it possible to exchange or introduce the gene of interest, using the modern tools of recombinant DNA (rDNA) techniques, within same or different species or also within kingdoms. Genetically altered foods are made from plants or animals that have had their deoxyribonucleic acid (DNA) changed in a way that does not happen naturally. Often, individual genes are transferred from one organism to another, even when the organisms are not related.

As the population on earth is expected to rise from 6.7 billion to 9 billion by 2050, this will increase the demand for food; hence, there is a need to increase food to 50 % by 2030. Because of many problems like availability of arable land which has affected due to urbanisation, desertification and environmental degradation. The second big challenge is the availability of water good enough for drinking and farming. Water resources are under severe strain in many parts of the world. Hence, the challenge of food production by 50 % must be tackled on the available land with water scarcity using biotechnology tools. Modification of plants by rDNA technology would be most beneficial.

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The need for better traits in the plant is the base of GM plants, and the skill and knowledge of transferring gene into plants indorsed the conception that some of the primary transgenic crops like tobacco, tomato, corn, soybean and cotton developed mainly for insect and herbicide resistance with single or multiple gene are under cultivation in different parts of the world. Apart from these qualities, researchers have also proposed on traits like increased vitamin and mineral contents of grains by enhanced amino acid profiles, better vegetable oils by improving nutritional composition of oilseed and crops with increased tolerance to biotic and abiotic stresses. The production of active medical compounds and industrial enzymes and bioplastics is an important trait for manipulation.

Keywords
Genetically modified (GM) crops • Gene transfer in plants • *Agrobacterium* • Particle gun • GM foods • Biosafety

27.1 Introduction

Genetic engineering, the term we know today, started with the discovery of *Agrobacterium tumefaciens* and its ability to integrate its own genome into host plant, and thereafter, particle gun bombardment and electroporation technique came in the game which facilitates insertion of gene of interest into the plant genome (Barton et al. 1983; Herrera-Estrella et al. 1983; Horsch et al. 1985; Fromm et al. 1985; Klee et al. 1987; Sanford et al. 1987). Deliberate alteration of the genome of an organism by introduction of one or a few specific foreign gene is referred to as 'genetic engineering' or 'genetic transformation', and the modified organism is described as a 'transformed' or 'transgenic organism'. In this highly sophisticated method of genetic modification of plants, the genetic material or DNA sequence coding for a desirable trait is identified in the donor organism by a variety of molecular technique and then cut out from the parental DNA using the 'molecular scissors', restriction endonucleases. The relatively small pieces of DNA are then introduced in the recipient plant cells by one of several possible methods, and the plant is regenerated from them. Such plants having transgene in their genome are called as genetically engineered plants, i.e. GE plants or genetically modified (GM) crops and food.

Genetic engineering makes it possible to exchange or introduce the gene of interest, using the modern tools of recombinant DNA (rDNA) techniques, within same species or from species into an organism or within kingdoms. Genetically altered foods are made from plants or animals that have had their deoxyribonucleic acid (DNA) changed in a way that does not happen naturally. Often, individual genes are transferred from one organism to another, even when the organisms are not related.

By manipulating the genes and genome of plants, scientists have created two types of genetically altered foods: first are those beneficial to the producer and second are those beneficial to the consumer. Manipulation in genome all depends upon the traits we want to introduce in the plant. The most important beneficial traits include insect resistance, viral resistance, herbicide resistance, increased yield or reduced time needed to ripen. Consumer-friendly traits include longer freshness, greater nutritional value or medicinal value.

27.2 Need for Genetically Modified Crops

The population on earth is expected to rise from 6.7 billion to 9 billion by 2050. This will increase the demand for food, and to accommodate this, agriculture production all over the world need to be increased by 50 %, by 2030 (Song et al. 2003). One of the biggest problems is availability of arable land. The percentage of agriculture land is limited, the remaining land lost to urbanisation, desertification and environmental degradation. Second big challenge is availability of water good enough for drinking and farming. Water resources are under severe strain in many parts of the world. In the last 6 years, there is a fourfold decrease in the availability of freshwater per person (Tzfira and Citovsky 2006). Seventy percent of the total available water is used for agriculture (The Royal Society 2009), and about 50 % of the world total wetland has already disappeared. Hence, the challenge of raising food production by 50 % must be tackled on the available land with water scarcity.

Climate change is the next big challenge which will put pressure on agriculture production. Greenhouse effect, glacier melting on poles and rise in the sea level ultimately submerge low-lying croplands. Temperature will rise above 30°, there will be increased heat and drought spells which ultimately lead to limited crop production (Sanford et al. 1987). Despite of these abiotic stresses, biotic stresses are also going to be challenging for food production. Pest and diseases are also expected to increase. The stress due to biotic and abiotic factors leads to 30–60 % yield losses each year globally.

Current situation is at critical stage and will be more demanding for creating more land and water for cultivation. We cannot increase the land availability for agriculture but what we can do is improve our plants to withstand these stresses. Thus, the important goal would be to adopt our existing food crops to higher temperature, decrease water availability, flooding, rising salinity and challenging pathogen and insect threat (Song et al. 2003). Such improvements will require diverse approach that will enhance the sustainability of our agriculture and farmers. These include more effective land and water management, integrated pest management of new generation of agriculture and crop tolerance to diverse stresses (Shah et al. 1986).

27.3 Classical Breeding Versus Genetic Engineering

Modifications made in plants by rDNA technology and classical breeding are having both similarities and differences. Similarities involve exchange of genome/genes, changes in sequence order, up- and downregulation of genes in an organism and utilisation of many of the enzymes. However, differences with the rDNA technique are, small amount of genetic material is modified (one or few genes). But in classical breeding technique, where whole genome of an organism is involved, which exchanges position of genes within organism (Fig. 27.1). The next important difference is that in rDNA technology we can precisely control expression and place of expression of gene, where this precision is not possible with classical breeding (Cho et al. 2000).

Third important difference is that classical breeding can be done in related species or genera. For example, a wild *Lycopersicon* variety can be crossed with cultivated tomato (*Lycopersicon esculentum*) varieties (Chetelat et al. 1995), and wheat (*Triticum aestivum*) can be crossed with rye (*Secale cereale*) to yield triticale (Stallknecht et al. 1996). But in many crosses, wild relatives and different genera or species are not compatible, or crosses can be made but the resulting embryos must be rescued by *in vitro* culture to obtain a plant (Shah et al. 1986). Exactly apposite to this, in rDNA approach, we can use genetic material from any

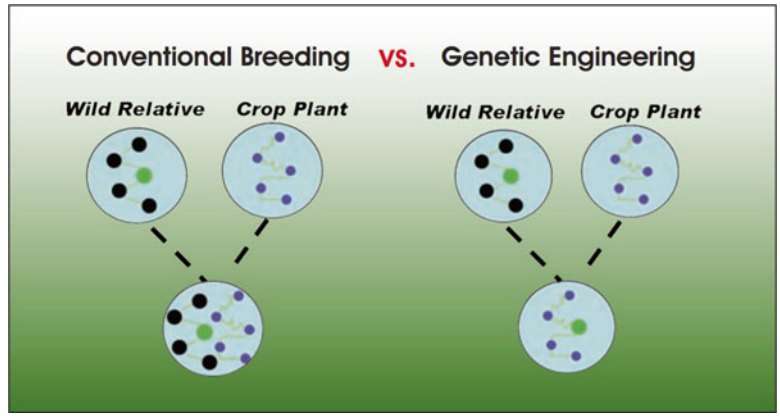

Fig. 27.1 Classical breeding vs rDNA/GM food (Source: Biotech Mentor's Kit 2003)

living organism. This gives us freedom to introduce DNA from bacteria, fungus, virus and animals to plants. Thus, we can come up with gene combinations never seen before.

27.3.1 Marker-Assisted Selection in Aid to Classical Breeding

We all know classical breeding is a cumbersome and tedious method which takes several years to develop a single variety with desirable character. To increase its speed and specificity, marker-assisted selection (MAS) comes in aid. When we know which specific gene or allele is responsible for certain characters in plants, then it can be used as a marker to assist selection of specific traits in breeding. This ability to mark or select the desirable character specifically and to avoid the deleterious ones in a fast and reliable manner is critical to the development of improved germplasm through breeding.

MAS can help to quickly identify plants with desirable traits in large populations. These markers are closely associated with the desirable trait and are indirectly used for trait selection in plants (Dubcovsky 2004). Nowadays with the knowledge of complete and partial genome of the organism, it is possible to identify marker within the causative gene itself rather than linked to it. For example, *Xa5* gene, which provides resistance to bacterial blight in rice (*Oryza sativa*), marker was found in gene itself, and its use prevented separation of the marker from the trait by recombination (Iyer-Pascuzzi and McCouch 2007).

MAS particularly comes handy for selection of traits when (a) phenotypic screening in the field is difficult or costly, e.g. drought/frost tolerance or resistance to exotic diseases or pests; (b) multiple alleles exist; and (c) recessive or low heritability traits exist that require progeny testing. MAS is used for some important traits, e.g. leaf rust resistance in wheat (Niederberger et al. 2004), erect panicle in rice (Kong et al. 2007), soybean (*Glycine max*) rust resistance (Hyten et al. 2007), drought adaptation in maize (Ribaut and Ragot 2007) and root-knot nematode resistance in cotton (*Gossypium hirsutum*) (Ynturi et al. 2006).

Organic farming is a new upcoming trend in international market. GM crop utilisation is banned in this practice. National Organic Standards specify that these varieties cannot be intentionally grown by organic farmers. But MAS can be used to introduce desired traits from wild or related species, and these species are acceptable to organic growers. Utilising MAS to create food crops has led to the term 'super organics' (Manning 2004, Super organics. Wired, May, Issue 1205 53), crops that are grown under certified organic conditions and were created through classical breeding using genomic information and MAS, not through rDNA methods.

27.4 Global Scenario of Genetically Modified Crops

The success of these GM crops can be measured by increase in their global acreage or farmer acceptance. Biotech crop hectares increased by more than 100-fold from 1.7 million hectares in 1996 to 175.2 million hectares in 2013. In 2013, 175.2 million hectares of biotech crops were grown globally. Hectare of biotech crops increased every single year from 1996 to 2013 with 12 years of double digit growth rates. In 2013, hectarage of biotech crops grew at an annual rate of 3 %, up by 5 million from 170.3 million hectares in 2012. The top biotech crops in order of hectarage are soybean, maize, cotton and canola (Fig. 27.2). Other biotech crops grown in 2013 are alfalfa, sugar beet, papaya, squash, poplar, tomato and sweet pepper. Traits also played major role in their acceptance worldwide as can be seen in Fig. 27.3. For the second consecutive year, developing countries planted more biotech crops than industrial countries (Fig. 27.4). Acceptance of some GM crops in comparison with non-GM counterparts can be seen in Fig. 27.5. Farmers from Latin America, Asia and Africa collectively grew 94.1 million hectares or 54 % of the global 175.2 million hectares of biotech crops in 2013, compared with industrial countries at 81.1 million hectares, or 46 % of the global total. Twenty-seven developing and industrial countries planted biotech crops in 2013. Of the 27 countries which planted biotech crops in 2013, 19 were developing and 8 were industrial countries (Figs. 27.6 and 27.7). The five lead developing countries in Latin America (Brazil, Argentina), Asia (India, China), and Africa (South Africa) grew 47 % of global biotech crops (James 2006 Global status of commercialised biotech/GM crops: 200642). Certainly these GE crops are successful.

27.5 Genetically Modified Crops and Their Traits

The need for better traits (characters) in the plant is the base of GM plant development, and the skill and knowledge of transferring gene into plants

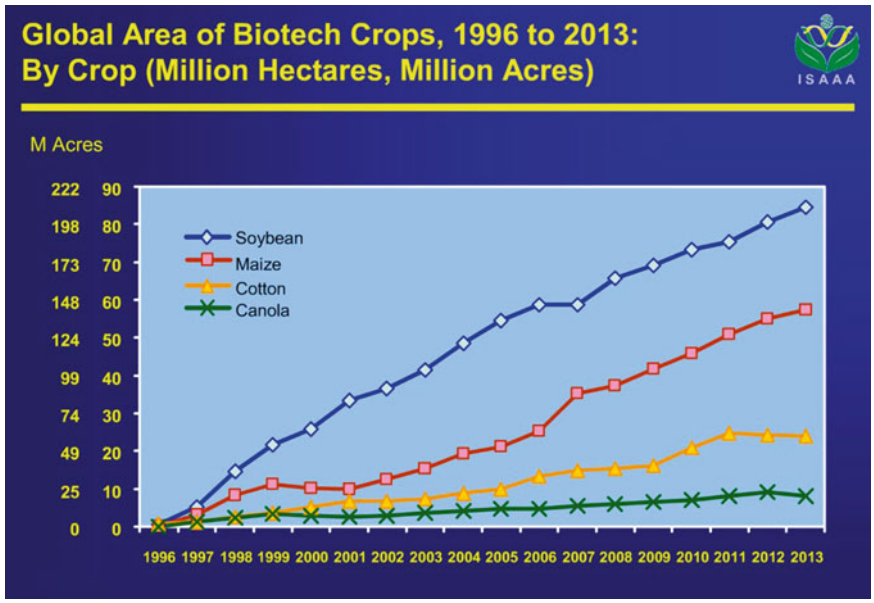

Fig. 27.2 Global area of biotech crops by crops (Source: James 2013)

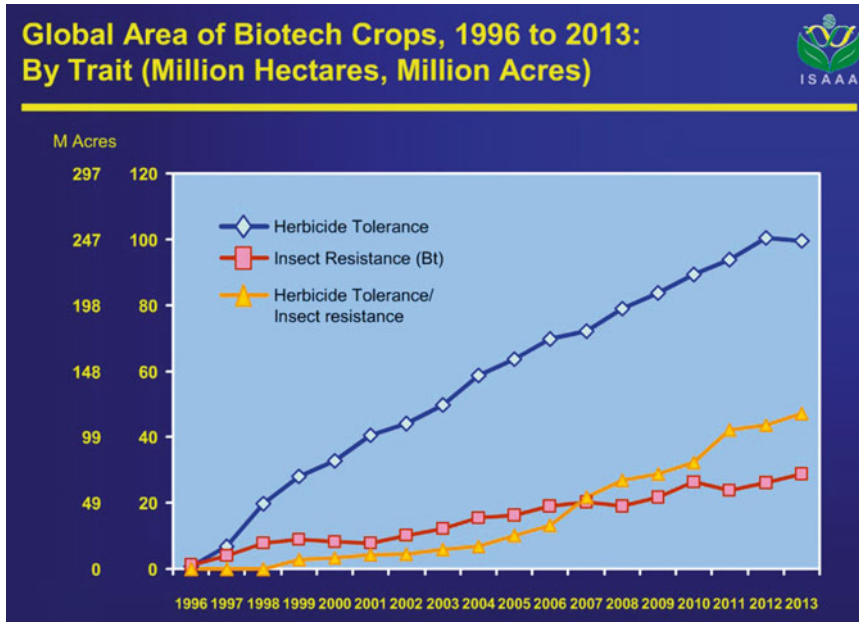

Fig. 27.3 Global area of biotech crops by traits (Source: James 2013)

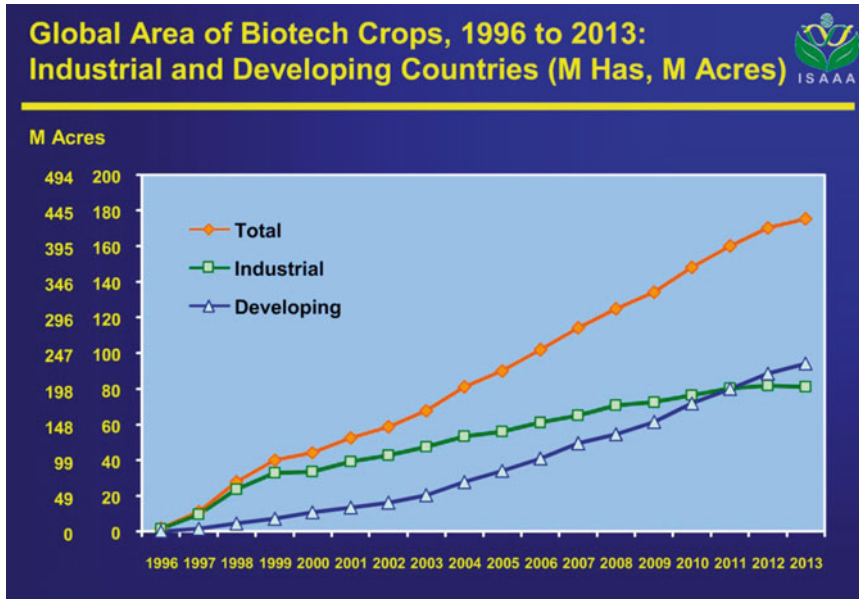

Fig. 27.4 Global area of biotech crops in industrial and developing countries (Source: James 2013)

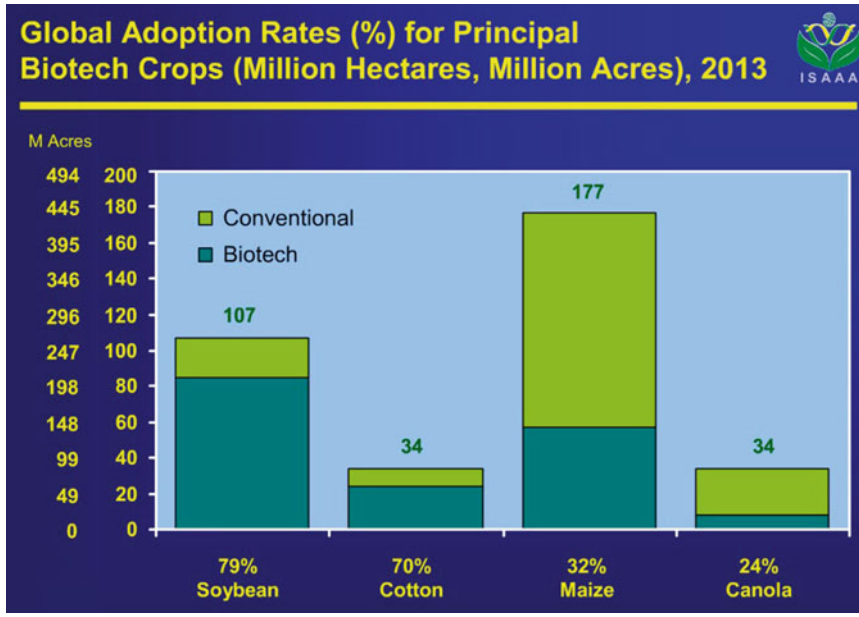

Fig. 27.5 Global adoption rate for main biotech crops (Source: (James 2013))

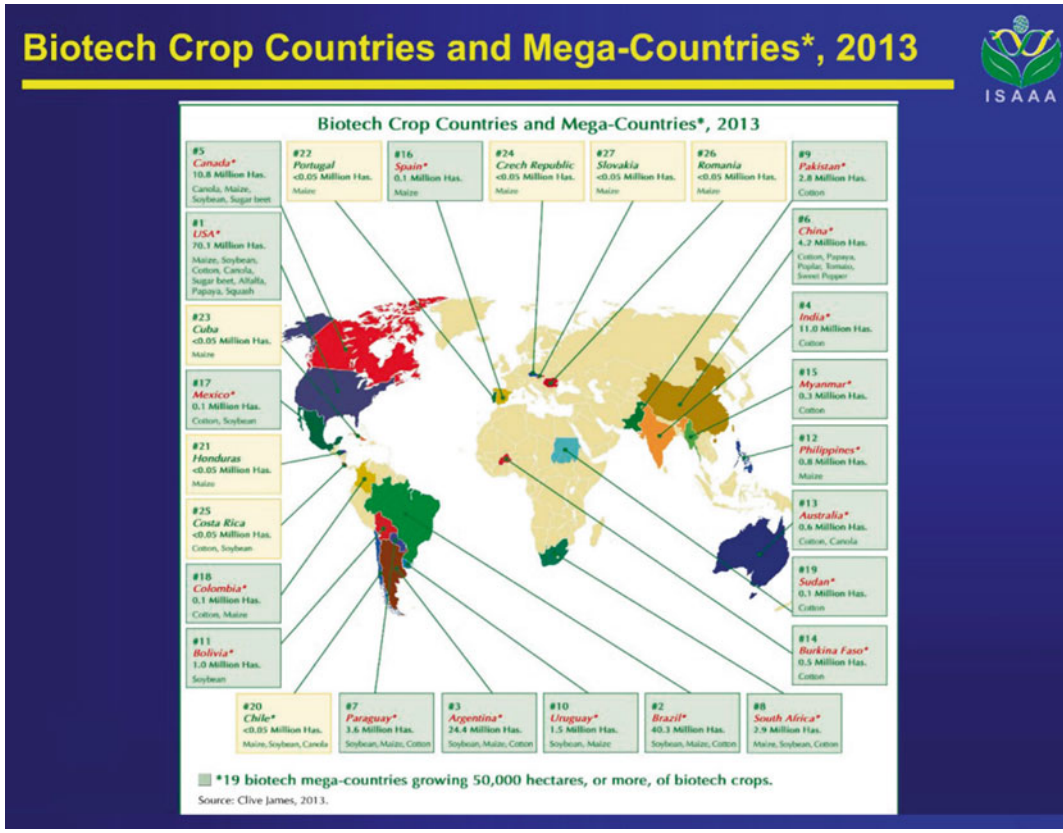

Fig. 27.6 Global map of biotech crop countries (Source: James 2013)

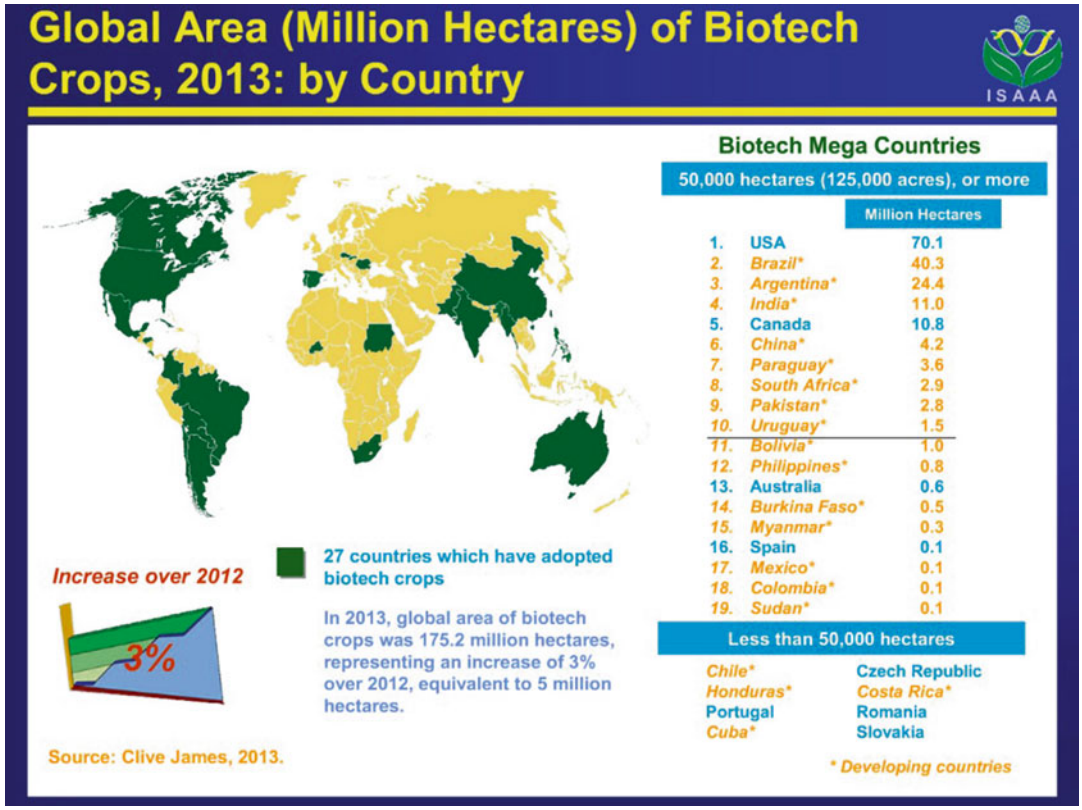

Fig. 27.7 Global area of biotech crop (Source: James 2013)

indorsed the conception of some of the primary transgenic crops like tobacco, tomato, corn, soybean and cotton (Bevan et al. 1983; Martineau 2001; Gordon-Kamm et al. 1990; McCabe et al. 1988; Umbeck et al. 1987; Nandeshwar et al. 2009) (Table 27.1). Bacterial genes were introduced in crops to provide defence against pests and diseases which ultimately condensed application of pesticides in the field (Gatehouse 2008). Herbicide-tolerant crops help in weed management practices a lot. This aided the acceptance of more environmentally friendly farming practices which lead to improved soil quality and diminished greenhouse gas emissions and fertiliser use, on the other hand refining the quality of water run-off to streams, rivers, lakes and aquifers (Cerdeira and Duke 2006; Fernandez-Cornejo et al. 2012; Marra et al. 2004; Qaim 2005; Raven 2010; Scheffe 2008; Shah et al. 1986).

Apart from these qualities, researchers have also proposed on traits like increased vitamin and mineral contents of grains by enhanced amino acid profiles (Lucca et al. 2006), better vegetable oils by improving nutritional composition of oilseed (Knauf 1987) and crops with increased tolerance to biotic and abiotic stresses (Wang et al. 2003). The production of active medical compounds and industrial enzymes (Davies 2010) and bioplastics (Poirier et al. 1995) (Table 27.2).

In the history of GE plants, GE tobacco was the first candidate to be reported in 1983 (Bevan et al. 1983) but not grown commercially. Flavr Savr™ tomato was the first GM crop commercialised in 1994 with longer shelf life (Paoletti et al. 2008; Martineau 2001). This was followed by other commercial GM crop candidates which move in the market such as canola, corn, cotton, soybean, alfalfa and others (Table 27.1). Table 27.3 provides the list of countries involved in GM crop production and import, and Table 27.4 shows the companies involved in research and development of GM crops.

Table 27.1 List of genetically modified crops

1.	Alfalfa (*Medicago sativa*)
2.	Argentine Canola (*Brassica napus*)
3.	Bean (*Phaseolus vulgaris*)
4.	Carnation (*Dianthus caryophyllus*)
5.	Chicory (*Cichorium intybus*)
6.	Cotton (*Gossypium hirsutum* L.)
7.	Creeping bentgrass (*Agrostis stolonifera*)
8.	Eggplant (*Solanum melongena*)
9.	Flax (*Linum usitatissimum* L.)
10.	Maize (*Zea mays* L.)
11.	Melon (*Cucumis melo*)
12.	Papaya (*Carica papaya*)
13.	Petunia (*Petunia hybrida*)
14.	Plum (*Prunus domestica*)
15.	Polish canola (*Brassica rapa*)
16.	Poplar (*Populus* sp.)
17.	Potato (*Solanum tuberosum* L.)
18.	Rice (*Oryza sativa* L.)
19.	Rose (*Rosa hybrida*)
20.	Soybean (*Glycine max* L.)
21.	Squash (*Cucurbita pepo*)
22.	Sugar beet (*Beta vulgaris*)
23.	Sugarcane (*Saccharum* sp.)
24.	Sweet pepper (*Capsicum annuum*)
25.	Tobacco (*Nicotiana tabacum* L.)
26.	Tomato (*Lycopersicon esculentum*)
27.	Wheat (*Triticum aestivum*)

Source: ISSA, GM Approval Database (http://www.isaaa.org/)

Though GE crops have considerable large acreage, they lack the diversity of crop types and traits in commercial production. Some of the limited minor acreage GE crops, which are commercially successful, are papaya (*Carica papaya*), certain types of squash (*Cucurbita* sp.) and sweetcorn (James 2006).

Most of the major GE crops are based on traits like pest protection via genes from Bt or HT, largely showing tolerance on Monsanto's RoundUp® herbicide and Bayer's Liberty® herbicide. Bt cotton resistance to bollworms is among the most accepted all over the world. Recently, maize engineered for rootworm and European corn borer resistance (both Bt based) and tolerance to RoundUp® herbicide.

The only engineered whole fruit available commercially in the USA today is GE papaya by the name 'Rainbow' and 'SunUp', and this papaya contains a viral coat protein gene (Gonsalves et al. 2000; Gordon-Kamm et al. 1990; Lius et al. 1997). GE squash (yellow crookneck, straight neck and zucchini) is another whole GE food available in the USA, and it is resistant to watermelon mosaic virus 2 (WMV2), zucchini yellow mosaic virus (ZYMV) and cucumber mosaic virus (CMV) (Tricoli et al. 1995; Gaba et al. 2004).

Table 27.2 List of traits/characters involved in GM crops

1.	2,4-D herbicide tolerance	2.	Altered lignin production
3.	Anti-allergy	4.	Antibiotic resistance
5.	Coleopteran insect resistance	6.	Delayed fruit softening
7.	Delayed ripening/senescence	8.	Dicamba herbicide tolerance
9.	Drought stress tolerance	10.	Enhanced photosynthesis/yield
11.	Fertility restoration	12.	Glufosinate herbicide tolerance
13.	Glyphosate herbicide tolerance	14.	Isoxaflutole herbicide tolerance
15.	Lepidopteran insect resistance	16.	Male sterility
17.	Mannose metabolism	18.	Mesotrione herbicide tolerance
19.	Modified alpha amylase	20.	Modified amino acid
21.	Modified flower colour	22.	Modified oil/fatty acid
23.	Modified starch/carbohydrate	24.	Multiple insect resistance
25.	Nicotine reduction	26.	Nopaline synthesis
27.	Oxynil herbicide tolerance	28.	Phytase production
29.	Sulfonylurea herbicide tolerance	30.	Viral disease resistance
31.	Visual markers	32.	

Source: ISSA, GM Approval Database (http://www.isaaa.org/)

Table 27.3 List of countries with GM crop approvals

1.	Argentina
2.	Australia
3.	Bangladesh
4.	Bolivia
5.	Brazil
6.	Burkina Faso
7.	Canada
8.	Chile
9.	China
10.	Colombia
11.	Costa Rica
12.	Egypt
13.	European Union
14.	Honduras
15.	India
16.	Indonesia
17.	Iran
18.	Japan
19.	Malaysia
20.	Mexico
21.	Myanmar
22.	New Zealand
23.	Norway
24.	Pakistan
25.	Panama
26.	Paraguay
27.	Philippines
28.	Russian Federation
29.	Singapore
30.	South Africa
31.	South Korea
32.	Switzerland
33.	Taiwan
34.	Thailand
35.	Turkey
36.	United States of America
37.	Uruguay

Source: ISSA, GM Approval Database (http://www.isaaa.org/)

Another GE food in USA is GE sweetcorn, engineered with a Bt gene which provides protection against earworms (*Helicoverpa zea*), very notorious in North America. Damage by earworm results in subsequent fungal and bacterial attack and quality loss (Horvath 2003). Bt expression reduces insect attack and as a result reduces damage of insects, and mycotoxigenic fungi numbers are decreased, and this results in lower levels of mycotoxins, such as fumonisins, which have toxic effects on humans (Westhuizen et al. 2003).

Pest resistance traits are very important in taming crop performance by protecting against pests. A gene from wild Mexican potato genome (*Solanum tuberosum*) put into cultivated potato, allowing the GM potato to survive exposure to the many races of *Phytophthora infestans*, causative agent for the Irish potato famine (Song et al. 2003). *Mi* gene native to tomato was upregulated, which provides resistance against the root-knot nematode (Rossi et al. 1998). Europe which is reluctant to GM crops also held first field trial of GE grapes (*Vitis vinifera*) which took place in the northern Alsace region of France in 2005. A coat protein gene from fan leaf virus was inserted into the grape rootstock (Bouquet et al. 2003).

Crop yield is another trait in GM crops. In 2001, transgenic rice plants exhibit higher photosynthesis due to expression of maize proteins pyruvate orthophosphate dikinase (PPDK) and phosphoenolpyruvate carboxylase (PEPC) (Ku et al. 2001).

Nitrogen use efficiency is also another important agronomic trait which aimed at reducing fertiliser usage and increasing sustainability. The plant-specific transcription factor Dof1, when introduced into the model plant species *Arabidopsis*, increased nitrogen content by ~30 %, improving growth under low-nitrogen conditions (Yanagisawa et al. 2004).

High salt and high and low water availability are the important abiotic stress factors for which we need to improve crops. In this league, *CBF* gene from cold response pathway of *Arabidopsis* induces expression of COR (cold regulator) genes which enhances the tolerance to freeze in non-acclimated plants (Liu et al. 2004). Similarly, GM tomato which is engineered to overexpress vacuolar Na^+/H^+ antiport is able to produce fruits in nearly 40 % of seawater concentration, i.e. around 200 mM sodium chloride, and fruits showed very less amount of sodium chloride (Zhang et al. 2001). Similarly GM rice was produced with incorporation of three genes, which enriched rice with vitamin A, well known as Golden Rice (Park et al. 2005).

Table 27.4 List of GM developers

1.	Agricultural Biotech Research Institute (Iran)
2.	Agritope Inc. (USA)
3.	BASF
4.	Bayer CropScience (including fully and partly owned companies)
5.	Bayer CropScience and MS Technologies LLC
6.	Bayer CropScience and Syngenta
7.	Beijing University
8.	Bejo Zaden BV (Netherlands)
9.	Central Institute for Cotton Research and University of Agricultural Sciences Dharwad (India)
10.	Centre Bioengineering, Russian Academy of Sciences
11.	Chinese Academy of Agricultural Sciences
12.	Cornell University and University of Hawaii
13.	Cotton and Sericulture Department (Myanmar)
14.	DNA Plant Technology Corporation (USA)
15.	Dow AgroSciences LLC
16.	Dow AgroSciences LLC and DuPont (Pioneer Hi-Bred International Inc.)
17.	DuPont (Pioneer Hi-Bred International Inc.)
18.	EMBRAPA (Brazil)
19.	Florigene Pty Ltd. (Australia)
20.	Genective S.A.
21.	Huazhong Agricultural University (China)
22.	Institute of Microbiology, CAS (China)
23.	JK Agri Genetics Ltd (India)
24.	Maharashtra Hybrid Seed Company (MAHYCO)
25.	MetaHelix Life Sciences Pvt. Ltd (India)
26.	Monsanto Company (including fully and partly owned companies)
27.	Monsanto Company and Bayer CropScience
28.	Monsanto Company and Dow AgroSciences LLC
29.	Monsanto Company and DuPont (Pioneer Hi-Bred International Inc.)
30.	Monsanto Company and Forage Genetics International
31.	Monsanto Company and Scotts Seeds
32.	Nath Seeds/Global Transgenes Ltd (India)
33.	National Institute of Agrobiological Sciences (Japan)
34.	Novartis Seeds and Monsanto Company
35.	Origin Agritech (China)
36.	PT Perkebunan Nusantara XI (Persero)
37.	Renessen LLC (Netherlands)
38.	Renessen LLC (Netherlands) and Monsanto Company
39.	Research Institute of Forestry (China)
40.	SEITA S.A. (France)
41.	Seminis Vegetable Seeds (Canada) and Monsanto Company (Asgrow)
42.	South China Agricultural University
43.	Stine Seed Farm, Inc. (USA)
44.	Suntory Limited (Japan)
45.	Syngenta
46.	Syngenta and Monsanto Company
47.	United States Department of Agriculture – Agricultural Research Service
48.	University of Florida
49.	University of Saskatchewan
50.	Vector Tobacco Inc. (USA)
51.	Zeneca Plant Science and Petoseed Company

Source: ISSA, GM Approval Database (http://www.isaaa.org/)

GM plants have also been engineered to focus problems related to human diseases. These include development of vaccine against pneumonic and bubonic plague (Alvarez et al. 2006); a potato-based vaccine for hepatitis B, shown to raise immunological responses in humans (Thanavala et al. 2005); a GM pollen vaccine that reduces allergy symptoms (Niederberger et al. 2004); and an edible rice-based vaccine targeted at alleviating allergic diseases such as asthma, seasonal allergies and atopic dermatitis (Takagi et al. 2006).

Development of alternative energy source is also an important trait which scientist wants to achieve, which helps to tackle non-renewable energy crises and greenhouse gas emissions. This involves engineering *Chlamydomonas reinhardtii* to produce hydrogen gas, a renewable energy source (Melis and Happe 2001).

27.6 GM Food Products

A number of processed foods are made by enzymes from the organisms or whole organism that change the nature of food, for example, yeast used for beer and wine, bacteria for yogurt and blue cheese made from fungi. These organisms and enzymes are modified to give better results.

Cheesemaking enzyme, i.e. rennin which is isolated from rennet of stomach of slaughtered calves. In cheesemaking, rennet is used to coagulate milk to separate the curds (solids) and whey (liquid). Recombinant rennin or chymosin was the first protein produced through rDNA means to be used in food (Green et al. 1985). The gene responsible for chymosin production was cloned in yeast and *Escherichia coli*. And these GM organisms were then used for large-scale production of enzyme. Engineered chymosin is currently used in approximately 60 % of US hard cheese products (Biotechnol Ind Organ (BIO) 2007). Other products produced by rDNA methods include food supplements, such as vitamin B2 (riboflavin) (Perkins et al. 1999), α-amylase (used to produce high-fructose corn syrup and dry beer) and lactase (added to milk to reduce the lactose content for persons with lactose intolerance).

27.7 Development of Transgenic Crops

For the development of transgenic crops, the biochemistry of the gene, its mode of action, expression pathway, safety of the gene and gene product must be known in detail. And that is the first and most important step of the procedure. Each step advancement represents a financial commitment on behalf of technology developers of between USD10 million and USD46 million (McDougall 2011).

The process of genetic engineering can be divided into the following steps:

27.7.1 Step 1: Gene Discovery

An idea of commercial product, once formulated, the work of the gene discovery process begins. A number of candidate genes are considered from variety of sources like genomes of plants, microorganisms, animals, study of naturally occurring biological processes and review of relevant scientific literature. A number of candidate genes are then tested in model plants like *Arabidopsis thaliana* and tobacco and in plant cell culture to understand their nature.

For example, *Cry* gene discovery from soil bacterium *Bacillus thuringiensis* (Bt) is a good case study for gene discovery. Bt have been used for many years for spraying to control insects on crops (Schlenkera and Robertsb 2009), and now Bt have been shown to have a number of genes which can be used for insect resistance trait (Scheffe 2008). The gene coding for the insecticidal toxin (Bt toxin) was then inserted into a crop plant, making it resistant to feeding damage by the target pest. Nowadays due to bioinformatics and proteomics studies, candidate genes can then be engineered to produce proteins with increased spectra of activity based on their structure and function.

27.7.2 Step 2: Nucleic Acid (DNA/RNA) Extraction

Nucleic acid, i.e. either DNA or RNA extraction, is the first step in the genetic engineering process. Most reliable and trusted methods are used for isolating these components from the cell. The normal isolation procedure starts with the disruption of the cell, then separation of cell debris from nucleic acids and precipitation of the DNA/RNA which form threadlike pellets.

27.7.3 Step 3: Gene Cloning

There are basically four stages in any cloning experiment: generation of DNA fragments (by PCR or restriction digestion), joining to a cloning vector, propagation in a host cell and selection of the required sequence (Fig. 27.8). In DNA extraction, all DNA from the desired organism is extracted. This genomic DNA is treated with specific enzymes called restriction enzymes cutting it into smaller fragments with defined ends to allow it to be cloned into bacterial vectors. Copies of the vector will then harbour many different inserts of the genome. These vectors are transformed into bacterial cells and thousands of copies are produced. Using information relating to

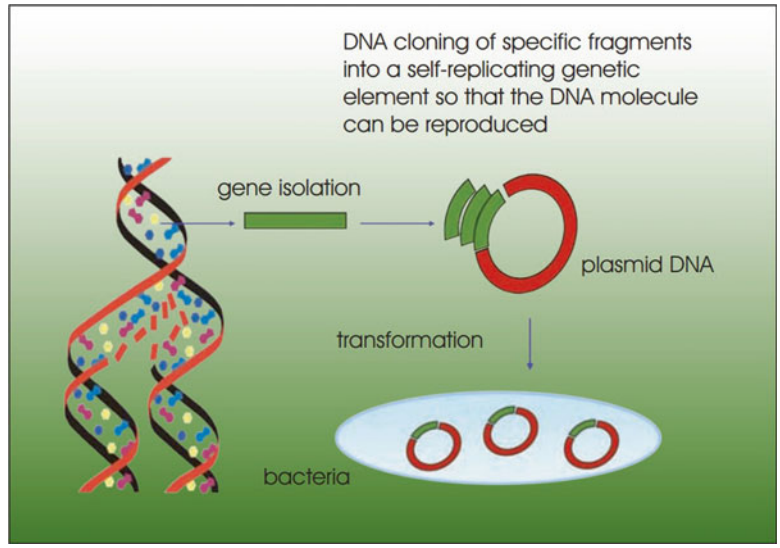

Fig. 27.8 Gene cloning procedure (Source: ISAAA, Agricultural Biotechnology Biotech Booklet-3 (http://www.isaaa.org/))

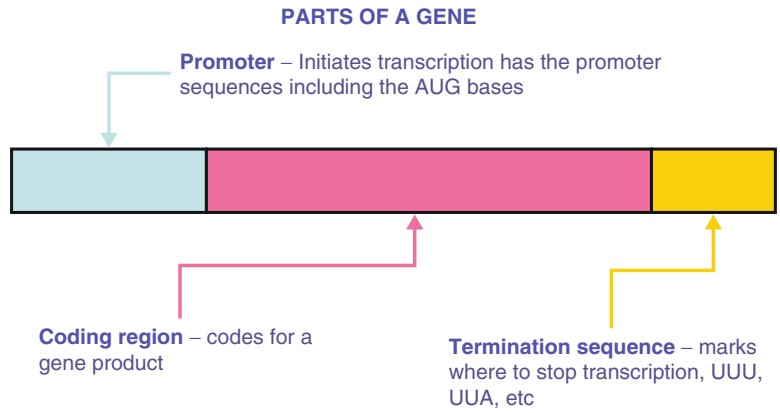

Fig. 27.9 Gene stacking (Source: ISAAA, Agricultural Biotechnology Biotech Booklet-3 (http://www.isaaa.org/))

specific molecular marker sequences and the desired phenotype, the vector harbouring the desired sequence is detected, selected and isolated and clones are produced. Restriction enzymes are again utilised to determine if the desired gene insert was cloned completely and correctly. Or one can design a gene specific primer or degenerated primer from the sequence data available on Internet and then can amplify the specific gene by PCR, and the PCR product can be cloned in cloning vector and then can be sequenced and characterised (Fig. 27.8).

27.7.4 Step 4: Gene Design and Packaging

Once the gene of interest has been cloned, it has to be linked to pieces of DNA that will control its expression inside the plant cell (Fig. 27.9). These pieces of DNA will switch on (promoter) and off (terminator) the expression of the gene inserted. Gene designing/packaging can be done by replacing an existing promoter with a new one, incorporating a selectable marker gene and reporter gene and adding gene enhancer fragments, introns and organelle-localising sequences, among others.

27.7.4.1 Promoters

Promoters allow differential expression of genes. For instance, some promoters cause the inserted genes to be expressed all the time, in all parts of the plant (constitutive), whereas others allow expression only at certain stages of plant growth, in certain plant tissues, or in response to external environmental signals. The amount of the gene product to be expressed is also controlled by the promoter. Some promoters are weak, whereas others are strong. Controlling the gene expression is an advantage in developing GM plants.

27.7.4.2 Selectable Marker Genes

Selectable marker genes are usually linked to the gene of interest to facilitate its detection once inside the plant tissues. This enables the selection of cells that have been successfully incorporated with the gene of interest, thus saving considerable expense and effort. Genetic engineers used antibiotic resistance and herbicide resistance marker genes to detect cells that contain the inserted gene. Cells that survive the addition of marker agents to the growth medium indicate the presence of the inserted gene. Although increase in antibiotic resistance in humans and animals is unlikely to occur using antibiotic resistance marker, genes coding for resistance to non-medically important antibiotics are preferred. In addition, alternative types of marker genes have been developed which are related to plant metabolism such as phosphomannose isomerase, xylose isomerase and others.

27.7.4.3 Reporter Genes

Reporter genes are cloned into the vector in close proximity to the gene of interest, to facilitate the identification of transformed cells as well as to determine the correct expression of the inserted gene. Reporter genes that have been used include the beta-glucuronidase gene (*gusA* gene) which acts on a particular substrate producing a blue product, hence making the transformed cells blue; the green fluorescent protein (*gfp*) which allows transformed cells to glow under a green light; and luciferase gene that allows cells to glow in the dark, among others.

27.7.4.4 Enhancers

Several genetic sequences can also be cloned in front of the promoter sequences (enhancers) or within the genetic sequence itself (introns, or non-coding sequences) to promote gene expression. An example is the cloning of the cauliflower mosaic virus promoter enhancers in front of the plant promoter.

Once the gene of interest is packaged together with the promoter, reporter and the marker gene, it is then introduced into a bacterium to allow for the creation of many copies of the gene package. The DNA isolated from the bacterial clones can then be used for plant cell transformation using particle bombardment. If however the use of bacteria *Agrobacterium tumefaciens* is preferred in the plant transformation, the whole gene package should be cloned in between two border sequences (left and right border) of a binary vector plasmid. This will allow processing of the *Agrobacterium* so that only the transfer DNA (T-DNA) will be incorporated into the plant genome (Fig. 27.10).

27.7.5 Step 5: Transformation

The most common methods used to introduce the gene package into the binary vector into plant cells in a process called transformation or gene insertion include biolistic transformation using the gene gun and *Agrobacterium*-mediated transformation (Altpeter et al. 2005; Tzfira and Citovsky 2006). There are many techniques of gene transformation in plants which include in-planta transformation procedure.

27.7.5.1 Particle Bombardment

Particle bombardment is a mechanical method of introducing the desired gene. The desired genetic sequence is cloned into a plant DNA vector and introduced into the plant using the gene gun or particle gun. As in the common gun, the gene gun uses minute particles of tungsten or gold as the bullet. These particles are coated with the DNA solution and fired to the plant cells through the force of the helium gas inside a vacuum-filled chamber. The DNA and the tungsten/gold

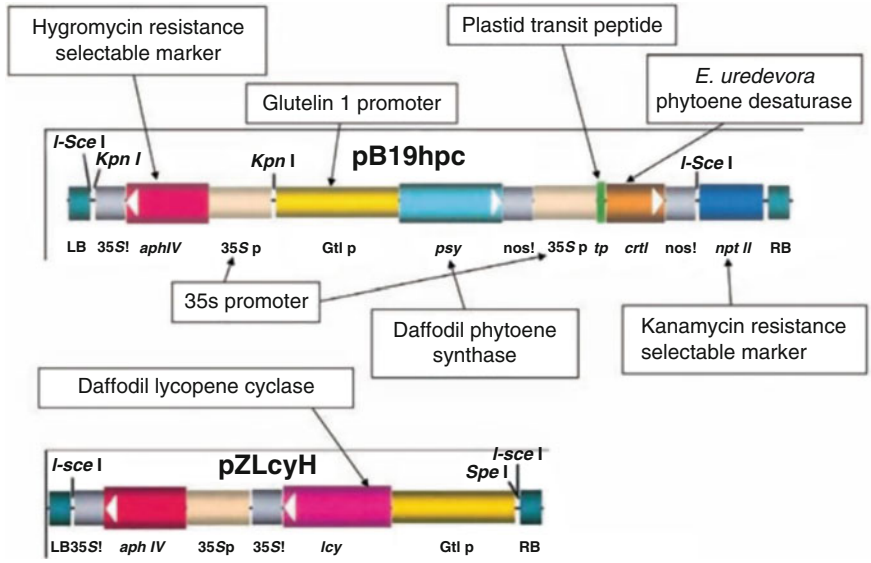

Fig. 27.10 Gene construct components (Source: ISAAA, Agricultural Biotechnology Biotech Booklet-3 (http://www.isaaa.org/). Source: Ye et al. (2000))

particles get inside the cell, and within 12 h, the inserted DNA gets inside the nucleus and integrated with the plant DNA. The tungsten/gold particles are sequestered to the vacuole and eliminated later. Transformed cells are cultured in vitro and induced to form small plants (regeneration) that express the inserted gene.

27.7.5.2 *Agrobacterium tumefaciens*-Mediated Transformation

The 'sharing' of DNA among living forms is well documented as a natural phenomenon. For thousands of years, genes have moved from one organism to another. For example, *Agrobacterium tumefaciens*, a soil bacterium known as 'nature's own genetic engineer', has the natural ability to genetically engineer plants. It causes crown gall disease in a wide range of broad-leaved plants, such as apple, pear, peach, cherry, almond, raspberry and roses. The disease gains its name from the large tumour-like swellings (galls) that typically occur at the crown of the plant, just above soil level.

Basically, the bacterium transfers part of its DNA to the plant, and this DNA integrates into the plant's genome, causing the production of tumours and associated changes in plant metabolism. Molecular biologists have utilised this biological mechanism to improve crops. The genes that cause the galls are removed and replaced with genes coding for desirable traits. Plant cells infected with the bacterium will not form galls but produce cells containing the desired gene, which when cultured in nutrient medium will regenerate into plants and manifest the desired trait.

The main goal in any transformation procedure is to introduce the gene of interest into the nucleus of the cell without affecting the cell's ability to survive. If the introduced gene is functional, and the gene product is synthesised, then the plant is said to be transformed. Once the inserted gene is stable, inherited and expressed in subsequent generations, then the plant is considered a transgenic.

27.7.6 Step 6: In Vitro Regeneration of Plant

The parts of the plant to be made transgenic, i.e. explants are treated with Agrobacterium containing desire gene then grown on selective media and regenerated into whole plant in vitro on MS media or other defined media. After complete regeneration and establishment, they are tested for

presence of a transgene using various techniques of molecular biology.

27.7.7 Step 7: Detection of Inserted Genes

Molecular detection methods have been developed to determine the integrity of the transgene (introduced gene) into the plant cell.

27.7.7.1 Polymerase Chain Reaction

Polymerase chain reaction or PCR is a quick test to determine if the regenerated transgenic cells or plants contain the gene. It uses a set of primers (DNA fragments), forward and backward primers, whose nucleotide sequences are based on the sequence of the inserted gene. The primers and single nucleotides are incubated with the single-stranded genomic DNA, and several cycles of DNA amplification are conducted in a PCR machine. Analysis of the PCR products in agarose gel will show if the plants are really transformed when DNA fragments equivalent in size with the inserted gene are present and amplified in the form of bands.

27.7.7.2 Southern Blot Analysis Determines the Integrity of the Inserted Gene

Southern blot analysis determines the integrity of the inserted gene whether the gene is complete and not fragmented, at the correct orientation, and with one copy number. The DNA coding sequence is the probe binding to the single-stranded genomic DNA of the transgenic plant which is implanted on a nitrocellulose paper. Autoradiography will reveal the transgenic status of the plant. One copy of the transgene is desired for optimum expression.

27.7.7.3 Northern Blot Analysis

Northern blot analysis determines whether the transcript or the messenger RNA (mRNA) of the introduced DNA is present and is correctly transcribed in the transgenic plant. The messenger RNA of the transgenic plants is isolated and processed to bind to the nitrocellulose membrane. Labelled DNA is used to bind to the mRNA and can be visualised through autoradiography.

27.7.7.4 Western Blot Analysis

Western blot analysis or protein immunoblotting is an analytical technique used to detect whether the transgenic plants produce the specific protein product of the introduced gene. Protein samples are extracted from the transgenic plants, processed into denatured proteins and transferred to a nitrocellulose membrane. The protein is then probed or detected using the antibodies specific to the target protein.

27.7.7.5 Quantitative PCR Analysis

Quantitative PCR analysis is the latest method where we can monitor real-time data of PCR amplification reaction. In this method we use very specific primers for the gene, and they were tagged by fluorescence dye which helps us to monitor real-time development in the reaction. This method can be used to study gene expression and copy number detection of the gene.

27.7.8 Step 8: Transgenic Plant Testing

Controlled-environment testing in a greenhouse or growth chamber allows for rapid screening of many transgenic events. For example, transgenic plants expressing a protein with insecticidal properties can be screened via a high-throughput insect assay in a greenhouse, and events that are being developed for herbicide tolerance can be treated with the appropriate herbicide to select for events that exhibit the desired level of tolerance. The controlled-environment screening can be combined with high-throughput imaging systems to measure and quantify plant growth and development under these specific conditions.

Transgenic events that meet performance criteria under controlled conditions are then tested in field trials and closely monitored under regulated conditions. These trials include efficacy tests to evaluate the performance of the developed trait; in the case of insect-control traits, the events are tested by infesting test plants with the

target insect, either artificially or via natural insect pressure, and then scoring the level of damage on the plants after insect feeding. The agronomic performance of the event is tested in addition to trait efficacy. To be considered for commercialisation, an event that shows excellent protection against insect-feeding damage must also demonstrate agronomic and phenotypic characteristics that are equivalent or superior to those of a non-transgenic isoline in the absence of or with minimal insect pressure.

27.7.9 Step 9: Backcross Breeding (If Needed)

Genetic transformation is usually conducted in elite or commercial varieties which already possess the desired agronomic traits but lack the important trait of the transgene. Thus, once successfully conducted, the genetically modified plant will be easily recommended for commercialisation if it shows stability in several generations and upon successfully passing and fulfilling varietal registration requirements.

However, some plant transformations may have been performed in plant varieties which are amenable to genetic transformation but are not adapted nor important in the target country. There may also be sterility problems in the transgenic plant. In such cases, conventional plant breeding is performed where the transgenic plant becomes the pollen source in the breeding programme and the elite lines or commercial varieties as the recurrent parent. Backcross breeding enables the combination of the desired traits of the recurrent parent and the transgenic line in the offspring.

The length of time in developing transgenic plant depends upon the gene, crop species, available resources and regulatory approval. It varies from 6 to 15 years before a new transgenic plant or hybrid is ready for commercial release.

27.8 Biosafety

The theoretical framework for safety evaluation of genetically engineered crops:

Through a structure of science-based risk assessment and risk management measures, regulatory agencies evaluate GM crops and derived products, globally. Assessment standards are established by regulatory agencies around the world. GM crops must meet all these standards. This process is composed of four steps: hazard identification, hazard characterisation, exposure assessment and integrative risk characterisation (Codex Alimentarius 2001; Eur Comm 2000; Eur Food Saf Auth 2006). The safety of GE crops is determined as with conventional crops, based on the standard that there must be 'a reasonable certainty that no harm will result from intended uses under the anticipated conditions of consumption' (Organ Econ Co-op Dev 1993a, b). This standard is important because no food, either conventional or genetically engineered, is safe in absolute terms. For example, many conventional foods, such as potatoes, soybeans and celery, contain toxins and/or anti-nutrients, and, like most traditional foods, they have not been subject to food or feed safety studies. Instead, the safety of these crops and foods has been established by virtue of their long history of safe use (Constable et al. 2007).

27.8.1 A Comparative Approach to Risk Assessment

Safety of a GE crop assessment historically begins with a comparative assessment, also referred to as the concept of 'substantial equivalence' (Chassy et al. 2004; Organ Econ Co-op Dev 1993a, b). This tactic helps categorise similarities and differences between the newly developed crop and a conventional counterpart that has a long history of safe use (Constable et al. 2007; Kok et al. 2008). Any diversion from the conventional plant will be focused of the food, feed and environmental safety assessment, which then prove whether GM crop can be considered as safe or not (Codex Alimentarius 2009; Food Agric Organ UN, World Health Organ 1996, 2000; Organ Econ Co-op Dev 1993a, b).

27.8.2 Safety Assessment Strategy for Genetically Engineered Crops

There is a rigorous process of safety insurance for GM crops when to use as food, as animal feed and as an integrated part of the environment. Many important assumptions were drawn during the safety studies undertaken during the development of the GM crops. In the history of mankind, GM crops go through much difficult and thorough safety assessments. This assessment is revived by multiple agencies under strict rules with national and international safety assessment guidelines (Paoletti et al. 2008).

27.8.3 Food and Feed Safety

A number of comparative and toxicological approaches and guidelines which are internationally approved are followed to assess the safety of food and feed of GM crop (Codex Alimentarius 2003a, b, 2009; Eur Food Saf Auth 2008; Faustman and Omenn 2008; (Food Agric Organ UN, World Health Organ 1996; Organ Econ Co-op Dev 1998). Safety and nature of the introduced protein is a main concern in this assessment.

Other important point is assessing any potential compositional differences in GM crop compared with a conventional control. This includes assessing the levels of numerous nutrients, antinutrients, toxins, allergens and other important compounds normally present in crops—e.g. macronutrients such as carbohydrates, proteins (including all amino acids), oils (including fatty acids) and vitamins and minerals—to ensure that the GE crops are compositionally equivalent to conventional varieties (Organ Econ Co-op Dev 2001, 2002, 2004). 90-day animal toxicity studies adapted from Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 are also performed using the grain or another harvested component of the GE crop (Organ Econ Co-op Dev 1998).

27.8.4 Environmental Risk Assessment (ERA)

Potential harmful effects on environment of GM crop product are assessed through ERA. It measures the ecological impact of GM crop on environment. The guidance from the US Environmental Protection Agency is consistent with other international scientific guidance documents (Aust Gov Dep Health Ageing Off Gene Technol Regul 2009; Can Food Insp Agency 2012; Eur Food Saf Auth 2010; Organ Econ Co-op Dev 1993a, b) and publications for ERAs of GE crops Nickson 2008; Raybould 2006, 2007; Wolt et al. 2010).

27.9 Pros and Cons of GM Crops

So far we have seen that there are great goals that can be achieved by this rDNA technology and GM crops. Increased production, greater nutritional value, greater resistance to biotic and abiotic stress, better taste and good for human health are some of the main characteristics/traits that GM crops can provide over natural crops. But, on the other hand, there is a great concern about the odd effects like unforeseen hostile health effects, potential damage to environment, commercial exploitation by private firms, etc.

27.9.1 Pros

27.9.1.1 Improved Tolerance to Biotic and Abiotic Stresses

As we have seen with the selection of proper candidate gene, crops can be made tolerant to biotic stresses like they are made resistant to insects, pest and diseases (like Bt cotton, Bt corn, etc.) which ultimately reduces the use of insecticides, fungicides, pesticides and other chemicals in the agriculture which helps to improve gross income of the farmers and also helps to reduce the load of harmful chemicals in the environment.

27.9.1.2 Biotech Crops Contribute to Food Security, Sustainability and Climate Change

From 1996 to 2012, economic gains at the farm level of US$116.0 billion were generated globally by biotech crops, due to reduced production costs and substantial yield gains. Biotech crops have reduced the amount of pesticides used by 497 million kg. In 2012 alone, fewer insecticide sprays reduced CO_2 emissions by 26.7 billion kg, equivalent to taking 11.8 million cars off the road for a year (James 2006).

27.9.1.3 Increased Crop Productivity

GM crops have significantly contributed to the food, feed and fibre security. GM crops have provided more affordable food by reducing production cost. GM crops allow less ploughing, less pesticide uses and less labour which help farmers to add in their gross income.

27.9.1.4 GM Crops Help in Conservation of Biodiversity

GM crops are a land-saving technology. We have GM crops that can provide more yield in the limited availability of land. This protects the deforestation needed for new agriculture land and helps to protect biodiversity.

27.9.1.5 Reduce Agricultural Eco-Footprint

Because of insect- and pest-resistant GM crops, the pesticide spraying was reduced by 503 million kg. This has tremendously reduced the chemical load in environment due to agriculture practices.

27.9.1.6 Resistance to Herbicides

Herbicide-resistant GM crops have made it easy to control the troublesome weeds in the field. This has limited the use of herbicides, which has decreased environmental impact from herbicides by 18.7 %.

27.9.1.7 Help Mitigate Climate Changes

Fewer uses of pesticides, insecticides and herbicides in the field help to reduce the fuel used in the process of development, transport and application. This reduced the CO_2 emission equivalent to removing 11.9 million cars from the road for completely 1 year.

27.9.1.8 Faster Growth

Crops can be engineered to grow faster, and they can be cultivated in the areas where shorter growing period is available and also multiple harvest seasons can be taken.

27.9.1.9 Improved Nutritional Quality

Crops can also be engineered to produce larger and better essential vitamins and minerals. They can also be altered to increase amount of carbohydrates, proteins and saturated and unsaturated fat content.

27.9.1.10 Medicine and Vaccine Production

GM crops can also produce medicines and vaccines, so that human treatment can be achieved cheaply and efficiently through diet in very poor countries.

27.9.1.11 Improved Taste

Crops can be engineered to produce better tasting food, which may encourage people to eat more healthy food that are currently not likable (Fig. 27.11).

27.9.2 Cons

27.9.2.1 Unexpected Side Effects

There are concerns about the unpredictable side effects of GM food on human health. The problem is allergic reactions; genetic modification often mixes or adds new proteins that weren't indigenous to the original plant, which may cause new allergic reactions to humans or livestock. Most of the GM crops have antibiotic resistance

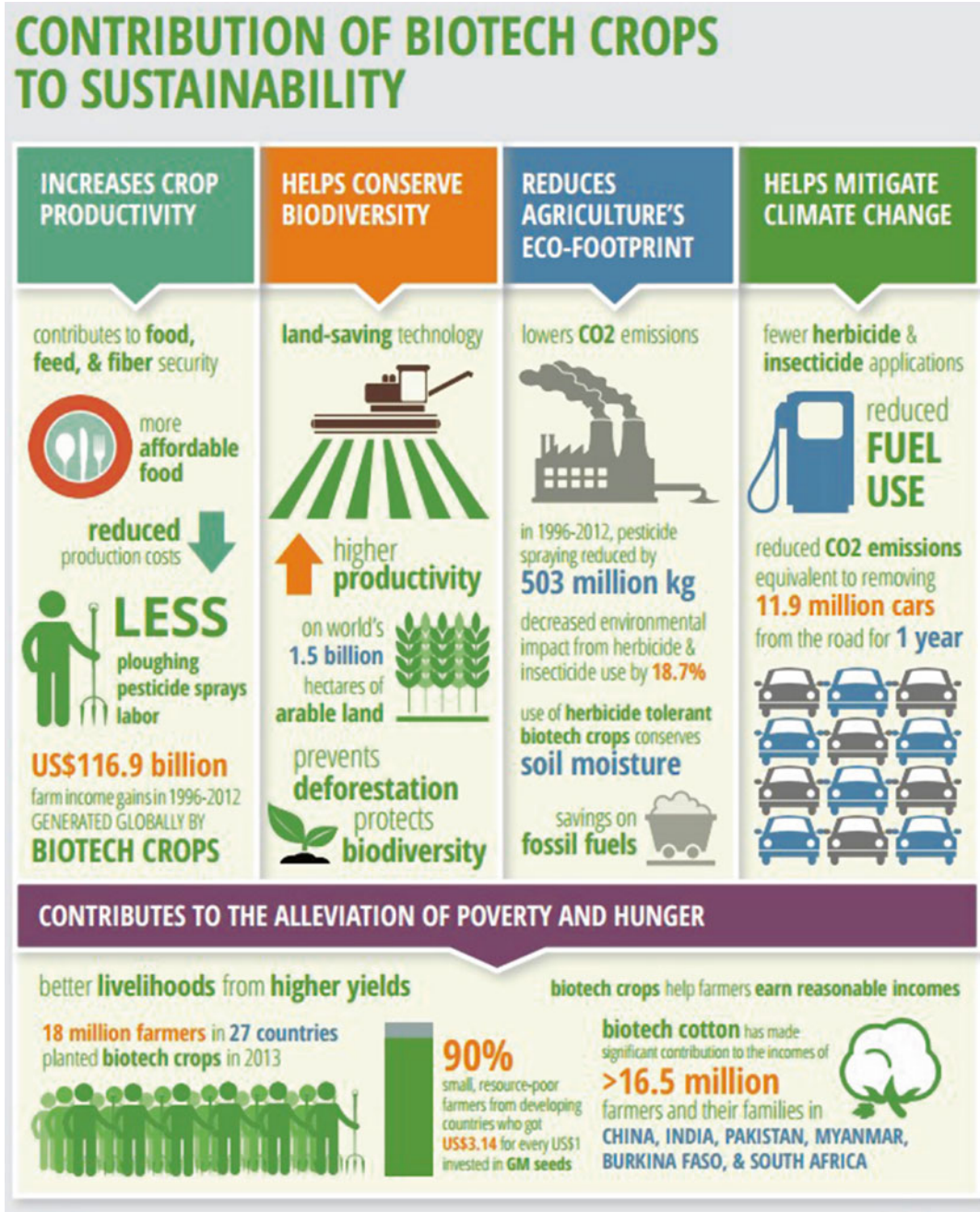

Fig. 27.11 Pros of GM crop shows us the promising positive effects of GM crops on human society and world environment (Source: Brookes et al. 2014; James 2013)

genes in them. It is speculated that when humans eat them, these antibiotic features persist in our bodies and make actual antibiotic medications less effective. But the possibility of this to happen is not there or very less. We eat vegetables and meat in our day-to-day life for thousands of years, but have we seen any example that a human or animal show characters of vegetables and animals? When we eat something, the nuclear material get digested and fabricated completely.

27.9.2.2 Labelling of GM Food

Labelling of GM food is a matter of concern. Nowadays so many products are available in the market; customer may never know what is he or she eating? It may be GM or non-GM food. Not all countries have strict rules on labelling of GM foods or their ingredients or GM-processed food. Vegetarians and vegans might unknowingly eat plant-based foods containing genes from animals.

27.9.2.3 Threat to Diversity

Toxic genes for specific insects may kill other beneficial insects, which may have effects on animals further up the food chain. Another risk is that these foreign genes may escape into wild. If like herbicide gene cross passed into wild weed species, it can produce super weed which will be very hard to control. Making plants resistant to bacteria, fungi, viruses and insects can cause these pathogens to become more virulent which will cause more damage to crops.

27.9.2.4 Effect on Non-GM Crops

Pollens from GM crops can transfer on non-GM counterpart which may genetically modify the non-GM crop which will spread in wild varieties.

27.9.2.5 Overuse of Herbicides

It is very essential to educate the farmers about the GM product they are planting. Herbicide-resistant crop may encourage them to spray more herbicides for quick results; it may increase the load of chemicals in the soil and pollute rivers and other waterways. This may poison the fish and other animals, birds and plants and can accumulate in all food chains in ecosystem causing lethal damage.

27.9.2.6 Benefits May Not Be Available to Everyone

Due to high price and top-end technology involved in the production and due to IPR issues, it may be possible that GM technology is being monopolised by a small number of private companies.

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Engineering of Plants for the Production of Commercially Important Products: Approaches and Accomplishments

28

Salah E. Abdel-Ghany, Maxim Golovkin, and A.S.N. Reddy

Abstract

For centuries humans have used plants as a source of food, fiber, fuel, and medicine because they have the ability to synthesize a vast array of complex organic compounds using light, carbon dioxide, and water. Advances in recombinant DNA and transgenic technologies during the last several decades have opened many new avenues to further exploit plants for production of many novel products. The potential to use plants to synthesize diverse native and nonnative industrial and pharmaceutical products coupled with the depletion of fossil fuels that are the source of many commercially important products and the adverse effects of chemical synthesis of platform chemicals on the environment have renewed considerable interest in using plants for large-scale production of chemicals and value-added compounds. To accomplish this, different genetic engineering and transformation strategies have been developed for introducing multiple genes (gene stacking), modulating their expression with regulatable promoters, and targeting the products to a specific compartment in the cells. Successful metabolic engineering of plants should lead to sustained production of platform chemicals, pharmaceuticals, and biopolymers. In

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this chapter, we present an overview of different methods that are currently used to introduce and manipulate expression of one or more genes into plants and discuss some of the recent achievements in producing value-added products and pharmaceuticals in plants.

Keywords
Metabolic engineering • Pharmaceuticals • Platform chemicals • Value-added compounds • Biomass • Butanetriol • Bioenergetics • Bioplastics • Transformation • Molecular pharming

28.1 Introduction

Plants, as photoautotrophs, convert light energy into chemical energy and use it for their growth, development, and synthesis of many complex organic compounds. Humans have been using plants for centuries as a renewable source of food, fiber, fuel, pharmaceuticals, and other biomaterials. Since plants naturally produce a vast array of complex organic compounds and secondary metabolites, they have been used extensively to produce many commercially important industrial and consumer products (United States Department of Agriculture 2008; Saito and Matsuda 2010; Yoon et al. 2013). During the last century, petroleum-based chemicals were widely used to produce diverse products ranging from plastic polymers to cosmetics. However, increasing demand for petroleum and petroleum-derived products due to population growth (United Nations 2013), the diminishing supplies of fossil fuels, and the adverse impact of producing industrial chemicals from fossil fuels on the environment renewed the interest in using plants to produce industrial chemicals and other value-added products in a sustainable and environmentally safe manner. It is anticipated that plants will play an important role in bio-based economy in reducing reliance on petrochemicals and in producing many unconventional chemicals that are currently produced from petrochemical feedstock and also in producing biofuels (United States Department of Agriculture 2008). This bio-based approach offers several advantages for the production of industrial chemicals. These include sustainability as plants are renewable sources and the likelihood of being highly cost-effective as they use light energy and CO_2 to produce sugars and a vast repertoire of organic compounds that can serve as precursors for the synthesis of desirable carbon compounds. Furthermore, bio-based synthesis of industrial chemicals is environmentally friendly as compared to chemical synthesis. In addition, the development of technologies in the 1980s to stably introduce and express genes in plants has opened new avenues to use plants to produce novel platform chemicals and pharmaceutical products that are not normally produced in plants (Poirier et al. 1992, 1995; Bohmert-Tatarev et al. 2011; Golovkin 2011; Abdel-Ghany et al. 2013).

Advances in plant biotechnology have allowed engineering of complex biochemical pathways not only by manipulating expression of a single gene but also by introducing multiple genes from other plants and non-plant systems including bacteria. Coordinated expression of multi-subunit proteins and manipulation of metabolic pathways have been widely used for diverse applications (Table 28.1). These include improvement of food and feed quality (Fitzpatrick et al. 2012; Tieman et al. 2012), increased tolerance of crop plants to abiotic and biotic stresses (Rhodes 1994; Nanjo et al. 1999; Munns 2005), development of weed and insect resistant crops, and use of plants for production of polymers (bioplastics), biofuels, platform chemicals, and pharmaceuticals (Nikolau et al. 2008; Andrianov et al. 2010; Bohmert-Tatarev et al. 2011; Abdel-Ghany et al. 2013; Golovkin 2011).

This chapter will focus on production of commercially important products such as platform chemicals and pharmaceuticals in plants using

Table 28.1 Some examples of different applications of plant biotechnology in agriculture, medicine, and other areas

Agriculture	Molecular farming	Other areas
Herbicide resistance	Pharmaceutical proteins	Phytoremediation
Pathogen resistance	Medicinal compounds	Biofuels
		Energetic materials
Manipulate growth and development	Industrial chemicals/proteins	
Yield and product quality	Polymers	Cosmetics
Senescence and stress control		

plant biotechnological approaches. Introduction and manipulation of foreign genes in plants is fundamental to all biotechnological approaches; hence, we will first provide an overview of different methods that are currently used to introduce and manipulate expression of one or more genes in plants. We will then discuss how these methods are used to engineer plants to produce many valuable products by manipulating existing metabolic pathways or introducing novel pathways.

28.2 Commonly Used Methods to Introduce Genes into Plants

Prior to the 1980s, people used to introduce desired genes/traits into crop species through conventional breeding between the domesticated varieties and the wild relatives that have the traits of interest, and then new cultivars with desirable characteristics are obtained by backcrossing (Yan et al. 2010; Powell et al. 2012). This is very laborious, time consuming, and limits gene transfer between sexually compatible plant species. However, genes of many desirable traits may not be found in crossable relatives of the interested crop. The development of a method in the early 1980s to stably introduce and express a foreign gene from other organisms into plants has changed the way to introduce new traits into plants. As discussed below, it is now possible to introduce multiple genes (gene stacking) from any organism into the nuclear or chloroplast genome of plants; produce proteins constitutively, in a particular cell type or in response to a chemical or environmental cue; and target the proteins to a desired cellular location. These improvements allowed high level of expression of multiple genes and allowed engineering of plant metabolism to produce desired chemicals. In Table 28.2, we summarized various methods to introduce and manipulate expression of genes in plants and their advantages and disadvantages.

28.2.1 *Agrobacterium*-Mediated Transformation

The most commonly used method for introducing a gene into a plant genome is *Agrobacterium-mediated* transformation using the soilborne pathogen *Agrobacterium tumefaciens* (Tzfira and Citovsky 2003; Valentine 2003; Gelvin 2012). In *Agrobacterium*-mediated transformation, the bacterium represents the biological machinery that delivers the gene(s) of interest into the plant genome. This process involves proteins derived from both the bacterial pathogen and the host plant (reviewed in Gelvin 2012). In nature, *Agrobacterium* infects some plant species and causes crown gall tumors. During infection, the *Agrobacterium* transfers a portion of the DNA, called T-DNA, from the tumor-inducing (Ti) plasmid into the host where it subsequently integrates into the host genome. Wild-type T-DNA encodes genes involved in hormone and opine biosynthesis that are expressed in host cells and cause cell proliferation and tumor formation (Gelvin 2012). To introduce desired genes into plants, the T-DNA was engineered by replacing the hormone and opine biosynthetic genes with selectable marker genes and multiple cloning sites for insertion of gene(s) of interest flanked by the T-DNA left and right border sequences. The engineered T-DNA was also moved into a small plasmid for easy cloning of genes using an *Escherichia coli* system, and then this vector with

Table 28.2 Summary of current plant transformation and expression systems

Major system		Expression levels	Advantages	Disadvantages
Stable	*Nuclear*			
	Constitutive expression Transgenic plants and cell cultures	Modest (<1 % TSP) (Seeds >1 %)	Inheritable gene Broad host range Possible direct consumption Plant-specific PTM (glycosylation) High yield and scale-up	Low expression Lengthy procedure Positional effect and gene silencing Environmental risks
	Chloroplast			
	Constitutive expression Transplastomic plants	High (>5 % TSP)	High expression and accumulation Inheritable gene No gene transfer though pollen No position effect and gene silencing Scale-up capacity	Prokaryotic machinery No glycosylation Narrow host range Somatic propagation Lengthy procedure
Transient	*Plant viruses*			
	Transient expression/ modern systems/ wild-type plants	High (>10 % TSP)	High expression and accumulation Easy molecular manipulations Simple and quick transformation Short production times High scale-up capacity Less environmental risks	Not inheritable Restricted to some hosts Need of purification (endotoxins) Some environmental risks

PTM posttranslational modification, *TSP* total soluble protein

genes of interest is introduced back into an *Agrobacterium* strain (helper strain) containing a Ti plasmid harboring the virulence (*vir*) genes but without T-DNA (Anami et al. 2013). To stably introduce gene(s) into plants, the *Agrobacterium* containing the engineered plasmid is cocultivated with the explant. During the cocultivation, *Agrobacterium* transfers T-DNA into the host cell, which then integrates randomly into the nuclear genome by nonhomologous recombination. Transgenic calli or somatic embryos are selected and regenerated on selection medium. In Arabidopsis, transgenic lines can be generated by submerging or spraying the floral buds with *Agrobacterium* culture and then selecting the transgenic lines by germinating the seeds on selection plates (Clough and Bent 1998). In nature, *Agrobacterium* mainly infects dicotyledonous plants (De Cleene and De Ley 1976). However, under laboratory conditions, it is the method of choice for genetic manipulation of many plant species including monocots (Tzfira and Citovsky 2006). Some genotypes and plant species are not transformable with *Agrobacterium*, a barrier that limits the use of *Agrobacterium-mediated* transformation in these species.

28.2.2 Direct Gene Transfer

Direct gene transfer by high-velocity microprojectiles (also called particle bombardment or biolistic method) and electroporation has also been widely used to introduce genes into plants, especially in those species that are not amenable for transformation with *Agrobacterium* (Altpeter et al. 2005; O'Kennedy et al. 2011). In case of particle bombardment, metal particles coated with plasmid DNA containing the gene(s) of interest are introduced into plant cells in a pressurized helium stream using a gene gun. Physical and chemical parameters such as rupture pressure, type of particles, particle travel distance, and DNA concentration can be optimized to different tissues and different species. In case of electroporation, plasmid DNA is introduced into protoplasts

by subjecting the cells to an electric shock (Anami et al. 2013). There are advantages and disadvantages of both *Agrobacterium-mediated* transformation and particle bombardment methods. The ability of Agrobacterium to introduce a single or a few copies of gene per genome is an advantage over particle bombardment that results in insertion of multiple copies, which might result in recombination and instability (Gao and Nielsen 2013). On the other hand, some advantages make particle bombardment a method of choice for engineering of recalcitrant plant species, different cell types, organelle transformation, and simultaneous transformation of multiple genes with no need for complex cloning or crossing of lines carrying different transgenes (Altpeter et al. 2005).

28.2.2.1 Stable Versus Transient Transformation

Once foreign DNA is integrated into the host genome (stable transformation) by either method, it is inherited and segregated in the progeny according to Mendelian laws (De Block et al. 1984; Horsch et al. 1984). However, during propagation of these lines, complete loss of function of the transgene or significant variation of gene expression among lines might occur as a result of gene silencing, multiple integration events, or complex genetic rearrangements (Slater et al. 2003; Schiermeyer et al. 2004). Analysis of a large number of independent transgenic lines for each gene is necessary to achieve the highest expression/accumulation of introduced gene product. In stable transformation, the effect of transgenes can be studied at the biochemical, physiological, morphological, genetic, and cell biological levels to gain insight into their functions and biological roles. This is different from transient transformation where gene expression, subcellular localization, or protein targeting can be tested within hours to days, thus avoiding *in vitro* plant regeneration procedures (Sheen 2001). Transient transformation can be performed by shooting DNA-coated beads into epidermal peels, cell cultures, and tissues, electroporation with protoplasts, or agroinfiltration of leaves (Kirienko et al. 2012; Mangano et al. 2014). Agroinfiltration of *Nicotiana benthamiana* leaves has been used widely to study protein-protein interactions by means of bimolecular fluorescence complementation technology (BiFC) and fluorescence resonance energy transfer (FRET) (Ali et al. 2008; Boruc et al. 2010). Recently, an Arabidopsis proteome chip prepared from thousands of transiently expressed Arabidopsis cDNAs in *Nicotiana benthamiana* leaves was used to perform protein-protein interaction studies, which resulted in identification of targets of calmodulin and MAP kinases (Popescu et al. 2007; Lee et al. 2011).

28.2.3 Organelle Transformation

There is a public concern about the insertion of a transgene in the nuclear genome as the transgenes could move – "transgene flow" – to wild species or to weedy relatives growing in natural or seminatural communities (Wilkinson et al. 2003; Singh et al. 2010). In recent years, researchers have begun to introduce genes into plastids as an alternative approach to nuclear transformation as it offers several advantages over transferring genes into nuclear genomes (Bock 2007; Wani et al. 2010). First, plastid transformation has the potential to eliminate the gene flow through pollen grains since plastids are maternally inherited in a majority of angiosperms (Wani et al. 2010). Second, plastid transformation allows high levels (up to 40 %) of protein expression compared to 0.5–3 % in case of nuclear transformation (Daniell et al. 2002). The high production of expressed protein in transplastomic plants (plants with transformed plastid genome) is possible due to the high copy number of plastids per plant cell, and also the expressed proteins are less affected by pre- and posttranscriptional gene silencing. Third, plastid transformation has the advantage of expressing multiple genes from polycistronic mRNA with the absence of epigenetic effect and gene silencing (Sidorov et al. 1999; Maliga 2001). However, there are a few drawbacks in introducing genes into the plastid genome. The expressed proteins in the plastids are not glycosylated because of the prokaryotic

nature of the plastids. This is important especially for production of pharmaceuticals (Golovkin 2011). Another problem is the lack of tissue-specific and developmentally regulated control mechanisms (Wang et al. 2009). This is necessary when expression of the transgene is desired to be limited to a specific tissue, organ, and/or developmental stage or the high level of foreign proteins in plastids may have deleterious pleiotropic effects (Magee et al. 2004). Recently, the toxic effects of transgene expression in plastids have been addressed by using an inducible expression system that requires coordination between nucleus and plastids. A T7 RNA polymerase gene (T7RNAP) fused to a chloroplast transit peptide at the N-terminus was cloned under the control of an alcohol-inducible promoter and introduced into the nuclear genome of tobacco. Induction of T7RNAP by ethanol application leads to its expression and transport to plastids where transcription of the plastid transgene under the control of the T7 promoter is turned on (Lossl et al. 2005). Also, an external control of plastid transgene expression was developed using modified promoters containing binding sites for bacterial *Lac* repressor. This allows chemical induction of plastid transgene in intact plants or after harvesting (Muhlbauer and Koop 2005).

Plastid transformation has been used to introduce new traits and also for basic research to understand processes in plastids such as RNA editing (Ruf and Bock 2011). Transgenic chloroplasts have been tested for the production of many therapeutic proteins. This method avoids the expensive fermentation procedures, and, in addition, the oral delivery of plastid-derived therapeutic proteins could eliminate the expensive purification, cold storage, and cold transportation (Singh et al. 2009). More than 20 vaccines were successfully expressed in chloroplasts (Golovkin 2011), suggesting that chloroplast contains the mechanisms that allow correct folding and disulfide bond formation that result in fully functional proteins. However, further studies are needed to establish a feasible production system for biopharmaceuticals in chloroplasts. If functional glycoproteins could be expressed in chloroplasts, then plastid engineering would be of great use in expressing many commercially important proteins. Plastid engineering was also used for enhancing the nutritional value of food crops such as producing fruits with elevated contents of β-carotene (Apel and Bock 2009), for phytoremediation (Ruiz et al. 2003), for reversible male sterility (Ruiz and Daniell 2005), for biotic and abiotic stress tolerance (Wani et al. 2010), and for production of platform chemicals such as the bioplastic polyhydroxybutyrate (PHB) (Nakashita et al. 2001; Lossl et al. 2003) (Sect. 28.7.4.2).

Although the first transgene delivery to plastids was achieved using the *Agrobacterium-mediated* method (Block et al. 1985), particle bombardment is the method of choice for plastid transformation. To obtain a genetically stable transplastomic line, all genome copies have to be uniformly transformed (homoplasmic), a process that takes a long time. Plastid transformation occurs through the following steps: First, introduction of the plasmid containing the selectable marker (e.g., an antibiotic resistance) and the gene of interest by the biolistic process or by polyethylene glycol; second, integration of the foreign DNA by homologous recombination into the plastid genome; third, gradual elimination of wild-type genome copies during repeated cell division on a selective medium; and fourth, regeneration of the transplastomic shoot. In some cases, the antibiotic resistance used in selection is not desirable in the final products; therefore, many strategies have been developed to eliminate the necessity of using these selectable markers (Maliga 2002, 2003; Wang et al. 2009).

28.2.4 Viral-Based Transient Expression of Proteins

Modular transient expression systems using plant viral vectors are offering a robust industrial method to achieve high yields of heterologous proteins. The advantage with this method is that the transgene is not heritable. Two strategies have been used for transient expression using viral vectors. The first is called a "full virus strategy." The protein of interest, e.g., small protein fragment such as immunogenic epitope, is expressed as a

fusion to the viral coat protein usually in tobacco (Grevich and Daniell 2005). The major limitation of this strategy is that inserts larger than 1 kb cannot usually be expressed. In the second strategy that uses so-called deconstructed viral vectors, there is no "20–25 amino acid barrier" for coat protein fusion and longer proteins up to 80 kDa can be expressed. In this strategy, only the viral elements required for efficient expression of the interested gene were maintained, while the components needed for infectivity, amplification/replication, cell-to-cell movement, assembly of viral particles, and systemic spread are provided using nonviral components (Gleba et al. 2007). Depending on the vector used and the host organism, the process takes 4–16 days, and depending on the gene of interest, up to 5 g recombinant protein per kg fresh leaf biomass can be produced (Gleba et al. 2007). The viral-based expression system has already been used to produce functional platform proteins of different origin such as biologically active human growth hormone and antigens (reviewed in Gleba and Giritch 2011). Overall, this industrial process has a rapid scale-up and high-yield production of diverse heterologous proteins, thereby greatly reducing the cost and time.

28.3 Manipulation of Transgene Expression

Unlike in bacteria, in plant metabolic engineering great care must be taken to control transgene expression. Plants have organelles that affect resources compartmentalization. Also, plants have numerous specialized and differentiated organs in which gene expression may differ dramatically. In addition, temporal and developmental processes can significantly influence when and where the transgene will be active. Moreover, the aim of the engineering process also influences the design strategy. For example, is the engineering intended to impact native physiological processes or synthesis of metabolites or is the goal to overproduce new products or proteins? Such considerations mandate the type of promoters that drive the transgene and its level of expression.

The promoter, a portion of DNA upstream of a gene transcription start site, controls the gene expression levels and its specificity i.e., constitutive, inducible, tissue-specific, or developmentally regulated. In transgenic plants, promoters are used to regulate the level and specificity of the transgene expression (Anami et al. 2013). Various types of promoters that differ in strength and regulation have been used in plant metabolic engineering.

28.3.1 Constitutive Promoters

Constitutive promoters are used to produce large quantities of the transgene product in all plant parts and throughout the growth and development period. Several constitutive promoters have been used successfully to express transgenes in plants. The most commonly used are those of viral origin such as the cauliflower mosaic virus 35S promoter (35S CaMV) that confers a high expression in most cell types except pollen. The expression of these promoters is independent of environmental conditions, and they are active in various monocotyledonous and dicotyledonous plants (Odell et al. 1985; Benfey and Chua 1990). Although a very high level of expression is achieved by using the constitutive promoters of viral origin, gene silencing via co-suppression has been reported (Koosha et al. 1989). Co-suppression phenomena can be avoided by using constitutive promoters of plant origin such as maize ubiquitin (maize *Ubi-1*) (Shrawat and Lorz 2006), rice actins (*OsAct1* and *OsAct2*) (McElroy et al. 1990), *Brachypodium distachyon* elongation factor, ubiquitin (*BdEF1α* and *BdUBI1*) (Coussens et al. 2012), and switchgrass ubiquitin promoters (*PvUbi1* and *PvUbi2*) (Mann et al. 2011). Other constitutive promoters that are also widely used are nopaline synthase (*nos*), octopine synthase (*ocs*), mannopine synthase (*mas*) (von Lintig et al. 1994), and maize alcohol dehydrogenase 1 (*Adh1*) (Kyozuka et al. 1994). To introduce more than one gene in a transgenic plant, different promoters and terminators should be used to avoid the co-suppression and silencing of the introduced genes (Abdel-Ghany et al. 2013).

28.3.2 Inducible Promoters

In some cases, constitutive expression of an introduced gene can have adverse effects on plant growth and development when the overproduction occurs at the wrong time in development or in a tissue where it is not normally expressed. For instance, in metabolic engineering of Arabidopsis for cellulose production, constitutive overexpression of glucanases and cellulases (cellulose-degrading enzymes) reduced cellulose crystallinity and the glucose content in the cell wall (Takahashi et al. 2009), modified leaf shape and growth (Petersen and Bock 2011), and reduced environmental stress tolerance (Taylor et al. 2008). Therefore, under certain circumstances, it is desirable to use inducible promoters to regulate foreign gene expression to a distinct developmental stage or for a certain duration (Cai et al. 2007). Inducible systems are usually based on two components. The first component is a native or chimeric transcription factor (activator) that when induced specifically binds to a specific promoter. The second component contains the binding sites for the activator to activate transcription of a gene fused to this promoter (Borghi 2010). Inducible promoters are activated in response to various external stimuli, which can be chemical factors such as tetracycline, ethanol, steroids, metal ions, and herbicides or physical factors such as heat, cold, and light (Zuo and Chua 2000). Those promoters that respond to specific chemical compounds, not found naturally in the organism of interest, are of particular interest in metabolic engineering because they offer very stringent regulation of gene expression. Tetracycline inducers are particularly attractive because they are small lipophilic compounds that enter easily into eukaryotic cells by passive diffusion. They have been used successfully to produce valuable pharmaceuticals or industrial proteins in plant cell suspension culture (Bortesi et al. 2012). Another antibiotic, which has been used widely in bioengineering of cell culture, is pristinamycin. The use of a pristinamycin-responsive promoter to drive transgene expression has the advantage of controlling bacterial contamination as well as easy induction of the transgene within the bioreactor (Frey et al. 2001). The ethanol-inducible system (Roslan et al. 2001), derived from the filamentous fungus *Aspergillus nidulans*, has been optimized for the production of proteins in plants and studying the function of proteins (Van Hoewyk et al. 2007). Steroid-inducible systems have also been used in studying the function of plant transcription factors that control plant developmental pathways (Aoyama et al. 1995; Gunl et al. 2009). Also, the promoter of the Arabidopsis heat shock protein (HSP18.2) has been used successfully to induce expression of desired genes in several plants after heat shock (37 °C) (Matsuhara et al. 2000; Masclaux et al. 2004). In the absence of heat stress, the HSP18.2 promoter is repressed (Yoshida et al. 1995).

28.3.3 Organ-, Tissue-, Domain-, and Cell-Type-Specific Promoters

Rather than overexpressing the transgene in all tissues, another option is to target the transgene expression to a specific organ or tissue that is rich in precursors of a desired metabolic pathway or is easy to harvest, e.g., leaves, tubers, or fruits (Lessard et al. 2002). Several endosperm-specific promoters have been used to express single or multiple enzymes of biochemical pathways in this tissue either to dissect a metabolic pathway or to improve the nutritional value of seeds (Naqvi et al. 2009; Coussens et al. 2012). Expression of phytoene synthase (*psy*) in indicia rice under the control of seed-specific glutelin promoter (Gt-1 P) in combination with lycopene β-cyclase (*lcy*) and phytoene desaturase (*crtI*) fused to the transit peptide sequence of the pea-Rubisco small subunit and, driven by the CaMV35S promoter, caused the seeds to accumulate high levels of carotenoids and become yellow (Datta et al. 2003). Other tissue-specific promoters that have been used in plant biotechnology are the tapetum-specific promoters for generation of male sterility in canola, brassica, tobacco, Arabidopsis, rice, and others (Mariani et al. 1990; Tsuchiya et al. 1995; Mitsuda et al. 2006; Zhan et al. 1996), the green tissue-specific Rubisco small subunit promoter

(Rubisco SSU) (Bakhsh et al. 2011; Abdel-Ghany et al. 2013), and the floral whorl-specific AP1 promoter (Byzova et al. 2004).

28.4 Suppression of Gene Expression

In addition to overexpression of transgenes that confer desirable traits, suppression of the levels of endogenous gene(s) expression is also of interest for plant biotechnology. Studies in gene silencing-related mechanisms started in early the 1990s based on a surprising observation in which the introduction of a transgene into the genome led to the silencing of both the transgene and homologous native gene (Napoli et al. 1990; van der Krol et al. 1990a), a phenomena known as posttranscriptional gene silencing (PTGS). Different methods have been applied for gene silencing in plants.

28.4.1 Antisense RNA

The antisense RNA strategy is a powerful tool for the selective suppression of gene expression in plants (reviewed in Bird and Ray 1991). It has been used to study genes involved in a wide range of physiological and biochemical processes in a variety of tissue types such as flower pigmentation (van der Krol et al. 1990b), photosynthesis (Stockhaus et al. 1990), fruit-ripening, and viral resistance (Hemenway et al. 1988; Powell et al. 1989). The principle of antisense RNA is based on the introduction of a gene corresponding to a desired gene in a reverse orientation so that an antisense RNA molecule that is complementary to whole or part of the sequence of the mRNA of the target gene is produced. Inside the nucleus, a duplex is formed between the sense and antisense transcript, which interferes with several aspects of gene expression at the transcriptional, post-transcriptional, and translational levels.

Antisense RNA has several limitations. Its effectiveness varies between cells and organs depending on the promoter employed even in the case of the 35S CaMV constitutive promoter because its expression was found to vary in different cell types (Wilkinson et al. 1997; Tang et al. 1999). A further limitation is that gene silencing methods will potentially target all homologous genes, either members of a family or closely related genes (Thorneycroft et al. 2001). Also, it is not possible to eliminate an enzyme completely by antisense RNA, and in many cases the transcript is reduced to undetectable levels without apparent phenotype, making it difficult to derive a firm conclusion about its function as it is usually argued that a small amount of residual enzyme is sufficient to fulfill the required role *in vivo* (Takaha et al. 1998).

28.4.2 Insertional Mutagenesis

Gene knockout is one of the important tools in plant functional genomics. It is based on the insertion of a foreign DNA (either T-DNA or transposon) into the gene of interest. Insertion of foreign DNA into a structural gene (whether into an exon or intron) in most cases will disrupt gene expression completely and result in a null mutation. In some cases, insertion of foreign DNA in the promoter or in the 3′-UTR reduces the expression (knockdown) rather than producing null mutation. The foreign DNA not only disrupts the expression of the gene into which it is inserted but also acts as a marker for subsequent identification of the mutation. One advantage of such insertional mutagenesis is that it allows the recovery of mutations in essential genes, as the plants can be maintained in the heterozygous state. Another advantage of this method is that only a single gene within a family of closely related genes is affected, allowing functional analysis of individual members in a gene family. Using *Agrobacterium*-mediated transformation, a large number of T-DNA-transformed lines have been generated, and, therefore, one has a very high chance of finding a plant carrying a T-DNA insert within any gene of interest. Many T-DNA lines have been developed in the two most important model plant species, Arabidopsis and rice (Krysan et al. 1999; Jeon et al. 2000; Alonso et al. 2003; Sallaud et al. 2004). These T-DNA

stocks have served as a foundation for functional characterization of thousands of genes in these two species. Unfortunately, in many plant species, the *Agrobacterium*-mediated transformation is not developed yet or is not efficient enough to produce a sufficient population of insertion lines that cover the whole genome (Duangpan et al. 2013). In these species, the transposable elements (TEs) are the choice for insertional mutagenesis. Several TEs have been used in plants including the *Activator* and *Mutator* transposons in maize (Walbot 1992), the *Tam3* transposon in *Antirrhinum majus* (Luo et al. 1991), the *Tos17* retrotransposon in rice (Hirochika 2010), and *Tnt1* retrotransposon in tobacco, potato, soybean, and other species (Grandbastien et al. 1989; Cui et al. 2013; Duangpan et al. 2013).

28.4.3 Artificial microRNAs (amiRNAs)

Since insertional mutagenesis approaches are available for a limited number of model plants, targeted gene silencing using amiRNA technology is a valuable tool for gene manipulation and functional analysis in plants (Li et al. 2013). amiRNA technology exploits the endogenous miRNA biogenesis and silencing machineries to generate miRNAs that direct silencing of a desired gene. In plants, the miRNA precursor transcript is 80–250 nucleotides in length with some structural features such as mismatches, bulges, and one or several imperfect foldbacks and when processed produces an miRNA/miRNA* duplex. When both duplex sequences are altered without changing the structural features, a high-level accumulation of miRNAs of a desired gene can be produced in different plant species (Parizotto et al. 2004; Alvarez et al. 2006; Schwab et al. 2006; Ossowski et al. 2008; Sablok et al. 2011). Therefore, artificial miRNA is built on a native miRNA precursor by replacing the original miRNA/miRNA* duplex with a customized sequence to silence a gene of interest, thereby generating a loss-of-function phenotype for the targeted gene (Parizotto et al. 2004; Alvarez et al. 2006; Schwab et al. 2006; Ossowski et al. 2008). A Web-based miRNA designer (WMD) (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Home;project=stdwmd), which works for >100 plant species, allows the design of gene-specific amiRNA candidates within a given transcriptome (Schwab et al. 2006; Ossowski et al. 2008). WMD computationally generates hundreds of amiRNAs for each target that are ranked based on the complementarity and hybridization energy without knowing the *in vivo* efficiency. The efficacy of amiRNAs has been tested in Arabidopsis (Schwab et al. 2006), rice (Warthmann et al. 2008; Schmidt et al. 2012), *Physcomitrella patens* (Khraiwesh et al. 2008), *Chlamydomonas reinhardtii* (Molnar et al. 2009), and other species. The advantages of amiRNA compared to other RNA silencing approaches such as RNA interference (RNAi) and virus-induced gene silencing (VIGS) are that it has minimal off-target effects and a unique capacity for multigene silencing (Alvarez et al. 2006; Niu et al. 2006; Schwab et al. 2006; Ossowski et al. 2008). Since miRNA silencing depends on many unpredictable *in vivo* factors such as target mRNA structure and mRNA binding proteins (Fabian et al. 2010; Pasquinelli 2012) and WMD prediction does not consider these factors, many amiRNAs should be tested to find an optimal amiRNA. Recently, an epitope-tagged protein-based amiRNA (ETPamir) screen was developed for efficient amiRNA gene silencing in plants, in which target mRNA encoding epitope-tagged proteins were coexpressed in protoplasts with amiRNA candidate targeting single or multiple genes (Li et al. 2013). These screens were applied to diverse plant species including Arabidopsis, tobacco, tomato, sunflower, maize, and rice with the potential for improving crop engineering with highly predictable and efficient amiRNAs (Li et al. 2013).

28.5 Metabolic Engineering Using Multiple Transgenes

In plants, metabolic pathways can be enhanced by overexpressing the rate-limiting enzymes or introducing enzymes that are insensitive to

negative feedback regulation. However, introduction of a novel pathway or metabolic engineering of a plant for production of platform chemicals often requires expression of multiple transgenes. In prokaryotes, multigene engineering is possible through coexpression of an operon as a polycistronic transcript (Steen et al. 2010). But in plant cells, except in chloroplasts, the polycistronic mRNA is not efficient in translation because of the scanning mode of the eukaryotic ribosome for recognizing the 5′ cap and the initiation codon in the transcript and the need for a proper sequence context in the 5′ UTR (Kozak 2005). Therefore, different strategies have been developed to introduce multiple transgenes into plants.

The common strategy for introducing more than one gene is stacking of expression cassettes in one vector. Each cassette consists of its own promoter, gene of interest, and a terminator. To avoid gene silencing through co-suppression, different promoters and terminators should be used and the cassettes are arranged in tandem and in the same transcriptional orientation (Hamilton and Baulcombe 1999; Kooter et al. 1999). For example, introduction of β-carotene pathway in rice was achieved by co-transformation with two plasmids, each with a two-gene cassette (Ye et al. 2000). One plasmid contained the phytoene synthase gene *PSY* under the control of the endosperm-specific glutelin promoter and the phytoene desaturase gene *crt1* under the control of the CaMV 35S promoter. The second vector contained the lycopene β-cyclase gene and the selectable marker gene. The three genes for carotenoid biosynthesis additionally contained an N-terminal transit peptide sequence to direct the proteins to the chloroplast, the sites for carotenoids biosynthesis. Another example is the introduction of the butanetriol metabolic pathway in Arabidopsis (Abdel-Ghany et al. 2013). Production of butanetriol from its precursor, xylose or arabinose, requires four enzymes. The genes for the first three enzymes in the pathway were cloned in binary vectors with each gene driven by a different promoter and terminator (Fig. 28.1). Each gene was cloned into a promoter vector, and then the gene with the promoter/terminator was moved sequentially into a binary vector that was used for *Agrobacterium-mediated* transformation (Abdel-Ghany et al. 2013).

Vectors containing different cassettes can also be introduced into plants by co-transformation, serial transformation, or crossing. Co-transformation with direct gene transfer is a straightforward method but more challenging if *Agrobacterium*-mediated transformation is to be used due to the requirement to maintain two plasmids in one *Agrobacterium* cell or the need to infect a single cell with two *Agrobacterium* strains (Ebinuma et al. 2001). In serial transformation, referred to as super transformation, a transgenic plant will be transformed successively with additional transgenes (Bock 2013). This method is more time consuming and requires a new selectable marker gene for each super transformation step unless the selectable marker gene is recycled using marker excision techniques (Hare and Chua 2002). Crossing is also a time-consuming process especially if the plant generation time is long, and this is not an option in crop plants that propagate vegetatively such as potato and sugarcane.

Another strategy for multigene transformation is combinatorial transformation (Zhu et al. 2008). This method takes advantage of the particle bombardment method, which induces a few double-stranded DNA breaks into which multiple copies of the transforming plasmid's DNA can co-integrate before DNA repair (Altpeter et al. 2005). Individual transgenes required for a metabolic pathway as well as the selectable marker are cloned in expression cassettes, mixed, and loaded onto gold or tungsten particles. Particle bombardment with these microprojectiles sometimes introduces copies of all constructs present in the mix. How many of them are introduced into the genome depends on the efficiency of the integration process as well as on the stability of the DNA released from the particles in the host cells. Following the selection, transgenes co-transformed with the selection marker can be analyzed, and transgenic lines can be generated. The most attractive feature of combinatorial transformation is that there is no theoretical limit to the number of transgene cassettes that can be co-transformed. Combinatorial transformation

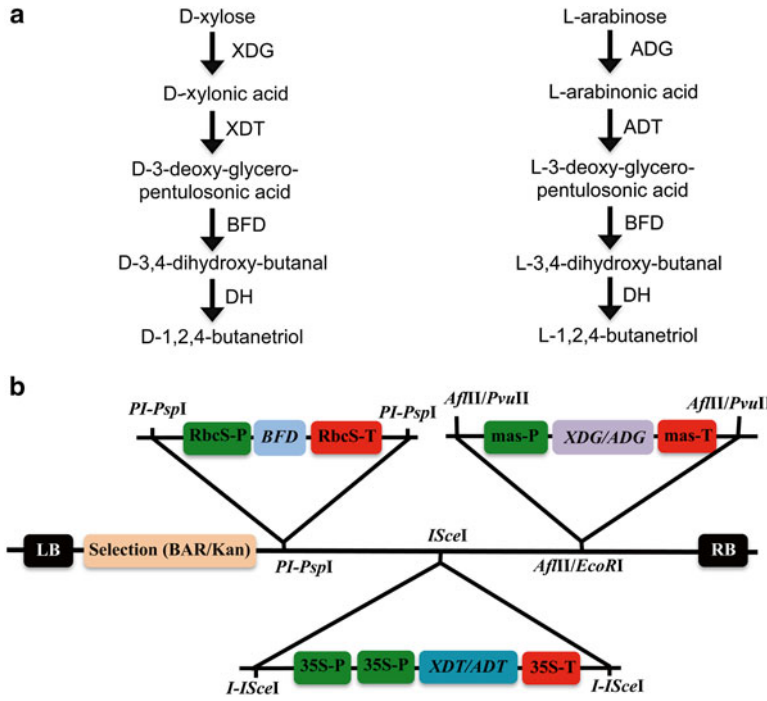

Fig. 28.1 Schematic diagrams of 1,2,4-butanetriol pathways and design of gene constructs for plant transformation. (**a**) Schematic representation of the biochemical pathways showing the synthesis of D-1,2,4-butanetriol and L-1,2,4-butanetriol from xylose and arabinose. XDG, D-xylose dehydrogenase; ADG, L-arabinose dehydrogenase; XDT, D-xylonate dehydratase; ADT, L-arabinonate dehydratase; BFD, benzoylformate decarboxylase; DH, dehydrogenase. (**b**) Binary vector construct containing the three genes encoding the first three enzymes used in the butanetriol synthesis pathways from xylose and arabinose. Each vector had a set of genes amplified from bacteria or codon-optimized synthetic genes for the xylose pathway or the set of codon-optimized synthetic genes for the arabinose pathway. *RbcS-P and RbcS-T* Rubisco small subunit promoter and terminator, respectively, *mas-P and mas-T* mannopine synthase promoter and terminator, respectively; and *35S-P and 35S-T* cauliflower mosaic virus promoter and terminator, respectively (From Abdel-Ghany et al. 2013)

has been used to improve the nutritional value of maize by introducing the biosynthetic pathways for three vitamins (vitamin A, C, and B9) (Naqvi et al. 2009).

28.6 Targeted Genome Engineering

Although the co-transformation, re-transformation, and sexual crossing methods have been proven to be useful for metabolic engineering of plants, these methods suffer from several flaws (reviewed in Dafny-Yelin and Tzfira 2007; Naqvi et al. 2010). For instance, the integration of genes in the genome is completely random, which can have marked effects on the host, such as inactivation of an endogenous gene, different levels of transgene expression among lines, and even silencing of the transgene when inserted into a heterochromatic region. To overcome these issues, a technology that allows stacking of a number of genes in a single multigene array, introducing it at a precisely identified chromosomal locus, and then manipulating the array by genome editing is needed. Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) offer potential for efficient targeted plant genome engineering (Bogdanove and Voytas 2011; Zeevi et al. 2012; Zhang et al. 2013). ZFNs and TALENs are sequence-specific nucleases that create *in vivo* double-stranded breaks at target loci followed by nonhomologous end joining (NHEJ) and homologous recombination, resulting

in targeted gene insertion or gene replacement (Wright et al. 2005; Zhang et al. 2013). Using these systems, binary vectors that can carry up to nine genes and integrate them in the genome were developed (Zeevi et al. 2012). In plants, the use of these sequence-specific nucleases for targeted genome modifications has many applications, ranging from dissecting gene function (Curtin et al. 2012) to creating plants with new traits such as disease resistance, altered metabolite profile, and tolerance to herbicides (Shukla et al. 2009; Townsend et al. 2009).

28.7 Applications of Metabolic Engineering

28.7.1 Improving the Nutritional Value of Crops

Multigene transformation for metabolic engineering helped to generate plants with high nutritional values. For example, to develop golden rice, the entire β-carotene (pro-vitamin A) biosynthetic pathway was introduced into rice endosperm (Ye et al. 2000). The transgenic rice produced seeds with yellow endosperm due to the accumulation of β-carotene (Ye et al. 2000). Substantial improvements of β-carotene were achieved by testing enzymes from different sources, some of which turned out to have superior catalytic activities (Paine et al. 2005). A similar technology has been used to improve the content of other vitamins that are limiting in diet and important in maintaining optimal health (DellaPenna 2007).

Iron biofortification is another application of metabolic engineering. Iron is the most commonly deficient micronutrient in the human diet and about 25 % of the world population suffers from iron deficiency. To increase the iron content in seeds, the iron storage protein ferritin and *Aspergillus* phytase were expressed in the endosperm of rice and maize, respectively, which resulted in significant increase in the levels of bioavailable iron (Goto et al. 2000; Lucca et al. 2002; Drakakaki et al. 2005; Aung et al. 2013). Similar technology was applied for lettuce (Goto et al. 2000). Other nutritionally improved traits that are introduced into agricultural crops and intended to provide health benefits for human and animals include protein quality and level, essential amino acids, oil and fatty acids, fibers, vitamins, and minerals (reviewed in Newell-McGloughlin 2008).

28.7.2 Enhancement of Biomass and Photosynthetic Efficiency

Metabolic engineering has also been employed to improve the photosynthetic efficiency of crop plants (Kebeish et al. 2007). Phosphoglycolate produced from the oxygenation reaction of Rubisco is recycled into phosphoglycerate during photorespiration, which is then used in the Calvin cycle. This recycling process results in net loss of fixed carbon, thereby reducing the yield of biomass and sugars (Kebeish et al. 2007). Introduction of a glycolate catabolic pathway in the chloroplast enabled the release of CO_2 in close proximity of the Rubisco enzyme, which in turn suppresses the unwanted oxygenation reaction and increases the CO_2 fixation capacity (Kebeish et al. 2007). The five transgenes required for the glycolate metabolic pathway were introduced into Arabidopsis chloroplasts by the re-transformation strategy (Kebeish et al. 2007). Transgenic lines showed reduced photorespiration rate and increased biomass production (Kebeish et al. 2007), a potential for improving the efficiency of photosynthesis in crop plants. Also, in a green house simulation experiment using antisense technology, it was possible to produce transgenic plants with reduced Rubisco activity (Makino et al. 1997). These plants showed 15 % higher photosynthetic rates and efficient nitrogen use in an elevated partial pressure of carbon dioxide and high irradiation, although 65 % reduction was observed under normal condition (Makino et al. 1997). Also, overexpression of other enzymes such as maize phosphoenolpyruvate carboxylase in rice and cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase in tobacco enhanced photosynthesis and growth (Ku et al. 1999; Miyagawa et al. 2001).

28.7.3 Resistance to Biotic and Abiotic Stresses

Several biotic and abiotic stresses such as pathogens, insects, salinity, drought, high temperature, and freezing severely limit plant growth and development. Plant biotechnology has allowed the development of transgenics that are more resistant to stresses. In response to osmotic stress, plants accumulate compatible solutes or osmolytes such as glycine betaine, proline, and sugar alcohols, suggesting that overproduction of these solutes in transgenic plants may result in improved stress tolerance. Overexpression of bacterial choline monooxygenase (CMO), the first enzyme in the betaine biosynthesis pathway, in plants resulted in increased resistance to various stresses such as salt and extreme temperature (Sakamoto and Murata 2001). *P5CS* ($\Delta^{1\text{-pyrroline}}$-5-carboxylate) is the rate-limiting enzyme in the proline biosynthetic pathway, and transgenic tobacco plants overexpressing the *P5CS* gene produced 10- to 18-fold more proline and exhibited better performance under salt and freezing stresses (Kishor et al. 1995; Konstantinova et al. 2002). Another way to increase the proline content is to downregulate its catabolism. Arabidopsis plants expressing the antisense mRNA for proline dehydrogenase (*AtProDH*), the first enzyme in the proline degradation pathway, constitutively accumulated proline and showed tolerance to freezing (−7 °C) and to NaCl (600 mM) (Nanjo et al. 1999). Rice plants engineered for trehalose overexpression showed higher capacity for photosynthesis, decrease in photooxidative damage, and tolerance to multiple abiotic stresses (Garget al. 2002).

The dehydration-responsive transcription factors (DREB) and C-repeat binding factors (CBF) are conserved transcription factors that are induced in response to stresses and induce the expression of stress-regulated genes. Increased expression of Arabidopsis *CBF1*, *CBF2*, and *CBF3* induced the expression of many cold-regulated genes and increased the freezing tolerance of non-acclimated plants (Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000). ABA signaling plays a vital role in drought response as many of the drought-inducible genes are induced by ABA (Wang et al. 2003). Constitutive overexpression of ABA-responsive element-binding proteins ABF3 and ABF4 in Arabidopsis demonstrated enhanced drought tolerance with altered expression of ABA- and stress-responsive genes (Kang et al. 2002). Similarly, overexpression of heat shock transcription factors *HSF1* and *HSF3* led to the expression of several *hsp* genes, conferring thermotolerance in transgenic plants (Lee et al. 1995), while the tomato plants expressing *HsfA1* antisense mRNA were extremely sensitive to elevated temperature (Mishra et al. 2002). Engineered plants overexpressing antioxidants such as catalases, ascorbate oxidases, and superoxide dismutases showed increased resistance to oxidative stress caused by high light, low temperatures, and UV radiation (Gupta et al. 1993; McKersie et al. 2000).

Application of plant biotechnology led to improved resistance to insects, diseases, and herbicides (reviewed in Muthurajan and Balasubramanian 2010). For example, transgenic plants that express both *Bt* and *cry* endotoxins were found to exhibit resistance against insects that feed by chewing or biting. Also, simultaneous introduction of three genes expressing insecticidal proteins *Cry1Ac*, *Cry2A*, and *gna* into indica rice controlled three of the major rice pests (Maqbool et al. 2001). Coexpression of two PR-protein genes (chitinase and glucanase) was shown to confer resistance to several fungal diseases (Muthurajan and Balasubramanian 2010).

28.7.4 Production of Platform Chemicals

According to the US Department of Energy, 12 chemicals and chemical classes could be produced from plants, and these chemicals can function as building blocks or platform chemicals from which many value-added compounds such as biopolymers, biofuels, energetic materials, and pharmaceuticals can be produced (United States Department of Agriculture 2008).

28.7.4.1 Energetic Materials

1,2,4-Butanetriol (butanetriol) can be nitrated to produce butanetrioltrinitrate (BTTN), an energetic material and a useful building block for the synthesis of various drugs and important chemicals (Niu et al. 2003; Sato et al. 2003; Yamada-Onodera et al. 2007; United States Department of Agriculture 2008). BTTN is used as a propellant and as an energetic plasticizer in mining and in a variety of Department of Defense and Department of Energy applications. In addition, it is anticipated that BTTN could replace nitroglycerin as a vasodilator for the treatment of angina. Advantages of BTTN over nitroglycerin are that it is less hazardous, less shock sensitive, less volatile, more thermally stable, and resistant to degradation by nitrate reductase, minimizing the number of metabolites generated from degradation.

Chemical synthesis of D, L-butanetriol needs petroleum-derived substrates and requires expensive metal catalysts that adversely impact the environment (Niu et al. 2003). On the other hand, bacterial synthesis of butanetriol from precursors D-xylose or L-arabinose requires continuous supply of these sugars (Niu et al. 2003), a process that limits the bacterial production. Four enzymes are necessary for synthesis of butanetriol from either xylose or arabinose: The first two enzymes D-xylose dehydrogenase (XDG) and D-xylonate dehydratase (XDT) for xylose pathway or L-arabinose dehydrogenase (ADG) and L-arabinonate dehydratase (ADT) for arabinose pathway are substrate specific, whereas the last two enzymes benzoylformate decarboxylase (BFD) and dehydrogenase (DH) are common for both pathways (Fig. 28.1).

Plants synthesize the pentose sugars (xylose and arabinose), which can be converted into butanetriol by expressing microbial enzymes allowing plant-based production of this platform chemical. To produce butanetriol in plants, the native bacterial genes or the codon-optimized ones for both xylose and arabinose pathways were cloned under different promoters and introduced into Arabidopsis plants (Abdel-Ghany et al. 2013). Stable transgenic lines accumulated butanetriol (Fig. 28.2) and showed an altered metabolic profile. Manipulation of the growth medium significantly increased the butanetriol production.

Another energetic bio-based intermediate with potential for production of propellants/explosives is phloroglucinol. In addition to being the starting material for biosynthesis of 1,3,5-triamino-2,4,6-trinitrobenzene (TATB), a stable energetic material used by the US military, phloroglucinol has medical, agricultural, and industrial applications. Chemical synthesis of phloroglucinol involves oxidation of 2,4,6-trinitrotoluene (TNT), a process that presents an explosion hazard and generates carcinogenic chromates as well as other waste streams. On the other hand, bacterial synthesis of phloroglucinol requires a continuous supply of glucose to generate the precursor malonyl-CoA. Plants have malonyl-CoA as the precursor for fatty acid, flavonoids, and phytoalexins biosynthesis (Baud et al. 2003) and, therefore, can be used for sustainable production of phloroglucinol. We generated transgenic plants expressing the bacterial *PhlD* gene targeted to either chloroplast or to cytosol for phloroglucinol production (Abdel-Ghany, Day, Heuberger, Broeckling, and Reddy, MS in preparation).

28.7.4.2 Polyhydroxyalkanoates (PHAs), Bioplastics

For a long time, there has been an interest in the development and production of biodegradable plastics to address problems associated with plastic waste and its harmful effect on the environment (Poirier et al. 1995). In 1992 Somerville's group demonstrated the production of polyhydroxybutyrate (PHB), a 100 % biodegradable polymer, in Arabidopsis by expressing two bacterial proteins required to convert acetoacetyl-CoA to PHB (Poirier et al. 1992). PHB is the most common and best-characterized member in a group of polyhydroxyalkanoates (PHAs), which are biopolymers made entirely from bacterial fermentation. In bacteria, three enzymes are necessary for PHB synthesis: first, a β-ketothiolase that catalyzes the reversible condensation of two acetyl-CoA molecules to form acetoacetyl-CoA; second, an acetoacetyl-CoA reductase

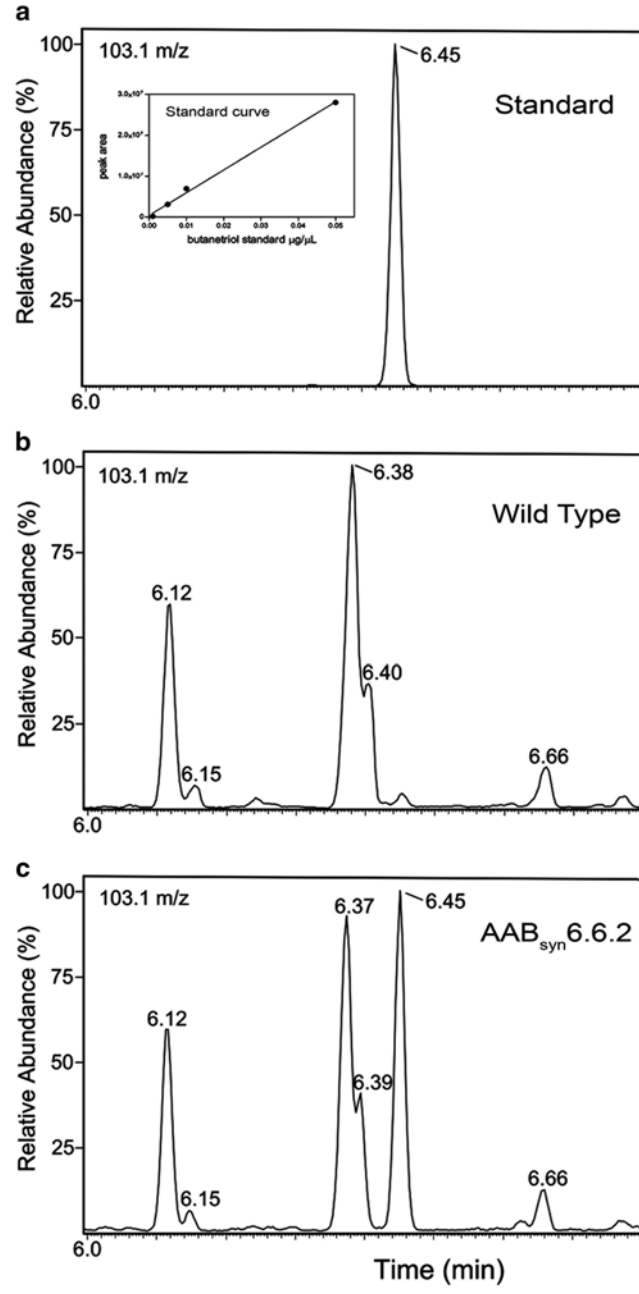

Fig. 28.2 Detection of 1,2,4-butanetriol in a transgenic line. Metabolites from vacuum-dried tissues of wild-type and transgenic lines were extracted and detected by GC-MS. (**a**) Extracted ion chromatogram (EIC, 103.1 m/z) of 1,2,4-butanetriol standard solution and calibration curve. Butanetriol eluted at 6.45 min. (**b**) EIC of wild type. (**c**) EIC chromatogram of AAB_{syn} 6.6.2 transgenic line showed large peak at 6.45 min with a mass spectrum that matches the commercial butanetriol standard (From Abdel-Ghany et al. 2013)

which reduces acetoacetyl-CoA to R-(−)-3-hydroxybutyryl-CoA; and third, a PHA synthase which subsequently polymerizes R-(−)-3-hydroxybutyryl-CoA to form PHB. By changing the carbon source and the bacterial strain used in the fermentation process, it was possible to produce biopolymers having physical properties ranging from stiff and brittle plastic to rubbery polymers (Anderson and Dawes 1990).

Despite its basic attractiveness as a substitute for petroleum-derived polymers, the major drawback of bacterial PHA synthesis is the high cost of bacterial fermentation, making bacterial PHAs five to ten times more expensive than the

synthetic polymer (Poirier et al. 1995). In this respect, crop plants were seen as an attractive system for the sustained production of large amounts of polymers at low cost as plants have the precursor acetyl-CoA and the enzyme β-ketothiolase in the cytoplasm. Through creation of novel metabolic pathways targeted to the cytoplasm, plastid, or peroxisome, varieties of PHAs with different physical properties have been produced in a number of transgenic plants. To date, the highest levels of PHB have been achieved in plastids, likely due to the high flux of the PHB pathway substrate acetyl-CoA through this organelle during fatty acid metabolism (Suriyamongkol et al. 2007; Van Beilen and Poirier 2008; Bohmert-Tatarev et al. 2011). In Arabidopsis plants engineered with plastid-targeted enzymes, PHB has been produced as granules at levels up to 40 % dry weight but with significant growth penalties such that plants were dwarf and produced no seeds (Bohmert et al. 2000). These deleterious effects might be caused by the depletion of acetyl-CoA and acetoacetyl-CoA from the endogenous flavonoid and isoprenoid pathways (Van Beilen and Poirier 2008). To circumvent the growth penalties associated with constitutive expression of the PHA biosynthetic pathway, the introduced genes were fused to an inducible promoter that can be activated at later stages of growth when the plant accumulates sufficient biomass for PHB production (Kourtz et al. 2007). PHAs synthesis in biomass crops of industrial interest such as switchgrass, sugarcane, maize, tobacco, cotton, flax, and others was also reported (reviewed in Bornke and Broer 2010), although more investigation is needed to channel the precursors toward PHA synthesis without deleterious effects on plant growth. In addition to being bioplastics, PHAs can also be converted through simple processes to a range of platform chemical intermediates such as acrylic acid, crotonic acid, propylene, and butanol (Zhong and Whitehouse 2005; Peterson and Fischer 2010), which have valuable industrial applications.

28.7.4.3 Cyanophycin, a Poly-Amino Acid Polymer

Cyanophycin is another important water-soluble and biodegradable polymer that contains only two amino acids, L-arginine and L-aspartate, in a ratio of 1:1 (Simon 1987; Elbahloul et al. 2005). It is synthesized via non-ribosomal polypeptide synthesis in many cyanobacteria in a polymerization reaction catalyzed by an enzyme cyanophycin synthetase (*cphA*). Cyanophycin is a major source of several platform chemicals that can be further processed into many other chemicals of industrial, agricultural, and medical applications (Joentgen et al. 2001) complementing the application ranges of PHB. Mild hydrolysis of cyanophycin results in homopolymers and copolymers of polyaspartate and L-arginine, which serve as the starting point for the syntheses of a range of compounds such as 1,4-butanediamine that can be used for the synthesis of nylon-4,6; 3-aminotetrahydrofuran that is used heavily in the polymer industry; fumaric acid that is used for polyester resin; and acrylamide which is used in papermaking and manufacturing dyes (Bornke and Broer 2010). Polyaspartate polymer could also replace the nondegradable synthetic polyacrylates in various processes such as water treatment and plastics (reviewed in Bornke and Broer 2010). For sustainable production of this important polymer in plants, the cyanophycin synthetase gene from cyanobacteria was introduced into tobacco and potato under the control of the 35S CaMV promoter with modest accumulation (0.24–1.14 % per unit DW) and deleterious effects on plants (Neumann et al. 2005). Targeting the cyanophycin synthesis to the plastids or to tubers significantly enhanced its accumulation up to 7.5 % DW without noticeable effect on plant growth (Huhns et al. 2008, 2009). Similar biotechnological approaches have been applied for bio-production of other important poly-amino acids such as poly-γ-glutamate and poly-lysine using bacterial operons (Oppermann-Sanio et al. 1999; Oppermann-Sanio and Steinbuchel 2002).

28.7.5 Production of Pharmaceuticals in Plants

The growing need for safer and cheaper drugs and the availability of tools to express foreign proteins of interest in plants led to the idea of using plants to produce vaccines and other pharmaceuticals. A schematic representation of the concept is shown in Fig. 28.3. Various pharmaceutical proteins can be produced *in planta* in their original or genetically modified form. The molecular design of the correspondent expression cassettes is largely dependent on the choices of expression system (Schiermeyer et al. 2004). The key considerations are the size, solubility, folding parameters, as well as possible glycosylation and other posttranslational modifications. Recombinant products can also be designed as protein fusions or chimeras with affinity tags that can aid in detection and purification (Gomord et al. 2004; Benchabane et al. 2008). Many proteins (>150) of interest to the pharma industry have already been tested for production *in planta*. The advantages of using plants for pharmaceutical production are that it is easy to scale up and is expected to incur low production costs. Furthermore, plants are considered as a safe and pathogen-free biomass source.

Plants have been tested for vaccine production, often referred to as "edible," "oral," or "green vaccines" (Mor et al. 1998; Streatfield and Howard 2003). The main idea is to express immunogenic antigens in edible parts of plants and feed animals or humans to immunize them. These antigens are capable of inducing strong and protective immune responses sufficient to neutralize the pathogens (Bae et al. 2009), especially the ones against the reemerging infectious diseases, cancer, autoimmune diseases, and others (Ulmer et al. 2006). The pioneering work of Dr. Charles Arntzen and his colleague Dr. Mason in this area has led to many studies and establishment of a novel branch of plant biotechnology (Goldstein and Thomas 2004; Schiermeyer et al. 2004; Ma et al. 2005a). Successful oral feeding experiments with some strong antigenic proteins such as *E. coli* enterotoxin B (LTB), *Cholera* toxin B (CT-B), and Hepatitis B surface antigen (HBsAg) (Tiwari et al. 2009) that do not require immune stimulators (adjuvants) (Reed et al. 2009) confirm the potential for using plant-based vaccines. Plant vaccines together with plant-derived functional

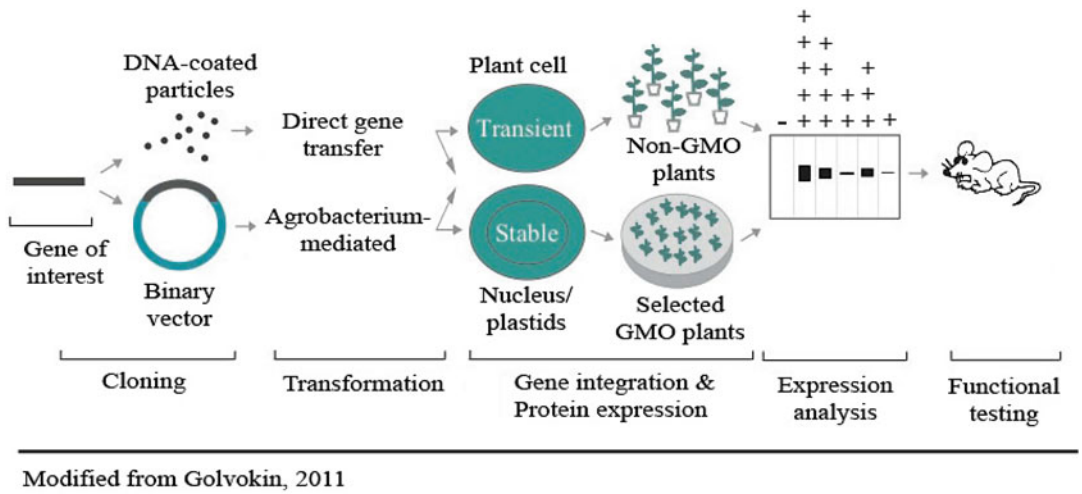

Fig. 28.3 Schematic representation of the key stages for production of pharmaceuticals in plants. The gene(s) of interest is cloned in a binary vector, which is then transferred into plant cells by either *Agrobacterium*-mediated transformation or direct gene transfer. In the case of stable transformation, the transgenic lines are selected and used for production of the recombinant protein. Production of recombinant protein is usually followed with functional testing in lab animals (Modified from Golovkin 2011)

antibodies (Hood et al. 2002; Stoger et al. 2002; Fischer et al. 2003) have reached the initial stages of testing in animals and humans (Table 28.3). The first officially approved trial was conducted in 1997 with LTB in potato tubers (Tacket et al. 1998). Some products can be used as a simple processed material directly in needle-free immunization (Ma et al. 2005b; Streatfield 2006; Floss et al. 2007; Tiwari et al. 2009). These groundbreaking studies and many other studies that followed suggest the potential for engineered plants in providing safe and, probably, cheap therapeutic products. The technology is now transitioning from academic labs to industrial production of valuable recombinant proteins like subunit vaccines, antibodies, and other therapeutic proteins.

The "edible" pharmaceutical concept has some issues that need to be addressed. For instance, the dosage control is difficult because of variable quantities of recombinant protein in plant tissues (fruits, vegetables, grains, etc.). Another concern is a potential environmental risk (Mascia and Flavell 2004; Peterson and Arntzen 2004). The release and use of edible crops, like vegetables, fruits, and grains, may lead to possible genetic cross-contamination of other agriculturally important crops (Sparrow and Twyman 2009). Special regulatory measures called "*c*urrent *g*ood *m*anufacturing *p*ractice" (cGMP) are currently in place (FDA 2002) to guide the entire research/production process. These regulatory measures are applicable to the plant-based pharmaceutical products also. A recent approval of the first US plant-based (tobacco) recombinant vaccine against Newcastle disease in chickens in 2006 (Vermij and Waltz 2006) indicates that plant vaccines are safe and efficacious at least for veterinary use. Simple downstream processing of biomass (dry or dry/freezing) could also help to normalize the active recombinant components, e.g., for low-concentration microbicides. The final products could be extracts, powders, capsules, tablets, and ointments (Daniell et al. 2005; Scott 2005; Yusibov and Rabindran 2008; Rybicki 2009; Tiwari et al. 2009). Scientists remain cautiously optimistic that the latest developments in this area will garner the attention of the pharma industry and bring needed support to establish plant biopharming.

28.8 Conclusions

As discussed above, significant progress has been made in recent years in engineering plants to produce commercially important products. This field has moved from a single-gene manipulation to introduction of whole new pathways from

Table 28.3 Plant-derived pharmaceuticals in clinical trials

Product	Targeted disease	Plant source
Vaccines		
E. coli enterotoxin B (LTB)	Diarrhea	Potato and maize
Hepatitis B surface antigen (HBsAg)	Hepatitis B	Potato and lettuce
Norwalk virus antigen	Sickness/diarrhea	Potato
Rabies glycoprotein G	Rabies	Spinach
Antibodies		
LSBC scFVs	Non-Hodgkin's lymphoma	Tobacco
Avicidin	Colorectal cancer	Maize
CaroRx	Dental caries	Tobacco
Other products		
Gastric lipase	Cystic fibrosis, pancreatitis	Maize
Human intrinsic factor	Vitamin B12 deficiency	Arabidopsis
Lactoferrin	Gastrointestinal infections	Maize
Human glucocerebrosidase	Gaucher disease	Carrot
Enzyme		

LTB Heat-labile enterotoxin B subunit, *LSBC* Lifesaver for Babies and Children, *scFVs* single-chain Fv fragments

heterologous systems. The current toolbox allows stacking of a large number of genes at the same chromosomal locus as a multigene array and its manipulation by genome editing. The choice of the tool used depends on several factors such as plant species, number of genes to be engineered into the plant, the desired expression level, cellular compartmentalization of the pathway of interest, and the available information about the metabolic pools, fluxes, and regulation of the metabolic pathway. Reduction in gene synthesis costs and progress in assembling larger pieces of synthetic DNA in recent years have made engineering complex networks possible.

Although much progress has been made in engineering plants to produce several commercially important products, there are still significant challenges in bringing these plant-based products to market. The main strategy that has been used for engineering plants is based on introduction of one or more genes under constitutive, regulatable, or tissue-specific promoters. This approach works well in producing mRNA from the introduced gene, but it does not always result in the required amount of protein due to many post-transcriptional and posttranslational regulatory mechanisms (e.g., mRNA stability, translatability of mRNAs, stability of proteins) that cells employ to modulate the levels of proteins and their function. Because of this, often-desired compounds are produced at very low levels. Incorporating elements that impact expression of a gene after mRNA biogenesis into gene construction design may lead to increased production of desired proteins. Also, production of native or novel chemicals and compounds in plants at high levels often is detrimental to plant growth and development. This is mainly due to lack of our understanding of the interconnections between metabolic pathways and insufficient knowledge about the multilevel regulation of metabolic pathways. These limitations must be addressed in order to make the outcome of metabolic pathway engineering more predictable and at the same time avoid unwelcome surprises from adverse phenotypes of transgenic plants. Comprehensive global studies on transgenic lines using "omics" approaches together with the knowledge obtained from systems biology should help in addressing some of the current issues associated with plant metabolic engineering. Furthermore, application of emerging tools in synthetic biology to manipulate existing gene circuits and create novel circuits to plant metabolic engineering is also expected to mitigate some of these issues.

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Genetic Engineering Strategies for Abiotic Stress Tolerance in Plants

29

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Abstract

Crop plants are affected by a variety of abiotic stresses such as salinity, drought, extreme temperatures, and oxidative stress and cause a significant yield loss (more than 50 %). In the near future, these abiotic stresses might increase because of global climate change. Abiotic stresses lead to dehydration or osmotic stress through reduced availability of water for vital cellular functions and maintenance of turgor pressure and also result in high production of reactive oxygen species (ROS). Plants are evolved with various mechanisms such as changes in cellular and metabolic processes to cope with the stress condition. Recent developments in molecular genetics have contributed greatly to our understanding of the biochemical and genetic basis of abiotic stress tolerance. This has led to the development of abiotic stress-tolerant plants with yield advantage by modulation of the expression of the genes that encode for enzymes involved in the biosynthesis of osmoprotectants (e.g., proline, sugars, sugar alcohol, glycine betaine, and polyamines), antioxidant enzymes, protective proteins (e.g., LEAs and HSPs), transporters, regulatory proteins, kinases, and transcription factors. More recently, posttranscriptional and posttranslational

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regulation mechanisms of the abiotic stress response, like microRNAs and ubiquitination, appear as promising new modulation targets to develop abiotic stress-tolerant plants and contribute to the development of more productive crops to feed the growing mass.

Keywords
Abiotic stress • Transgenic plants • Abiotic stress tolerance • Osmolytes • Polyamines • Carbohydrates • Antioxidant enzymes • Late embryogenesis abundant proteins • Heat shock proteins • Transporters • Kinases • Transcription factors • miRNAs

29.1 Introduction

Plants, as sessile organisms, have had the need both to develop and evolve mechanisms that allow them to be informed about their changing environment, as well as systems and strategies to react and adapt to these changes. These capabilities have allowed plants not only to survive against environmental and biological threats but also have accorded them the ability to face different types of environment and, thus, colonize many habitats on earth.

Abiotic stress is a term that refers to those unfavorable environmental conditions that adversely affect plant growth and development. Some examples of abiotic stresses that a plant may face include decreased water availability (due to drought or osmotic stress), soil salinity (salt stress), or extreme temperatures (heat or cold stress), which are considered to be among the most threatening environmental conditions for crop yield. To mitigate and recover from damaging effects of abiotic stress, plants have evolved various adaptive strategies at the cellular and metabolic levels that come into play at the onset of stress.

29.1.1 Abiotic Stress Effects

One of the most threatening situations that plants have to face is water deficit, due to drought or osmotic stress, which can result from low and erratic rainfall, low water-holding capacity of soil, and situations where transpiration rate exceeds the rate of water uptake. Drought implies changes in water potential gradients between soil and plant, such that the plant's ability to absorb water is compromised, leading to loss of cell turgor, change in cell volume, denaturation of proteins, and changes in several physiological and molecular components as well as membrane integrity. In response to this situation, the plant triggers cellular and metabolic processes including inhibition of cell expansion, cell wall synthesis, protein synthesis, stomata conductance, and photosynthetic activity. Also, the plant triggers adaptation mechanisms that include biosynthesis of the phytohormone abscisic acid (ABA) and production of compatible solutes as well as gene products involved in osmotic adjustment and protection/repair of cellular structures (Bartels and Sunkar 2005; Mahajan and Tuteja 2005; Bhatnagar-Mathur et al. 2008; Janska et al. 2009).

Salt stress develops from excessive concentration of salt, especially sodium chloride (NaCl) in the soil. In a situation of high salinity, soil water potential decreases, making the plant face an osmotic stress situation initially, which triggers responses similar to drought. Later, the salt that has entered the root is transported via the xylem to the shoot and additional ion-specific toxicity effects arise, contributing to growth inhibition and accelerated leaf senescence. To face this second phase, the plant sets into motion mechanisms concerning ion uptake and compartmentalization, with an objective of reestablishing ion homeostasis (Munns and Tester 2008).

Salinity and drought stress are often associated with temperature situations above the

normal/optimum, causing heat stress. In this stress situation, many proteins suffer denaturation and dysfunction, membrane fluidity, and permeability; cellular homeostasis is disturbed, leading to severe retardation in growth, development, and even death (Hasanuzzaman et al. 2013).

Cold or low-temperature stress in normally faced by plants growing in temperate and frigid areas. Chilling (<20 °C) or freezing (<0 °C) temperatures can induce ice formation in plant tissues, also leading to a situation of water deficit in the cell. Also, as enzyme activities are extremely temperature dependent, low temperatures affect the rate of most biochemical reactions of the plant cell, including photosynthesis. On the other hand, membrane fluidity properties also change. Some of the responses triggered by cold stress include production of more energy by activation of primary metabolism and induction of molecular chaperones, which contribute by stabilizing proteins against freeze-induced denaturation (Chinnusamy et al. 2007; Theocharis et al. 2012).

29.1.2 Abiotic Stress Perception and Response

The very first event experienced by a plant, when one or more environmental factors change from their optimum, is sensing. Abiotic stress sensing is a complex issue where there is no single sensing mechanism common to all stresses. In the case where a chemical agent is related to the stress situation (like heavy-metal stress or nutrient depletion), a model of ligand-receptor could be hypothesized. This model, however, could not be applied in the same way to situations where stress is produced by changes in physical parameters. For example, primary sensing of temperature stress (heat stress or chilling/freezing conditions) does not involve any chemical ligand, although the existence of receptor proteins that change its conformation with temperature could be hypothesized. In this sense, several evidences support the existence of membrane-associated thermosensors that can perceive and respond appropriately to mild changes in the fluidity of the membrane, which could be generated by changes in temperature or in the lipid composition of those membranes (Saidi et al. 2011). In addition, as stated above, high salinity, drought, and cold (freezing) situations produce similar changes in the osmosis cell conditions, increasing water potential inside the plant cell, and thus cell turgor decreases. Yeast membrane protein SLN1 acts as an osmosensor by reacting to changes in cell turgor pressure and triggers a two-component system, which activates mitogen-activated protein kinase (MAPK) pathways (Maeda et al. 1994; Reiser et al. 2003). The isolation of the *Arabidopsis thaliana* histidine kinase (HK) receptors ATHK1, homologue to SNL1 (Urao et al. 1999), as well as the membrane cytokinin receptor CRE1, which is able to act as an osmosensor when expressed in yeast (Reiser et al. 2003), has opened the possibility of the existence of osmosensor systems similar to yeast in higher plant cells (Fig. 29.1).

Sensing mechanisms activate a complicated interplay of downstream signaling cascades, which transmit, amplify, and finally trigger cell responses that lead to counterreactions against harmful effects of the stress condition. The knowledge of stress signaling pathways leading from stimulus to the end response in plants has increased over the recent years. These downstream signaling pathways are composed by several active participators including second messengers, phytohormones, and phosphoprotein (protein kinases and protein phosphatases) cascades.

Second messengers are small intracellular signaling molecules or ions, normally located in the cytoplasm of a cell, whose levels change in response to a signal received by a membrane sensor. Second messengers spread by diffusion and regulate activity of proteins, helping to distribute and amplify the signal received. Second messengers involved in stress signaling pathways include calcium (Ca^{2+}) (Tuteja and Mahajan 2007), reactive oxygen species (ROS) (Suzuki et al. 2012), cyclic nucleotides, and polyphosphoinositides, among others (Tuteja and Sopory 2008).

On the other hand, phytohormones activate signaling pathways that could act independently

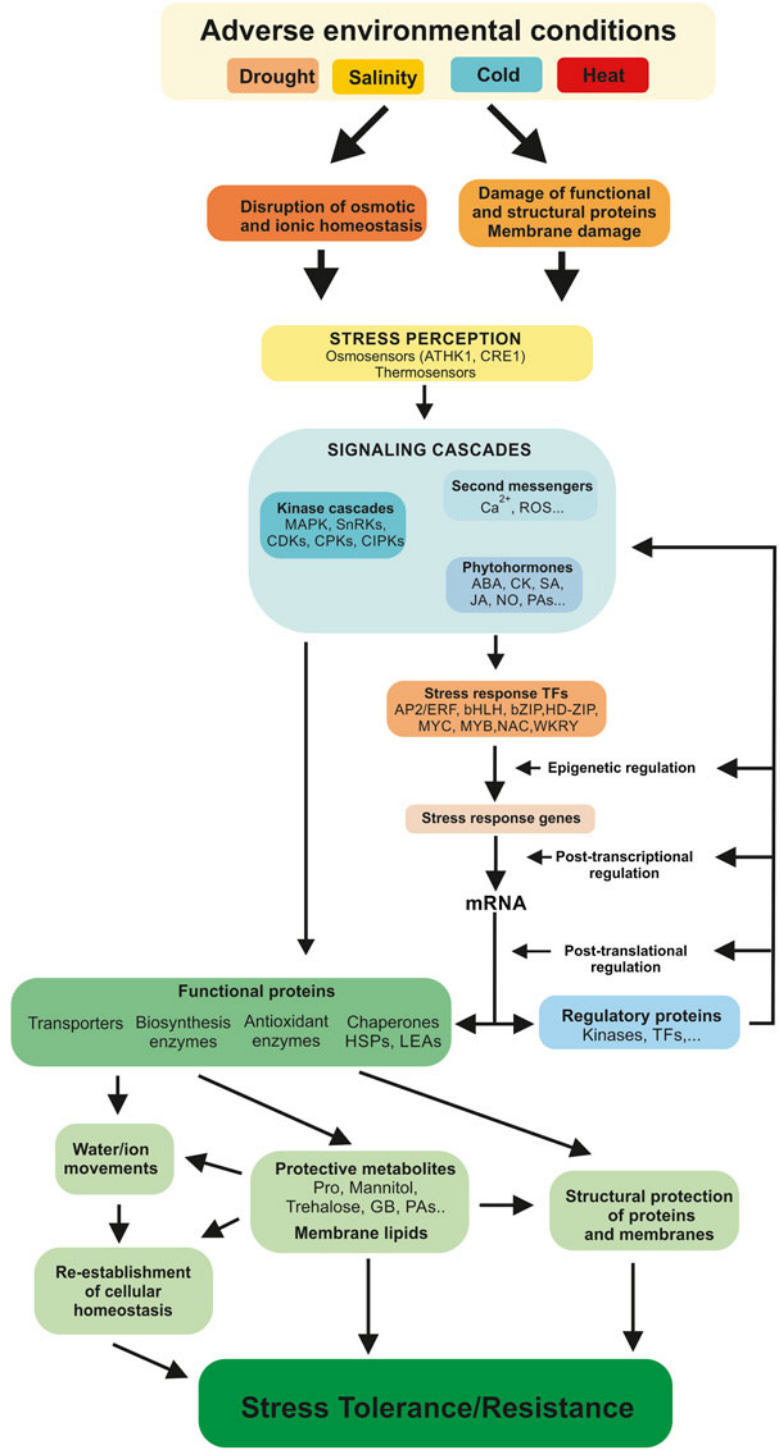

Fig. 29.1 Schematic overview of plant molecular response to abiotic stress factors. When exposed to adverse environmental conditions, a complex regulatory network is triggered by plant sensor mechanisms. Downstream signaling cascades lead to the production of regulatory and functional proteins with roles in mechanisms that reestablish cellular homeostasis, eliminate toxic compounds, and protect and repair damaged structures

or synergistically with other stress-triggered pathways. In this sense, ABA constitutes the most studied stress-responsive hormone and plays a central role in abiotic stress responses. Other phytohormones and signaling molecules such as cytokines (CK), salicylic acid (SA), ethylene (ET), jasmonic acid (JA), nitric oxide (NO), sugars, and polyamines (PAs) play a substantial direct or indirect role in abiotic stress response (Tuteja and Sopory 2008).

Protein kinases and phosphatases play a fundamental role in coordinating the activity of many known signal transduction pathways. Those proteins control phosphorylation status of their target proteins by catalyzing reversible phosphorylation processes. Cell signal transduction pathways often rely on phosphoprotein cascades, including stress responses. They are divided into several categories according to their structural and functional characteristics. Several families are known to be involved in downstream stress signaling cascades in plants, including MAPK cascades, sucrose non-fermenting 1(SNF1)-related kinases (SnRKs), cyclin-dependent protein kinases (CDKs), Ca^{2+}-dependent protein kinases (CPKs), and calcineurin B-like(CBL)-interacting protein kinases (CIPKs), among others (Boudsocq and Laurière 2005; Boudsocq and Sheen 2013).

All these elements also interact among themselves, forming a complex network, whose end result is modification of target proteins that may have enzymatic or structural function, leading to quick physiological cell responses. Alternately, signaling pathways can also lead to production on new proteins by modifying the activity of transcription factors (TFs) which modulate gene expression by binding to specific DNA sequences in the promoters of target genes. TFs classify as a big family depending upon the presence of known DNA-binding domains. Stress-related TFs include members of APETALA2/ethylene-responsive factor (AP2/ERF), basic helix-loop-helix (bHLH), basic leucine zipper (bZIP), homeodomain leucine zipper (HD-ZIP), myelocytomatosis (MYC), myeloblastosis (MYB), and NAC and WRKY families (Lindemose et al. 2013).

Transcriptome studies have revealed that the genes induced by those signaling cascades triggered by abiotic stress could be divided into two major groups according to the functions of their products (Bohnert et al. 2001; Fowler and Thomashow 2002; Seki et al. 2002). The first group, often called functional proteins, consists of a large number of proteins with enzymatic and structural functions which help to protect the cells from the diverse effects of stress, such as membrane proteins that help to reestablish cell homeostasis (as water channel proteins and membrane transporters), biosynthesis enzymes of metabolites for osmotic adjustment or with protective functions (also called compatible solutes), ROS detoxification enzymes (as glutathione S-transferases, hydrolases, superoxide dismutases, and ascorbate peroxidases), and other proteins for macromolecular protection [such as late embryogenesis abundant (LEA) proteins, chaperones, and mRNA-binding proteins]. The second group comprises a variety of regulatory proteins (transcription factors, protein kinases, receptor protein kinases, ribosomal-protein kinases, and transcription-regulation protein kinase) and signal transduction proteinases (phosphoesterases and phospholipase C) involved in the regulation of signaling cascades that control expression of additional genes whose products could belong, in turn, to each of the two groups (Agarwal et al. 2006; Shinozaki and Yamaguchi-Shinozaki 2007).

Apart from TFs, modulation of gene expression in response to abiotic stress may be performed at other levels. In this sense, there are some reports where involvement of epigenetic mechanisms has been pointed out, as the alteration of methylation status of DNA or modifications of nucleosomal histones, in the response to environmental cues and to different types of abiotic stresses (Boyko and Kovalchuk 2008; Kim et al. 2010). A second level of tuning of gene expression could be performed by posttranscriptional regulation mechanisms. In this sense, most of the alternative splicing events that have been described in response to abiotic stress concern genes with regulatory roles (Mastrangelo et al. 2012; Seo et al. 2013). Additionally, the role of

miRNAs in stress responses has been demonstrated (de Lima et al. 2012). By the other hand, posttranslational modification of the gene products constitutes a third level of control. Apart from protein phosphorylation, it has been described that protein sumoylation (Miura et al. 2007; Xu and Yang 2013) and protein ubiquitination (Lyzenga and Stone 2012; Mazzucotelli et al. 2008) are processes that play major roles in the modulation of plant response to abiotic stress (Fig. 29.1).

The concerted actions of those control mechanisms ensures that gene expression events downstream stress perception could be carried out in adequate spatial and temporal patterns and thus shape a specific transcriptome and proteome response by the plant, adjusted to each stress situation and focused to reach to a new balance between growth, development, and survival. The complete understanding of the interplay of these regulatory systems is crucial for the comprehension of the molecular mechanisms governing plant adaptation to environment as well as for the design of strategies to improve plant stress tolerance.

29.1.3 Strategies to Improve Plant Resistance

As previously stated by Mizoi and Yamaguchi-Shinozaki (2012), there are three possible strategies that could be undertaken to improve plant stress resistance:

1. A stress *escape* strategy that implies to change the plant life cycle, e.g., by making plants grow and flower only during the more favorable seasons
2. An *avoidance* strategy that implies to modify the architecture of organs or tissues in order to prevent the formation of stressful internal environments in plants, such as extremely high-salt concentrations or high osmotic potentials inside tissues
3. A *tolerance* strategy that implies to improve tissue and plant cell viability in order to tolerate stressful internal environments

Regarding abiotic stress, a majority of the physiological and molecular biology studies on plant tolerance have been undertaken using the third strategy above, by researching whose are the cellular responses to abiotic stress and how they are regulated. In some cases, knowledge has been gained using transgenic approaches, which also resulted in generation of stress-resistant plants. In other cases the knowledge gained has served as basis for the design of alternative transgenic strategies that also had led to the production of resistant plants.

29.2 Transgenic Approaches for Improving Abiotic Stress Tolerance

29.2.1 Overproduction of Functional Proteins

The adaptation of the plant to abiotic stress includes a series of cell physiological processes including the production of metabolites and proteins involved in protective functions. Therefore, some of the strategies used for obtaining plants with increased stress tolerance have been aimed at the modulation of the expression of genes involved in the biosynthesis of these metabolites, as well as those coding for those proteins that play a direct part in the mechanisms of protective function of the plant cell (Fig. 29.1).

29.2.2 Enzymes for Production of Protective Metabolites

In response to different stresses, plants use a common physiological survival mechanism that consists in the accumulation of low-molecular-weight organic compounds that are highly soluble in water and nontoxic at high concentrations. Those compounds, often called osmoprotectants or compatible solutes, protect plants from stress by contributing to cellular osmotic adjustment as well as by helping to protect cell structures from damage and denaturation by acting as ROS

scavengers and/or as low-molecular-weight chaperones that protect membrane integrity and stabilize proteins (Chen and Murata 2002). A wide range of metabolites have been identified as osmoprotectants, including amino acids (e.g., proline), quaternary and other amines [e.g., glycine betaine (GB) and polyamines (PAs)], and a variety of sugars and sugar alcohols (e.g., mannitol and trehalose). Overproduction of such osmoprotectants has been extensively used as strategy to attempt to improve tolerance to abiotic stresses (Bhatnagar-Mathur et al. 2008).

29.2.3 Proline

It is well known that many plants and other organisms, including eubacteria, protozoa, as well as marine invertebrates, accumulate proline to a high concentration in response to various stress conditions. Apart from acting as an osmolyte and contributing to the maintenance of cell turgor or osmotic balance, other roles have been proposed for proline in plant stress response, such as molecular chaperone, being a metal chelator, forming part of the antioxidative defense mechanism, or even playing a role in signaling. On the other hand, proline accumulation could also be a sink of carbon and nitrogen for use after relief of water deficit (Ashraf and Foolad 2007; Verbruggen and Hermans 2008). Plants are able to synthesize proline from two different precursors. One biosynthesis pathway converts glutamate to proline by two successive reductions catalyzed by pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR), respectively (Delauney and Verma 1993). Proline could also be obtained from ornithine (Orn), which is transaminated to P5C by Orn-δ-aminotransferase (OAT), a mitochondrial enzyme. By the other hand, proline is degraded by a process reverse to proline biosynthesis and catalyzed by proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH). Proline biosynthesis is a cytosolic and plastidial process, while proline degradation takes place in mitochondria (Verbruggen and Hermans 2008).

The accumulation of proline in plants under stress is caused either by the induction of expression of proline biosynthesis genes or by the repression of the genes of its degradation pathway. During osmotic stress, glutamate pathway is the main route used by plants for proline accumulation, although in young *Arabidopsis* plants, the Orn pathway seems also to contribute by enhancement of OAT activity (Verbruggen and Hermans 2008). Proline overproduction by transgenic approaches has been achieved by the same strategies: favoring proline synthesis by overexpression of *P5CS*, *P5CR*, or *OAT* genes or inhibition of proline degradation by *PDH* silencing by antisense or knockout mutants. An excellent review by Kavi Kishor and Sreenivasulu (2014) includes an updated list of 65 transgenic plants where some of these strategies have led to proline accumulation and abiotic stress tolerance.

29.2.4 Amines and Polyamines

GB is a quaternary amine compound widely distributed in higher plants and is synthesized in many plant species at elevated rates in response to various types of environmental stress (Chen and Murata 2008). In plants, GB is synthesized from choline via two sequential enzymatic reactions catalyzed by choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH), while in the soil bacterium *Arthrobacter* sp., GB synthesis from choline is performed by a single enzyme, choline oxidase, which is encoded by the *codA* gene (Chen and Murata 2002). Also, in extreme-halophytic phototrophic bacteria *Actinopolyspora halophila* and *Ectothiorhodospira halochloris*, GB is synthesized by a third pathway from glycine catalyzed by two enzymes, namely, glycinesarcosine methyltransferase (GSMT) and sarcosine dimethylglycine methyltransferase (SDMT), by a three-step N-methylation reaction (Chen and Murata 2002). Several plants species, known as natural accumulators of GB, normally contain low levels of GB, but they increase significantly their GB levels when subjected to abiotic stress. Indeed, many of those plants are

salt-tolerant plants (Chen and Murata 2008; Rhodes and Hanson 1993). In contrast, many other plant species including *Arabidopsis*, rice, tobacco, potato, and tomato show no detectable GB levels under normal or stressful conditions and consequently have been chosen as good candidates for the engineering of GB biosynthesis. In a variety of cases transgenic lines generated from several plant species, being most of them from natural non-accumulators of GB plants, have been obtained overexpressing GB-biosynthetic genes from bacteria and plants. The various transgenic plants accumulate GB at a variety of levels and exhibit enhanced tolerance to various types of stresses (Park et al. 2007; Zhou et al. 2008; He et al. 2010; Zhang et al. 2011a, b). An extensive list could be found in the reviews by Chen and Murata (2011) and Wani et al. (2013). Several mechanisms have been proposed to explain this enhanced tolerance. By one side, GB could act as a stabilizer of certain protein complexes, preventing its denaturation when plant cells are exposed to stress conditions. Also, it has been observed that GB is an inducer of the expression of specific genes that encode ROS-scavenging enzymes and thus could prevent the accumulation of excess ROS that could accumulate when plant are exposed to abiotic stress. Combination of both actions will result in conservation of the integrity of cell membranes and protein complexes such as photosynthetic machinery, ion channels, or translation machinery, helping to prevent their denaturation when exposed to abiotic stress conditions (Chen and Murata 2011; Wani et al. 2013).

PAs are a group of low-molecular-weight amine-containing aliphatic molecules. The most widespread forms of PAs in living organisms are the diamine putrescine (Put), triamine spermidine (Spd), and tetramine spermine (Spm), which differ in the number of aminopropyl moieties added to the carbon skeleton of Put and thus in their number of positively charged amine groups at physiological pH. PAs have been implicated as key players in a wide array of plant growth and developmental processes as well as in biotic and abiotic stress responses (Bhattacharya and Rajam 2006; Alcázar et al. 2010; Kusano et al. 2008; Takahashi and Kakehi 2010).

Intracellular levels of PAs in plants are mostly regulated by anabolic and catabolic processes as well as by their conjugation to hydroxycinnamic acids and macromolecules like proteins and DNA. Plants synthetize the essential diamine Put either directly from ornithine (Orn) decarboxylation in a reaction catalyzed by ornithine decarboxylase (ODC) or by an alternative pathway that involves arginine (Arg) decarboxylation by arginine decarboxylase (ADC) followed by two additional successive steps catalyzed by agmatine iminohydrolase (AIH) and *N*-carbamoylputrescine amidohydrolase (CPA). Spd and Spm are the result of sequential additions of aminopropyl moieties to Put and Spd, respectively, by the enzymes Spd synthase (SPDS) and Spm synthase (SPMS). Decarboxylated S-adenosylmethionine (dcSAM) is used by both enzymes as donor molecule of aminopropyl groups and is synthetized from the decarboxylation of *S*-adenosylmethionine (SAM) in a reaction catalyzed by SAM decarboxylase (SAMDC). Spd and dcSAM can also form a structural isomer of spermine, thermospermine (tSpm) in a reaction catalyzed by tSpm synthase (tSPMS). PAs are catabolized through oxidative deamination in reactions catalyzed by diamine oxidase (DAO) and polyamine oxidase (PAO) activities. DAOs display high affinity for diamines like Put, producing Δ^1-pyrroline, hydrogen peroxide (H_2O_2), and ammonia, while PAOs oxidize secondary amine groups from Spd and Spm, leading to the formation of 4-aminobutanal or (3-aminopropyl)-4-aminobutanal, along with 1,3-diaminopropane (DAP) and H_2O_2. PAOs are also able to catalyze the back-conversion of Spm to Spd, producing 3-aminopropanal and H_2O_2. Some PAO isoforms are also involved in back-conversion processes of tSpm to Spm, and Spm to Put, which lead to the production of H_2O_2 (Alcázar et al. 2010; Bitrián et al. 2012; Moschou et al. 2012).

A large number of studies have shown increases in PA levels in different plant species under a number of environmental stress conditions, and its accumulation correlates with enhanced stress tolerance (Bouchereau et al. 1999; Alcazar et al. 2006; Bhattacharya and Rajam 2006; Groppa and Benavides 2008). Gene

expression studies have also been pointed out that the expression of some of the PA biosynthesis genes increases in response to stress, although with different levels, suggesting a differential regulation of PA pathway genes during stress, consistent with different pathways involved in the regulation of PA biosynthesis (Alcázar et al. 2010).

Modulation of PA metabolism has also been achieved by heterologous or homologous overexpression of *ODC*, *ADC*, *SAMDC*, *SPDS*, and *SPMS* in tobacco (Kumria and Rajam 2002; Waie and Rajam 2003), eggplant (Prabhavathi and Rajam 2007), tomato (Hazarika and Rajam 2011), pear, sweet potato, *Arabidopsis*, and rice (Pujni et al. 2007) (a detailed list could be found in Marco et al. 2012). Although the degree of modification of one or more specific PAs obtained is not the same in all plants, all transgenic lines share a common enhancement of the tolerance against one or more abiotic stresses. Indeed, the improvement in tolerance observed always correlates with elevated levels of Put and/or Spd and Spm (Kumar et al. 2006; Marco et al. 2012). Thus, the elevation of PA levels in the plant as a response to stress seems to have a protective role. However, the question about what are the precise mechanisms of action by which PAs are able to exert this protection against challenging environmental conditions remains still unresolved, although some roles have been put forward (Alcázar et al. 2010; Gill and Tuteja 2010). Several protective roles proposed have been related to their chemical structure. As polycations, PAs are able to modulate ion balance of the cell and interact with anionic molecules such as DNA, RNA, proteins, and membrane lipids (Feuerstein and Marton 1989; Schuber 1989). In this sense, PAs could act as chaperone molecules, which bind to those macromolecules not only to protect them from degradation but also to help to maintain the most stable conformation under stress conditions. On the other hand, the dual anion- and cation-binding properties of PAs confer them radical-scavenging properties (Bors et al. 1989) and open the possibility that PAs could play an antioxidative role in the cell. Additionally, PA catabolism produces H_2O_2, which could act as a signaling molecule and trigger the activation of the antioxidative defense response of the plant. However, the fact that this peroxide production could also be a source of oxidant species makes the PA antioxidant role still a matter of controversy (Groppa and Benavides 2008). More recently, gene expression studies have pointed out that modification of endogenous PA levels alters the expression of some important genes, most of them stress related. Therefore, other mechanisms by which PAs can perform their protective action against stress could be through the activation at transcriptional level of plant defense mechanisms, which in turn would render stress tolerance. In this regard, some evidences point out that some of the changes in gene expression observed could be a direct or indirect consequence of cross talking between PAs with other signaling pathways, including ABA, JA, gibberellic acid (GA), Ca^{2+}, and NO, among others (Alcázar et al. 2010; Marco et al. 2012).

29.2.5 Sugars and Sugar Alcohols

Mannitol is a sugar alcohol that accumulates upon salt and water stress in plants and appears to function in osmotic stress tolerance by serving as a compatible solute or osmoprotectant (Bohnert and Jensen 1996). Among plants, celery (*Apium graveolens* L.) is the major mannitol producer, as well as a salt-tolerant plant. Indeed, mannitol biosynthesis in celery is increased by salt stress (Everard et al. 1994). The main transgenic approach to obtain plants with elevated mannitol levels has been by the introduction of the bacterial mannitol-1P dehydrogenase (encoded by *mtlD* gene in *E. coli*) in several plant systems. Mannitol accumulation has been observed when *mtlD* gene is overexpressed in tobacco (Tarczynski et al. 1992; Karakas et al. 1997), *Arabidopsis* (Thomas et al. 1995), eggplant (Prabhavathi et al. 2002), poplar (Hu et al. 2005), wheat (Abebe et al. 2003), and rice (Pujni et al. 2007). The simultaneous expression of two genes, mtlD and glucitol-6-phosphate dehydrogenase, in transgenic loblolly pine has conferred

enhanced tolerance to salt stress (Tang et al. 2005). Interestingly, marker-free transgenic tomatoes were also developed for tolerance to multiple abiotic stresses like salinity, drought, and heavy metal by engineering mannitol accumulation (Gupta and Rajam 2013). An alternative successful approach has been the transformation of *Arabidopsis* plants with the celery gene for mannose-6P reductase (M6PR) that usually converts mannose-6P to mannitol-1P as part of the path to mannitol biosynthesis (Zhifang and Loescher 2003). In all cases, transgenic lines showed an increase in mannitol levels and an improved tolerance to water and/or salinity stress. However, another common observation on those transgenic plants is that the amount of accumulated mannitol is too small to account for its effect as an osmolyte, and some authors suggest that this protective effect could be exerted through its ROS quencher properties (Abebe et al. 2003; Shen et al. 1997). More recently, transcriptome analysis of *Arabidopsis* overexpressing celery M6PR gene indicates that mannitol-enhanced stress tolerance is due at least in part to increased expression of a variety of stress-inducible genes, including members of the ROS-detoxifying system (Chan et al. 2011).

Trehalose is a nonreducing disaccharide consisting of two units of glucose bound by an α-α-(1→1) linkage. It is present in diverse organisms including algae, fungi, bacteria, lower and higher plants, as well as insects and invertebrates (Elbein et al. 2003). In some organisms, like bacteria and yeast, this sugar accumulates in response to osmotic stress, heat stress, and drought stress, playing a protective role (Iordachescu and Imai 2008). Trehalose is also present in high concentrations in anhydrobiotic organisms (Drennan et al. 1993). Those organisms, among which are those called "resurrection plants", are able of surviving the loss of most of their water content until a quiescent stage is achieved and upon watering rapidly revive and are restored to their former state. In those plant species, trehalose accumulation helps to survive complete desiccation by stabilizing dehydrated proteins, lipid membranes, and biological structures. While in resurrection plants trehalose constitutes the main soluble sugar, the levels of this sugar in other higher plants are very low to consider that could exert the same full-scale protective role, and it has been suggested that in most plant species sucrose has taken over the role of trehalose as a preservative molecule during desiccation (Goddijn and van Dun 1999). Besides, since *in vitro* studies have demonstrated that trehalose can stabilize dehydrated biological structures such as lipid membranes or enzymes, more effectively than other sugars, in recent years there has been a growing interest in trehalose metabolism as a means of engineering stress tolerance in higher plants, including crops. Higher plants, as well as various eukaryotic and prokaryotic organisms, synthesize trehalose from UDP glucose by a two-step pathway catalyzed by trehalose phosphate synthase (TPS) and trehalose phosphate phosphatase (TPP). On the other hand, trehalose degradation is performed by the enzyme trehalase (TreH), forming two glucose residues. Different groups have attempted to create stress-tolerant plants by introducing TPS genes in tobacco (Almeida et al. 2005), potato (Stiller et al. 2008), tomato, *Arabidopsis*, and rice (Redillas et al. 2012) (a detailed list could be found in Iordachescu and Imai 2008). In most cases, an improvement in abiotic stress tolerance, mainly drought, has been reported. However, the levels of trehalose accumulated in these transgenic plants are generally low (compared to those measured in resurrection plants) and do not correlate well with the level of tolerance, suggesting that trehalose in plant stress response may have a function other than direct protective roles (Iordachescu and Imai 2008). Some possible roles have been proposed, like ROS scavenger, signaling molecule, an antiapoptotic role, or acting as compatible solute but localized in specific organs, cells, or organelles (Fernandez et al. 2010).

29.2.6 Enzymes for Membrane Lipid Biosynthesis

Membranes are critical sites of injury by chilling, freezing, and heat and osmotic stresses. Hence, transgenic approaches have also been attempted

to engineer cell membranes with an improved stress tolerance. Adaptation of living cells to low temperatures involves alterations in the membrane lipid composition, for example, by decreasing membrane fluidity through increase of their unsaturated fatty acid percentages (Nishida and Murata 1996). Therefore, several researchers have tried to modulate the level of membrane lipid unsaturation by transgenic approaches. Plastidial *cis*-unsaturated phosphatidylglycerol levels have been increased by overexpression of chloroplastic glycerol-3-phosphate acyltransferase (GPAT) in tobacco (Murata et al. 1992), rice (Yokoi et al. 1998; Ariizumi et al. 2002), and tomato (Sui et al. 2007), leading to plants with an improved photosynthesis tolerance to cold. On the other hand, modulation of unsaturated membrane lipids have also been achieved by overexpression, co-suppression, or mutation of fatty acid desaturase genes, leading to changes in cold or heat tolerance of several plants (Matsuda and Iba 2005; Upchurch 2008).

29.2.7 Antioxidant Enzymes

Although plant cells use reactive oxygen species (ROS) as a part of their signal transduction pathways (Miller et al. 2010, 2011), its accumulation above a certain threshold causes oxidative damage to cell components and leads to cellular impairment (Gill and Tuteja 2010; Suzuki et al. 2012). To protect against oxidative stress, plant cells have developed an extensive ROS-scavenging network, which involves nonenzymatic antioxidants, including vitamin C, vitamin E, glutathione, carotenoids, and flavonoids, and also numerous enzymatic mechanisms such as multiple ascorbate peroxidases (APX), dehydroascorbate reductase (DHAR), catalases (CAT), superoxide dismutases (SOD), glutathione peroxidases (GPX), glutathione S-transferases (GST), alternative oxidases, and peroxiredoxins (Halliwell 2006). Since the majority of abiotic stresses induce the accumulation of active ROS, which in turn cause oxidative stress (Apel and Hirt 2004; Laloi et al. 2004), one of the classical approaches explored toward obtaining increased stress tolerance has relied on boosting cell enzymatic and nonenzymatic ROS-scavenging systems (Gill and Tuteja 2010; Miller et al. 2010).

In this sense, an improved stress tolerance has been observed in several transgenic plants that overexpress several antioxidant enzymes such as GPX, SOD, APX, and GST (John et al. 2010). SOD enzymes catalyze the conversion of anion superoxide O_2^- to peroxide H_2O_2 and are present in the plant cell as two isoforms, one found in the chloroplast (Cu/ZnSOD), whereas a Mn-containing enzyme is located in the mitochondria (MnSOD). Tobacco plants transformed with pea Cu/ZnSOD under the control of a constitutive promoter show an increased tolerance to chilling (Gupta et al. 1993). Also, overexpression of *Avicennia marina* Cu/ZnSOD enhances salt and drought tolerance of transgenic rice plants (Prashanth et al. 2008). Drought tolerance has also been observed in pepper plants overexpressing a pea Cu/ZnSOD (Chatzidimitriadou et al. 2009). Similarly, overexpression of MnSOD enhances salt and cold tolerance in *Arabidopsis* (Wang et al. 2004) as well as improves drought tolerance in rice (Wang et al. 2005), alfalfa (McKersie et al. 1996), *Brassica napus* (Gusta et al. 2009), and potato (Waterer et al. 2010).

H_2O_2 generated by SOD or by other metabolic processes could be detoxified by the action of APX/DHAR/GST enzymes in the glutathione-ascorbate cycle, which decomposes peroxide to water, by the action of GPX, leading to the production of oxidized glutathione, or by a dismutation reaction catalyzed by CAT. Induction of the genes that code for APX isoenzymes in response to salinity and drought has been reported in several plant species, and overexpression of APX genes has led to stress tolerance in tobacco and *Arabidopsis* (Badawi et al. 2004; Kavitha et al. 2008; Lu et al. 2007). Also, transgenic *Arabidopsis* plants overexpressing a rice DHAR are resistant to salt stress (Ushimaru et al. 2006). On the other hand, tobacco seedlings overexpressing a cDNA encoding an enzyme with both GST and GPX activities show a better growth when control seedlings are exposed to chilling and salt stress (Roxas et al. 1997). Also, overexpression of a GST in *Arabidopsis* improves seed germination and seedling salt tolerance (Qi et al.

2010). Moreover, heterologous overexpression of *E. coli* catalase *KatE* gene leads to improved salt tolerance in rice (Moriwaki et al. 2008) and tobacco (Al-Taweel et al. 2007), while transgenic rice overexpressing a wheat catalase cDNA displays increased capability to maintain low levels of peroxide and enhanced tolerance to low-temperature stress (Matsumura et al. 2002).

Simultaneous overexpression of more than one ROS-detoxifying enzyme has also been reported in some studies. Transgenic rice plants constitutively co-expressing glutathione S-transferase (GST) and CAT genes show enhanced tolerance to salinity and oxidative stresses at the vegetative stage (Zhao and Zhang 2006). Moreover, tobacco plants that simultaneously express CuZnSOD, APX, and DHAR in their chloroplasts are more tolerant to salt and oxidative stress as compared to those transgenic lines expressing single- or double-gene transfer (Lee et al. 2007).

Manipulation of ROS levels and improvement of stress tolerance have also been approached by using other proteins related to antioxidant mechanisms. Aldehyde dehydrogenase (ALDH) catalyzes the oxidation of toxic aldehydes, which accumulate as a result of side reactions of ROS with lipids and proteins (Kirch et al. 2004). Overexpression of the aldehyde dehydrogenase AtALDH3 gene in *Arabidopsis* conferred tolerance to dehydration, salt, and heavy metals, suggesting that ALDH can maintain membrane integrity under osmotic stress (Sunkar et al. 2003). On the other hand, it has also been shown that ectopic expression of the cotton stress-responsive metallothionein MT3a gene, involved in the regulation of metal homeostasis and oxidative stress, increases tolerance to salt, drought, and low temperature in tobacco (Xue et al. 2009).

29.2.8 Protective Proteins: LEAs, HSPs, and Other Chaperones

LEA proteins constitute a diverse group of families of very hydrophilic proteins whose expression was first described during seed maturation (Galau et al. 1986). Their intracellular accumulation is tightly correlated with acquisition of desiccation tolerance in seeds of anhydrobiotic plants (Bartels 2005) as well as in response to other abiotic stress conditions such as drought and cold (Battaglia et al. 2008). LEA-type proteins are encoded by *RD* (responsive to dehydration), *ERD* (early responsive to dehydration), *KIN* (cold inducible), *COR* (cold regulated), and *RAB* (responsive to ABA) genes in different plant species (Shinozaki and Yamaguchi-Shinozaki 2000; Zhu 2002). Although their actual mode of function is unclear, it has been proposed that LEAs could perform their protective role due to their highly hydrophilic nature that could give them the capacity of sequestering ions that are concentrated during cellular dehydration, as well as performing a chaperone-like function by retaining water molecules and thus preventing protein aggregation and protecting cellular components from inactivation and denaturation during the water deficit situation common to high-salt, drought, and freezing conditions (Bartels and Sunkar 2005; Goyal et al. 2005; Vinocur and Altman 2005).

Overexpression of some *LEA* genes has been correlated with desiccation and salt tolerance in several cases. Ectopic expression of the group 3 LEA protein HVA1 from barley confers tolerance to drought and salt stress in rice (Rohila et al. 2002; Xu et al. 1996) and improves biomass productivity and water use efficiency in transgenic wheat under water deficit conditions (Bahieldin et al. 2005; Sivamani et al. 2000) as well as leads to an enhancement of drought and salinity tolerance in mulberry (Checker et al. 2012). Also, overexpression of *Brassica napus* gene *MEleaN4*, which encodes another group 3 LEA protein, in transgenic Chinese cabbage plants enhances their growth ability under salt and drought stress conditions (Park et al. 2005). In addition, homologous overexpression of *OsLEA3-1* gene improves transgenic rice yield under drought stress (Xiao et al. 2007). On the other hand, it has been reported in some cases that overexpression of dehydrins, a subfamily of group 2 LEA proteins, confers tolerance to freezing (Battaglia et al. 2008). Homologous overexpression of *Arabidopsis* dehydrin COR15a

enhances the freezing tolerance of chloroplasts and protoplasts (Steponkus et al. 1998). Overexpression of other dehydrins from *Arabidopsis*, such as RAB18, COR47, LTI29, and LTI30, also leads to transgenic plants with an improved freezing tolerance (Puhakainen et al. 2004). Further, tobacco plants overexpressing citrus dehydrin CuCOR19 show earlier germination and better seedling growth under low temperature than control plants (Hara et al. 2003), and leaves from transgenic strawberry plants overexpressing a wheat dehydrin WCOR410 show an improved freezing tolerance (Houde et al. 2004). More recently, it has been reported that overexpression of JcLEA, a group 5 LEA protein identified in *Jatropha curcas*, confers an increased resistance to both drought and salt stresses in *Arabidopsis* transgenic plants (Liang et al. 2013).

The production of heat shock proteins (HSPs) is one of the most prominent responses of virtually all cells under conditions of high temperature. Five families of HSP have been described (Hsp100/ClpB, Hsp90/HtpG, Hsp70/DnaK, Hsp60/GroEL, and small HSP), and they are thought to act as molecular chaperones. Many plant HSPs are induced by heat stress, and their role in heat tolerance has been shown in transgenic or mutant plants (Kotak et al. 2007). HSPs are also induced by water stress in several plants (Bartels and Sunkar 2005). It has been described in some cases that HSP overexpression enhances heat tolerance in plants. For instance, the cultured cells of carrot overexpressing *HSP17.7* gene perform with better growth rate than control cultures at 37 °C (Malik et al. 1999). Also, the constitutive expression of *HSP101* gene in *Arabidopsis* helps seedlings tolerate sudden shifts to extreme temperatures better than the untransformed control plants (Queitsch et al. 2000), and its overexpression in rice also enhances the heat tolerance of the transgenic lines (Katiyar-Agarwal et al. 2003). In other cases, this protective effect is extended to other stress conditions. In this sense, *Arabidopsis* plants overexpressing small heat shock protein show enhanced tolerance to drought and salinity (Sun et al. 2001). Also, overexpression of *sHSP17.7* in rice leads to transgenic plants with an enhanced drought and osmotic stress tolerance (Sato and Yokoya 2008). More recently, it has been demonstrated that constitutive expression of a cotton HSP, GHSP26, enhances drought tolerance in transgenic cotton plants (Maqbool et al. 2010).

Other reports have pointed out that stress tolerance could be achieved by overexpressing other proteins with chaperone activity. The expression of two bacterial RNA chaperones, *E. coli* CspA and *B. subtilis* CspB, resulted in enhanced tolerance to abiotic stress, by maintaining growth, photosynthesis, and development in rice, maize, and *Arabidopsis* (Castiglioni et al. 2008). More recently, heat tolerance has been obtained in *Arabidopsis* by overexpression of NADPH-thioredoxin reductase, type C (NTRC), a component of a high-molecular-weight complex with redox-dependent holdase-chaperone activity (Chae et al. 2013). On the other hand, tobacco plants overexpressing calnexin, a Ca^{2+}-dependent molecular chaperone, exhibit better germination under osmotic stress and survival under dehydration stress conditions (Sarwat and Naqvi 2013).

29.2.9 Transporters

One of the main strategies for achieving greater tolerance to abiotic stress is to improve the capacity of plants to reestablish both ionic and osmotic homeostasis that are altered under stressful environments, like high-salt environments. Under saline conditions, Na^+ and Cl^- are the predominant toxic ions for cell metabolism affecting plant growth and development, and the maintenance of a high cytosolic K^+/Na^+ ratio is essential for plant salt tolerance (Glenn et al. 1999). Na^+ transporters are involved in reestablishing ionic homeostasis after salt stress, preventing Na^+ accumulation in cells either by increasing Na^+ storage in the vacuole or by improving their excretion out of the root (Tuteja 2007). Manipulation of tonoplast Na^+ transport in order to improve salt tolerance has been one of the genetic engineering approaches with greatest success. *Arabidopsis* vacuolar Na^+/H^+ antiporter AtNHX1 has been overexpressed in *Arabidopsis*

(Apse et al. 1999), as well as in tomato (Zhang and Blumwald 2001), *Brassica* (Zhang et al. 2001), cotton (He et al. 2005), wheat (Xue et al. 2004), beet (*Beta vulgaris*) (Yang et al. 2005), and tall fescue (*Festuca arundinacea*) (Zhao et al. 2007), with a common result of increased salt tolerance. Likewise, overexpression of the rice ortholog, *OsNHX1*, in rice (Fukuda et al. 2004) and maize (Chen et al. 2007) showed improved salt stress tolerance. Similar results were observed when cotton *GhNHX1* gene is overexpressed in tobacco (Wu et al. 2004). NHKs from halophytes *Atriplex gmelini* AgNHX1 (Ohta et al. 2002) and *Suaeda salsa* SsNHX1 (Zhao et al. 2006a) also improved salt tolerance when overexpressed in transgenic rice. Other members of the NHX family from *Arabidopsis* have also been tested, like AtNHX3, whose overexpression in sugar beet confers an increased resistance to high salinity (Liu et al. 2008), as well as AtNHX5, which enhances salt and drought tolerance in rice (Li et al. 2011c) and paper mulberry (*Broussonetia papyrifera*) (Li et al. 2011b).

On the other hand, the plasma membrane Na^+/H^+ antiporter salt overly sensitive 1 (SOS1) is involved in Na^+ extrusion and long-distance Na^+ transport from root to shoot (Shi et al. 2002) and improves salt tolerance of transgenic *Arabidopsis* when overexpressed (Shi et al. 2003). Also, heterologous overexpression of plasma membrane Na^+/H^+ antiporter SOD2 from yeast (*Schizosaccharomyces pombe*) in transgenic rice increases salt tolerance (Zhao et al. 2006b).

Previously, another transporter from yeast, which facilitates intracellular K^+ accumulation and decrease of intracellular Na, HAL1, was overexpressed in tomato, leading to plants with increased fruit yield and enhanced K/Na^+ selectivity in leaves (Rus et al. 2001).

Vacuolar proton pumps, which generate a proton electrochemical gradient across tonoplast that is used by other vacuolar co-transporters, have been another point where transgenic strategies have been attempted to improve the plant capacity to withstand against osmotic disturbances. In this sense, transgenic plants expressing higher levels of the vacuolar proton-pumping pyrophosphatase (H^+-PPase), AVP1, have an increased capacity to retain more solutes and water and are more resistant to salt and drought than are wild-type plants (Gaxiola et al. 2001). AVP1 has also been overexpressed in cotton, leading to similar results (Pasapula et al. 2011). On the other hand, other reports have used H^+-PPase *TsVP* from halophyte *Thellungiella halophila*. Overexpression of *TsVP* in tobacco leads to transgenic plants that accumulate more solutes than wild-type plants and are more resistant to saline conditions (Gao et al. 2006). *TsVP* overexpression also increases cell solute content and improves their resistance to saline stress in cotton (Lv et al. 2008). Cotton *TsVP*-overexpressing plants are also resistant to drought (Lv et al. 2009), and similar results were also obtained in maize (Li et al. 2008).

Also, co-overexpression strategies of vacuolar transporters have been tried. The overexpression of the *Suaeda salsa SsNHX1* and *Arabidopsis AVP1* in rice transgenic plants exhibited an enhanced salt tolerance as compared to plants overexpressing *SsNHX1* alone (Zhao et al. 2006a).

Plants exposed to saline environments not only face Na^+ toxicity, but also water loss caused by osmotic stress. Thus, the ability to maintain water content under stress conditions is critical for plant survival. Aquaporins are intrinsic membrane proteins implicated in water diffusion and regulated in response to environmental cues (Li et al. 2014). Overexpression of aquaporins has resulted in contrasting results, with improvement of tolerance to salt and drought in *Arabidopsis* when aquaporins PgTIP1 from *Panax ginseng* (Peng et al. 2007), TaNIP from wheat (Gao et al. 2010), cotton *GhPIP2;7* (Zhang et al. 2013), and OsPIP1 (OsPIP1-1) or OsPIP2 (OsPIP2-2) from rice (Guo et al. 2006) are overexpressed. Tolerance to salt is also observed in tobacco plants overexpressing *rice TaAQP8* (Hu et al. 2012), as well as a better performance in water deficit conditions in the case of rice plants overexpressing aquaporin RWC3 (Lian et al. 2004). However it has been reported in other cases that overexpression of aquaporins increased plant susceptibility to stress, like the drought-sensitive tobacco plants overexpressing

Arabidopsis PIP1b (Aharon et al. 2003) or the salt-sensitive rice plants overexpressing wheat HvPIP2 (Katsuhara et al. 2003). Also, *Arabidopsis* plants that overexpress *Glycine soja* GsTIP2 plants show an increased water loss under drought stress conditions (Wang et al. 2011). Since negative effects on stress resistance were rather seen when an aquaporin of interest was overexpressed in a heterologous plant species, it has been speculated that in those cases the foreign aquaporin may not be properly recognized by the endogenous stress response machinery (Li et al. 2014).

29.2.10 Overproduction of Regulatory Proteins

The mechanisms of response and adaptation of plants to stress generally involve a combination of processes, which must occur by temporally and spatially appropriate patterns. To do that, the activities of the proteins that ultimately perform these processes are modulated by signaling cascades that ensure those patterns. Therefore, another transgenic strategy for obtaining stress-resistant plants has been the modulation of the expression of genes encoding the regulatory proteins that are part of those signal transduction pathways, in order to mimic or even enhance the plant adaptation mechanisms.

29.2.11 Kinases

Protein kinases are involved in various stages of plant development and adaptation. Among them, MAPK plays an essential role in the signal transduction pathways that link cell stimulus perception with cell response. Indeed, MAPKs constitute one of the most studied signaling mechanisms in plants. The MAPK cascades are generally organized as modular pathways in which the activation of upstream MAPKKKs leads to the sequential phosphorylation and subsequent activation of downstream MAPKKs and MAPKs. Through their kinase activity, MAPK cascades are an integral part of the mechanisms that translate incoming environmental cues into posttranslational modifications of target proteins, triggering a myriad of transcriptomic, cellular, and physiological responses in the plant. In this sense, MAPK pathways are known to be activated by diverse abiotic stresses such as cold, salt, heat, drought, wounding, UV irradiation, osmotic shock, ozone, or heavy-metal intoxication (Šamajová et al. 2013).

Modulation of MAPK cascades by transgenic modification of the expression and/or activity of some of its members has led to abiotic stress tolerance in some cases. Tobacco MAPKKK NPK1 confers resistance to multiple environmental stress conditions when an active version of this MAPKKK is constitutively expressed in this plant (Kovtun et al. 2000). Moreover, the constitutive low-level of expression of this active tobacco NPK1 in maize, increases its tolerance to freezing (Shou et al. 2004a) and drought (Shou et al. 2004b). More recently, it has been reported that *Arabidopsis* plants overexpressing MAP3Kδ4 exhibit enhanced tolerance to salt stress (Shitamichi et al. 2013). In addition, the overexpression of another rice MAPKKK from rice DSM1 increases the tolerance of transgenic rice seedlings to dehydration (Ning et al. 2010).

Regarding *MAPKK* genes, *Arabidopsis* plants overexpressing AtMKK2 exhibit constitutive AtMPK4 and AtMPK6 activity, as well as a constitutively upregulated expression of stress-induced genes, and show an increased tolerance to salt and cold (Teige et al. 2004). Also in *Arabidopsis*, AtMKK1 overexpression enhances drought resistance (Xing et al. 2008), and plants overexpressing AtMKK3 exhibit an enhanced tolerance to salt and an increased ABA sensitivity (Hwa and Yang 2008). In addition, maize MAPKK ortholog ZmMKK1 confers chilling resistance when overexpressed in transgenic tobacco (Cai et al. 2014), while overexpression of cotton GhMKK1 increases salt and drought stress tolerance of *Nicotiana benthamiana* plants (Lu et al. 2013). On the other hand, tolerance to salt and cold stress is obtained in *Arabidopsis* plants by overexpressing maize ZmMKK4 (Kong et al. 2011). Overexpression of an active OsMKK6 enhances tolerance to salt stress in rice (Kumar and Sinha 2013).

Overexpression of *MAPK* genes has also lead to stress tolerance in other cases. Thus, ZmMPK7 enhances protection against ROS-mediated injury during osmotic stress when it is overexpressed in transgenic tobacco (Zong et al. 2009), while *Arabidopsis* plants constitutively overexpressing ZmSIMK1 have an improved resistance against salt stress (Gu et al. 2010). Moreover, homologous overexpression of OsMAPK5 leads to transgenic plants with increased multiple-stress tolerance (Xiong and Yang 2003).

CPKs are Ca^{2+} sensors involved in Ca^{2+}-mediated signal transduction in plant cells and play vital regulatory roles in plant development and stress responses (Boudsocq and Laurière 2005; Boudsocq and Sheen 2013; Valmonte et al. 2014). Overexpression of CPK genes *AtCPK4* and *AtCPK11* renders *Arabidopsis* plants with an enhanced ABA-induced stomata closure and an improved capacity to retain water that makes them more tolerant to drought stress (Zhu et al. 2007b). Transgenic plants with similar phenotype have also been obtained by heterologous overexpression of maize genes *ZmCPK4* (An et al. 2008) and *ZmCPK12* (Wang and Song 2013). On the other hand, *Arabidopsis* plants overexpressing AtCPK6 have an increased survival rate to salt and drought stress (Xu et al. 2010). As an example of a different approach, disruption of *AtCKP23* gene expression by T-DNA insertion increased *Arabidopsis* tolerance to drought and osmotic stresses, suggesting its role as a negative regulator in plant signaling cascades involved in the response to those stresses (Ma and Wu 2007). CPK modulation by transgenic approaches has also been described in rice, where overexpression of *OsCDPK7* confers both cold and salt/drought tolerance (Saijo et al. 2000), whereas plants overexpressing *OsCDPK21* (Asano et al. 2011) and *OsCDPK12* (Asano et al. 2012) show an enhanced salt tolerance. Also, it has been described that *OsCDPK13* overexpression leads to rice plants with improved recovery rates after cold stress (Abbasi et al. 2004).

Ca^{2+}-mediated signal transduction pathways could also be mediated by CBLs (Boudsocq and Laurière 2005). Binding of Ca^{2+} to CBLs changes their conformation, making them able to interact with a specific group of protein kinases designed as CIPKs (Kim et al. 2000). CBL-CIPK signaling pathways have been thoroughly studied in the salt stress response of *Arabidopsis*. CBL SOS3 and CIPK SOS2 are part of the SOS signaling pathway, which leads to sodium ion homeostasis in response to salt stress. The CBL-CIPK complex SOS2/SOS3 activates the Na^{+}/H^{+} antiporter SOS1 by phosphorylation (see section 29.2.9) (Qiu et al. 2002). *Arabidopsis* plants overexpressing single genes *SOS1*, *SOS2*, and *SOS3* or co-expressing them have been generated (Yang et al. 2009). The transgenic plants overexpressing *SOS3* or *SOS1* did exhibit a clear increase in salt tolerance compared to the control plants. However, overexpression of *SOS2+SOS3* or *SOS1+SOS2+SOS3* did not result in an improvement of plant tolerance as compared to transgenic plants overexpressing only *SOS3* or *SOS1*.

Regarding other CBLs, it has been described that overexpression of *AtCBL1* triggers an increase of the expression of drought and salt stress-related genes, which is correlated with an enhanced tolerance to both these stresses, whereas T-DNA knockout mutants display the opposite phenotype (Albrecht et al. 2003; Cheong et al. 2003). Similar results were obtained in *Arabidopsis* plants overexpressing *AtCBL5* (Cheong et al. 2010). Heterologous overexpression of CBLs in *Arabidopsis* has also been reported. In this regard, maize ZmCBL4 overexpression enhances the salt tolerance of *Arabidopsis* at the germination and seedling stages (Wang et al. 2007). Also, *Arabidopsis* plants overexpressing poplar CBLs PeCBL6 or PeCBL10 show enhanced tolerance to high salinity, drought, and low temperature (Li et al. 2013).

With regard to other CIPKs, transgenic rice plants overexpressing *OsCIPK03*, *OsCIPK12*, and *OsCIPK15* genes display a significantly improved tolerance to cold, drought, and salt stress, respectively (Xiang et al. 2007). Also, chickpea CaCIPK6 overexpression increases salt tolerance and root development in tobacco (Tripathi et al. 2009). Overexpression of another CIPK isolated in apple *MdCIPK6L* also confers tolerance to salt, drought, and chilling stress in transgenic *Arabidopsis*, tomato, and apple (Wang et al. 2012). More recently, it has been reported that the identification of wheat

TaCIPK29, a new member of *CIPK* gene family, and its overexpression in tobacco transgenic plants displayed higher germination rates, longer root lengths, and better growth status than wild-type plants under salt stress conditions (Deng et al. 2013).

Another family of kinases induced by hyperosmotic and saline stresses in *Arabidopsis* and rice is the SNF1-related protein kinase 2 (SnRK2) family (Boudsocq and Laurière 2005). Two cases of transgenic overexpression of SnRK2 genes have been described. Overexpression of *SRK2C* gene in *Arabidopsis* enhances drought tolerance significantly in concert with the upregulation of stress-responsive gene expression (Umezawa et al. 2004). In addition, rice SnRK2 gene *SAPK4* has also been overexpressed in rice, which results in an improvement of germination and growth rates under salt stress (Diedhiou et al. 2008).

29.2.12 Transcription Factors

Transcription factors (TFs) are essential members of the cell signal transduction pathways that transduce the information carried previously by upstream signals (second messengers, kinases) to changes in gene expression. The importance of TFs as regulatory proteins in plants is evidenced by the fact that they represent an important part of their genomes, as in the case of *Arabidopsis*, which has 1,500 TFs in its genome (Ratcliffe and Riechmann 2002). Moreover, TF gene families have experienced higher expansion rates during evolution of plants, suggesting their main role in the adaptation of plants to various environments (Shiu et al. 2005). Those features have made TFs to be considered as good target candidates for the generation of stress-tolerant plants.

Each single TF can control the expression of many target downstream genes in parallel through the specific union of the TF to cis-acting elements in the promoter regions of their respective target genes, forming a transcription unit called "regulon" (Nakashima and Yamaguchi-Shinozaki 2006). Several regulons have been described in the gene response of plants to abiotic stresses. The study of the expression mechanisms of osmotic stress- and cold stress-responsive genes in *Arabidopsis* has allowed to identify multiple regulons. Among them, the dehydration-responsive element-binding protein/C-repeat (CRT)-binding factor (DREB1/CBF) regulon is involved in both osmotic stress- and cold stress-responsive gene expression, while other regulons, including DREB2, AREB/ABF, MYB/MYC, and NAC, are involved in osmotic stress-responsive gene expression (Nakashima and Yamaguchi-Shinozaki 2006).

DREB1/CBF and DREB2 are TF members of the AP2/ERF family that bind to the cis-element DRE/CRT. DREB1/CBF refers to a set of three TFs (DREB1A, DREB1B, and DREBIC) highly similar in amino acid sequences and whose genes are located in tandem within the *Arabidopsis* genome (Nakashima and Yamaguchi-Shinozaki 2006). Cold stress immediately induces the *DREB1/CBF* genes, whose products activate the expression of multiple stress-inducible target genes. Dehydration induces *DREB2* genes, leading to the expression of various genes that are involved in drought stress tolerance (Liu et al. 1998; Nakashima et al. 2000). Therefore, these TFs have been used as preferred targets to generate transgenic plants with resistance to cold and drought stress. Thus, transgenic *Arabidopsis* lines overexpressing DREB1A, DREB1B, and DREB1C under control of constitutive promoters exhibit remarkably enhanced salt, drought, and cold tolerance (Kasuga et al. 1999; Liu et al. 1998). *Arabidopsis* DREB1/CBF genes have also been found in a variety of plant species and have been shown to enhance abiotic stress tolerance in other plants either by heterologous or homologous overexpression (an extensive list of examples could be found in Hussain et al. 2011 and Agarwal et al. 2013). The DREB1/CBF genes apparently produce abiotic stress tolerance by upregulating a suite of native stress-responsive genes whose products produce physiological adaptations that enable the plant cells to cope with osmotic stress, but their ectopic expression usually results in dwarf phenotypes (Agarwal et al. 2013; Nakashima and Yamaguchi-Shinozaki 2005). To overcome this problem, stress-inducible promoters with low background expression under normal growth conditions have been used to drive expression of the *DREB1/CBF*

genes to achieve increased stress tolerance without growth retardation. This approach has succeed by using stress-inducible *RD29A* promoter and *DREB1A* in *Arabidopsis* (Kasuga et al. 1999), tobacco (Kasuga et al. 2004), wheat (Pellegrineschi et al. 2004), and peanut (Bhatnagar-Mathur et al. 2007). Genes coding for DREB2 homologues have also been isolated from several plant species. However, in contrast to the *DREB1*/*CBF* genes, overexpression of *DREB2* in *Arabidopsis* does not improve stress tolerance, suggesting that DREB2 proteins are tightly regulated by upstream mechanisms, including posttranscriptional modifications (Liu et al. 1998). DREB2A possess a negative regulatory domain that could be eliminated by deletion, generating a constitutive active form (DREB2A-CA). Transgenic *Arabidopsis* plants overexpressing DREB2A-CA showed growth retardation and improved tolerance to drought and heat stress (Sakuma et al. 2006a, b). Similar results are obtained when maize *ZmDREB2A* (Qin et al. 2007) and rice *OsDREB2B* (Matsukura et al. 2010) are overexpressed in the same plant.

The basic leucine zipper TFs ABRE-binding protein/ABRE-binding factor (AREB/ABF) bind to cis-element ABRE and activate ABA-dependent gene expression (Nakashima and Yamaguchi-Shinozaki 2006). *Arabidopsis AREB1*/*ABF2* (*AREB1*) and *AREB2*/*ABF4* (*AREB2*) are induced by drought, NaCl, and ABA treatment and are activated by an ABA-dependent phosphorylation process (Uno et al. 2000). *Arabidopsis* plants overexpressing AREB2 show an enhanced sensitivity to ABA, as well as reduced transpiration rate, and enhanced tolerance to drought (Kang et al. 2002). On the other hand, AREB1 is required for normal glucose response in *Arabidopsis*, and its overexpression affects stress tolerance to salt, drought, and heat (Fujita et al. 2005; Kim et al. 2004). AREF/ABF rice homologues TRAB1 and its close relative OsbZIP23 have also been described (Hobo et al. 1999; Xiang et al. 2008). Overexpression of OsbZIP23 renders transgenic plants with ABA hypersensitivity and increased tolerance to drought and salt (Xiang et al. 2008).

AtMYB and AtMYC have been described as TFs that recognize distinct cis-elements present in the promoter of the drought-inducible gene *RD22* and activate it in a cooperative way (Abe et al. 1997). Since both TFs are synthetized after accumulation of ABA levels, it has been proposed that they act in a late stage of the stress responses. Transgenic plants overexpressing AtMYC2 and/or AtMYB2 cDNAs have higher sensitivity to ABA and an improved osmotic stress tolerance (Abe et al. 2003). Analysis of the transcriptome of those transgenic plants has allowed the identification of other target genes for AtMYB and AtMYC, including other ABA- and JA-inducible genes.

The group of proteins NAM, ATAF, and CUC (NAC) were first identified as a group of TFs with roles in plant development. Several members of the NAC family have also been shown to be involved in abiotic stress responses in *Arabidopsis*, regulating both ABA-dependent and ABA-independent genes (Fujita et al. 2004; Tran et al. 2004). Three NAC TFs have been isolated by its binding ability to the promoter of the early responsive to dehydration 1 (*ERD1*) gene. Transgenic plants overexpressing those NAC cDNAs show an upregulation of several drought-inducible genes, as well as an increased drought tolerance, although *ERD1* is not induced in those plants (Tran et al. 2004). Another NAC from rice, OsNAC6, is induced by ABA and abiotic stresses such as high salinity, cold, and drought (Nakashima et al. 2007) and when overexpressed under the control of both constitutive and stress-inducible promoters yields transgenic rice plants with an improved drought and salinity tolerance (Nakashima et al. 2007). Water-stress tolerance has also been observed in transgenic rice plants overexpressing other stress-inducible NAC genes, *SNAC1* (Hu et al. 2006) and *SNAC2* (Hu et al. 2008). In addition, overexpression of another rice NAC gene, *ONAC045*, increases tolerance of the rice plants to drought and salt without negative effects on growth (Zheng et al. 2009).

Other transcription factors that do not belong to those regulons have also been used successfully to obtain plants with an abiotic stress resistance. Additional examples could be found in the reviews of Agarwal et al. (2013) and Hussain et al. (2011).

29.2.13 Other Levels of Regulation

As described in section 29.1.2, recent findings have pointed out that gene expression in response to stress could be fine-tuned by posttranscriptional and posttranslational mechanisms. Some authors have reported successful approaches where components of those regulatory systems have been targeted to develop transgenic plants with stress tolerance.

29.2.14 miRNAs

miRNAs are the most abundantly expressed and well-studied class of the noncoding regulatory small RNAs (21–24 nucleotides) that are accumulated and produced in plants. miRNAs regulate gene expression in plants by causing degradation or inhibition of the translation of their complementary mRNA targets (Sunkar 2010). In plants, it has been pointed that miRNAs have diverse biological functions, playing roles in the regulation of optimal growth and development as well as in abiotic and biotic stress responses (de Lima et al. 2012; Khraiwesh et al. 2012; Sunkar et al. 2012). Interestingly, TF genes are the major targets for several stress-regulated miRNAs, making them good candidates for new transgenic approaches focused to alter stress response. In this sense, tomato plants overexpressing *Sly-miR169* are more tolerant to drought stress (Zhang et al. 2011a, b). Also, overexpression of *Osa-miR319* yields rice plants resistant to cold (Yang et al. 2013), as well as transgenic creeping bentgrass tolerant to salt and drought (Zhou et al. 2013). The details on the role of miRNAs in abiotic stress tolerance have been covered in Chap. 36 of this book.

29.2.15 Ubiquitination

Defective proteins and proteins which are no longer required for cell are tagged by covalent union of chains formed by multiple units of the small protein ubiquitin. Ubiquitin attachment is mediated by the sequential action of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes, being E3 the enzyme which recognizes the target proteins and ensures ubiquitination specificity. A number of studies have pointed out the relevance of the ubiquitin-dependent protein degradation in stress-related signaling and response mechanisms (Zhang and Xie 2007; Zhu et al. 2007a). In fact, transcriptome and proteome studies from several plant species indicate that hundreds of ubiquitination-related transcripts/proteins are modified after exposure to abiotic stresses, suggesting a role for ubiquitination in stress responses (Mazzucotelli et al. 2008).

Particularly, many components of the ubiquitination pathway have been identified or predicted to be essential in ABA homeostasis and signaling (Lee and Kim 2011), and some examples of upregulation of E3 ubiquitin ligases have led to transgenic plants with stress tolerance through ABA-related mechanisms. XERICO, an H2-type zinc-finger E3 ubiquitin ligase, controls the level of ABA by enhancing the transcription of the key ABA biosynthesis gene *AtNCED*3. Thus, upregulation of the *XERICO* gene in *Arabidopsis* enhances ABA levels and results in improved drought tolerance due to an increased stomatal closure (Ko et al. 2006). Moreover, TF ATHB6 is a negative regulator of ABA response, whose degradation is promoted by MATH-BTB, a component of an E3 ubiquitin ligase complex. Overexpression of MATH-BTB leads to *Arabidopsis* plants with an increased drought tolerance with minimal phenotypic alterations (Lechner et al. 2011). Also, *Arabidopsis* E3 ligase SDIR1 is induced by drought and salt stress, and it has been identified as a positive regulator in ABA response. SDIR1 overexpression in *Arabidopsis* confers an enhanced ABA-induced stomatal closure, as well as a drought tolerance phenotype, which is also observed when this *Arabidopsis* gene is overexpressed in tobacco and rice (Zhang et al. 2008). On the other hand, AtAIRP1 and RHA2b have also been identified as other *Arabidopsis* E3 ubiquitin ligases that act as a positive regulator in ABA-mediated drought responses, and its respective overexpression leads to transgenic plants with an improved resistance to drought stress (Li et al. 2011a; Ryu et al. 2010).

Regarding other drought-response mechanisms, it has been suggested that hot pepper E3 ligase Rma1H1 and its *Arabidopsis* homologue Rma1 target *Arabidopsis* aquaporin PIP2;1, inhibiting its traffic from the ER to the plasma membrane, and play a positive role in drought tolerance mechanisms. In fact, *Rma1H1* overexpression in *Arabidopsis* increases tolerance to drought stress (Lee et al. 2009).

Ubiquitination mechanisms have also been targeted to improve cold stress tolerance. ICE1 is a TF involved in the expression of cold-inducible regulon CBF/DREB1. Degradation of ICE1 under cold is mediated by ubiquitin E3 ligase HOS1, leading to the attenuation of plant cold response. The overexpression of a point-mutant version of ICE1 resistant to ubiquitination leads to *Arabidopsis* transgenic plants with high levels of this TF and an improved cold tolerance (Miura et al. 2011).

Apart from E3 ligases, it has been reported that constitutive overexpression of peanut E2 enzyme AhUBC2 in *Arabidopsis* upregulates the expression of ABA-independent stress-responsive genes, increases the synthesis of Pro, and renders plants with improved drought tolerance (Wan et al. 2011). Similar results were observed in *Arabidopsis* plants overexpressing soybean E2 gene *GmUBC2* (Zhou et al. 2010). Additionally, drought tolerance has also been observed in tobacco plants overexpressing wheat polyubiquitin gene *Ta-Ub2* (Guo et al. 2008).

29.3 Limitations and Future Prospects of Transgenic Strategies

As seen in the previous section, many genes associated with plant response(s) to abiotic stresses have been identified and used for generating stress-tolerant plants, thereby demonstrating the potential of transgenic strategies. However, it has also been noted by some authors that these strategies are not exempt from downsides.

A majority of the overexpression strategies have been based on the use of constitutive and strong promoters, such as cauliflower mosaic virus 35S (*CaMV35S*) (Odell et al. 1985), ubiquitin 1 (*UBI1*) (Holtorf et al. 1995), and actin (McElroy et al. 1990) promoters, which often render plants, though resistant to stress, to show undesired pleiotropic effects like growth retardation and reduced fruit/seed number and fresh weight under normal conditions (Wang et al. 2003; Cabello et al. 2014). For strategies that involve manipulation of signaling pathways, cross talk with other regulatory pathways (such as development) increases the probability of appearance of pleiotropic effects. In other cases, undesired effects could come from toxic levels of the overproduced protein or metabolite. Furthermore, although these pleiotropic effects may be absent, constitutive activation of stress response pathways imposes on the plant metabolic and energetic costs that have been compensated by diverting the plant from developmental programs, thus resulting in yield penalties and consequent limited benefit for agricultural crops. In addition, the use of strong constitutive promoters could accelerate the process of RNA silencing of the transgene (Dietz-Pfeilstetter 2010). The most common alternative proposed in literature to solve this problem is to use stress-inducible promoters that ensure activation of the desired gene only when needed. In this regard, some of the examples cited above have successfully used promoters of stress-inducible genes such as the *Arabidopsis RD29A* promoter, or barley *HAV22* promoter, for obtaining transgenic plants with desired stress tolerance and without the undesired effects in control conditions. However, widespread use of this strategy requires isolation, study, and development of a more extensive catalog of stress-induced promoters with a better knowledge of their characteristics, such as threshold stress level under which the promoter can be activated, levels of expression, reversibility, and dose-dependence characteristics.

Furthermore, although a majority of these transgenic lines have served as useful tools for gaining knowledge in the mechanism of plant response to stress, and this has even been applied in plants of agronomic interest such as crops,

there are still few reports of transgenic lines that maintain tolerance to stress in field trials. With the exception of drought-resistant maize line MON87460, developed by Monsanto Company (Monsanto 2012), there have not been any more reports of release to the market of stress-tolerant genetically engineered varieties. Some explanations for this limited success have been given. Most of the studies have been conducted under laboratory growth conditions focusing on short-term harsh, single stress conditions where stress tolerance/resistance is estimated as the survival rate (which is easier to screen). Moreover, in most studies, stress tolerance is tested at the vegetative stages (seedlings) only. A majority of the crops, in contrast, are exposed to heterogeneous conditions during their life cycle in the field, which include mild episodes of combined stress conditions (Mittler 2006; Mittler and Blumwald 2010), and are particularly susceptible to stress at their reproductive stage (during flowering and seed/fruit/grain maturation), which constitutes the most critical stage determining crop yield. Some authors have pointed out the need for standardized protocols to assay stress tolerance and to phenotype plants which mimic better the situations that plants have to face in the field (Bhatnagar-Mathur et al. 2008; Cabello et al. 2014). On the other hand, since the plants in the field face stresses of diverse nature simultaneously, additional efforts in the study of cross talk between different plant stress response pathways should be addressed. In this sense, application of new approaches as systems biology (Cramer et al. 2011) could help gain better knowledge of plant response mechanisms to stress as well as lead to isolation of novel targets for transgenic modification to enable development of plants without growth penalties under normal conditions and which are able to maintain high productivity under stress.

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Genetic Engineering Strategies for Biotic Stress Tolerance in Plants

30

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Abstract

Crop plants are exposed to a plethora of biotic and abiotic stresses. Biotic stresses such as pathogens (viruses, bacteria and fungi), insect pests, nematode parasites and weeds cause a significant loss of crop yield and quality. Although conventional strategies like breeding for resistant varieties and agrochemicals and biocontrol agents for control of diseases and pests have been in use for a long time, these have been met with limited success. During the last 10 years, technological advancements in genetic engineering have led to the development of transgenic crop varieties resistant to various biotic stresses. A large number of transgenic crops have been developed and more are underway; however, the number of biotech crops reaching the field from labs is still limited. Transgenic crops developed against insect resistance and/or herbicide tolerance have been commercial success stories, an example being Bt cotton.

Keywords

Transgenic crops • Biotic stress • Disease resistance • Coat protein • Pathogen-related proteins • Insect resistance • Bt crops • Nematode resistance • Herbicide tolerance • RNAi

30.1 Introduction

In the present-day scenario, food availability, quality and safety are given top priority, keeping in view the changing climate which will, in the near future, reduce availability of fertile land for agriculture (Easterling et al. 2000) besides directly affecting agricultural yield. Crop yield losses due to biotic stresses like pest infestation, weed overtake and disease occurrence together with abiotic factors like drought, salinity and

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extreme temperature diminish considerably food production economics in many parts of the world. With climate change predicted to alter conditions in various parts of the world, researchers started developing models to forecast adverse effects of abiotic and biotic factors on crop productivity. In a study by Collier et al. (2008), it was deduced that changes in life cycle of a pest with increased number of generations or with increase in number of survivors of a particular stage of instar could be a consequence of the changing climatic conditions in the future. Such changes are also predicted for plant pathogens (Lonsdale and Gibbs 2002; Turner 2008). Apart from this, the tremendous increase in human population is bound to elevate food demand in terms of both quality and quantity. In fact, it has been suggested that food production needs to be doubled by 2050 with available arable land to ensure food security for future generations (Baulcombe 2010; Datta 2013). Further, it was estimated that in wheat alone, production needs to increase by 2 % annually (Singh and Trethowan 2007).

Enhancement of food production can be achieved by various strategies: (1) increasing the land area under cultivation, (2) developing improved varieties by traditional breeding methods, (3) adopting better agricultural techniques and tools and (4) adopting transgenic approaches. The first approach is neither feasible nor sustainable, as we can no longer convert scarce forest areas into arable lands. Although conventional breeding has contributed its share, it has limitations such as non-precise gene transfer, transfer of unwanted genes, time-consuming and laborious (Rajam et al. 1998; Bhatnagar-Mathur et al. 2008; Hazarika and Rajam 2011). At the same time, increasing use of land for purposes other than agriculture (like housing, industrial building, forest conservation and so on) also needs to be considered (Evans 2009). In the present context, an effective and challenging task is to maximise land use efficiency by increasing crop productivity per unit area. Therefore, better tools and techniques can only compliment conventional agricultural productivity by use of other novel approaches.

In this regard, transgenic technology has gained relevance for crop improvement and has added a new thrust to agriculture. Better understanding of biotic and abiotic factors involved in crop improvement, and their manipulation for better results, is developing as a potential strategy. Area under cultivation of genetically modified (GM) crops is increasing year by year (James 2013). Although this is a positive sign for increasing acceptance of GM crops in the commercial market (with a notion that the productivity of these crop varieties can deal with the demand for quantity and quality of food) (Chakraborty et al. 2000; Datta 2012), concerns regarding the effects of these GM products on human health do persist (Dona and Arvanitoyannis 2009). It is to be noted here that in both conventional breeding techniques and transgenic crops, change in the genetic make-up of the plant may be involved. However, in the production of GM varieties via the transgenic technology, genetic changes that are brought about are small and defined, whereas in conventional breeding, these changes can be relatively large and may involve a set of ill-defined, uncharacterised genes (Datta 2013). In this chapter, we have given a general overview of biotic factors and transgenic approaches being used for alleviating these stresses for improving crop health and productivity.

30.2 Types of Biotic Stress

Plant species are under a constant threat from a number of biotic stresses that exist simultaneously in nature. Attack by pathogens and parasites such as bacteria, fungi, viruses and nematodes plays a crucial role in plant growth and productivity. Apart from these, insect herbivory and theft of nutrients by weeds also cause serious economic losses in crop yield. These stresses very often coexist, leading to multiple stresses which might elevate the level of biotic stress incident on crop plants.

30.3 Conventional Strategies to Alleviate Biotic Stresses

To protect plants against biotic stresses, various prevention and control strategies have been tested over the ages. Conventional breeding practices for the generation of disease-resistant lines against diverse biotic stresses in plants have been widely employed over several decades now. Besides conventional agricultural practices, farmers depend on chemical pesticides and herbicides to a great extent which leads to significant increase in crop yields. However, the ever-increasing environmental and health concerns against use of chemicals in agriculture paved the way for the development of biological control agents (BCAs). Biological control measures include use of BCAs, i.e. use of antagonists that suppress plant diseases; use of predatory insects, entomopathogenic fungi, bacteria and nematodes for control of insect pests; and use of weed-specific pathogens for control of populations of specific weeds. Formulated natural plant products are also used in integrated pest management (IPM) strategies.

30.4 Why the Need for an Alternative?

Although BCAs and resistant lines of crop plants are successfully used for some plant disease and insect pest control, emergence of resistance-breaking biotypes is rapid in pace. Conventional plant breeding techniques are laborious and time-consuming. To keep pace with the emergence of resistant biotypes of pests, breeders need to look for new resistant traits that are more durable. However, keeping the time required for developing a new cultivar in view, it becomes difficult to rely wholly on such a strategy. Advancements in the field of genetic engineering and molecular biology over the past two decades have led to the use of transgenic technology in crop improvement.

30.5 Transgenic Approaches to Biotic Stress Tolerance in Plants

The high potential of genetic engineering in developing disease-resistant transgenic crop plants has been successfully tapped as a strategy alternative to conventional breeding. Over the years, success stories have thrown up transgenic plants resistant to various plant pathogens and insect pests, and these engineered crops have moved to the field (James 2013) instead of remaining as just laboratory curiosities. Thus, transgenic crop plants engineered for resistance against bacteria, fungi (Wally and Punja 2010), virus (Prins et al. 2008), pathogens and nematodes (Atkinson et al. 2003; Tamilarasan and Rajam 2013) have become a reality, so also resistance against insect herbivory (Christou et al. 2006; Gatehouse 2008) and for dealing with the unwanted weeds (Herouet-Guicheney et al. 2009).

30.5.1 Viruses

Plant viruses cause significant losses in crop yield and productivity on a global scale. The most devastating infectious plant diseases are caused by viruses (Anderson et al. 2004) belonging to the *Tospovirus* group, causing crop losses of more than a billion dollar a year (Prins and Goldbach 1998). In view of the limited effectiveness of control mechanisms like the use of virus-free seeds, prevention by phytosanitation, breeding for resistant lines or prevention of vector transmission (Mawassi and Gera 2012), the transgenic approach has been used. Introduction of natural virus-resistant genes into an existing, desirable cultivar can be achieved using genetic engineering. Pathogen-derived resistance (PDR) is the most commonly used phenomenon. Here, transgenic plants expressing genes of a viral pathogen show resistance against the same or related viruses (Slater et al. 2003). The first

application of PDR phenomenon with respect to plant viruses was demonstrated by Powell-Abel et al. (1986) who showed that transgenic tobacco plants expressing tobacco mosaic virus coat protein gene were resistant to attack by the tobacco mosaic virus. Examples of pathogen-derived genes include the replicase protein (Rubino and Russo 1995), coat protein (Lindbo and Dougherty 1992; Nelson et al. 1993; Ganesan et al. 2009), proteinase (Vardi et al. 1993), movement protein (Cooper et al. 1995) or genes coding for host cell lethal proteins like antiviral compounds (ribosome-inhibiting proteins) (Wang et al. 1998) or those that target the viral genome or particle directly, viz., ribozymes and ribonucleases (Sano et al. 1997). Resistance against viruses can also be obtained by presetting the RNA silencing system in plants by introducing and expressing fragments of sense and antisense viral nucleic acid sequences into chromosomes of the plants (Prins et al. 2008; Tyagi et al. 2008; Duan et al. 2012), or by expressing the virus mini-replicon (Brumin et al. 2009), or by expression of artificial microRNA (Niu et al. 2006). Management of papaya ringspot virus (PRSV) is one of the remarkable success stories (Gonsalves et al. 2004).

30.5.2 Bacterial and Fungal Pathogens

Research over the years has led to the use of various strategies for the development of transgenic plants resistant to bacterial and fungal pathogenic attack (Salomon and Sessa 2012), the first among these being expression of resistance genes (R-genes). R-genes code for nucleotide-binding peptides harbouring leucine-rich repeats (LRRs) which are transmembrane in nature (Bent and Mackey 2007). These peptides recognise products of avirulence (*Avr*) genes produced by the pathogen and subsequently induce activation of diverse downstream processes that include activation of pathogenesis-related genes (PR genes), production of high amounts of inhibitory molecules and their accumulation and induction of the hypersensitive response (Kiraly et al. 2007). Introduction of R-gene *Rxo1* from maize into rice led to the latter's resistance to bacterial streak disease caused by *Xanthomonas oryzae* pv. *oryzicola* (Zhao et al. 2005a), while expression of R-gene (RPI-BLB2) from wild potato conferred resistance to the cultivated potato against attack by *Phytophthora infestans* (van der Vossen et al. 2005).

Many fungal pathogens produce toxins called mycotoxins in general. *Fusarium culmorum* and *F. graminearum* produce deoxynivalenol, a trichothecene mycotoxin, which confers virulence to these fungal strains and also poses toxicity threat to humans and animals (Kimura et al. 2006). Looking into the way in which the fungus deals with the mycotoxin, researchers developed transgenic lines of wheat, rice and barley. These genetically modified (GM) plants overexpressed the trichothecene 3-O-acetyltransferase (*tri101*) gene, whose product is involved in acetylating deoxynivalenol which results in a much less active form of the toxin (Manoharan et al. 2006). The transgenic wheat line showed reduced wheat spike infection (Okubara et al. 2002), and transgenic rice (Ohsato et al. 2007) and barley (Manoharan et al. 2006) yielded grains with far less mycotoxin contamination than in the wild type.

Use of antimicrobial peptides and PR-proteins in engineering plants for resistance against pathogens is the most common strategy. These proteins are expressed constitutively in plants; however, their levels shoot up upon pathogen attack (Rao et al. 1999; Wally and Punja 2010). Antimicrobial metabolites thus produced are involved in degrading fungal components like the cell wall, cell membrane or RNA, or these induce secondary metabolite production or increase physical barriers in a plant cell (Ferreira et al. 2007). A wide range of such peptides have been overexpressed in plants for conferring resistance against different plant pathogens, to name a few, chitinases, β1-3 glucanases, defensins, thionins, thaumatin-like proteins (Rajam et al. 2007), lipid-transfer proteins and phenylalanine ammonia lyase (reviewed by Wally and Punja 2010). Recent reports show that introduction of alfalfa glucanase into eggplant led to increased resistance

against pathogenic fungi like *Verticillium dahlia* and *Fusarium oxysporum* (Singh et al. 2014).

Interestingly, polyamines like diamine putrescine, triamine spermidine and tetra-amine spermine have been implicated in abiotic (Rajam et al. 1998) and biotic (Hazarika and Rajam 2011) stress tolerance (Hussain et al. 2011) besides their involvement in the regulation of a wide variety of biological processes including cell division, growth and development, stabilisation of DNA and gene regulation (Igarashi and Kashiwagi 2010). Although considerable amount of work has been done on genetic manipulation of polyamine biosynthesis genes for abiotic stress tolerance in various plants (Marco et al. 2012), engineering polyamine biosynthesis for biotic stress tolerance has not been explored much so far, as evidenced by scarce literature on the subject. For instance, enhanced fungal resistance was reported by overexpressing the polyamine biosynthesis gene, S-adenosylmethionine decarboxylase, in tobacco (Waie and Rajam 2003) and tomato (Hazarika and Rajam 2011) and arginine decarboxylase in eggplant (Prabhavathi and Rajam 2007).

RNA interference (RNAi) strategy involving plant-mediated silencing of fungal genes shows promise for developing potential tools in the fight against fungal attack on crop plants in the near future. Fungal uptake of synthetic siRNA specific to the ornithine decarboxylase (*ODC*) gene led to downregulation of *ODC* gene and in turn caused significant decrease in mycelial growth and sporulation in the fungal pathogen, *Aspergillus nidulans* (Khatri and Rajam 2007). Transgenic tobacco plants that expressed antisense RNA of heterologous (mouse) ornithine decarboxylase gene were demonstrated to be more resistant to *Verticillium* wilt compared to the wild type plants (Rajam 2012a). Also, development of powdery mildew causing fungus in wheat and barley, *Blumeria graminis*, was affected adversely by *in planta* expression of antisense transcripts and dsRNA specific to the fungus (Nowara et al. 2010). These initial steps might pave the way for the development of a promising tool for control of fungal plant pathogens (Rajam 2012b). However, success of transgenic crops showing resistance to bacterial and fungal pathogens is limited in comparison to insect-resistant transgenic crops (Punja 2006).

30.5.3 Insect Pests

Success story of the transgenic plants for insect resistance started with transforming plants with *cry* genes from *Bacillus thuringiensis* (Vaeck et al. 1987). These genes code for highly insecticidal crystal proteins called Bt toxins. Upon ingestion, the toxin binds to specific receptors in the insect midgut epithelium, and further action of the proteases activates the toxin. This active toxin induces lytic pore formation in the midgut epithelial cells, leading to their lysis, and subsequently kills the insect larva (Daniel et al. 2000). Over 400 genes coding for these toxin variants have been identified from different strains of *B. thuringiensis* (Crickmore et al. 2007). Insecticidal spectrum of each of these toxin variants is distinct. Since the first transgenic tobacco and tomato plants were developed carrying *cry* genes (Vaeck et al. 1987), tissue-specific or constitutive expression of Bt toxins has been established in many crop plants against specific pest species (Pierpoint and Hughes 1996). Bt cotton is one among them and is a commercial success. Apart from Bt toxin, *B. thuringiensis* also expresses other proteins like vegetative insecticidal protein (Vip) which is, obviously, insecticidal in nature. Ingestion of Vip proteins leads to the swelling and osmotic lysis of midgut epithelial cells, causing death of the target insect. Unlike *cry* toxins, these are expressed both during vegetative growth and sporulation of the bacterium (Estruch et al. 1996; Crickmore et al. 2007). Like Bt, different Vip toxins act against specific groups of insects. Although Vip3 showed very high insecticidal activity against major lepidopteran pests attacking maize and cotton (Estruch et al. 1996; Fang et al. 2007), licence granted for release of GM Vip cotton is limited and controlled (Crothers 2006). Other entomopathogenic bacteria like *Photorhabdus luminescens* also express insecticidal toxin complexes whose genes have been introduced into plants

(Liu et al. 2003). Transgenic *Arabidopsis thaliana* plants expressing *tcdA* gene encoding for toxin A showed resistance against tobacco hornworm and southern corn rootworm.

Apart from genes from microbes, genes from the insect itself are used for killing the pest. One such gene is chitinase, which is a development-associated gene in insects and is expressed during larval moulting, i.e. shedding of the peritrophic membrane and exoskeleton. Insect pests feeding on transgenic plants constitutively expressing insect chitinase are exposed to a constant level of this enzyme all through their development cycle, which disturbs their process of regular moulting. Ding et al. (1998) showed that transgenic tobacco plants expressing chitinase conferred resistance to lepidopteran pests.

Plant transformation with plant genes encoding for insecticidal secondary metabolites like proteinase inhibitors, alpha-amylase and lectins has also been shown to confer resistance to insect attack. In insects, proteinases are among the digestive enzymes essential for catalytic release of amino acids from the ingested proteins which, thereby, contribute to the insect's growth and development. Hence, ingestion of proteinase inhibitors could prove lethal to insects. Proteinase inhibitors are produced in high amounts and are accumulated by plants in response to herbivorous attack or mechanical injury (Ryan 1990). Although plants expressing different proteinase inhibitors, viz., BTI-CMe (barley trypsin inhibitor) and MTI-2 (mustard trypsin inhibitor), showed significant insect resistance (Altpeter et al. 1999; De Leo et al. 2001), genes for proteinase inhibitors like cowpea trypsin inhibitor (CpTI) have been used in combination with Bt toxins to enhance protection of the transgenic crop against insects (Gatehouse 2011).

Expression of protein inhibitors of proteases (Wolfson and Murdock 1987; Thomas et al. 1995) in plants had a significant effect on reproductive output of target insect pests. Exploiting the neuropeptides and inhibitors of polyamine biosynthesis with potential insecticidal activity might prove beneficial for developing insect resistance in plants (Rajam 1991; Raina et al. 1994; Kumar et al. 2009).

With ever-increasing knowledge in the field of RNAi, RNA-mediated (sense or antisense RNA, dsRNA, artificial microRNA) strategies are being employed to induce RNAi in transgenic plants as an effective defence mechanism against insect attack (Mao et al. 2007; Baum et al. 2007; Terenius et al. 2011). One of the major achievements is the use of this strategy to control sap-sucking insects like aphids. Recent success with the use of plant-generated dsRNA and other small RNA-mediated approaches, for conferring resistance against aphids, is remarkable (Pitino et al. 2011; Guo et al. 2014).

30.5.4 Nematode Parasites

Plant parasitic nematodes can cause crop yield losses up to 20 % in a single crop, and in most cases, these losses are caused by attack of root-knot nematodes and cyst nematodes (Koenning et al. 1999; Bird and Kaloshian 2003; Tamilarasan and Rajam 2013). Conventional strategies like crop rotation do not hold good for control of nematodes with a wide host range, and chemical practices are expensive (Abad et al. 2003). Alternate transgenic approaches and RNAi, which was in fact discovered in the nematode *Caenorhabditis elegans* (Fire et al. 1998), provide a better scope for control of plant parasitic nematodes. Some of the strategies used for developing transgenic plants with resistance to nematodes include anti-invasion and migration, anti-nematode feeding and development and feeding-cell attenuation strategies (Atkinson et al. 2003). Expression of cysteine proteinase inhibitor (cystatin) in transgenic plants helped potato plants resist nematode attack (Cowgill et al. 2002). The parasitism genes express parasitism proteins which help the nematode infect the root and parasitise the plants (Davis et al. 2004). Using RNAi approach, *Arabidopsis* plants were bioengineered to express dsRNA of one of the parasitism genes, *16D10*. These transgenic plants were resistant to four species of root-knot nematodes (Huang et al. 2006). Therefore, this strategy can be applied to agriculturally important crops for developing resistance against

nematodes. Similarly, the nematode genes involved in its development and reproduction and the nematode-responsive host plant genes have been targeted for developing effective methods of plant nematode control (for review, Tamilarasan and Rajam 2013).

30.5.5 Weeds

Herbicides are chemicals which are used to kill the weeds, but at the same time, they are deadly to the cultivated crop plants also. Hence, development of transgenic plants resistant to herbicides was taken up. One of the most commonly used herbicide, glyphosate, inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. Overexpression of this enzyme in transformed *Petunia* plants conferred resistance to this herbicide (Shah et al. 1986). Expression of an insensitive form of EPSP synthase also showed similar results (Herouet-Guicheney et al. 2009). Since then, this strategy has been used for several crop plants. Glyphosate-tolerant soybean was successfully commercialised in 1996, and later, GM crops like herbicide-tolerant maize, cotton, etc. were also accepted by the farmers (James 2013). Recent studies showed that transformation with two genes, glyphosate acetyl transferase *gat* and EPSPS *G2-aroA* genes, conferred high level of tolerance towards glyphosate in tobacco plants (Din et al. 2014). Further, stacking of genes for herbicide tolerance and insect resistance has also been reported in crops like cotton, and these are being commercialised (James 2013).

30.6 Concerns About Transgenic Plants and Novel Alternative Strategies

In spite of the various advantages of the use of transgenic technology, there are some limitations. One major concern, in general, about the release of transgenic plants into the natural environment is transgene flow. Gene flow is a natural process among related species (vertical gene flow) or related genera within a family (diagonal gene flow) or, even, among unrelated species (horizontal gene flow). However, transgenes that code for traits not found in related species, or even genera, would not integrate into these plants had there not been a transgenic. This unnatural event might pose a threat to the natural gene pool over time (Gressel 2012). The most common cases reported in this context are flow of herbicide-resistant genes from crops to weeds as reported in transgenic glufosinate-resistant rice (Song et al. 2011). The ability to control weeds in such cases, thus, becomes close to nil. Various techniques for transgene containment to prevent such transgene flow have been discussed recently (Gressel 2012). In the case of a leaky containment mechanism where such a transgene movement takes place, methods of transgene mitigation to suppress its spread and establishment in the population have also been highlighted (Gressel 1999, 2012).

Development of resistant pest populations is another shortcoming of transgenic crops expressing insecticidal genes. This has been well noted in Bt crops (Shelton et al. 2003; Moar et al. 2008). Although development of resistant target pest populations against Bt crops has been slower than against that of Bt sprays (Fox 2003; Gressel 2012), there are field reports of Bt-resistant pest populations, as observed in *Spodoptera frugiperda* and *Helicoverpa zea* (Moar et al. 2008; Tabashnik et al. 2008). To this problem, 'refugia' have worked as a solution. Planting regular rows of Bt and non-Bt crops allows survival of the susceptible pests. Bt-resistant trait is recessive, and as long as the susceptible mating population is within reach, selection of resistant genes in the population is expected to be very meagre (Bates et al. 2005). Pyramiding of two or more *cry* genes, or different insecticidal genes, is expected to delay eruption of resistant pest species, as the pest needs to then evolve resistance against two or more insecticidal toxins simultaneously (Zhao et al. 2005b; Watkins et al. 2012).

A more recent advancement in this field is the RNAi approach, which is an alternative to expression of transgenes and might prove to be advantageous over the latter, considering the

limitations and concerns of the transgenic approach. RNAi technology can be applied for control of those insect pests where effective Bt genes are not available, e.g. sucking pests like aphids and jassids. One other important point is its species specificity which reduces the risk of negative effects on nontarget organisms (Huesing et al. 2009). Unlike the existing transgenic crops which are based mainly on transgene protein, RNAi strategy works at the post-transcriptional level where there is no protein in the transgenic plant, and therefore, it is very unlikely that the target pathogen or pest would gain resistance in the absence of the transgene protein (Rajam 2011; Rajam and Singh 2011). A case in point is Bt crops and other transgenic plants where target pathogens or pests have been shown to be resistant after a few generations (Moar et al. 2008; Rajam and Singh 2011). Further, a major concern with transgenic plants is the possible allergic response in the consumers against the expressed transgene protein (Niblett and Bailey 2012) which, on the other hand, in RNAi plants, might not be the case due to absence of any transgene protein. This makes these RNAi plants and their products safe for human and animal consumption and greatly reduces the regulatory issues related with their commercialisation (Auer and Frederick 2009). The beauty of RNAi approach is that more than one gene can be effectively targeted using chimeric RNAi constructs (Gupta et al. 2013; Sinha and Rajam 2013) to the pathogen or pest by this technique. This would also avoid development of resistance by target organisms and would allow targeting of more than one pathogen or pest, as chimeric RNAi construct contains sequences of more than one target gene (Rajam 2011; Rajam and Singh 2011). However, RNAi approach has at least one limitation, i.e. the sequence(s) introduced can have off-target effects on the host plant or other organisms, depending upon the extent of homology between the introduced sequence and the sequence of nontarget genes (Du et al. 2005).

Details on the use of RNAi technology for crop improvement, including control of diseases and pests, are available in the following chapter.

30.7 Conclusions and Future Prospects

The most important point which needs consideration in the use of transgenic approach for alleviating biotic stress in plants is stability of the resistance in engineered plants. Although a large number of transgenic plants resistant to various biotic stresses are under development, only a few have become commercially successful. Examples of such commercial successes are Bt crops. One of the main features responsible for acceptance of Bt crops in the commercial market by consumers was the proof provided for Bt toxin as having no effect on birds and mammals, including humans (Goldberg and Tjaden 1990; Mendelsohn et al. 2003). For large-scale public acceptance of transgenic technologies, the transgenics need to be of proven safety for human and animal consumption; also, the economic balance, i.e. cost involved in developing, raising and registering the transgenic crops, should be balanced with profit margins of their producers, and finally, ease of their availability to the consumer must be ensured.

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RNAi for Crop Improvement

31

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Abstract

Scientific breakthroughs bring about significant advances in basic research, which ultimately lead to their applications for human welfare. One such discovery is RNA interference (RNAi), a highly conserved gene regulatory mechanism that controls the expression of genes at posttranscriptional level. Since the discovery of RNAi, it has become evident that RNAi has immense potential for crop improvement. The technology has been employed successfully to study the gene function and to alter the gene expression in plants for desired traits. RNAi has proved to be a novel and potential tool in combating abiotic and biotic stresses. Enriched nutritional quality, male sterility, delayed ripening, and secondary metabolite manipulation add to the list of successful applications of RNAi technology. This chapter focuses on the potential of RNAi technology to produce improved crop varieties.

Keywords

RNA interference • Gene silencing • Transgenic plants • Crop improvement

31.1 Introduction

The crop yield is facing drastic reduction because of scarcity of land, water resources, climatic changes, and damages caused by abiotic and biotic stresses. Increasing population is also posing the demand for global sustainable agricultural practices. To address this demand, conventional breeding is being employed from ages as one of the effective ways to raise quality plants, but this process is time consuming and laborious and also has other ecological, physiological, and biological constraints. However, genetic engineering is playing pivotal role in crop improvement. Novel traits can be effectively incorporated into crops through genetic manipulations, leading to increased yield, nutritional value, and also tolerance to biotic and abiotic

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stresses. But, transgenic crops always had ethical issues in terms of biosafety especially in case of edible crops. Hence, safer strategies have to be developed for crop improvement.

RNA interference (RNAi) technology is proven to be a potential alternative for crop improvement, probably with less biosafety issues as no transgene protein is expressed in transgenic lines (Rajam 2012). RNAi pathway mainly comprises small-interfering RNAs (siRNAs) and microRNAs (miRNAs). Both siRNAs and miRNAs are produced by the cleavage of double-stranded (dsRNA) precursors by a member of the RNase III family Dicer and Dicer-like enzyme, respectively (Bernstein et al. 2001; Hutvagner et al. 2001). Finally, the small noncoding RNAs (siRNAs and miRNAs) in association with RNA-induced silencing complex (RISC), Argonaute (AGO), and other effector proteins lead to gene silencing. By this means these small RNAs regulate various aspects of plant growth and development. This gave biotechnologists an opportunity to use this easy but highly effective way to modulate gene expression to get desired traits (Mittal et al. 2011). The present chapter therefore focuses on the discovery of RNAi and its mechanism of action and applications for crop improvement.

31.2 RNAi: Discovery and Mechanism of Action

It all began in 1990 when chalcone synthase gene (*CHS A*) encoding a key enzyme in anthocyanin biosynthesis pathway, introduced in *Petunia hybrida* L. for enhancing anthocyanin pigments, produced white or chimeric flowers instead of dark purple flowers in transgenic *Petunia*. This suggested that the introduced transgene was functionally inactive and also suppressed the endogenous gene expression and the phenomenon came to be known as "cosuppression" (Napoli et al. 1990; Hannon 2002; Campbell and Choy 2005). A similar phenomenon named "quelling" was then discovered in the fungus *Neurospora crassa* (Romano and Macino 1992; Cogoni et al. 1996). Later on, the term RNAi came into picture for the first time when it was elucidated in the nematode, *Caenorhabditis elegans* (Fire et al. 1998).

The process of RNAi in plants is initiated by 21- to 24-nucleotide-long (nt), small interfering RNAs (siRNAs), which are generated from long endogenous or exogenous dsRNA molecules through the cleavage by a ribonuclease III-type enzyme called Dicer (Hamilton and Baulcombe 1999; Zamore et al. 2000). These siRNAs (21–24 nt) are then incorporated into a multiprotein complex called RISC which contains AGO proteins (Baumberger and Baulcombe 2005; Vaucheret 2008). The ATP-activated RISC unwinds the double-stranded siRNA. The sense strand of the siRNA duplex is degraded by RNA helicase activity and the antisense strand of siRNA molecule is retained in the RISC complex (Kusaba 2004). RISC with antisense siRNA then targets the homologous mRNA by complementary base-pairing and cleaves the mRNA leading to inhibition of protein synthesis (Bartel 2004) (Fig. 31.1).

siRNAs also regulate the gene expression at transcriptional level by regulating the chromatin modification. siRNA recruits several DNA- and histone-modifying proteins including the cytosine methyltransferase CHROMOMETHYLASE3 (CMT3) and maintains the chromatin in a minimal transcriptional activity state, leading to transcriptional gene silencing (TGS) (Ossowski et al. 2008; Fagegaltier et al. 2009; Burkhart et al. 2011).

31.3 Applications of RNAi Technology in Agriculture

Increasing world population is facing threat due to huge crop losses because of several factors that affect the proper production and distribution of crops. There is an urgent need to address this problem which can be achieved by amalgamation of conventional and cutting edge technologies. RNAi technology has emerged as one of the most potential and promising strategies for producing improved quality plants. This biological phenomenon has been assessed in a number of plant systems and has been successfully used to silence the genes to get better traits. The examples listed

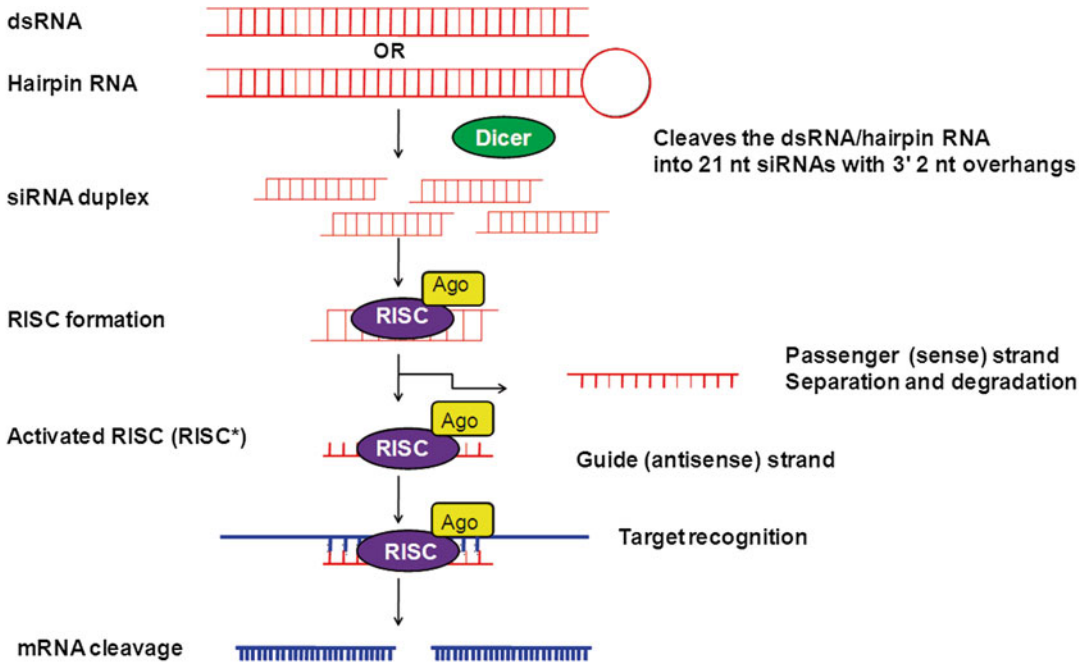

Fig. 31.1 Biogenesis and mechanism of action of siRNAs

below illustrate the possibilities of this intrinsic biological mechanism for commercial exploitation.

31.3.1 Biotic Stress Tolerance

Total agricultural production is greatly affected by biotic stresses, which include viral, bacterial, and fungal pathogens, insect pests, and nematode parasites. There is a need to address this problem more efficiently. We have discussed the importance of RNAi as a promising solution to biotic stress management.

31.3.1.1 Viral Diseases

Viruses cause major loss of plant productivity and transmit the disease either directly from parent to progeny or indirectly through insect vectors and hence their control becomes very difficult. "Pathogen-derived resistance" (PDR), where resistance to a determined pathogen could be obtained from its own genetic material, has been used to develop disease-resistant plants. This approach comprises (1) the expression of viral coat protein (CP) and replication-associated proteins (Reps) and (2) gene silencing by antisense and hpRNA (Shepherd et al. 2009).

RNAi has revealed a way for obtaining virus-resistant trait in many crop plants. It was first reported in potato, where simultaneous expression of both sense and antisense transcripts of the helper-component *proteinase* (*HC-Pro*) gene showed complete resistance to potato virus Y (PVY) (Waterhouse et al. 1998). Silencing of viral coat protein through RNAi is an effective method for generation of resistant plant that has been successfully reported in potato and medicinally important papaya plants (Missiou et al. 2004; Kertbundit et al. 2007).

An important class of viruses includes geminiviruses, the DNA viruses, which are responsible for a significant amount of crop damage. Expression of hairpin construct of noncoding intergenic region of *mungbean yellow mosaic India virus* (MYMIV) under the control of 35S promoter in MYMIV-infected black gram plants resulted in recovery from the infection which

lasted till senescence (Pooggin et al. 2003). Apart from that, when siRNAs designed to the replicase (Rep)-coding sequence of *African cassava mosaic virus* (ACMV) were cotransfected with protoplasts, it showed 99 % reduction of Rep transcripts and 66 % reduction of viral DNA (Vanitharani et al. 2003). In another report, transgenic cassava lines with high levels of AC1-homologous small RNAs showed resistance to ACMV (Vanderschuren et al. 2009).

RNAi-mediated resistance to cassava brown streak disease (CBSD) in Cassava was first demonstrated by Patil et al. (2011). They observed resistance against very distant isolates of causative organism cassava brown streak virus (CBSV) and cassava brown streak Uganda virus (CBSUV).

31.3.1.2 Bacterial Diseases

Although considered structurally simple, bacteria are extremely diverse from a metabolic standpoint and are found almost everywhere on earth in vast numbers—from those living in jet fuel and on the rims of volcanoes to those thriving in hydrothermal vents deep on the ocean floor. The first bacterial disease ever discovered was anthrax (caused by *Bacillus anthracis*) of cattle and sheep in 1876. The discovery of anthrax in cattle was immediately followed by the discovery of fire blight of pear and apple (caused by *Erwinia amylovora*) by T. J. Burrill from the University of Illinois (1877–1885). Escobar et al. (2001) showed the striking example of bacterial disease management where RNAi showed a remarkable type of gene regulation. They developed a crown gall disease management strategy that targets the process of tumorigenesis (gall formation) by initiating RNAi of the *iaaM* and *ipt* oncogenes. Expression of these genes is a prerequisite for wild-type tumor formation. RNAi constructs, targeting *iaaM* and *ipt* gene(s) in *Arabidopsis thaliana* and *Lycopersicon esculentum*, showed resistance to crown gall disease (Dunoyer et al. 2007).

Infection by *Pseudomonas syringae* pv. *tomato* in *Arabidopsis* induced the production of natsiRNA (nat-siRNAATGB2) which downregulates a PPRL gene that encodes a negative regulator of the RPS2 disease-resistance pathway. As a result, the induction of nat-siRNAATGB2 increases the RPS2-mediated race-specific resistance against *P. syringae* pv. *tomato* in *Arabidopsis* (Katiyar-Agarwal et al. 2007).

31.3.1.3 Fungal Diseases

Homology-based gene silencing induced by transgenes (cosuppression), antisense, or dsRNA has been successfully demonstrated in many plant pathogenic fungi, including *Cladosporium fulvum* (Hamada and Spanu 1998), *Magnaporthe oryzae* (Kadotani et al. 2003), *Venturia inaequalis* (Fitzgerald et al. 2004), *Neurospora crassa* (Goldoni et al. 2004), *Aspergillus nidulans* (Hammond and Keller 2005; Khatri and Rajam 2007), *Fusarium oxysporum* (Singh 2011), and *Fusarium graminearum* (Nakayashiki 2005). The efficient method for rapid characterization of fungal genes using diced siRNAs has been reported in model filamentous fungus *A. nidulans* (Natarajaswamy et al. 2013).

RNAi-mediated downregulation of GST (glutathione *S*-transferases) enzyme, which catalyzes a variety of reactions, resulted in significant increase in resistance in *Nicotiana tabacum* against *Phytophthora parasitica* var. *nicotianae* (Hernández et al. 2009). The transgenic tobacco plants expressing heterologous (mouse) ornithine decarboxylase (ODC) antisense RNA have been shown to have higher resistance to *Verticillium* wilt as compared to wild-type plants (Kumria 2000). The expression of dsRNA and antisense transcripts specific to powdery mildew fungi *Blumeria graminis* in wheat and barley affected the growth of the fungus (Nowara et al. 2010). Virus-induced gene silencing (VIGS) has been used to introduce the gene fragments from the rust fungi *Puccinia striiformis* f. sp. *tritici* or *P. graminis* f. sp. *tritici* to plant cells leading to reduced expression of the corresponding genes in the rust fungus (Yin et al. 2011).

In cereals, *barley stripe mosaic virus* (BSMV)-induced RNAi has emerged as a useful tool for loss of function studies. Three genes, a MAP kinase (PtMAPK1), a cyclophilin (PtCYC1), and calcineurin B (PtCNB), predicted to be involved in pathogenicity, have been targeted by

BSMV-mediated host-induced gene silencing (HIGS) in the wheat leaf rust fungus *Puccinia triticina* (Pt). BSMV RNAi constructs were then inoculated in the wheat plant leaves. Subsequent Pt inoculation resulted in a suppressed disease phenotype and reduced endogenous transcript levels of the targeted fungal genes indicating translocation of siRNA molecules from host to fungal cells (Panwar et al. 2013b). The same group has shown the use of *Agrobacterium tumefaciens*-mediated *in planta* induced transient gene silencing (PITGS) assay for use in *Triticum* spp. (wheat). *Agroinfiltration* effectively delivered hairpin silencing constructs of the three genes mentioned above in wheat, leading to the generation of fungal gene-specific siRNA molecules in infiltrated leaves and resulting in up to 70 % reduction in transcription of the endogenous target genes (Panwar et al. 2013a).

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense* (Foc), is among the most destructive diseases of banana (*Musa* spp.). RNAi-mediated knockdown of vital genes of fungus (*velvet* and *Fusarium transcription factor 1*) has shown effective resistance against Foc. Transformed banana lines were found to be free of external and internal symptoms of Foc after 6-week-long greenhouse bioassays. The five selected transgenic lines for each construct showed resistance to Foc for 8 months postinoculation (Ghag et al. 2014).

31.3.1.4 Insect Attack

Insects can cause considerable damage to crop plants. By defoliating plants or sucking out their sap, insects slow down their growth by weakening and sometimes killing them. The use of RNAi for the insect control seems to be the consequence of success of Cry toxins from Bt as an insecticide. The sensation of RNAi in insect control began when two exciting reports came into scientific community. Mao et al. (2007) targeted cotton bollworm gut-specific cytochrome P450 gene *CYP6AE14*, which confers resistance to gossypol, a polyphenol compound of cotton plants. Cotton bollworm larvae when fed on transgenic tobacco and *Arabidopsis* plants that expressed the *CYP6AE14*-specific dsRNA showed sensitivity to gossypol in artificial diets. Whereas, Baum et al. (2007) fed Western corn rootworm larvae (WCR, *Diabrotica virgifera*) 290 different dsRNAs and observed that 14 of them caused significant mortalities at doses $\leq$5.2 ng/cm^2. Transgenic corn expressing dsRNA specific to the gene encoding the A subunit of the V-type ATPase proton pump showed significant reduction in WCR-feeding damage. Both reports showed that RNAi pathway can be exploited to control insect pests via *in planta* expression of dsRNA against well-chosen target genes of insects (Baum et al. 2007; Mao et al. 2007). *v-ATPaseA* gene has also been shown to be a potent target to control the whitefly population. Plant-mediated pest resistance was achieved against whiteflies by genetic transformation of tobacco expressing siRNAs against the whitefly *v-ATPaseA* gene. The transcript level of *v-ATPaseA* in whiteflies was reduced up to 62 % after feeding on the transgenic plants, leading to their mortality (Thakur et al. 2014).

Thereafter, a number of reports suggested the success of this technology for the control of insect pests. Transgenic tobacco plants expressing dsRNA against *EcR-USP* (ecdysone receptor-ultraspiracle particle) (Zhu et al. 2012), *AChE* (acetylcholinesterase) (Kumar 2011), and *HR3* (Xiong et al. 2013) involved in regulation of molting and development in *H. armigera* resulted into resistant plants, with larvae fed upon them showing developmental deformities and lethality. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, *HMGR*) gene, a key enzyme in the mevalonate pathway in insects, has also been shown to be a potential target for insect control using RNAi (Wang et al. 2013).

Apart from its application in generating resistant plants, RNAi has been extensively used for functional studies in insects. The feeding of dsRNA and siRNA solutions for knockdown of target pest genes has been successfully shown in *Apolygus lucorum* (Zhou et al. 2014), *Nilaparvata lugens* (Chen et al. 2010), *Bemisia tabaci* (Upadhyay et al. 2011), and *Helicoverpa armigera* (Kumar et al. 2009) which led to a strong decline in the expression of the target gene and can be used to explore gene functions. Recently,

oral delivery of dsRNA molecules to *Spodoptera littoralis* against a gene highly similar to *P102* of *Heliothis virescens* strongly suppressed the encapsulation and melanization response, suggesting that the protein is functionally conserved and plays role in insect immunity (Lelio et al. 2014).

31.3.1.5 Nematode Attack

A number of genera and species of nematodes are highly damaging to a great range of hosts, including foliage plants, agronomic and vegetable crops, fruit and nut trees, turf grass, and forest trees. Some of the most damaging nematodes are root knot (*Meloidogyne* spp.), cyst (*Heterodera* and *Globodera* spp.), root lesion (*Pratylenchus* spp.), spiral (*Helicotylenchus* spp.), burrowing (*Radopholus similis*), bulb and stem (*Ditylenchus dipsaci*), reniform (*Rotylenchulus reniformis*), dagger (*Xiphinema* spp.), and bud and leaf (*Aphelenchoides* spp.) (Tamilarasan and Rajam 2013). Oral delivery of dsRNA was first demonstrated in *C. elegans* (Fire et al. 1998).

Plant-mediated RNAi in plant parasitic nematodes through dsRNA targeting has been shown in RKN which resulted in effective resistance (Yadav et al. 2006; Huang et al. 2006). The host-induced RNAi in *Arabidopsis thaliana*, which is a host for the sugar beet cyst nematode *Heterodera schachtii,* led to reduction in the number of mature nematode females. Although no complete resistance was observed, the reduction of developing females ranged from 23 to 64 % in different RNAi lines (Sindhu et al. 2009). Fewer females (reduced by 81–93 %) were observed on the transgenic roots obtained by hairy root cultures engineered to silence either two of ribosomal proteins, a spliceosomal protein or synaptobrevin, of *H. glycines* by RNAi (Klink et al. 2009). Similarly, high reduction in egg production was achieved by targeting mRNA splicing factor prp-17 or an uncharacterized gene cpn-1 (Li et al. 2010).

The success reports of RNAi for controlling the nematode infection also include the following research outcomes. Targeted silencing of conserved region of *M. incognita* gene acetylcholinesterase (*AChE*) involved in neurotransmission and also in many cellular processes, through host-derived RNAi, resulted in reduced fecundity. The enhanced resistance to nematode infection displayed by different lines strongly suggests their utilization in nematode control (Tamilarasan 2013). A recent study also demonstrated that *MiASB* (*M. incognita* mitochondrial ATP synthase b subunit) silencing had a positive effect on the control of root-knot nematodes, and the gene may be associated with the formation of galls caused by the nematode (Huang et al. 2013).

31.3.2 Abiotic Stress Tolerance

Plants are constantly affected by abiotic factors such as high salinity, flood, drought, heavy metal, and variable temperatures which considerably reduce the productivity. Functional genomics studies have come up with novel genes involved in stress adaptation in plants, which can be manipulated to get tolerance (Pardo 2010). Poly (ADP-ribose) polymerase (PARP) is induced by stress in animals and is responsible for energy depletion. The enzyme PARP1 and to a lesser extent PARP2 are primarily responsible for stress-induced poly (ADP-ribosyl)ation activity. Upon induction, polymers of ADP-ribose are synthesized by a range of nuclear enzymes using NAD^+ as substrate, while overproduction of PARP leads to a rapid breakdown of the NAD^+ pool. ATP molecules are required for resynthesis of NAD^+, and as a consequence the cellular ATP is depleted which leads to necrotic cell death (De Block et al. 2005). Since PARP1 and PARP2 homologues are found in plants, PARP was targeted by RNAi for abiotic stress tolerance in plants (De Block et al. 2005; Vanderauwera et al. 2007).

RNAi-mediated downregulation of RACK1, receptor for activated C-kinase 1, which is a highly conserved scaffold protein with flexible functions, plays important roles in plant growth and development indicating its possible role in drought stress response in rice compared to non-transgenic plants (Da-Hong et al. 2009).

Similarly, disruption of a rice farnesyltransferase/squalene synthase (SQS) by maize squalene

synthase through RNAi improved drought tolerance at both the vegetative and reproductive stages (Manavalan et al. 2012). Expression of OsTZF1, a member of the CCCH-type zinc finger gene family in rice, was induced by drought, high-salt stress, and hydrogen peroxide. OsTZF1-RNAi plants were susceptible to abiotic stress demonstrating that OsTZF1 positively regulates high-salt and drought stress tolerance in rice plants (Jan et al. 2013).

31.3.3 Development of Male Sterile Plants

A hybrid production system is based on the mechanism for inducing male sterility in one of the parental lines so as to ensure purity of the resultant hybrid seed. Male sterility trait has been an important aspect in agriculture to improve the crop productivity by the hybridization process (Duvick 1999). Moreover, its value in gene containment of the genetically modified crops has increased its importance (Moon et al. 2010). Natural male sterile mutants, cytoplasmic male sterile (CMS) mutants, and nuclear male sterile (NMS) mutants have been used for the hybrid seed production (Duvick 2005; Wang et al. 2005).

TA29, an anther-specific gene, is expressed exclusively in anthers at the time of microspore development. Downregulation of TA29 of tobacco (*N. tabacum* cv. Samsun) by RNAi produced male sterile lines (Nawaz-ul-Rehman et al. 2007). Nucleases are enzymes playing vital role in nucleic acid metabolism. Rice transgenic plants expressing hairpin RNA for OsGEN-L (OsGEN-like) gene, a new member of the RAD2/XPG nuclease family, exhibited low fertility and were male sterile (Moritoh et al. 2005). Silencing a male-specific gene, *Bcp1*, in the model host *A. thaliana* resulted in male sterile lines. *Bcp1*, an anther-specific gene, is active in both diploid tapetum and haploid microspores. Transgenic plants were phenotypically indistinguishable from nontransgenic plants, and by crossing with nontransgenic fertile pollens, successful seed set was observed (Tehseen et al. 2010).

S-Adenosylmethionine decarboxylase (*SAMDC*), a key gene involved in polyamine biosynthesis, when targeted in tapetal tissue of tomato under the control of tapetal-specific A9 promoter using RNAi, resulted in male sterile lines. These transgenics had sterile pollen and failed to set fruits, but female fertility was unaffected as cross-pollination resulted in fruit setting (Sinha and Rajam 2013).

31.3.4 Nutritional Improvement

Plants provide most of the nutrients required in the human diet, although the major staple crops are often deficient in some of these nutrients. RNAi technology has also been used in several plants to improve their nutritional quality. A dominant high-lysine maize variant was produced by knocking out the expression of the 22-kDa maize zein storage protein, a protein that is poor in lysine content (Segal et al. 2003). A recessive lysine-rich mutant called opaque 2 (O2) has been obtained by traditional breeding. The O2 gene encodes a maize basic leucine zipper transcriptional factor that controls the expression of a subset of storage proteins, including the 22-kDa zein storage protein. Opaque 2 mutant was lysine-rich but showed poor seed quality and yield. Downregulation of lysine-poor zein gene via RNAi generated normal and quality seeds with high levels of lysine without altering the general functions of O2 (Angaji et al. 2010). Fatty acid composition of cotton seed oil was manipulated by hpRNA-mediated gene silencing of two fatty acid desaturase genes, stearoyl-acyl-carrier protein D9-desaturase and oleoylphosphatidylcholine u6-desaturase. Downregulation of one gene substantially elevated stearic acid level from 2 to 3 % up to as high as 40 %, and silencing of the other gene enhanced oleic acid content, up to 77 % compared with about 15 % in seeds of untransformed plants (Liu et al. 2002).

RNAi technology was used to enhance β-carotene content in potato by silencing the β-carotene hydroxylase gene (*BCH*), which converts β-carotene to zeaxanthin. RNAi constructs having the tuber-specific granule-bound starch

synthase (GBSS) promoter and the other containing the strong constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter were introduced into potato by *Agrobacterium*-mediated transformation. The transformants derived from the GBSS construct contained more β-carotene than CaMV 35S transformants. These results showed that BCH silencing can increase the content of carotenoids, β-carotene, and lutein in potato which will provide a tool for combating the incidence of vitamin A deficiency in populations (Eck et al. 2007). Silencing of DE-ETIOLATED1 (*DET1*) in *Brassica napus* resulted in seeds with increased levels of lutein, β-carotene, and zeaxanthin relative to nontransgenic seeds (Wei et al. 2009). *DET1* suppression also led to reduced levels of sinapate esters responsible for bitter taste, poor meal palatability, and unpleasant flavor to the meat and milk of animals fed on a *B. napus* seed meal diet.

Tomatoes are a principal dietary source of carotenoids and flavonoids, both of which are highly beneficial for human health. Overexpression of genes encoding biosynthetic enzymes or transcription factors has resulted in tomatoes with improved carotenoid or flavonoid content, but never with both. Increased nutritional value was obtained by suppressing an endogenous photomorphogenesis regulatory gene, *DET1*, using fruit-specific promoters combined with RNA interference (RNAi) technology. Both carotenoid and flavonoid contents were increased significantly, whereas other parameters of fruit quality were largely unchanged (Davuluri et al. 2005).

Starch, a major plant carbohydrate, is composed of amylase and amylopectin. Amylose molecules tend to efficiently form digestion-resistant complexes when the cooked food undergoes the process of cooling (Crowe et al. 2000). To increase the amylase content in wheat, RNAi constructs designed to silence the genes encoding the two starch-branching isozymes of amylopectin synthesis were expressed under a seed-specific promoter which resulted in increased grain amylase content to over 70 % of total starch content (Regina et al. 2006; Tang et al. 2007). RNAi constructs have been used in *Zea mays* and *A. thaliana* to modify the levels of phosphate metabolism involved in leaf starch degradation. Phosphate manipulation led to increase in starch content (Weise et al. 2012).

31.3.5 Flower Color Modification

Floriculture, or flower farming, is concerned with the cultivation of flowering and ornamental plants for gardens and for floristry. Flower color modification is one of the most desirable traits in floral industry. RNAi can be used as a tool to silence the pigment synthesis genes, which can lead to different flower color patterns. *CHI* (chalcone isomerase) gene silencing in tobacco by RNAi showed decreased pigmentation and change of flavonoid components in flower petals. Plants showed yellow coloration due to accumulation of high levels of chalcone in pollens (Nishihara et al. 2005).

Nakatsuka et al. (2008) performed RNAi-mediated suppression of three anthocyanin biosynthetic genes—chalcone synthase (*CHS*), anthocyanidin synthase (*ANS*), and flavonoid 3′5′-hydroxylase (*F3′5′H*)—in gentian plant. In *CHS* suppressed transgenics, petals exhibited pure white to pale-blue color, whereas in *ANS* suppressed transgenics, petals were only pale-blue. Suppression of the *F3′5′H* gene decreased delphinidin derivatives and increased cyanidin derivatives and led to magenta flower colors. The same group demonstrated that RNAi-mediated downregulation of anthocyanin 5, 3′-aromatic acyltransferase (5/3′AT) and flavonoid 3′,5′-hydroxylase (F3′5′H) activities in gentian plant produced modified flower color (Nakatsuka et al. 2010).

The flower color of *Torenia hybrid,* an important garden plant, was successfully modulated by RNAi. Downregulation of chalcone synthase (*CHS*) gene by using each of the coding region and the 3′-untranslated region of the CHS mRNA as an RNAi target led to modulation of flower color from blue to white and pale (Fukusaki et al. 2004).

Roses are the most important cut flower commercially and have played a major role in human culture from ancient time. RNAi-mediated

silencing of the cyanidin genes in rose and introduction of delphinidin genes produced flowers that accumulated delphinidin-based anthocyanins exclusively with a concomitant color change toward blue (Tanaka et al. 2009; Katsumoto et al. 2007).

31.3.6 Secondary Metabolite Manipulation

Plant secondary metabolites are economically important as drugs, fragrances, pigments, food additives, and pesticides. Secondary metabolite production, however, sometimes is blocked by undesirable compounds, which can be suppressed by RNAi. The versatility of RNAi for controlling multigenes responsible for metabolite production has been well recognized as an effective strategy (Borgio 2009).

The first commercially useful cultivar produced by RNAi was the rice mutant line LGC-1 (low glutelin content-1), thus making it useful for patients who must restrict protein intake such as kidney disease patients (Mochizuki and Hara 2000). LGC-1 and some cultivars developed using LGC-1 as a cross parent are beginning to be used for this type of diet therapy (Kusaba et al. 2003). This dominant mutation produced hpRNA from an inverted repeat for glutelin, the gene for the major storage protein glutelin, leading to lower glutelin content in the rice through RNAi. Gil-Humanes et al. (2008) used RNAi technology to silence the expression of specific γ-gliadins and demonstrated the feasibility of systematically silencing specific groups of gluten proteins without affecting fertility, grain morphology, and seed weight when compared to the control lines.

RNA-mediated suppression of tryptamine biosynthesis in *Catharanthus roseus* during hairy root culture eliminated the production of monoterpene indole alkaloids (a class of natural products derived from two starting substrates), tryptamine and secologanin. To utilize this chemically silent background, they introduced an unnatural tryptamine analog into the production media and demonstrated that the silenced *C. roseus* culture could produce a variety of novel products derived from this unnatural starting substrate (Runguphan et al. 2009).

Transformation of *Papaver somniferum* with RNAi construct designed to reduce the levels of the gene encoding the morphine biosynthetic enzyme salutaridinol 7-O-acetyltransferase (SalAT) led to the accumulation of the intermediate compounds, salutaridine and salutaridinol, in a ratio ranging from 2:1 to 56:1 (Kempe et al. 2009). California poppy (*Eschscholzia californica*) cells were transformed with RNAi construct harboring berberine bridge enzyme (*BBE*) gene to suppress the activity of the enzyme and resulted into reticuline accumulation at a maximum level (Fujii et al. 2007). The artemisinin content of transgenic *Artemisia annua* L plants was significantly increased by 3.14-fold as compared to untransformed control plants by suppressing the expression of *SQS* (squalene synthase), a key enzyme of sterol pathway, by means of a hairpin RNA (Zhang et al. 2009).

Over 10 % of the coffee on the world market is shared by decaffeinate coffee (DECAF). Caffeine is a stimulant of the central nervous system, the heart muscle, and the respiratory system and has a diuretic effect. Its adverse side effects include insomnia, restlessness, and palpitations. Modulation of caffeine biosynthesis *in planta* was done by suppression of CaMXMT1 (7-N-methylxanthine methyltransferase or theobromine synthase) by the double-stranded RNA method. The caffeine content of transgenic plants was reduced by up to 70 %, indicating that it is possible to produce decaffeinated coffee beans using RNAi (Ogita et al. 2003, 2004).

Cotton is a major cash crop which produces fibers and oil. The cotton seeds that remain after fiber extraction could be extensively used as sources of protein and calories, but they are largely underutilized because they contain a toxic gossypol terpenoid. Gossypol is also produced in vegetative cotton tissues where it protects cotton plants from insects and other pathogens. Transgenic cotton plants expressing RNAi construct of the d-cadinene synthase gene of gossypol synthesis fused to a seed-specific promoter caused seed-specific reduction of this metabolite,

while its content in nonseed tissues was comparable to the control plants (Sunilkumar et al. 2006).

Cassava is a major staple food in tropical countries but contains unnecessary glucosides. Jørgensen et al. (2005) used RNAi to prevent production of the cytochrome P450 enzyme that makes the first committed step in the biosynthesis of linamarin and lotaustralin, and generated transgenic cassava (*Manihot esculenta*) plants with elimination of cyanogenic glucosides in the leaves (<1 % of nontransgenic amounts) and a 92 % reduction of cyanogenic glucoside amount in tubers.

31.3.7 Enhanced Fruit Shelf Life

Fruit ripening has received considerable attention because of the dramatic changes in the metabolic processes that take place before and after this event, as well as due to its commercial importance. Fruits are an important dietary supplement. The quality of fruit is determined by a wide range of desirable characteristics such as nutritional value, flavor, processing qualities, and shelf life. The massive losses accrue during transportation and post-harvest handling of the fruit which run into billions of dollars worldwide. Therefore, there is a need to increase the shelf life of fruits so as to minimize the agronomic loss.

Ethylene, unlike the rest of the plant hormone compounds, is a gaseous hormone inducing several responses during ripening through a signaling cascade (Crocker et al. 1935). The shelf life of tomato has been increased by targeting the genes coding for ethylene biosynthesis pathway. The dsRNA of tomato ACC oxidase expression cassette was successfully introduced into tomato cultivar Hezuo 906 under the control of cauliflower mosaic virus 35S promoter by *A. tumefaciens-mediated* transformation. Transgenic plants produced had fruits having traces of ethylene and had a prolonged shelf life of more than 120 days with similar levels of total soluble sugar, titratable acid, amino acids, and total soluble solids as the control plants (Xiong et al. 2005). Similarly, delayed ripening tomatoes were generated by silencing three homologues of 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) gene, catalyzing the rate-limiting step in ethylene biosynthesis during the course of ripening, using RNAi technology. The chimeric RNAi-ACS construct designed to target ACS homologues effectively repressed the ethylene production in tomato fruits. Fruits from such lines exhibited delayed ripening and extended shelf life for 45 days, with improved juice quality (Gupta et al. 2013).

Recently, SlSGR1 (encoding a STAYGREEN protein that plays a critical role in the regulation of chlorophyll degradation in tomato leaves and fruits)-repressed lines reduced H_2O_2 levels and inhibited ethylene signal transduction during fruit ripening, promoting the retention of firmness and sustained cell membrane integrity and resulting in delayed fruit senescence during storage and an enhanced shelf life from 25 to 45–58 days when harvested at the breaker (Br) stage and stored at room temperature (Luo et al. 2013).

31.3.8 Pros and Cons of RNAi Technology

RNA silencing has emerged as an area of thorough investigations leading to new discoveries. RNAi-mediated gene silencing is a valuable technology for the development of transgenic crop plants, with a focus on nutritional enrichment and plant protection from bacteria, nematodes, fungi, and insects pests, which are two major hurdles in production and productivity of agriculture crops. RNAi strategy has certain advantages over other approaches. For instance, the silencing is sequence-specific and more than one gene can be targeted. Additionally, the extent of the gene silencing can be controlled, so that the essential genes will only be silenced at desired stage and tissue. As there is no transgene protein expression in RNAi approach, there would not be any extra metabolic load on the transgenic plants. Further, in the absence of transgene protein, there is less likelihood of development of resistance by the target pest or pathogen, and RNAi

plants would pose minimal biosafety issues (Rajam 2011).

However, there are also some limitations to RNAi technology. Although it is a method of sequence-specific targeting, there may be issues of off-target effects leading to undesirable traits. There does exist a concern that inadvertent secondary effects could be generated by using non-coding small RNA-mediated gene silencing, especially when this approach is used to engineer broad spectrum resistance into plants against pathogens/pests. Delivery methods for the dsRNA are a limiting step for a number of species for which RNAi-based approaches cannot be used easily. There still remain many significant challenges in development and commercialization of GM crops utilizing RNAi-based technology. Not only tremendous efforts are required for achieving scientific breakthroughs but also promotion of public acceptance of GM crops among other complicated ethical issues has to be taken care of.

We, hence, conclude that agricultural biotechnology, including RNAi technology, would serve as one of the most important measures for crop improvement, which will contribute to agriculture productivity to a great extent.

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Plant MicroRNAs: Biogenesis, Functions, and Applications

32

Manish Pareek, Sneha Yogindran, S.K. Mukherjee, and Manchikatla Venkat Rajam

Abstract

MicroRNAs (miRNAs) are small 21-nt-long noncoding RNAs, involved in posttranscriptional regulation of mRNA either by direct cleavage or translational repression. They are mainly transcribed as independent transcriptional units and their expression differs during different stages of plant development. Plant miRNAs have been well studied during developmental stages and different environmental stress conditions, but their functions and regulatory networks have yet to be understood. Understanding their regulatory roles may open new avenues for crop improvement which is necessary for increased food production to feed the growing population. MicroRNA interference (miRNAi) technology provides an efficient platform for functional studies and agricultural applications.

Keywords

miRNAs • Plant development • Stress • Artificial miRNAs (amiRNA) • MicroRNA-induced gene silencing (MIGS) • miRNA interference (miRNAi)

32.1 Historical Perspective

During the last decade, the "RNA world" has been transformed into an "RNA molecular world" where RNA is not only restricted to the enzymatic functions but also regulates gene expression. This transformation has been carried out along with many fronts, but one of the most significant advances has been the discovery of small noncoding RNAs. Small interfering RNA (siRNA) and microRNAs (miRNAs/miRs) are the two major classes of plant small noncoding RNAs. siRNAs are formed from dsRNA while miRNAs originate from single-stranded RNAs transcribed from miRNA loci (Voinnet 2009). Plants have highly conserved and more recently evolved species-specific miRNAs to control a vast array of biological processes.

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The first miRNA *lin-4* was discovered in 1993 by Victor Ambros and his colleagues in *Caenorhabditis elegans* that has been shown to repress the protein levels of *lin-14*, a gene that functions in the same developmental pathway. The *lin-4* gene does not encode any protein but instead makes a pair of small RNAs. One RNA is about 21-nt long and the other is approximately 60-nt long. The longer RNA which folds into a stem-loop structure was predicted to be the precursor of the 21-mer RNA. This shorter *lin-4* RNA was actually the founding member of the abundantly available class of small regulatory RNAs known today as miRNA. The *lin-4* RNA bound with partial antisense complementary sequences found in the 3′-untranslated region (3′-UTR) of *lin-14* mRNA and repressed its translation without reducing the level of *lin-14* transcript (Lee et al. 1993). After 7 years of discovery of *lin-4*, another miRNA called *let-7* was discovered in 2000 in the same organism (Reinhart et al. 2000). miRNA *let-7* regulates the expression of gene *lin-41* which is required for the transition from late larval to adult cell fate. The *let-7* miRNA was found in almost all animal phyla, establishing biological importance of miRNAs.

Later on, several investigators have cloned miRNAs from various organisms. Cloning of small RNAs also revealed their ubiquitous presence in plants. The first plant miRNA was discovered in *Arabidopsis thaliana* in 2002. Llave et al. (2002) cloned a large set of miRNAs of predominantly 21- to 24-nt length from *Arabidopsis*. Among the 125 sequences identified, about 90 % originated from the intergenic regions and a few others from the protein-coding genes. The accumulation of some of these miRNAs was developmentally regulated. A set of miRNAs surprisingly mapped in the transposable elements of the *Arabidopsis* genome (Mette et al. 2002). Differential expression pattern of 16 *Arabidopsis* miRNAs was observed, of which 8 were conserved in the rice genome. CARPEL FACTORY, an *Arabidopsis* Dicer (RNase III family enzyme) homologue, when mutated, prevented the miRNA accumulation. This observation led to the conclusion that a similar mechanism also exists in plants that direct miRNA processing as in animals (Park et al. 2002; Reinhart et al. 2002).

Despite some similarities between the plant and animal miRNAs, the divergences between these two groups were more noticeable. It includes the sizes of the mature miRNAs, gene structure, 5′ nucleotide preferences, and most significantly absence of any genetic conservation between the two groups. As the mechanistic studies emerged strongly, it became clear that these two groups differ significantly in the modes of their biogenesis and functions. Taken all together, it appears that these two groups had dual origins in their evolutionary lineages.

32.2 Biogenesis and Activity of MicroRNAs

miRNAs are transcribed from noncoding nuclear genes, a majority of which are located in intergenic and intragenic (intron) regions, and very few of them are located in 5′-UTR or 3′-UTR regions. Plant miRNAs are generally transcribed by RNA Pol II which produces capped and polyadenylated long primary miRNA transcripts (pri-miRNAs), which are then processed into precursor miRNA (pre-miRNA) of about 80–500 nucleotides in length, by an enzyme, DICER-LIKE 1 (DCL1), with RNase III activity (Kurihara et al. 2006). A part of the pri-miRNA folds to form a stem-loop structure which is cropped by the DCL1 complexes. A general feature of the pri-miRNA is shown in Fig. 32.1. DCL1-mediated processing occurs mostly at the lower stem or base, while in a few cases, the terminal loop controls the processing. With long-stem or terminal loop-branched pri-miRNAs, the processing could be bidirectional, i.e., from base to loop and loop to base, resulting in productive and abortive processing of miRNAs, respectively. DCL1 complexes generally cut pri-miRNAs at a distance of 16–17 bp from the single-strand–double-strand junction (Zhu et al. 2013). Pri- to pre-miRNA conversion also requires the double-stranded RNA (dsRNA)-binding protein HYPONASTIC LEAVES1 (HYL1) and the C2H2 zinc finger protein SERRATE (SE) along

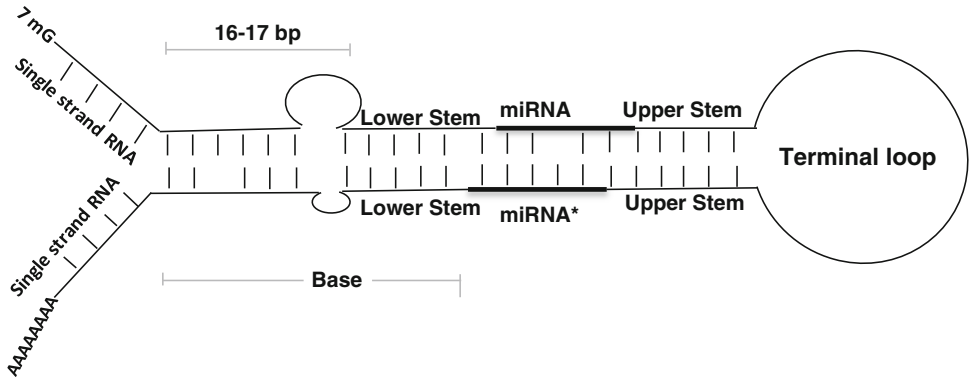

Fig. 32.1 General features of primary miRNA (pri-miRNA)

with other factors like CBC, DDL, and TOUGH (Yang et al. 2006a; Parry et al. 2007; Yu et al. 2008; Ren et al. 2012). These proteins are located in nuclear processing centers called D-bodies or SmD3-/SmB-bodies (Kurihara et al. 2006; Fang and Spector 2007).

Pre-miRNA is unstable in nucleus and processed into miRNA–miRNA* duplex by DCL1 and stabilized by S-adenosyl methionine-dependent methyltransferase HUA ENHANCER 1 (HEN1), which methylates all plant-silencing small RNAs. Methyl groups deposited on the 3′ terminal nucleotides of each strand prevent their uridylation and subsequent degradation by the SMALL RNA DEGRADING NUCLEASE (SDN) class of exonucleases (Li et al. 2005; Yang et al. 2006b). The modified miRNA–miRNA* is exported into cytoplasm by HASTY (an ortholog of exportin-5 protein) and processed into mature miRNA (Park et al. 2005). The miRNA-programmed silencing complex is often referred to as RNA-induced silencing complex (RISC) (Hammond et al. 2000; Llave et al. 2002; Tang et al. 2003) (Fig. 32.2). Cloning and expression data indicate that the miRNA strand of this duplex, also known as guide strand, accumulates to much higher levels in vivo than does the miRNA* or the passenger strand (Reinhart et al. 2002; Lim et al. 2003). This asymmetric accumulation is accomplished by preferential loading of the miRNA strand into the silencing complex, where it is protected from degradation, whereas the miRNA* strand is preferentially excluded from the silencing complex and is degraded. Bioinformatics and biochemical studies of functional siRNA duplexes gave insights into the asymmetry of RISC loading. The siRNA duplex strand with less stable 5′ end pairing is selectively loaded into RISC, where it guides silencing, whereas the strand with the more stable 5′ end pairing is excluded from RISC. Most miRNA–miRNA* duplexes also appear to have energetic asymmetry; the 5′ ends of most miRNAs are less stably paired than are the 5′ ends of the corresponding miRNA*s (Khvorova et al. 2003; Schwarz et al. 2003). Hence, the final product of the miRNA biogenesis pathway is a small single-stranded RNA incorporated into a silencing complex (Fig. 32.2).

A central component of the silencing complex is a member of the Argonaute protein family. Argonaute proteins contain two conserved regions, the PAZ and PIWI domains (Carmell et al. 2002). The PAZ domain appears to be an RNA-binding domain (Lingel et al. 2003; Song et al. 2003; Yan et al. 2003), and the PIWI domain is structurally and functionally similar to RNase H enzymes (Liu et al. 2004; Song et al. 2004). Many organisms contain multiple members of the Argonaute family; in some cases, there is evidence for functional diversification of the different Argonautes. AGO1 is the only Argonaute gene known to be required for miRNA function in *Arabidopsis*. *Arabidopsis* AGO1 binds miRNAs and catalyzes target cleavage in vitro (Baumberger and Baulcombe 2005;

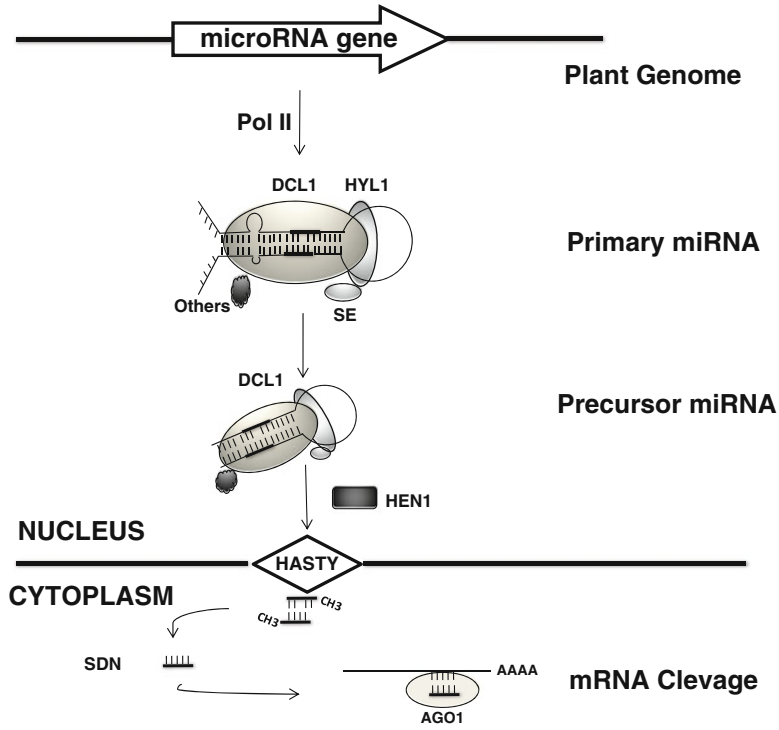

Fig. 32.2 Biogenesis of plant miRNA: the primary miRNA (pri-miRNA) transcript is transcribed from the microRNA gene by RNA polymerase II (Pol II). Pri- to pre-miRNA conversion requires many proteins like DCL1, HYL1, SE, HEN1, and others (TOUGH, DDL, and CBC). HASTY is involved in miRNA transport from nucleus to cytoplasm. SDN degrades the passenger miRNA strand and AGO1 is responsible for mRNA cleavage

Qi et al. 2005), and *ago1* mutants have elevated levels of miRNA targets in vivo (Vaucheret et al. 2004).

The cleavage of target mRNA occurs at the 10th or 11th nucleotide from the 5′ end of the miRNA, regardless of the miRNA length, and requires the base pairing of the 5′ end of the miRNA with the target mRNA (Palatnik et al. 2003; Xie et al. 2003; Floyd and Bowman 2004; Jones-Rhoades and Bartel 2004; Mallory et al. 2004). The resulting cleavage products have 3′ hydroxyl and 5′ phosphate groups, similar to the products of other enzymes with "slicer" activity, such as RNase H (Martinez and Tuschl 2004; Schwarz et al. 2004; Song et al. 2004).

The plant miRNAs are mostly rich in U at the 5′ end and are consequently sorted in the AGO1-mediated RISC complexes (Voinnet 2009). A few *Arabidopsis* miRNAs have U to A mutation at the 5′ end which are sorted in AGO2 complexes in vivo leading to loss of their silencing potential (Mi et al. 2008; Vazquez et al. 2008). However, a bioinformatics analysis of the next-generation sequencing (NGS) data revealed that there is a wide range of variations in the occurrence of U at the 5′ position in monocots (45–85 %) and dicots (45–70 %). In potato, U and A dominate almost equally at the 5′ position. The effect of differential sorting in silencing has not been widely reported in plants other than *Arabidopsis* and rice. The 5′ A-variant of 24-mer miRNAs are expected to sort in the AGO4 complex which causes heterochromatinization (Takeda et al. 2008).

The NGS data on plant miRNAs also show that a small percent of many miRNAs undergo modifications both at the 5′ and 3′ ends with 3′ end modifications being more common (Ameres and Zamore 2013). These modifications are either additions or deletions but its biochemical causes are still uncertain. These modifications arise either due to faulty processing of DCL1 or could be the result of post-maturation processes of miRNAs (Voinnet 2009). More than 80 % of the modified *Arabidopsis* and rice miRNAs are found in the AGO1 and AGO4 complexes, indicating that the modified miRNAs have a functional role.

Small interfering RNAs (siRNAs) are cell autonomous and migrate from their place of

origin to distant locations to cause repression of the target genes. However, plant miRNAs are generally considered as sluggish and do not tend to move out from their place of origin as revealed by the in situ hybridization data (Valoczi et al. 2006) or cell-specific expression of artificial miRNA (Tretter et al. 2008). Though, recent reports indicate that a few miRNAs are really mobile. The tomato miR172, produced either in the floral or leaf tissues, migrates down to the roots of the plant and helps to acquire resistance against the nematode-induced damage. Few other miRNAs produced in leaf tissues are also found in phloem and roots which enable the plants to defend against the stress responses. Sensing low levels of nutrients like sulfur and phosphate, the miR395 and miR399 are known to translocate from the leaves to the roots to help ameliorate the nutrient deficiency (Voinnet 2009).

32.3 miRNA Families, Conservation, and Evolution

Since the advent of NGS techniques and advanced computational facilities, the small RNA database is flooded with plant miRNA sequences. The miRBase v21 (http://www.mirbase.org/index.shtml) reports the presence of 7,057 miRNAs in 73 different plant species. The miRNAs of same or nearly identical sequences can be produced by many pre-miRNAs having otherwise very different base sequences and can be clubbed together as members of a family. The miR169 family of *Arabidopsis* has 14 members. A recent study has computationally identified over 2,000 miRNAs from six Solanaceae plants based on the genomic sequences and PUT (PlantGDB-assembled unique transcripts, EST Assemblies). The 982 potato miRNAs have been classified in 71 miRNA families, and the miR1128 family is predicted to have 141 members and thus is considered to be the largest known family in the plant kingdom (Gu et al. 2014). Sometimes the uniqueness of the plant species can also be discerned by the presence of distinct miRNA family(ies).

Initially, miRNAs discovered in *A. thaliana* and rice (*Oryza sativa*) were from families that are conserved between the two species (Llave et al. 2002; Park et al. 2002; Jones-Rhoades and Bartel 2004; Sunkar and Zhu 2004). Computational prediction and sequencing techniques resulted in the identification of miRNAs from ancient families (Axtell and Bartel 2005). miR396, miR397, miR398, miR828, miR2111, and miR403 are few miRNA families which are conserved in plants ranging from vascular plants to seed plants (Cuperus et al. 2011). miR156, involved in the transition from the juvenile to the adult phase of shoot development in plants, has been found to be much conserved from angiosperm to lower plants. miR166 and miR171 have also been found to be conserved among different plant species. However, there are miRNAs such as miR415 and miR417 which have been reported only in angiosperms and hence are not conserved. Interestingly, in single-cell alga *Chlamydomonas reinhardtii*, no miRNAs have been reported which are otherwise much conserved across the plant species suggesting its early evolutionary divergence from higher plants (Table 32.1). miRNA families also show cross-kingdom conservation. Two miRNA annotated families (miR854 and miR855) were reported as conserved between the plant and animal lineages (Arteaga-Vázquez et al. 2006). However, loci from these families lie in retrotransposons.

Apart from conserved miRNAs, species-specific or non-conserved miRNAs have also been identified. Deep sequencing of *A. thaliana* small RNA populations suggested that, in spite of conserved miRNAs, there are miRNAs restricted to the family Brassicaceae (Lu et al. 2006; Rajagopalan et al. 2006; Fahlgren et al. 2007, 2010; Ma et al. 2010). A large number of species-specific miRNA genes were observed in *Physcomitrella patens* and *Selaginella moellendorffii* (Axtell et al. 2007), rice (Heisel et al. 2008; Lu et al. 2008a; Sunkar et al. 2008; Zhu et al. 2008), *Medicago truncatula* (Szittya et al. 2008; Lelandais-Brie`re et al. 2009), and *Glycine max* (Subramanian et al. 2008). The presence of a large number of non-conserved miRNAs in plants also suggests that plants carry recently evolved miRNA loci.

The miRNA region can be divided into two regions, evolutionary stable and variable, which are the outcomes of evolution over a long period. The region including the mature miRNA and

Table 32.1 Representative plant miRNAs and their conservation across the major plant phyla (based on miRBase v21)

	Angiosperms										Gymnosperms			Bryophyte	Alga
miRNAs	Ath	Sly	Gma	Bna	Nta	Osa	Tae	Hvu	Zma	Sbi	Pta	Pab	Cln	Ppa	Cre
miR156	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−
miR159	+	−	+	+	+	+	+	+	+	−	+	−	−	−	−
miR160	+	+	+	+	+	+	+	−	+	+	−	+	−	+	−
miR162	+	+	+	+	+	+	−	−	+	+	−	−	+	−	−
miR164	+	+	+	+	+	+	+	−	+	+	−	−	+	−	−
miR166	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−
miR167	+	+	+	+	+	+	+	−	+	+	−	−	−	+	−
miR168	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−
miR169	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−
miR171	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−
miR172	+	+	+	+	+	+	−	−	+	+	−	−	−	−	−
miR319	+	+	+	−	+	+	+	−	+	+	+	−	−	+	−
miR390	+	+	+	+	+	+	−	−	+	+	+	−	−	+	−
miR393	+	−	+	+	−	+	−	−	+	+	−	−	−	−	−
miR394	+	+	+	+	+	+	−	−	+	+	−	−	−	−	−
miR395	+	+	+	+	+	+	+	−	+	+	−	+	−	+	−
miR396	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−
miR397	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−
miR398	+	−	+	−	+	+	+	−	+	+	+	−	−	−	−
miR399	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−
miR403	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
miR408	+	−	+	−	+	+	+	−	+	+	+	−	−	+	−
miR413	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
miR414	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−
miR415	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
miR416	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
miR417	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
miR418	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
miR419	+	−	−	−	−	+	−	−	−	−	−	−	−	+	−
miR420	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
miR426	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
miR472	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
miR477	−	+	−	−	+	−	−	−	−	−	−	−	−	+	−
miR479	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
miR482	−	+	+	−	+	−	−	−	+	−	−	+	−	−	−
miR529	−	−	−	−	−	+	−	−	+	+	−	−	−	+	−
miR535	−	−	−	−	−	+	−	−	−	−	−	+	−	+	−
miR536	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
miR783	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Ath *Arabidopsis thaliana*, Sly *Solanum lycopersicum*, Gma *Glycine max*, Bna *Brassica napus*, Nta *Nicotiana tabacum*, Osa *Oryza sativa*, Tae *Triticum aestivum*, Hvu *Hordeum vulgare*, Zma *Zea mays*, Sbi *Sorghum bicolor*, Pta *Pinus taeda*, Pab *Picea abies*, Cln *Cunninghamia lanceolata*, Ppa *Physcomitrella patens*, Cre *Chlamydomonas reinhardtii*

miRNA* is stable, whereas the other region, which represents most of the miRNA genes including the loop and other sequences, belong to the variable region (Bullini and Coluzzi 1972).

Over the geological periods, plant species have evolved in their lifestyle and behavior. Since the miRNAs govern the plant behavior, it is no wonder that the plant miRNAs have also evolved accordingly. The rate of miRNA evolution is determined by several factors including the time intervening the emergence and continuance of a given plant species. For example, the moss *Physcomitrella patens* and the eudicot *A. thaliana* are widely different in their characteristics and have diverged about half a billion years ago. However, both the lineages have a comparable number of miRNA families, 67 and 66 in moss and *A. thaliana*, respectively (Taylor et al. 2014). Recent analysis reveals that the evolution of the novel miRNA family did not occur at a continuous speed in the geological scale of time, but it resulted due to sudden bursts of phenotypic evolution such as the establishment of plant life on lands. Indeed such an expansion in the miRNA families within the angiosperm lineages has been speculated from the birth of flowering plants (Nozawa et al. 2012).

Among other interesting features of miRNA evolution, the most notable one would possibly be the origin of the miRNA gene itself. A closer look at the pre-miRNA sequences outside the mature miRNA region of a given pre-miRNA reveals a good amount of their similarity with the miRNA target gene (protein-coding) sequence. Based on this observation, the evolution of miRNA genes with the inverted duplication of miRNA target gene or its fragments has been proposed (Voinnet 2009; Cuperus et al. 2011). In fact three possible pathways of formation of miRNA genes are known till now. (a) The theory of formation of plant miRNA genes based on the inverted duplication of protein-coding genes have been advanced by a number of investigators (Rajagopalan et al. 2006; Fahlgren et al. 2007, 2010). This theory is attractive as it readily explains the target selection by miRNAs and the consequent silencing. The processing aspects have also undergone evolution from the DCL4-mediated processing to the DCL1-centered activity. (b) Miniature inverted-repeat transposable elements (MITEs) could have given rise to miRNA genes as they harbor potential hairpin structures. Many *Arabidopsis* and rice miRNAs might have evolved through MITEs (Piriyapongsa and Jordan 2008). (c) Duplication of preexisting miRNA genes with accumulation of mutations might have brought in new miRNA genes. As the plant miRNAs are often clubbed as members of the miRNA family, consisting of several miRNA genes, this theory has received particular attention (Li and Mao 2007). All of these factors contribute to different extents in the evolution of plant miRNAs (Nozawa et al. 2012).

Irrespective of pathways of evolution, a few miRNA genes which are highly conserved and expressed across the plant kingdom is classified as the old class of miRNA genes, although these are few in numbers. Most other miRNA genes are phylogenetically restricted and have evolved relatively recently and expressed in either low or moderate copies in plants. These latter miRNA genes are known as the young ones. A few of them might not have their targets at all, and these have evolved most recently in the time scale. If they cannot acquire their target genes, they might eventually dissipate (Cuperus et al. 2011).

32.4 Role of MicroRNAs in Gene Regulation

miRNAs regulate the target gene expression either by mRNA cleavage or by translational repression. Choice of the posttranscriptional mechanisms is determined by the identity of the target. Once incorporated into a cytoplasmic RISC, the miRNA will specify cleavage if the mRNA has sufficient complementarity to the miRNA, or it will repress the translation if the mRNA does not have sufficient complementarity to be cleaved but does have a suitable clustering of miRNA-binding sites.

32.4.1 miRNA-Directed Target mRNA Cleavage/Degradation

Plant miRNAs recognize full or near complementary mRNA targets and direct endonucleolytic

mRNA cleavage (Llave et al. 2002; Rhoades et al. 2002; Tang et al. 2003; Voinnet 2009). Cleavage occurs between nucleotides in the 10th and 11th nucleotide position, opposite the miRNA strand, and is catalyzed by the AGOs. The resulting 5′ and 3′ mRNA fragments are degraded from the newly generated 3′ and 5′ ends, by the activity of nonspecific nucleases (Souret et al. 2004).

The register of cleavage does not change when the miRNA is not perfectly paired to the target at its 5′ terminus (Kasschau et al. 2003; Palatnik et al. 2003). Therefore, the cut site appears to be determined relative to miRNA residues, not miRNA: target base pairs. After cleavage of the mRNA, the miRNA remains intact and can guide the cleavage of additional mRNAs (Hutvágner and Zamore 2002; Tang et al. 2003). These studies indicated that endonucleolytic cleavage of highly complementary targets is a prominent mechanism of miRNA-based regulation in plants.

32.4.2 miRNA-Directed Translational Repression of Target Genes

miR156/miR157, which targets SBP-box gene SPL3, and miR172, which targets APETALA 2, have been reported to repress translation in plants (Aukerman and Sakai 2003; Chen 2004; Gandikota et al. 2007). *A. thaliana* mutants defective in silencing of a GFP reporter containing miR171-binding site in the 3′-UTR showed higher expression of GFP protein, while the mRNA levels were not restored. This suggested that the mutations suppressed miRNA-mediated translational repression but not mRNA cleavage. Nevertheless, the observations suggest that plant miRNAs can repress translation in the absence of target degradation (Brodersen et al. 2008).

Enhancer of decapping 4 (eDC4; also known as Ge1 and HeDlS), which is known as vARICOSe in *A. thaliana*, is required for miRNA-mediated mRNA decay but not translational repression in animals (Eulalio et al. 2007). In *Drosophila melanogaster* cells, eDC4 is known as a suppressor of miRNA-mediated gene silencing by depleting eDC4-suppressed mRNA degradation mediated by miRNAs, restoring transcript levels (Eulalio et al. 2007), whereas, in plants, vARICOSe mutants showed increased levels of protein expression but no increase in mRNA levels (Brodersen et al. 2008).

32.4.3 miRNA-Directed DNA Methylation

miRNAs are also involved in DNA methylation, a well-known epigenetic regulation, and change the expression pattern of genes. There are miRNA variants which associate with AGO proteins involved in RNA-directed DNA methylation (Law and Jacobsen 2010). AGO4, AGO6, and AGO9 have been reported in *A. thaliana* to direct methylation of target DNA by binding with 24-nt siRNAs having 5′ adenosine generated by DCL3 from dsRNAs produced through the activities of RDR2- and DNA-dependent RNA polymerase IV (Qi et al. 2006; Mi et al. 2008; Havecker et al. 2010; Law and Jacobsen 2010). In rice, DCL3a has been shown to process multiple miRNA fold-backs, yielding 24-nt miRNA-like siRNAs which preferentially associate with rice AGO4a and AGO4b, and like 24-nt siRNAs, they can guide the methylation of target (Wu et al. 2010). In moss *Physcomitrella patens dcl1b* mutants, there was accumulation of miRNA–target RNA duplexes and hypermethylation of the genes encoding target RNAs, leading to gene silencing (Khraiwesh et al. 2010). Therefore, it can be concluded that apart from the function of mRNA repression, miRNA and miRNA–AGO complexes can function at transcriptional silencing level.

32.5 Regulatory Role of miRNAs in Plant Growth and Development

Plant development is a highly regulated process and controlled at many levels. The discovery of miRNAs and their roles in plant development has opened a new frontier to further understand the mechanisms underlying this regulation. miRNA

target genes are mainly transcription factors and F-box proteins, involved in major plant developmental pathways such as leaf development, patterning and polarity, floral identity and flower development, flowering time, developmental-phase transition, shoot and root development, hormone signaling, and stress responses (Jones-Rhoades et al. 2006).

Developmental defects of the *A. thaliana* mutants *ago1*, *dcl1*, *hen1*, *hyl1*, and *hst* provided clues for the key roles of miRNAs in plant development (Telfer and Poethig 1998; Bohmert et al. 1998; Jacobsen et al. 1999; Lu and Fedoroff 2000; Chen et al. 2002). Reduced level of miRNA and many developmental abnormalities were observed in loss-of-function *dcl1* gene (Park et al. 2002; Reinhart et al. 2002; Dugas and Bartel 2004). HASTY, a protein required for transporting miRNA–miRNA* duplex from the nucleus to the cytoplasm, when downregulated, caused many pleiotropic phenotypes including disrupted leaf shape and flower morphology, reduced fertility, accelerated vegetative phase change, and disrupted phyllotaxis of the inflorescence (Bollman et al. 2003). These findings clearly indicate that miRNAs are involved in a variety of developmental processes in plants.

32.5.1 Leaf Development and Patterning

Plant leaves exhibit adaxial/abaxial (upper/lower) axis pattern which is controlled by the expression of class III homeodomain-leucine zipper (HD-ZIP) (Juarez et al. 2004). PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV) are three closely related *Arabidopsis* HD-ZIP transcription factors. Using computational approaches, miR165/miR166 has been implicated in the functioning of these transcription factors. In phb-d, phv-d, and rev-d mutants, the mutations are found to be in the region of miR165/miR166 complementary sites which decrease the efficiency of miRNA-directed cleavage in vitro (Tang et al. 2003). Another member of class III HD-ZIP transcription factors, INCURVATA4/ATHB15/CORONA, was found to participate in the control of leaf polarity, as well as in shoot and root apical meristem patterning and stem vascular differentiation (Prigge et al. 2005; Williams et al. 2005; Ochando et al. 2006). Gain-of-function icu4 mutants bear the same nucleotide substitution similar to phabulosa, phavoluta, and revoluta mutants. This mutation affects the miR165/miR166 complementarity in target mRNAs, resulting in impaired cleavage of the mutant transcripts. Study also revealed that miR164 is another regulator of leaf patterning (Nikovics et al. 2006). miR164a and the transcription factor CUP-SHAPED COTYLEDON2 (CUC2) gene controls leaf margin serration in *Arabidopsis*.

In addition to miR166 and miR165, miR159/miRJAW also regulates leaf development by targeting a subset of TCP transcription factor genes. Overexpression of miRJAW resulted in low levels of all tested TCP mRNAs and caused jaw-D phenotypes, including uneven leaf shape and curvature. In contrast, overexpression of miRJAW-resistant TCP mutants indicated that miRJAW-guided mRNA cleavage was sufficient to restrict TCP function (Palatnik et al. 2003). Lately, it has been shown that miR396 which regulates growth-regulating factors (GRFs transcription factors), when overexpressed, affected the cell proliferation in the meristem and developing leaves and showed reduced leaf size (Mecchia et al. 2013).

32.5.2 Flower Development

Flower development is one of the most important stages of plant development, especially for flowering plants. Mature flowers consist of carpels, stamens, petals, and sepals. The ABC model best explains the origin of flower (Lohmann and Weigel 2002; Jack 2004). According to this model, four flower organs are controlled by the combinatorial actions of three classes (A, B, and C) of transcription factors (Lohmann and Weigel 2002). APETALA 2 (AP2) is one of the class A genes that play an important role in flowering time and flower morphology. It has been demonstrated that AP2 is one of the targets of miR172

(Aukerman and Sakai 2003; Chen 2004). Overexpression of miR172 inhibited the translation of the AP2 gene and AP2-like genes (target of eat 1, TOE1) and resulted in early flowering and disrupting the specification of floral organ identity similar to loss-of-function *ap2* gene mutants (Aukerman and Sakai 2003; Chen 2004; Luo et al. 2013). Another important feature of flower development is phase change, i.e., the change from vegetative growth to reproductive growth. This transition (including the transition from a juvenile to adult leaf) is also regulated by the AP2-like gene glossy15 (gl15) in maize. Overexpression of miR172 resulted in decreased gl15 expression through gl15 mRNA cleavage and resulted in delayed phase change from vegetative to reproductive (Lauter et al. 2005).

Formation of extra petals in early-arising flowers was observed in *Arabidopsis eep1* mutant which is the target of miR164c, one of the three known members of the miR164 family (Baker et al. 2005). The other two members of the miR164 family are miR164a and miR164b, which negatively regulate several members of the NAC family of transcription factors that are required for plant organ formation and boundary establishment (Reinhart et al. 2002; Rhoades et al. 2002; Jones-Rhoades and Bartel 2004).

GAMYB, gibberellin-modulated MYB transcriptional activator and a positive regulator of LEAFY, has been reported to play an important role in flower development (Achard et al. 2004; Kaneko et al. 2004; Millar and Gubler 2005). GAMYB is one target of miR159. Overexpression of miR159 resulted in a reduction of LEAFY transcript levels and further perturbed anther development and delayed flowering in short-day photoperiods (Achard et al. 2004; Schwab et al. 2005). miR156 is also involved in floral development and phase change by targeting members of squamosa promoter binding protein-like (SPL) plant-specific transcription factors. Overexpression of miR156 affects phase transition from vegetative growth to reproductive growth, including early initiation of rosette leaves, a severe decrease in apical dominance, and a moderate delay in flowering (Schwab et al. 2005).

Cardamine flexuosa, an herbaceous biennial plant, requires an age-dependent vernalization. miR156 and miR172 have been shown to control the timing of sensitivity to vernalization by regulating the MADS-box gene CfSOC1 and hence regulate flowering. The transgenic plants with the highest miR172 level flowered without exposure to cold temperature (Zhou et al. 2013a).

32.5.3 Organ Development

Plant shoots and roots develop from the shoot apical meristems and the root apical meristems, respectively. cuc genes encode the transcription factors of the NAC family known to be involved in shoot apical meristem formation and cotyledon separation during embryogenesis in *Arabidopsis*. Members (e.g., CUC1, CUC2, and NAC1) of the NAC family are targets of the miR164 family (Laufs et al. 2004; Mallory et al. 2004; Guo et al. 2005; Schwab et al. 2005). Plants overexpressing miR164 show a range of developmental abnormalities similar to *nac* gene family mutants. In contrast, loss-of-function miR164 mutants accumulate higher levels of nac mRNA, resulting in more lateral root formation (Guo et al. 2005). Thus, in the *Arabidopsis* meristem, miR164 restricts the boundary expansion by degrading cuc1 and cuc2 mRNAs during organ initiation.

32.6 Role of miRNA in Plant Pathogen/Pest Interaction

Plants in their native environment interact with many microorganisms like virus, bacteria, fungi, insects, nematodes, etc. Interacting microorganisms may be beneficial or pathogenic to the plants. This brings about many changes in the expression patterns of genes involved in plant pathogen interaction. miRNAs have been shown to play an important role in regulating the genes during stress responses in plants. This section provides a brief review of miRNAs associated with plant pathogen and pest attack.

32.6.1 miRNAs During Viral Infection

MicroRNAs responsive to plant infection by viruses were identified in many plant species, e.g., *Brassica rapa* (He et al. 2008), rice (Du et al. 2011), *Arabidopsis* (Bazzini et al. 2009; Blevins et al. 2011; Hu et al. 2011), and tomato (Lang et al. 2011). Turnip mosaic virus (TuMV)-encoded RNA silencing suppressor, P1/HC-Pro, was reported to interfere with miR171, which targets Scarecrow-like transcriptional factors and inhibits the miR171-guided target mRNA cleavage. Eight out of ten miR171 targets showed elevated accumulation in the presence of P1/HC-Pro, but the expression of miR171 was not affected due to the suppressor impacting steps downstream the maturation of miR171 (Kasschau et al. 2003). In *Arabidopsis*, miR156 and miR164 were induced by infection with the virus TuMV and were induced in transgenic *Arabidopsis* plants expressing the viral silencing suppressor P1/HC-Pro (Kasschau et al. 2003). Upon virus infection, new miRNAs (miR158 and miR1885) were identified in *Brassica*; miR158 and miR1885 were specifically induced by TuMV but not by cucumber mosaic virus or tobacco mosaic virus (He et al. 2008).

32.6.2 miRNAs During Bacterial Infection

Differential miRNA expression pattern during bacterial infection was first observed in tumor induced by *Agrobacterium tumefaciens* infection, in which *At*-miR393 and *At*-miR167 were downregulated. The predicted targets of these miRNAs are ARF8 and TIR1 proteins which are known to positively regulate auxin response in plants (Dunoyer et al. 2006). Concurrent report has showed that miRNA393 expression was increased in flg22-elicited (PAMP associated with *Pseudomonas syringae*) *Arabidopsis* seedlings. *At*-miR393 targets the auxin receptor TIR1 and AFB transcripts leading to repression of the auxin-signaling pathway. Further overexpression of *At*-miR393 resulted in phenotypes similar to auxin-signaling mutants and showed increased resistance against bacterial infection (Navarro et al. 2006). Using direct sequencing approach, expression profile of small RNA of *Arabidopsis* leaves treated with *P. syringae* pv. tomato (*Pst*) identified three miRNAs (miR160, miR167, and miR393) that were highly induced and one that was downregulated (miR825) after infection. miR167 and miR160 target the transcripts encoding transcription factors involved in auxin-signaling pathways (Fahlgren et al. 2007). These reports indicate that miRNA-mediated downregulation of auxin signaling is involved in plant immunity against bacterial infections.

ago1 and *dcl1* mutants are compromised in flg22-mediated bacterial resistance, providing their role in PTI response. RNA sequencing associated with AGO1 shows accumulation of miR160, miR167, miR393, miR396, and miR824 and reduction of miR398 and miR773. Overexpression of miR160, miR398, and miR773 shows that miR160 positively regulates while miR160 and miR398 negatively regulate PTI response (Li et al. 2010).

Moreover, it has been also reported that miR393* accumulates during *Pst* infection and is highly enriched in AGO2 protein in *Arabidopsis* seedlings. The miR393*–AGO2 complex targets a SNARE protein MEMB12 which regulates secretion and accumulation of PR1 protein governing the bacterial resistance to plants. Thus, miR393–miR393* pair is an amusing example that contributes to antibacterial resistance using different AGO proteins (AGO1/AGO2) and targeting different pathway genes (Zhang et al. 2011b).

Global miRNA expression profiling using small RNA libraries of the *Arabidopsis* plant treated with the different strains of *Pst* shows the involvement of miRNA-mediated regulation of different plant signaling pathways. Among them, miR160, miR167, miR390, and miR393 regulate auxin signaling, and miR319 interfere in jasmonate pathway genes. Moreover, miRNA159 suppresses abscisic acid signaling and activates salicylic acid signaling pathway genes via targeting *MYB33* and *MYB101* transcripts (Zhang et al. 2011a).

32.6.3 miRNAs During Fungal Infection

Various miRNA families were shown to differentially express in response to infection by the rust fungus *Cronartium quercuum* f. sp. *fusiforme*, which causes fusiform rust disease in pines; 10 of 11 miRNA families were downregulated, including seven that were pine specific. For example, miR156 and miR160 were repressed in pine stems infected with the rust fungus (Lu et al. 2007). Moreover, miRNAs may affect the response of wheat (*Triticum aestivum* L.) to powdery mildew infection; some of the conserved miRNAs showed differential expression patterns in response to powdery mildew infection: miR156, miR159, miR164, miR171, and miR396 were downregulated, and miR393, miR444, and miR827 were upregulated, respectively (Xin et al. 2010).

Next-generation sequencing approach was used to identify the novel and conserved miRNA in soybean during different stress conditions. The study revealed the downregulation of miR166, miR169, miR397, and MIR-Seq13 genes in the susceptible genotype during infection with *Phakopsora pachyrhizi* (Kulcheski et al. 2011). Three different cultivars of soybean were analyzed for the miRNA expression using microarray technology. Analysis showed that miR1507, miR1508, miR1510, miR159, miR319, miR396, and miR482 are negatively regulated, whereas families of miR156, miR166, and miR171 were positively regulated by *Phytophthora sojae* infection (Guo et al. 2011).

Tomato miRNAs regulated by *Botrytis cinerea* have been identified through microarray-based analysis. miR160 and miR171a were downregulated and miR169 was upregulated during *Botrytis* infection (Jin et al. 2011). Microarray analysis of *Populus trichocarpa* plantlets infected with the poplar stem canker pathogen, *Botryosphaeria dothidea*, revealed upregulation of 12 miRNAs, namely, miR156, miR159, miR160, miR164, miR166, miR168, miR172, miR319, miR398, miR408, miR1448, and miR1450, but no downregulation of any miRNA was observed (Zhao et al. 2012).

Verticillium wilt, caused by the fungal pathogen *Verticillium dahliae*, is a vascular disease which threats many economically important crops including cotton and eggplant. Small RNA libraries were constructed by cotton root tissue inoculated with the fungal spores; on comparison with the control and other cultivar tissues, 215 miRNA families were identified including the 14 novel miRNA families. During the fungal infection, more than 65 miRNA families showed change in their expression among the libraries. Similarly, in eggplant during infection by *V. dahlia*, mock control and infected seedling were used for deep sequencing of small RNA. Sequencing results showed the presence of 99 known miRNA families and two putative miRNAs in eggplant. However, six miRNAs changed their expression during the fungal infection in eggplant. During the *Verticillium* infection, the small RNA pull is shifted from 21-nt to 24-nt reads in comparing with mock to infected samples. The same phenomenon was observed in both cotton and eggplant, representing the involvement of different small RNA silencing pathways during fungal infection (Yin et al. 2012; Yang et al. 2013b). A recent study on miRNA involvement in plant immunity was conducted in rice to reveal the role of miRNAs in plant response to the blast fungus *Magnaporthe oryzae*. A new rice miRNA, *Osa-miR7695*, was discovered and found to negatively regulate a natural resistance-associated macrophage protein 6 (OsNramp6). Overexpression of *Osa*-miR7696 in transgenic rice plants conferred enhanced resistance to rice blast infection. The study also highlights a miRNA-mediated regulation of OsNramp6 in disease resistance while illustrating the existence of a novel regulatory network that integrates miRNA function and mRNA processing in plant immunity (Campo et al. 2013).

32.6.4 miRNAs During Insect Attack

Very few reports are available for miRNA expression profiling after insect attack. Pandey et al. (2007) reported herbivory-induced change in small RNA transcriptome of plants. *Myzus persicae* (green peach aphid) produced signifi-

cantly less progeny on *Arabidopsis* miRNA pathway mutants, suggesting an important role of miRNAs during phloem-feeding insect, the green peach aphid (*Myzus persicae*), attack. Plants unable to process miRNAs respond to aphid infestation with increased induction of PHYTOALEXIN DEFICIENT3 (PAD3) and production of camalexin. Aphids ingest camalexin when feeding on *Arabidopsis* and were more successful on pad3 and cyp79b2/cyp79b3 mutants defective in camalexin production. Aphids produced less progeny on artificial diets containing camalexin. miRNAs important for the growth and development of pests can be a potential target for raising resistant cultivars. This work also highlights the extensive role of the miRNA-mediated regulation of secondary metabolic defense pathways with relevance to resistance against a hemipteran pest (Kettles et al. 2013).

32.6.5 miRNAs During Nematode Infestation

miRNAs were also suggested to be involved in plant–nematode interactions. miR161, miR164, miR167a, miR172c, miR396c, miR396a, miR396b, and miR398a were downregulated in *Arabidopsis* in response to infection by the nematode *Heterodera schachtii* (Hewezi et al. 2008; Khraiwesh et al. 2012). Comparative analysis of miRNA profiling in soybean indicated that 101 miRNAs belonging to 40 families were responsive to the infection of the soybean cyst nematode (SCN; *Heterodera glycines*), the most devastating pathogen in soybean. Twenty miRNAs were differentially expressed between SCN-resistant and SCN-susceptible soybean cultivars (Li et al. 2012).

32.7 Role of miRNAs During Abiotic Stress Responses

Abiotic stresses are the major constraint for the agricultural productivity. Also, to combat the oversized population and exploitation of fertile land, we need to use the unusable land for farming in geographically different areas. Many abiotic stresses cause decrease in plant productivity among which drought, salinity, cold, and heat stress are common. miRNAs are involved in abiotic stress responses in plants and are well studied. The following part provides some examples of miRNAs and their role during abiotic stresses.

32.7.1 Drought and Salinity Stress

Drought and salinity are two major constraints to agricultural productivity worldwide. In addition to protein-coding genes, the expression of miRNAs in plants is altered during stress conditions (Sunkar and Zhu 2004; Zhao et al. 2007; Trindade et al. 2010; Kulcheski et al. 2011; Sunkar et al. 2012). The miRNAs that have been identified as stress responsive in diverse plant species are too numerous. We have listed below the recently identified miRNAs which have been reported to be involved in stress response regulation.

Nuclear factor Y (NF-Y) is a heterotrimeric transcription factor composed of NF-YA, NF-YB, and NF-YC proteins. *GmNFYA3* encodes the NF-YA subunit of the NF-Y complex in soybeans (*Glycine max* L.). Coexpression in *Nicotiana benthamiana* and 5′ RACE assays indicated that miR169 directs GmNFYA3 mRNA cleavage in vivo. miR169 was demonstrated to be a positive modulator of drought stress tolerance in transgenic *Arabidopsis* plants (Ni et al. 2013). In tomato, miR169 was induced by drought stress, and constitutive overexpression of tomato miR169c led to reduced stomatal openings and transpiration rate and leaf water loss, thus enhancing drought tolerance in transgenic plants compared with wild-type controls. The transgenic tomato plants did not show any morphological and developmental variations under field conditions, demonstrating that miR169 or its targets could be potential candidate genes for genetic engineering to achieve enhanced abiotic stress tolerance in plants (Zhang et al. 2011c). Contrasting roles of miR169, i.e., positive regulator in tomato and negative regulator in *Arabidopsis*, suggest that the function of conserved miRNAs may vary from species to species.

miR319 is one of the first characterized and conserved miRNA families in plants and has been demonstrated to target TCP (for TEOSINTE BRANCHED/CYCLOIDEA/PROLIFERATING CELL FACTORS [*PCF*]) gene-encoding plant-specific transcription factors. The role of miR319 during abiotic stress was demonstrated using transgenic creeping bent grass (*Agrostis stolonifera*) overexpressing a rice (*Oryza sativa*) miR319 gene, *Osa*-miR319a. Transgenic plants overexpressing *Osa*-miR319a displayed morphological changes and exhibited enhanced drought and salt tolerance. Gene expression analysis indicated that at least four putative miR319 target genes, *As*PCF5, *As*PCF6, *As*PCF8, and *As*TCP14, and a homologue of the rice NAC domain gene *As*NAC60 were downregulated in transgenic plants. The results clearly demonstrate that miR319 controls plant responses to drought and salinity stress. The enhanced abiotic stress tolerance in transgenic plants can be related to significant downregulation of miR319 target genes, implying their potential for use in the development of novel molecular strategies to genetically engineer crop species for enhanced resistance to environmental stress (Zhou et al. 2013b).

32.7.2 Cold and Heat Stress

Temperature fluctuations over the course of each day and during changes in the seasons are common, and plants have to reprogram their gene expression to adjust to such dramatic shifts in temperature. miRNAs have been identified in plants recently in response to temperature stress. Cold-responsive gene regulatory networks are regulated by a number of miRNAs. Comparative profiling of miRNA expression among different species [*Arabidopsis*, *Brachypodium*, and poplar (*Populus trichocarpa*)] during cold stress revealed similarities as well as differences in miRNA regulation. miR397 and miR169 were upregulated in all the three, while miR172 upregulation was observed in *Arabidopsis* and *Brachypodium* but not in poplar (Zhang et al. 2009). Opposing patterns of miRNA expression during cold stress have also been reported in other plant species. During cold stress, miR168 levels were upregulated in poplar and *Arabidopsis* (Lu et al. 2008b; Liu et al. 2008) but downregulated in rice (Lv et al. 2010). Similarly, miR171 was downregulated in rice (Lv et al. 2010) but upregulated in *Arabidopsis* (Liu et al. 2008) in response to cold stress. Some of these conflicting reports could be genuine responses that may be attributed to the differential responses of unrelated plant species.

The miR319 family is one of the conserved miRNA families among diverse plant species which is reported to regulate plant development in dicotyledons, but little is known at present about its functions in monocotyledons. In rice, the miR*319* gene family comprises two members, *Osa*-miR319a and *Osa*-miR319b. Overexpression of *Osa*-miR319a and *Osa-miR319b* in rice both resulted in wider leaf blades. Leaves of *Osa*-miR319 overexpression transgenic plants showed an increased number of longitudinal small veins, which probably accounted for the increased leaf blade width. In addition, overexpressing *Osa*-miR319 led to enhanced cold tolerance (4 °C) after chilling acclimation (12 °C) in transgenic rice seedlings. Notably, under both 4 and 12 °C low temperatures, *Osa*-miR319a and *Osa*-miR319b were downregulated, while the expression of miR319-targeted genes was induced. Furthermore, downregulating the expression of *OsPCF5* and *OsPCF8* (target genes of miR319) in RNA interference (RNAi) plants also resulted in enhanced cold tolerance after chilling acclimation. The study demonstrated that miR319 plays important roles in leaf morphogenesis and cold tolerance in rice (Yang et al. 2013a).

Powdery mildew infection and high temperature are important limiting factors for yield and grain quality in wheat production. Long non-protein-coding RNAs (npcRNAs) are developmentally regulated and play roles in development and stress responses of plants. Several putative wheat stress-responsive long npcRNAs (approx. 125) were computationally identified which were not conserved among plant species. Among them, some were precursors of small RNAs such as miRNAs and siRNAs, two

long npcRNAs were identified as signal recognition particle (SRP) 7S RNA variants, and three were characterized as U3 snoRNAs. Wheat long npcRNAs showed tissue-dependent expression patterns and were responsive to powdery mildew infection and heat stress (Xin et al. 2011).

Heat stress rapidly induces miR398 and reduces transcripts of its target genes CSD1, CSD2 (copper/zinc superoxide dismutase), and CCS (a gene encoding a copper chaperone for both CSD1 and CSD2). Transgenic plants expressing miR398-resistant forms of CSD1, CSD2, and CCS under the control of their native promoters were more sensitive to heat stress (as indicated by increased damage at the whole-plant level and to flowers) than transgenic plants expressing normal coding sequences of CSD1, CSD2, or CCS under the control of their native promoters. In contrast, *csd1*, *csd2*, and *ccs* mutant plants were more heat tolerant (as indicated by less damage to flowers) than the wild type. Expression of genes encoding heat stress transcription factors (HSF genes) and heat shock proteins (*HSP* genes) was reduced in heat-sensitive transgenic plants expressing miR398-resistant forms of CSD1, CSD2, or CCS but is enhanced in the heat-tolerant *csd1*, *csd2*, and *ccs* plants. The study indicated that miR398 triggers a regulatory loop that is critical for thermotolerance in *Arabidopsis* (Guan et al. 2013).

32.8 miRNA Interference: Applications in Agriculture

Food security is a major problem across the globe to fulfill the needs of a growing population. The limitation of fertile land and use of harmful chemicals for agriculture practices also pose a major constraint to the environment. To overcome these problems, resistant and high-yielding cultivars are in demand. The transgenic crops using novel and advanced technologies ensure us to give high-yielding crop and utilization of unfertile land with minimum environmental harms. miRNA interference (miRNAi) is a recent and novel approach (Wang 2009) which can be used to overcome agricultural harms by manipulating expression of miRNA genes or the endogenous genes (Rajam 2012).

32.8.1 Deregulation of miRNAs (Overexpression and Downregulation)

miRNAs can be studied by changing their expression levels either by overexpression or downregulation of miRNA genes which ultimately regulate target transcript levels and result in particular phenotype. miRNA genes are present in genome as independent transcriptional units having upstream promoter and cis-acting elements. Overexpression studies can be done by using binary vectors or any plant transformation vector having the functional miRNA gene with upstream native or constitutive promoter. Downregulation of miRNA can be possible either by expressing antisense miRNA (antimiR) or by overexpressing the miRNA-resistant target transcript in plants. antimiR functions by simply binding with mature miRNAs, whereas miRNA-resistant target transcript does not cleave because of nucleotide substitution at miRNA-binding site (Rajam 2012; Guo et al. 2005).

Most of the miRNA overexpression studies were carried out in *A. thaliana* as it is a model plant system for molecular studies. Various kinds of overexpression studies were carried out to functionally characterize miRNA genes. For instance report, inducible expression of miR164 in wild-type plants led to reduction in NAC1 mRNA levels and reduced lateral root emergence, while miR164 mutant showed low level of miR164 and increased NAC1 transcript resulting in more lateral roots (Guo et al. 2005). Similarly, stomatal density on epidermis in *Arabidopsis* is regulated by Agamous-like16 (AGL16) gene, a member of the MADS-box protein family, which is in turn regulated by miRNA824. Overexpression of miR160 resulted in agravitropic roots and increased the number of lateral roots in *Arabidopsis* by manipulating auxin-signaling genes (Wang et al. 2005). In another study, overexpression of miR824-resistant AGL16 shows increased incidence of sto-

mata, while overexpression of miR824 resulted in decreased incidence of stomata in higher-order complexes (Kutter et al. 2007). *At*-miR159 is involved in the regulation of short-day photoperiod flowering time and anther development. Overexpression of *At*-miR159 resulted in delayed flowering in short-day photoperiods and enhanced male sterility (Achard et al. 2004).

Rice is a major food crop worldwide and can be a good model crop to study novel molecular practices for crop improvement. Overexpression of *Osa*miR397 resulted in enlarged grain size and promoted panicle branching. It has been shown that *Osa*miR397 regulates the brassinosteroid signaling which resulted in increased grain yield (Zhang et al. 2013). In another report, overexpression of miR319a and miR319b resulted in wider leaf blades and enhanced cold tolerance in rice seedlings (Yang et al. 2013a).

Sly-miR169 accumulates during drought stress while their targets' nuclear factor Y subunit genes (SlNF-YA1/2/3) and one multidrug resistance-associated protein gene (SlMRP1) levels were reduced significantly in tomato. Overexpression of *Sly*-miR169 in tomato plants resulted in reduced stomatal opening, decreased transpiration rate, lowered leaf water loss, and enhanced drought tolerance (Zhang et al. 2011c).

These reports on different plant systems showed that miRNA overexpression may lead to some agriculturally important phenotypes that can be useful to develop high-yielding crops. Similarly, the miRNA genes of the target pathogens or pests essential for their growth and development can be suppressed by plant expression of an antisense RNA specific to their miRNA sequences. Downregulation or inhibition of miRNA gene expression can also provide a novel tool for agricultural application but has not been explored in plants (Rajam 2012).

32.8.2 Artificial MicroRNAs (amiRNAs)

The artificial microRNA (amiRNA) technology exploits endogenous miRNA precursors to generate small RNAs (sRNAs) that direct gene silencing in either plants or animals (Alvarez et al. 2006; Niu et al. 2006; Schwab et al. 2006). miRNA precursors preferentially produce the miRNA–miRNA* duplex. When both sequences are altered without changing structural features such as mismatches or bulges, it will lead to the accumulation of miRNA of desired sequence. amiRNAs were first generated and used in human cell lines (Zeng et al. 2002) and later in *Arabidopsis* (Parizotto et al. 2004), where they were shown to effectively interfere with reporter gene expression. Later on, it was demonstrated that not only reporter genes but also endogenous genes can be targeted with amiRNAs and also seem to work with similar efficiency in other plant species (Alvarez et al. 2006; Schwab et al. 2006).

amiRNA sequences are designed according to the determinants of plant miRNAs so that the selected 21 mer specifically silences its intended target genes. There are three major criteria which have to be taken care while designing the amiRNAs. Firstly, they should start with a U (found in most plant and animal miRNAs); secondly, they should display 5′ instability relative to their amiRNA* (the nucleotides pairing to the miRNA in the precursor); and thirdly, their tenth nucleotide should be either an A (Reynolds et al. 2004) for artificial siRNAs and overrepresented in plant miRNAs (Mallory et al. 2004) or a U.

So far, most of the amiRNAs in plants are based on the natural precursor structures of *Ath-miR159a*, *Ath*-miR164b, *Ath*-miR172a, *Ath-miR319a*, and *Osa*-miR528. Liu et al. (2010) developed a simple amiRNA vector (pAmiR169d) based on the structure of *Arabidopsis* miR169d precursor (pre-miR169d). amiRNAs are effective when expressed from either constitutive or tissue-specific promoters.

amiRNAs have shown its potential in the development of virus-resistant plants. Cotton leaf curl disease, caused by single-stranded DNA viruses of the genus *Begomovirus* (family *Geminiviridae*), is a major constraint to cotton cultivation. Two amiRNA constructs, (P1C) having the miR169a sequence with the exception of the replaced 21 nt of the V2 gene sequence of *Cotton leaf curl Burewala virus* (CLCuBuV) and

(P1D) in which the sequence of the miRNA169a backbone was altered to restore some of the hydrogen bonding of the mature miRNA duplex, were used to transform the *Nicotiana benthamiana*. P1C transgenic plants showed good resistance against CLCuBuV, whereas P1D plants showed overall poorer resistance to challenge with all viruses tested (Ali et al. 2013).

amiRNAs also offer a promising strategy to control the multiplication and spread of geminiviruses in host plants. amiRNAs target the middle region of the AV1 (coat protein) transcript (amiR-AV1-3) and the overlapping region of the AV1 and AV2 (pre-coat protein) transcripts (amiR-AV1-1). Our analyses demonstrate that transgenic tomato plants expressing amiR-AV1-1, targeting the coat protein of geminivirus, *Tomato leaf curl virus* (ToLCV), were highly tolerant to *Tomato leaf curl New Delhi virus* (ToLCNDV) (Vu et al. 2013).

The results show that the amiRNA approach can deliver efficient resistance in plants against viral infection and that this has the potential to provide broad-spectrum protection from a number of viruses.

32.8.3 miRNA-Induced Gene Silencing (MIGS)

Plants have mainly two kinds of small RNA, siRNAs and miRNAs; besides these, there is another class of siRNA which is generated by the pathway other than the previously known siRNA formation mechanism. The special class of siRNA is known as tasiRNAs, as they are generated by the miRNA-directed cleavage of TAS transcripts in *Arabidopsis* plant. The generation of tasiRNA required component of microRNA (miRNA) pathway (AGO1, DCL1, HEN1, and HYL1) and component of cosuppression pathway (RDR6 and SGS3) (Vazquez et al. 2004; Chapman and Carrington 2007).

Arabidopsis has two genes *TAS1* and *TAS2* which are the target of miR173 and produced 21 nt which targets the genes having partial similarity with *TAS* transcript. tasiRNAs regulate gene expression by the activity of SGS3, RDR6, and DCL4 (Vazquez et al. 2004; Yoshikawa et al. 2005). In similar fashion, miRNA390 cleaves *TAS3* transcript and produces tasiRNAs which have their target site in auxin-responsive factors. miRNA390 generated tasiRNAs which repressed the expression of the ARF2, ARF3, and ARF4, thus releasing repression of lateral root growth (Marin et al. 2010).

miRNA-directed cleavage of the transcript having *TAS* site upstream to the gene of interest can be exploited to target the endogenous gene for the functional study and for crop improvement; the strategy is now popularly known as miRNA-induced gene silencing (MIGS). Felippes and Weigel (2009) reported that a fragment of *CHLORATA42* (*CH42*) linked to a miRNA173 site lead to silencing of the endogenous *CH42* gene. It shows that only the miRNA target site of *TAS* transcript is sufficient for the production of tasiRNAs and silencing of the endogenous transcripts. This hypothesis was further supported by generating the chimeric constructs of genes *AGAMOUS* (*AG*), *EARLY FLOWERING 3* (*ELF3*), *FLOWERING LOCUS* (*FT*), and *LEAFY* (*LFY*) with upstream miRNA173 target site (22 nt). The *Arabidopsis* plant expressing chimeric RNA of *AG*, *ELF3*, *FT*, and *LFY* shows the silencing of the endogenous copy of genes and shows phenotype similar to loss-of-function mutants. The miRNA173-directed cleavage of the chimeric mRNA produces specific tasiRNAs that silence the endogenous gene (Felippes et al. 2012).

MIGS has many advantages over the hpRNA and amiRNA silencing strategy. The first advantage of MIGS is silencing construct preparation is very easy by only placing the miRNA173 target site (22 nt) on upstream of the partial or full region of desired gene. The chimeric construct produces specific tasiRNA and effectively silences the gene of interest. The second advantage is that MIGS provides an easy way to silence more than one diverse gene by simple preparation of a chimeric gene placed behind the miRNA target site. However, miR173 is only reported in the *Arabidopsis* plant; it limits the use of miR173-based MIGS in other crop plants. This problem can be overcome by overexpressing the

Ath-miR173 and then expressing chimeric transcript for silencing of desired genes in other systems. *Ath*-miR173 is not well conserved and its overexpression may not harm other plant systems.

32.9 Conclusions and Future Perspectives

MicroRNAs are a class of small noncoding RNAs implicated in a wide range of biological processes by means of regulation of gene expression. The majority of plant miRNA targets comprise the transcription factors and other regulatory proteins, indicating that miRNAs are major regulators of gene expression. miRNAs provide a promising tool for functional genomics to better understand the basic mechanisms underlying various plant developmental events. Mutants of miRNA biogenesis genes have shown severe pleiotropic abnormalities. Overexpression of miRNAs and miRNA-resistant versions of particular miRNA targets shows a range of unusual phenotypes (Jones-Rhoades et al. 2006).

miRNAs are not only reported as crucial regulator of developmental processes, but they are also involved in plant abiotic and biotic stress responses. Many miRNA families are known to play important roles during abiotic stress such as cold, heat, drought, salt, etc. (Sunkar et al. 2012), and also during biotic stress like viral, bacterial, fungal, insect, and nematode attack (Balmer and Mauch-Mani 2013). miRNAs regulating stress-responsive genes can help to understand the way plants work under extreme environmental conditions and may be used for fine tuning the gene expression to make plants more stress tolerant. Over the years, miRNAs have emerged as an important tool for crop improvement. Indeed, work has already been successfully undertaken in this direction in plants like *Arabidopsis* and tomato. This may help to reduce food insecurity by breeding improved crop cultivars with enhanced agronomic traits.

Although miRNAs are potential tools for crop improvement, undesirable phenotypic changes in transgenic plants can be problematic. Thus, it is mandatory to study the underlying mechanisms of miRNA-mediated regulation in plants for proper execution of transgenic strategies so as to obtain desired traits with minimal off-target effects.

Hence, we can conclude that agricultural biotechnology along with miRNA-based techniques would serve as an important measure for improvement of crop plants.

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Environmental Biotechnology: A Quest for Sustainable Solutions

33

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Abstract

The most important component for subsistence of both biotic and abiotic components on earth is the environment. The survival and well-being of both these components depend upon a healthy physical environment. The solution for today's deteriorating environment is not only removal of the contaminants but also recycling and reuse of hazardous substances by converting waste to wealth in an aesthetic and eco-friendly manner. At present, an emerging way for the management of wastes is the surfacing of environmental biotechnology. In simple words, environmental biotechnology can be defined as "the integration of natural sciences and engineering in order to achieve the application of organisms, cells, parts thereof and molecular analogues for the protection and restoration of the quality of the environment". It can be used as a tool to maintain healthy equilibrium between environment and its living and nonliving components. This chapter deals with different domains of environmental biotechnology related to decontamination of polluted environment that serve as sustainable solutions for many associated environmental problems.

Keywords

Contamination • Environmental biotechnology • Restoration • Sustainable solutions

33.1 Introduction

There has been a tremendous increase in the amount of stress created on environment due to the unstoppable and unavoidable increase in industrialisation and urbanisation. The ever increasing demands of humans for better living have made the situation more complex. In the race for better living, man himself has led to problems like bio-

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magnification, emission of carbon dioxide leading to acidification of oceans, global warming as a result of release of greenhouse gases, introduction of foreign plant species (invasive species) leading to disturbed biodiversity, etc. Production of smog due to ever increasing air pollution has reduced the amount of sunlight reaching earth affecting the photosynthesis process in plants. The environment has gone through undesirable changes due to man-made activities like agricultural, industrial, constructional, exploration and recreational activities. Industrial activities comprise of oil refineries that are the biggest pollution generators in present era which process crude oil to obtain valuable products like LPG, kerosene, petrol, jet fuel, diesel oil, etc., resulting in generation of hazardous waste as an end product called oil sludge. This oil sludge is a major waste generated by oil refineries. Also, contamination occurs during its transport, storage and disposal. This sludge contains persistent compounds like PAH and aliphatic hydrocarbons that remain in the environment over a period of time and cause harmful effects on humans. They are known to impose carcinogenic, mutagenic and teratogenic properties on human health (Bamforth and Singleton 2005). Oil spills also have an adverse effect on plants and animals as well. Lungs, liver, kidney and intestines of aquatic birds and animals may get damaged due to exposure to crude oil. Furthermore, plants covered with oil are unable to photosynthesise (Mandal et al. 2011).

Mining activities especially opencast mining significantly devastate the land ecosystem, in which the land gets degraded and contaminated which subsequently results in pollution of nearby waterbodies, streams and groundwater leading to frightening environmental problems. Opencast mining activities involve the removal of large quantities of overburden, dumping and backfilling of the excavated area (Juwarkar and Jambhulkar 2008). Metalliferous mining and processing usually produce severe pollution of heavy metals. These processes leave the land infertile or barren which is more prone to erosion and leaching which further distributes pollutants in the nearby environment and, thus, disturbs the surrounding ecological balance.

The agricultural activities encompass the use of pesticides and chemical fertilisers for pest control and increased crop production which has led to land contamination rendering the soil to be infertile. The run-off of excess pesticides in local waterbodies has manifested several health problems such as headache, body weakness, blurred vision, vomiting, irritability, impaired concentration and abdominal pain. Other severe effects caused due to pesticides include suppressed immune system, asthma, reduced sperm concentration and vigour, liver diseases and nerve damage. The pesticide methyl bromide has been identified to have ozone-depleting properties (Ibitayo 2006).

Furthermore, one of the greatest challenges for the scientific community for the past few decades has been the environmental contamination caused due to wastewater. The wastewater generated by industries or during domestic and agricultural purposes has been reported to contain many harmful and toxic compounds which, upon unscientific discharge/disposal, leads to the contamination of nearby environment.

Owing to these detrimental effects of different contaminants, it is necessary to sustain the environment. Environmental biotechnology can serve as a solution to this nuisance. Environmental biotechnology is defined as "the integration of natural sciences and engineering in order to achieve the application of organisms, cells, parts thereof and molecular analogues for the protection and restoration of the quality of our environment" (Ezeonu et al. 2012) . This chapter concentrates on different technologies developed till date to sustain a healthy environment by remediating and reclaiming polluted environment by the use of plants and microorganisms as acting agents.

33.2 Acting Domains of Environmental Biotechnology

Figure 33.1 below depicts the domains, followed by a detailed description.

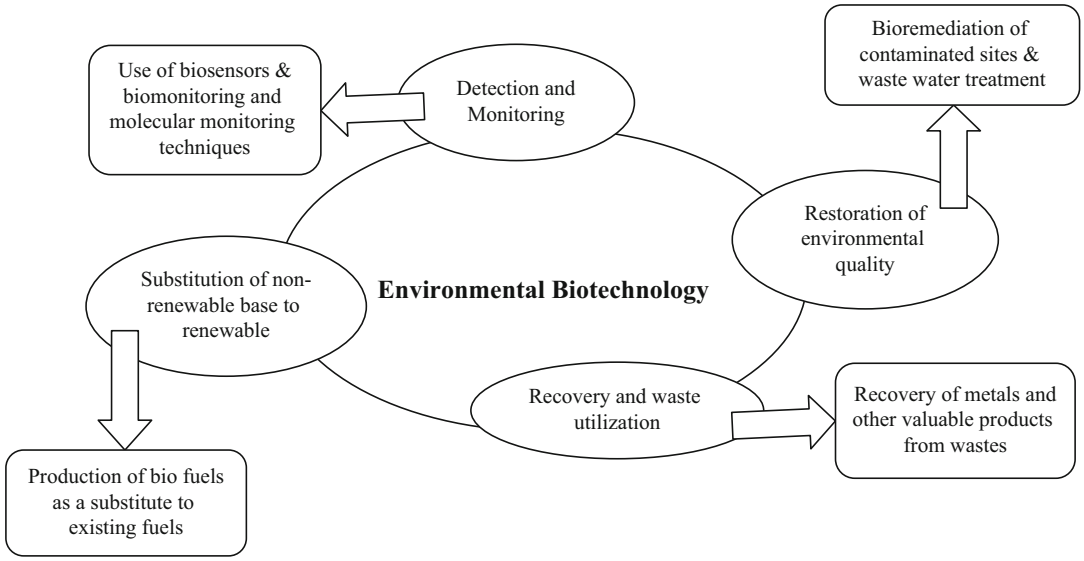

Fig. 33.1 Acting domains of environmental biotechnology

33.2.1 Restoration of Environmental Quality

The quality of environment is deteriorating due to attack from different hazardous chemicals and their components. The land resources were thought to be abundant in early times; however, our carelessness and negligence in using them have resulted in rendering these lands as barren and degraded. These lands are an effect of increased industrial activities in past few decades and disposal of hazardous substances. In response to the adverse health effects on humans as well as other living organisms created by contaminated sites, worldwide efforts are being undertaken by the scientific and regulatory community. The physico-chemical methods are not feasible in giving root solution to the problem of land contamination as it transfers the contaminant to other places and leads to formation of toxic breakdown products. Keeping in mind the need of present era, productive and clean land, an eco-friendly and sustainable technology needs to be introduced. Bioremediation technology can be an answer to the questions regarding the contaminated land-related questions. Bioremediation can be defined as "the use of living organisms to degrade the environmental contaminants into less toxic forms" (Vidali 2001). The living organisms are generally plants and microorganisms. By using bioremediation technology, the contaminated land can be redeveloped for use in an economic and environment-favouring approach. Bioremediation can be divided into different branches such as biostimulation, bioaugmentation, biodegradation, phytoremediation, biostabilisation, etc. Bioremediation can be in situ or ex situ. Ex situ remediation involves excavation of contaminated soil and treating it away from the site of contamination. In situ techniques are mostly favoured as it reduces the chances of further contamination during transport and also cuts the cost of carrying the contaminant to a desired place. In situ techniques generally remediate the contaminant in the same place either by addition of microorganisms (bioaugmentation) or by adding nutrients (biostimulation). Apart from these two techniques, plants can also be used to further reduce the contamination resulting in complete remediation of the site which is called phytoremediation (Juwarkar et al. 2010). Bioremediation processes generally make use of microorganism that uses the organic contaminates as their carbon source. Microorganisms during their co-metabolism process break down the contaminant to form carbon dioxide and water

resulting in its complete mineralisation. However, sometimes complete mineralisation may not occur, but the product formed may be either non-toxic or less toxic as compared to the parent contaminant (Suthersan 2010). These reactions are generally oxidation reactions. Microbes synthesise certain enzymes that catalyse these reactions during which the carbon double bond of organic compound is broken and the adjacent hydrogen atom is transferred to hydrogen receptor (Nanekar et al. 2015). Similarly, plants are also known to degrade and hold on to toxic components and help in remediation. Plants also secrete certain nutrient-rich exudates which contain enzymes, amino acids, organic acids, sugars and phenolics (Bias et al. 2006). These act as food for the organisms that thrive in the rhizosphere, fastening the degradation process. Also, they have the potential to accumulate metals and other organic compounds in the tissues and roots. Many contaminants with industrial and mining origin have been remediated using bioremediation technology. Juwarkar and Singh (2010) have successfully remediated zinc mine spoil dumps using this integrated biotechnological approach that has restored the productivity and fertility of the spoiled land. Also, organisms are being modified genetically to increase their degradation efficiency towards different contaminants. The bottlenecking in remediation of persistent contaminants can be circumvented by combination of different metabolic activities of different organisms in single organism or by enhancing the expression of gene responsible for degradation. Table 33.1 represents different bacteria and plants used for remediation purposes of different contaminants.

Biotechnological tools are already being used for wastewater treatment because these treatments can manage a broad range of effluents effectively as compared to chemical or physical methods. It is usually suitable for the treatment of wastewater having common organic pollutants. The use of microorganisms for wastewater treatment mainly depends on the source and characteristics of wastewater. Three types of wastewater usually categorised on the basis of their source are domestic/municipal wastewater, industrial/commercial wastewater and agricultural wastewater. The components in the effluent may be of physical, chemical or biological nature which can pose certain hazardous impacts on the environment which include changes in habitat of nearby vicinity especially aquatic habitat, changes in biodiversity and water quality, etc. Owing to very diverse parameters, it is very difficult to form the most effective microbial consortia and treatment strategy for wastewaters. Usually, these wastewaters require pretreatment as many of the components in wastewater are toxic to the microorganisms. The unstable organic matter of wastewater is converted into stale products such as CO, CO_2, CH_4, NH_3, etc. Molecular techniques can be used for biotreatment of wastewater and are a quite new field. The major application of these techniques may include enhanced degradation of xenobiotic contaminants and detection of pathogens and parasites using nucleic acid probes. The process of wastewater treatment process can be enhanced by selection of novel organisms so as to perform a certain action (Gavrilescu 2010).

33.2.2 Recovery and Waste Utilisation

Environmental biotechnology is creating new revolution by introducing genetically engineered organisms to extract their potential in production of valuable products from wastes. Also, waste can be utilised for synthesis of products like biosurfactants for use in remediation. Dubey and Juwarkar (2001) have studied biosurfactant production using distillery waste and whey waste by *Pseudomonas aeruginosa* species isolated from oil sludge. According to their observations, the culture produced 0.91 and 0.92 g/l biosurfactant using distillery and whey wastes for its growth, respectively.

Crop waste can be used as raw material for ethanol production using genetically modified organisms that form sugars and ultimately ethanol by synthesising increased amounts of cellulosic enzymes. This bioethanol may reduce the use of fossil fuels. Also, as it is derived from wastes, bioethanol can be used for carbon dioxide recycling in a closed loop, i.e. from the atmosphere to plant and from plant back to ethanol (Kim and Dale 2004).

Table 33.1 Genetically modified plants/bacteria and their role

Sr. no.	Species (plant/microorganism)	Role	Reference
	Genetically modified bacteria		
1	*Pseudomonas fluorescens* HK44	Naphthalene degradation	Sayler and Ripp (2000)
2	*Pseudomonas fluorescens* F113rifpcbrrnBP1::gfpmut3	Chlorinated biphenyl degradation	Boldt et al. (2004)
3	*Pseudomonas putida* PaW85	Petroleum degradation	Jussila et al. (2007)
4	*Escherichia coli*	Bioaccumulation of mercury	Deng and Wilson (2001)
5	*Escherichia coli* strain	Remediation of arsenic-contaminated water	Kostal et al. (2004)
6	*Achromobacter* sp. *AO22*	In situ bioremediation of Hg-contaminated sites	Ng et al. (2009)
7	*Bacillus subtilis BR151* (*pTOO24*)	Cd (naturally polluted soils)	Ivask et al. (2011)
8	*Pseudomonas fluorescens 4F39*	Remediation of Ni	Lopez et al. (2002)
9	*Pseudomonas putida*	Remediation of Cr	Ackerley et al. (2004)
	Transgenic plants		
10	Transgenic maize	Enhanced provitamin A content	Aluru et al. (2008)
11	Transgenic maize	Stress tolerance	James (2006)
12	GM tobacco	Chloroacetanilide herbicide phytoremediation	Macek et al. (2007)
13	*Lycopersicon esculentum*	Remediation of phenol	Oller et al. (2005)
14	*Nicotiana tabacum*	Remediation of pentachlorophenol	Limura et al. (2002)
15	*N. tabacum*	Remediation of bisphenol A and pentachlorophenol	Sonoki et al. (2005)
16	*N. tabacum* and *Arabidopsis thaliana*	Tolerance to herbicide	Didierjean et al. (2002)

Many bacteria and fungi are efficient in solubilising metals that can be extracted from metal-rich soils under appropriate environmental conditions (Krebs et al. 1997). The principles behind the ability of microbes to leach and mobilise metal are redox reactions, formation of organic or inorganic acids and excretion of complexing agents. Metals are extracted by the principle of either electron transfer from metal to the microorganism or on bacterial oxidation of metal ion (Torma 1991). Abidin et al. (2011) in their studies concluded that *Durio zibethinus* husk is an efficient adsorbent for gold (III) and can be used as a low-cost alternative to adsorb gold (III) in wastewater treatment.

33.2.3 Substitution of Non-renewable Base to Renewable

The endless need of fossil fuels as energy resources has created two problems, i.e. scarcity of available resources and pollution created by its burning. Also as it is a non-renewable source of energy, it will end at some time or other. Hence, there is a need to find a substitute for these energy-rich fuels. Biogas can serve as a substitute for conventional fuels which is also considered as economic over LPG. Ananthakrishnan and his co-workers (2013) have installed a kitchen waste biodigester and have found it economical and technically viable over LPG.

The importance of biologically produced fuels has increased as a result of dramatic variations in oil prices and global apprehension for climatic changes. Biofuels are being encouraged as the most capable substitutes by deducing the dependence on fossils fuels, supporting local economy and reducing CO2 emissions. Production of biofuels from renewable resources can be seen as a desirable means for meeting the needs of aviation and other transport facilities. Immense interest is concentrated on biofuels as they are produced from photosynthetic plants which store lipids in the form of triacylglycerols (TAGs). These TAGs can be used for synthesis of biodiesel through a transesterification process (Smith et al. 2009). An igniting interest is developing in algaculture for

making biodiesel, biogasoline, biomethanol and many other biofuels owing to the energy and food crisis worldwide making use of land that is either not under agricultural use or is not suitable for it. The algal fuels are gaining interest due to attractive characteristics that they can be grown using ocean and wastewater with minimal impact on freshwater and are relatively harmless to the environment if spilled owing to their biodegradable nature.

Potts and his coresearchers (2011) have successfully synthesised biobutanol from macroalgae growing in Jamaica Bay, New York City. The algae growing in the Bay containing nitrates, phosphates and carbon dioxide coming from the atmosphere were dried, grinded and subjected to hydrolysis to extract carbohydrate ultimately forming algal sugar solution. The butanol formed was removed by distillation process. Bacterial strains *Clostridium beijerinckii* and *C. saccharoperbutylacetonicum* were also used in the process to reach a final concentration of 0.29 g butanol/g sugar.

Chisti (2007) in his work mentioned that microalgae can be used for production of biodiesel. Microalgae are cell factories that use sunlight to convert carbon dioxide to biofuels, foods and high-value bioactivities. Making use of this quality, it can be used to produce a variety of biofuels like methane by anaerobic digestion of algal biomass biodiesel derived from microalgal oil and photobiologically produced biohydrogen.

33.3 Detection and Monitoring

33.3.1 For Pollution/Pollutants

Detection and monitoring of pollution is equally important as treatment or remediation. The direct means to detect pollution are keeping a record of the amount of pollutant, whereas the indirect ones include recording of changes in biotic and abiotic factors. For example, the estimation of biochemical oxygen demand (BOD) can give a direct estimation of pollution levels of a waterbody as the greater the amount of pollutant organic matter present in the waterbody, the greater the amount of microorganisms to break it and greater the amount of dissolved oxygen is required. The indirect means include biotic index which is a scale that gives measure of an ecosystem quality by presence, absence or abundance of a particular species. Organisms surviving in the environment are exposed to many chemicals and tend to accumulate these chemicals in varying concentrations. Gerdol et al. (2000) performed a survey for deposition of heavy metal in mountain territories of northern Italy. The moss samples (*Hylocomium splendens*) were collected from dense network sites, and the data was correlated to metal concentration and climatic factors.

A variety of biological methods are available for monitoring of pollutants and detection of pollution at different places. Many bacteria and viruses are used to monitor aquatic environment where water is used for drinking. *Escherichia coli* is an indicator species for contamination of water by domestic and industrial sewage discharges (Borrego et al. 1987). The use of living agents as monitoring tools is known as biomonitoring. The recent advancement in the field of monitoring includes methods like biosensors and immunoassays.

Biosensors are analytical application of biocatalysts such as isolated enzymes, tissues or organelles or whole cells. These biocatalysts are immobilised on physico-chemical device that monitors the activity by chemical transformation of the substance/substrate (Neujahr 1984). Biosensors are analytical tools and have advantages of being highly sensitive, rapid, reproducible and easy to use. Owing to their poor stability in solutions, immobilisation plays an important role to develop a stable biocomponent with transducers for integration (Kumar and D'Souza 2012). An optical microbial biosensor was described by Kumar et al. (2006) for the detection of methyl parathion. The biocomponent consisted of whole cells of *Flavobacterium* sp. immobilised by trapping in glass fibre filter. The immobilised species produced organophosphorus hydrolase that hydrolyses methyl parathion into detectable product p-nitrophenol. For this reason, the use of disposable microbial biocomponent for the detection of methyl parathion with optical biosensor

was a simple, single-step and direct measurement of very low quantity of the sample also.

33.3.2 For Bioremediation

Bioremediation, as mentioned above, is intended to improve the quality of contaminated environment. For an effective bioremediation to take place, survival of the augmented organisms is essential as they are the degrading army. For this, several monitoring assays have been listed till date. Luciferase markers are useful in monitoring inoculated consortiums. The bacteria tagged with bacterial luciferase genes (*luxAB*) can easily be detected and counted with their luminescent colonies on agar plates (Jansson et al. 2000).

The green fluorescent protein (GFP) which is coded by gfp gene from a jellyfish *Aequorea victoria* is also used as a marker to monitor bacterial cells in the environment. Southern blot techniques have been used to monitor naphthalene degraders in soil mesocosms where probes specific for the *nah* operon were used to determine naphthalene degradation potential of the microbes. Dot-blot hybridisations have been used to measure the level of PCB-degrading organisms in soil environments with isolated polychlorinated biphenyl (PCB) catabolic genes (Widada et al. 2002).

Fazi et al. (2008) in their study have mentioned that PCR-based methods are used for monitoring the presence of *Dehalococcoides* sp. which is capable of completely dechlorinating chloroethenes to harmless ethene. Also at field level, real-time quantitative PCR (qPCR) for the 16S rRNA gene is the most widely used technique. Another powerful tool for the detection and quantification of key degradative microorganisms is fluorescence in situ hybridisation (FISH).

33.4 Present and Future of Environmental Biotechnology

The key service that environmental biotechnology provides to the developing era is detoxification of different contaminated matrices to retrieve lost assets and converting them to forms that can be used by the society with ease. The emergence of environmental biotechnology has created a robust paradigm when it comes to the fields like remediation and treatment of contaminated sites, production of wealth from waste, improvisations in technologies to monitor both pollutant and pollution, etc. However, there is still a wide scope to intertwine molecular biology and microbiology as the key science lying underneath environmental biotechnology is microbial ecology. The awareness and management of different fundamental biological processes can be achieved by probing the environment and using different methods on genomic, proteomic and metabolomic levels. To ensure that the microbial communities provide the desired services, they have to be managed. Genetic modification of organisms and developing different monitoring tools are the most anticipated areas of work in the field of environmental biotechnology. Introducing these newly developed techniques at field level will help to lessen the problems created due to contaminants and cure the environment.

33.5 Conclusions

Biotechnology has established itself in many areas and upcoming developments assure to widen its scope. With new developments in treating the contaminated environment, the emerging list of what can be practised or remediated by simple and feasible means is increasing. As we move on to the next millennium, the problems will grow as urbanisation and industrialisation continue to mount. With this increasing load of pollution and contamination, the role of environmental biotechnology as a tool also grows for remediation and environmentally sensitive industry. Environmental biotechnology, so far, has proven its potential in many areas like wastewater treatment and contaminated land reclamation, production of economically important products by genetic modifications, monitoring the contaminants making use of biosensors, etc. Therefore, environmental biotechnology is well positioned to add to the development of a more sustainable

environment. The quest continues as time runs introducing new problems to deteriorate the quality of environment giving an opportunity for environmental biotechnology to discover new horizons.

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Phytoremediation: General Account and Its Application

34

Jitendra K. Sharma and Asha A. Juwarkar

Abstract

Developmental activities have resulted in contamination of the environment and thus disturbed the ecosystem. One of the main agenda for scientific community has been remediation of contaminated sites which has been expensive and intrusive to the ecosystem. Phytoremediation is a set of different processes/techniques which uses plants for containment, destruction, or extraction of contaminants. Various processes involved in phytoremediation are phytodegradation, phytoextraction, rhizofiltration, phytostabilization, phytovolatilization, and hydraulic control. This chapter highlights various processes involved in phytoremediation along with the essential components of phytoremediation. Emphasis is also given on recent advancements in phytoremediation in which microbe-assisted phytoremediation and use of transgenic plants have been discussed. This technology has been receiving attention because of its cost-effectiveness and environment-friendly approach. Therefore, phytoremediation may open a new avenue for a broader and more efficient and sustainable solution for the remediation of contaminated sites.

Keywords

Contamination • Phytoremediation • Heavy metals • Microbe-assisted phytoremediation • Transgenic plants

34.1 Introduction

Ever since man has started developmental activities, it has made his life more and more convenient. However, these activities are also having adverse impact on the environment owing to exploitation of natural resources, destruction of biodiversity, and use of chemicals. This has led to the disturbance in the ecosystem. The developmental activities such

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as manufacturing, transportation, and construction not only exhaust the natural resources but also lead to production and release of huge quantity of waste material which in turn leads to environmental pollution. The dispersion of various contaminants generated by various industrial activities has led to contamination of soil and water. Environmental pollution is not only a health issue but also an important social issue in which pollution has the capabilities to devastate homes and communities. Untreated or inappropriately treated waste is a main reason of pollution and environmental degradation which ultimately causes ill effects on human and environmental health.

A range of organic and inorganic chemicals are responsible for environmental contamination. These include heavy metals, hazardous wastes, explosives, pesticides, petroleum products, etc. (Ghosh and Singh 2005). Major constituent of inorganic pollutants is heavy metals which present various problems to the environment, while organic contaminants pose different kinds of problems to the environment. While many metals are essential, they are toxic at higher concentrations as they lead to oxidative stress because of the formation of free radicals. Although numerous regulatory measures have been enforced to reduce or limit the discharge of pollutants in soil, they are not adequate for checking the pollution.

Therefore, the need for the management and remediation of contaminated sites has been an issue of global concern, and researchers all over the globe are working extensively on formulating cost-effective and environmentally sound technologies for decontamination of these sites. Several methods are developed to manage or remediate these pollutants which include physical, chemical, and biological methods. Biological methods have an advantage over physical and chemical methods being cost incurred and environment friendly but have limitations with respect to efficiency (Juwarkar et al. 2014; Sharma et al. 2014). Soil microflora is able to degrade most of the organic contaminants, whereas metals need immobilization or physical removal. All of us should be aware of the cost that has to be paid to remediate the mess that has resulted from our careless practices. However, it is also obvious that extensive research is essential to develop cost-effective and more efficient methods for decontamination of contaminated soils. Bioremediation is one such technology that is gaining global attention which uses biological organisms such as microbes or plants for cleanup of contaminated sites (Juwarkar et al. 2014).

Microbes offer practical solutions to the environmental contamination, whereas plants of many different diversities present different solutions. The utility of plants seems to be eternal. They provide oxygen to breath; they are the basis of agriculture, medicines, fuel, fiber, oils, and industries. Besides this, plants can also serve as a tool for cleanup of contaminated environment as well as CO_2 sink. The use of plants for remediation of environmental contamination is termed as *phytoremediation*. Phytoremediation has also been expressed as green remediation, agroremediation, botano-remediation, and vegetative remediation. Phytoremediation implies the use of natural or genetically engineered/modified plants, many a times, together with their associated rhizospheric microbes which encourage plant growth and decontaminate the contaminated soil in combination with the plants. Plants extract heavy metals, naturally occurring aromatic and hydrocarbon compounds, and anthropogenic chemicals (pesticides, herbicides, fungicides, industrial chemicals, etc.). This approach has been established to be relatively economical, efficient, and environment friendly.

Plants survive by utilizing the resources available in their surrounding environment as they struggle for light, nutrients, and water. Phytoremediation involves understanding and exploiting specific plants in a best way to clean up the chemical imbalance created in the environment. The understanding of site's physiochemical properties, climatic conditions, type of contamination, site-specific plants and associated microbes, etc., helps to develop an effective phytoremediation system. Phytoremediation has been successfully adopted for remediation of several contaminants around the globe. Phytoremediation covers a variety of methods/processes which can lead to degradation, removal (through accumulation), or immobilization of the contaminant.

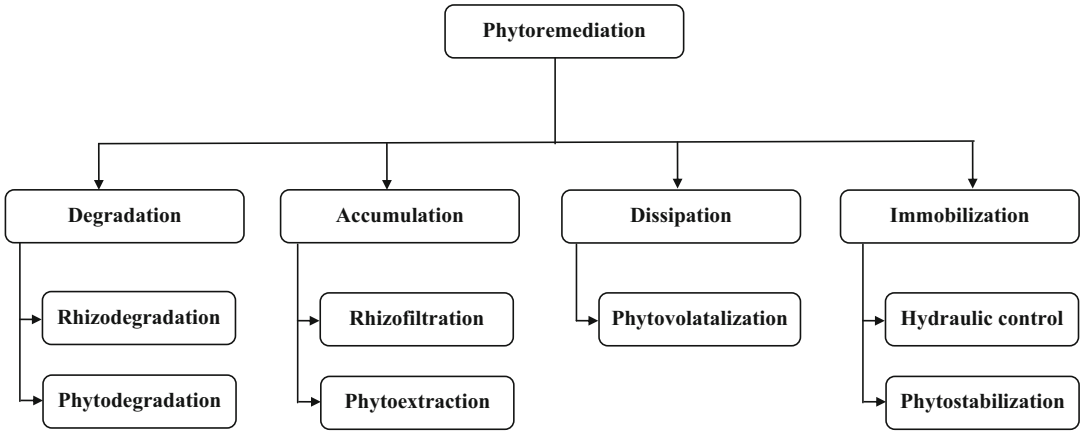

Fig. 34.1 Various processes of phytoremediation

Figure 34.1 depicts various processes involved in phytoremediation. The process of phytoremediation depends mainly on the type of plant and nature of the contaminant. Different processes involved in phytoremediation will be discussed individually along with their application in detail in this chapter. With each phytoremediation process/method, it is essential that the undesired transfer of contaminant to other media should be avoided. Phytoremediation is efficient in the removal or cleanup of different types of contaminants such as petroleum hydrocarbons, heavy metals, pentachlorophenol (PCP), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), etc. (Pivetz 2001). Simple plantation and/or vegetation is not sufficient for phytoremediation. Selection of appropriate plant species, its resistance to the contaminant, and role of different amendments in plant growth play a key role in effective implementation of phytoremediation.

Although thousands of plants have been studied by various researchers for their phytoremediation potential of various contaminants, a limited number of plants have shown significant results which can be applied on the field. For successful phytoremediation to occur, screening studies are an important aspect in selecting a suitable plant species (Vishnoi and Srivastava 2008). The results can be extrapolated from the data of hydroponic or greenhouse studies which can be then transferred to the field. Verification of applicability and efficacy of different methods of phytoremediation is needed on site-specific manner which is required to establish a solid technology for remediation. Nevertheless, phytoremediation necessitates dedication of resources and time but has shown the possibility to present a cost-effective, environmentally sound alternative to conventional remediation technologies at suitable sites.

34.2 Essential Plant's Metabolic Processes for Phytoremediation

Phytoremediation takes benefit of inherent processes of plants. These processes include uptake of water and chemicals, metabolism inside the plant, release of exudates into the soil, and biochemical and physical impacts of plant roots. Growth of plants mainly depends on photosynthesis, in which water and carbon dioxide are transformed into carbohydrates and oxygen by means of the energy from sunlight. The roots of plants are effective in extracting water held in soil and are also capable of water uptake even from negative osmotic potential through xylem.

Transpiration is another process which occurs primarily at the stomata in which there is loss of water from plant into the atmosphere in the form of vapor. Carbon dioxide uptake from atmosphere takes place via stomata, along with release of oxygen. Respiration carbohydrates formed

through photosynthesis and production of ATP are essential for the active transport of nutrients and require oxygen. Plants need macronutrients (N, P, K, Ca, Mg, S) and micronutrients (B, Cl, Cu, Fe, Mn, Mo, Zn, Co, Ni, Se, Si, V) for optimum growth (Vishnoi and Srivastava 2008). Nutrient uptake pathways can extract contaminants which are similar in chemical behavior or form to the required nutrients. The uptake of contaminant by plants largely depends on the hydrophobicity (Vangronsveld et al. 2009), polarity, type of plant, age of contaminant, and physicochemical properties of soil. Also, exudates released by plant roots along with the root-associated bacteria affect the uptake of various contaminants by the plant (Sun et al. 2010).

34.3 Different Processes/ Techniques in Phytoremediation

There are a number of different processes which will be discussed in this chapter along with their application. Table 34.1 briefs various processes/ techniques of phytoremediation along with the mechanisms involved and contaminants treated. Defining these processes is useful to elucidate and understand different mechanisms that may occur due to vegetation. Fate of the contaminant and necessary steps to be taken for effective phytoremediation must be defined. Different types of contaminants and contaminated media may require different types of phytoremediation processes specific to the type of contamination. Table 34.2 highlights various plants used for phytoremediation of various contaminants by various researchers over the period of time. Various processes involved in phytoremediation are described below.

34.3.1 Degradation

Degradation is usually accounted for organic contaminants. It is the breakdown or transformation of complex organic compound to simple and less toxic compounds. In phytoremediation, two types of degradation may occur depending upon the type of contaminant and plant: phytodegradation and rhizodegradation.

34.3.2 Phytodegradation

The process of phytodegradation usually implies uptake, metabolism, and degradation of contaminants inside the plant (Wenzel 2009). It may also happen in the soil, sediments, sludges, surface water, or groundwater by means of enzymes released by plant. Phytodegradation is a contaminant destruction process which is also known as phytotransformation. It is reported that the degradation of organic pollutants is driven by

Table 34.1 Overview of phytoremediation

Technique	Mechanism	Media	Contaminant
Phyto/rhizodegradation	Enzyme-catalyzed destruction of contaminants	Soil, sediment, sludges, groundwater	Organic compounds: PAHs, pesticides, PCBs, chlorinated solvents, etc.
Phytoextraction	Uptake and accumulation of contaminants in the plant	Soil, sediment, sludges	Metals: As, Cr, Cd, Cu, Hg, Pd, Mn, Ni, etc.
Rhizofiltration	Uptake/extraction of contaminants into the plant roots	Groundwater, surface water	Metals: Cr, Cd, As, etc.
Phytostabilization	Containment of contaminant	Soil, sediment, sludges, mine tailing	Metals: As, Cr, Cd, Cu, Hg, Pd, Mn, Ni, Zn, etc.
Phytovolatilization	Removal of contaminants by evapotranspiration	Soil, sediment, sludges, groundwater	Chlorinated solvents and inorganics such as Se, Hg, etc.
Hydraulic control	Degradation and/or containment of contaminant	Groundwater, surface water	Water-soluble organics and inorganics

Table 34.2 Plants used for phytoremediation of different contaminants

Genus/species	Contaminant	Reference
Eucalyptus urophylla X E. *grandis*	Pb	Peng et al. (2012)
Medicago sativa	Benzopyrene, PAEs, and PAHs	Sun et al. (2011a), Fu et al. (2012)
Nephrolepis exaltata	Hg	Chen et al. (2009)
Pteridium esculentum and *Silene vulgaris*	As, Zn, and Cd	Robinson et al. (2006)
Pteris vittata	As, Hg, Cs, and Sr	Chen et al. (2009), Wang et al. (2002)
Raphanus sativus	Cu	Choudhary et al. (2009)
Thlaspi caerulescens	Cd and Zn	McCutcheon and Schnoor (2003)
Brassica juncea	Phorate	Rani and Juwarkar (2012), Rani et al. (2012)
Panicum virgatum and *Populus deltoides*	PCBs	Meggo et al. (2013)
S. plumbizincicola, *E. splendens*, and *M. sativa*	PCBs, Cd, Cu	Wu et al. (2012)
Jatropha curcas	As, Cr, Zn	Kumar et al. (2008)
Medicago sativa L, *Festuca arundinacea* Schreb., and *Vetiveria zizanioides*	PAHs	Sun et al. (2011b), Nanekar et al. (2013)

metabolic processes of plants (Prasad and Freitas 2003). Contaminants such as chlorinated solvents, herbicides, pesticides, and other organic contaminants may be eliminated or reduced by employing phytodegradation process.

In this process, plant metabolism contributes to the reduction of contaminant by means of different metabolic processes such as transformation, breakdown, or volatilization, and the simpler breakdown products then are incorporated into plant tissues. Certain enzymes in plants such as dehalogenases, oxygenases, and reductases are responsible for the breakdown of various pollutants into simpler forms (Ghosh and Singh 2005).

34.3.3 Rhizodegradation

Rhizodegradation is the breakdown of contaminants through the influence of plant roots which ideally results in destruction of contaminant. Naturally occurring microorganisms such as bacteria, fungi, and actinomycetes can breakdown the organic contaminants in soil into less toxic products or sometimes completely mineralize to carbon dioxide and water. Plant roots have an ability to enhance the number and variety of microbial populations in the rhizosphere which ultimately leads to enhanced degradation of contaminant (Rani and Juwarkar 2012). Plant roots release exudates in the form of sugars, amino acids, organic acids, fatty acids, sterols, growth factors, nucleotides, flavanones, enzymes, etc., which increase the activity of the rhizospheric microflora subsequently resulting in increased contaminant biodegradation. Rhizodegradation primarily occurs in soil. Alteration in soil geochemical conditions such as pH takes place because of the release of exudates that may result in alteration in the transportation of contaminants (Pivetz 2001). The plant roots also positively affect the soil properties such as water content, water and nutrient transport, aeration, structure, temperature, etc., which facilitate more favorable conditions for soil microflora, besides the release of exudates.

34.3.4 Phytoextraction

Phytoextraction is one of the best processes for the removal of contaminants from soil which implies uptake of contaminant by plant roots with subsequent accumulation of the contaminant in the plant's aboveground portion, which is usually followed by harvest and eventual disposal of plant biomass (Wenzel 2009). It is also called as phytoaccumulation. It is a process of contaminant removal. Phytoextraction is a process which generally applies to metals (e.g., Ag, Cd, Co, Cr,

Cu, Hg, Mn, Mo, Ni, Pb, Zn), metalloids (e.g., As, Se), radionuclides (e.g., 90 Sr, 137 Cs, 234 U, 238 U), and nonmetals (e.g., B) (Salt et al. 1995; Pivetz 2001), because these contaminants in general are not further degraded within the plant. Phytoextraction/phytoaccumulation has also been referred as phytomining or biomining. A brief definition of phytomining is the utilization of plants to achieve an economic return from metals extracted by a plant.

Phytoextraction usually has not been considered for uptake of organic contaminants as these can be metabolized or volatilized by the plant and thus prevents the accumulation of contaminant within the plant. The uptake of a specific compound is determined by its lipophilicity described by its octanol water partition coefficient (K_{ow}). Therefore, hydrophobicity of a contaminant increases with increase in K_{ow}, and it has been studied that the compounds with K_{ow} value >3 are usually adsorbed by the roots and not subjected to uptake (Gaskin 2008; Imfeld et al. 2009). However, there have been few studies reporting the phytoaccumulation of organic contaminants. Researchers have shown interest in studying the metal-accumulating plants termed as hyperaccumulators which can accumulate a higher degree of metals for phytoremediation studies. The target medium in this process is usually soil; however, sediments, sludges, and groundwater can also be subjected to phytoextraction in conjunction with rhizofiltration. Zhang et al. (2009) reported reduction in exchangeable Cd in the planted soil after phytoextraction experiment using maize. Similarly, the decreased level of Cd in soil planted with maize was also documented (Mojiri 2011).

In order to craft this process economically and environmentally feasible, the plants must accumulate large concentrations of contaminants (e.g., heavy metals) into their roots with its subsequent translocation to surface biomass followed by generation of large quantity of plant biomass. Factors such as growth rate, disease resistance, element selectivity, etc., are also imperative in phytoextraction (Ghosh and Singh 2005).

34.3.5 Rhizofiltration

Rhizofiltration is the process which implies the removal of contaminants by plant roots by precipitating, concentrating, and absorbing the contaminant (Juwarkar et al. 2010). This process usually applies to surface water, wastewater, or extracted groundwater either through adsorption or on the roots or through absorption into the roots. The roots of plants release secondary metabolites called as root exudates which may lead to the biogeochemical conditions resulting in precipitation of contaminants into the water body or on the roots. Depending on the type of contaminant, its concentration, and plant species, the contaminant may remain adsorbed on the root, absorbed into the roots, or translocated into the aboveground portion of the plant. Rhizofiltration can be carried out in situ to remediate contaminated surface water bodies or ex situ, in which the engineered system of tanks is used to hold the externally supplied contaminated water and the plants. Both the systems necessitate an understanding of the contaminant speciation and their interactions within the system. Rhizofiltration and phytoextraction are having similarity for accumulation of contaminants on or in the plant. While rhizofiltration accumulates contaminants in or on the roots with a portion of contaminant subsequent translocation into other parts of the plants, phytoextraction occurs above the ground and not in the roots. Additionally, rhizofiltration differs from phytoextraction as the contaminant is primarily in water, rather than in soil. In a hydroponic experiment carried out by Abhilash et al. (2009b), the potential of *Limnocharis flava* (*L.*) for phytofiltration of Cd in contaminated water with low concentrations of Cd was investigated.

34.3.6 Phytostabilization

Phytostabilization is the use of plants to restrain soil contaminants in situ, through alteration of the chemical, biological, and physical conditions in the soil. Phytostabilization is also known as

phytoimmobilization (Wenzel 2009). Phytostabilization can take place through sorption, precipitation, complexation, or metal valence reduction (Ghosh and Singh 2005). The plants' key purpose is to reduce the amount of water percolating in the soil environment, which may lead to the formation of hazardous leachate and prevent erosion of soil to reduce the chances of distribution of hazardous contaminants to nearby area. The transport of contaminant in soil, sediments, or sludges can be reduced through absorption and accumulation in roots, adsorption on the roots, and complexation, precipitation, or metal valence reduction in soil. Further, the plantation reduces wind and water erosion of soil, thereby reduces spreading of the contaminant because of runoff, and may also prevent leachate generation. Phytostabilization research has generally focused on metal contamination such as lead, chromium, and mercury which are being identified among top potential candidates for phytostabilization (Pivetz 2001).

Alvarenga et al. (2009) studied the effect of the sewage sludge, organic residues, garden waste compost, and municipal solid waste compost on the phytostabilization of a metal-contaminated soil. Similarly, municipal solid waste was utilized for reclamation of coal mine spoil dump (Juwarkar and Singh 2007).

34.3.7 Phytovolatilization

Phytovolatilization employs an uptake of a contaminant by the plant and subsequent release of a volatile contaminant which is a volatile degradation product of original contaminant or a volatile form of a primarily nonvolatile contaminant (Wenzel 2009; Juwarkar et al. 2010). Phytovolatilization takes place as growing trees or plants absorb water along with organic and inorganic contaminants. Phytovolatilization is mainly a contaminant removal process, in which the transfer of the contaminant takes place from the original matrix, i.e., soil or groundwater, to the atmosphere. The metabolic processes occurring in the plant are responsible for alteration of contaminant to less toxic form. In some cases, the transfer of contaminant to the atmosphere enables much more efficient and effective natural degradation to occur, for example, photodegradation of the volatilized contaminants. While phytovolatilization leads to transfer of contaminants into the atmosphere, a risk analysis of this process on the ecosystem including humans becomes essential. Phytovolatilization has been mainly employed for the elimination of mercury, in which there is a transformation of mercuric ion into less toxic elemental mercury that takes place (Henry 2000). This process also depends on the type of contaminant, climatic condition, type of plant, and the soil type.

34.3.8 Hydraulic Control

Hydraulic control (or hydraulic plume control) is the use of plants to control the movement of groundwater and/or soil water, because of uptake and consumption of large quantity of water. Hydraulic control may influence and potentially hold the movement of a groundwater plume, lessen or prevent infiltration and leaching, and encourage upward flow of water from the water table (Pivetz 2001). Other phytoremediation processes, such as phytodegradation, rhizodegradation, and phytovolatilization, may take place as the contaminated water is brought to and into the plant. Water uptake by vegetation and transpiration rates is vital for hydraulic control and remediation of groundwater. This water uptake and the transpiration rate depend upon the species, age, mass, size, leaf surface area, and growth stage of the vegetation. They are also affected by various climatic factors, such as temperature, humidity, precipitation, etc.

34.4 Things to Be Considered for Successful Phytoremediation Implementation

Prior to initiating the phytoremediation for a contaminated site, it is essential to evaluate whether natural attenuation processes such as

biodegradation, photodegradation, dispersion, sorption, or volatilization would attain site-specific remediation in a particular time frame. If site-specific natural attenuation processes are capable of reducing the contaminant concentration, then that site may not necessarily be the candidate for phytoremediation.

Successful plant growth strongly depends on climatic conditions. Proper timing of precipitation, optimum amount of sunlight, shade, wind, proper air temperature, and growing season length are required to ensure optimum growth of plants. The study of site-specific plants to select a proper species can prove to be very beneficial. Soil amendment is a key requirement for the successful implementation of phytoremediation on contaminated sites. The maintenance of proper pH, nutrients, and soil water content is an essential factor for plant growth. Amendments such as fly ash, pig manure, sewage sludge, etc., are effective in lowering the toxicity of contaminant in soil and offer a slow release of nutrients such as N, P, and K to support growth of plants. However, potential adverse effects of their addition must be considered before they are added (Juwarkar and Singh 2010). Also, the type and concentration of contaminant is an imperative aspect to be studied before implementation of phytoremediation.

34.5 Advantages and Limitations of Phytoremediation

Phytoremediation on the one hand has advantages over conventional remediation technologies, while on the other hand, it has few disadvantages. The advantages and disadvantages of phytoremediation are described below.

34.5.1 Advantages

- Phytoremediation is an in situ technique which limits environmental disturbance and reduces cost.
- It is particularly well studied for the treatment of large area of surface contamination especially where other methods may not be cost-effective.
- Phytoremediation involves exploiting the natural ability of the environment to restore itself; therefore, it is a widely acceptable method for remediation of contaminants.
- Plant sample can be used as indicators to know the extent of remediation or conversely contamination.
- Plants help to contain the region of contamination by removing water from soil, thereby keeping the contaminants from spreading or confining them within or near the root system.
- Some wetland plants can transport oxygen to the rhizosphere that is available to microorganisms that allows aerobic degradation of a wide variety of organic contaminants.
- Phytoremediation leads to the improvement of soil quality by improving soil structure.
- Phytoremediation increases the porosity and therefore water infiltration, providing nutrients (nitrogen-fixing legumes) and accelerating nutrient cycling, and increases soil organic carbon.
- The use of plant in remediation efforts stabilizes the soil, thus preventing the soil erosion.
- Phytoremediation eliminates secondary air or waterborne waste; an example is the accumulation of PAHs from the atmosphere.
- It has the potential as a sink to CO_2, a known greenhouse gas that causes global warming.
- Apart from above benefits, it helps in greenbelt formation, thus improving the floral diversity.

34.5.2 Limitations

- Phytoremediation is slower than other ex situ remediation methods for the removal of contaminants from the environment.
- Phytoremediation requires long period and several seasons for site cleanup.
- Because it is slow, phytoremediation is not an appropriate method where the target contaminant poses an immediate threat to human health or the environment.
- If the contaminant is bound tightly on the soil particles or organic matter, it may not be available to plants for degradation.

- Environmental conditions like soil texture, pH, salinity, oxygen availability, temperature, and level of nonhydrocarbon contaminants such as metals must be within limits tolerable to plants.

34.6 Recent Advancements in Phytoremediation

Researchers working on biological remediation of toxic contaminants have shown their faith in phytoremediation owing to its additional advantages such as enhanced efficiency and CO_2 sequestration capability. In recent times, considerable work has been going on throughout the globe to enhance the phytoremediation efficiency. Genetic engineering has been employed to produce transgenic plants with enhanced efficiency for phytoremediation since past few years. Microbe-assisted phytoremediation (MAP), in which the use of selected plant species and site-specific bacteria is employed for enhanced remediation potential, has also been proved to produce significant results.

34.6.1 Microbe-Assisted Phytoremediation (MAP)

The limitations of phytoremediation, predominantly those associated with low efficiency and restrictions to the use of contaminant-containing biomass, could be dealt through novel biotechnological approaches that exploit recent advances in our understanding of interactions between plants and rhizospheric microorganisms and within plant tissues. Many plants employ interactions with rhizospheric microbes to survive in toxic and nutrient-restricted environments (Weyens et al. 2009; Abhilash et al. 2012). Plants secrete a wide range of chemicals in root exudates which usually help in the enhanced growth of rhizospheric microbes. These root exudates include organic acids, phenolic compounds, amino acids, etc., which play an important role in plant-microbe interactions (Tanimoto 2005; Crowley and Kraemer 2007). This process has been termed as microbe-assisted phytoremediation (MAP) technology which is a modern, very efficient, and successful phytoremediation technology for the remediation of degraded lands (Juwarkar and Singh 2010). Figure 34.2 shows schematic representation of MAP. This process mainly involves bioaugmentation with site-specific microorganisms to promote faster degradation of the contaminant along with plant. It can be best applied at sites with comparatively shallow contamination of pollutants that are amenable to the processes such as phytotransformation, rhizosphere bioremediation, phytostabilization, phytoextraction, and/or rhizofiltration. MAP has been successfully implemented for restoration of different types of contaminated lands (Juwarkar 2012).

34.6.2 Transgenic Plants in Phytoremediation

Plant breeds having superior potential for phytoremediation with elevated biomass production can be an option to improve phytoremediation.

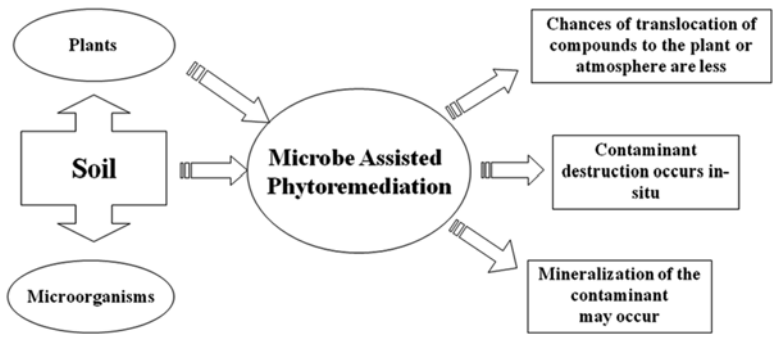

Fig. 34.2 Schematic representation of microbe-assisted phytoremediation

The productivity of plant is controlled by several genes. The development of proteomics, genomics, and metabolomics has contributed greatly to boost or manipulate the plant metabolism of several xenobiotic pollutants. Genetic engineering has potential to amplify the efficiency and use of phytoremediation, in which plants can be genetically modified using specific bacterial, fungal, or plant genes which possess useful properties for contaminant degradation, uptake, and transformation. Initially, the transgenic plants were used to improve crop productivity and reduce the use of pesticide. Genetic engineering techniques to incorporate more efficient hyperaccumulator gene into plants have been recommended by many authors (Chaney et al. 2000; Abhilash et al. 2009a; Juwarkar 2012). Insertion of multiple genes such as cytochrome P450s, GSH, and GT for complete degradation of xenobiotics in plant is among the promising development in transgenic technology (Aken 2008; Doty 2008; Abhilash et al. 2009a). Another approach which can be adopted is the development of genetically engineered plants with the ability to release degradation-promoting enzymes in the rhizospheric zone. Genetically modified transgenic plants were first developed for phytoremediation to remediate heavy metal-contaminated soils, for example, *Nicotiana tabacum* expressing a yeast metallothionein gene for higher tolerance to cadmium or *Arabidopsis thaliana* overexpressing a mercuric ion reductase gene for higher tolerance to mercury (Abhilash et al. 2009a).

34.7 Future Prospects and Conclusions

Phytoremediation is a quite new and attractive technology which has emerged in recent years and offers practical advantages over traditional remediation technologies. It offers tremendous perspectives for the development of cost-effective and efficient processes for decontamination of a wide range of pollutants. It has proved to have high potential for degradation of organic pollutants in soil, sediments, and groundwaters. Also the capability of plants to hyperaccumulate metals can be deployed in heavy metal-contaminated sites. As highlighted above, it offers several ways to get rid of contaminants in soil, sediments and/or, contaminated water. It is a solar driven, cost-effective, and natural cleanup technology which works best even in shallow levels of contamination. However, there are certain limitations in implementation of phytoremediation for which more research is required. Scientific community now posses a very good understanding of the biochemical processes involved in the detoxification of contaminants within plant cells. One of the most important aspects for researchers is to improve the efficiency of phytoremediation as it is essential to recognize the prices incurred in remediation process. After all, money matters! There are challenges which are required to be addressed especially when specific sites require customized approach for decontamination owing to the unique complexity of the location. The use of nanoparticles also can be proved effective in enhancing the efficiency of phytoremediation (Abhilash et al. 2012). Researchers have studied that CeO_2 and ZnO nanoparticles showed a positive impact on growth of root and shoot in edible plants like soybean, wheat, corn, and alfalfa (López-Moreno et al. 2010a, b), suggesting that nanotechnology can significantly enhance the efficiency of phytoremediation. The use of transgenic, MAPT, and nanotechnological approach can lead to the development of effective and efficient phytoremediation technology which can deal with a wide range of contaminants. Future interest will require receiving public and regulatory acceptance for successful implementation of phytoremediation using transgenic plants.

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Marine Biotechnology: Potentials of Marine Microbes and Algae with Reference to Pharmacological and Commercial Values

35

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Abstract

Marine biotechnology explores marine organisms as a prolific source for attractive biomolecules with broad biotechnological applications. This review dictates the potentials from natural products of both free-living and associated forms of marine bacteria, actinobacteria, cyanobacteria, fungi, and algae towards pharmacological and industrial importance. In accordance with the pharmacological perspectives, secondary metabolites, explored during the recent decade of 2003–2013, with significant cytotoxicity on human and murine cancer cells, antimicrobial and antiprotozoal properties, inhibition of metabolic key enzymes/proteins, and anti-inflammatory and antioxidant activities were emphasized here. Recent studies (2010–2014) towards biofuel and antifouling in the deal of industrial applications were highlighted. Undoubtedly, marine animals, viz., sponges, corals, mollusks, prochordates, etc., are the potential source of bioactive natural products with wider applications, but extensive exploration for their valuable chemicals left them in the dangerous zone. In order to save the bioresources and extend the research in marine microbes and macroalgae, this review is confined within marine bacterial, fungal, and algal applications. But the tool of metagenomics is devoid of such limitations; metagenome sample from marine source is sufficient to construct metagenome library so as to investigate the potentials by employing either activity-based screening or sequence-based screening approaches. Furthermore, many of marine microbes and algae are actively engaged in genetic engineering studies in applied aspects.

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Keywords

Marine bacteria • Cyanobacteria • Fungi • Algae • Cytotoxicity • Antimicrobial • Antiprotozoal • Antifouling • Anti-inflammatory • Antioxidants • Biofuel • Metagenomics

35.1 Introduction

Marine environment is a hidden treasure to be discovered a lot and biotechnology is one of the tools aiding to explore. Marine organisms adapted to survive in their environment by synthesizing unique chemicals in order to compete for nutrition, offense, and defense and manage varying physical and chemical factors. Such products can alternatively be used for our pharmacological and industrial needs. Among the potential genera identified for various biotechnological applications, the following marine forms are found to be promising candidates: marine bacteria, *Bacillus*, *Erythrobacter*, *Halomonas*, and *Rapidithrix*; marine actinobacteria, *Nocardia*, *Streptomyces*, *Salinispora*, etc.; marine cyanobacteria, *Lyngbya*, *Leptolyngbya*, *Oscillatoria*, *Symploca*, *Rivularia*, etc.; marine fungi, *Acremonium*, *Acrostalagmus*, *Aigialus*, *Apiospora*, *Arthrinium*, *Aspergillus*, *Chaetomium*, *Coniothyrium*, *Diaporthe*, *Emericella*, *Exserohilum*, *Fusarium*, *Gliocladium*, *Gymnascella*, *Metarhizium*, *Neosartorya*, *Penicillium*, *Phoma*, *Scytalidium*, *Spicaria*, *Trichoderma*, *Zygosporium*, etc.; and marine algae, *Ulva*, *Sargassum*, *Turbinaria*, *Laminaria*, *Padina*, *Rhodomela*, etc. Bioactive metabolites produced by these organisms are pertaining to various chemical groups including short peptides (linear peptide, cyclic peptide, and cyclopeptide – head to tail linkage of amino acids/its derivatives; glycolipopeptide and depsipeptide – one or more amide bonds (-CO-NH-) are replaced by ester bonds (-CO-O-); peptolide – cyclodepsipeptide), terpenes (containing isoprene units; diterpene contains four isoprene units, meroterpene is a partial terpenoid, and sesterterpene contains five isoprene units), terpenoid (in addition to isoprene units, it also bears other functional groups), azoles (five-membered heterocycle containing nitrogen; thiazole/thiazoline are sulfur-containing azoles), lactones (cyclic ester), macrolides (macrocyclic lactones), polyketides (containing alternate carbonyl and methylene (-CH2-) groups), fatty acid amide, quinones (even number of methine (=CH-) group in benzene ring is replaced by carbonyl (-C(=O)-) group to yield quinone; naphthoquinone, naphthalene containing; anthraquinone, anthracene containing), heterocycles (cyclic ring made by at least one atom other than carbon), alkaloids (basic nitrogenous heterocyclic compounds), carboline (pyrido indole), quinoline (nitrogen-containing heterocycle), exopolysaccharides (polymer of one/more sugar residues), etc. Many of such marine metabolites display notable anti-neoplastic property by exerting cytotoxicity on various human and murine cancer cell lines (Pettit et al. 2009; Shao et al. 2010; Hu and Macmillan 2011; Balunas et al. 2012 ; Boudreau et al. 2012; Eamvijarn et al. 2013; Huang et al. 2012; Shaala et al. 2013; Wang et al. 2012). Certain enzymes/proteins are specifically responsible for pathogenicity and diseases and are targeted with novel drugs. A few of such drug targets including proteases, protein tyrosine phosphatases, p53/MDM2 interaction, SrtA, HDAC, voltage-gated channels, AchE, P450, etc., are effectively inhibited by marine-derived compounds (Wen et al. 2008; Pontius et al. 2008b; Malloy et al. 2012a; Nunnery et al. 2012; Julianti et al. 2013; Liu et al. 2013; Salvador et al. 2013; Pavlik et al. 2013).

Tuberculosis, candidiasis, flu, malaria, leishmaniasis, etc., are prevalent in India and often pose increased risk on human health. Developing resistance against the drugs is a characteristic of causative agents. This urges to develop new drugs for the treatment, and marine microbes provide a

plenty of scope as potential resource. These marine forms also render anti-inflammatory products to mitigate the host inflammatory response, since inflammatory disorders result in hypersensitive reaction, viz., allergies, autoimmune diseases, etc. (Huang et al. 2007; Kong et al. 2010; Hwang et al. 2011; Choi et al. 2012). Drugs/toxins and normal cellular respiration will induce the production of reactive oxygen species (ROS) free radicals that damage cell membrane, DNA, and proteins. Antioxidants quench free radicals and prevent ROS-mediated cell injury. Specifically, marine algae are enriched with antioxidant metabolites/pigments and thus serve as a good biosource of antioxidants (Chen et al. 2011; Mohankumar et al. 2012; Wang et al. 2013a; Li et al. 2014).

Large complex biofilm formation (biofouling) on the vessels in marine environment is annoying ship transport and a causative of huge economic loss for marine industries. Inorganic biocides not only prevent biofouling but also hurt harmless marine creatures. Demand of ecosafe antifoulants can be met with potential marine products. Global demand for the fuel is exponentially increasing; biotechnology offers fuel production from biological sources. Both marine micro- and macroalgae are being used for the biofuel production (Qin et al. 2005; Kraan 2013; Merlin et al. 2013).

Marine metagenomics offers the mining of novel bioactive products and industrially valuable macromolecules from the uncultivable marine microbes and marine animals either through functional-based or sequence-based approaches (Donia et al. 2011; Felczykowska et al. 2012; He et al. 2012b, 2013b). For this purpose, metagenomic sample is sufficient rather than exploiting the biomass of the source organisms in a large quantity; thereby, bioresources will not be depleted. Moreover, genetic engineering utilizes the marine microbes and algae for the research and industrial values (Filonov et al. 2011; Montsant et al. 2004; Zaslavskaia et al. 2001). Hence, this review focuses the products from marine bacteria, fungi, and algae with pharmacological and industrial values.

35.2 Pharmacological Potentials

Natural products from marine microbes were determined to have various pharmacological values including anticancer/cytotoxicity and inhibition of specific drug target (enzymes/proteins), and antimicrobial, anti-inflammatory, and antioxidant properties are detailed.

35.2.1 Cytotoxicity/Antineoplastic Activity

Cytotoxicity of the marine microbial metabolites was observed on the various human cancer cell lines, viz., oral (KB), gastric (HM02, NUGC-3, GXF251L), hepatic (HepG2, SMMC-7721), breast (MCF7, MDA-MB-231, MAXF401NL), lung (A549, NCI-H460, NCI-H23, A549, H460, HuCCA-1), non-small-cell lung cancer (NSCLC) (H1325, H2122, HCC44, HCC366, LXF529L), pancreas (BXPC-3, PAXF1657L), CNS (SF-268), colon (KM20L2), prostate (DU-145, PC3), cervix (HeLa S3 cells, HeLa, Caski), ileocecal colorectal (HCT8), colorectal (HT-29, HCT116), ovary (SK-OV, COC1), leukemia (CCRF-CEM), lymphoblastic leukemia (MOLT-3), lymphoma (U937), melanoma (MEXF462NL), renal (RXF486L, RXF 393), blood and bone marrow (promyelocytic leukemia HL60), etc., and also on certain murine cancer cells, viz., mouse neuro-2a blastoma cells, murine leukemia (P388), mouse melanoma (B16), mouse fibroblast (NIH-3T3), mouse lymphocytic leukemia (L1210), etc. (Table 35.1).

35.2.1.1 Marine Bacterial Compounds

Marine bacterial cytotoxins are mainly falling in the groups of peptides, azoles, and terpenoids. Some of the compounds with cytotoxic properties at low concentration are discussed in detail. Pettit et al. (2009) have isolated two cyclodepsipeptides, bacillistatins 1 and 2, from marine bacteria *Bacillus silvestris*, which are obtained from a Pacific Ocean (southern Chile) crab and are found to be potential antineoplastic agents that

Table 35.1 Cytotoxic effect of marine microbial metabolites on various cancer cell lines

Metabolite	Chemical nature	Source	Target cell line	Concentration/effect	Reference
Marine bacterial metabolites					
2-aminophenoxazin-3-one (**1**), 2-amino- 6-hydroxyphenoxazin-3-one (**2**), and 2-amino-8-benzoyl-6-hydroxyphenoxazin-3-one (**3**)	Aminophenoxazin derivatives	γ-proteobacteria, *Halomonas* sp. strain GWS-BW-H8hM, East Frisian Wadden Sea	HM02 (gastric adenocarcinoma), HepG2 (hepatocellular carcinoma), and MCF7 (breast adenocarcinoma)	GI_{50} values of (**1**) 0.95, 1.4, and 0.13 μg mL^{-1} respectively; of (**2**) 1.6, 4.3, and 1.6 μg mL^{-1} respectively; and of (**3**) 1.4, 3.2, and 2.0 μg mL^{-1} respectively	Bitzer et al. (2006)
Ariakemicins A and B (mixture)	Linear peptides	Gliding bacterium, *Rapidithrix* sp.	A549 (human lung cancer cells) and BHK (baby hamster kidney cells)	IC_{50} values of 25 and 15 μg mL^{-1} respectively	Oku et al. (2008a)
Bacillistatins 1 (**a**) and 2 (**b**)	Cyclodepsipeptides	*Bacillus silvestris*	Human cancer cell lines: BXPC-3 (pancreas), MCF-7 (breast), SF-268 (CNS), NCI-H460 (lung); KM20L2 (colon) and DU-145 (prostate)	GI_{50} values of (**a**) 0.00095, 0.00061, 0.00045, 0.00230, 0.00087, and 0.00150 μg mL^{-1} respectively and of (**b**) 0.00034, 0.00031, 0.00180, 0.00045, 0.00026, and 0.00086 μg mL^{-1} respectively	Pettit et al. (2009)
Erythrazole B	Benzothiazole	*Erythrobacter* sp.	Non-small-cell lung cancer (NSCLC) cell lines: H1325, H2122, and HCC366	IC_{50} values of 1.5, 2.5, and 6.8 μM respectively	Hu and Macmillan (2011)
Erythrolic acid D	Meroterpenoid	*Erythrobacter* sp., isolated from marine sediment sample of Galveston, Texas	NSCLC cell lines: HCC44 and HCC366	IC_{50} values of 2.4 and 3.4 μM respectively	Hu et al. (2012)
Ieodoglucomide B	Glycolipopeptide	*Bacillus licheniformis*	NCI-H23 (lung cancer) and NUGC-3 (stomach cancer cells)	GI_{50} values of 25.18 and 17.78 μg mL^{-1} respectively	Tareq et al. (2012)
Marine actinobacterial metabolites					
N-(2-hydroxyphenyl)-2-phenazinamine (NHP)	Phenazines	*Nocardia dassonvillei*	HepG2, A549, HCT-116, COC1 cells	IC_{50} values of 40.33, 38.53, 27.82, and 28.11 μg mL^{-1} respectively	Gao et al. (2012)
Daryamides A, B, and C and (2E,4E)-7-methylocta-2,4-dienoic acid amide	Daryamides	*Streptomyces* strain CNQ-085	HCT-116 cells	IC_{50} values of 3.15, 9.99, 10.03, and 21.69 μg mL^{-1} respectively	Asolkar et al. (2006)

Mayamycin	Benz[a]anthracene	*Streptomyces* sp. strain HB202	HepG2 (hepatocellular carcinoma), HT-29 (colon adenocarcinoma), GXF251L (gastric cancer), LXF529L (non-small-cell lung cancer), MAXF401NL (mammary cancer), MEXF462NL (melanoma cancer), PAXF1657L (pancreatic cancer), RXF486L (renal cancer), NIH-3T3 (mouse fibroblast cell lines)	IC_{50} values of 0.2, 0.3, 0.2, 0.16, 0.29, 0.13, 0.15, 0.3, and 0.22 μM respectively	Schneemann et al. (2010)
Tetracenoquinocin (**1**), 5-iminoaranciamycin (**2**), and aranciamycin (**3**)	Anthracyclines	*Streptomyces* sp. Sp080513GE-26	HeLa cells and human acute myelogenous leukemia (LH-60) cells	IC_{50} values of (**1**) 120 and 210 μM, (**2**) IC_{50} >200 μM and (**3**) 2.7 and 4.1 μM respectively	Motohashi et al. (2010)
Lajollamycin	Nitro-tetraene spiro-*β*-lactone-γ- lactam	*Streptomyces nodosus* (NPS007994)	B16-F10 (tumor cells)	EC_{50} value of 9.6 μM	Manam et al. (2005)
Marine cyanobacterial metabolites					
Apratoxin H and apratoxin A sulfoxide	Depsipeptides	*Moorea producens*, collected from the Nabq mangroves, Red Sea	NCI-H460 (lung cancer cells)	IC_{50} values of 3.4 and 89.9 nM, respectively	Thornburg et al. (2013)
Bisebromoamide and norbisebromoamide	Peptides	*Lyngbya* sp.	HeLa S3 cells (cervix cells)	IC_{50} values of 40 and 45 ng mL^{-1} respectively	Sasaki et al. (2011)
Biselyngbyaside, biselyngbyolide A and biselyngbyaside B	Glycosidic macrolides	*Lyngbya* sp.	(**a**) HeLa S3 cells (**b**) HL60 cells (leukemia)	IC_{50} values against (**a**) 3.29, 0.22, and 3.5 μM respectively and against (**b**) 0.29, 0.027, and 0.82 μM respectively	Morita et al. (2012)
Coibacins A-D	Polyketides	*Oscillatoria* sp.	NCI-H460 cells	IC_{50} values of 31.5, 17.0, 21.3 and 11.4 μM respectively	Balunas et al. (2012)
Grassypeptolides D and E	Cyclic depsipeptides	*Leptolyngbya* sp., Red Sea	(**a**) HeLa (**b**) mouse neuro-2a blastoma cells	IC_{50} value against (**a**) 335 and 192 nM, respectively and against (**b**) 599 and 407 nM, respectively	Thornburg et al. (2011)

(continued)

Table 35.1 (continued)

Metabolite	Chemical nature	Source	Target cell line	Concentration/effect	Reference
Isomalyngamide A	Fatty acid amide	*Lyngbya majuscula*, Taiwan	MCF-7 and MDA-MB-231cells	IC_{50} values of 4.6 ± 1.0 and 2.8 ± 0.1 μM respectively	Chang et al. (2011)
Lagunamide C	Cyclodepsipeptide	*Lyngbya majuscula*, Pulau Hantu Besar, Singapore	P388 (murine leukemia), A549 (lung carcinoma), PC3 (prostate cancer), HCT8 (ileocecal colorectal adenocarcinoma), and SK-OV (ovarian) cancer cell lines	IC_{50} values of 24.4, 2.4, 2.6, 2.1, and 4.5 nM, respectively	Tripathi et al. (2011)
Malyngamide 4	Fatty acid amide	*Moorea producens*	MDA-MB-231, A549, and HT-29 cells	GI_{50} values of 44, 40, and 50 μM respectively	Shaala et al. (2013)
Pitipeptolides A and B	Cyclodepsipeptides	*Lyngbya majuscula*, Guam	(**a**) HT-29 (**b**) MCF7 cells	IC_{50} values against (**a**) 13 and 13 μM respectively and against (**b**) 13 and 11 μM respectively	Montaser et al. (2011)
Santacruzamate A	Amide	*Symploca* sp.	HCT116 and Hut-78 cells	GI_{50} values of 29.4 and 1.4 μM respectively	Pavlik et al. (2013)
Veraguamide A	Cyclic depsipeptide	*Oscillatoria margaritifera*, Panama	H-460 (human lung cancer cells)	LD_{50} of 141 nM	Mevers et al. (2011)
Veraguamides C, D, E, and G	Cyclic depsipeptides	*Symploca* cf. *hydnoides*, Guam	(**a**) HT-29 (**b**) HeLa cancer cells	IC_{50} values against (**a**) 5.8, 0.84, 1.5, and 2.7 μM and against (**b**) 6.1, 0.54, 0.83, and 2.3 μM respectively	Salvador et al. (2011)
Viequeamide A	Cyclic depsipeptide	*Rivularia* sp.	H-460 cells	IC_{50} value of 60 ± 10 nM	Boudreau et al. (2012)
Marine fungal metabolites					
07H239-A	Eremophilane sesquiterpene	Xylariaceous fungus, LL-07H239	CCRFCEM leukemia	IC_{50} value of 0.9 μg mL^{-1}	McDonald et al. (2004)
Anhydrofusarubin	Naphthoquinone	*Fusarium* sp. (no. b77)	HepG2 and Hep2 cells	IC_{50} values of 1.0 and 2.5 μg mL^{-1}, respectively	Shao et al. (2010)
Aspergilone A	Benzylazaphilone derivative	*Aspergillus* sp.	HL-60, MCF-7, and A-549 cells	IC_{50} values of 3.2, 25.0, and 37.0 μg mL^{-1}, respectively	Shao et al. (2011b)
Aspergiolide A	Anthraquinone derivative	*Aspergillus glaucus*	A-549, HL-60, BEL-7402, and P388 cell lines	IC_{50} values of 0.13, 0.28, 7.5, and 35.0 μM, respectively	Du et al. (2007)

Aspergillides A, B, and C	14-membered macrolides	*Aspergillus ostianus* strain 01F313	Mouse lymphocytic leukemia cells (L1210)	LD_{50} values of 2.1, 71.0, and 2.0 μg mL^{-1}, respectively	Kito et al. (2008)
Aspergiolide B (**1**) and (trans)-emodin-physcion bianthrone (**2**)	Aromatic polyketides	*Aspergillus glaucus*	HL-60 and A-549 cell lines	IC_{50} values of (**1**) 0.51 and 0.24 μM, respectively and of (**2**) 7.8 and 9.2 μM, respectively	Du et al. (2008)
Asperterrestide A	Cyclic tetrapeptide	*Aspergillus terreus* SCSGAF0162	Human carcinoma U937 and MOLT4 cell lines	IC_{50} values of 6.4 and 6.2 μM, respectively	He et al. (2013a)
Aszonapyrone A (**1**) and analog of chevalone C (**2**)	Indole alkaloids	(**1**) From coral-derived fungus *Neosartorya laciniosa* (KUFC 7896) and (**2**) from sponge-associated fungus *Neosartorya tsunodae* (KUFC 9213)	MCF-7, NCI-H460, and A375-C5 cells	GI_{50} values of (**1**) 13.6 ± 0.9, 11.6 ± 1.5, and 10.2 ± 1.2 μM respectively and of (**2**) 17.8 ± 7.4, 20.5 ± 2.4, and 25.0 ± 4.4 μM respectively	Eamvijarn et al. (2013)
Chloctanspirones A (**1**) and B (**2**)	Chlorinated polyketides	*Penicillium terrestre*	(**a**) HL-60 and A-549 cells (**b**) HL-60 cells	IC_{50} values of (**1**) for (**a**) 9.2 and 39.7 μM, respectively and of (**2**) for (**b**) 37.8 μM	Li et al. (2011)
Citrinadin A	Pentacyclic alkaloid	*Penicillium citrinum* N059 strain	L1210 (murine leukemia) and KB (human epidermoid carcinoma) cells	IC_{50} values of 6.2 and 10 μg mL^{-1}, respectively	Tsuda et al. (2004)
Citrinadin B	Pentacyclic alkaloid	*Penicillium citrinum* N059 strain	L1210 (murine leukemia) cells	IC_{50} value of 10 μg mL^{-1}	Mugishima et al. (2005)
Cordyheptapeptides C (**1**) and E (**2**)	Cyclic heptapeptides	*Acremonium persicinum* SCSIO115	SF-268 (human glioblastoma), MCF-7 (human breast cancer), and NCI-H460 (human lung cancer) cell lines	IC_{50} values of (**1**) 3.7 ± 0.3, 3.0 ± 0.2, and 11.6 ± 0.5 μM, respectively and of (**2**) 3.2 ± 0.2, 2.7 ± 0.2, and 4.5 ± 0.3 μM, respectively	Chen et al. (2012c)
Cytoglobosins C and D	Cytochalasins	*Chaetomium globosum* QEN-14	A-549 cell line	IC_{50} values of 2.26 and 2.55 μM, respectively	Cui et al. (2010)
Dankastatin C	Polyketide	*Gymnascella dankaliensis*	Murine P388 cell line	ED_{50} value of 57 ng mL^{-1}	Amagata et al. (2013)
Diaporthelactone	Lactone	*Diaporthe* sp.	KB and Raji cell lines	IC_{50} values of 6.25 and 5.51 μg mL^{-1}, respectively	Lin et al. (2005)

(continued)

Table 35.1 (continued)

Metabolite	Chemical nature	Source	Target cell line	Concentration/effect	Reference
Dihydrotrichodimerol and tetrahydrotrichodimerol	Bisorbicillinoids	*Penicillium terrestre*	(**a**) P388 and (**b**) A-549 cell lines	IC_{50} values against (**a**) 2.8 and 2.1 μM respectively and against (**b**) 8.8 and 4.3 μM respectively	Liu et al. (2005)
Disydonols A (**1**) and C (**2**)	Phenolic bisabolane sesquiterpenoid dimers	*Aspergillus* sp.	HepG2 (human hepatoma) and Caski (human cervical cells)	IC_{50} values of (**1**) 9.31 and12.40 μg mL^{-1}, respectively and of (**2**) 2.91 and 10.20 μg mL^{-1}, respectively	Sun et al. (2012)
Drimane sesquiterpenoids 6, 7 and RES-1149-2	Drimane sesquiterpenoids	*Aspergillus ustus*	(**a**) L5178Y, (**b**) PC-12, and (**c**) HeLa cells	EC_{50} values against (**a**) 5.3, 0.6, and 1.9 μg mL^{-1}, respectively, against (**b**) >10, 7.2 and >10, respectively, and against (**c**) >10, 5.9 and 7.5 μg mL^{-1}, respectively	Liu et al. (2009)
Expansol A (**1**) and B (**2**)	Polyphenols	*Penicillium expansum* 091006, isolated from mangrove *Excoecaria agallocha*	(**a**) HL-60 cell line and (**b**) A549 and HL-60 cells	IC_{50} values of (**1**) for (**a**) 15.7 μM and of (**2**) for (**b**) 1.9 and 5.4 μM, respectively	Lu et al. (2010)
(+)-Epiepoxydon	Phenyl compound with epoxy group	*Apiospora montagnei*, isolated from North sea alga, *Polysiphonia violacea*	Human cancer cell lines: HM02, HepG2, and MCF7	IC_{50} values of 0.7, 0.75, and 0.8 μg mL^{-1}, respectively and TGI (total growth inhibition) values of 1.0, 4.6, and 1.5 μg mL^{-1}, respectively	Klemke et al. (2004)
Epoxyphomalin A (**1**) and B (**2**)	Prenylated polyketides	*Phoma* sp.	BXF 1218 L (bladder), CXF HCT116 (colon), GXF 251 L (stomach), LXF 1121 L (lung), MAXF 401NL (breast), OVXF 1619 L (ovary), PAXF 1657 L (pancreas), PRXF 22RV1 (prostate), RXF 1781 L (kidney), and UXF 1138 L (uterus)	IC_{50} values of (**1**) 0.017, 0.329, 0.034, 0.381, 0.010, 0.258, 0.027, 0.034, 0.469, and 0.031 μg mL^{-1}, respectively and IC_{50} values of (**2**) 0.606, 2.245, 11.420, 1.584, 0.501, 0.278, 1.481, 0.589, 2.656, and 1.811 μg mL^{-1}, respectively	Mohamed et al. (2009)
Efrapeptins Eα, F and G	Linear pentadecapeptides	*Acremonium* sp.	H125 cells	IC_{50} values of each 1.3 nM	Boot et al. (2007)
Destruxin E2 chlorohydrin	Cyclic depsipeptide	*Metarhizium* sp.	HCT-116 cells	IC_{50} of 160 nM	Boot et al. (2007)
Herqueidiketal	Naphthoquinone	*Penicillium* sp.	A549 (lung carcinoma cell line)	LC_{50} value of 17.0 μM	Julianti et al. (2013)

12′-hydroxyroridin E (**1**), roridin Q (**2**) and roridin R (**3**)	Macrocyclic trichothecenes	(**1**) and (**2**) from *Myrothecium roridum* TUF 98 F42 and (**3**) from *Myrothecium* sp. TUF 02F6	L1210 (murine leukemia cell line)	IC_{50} values of 0.19, 31.2, and 0.45 μM, respectively	Xu et al. (2006a)
Hypothemycin and 4-*O*-demethylhypothemycin	Polyketides	*Aigialus parvus* BCC 5311	(**a**) NCI-H187 and (**b**) Vero cells	IC_{50} values for (**a**) 2.0 and 3.6 μg mL^{-1}, respectively, and for (**b**) 2.1 and 0.77 μg mL^{-1}, respectively	Isaka et al. (2009)
4-keto-clonostachydiol, clonostachydiol, and two oxidized derivatives of clonostachydiol	Polyketides	*Gliocladium* sp.	P388 cells	IC_{50} values of 0.55, 25, 1.9, and 3.9 μM respectively	Lang et al. (2006)
Luteoalbusins A (**1**) and B (**2**)	Indole diketopiperazines	*Acrostalagmus luteoalbus* SCSIO F457	SF-268, MCF-7, NCI-H460, and HepG-2 cells	IC_{50} values of (**1**) 0.46 ± 0.05, 0.23 ± 0.03, 1.15 ± 0.03, and 0.91 ± 0.03 μM respectively and of (**2**) 0.59 ± 0.03, 0.25 ± 0.00, 1.31 ± 0.12, and 1.29 ± 0.16 μM respectively	Wang et al. (2012)
Leptosphaerone C (**1**) and penicillenone (**2**)	Polyketides	*Penicillium* sp. JP-1	(**a**) A-549 (**b**) P388 cells	IC_{50} values of (**1**) against (**a**) 1.45 μM and of (**2**) against (**b**) 1.38 μM	Lin et al. (2008)
6-*O*-methyl-7-chloroaveratin	Chlorinated anthraquinone	*Aspergillus* sp. SCSIO F063	SF-268, MCF-7, and NCI-H460 cells	IC_{50} values of 7.11, 6.64, and 7.42 μM, respectively	Huang et al. (2012)
Ophiobolin K	Sesterterpene	*Emericella variecolor* GF10	T-47D, MDA-MB-231, HOP18, NCI-H460, HCT116, ACHN P388, and adriamycin resistant P388 cells	IC_{50} values of 0.35, 0.57, 0.65, 0.57, 0.33, 0.27, 0.51, and 0.36 μM respectively	Wei et al. (2004)
Penicacids A–C, mycophenolic acid, and 4′-hydroxy-mycophenolic acid	Mycophenolic acid and its derivatives	*Penicillium* sp. SOF07	Mouse splenocyte proliferation	IC_{50} values of 2.46 ± 0.32, >20, >30, 0.32 ± 0.06, and 1.10 ± 0.21 μM, respectively	Chen et al. (2012d)
Rostratins A-D	Disulfide alkaloids	*Exserohilum rostratum* (Drechsler)	HCT-116 (human colon carcinoma cells)	IC_{50} values of 8.5, 1.9, 0.76, and 16.5 μg mL^{-1}, respectively	Tan et al. (2004)
Scytalidamides A and B	Cyclic heptapeptides	*Scytalidium* sp.	HCT-116 cells	2.7 and 11 μM, respectively	Tan et al. (2003)
Spirotryprostatin E	Prenylated indole diketopiperazine alkaloid	*Aspergillus fumigatus*	MOLT-4, A549, and HL-60 cancer cells	IC_{50} values of 3.1, 2.3, and 3.1 μM respectively	Wang et al. (2008)

(continued)

Table 35.1 (continued)

Metabolite	Chemical nature	Source	Target cell line	Concentration/effect	Reference
Trichodermamide B	Dipeptide	*Trichoderma virens*	HCT-116 cells	IC_{50} value of 0.32 μg mL^{-1}	Garo et al. (2003)
Tropolactones A–C	Meroterpenoids	*Aspergillus* sp.	HCT-116 cells	IC_{50} values of 13.2, 10.9, and 13.9 μg mL^{-1} respectively	Cueto et al. (2006)
(-)-Trypethelone	Polyketide-type alkaloid	*Coniothyrium cereale*	Mouse fibroblast cells	IC_{50} value of 7.5 μM	Elsebai et al. (2011)
Ustusorane E	Isochromane derivative	*Aspergillus ustus* 094102	HL-60 cells	IC_{50} value of 0.13 μM	Lu et al. (2009)
Variecolorquinones A (**1**) and B (**2**)	Quinones	*Aspergillus variecolor* B-17	(**a**) A-549 and (**b**) HL-60 and P388 cells	IC_{50} values of (**1**) against (**a**) 3.0 μM and of (**2**) against (**b**) 1.3 and 3.7 μM, respectively	Wang et al. (2007)
Vermelhotin	Heterocyclic compound	Unidentified fungus CRI247-01 (order Pleosporales)	HuCCA-1, KB, HeLa, MDA-MB231, T47D, HepG2, A549, HCC-S102, HL-60, and P388 cells	IC_{50} values of 2.90 ± 0.17, 0.50 ± 0.00, 0.33 ± 0.08, 0.31 ± 0.08, 1.25 ± 0.35, 2.50 ± 0.70, 8.20 ± 2.54, 13.5 ± 0.70, 1.60 ± 0.03, and 1.23 ± 0.02 μg mL^{-1}, respectively	Kasettrathat et al. (2008)
Zygosporamide	Cyclic depsipeptide	*Zygosporium masonii*	SF-268 (CNS cancer cell line) and RXF 393 (renal cancer cell line)	GI_{50} values of 6.5 and ≤5.0 nM, respectively	Oh et al. (2006)

strongly inhibit human cancer cell lines including BXPC-3 (pancreas), MCF-7 (breast), SF-268 (CNS), NCI-H460 (lung), KM20L2 (colon), and DU-145 (prostate) and one murine cell line, P388 (lymphocytic leukemia). Bacillistatin 1($C_{57}H_{96}N_6O_{18}$), cyclodepsipeptide, was constituted with a head to tail linkage of three repeated sequence of *R*-valine, *S*-lactic acid, *S*-valine, and *2R*-hydroxy-*3S*-methylvaleric acid. Bacillistatin 2 shares the same molecular weight with bacillistatin 1 but slightly differs in one of its components; i.e., of the three *2R*-hydroxy-***3S***-methylvaleric acid, one is replaced with *2R*-hydroxy-*4S*-methylvaleric acid. Difference in the position of methylation (fourth carbon in one of valeric acids in bacillistatin 2 and third carbon in compound 1) influenced slightly in the cytotoxicity, i.e., compound 2 is slightly more active.

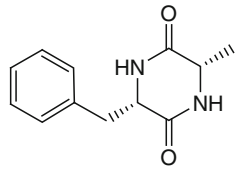
Erythrobacter sp., isolated from the mangrove sediment of Trinity Bay, was found to produce an unusual natural product with mixed biosynthetic origin, erythrazole B. The compound is very rare because of the presence of both benzothiazole and terpene groups. Its chemical components are glycine, tetrasubstituted benzothiazole, and 22 carbons containing unusual diterpene terminated with carboxylic acid. This compound exerts potent cytotoxicity against non-small-cell lung cancer (NSCLC) cell lines (H1325, H2122, and HCC366) (Hu and Macmillan 2011). Likewise, Hu et al. (2012) isolated *Erythrobacter* sp. from marine sediment of Galveston, Texas. Cytotoxic erythrolic acid D was purified from this bacterium, comprised of 4-hydroxybenzoic acid and an unusual 17 carbon-containing diterpene terminated with carboxylic acid. This compound was also found to exhibit cytotoxicity against NSCLC cell lines.

35.2.1.2 Marine Actinobacterial Compounds

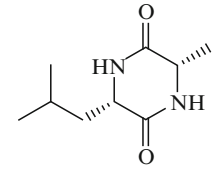
The marine actinomycete *Nocardia dassonvillei* which was isolated from the Arctic Ocean with extreme variations in pressure, salinity, and temperature showed cancer cell cytotoxicity and antifungal activity (Gao et al. 2012). The novel natural product N-(2-hydroxyphenyl)-2-phenazinamine (NHP) (Fig. 35.1) isolated from *N. dassonvillei* is the key for cancer cell cytotoxicity against HepG2, A549, HCT-116, and COC1 cells and also for antifungal activity against *Candida albicans*. The extreme environmental conditions in the Arctic such as low temperature, UV radiation, and variations in sunlight provoke not only the habitual adaptation but also the production of potential chemical compounds with significant biological activities. Earlier studies revealed that the NHP molecules exhibit activity by permeating the tissues and organs as well as affecting multiple pathways and/or multiple targets.

Asolkar et al. (2006) reported the cytotoxic compounds such as daryamides A, B, and C and (*2E*,*4E*)-7-methylocta-2,4-dienoic acid amide from the marine actinomycete *Streptomyces strain* CNQ-085, which was isolated from a marine sediment collected at a depth of ca.50 m off San Diego, CA, in which daryamide A exhibited significant cytotoxicity against human colon carcinoma cell line (HCT-116). These compounds are related to the manumycins, a microbial natural product which was reported for its antimicrobial, cytotoxic, and other biological activity potentials. Manumycin and its analogs inhibit Ras farnesyl transferase and the growth of Ki-ras-activated murine fibrosarcoma in mice; hence it is anticipated that the daryamides may also have such activity.

Mayamycin is a novel benz[a]anthracene derivative (Fig. 35.1) which was derived from the marine sponge *Halichondria panicea* associated with *Streptomyces* sp. strain HB202 (Schneemann et al. 2010). The compound mayamycin data revealed that the chemical nature was an angucycline-type polyketide with a C-glycosidically bound amino sugar. Hence, the production of aromatic polyketides was demonstrated by genetic analysis and it confirms the presence of type II polyketide synthase. This polyketide-derived tetracyclic benz[a]anthraquinone holds a wide variety of biological activities such as antibacterial, enzyme inhibition, and cytostatic effects. Therefore, mayamycin was tested for its biological significance, and it exhibits an excellent cytotoxicity against different human cell lines as well as pathogenic bacterial strains including antibiotic-resistant strains.

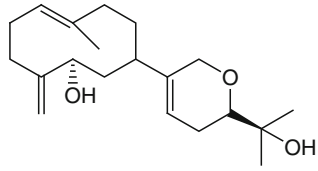


(6S,3S)-6-benzyl-3-methyl-2,5-diketopiperazine

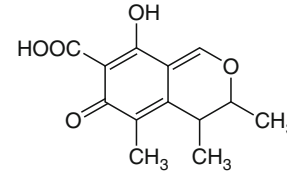


(6S,3S)-6-isobutyl-3-methyl-2,5-diketopiperazine

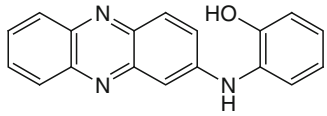
(Cho et al., **2012a**)



Lobocompactol (Cho et al., **2012b**)

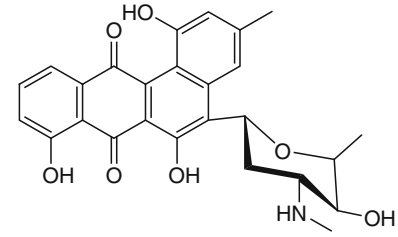


Citrinin (Subramani et al.,**2013**)

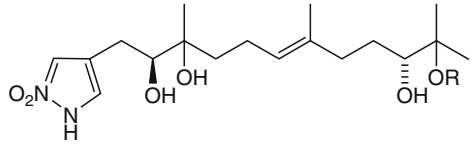


N-(2-hydroxyphenyl)-2-phenazinamine(NHP)

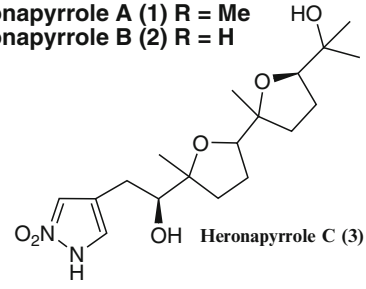
(Gao et al., **2012**)



Mayamycin (Schneemann et al., **2010**)



Heronapyrrole A (1) R = Me
Heronapyrrole B (2) R = H



Heronapyrrole C (3)

Heronapyrroles A-C (1-3) (Raju et al., **2010**)

Fig. 35.1 Marine actinobacterial metabolites

Motohashi et al. (2010) isolated the two new anthracyclines, namely, tetracenoquinocin (1) and 5-iminoaranciamycin (2), in addition to the known compounds aranciamycin (3) and antibiotic SM 173B from the marine sponge *Haliclona* sp. associated with *Streptomyces* sp. Sp080513GE-26. The compounds 1–3 were checked for its cytotoxic potential against human cervical carcinoma HeLa cells and human acute myelogenous leukemia LH-60 cells.

Comparatively, compound 3 (aranciamycin) exhibits potential inhibition than the compounds 1 and 2 with IC_{50} values of 2.7 and 4.1 μM against HeLa and HL-60 cells, respectively. The functional group ketone at C-5 in the compounds 1 and 3 is majorly responsible for cytotoxicity.

Lajollamycin, a nitro-tetraene spiro-*β*-lactone-γ-lactam antibiotic, was isolated from the marine *Streptomyces nodosus* (NPS007994) which is collected from Scripps Canyon, La Jolla, California, by Manam et al. (2005). It inhibits the tumor cell B16-F10 and the growth of both drug-sensitive and drug-resistant Gram-positive bacteria.

35.2.1.3 Marine Cyanobacterial Compounds

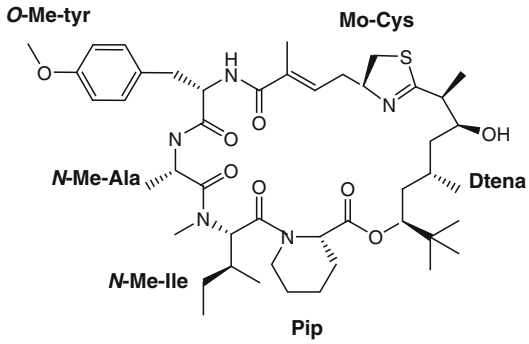
Intriguingly, marine cyanobacteria produce secondary metabolites that collectively contain both non-ribosomal peptide and polyketide moieties. The non-ribosomal peptide part is biosynthesized by non-ribosomal peptide synthetase mainly with non-proteinogenic and/or modified amino acids, while the polyketide part is made by polyketide synthase with unusual hydroxylated fatty acid. Hence, these metabolites are said to be of mixed biosynthetic origin, having pronounced bioactivity. Some of such cytotoxins isolated very recently during 2011–2013 are alone described here. Nagarajan et al. (2012) have already elaborated about the bioactive natural products isolated from marine cyanobacteria during 2000–2011.

Apratoxins are potent cytotoxic cyclodepsipeptides isolated from marine cyanobacteria *Lyngbya*. Apratoxins A–G were identified during the period of 2001–2010 and their structural and pharmacological characteristics were described by Nagarajan et al. (2012). Recently, Thornburg et al. (2013) have isolated apratoxin H and apratoxin A sulfoxide from marine cyanobacterium *Moorea producens* (*Lyngbya majuscula*), collected from the Nabq Mangroves in the Gulf of Aqaba (Red Sea). Apratoxin H ($C_{46}H_{71}N_5O_8S$) is made up of head to tail linkage of pipecolic acid, *N*-methyl-isoleucine, *N*-methyl-alanine, *O*-methyl tyrosine, modified cysteine, and dihydroxylated fatty acid moiety 3,7-dihydroxy-2, 5,8, 8-tetramethylnonanoic acid (Dtena). Apratoxin H differs from A sulfoxide by the presence of pipecolic acid in place of proline residue. Sulfur in the modified cysteine residue of apratoxin A undergone oxidation to yield apratoxin A sulfoxide ($C_{45}H_{69}N_5O_9S$). Apratoxin H seems more potent than apratoxin A sulfoxide against lung cancer cells (NCI-H460) (Fig. 35.2).

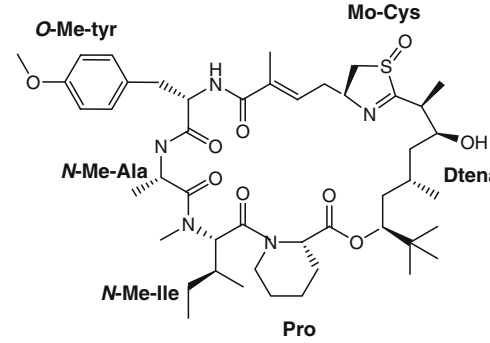
Grassypeptolides D and E (Fig. 35.2) are yet other potent cytotoxic cyclic depsipeptides isolated from marine cyanobacterium *Leptolyngbya* sp. that were collected from a shipwreck in the Red Sea. Both compounds possess the same molecular formula ($C_{57}H_{81}N_9O_{10}S_2$) and the same components, viz., β-amino acid residue 2-methyl-3-aminobutyric acid (Maba), threonine, *N*-methyl leucine, 2-aminobutyric acid-derived thiazoline carboxylic acid (Aba-thn-ca) residue, *N*-methylphenylalanine-derived 2-methylthiazoline carboxylic acid (*N*-Me-Phe-4-Me-thn-ca), proline, *N*-methyl valine, and phenyllactic acid (Pla). They differ in the orientation of carbon (7th) in threonine and carbon (11th) in *N*-methyl leucine (Thornburg et al. 2011).

Strong cytotoxic cyclodepsipeptide lagunamide C ($C_{46}H_{73}N_5O_{10}$) was isolated from *L. majuscula* from the western lagoon of Pulau Hantu Besar, Singapore. Head to tail-linked (-(*N*-methyl-alanine)-isoleucine-(*N-methyl-glycine*)-(*N*-methyl-phenylalanine)-alanine-(2-hydroxyisoleucic acid (Hila))-5,8- dihydroxy-2,6,9-trimethyl-undec-2-enoic acid (Dtuea)-) constitute this cyclodepsipeptide (Fig. 35.2). Hydroxy acid Hila and a polyketide (fatty acid) moiety Dtuea along with methylated and non-methylated amino acids made this compound to potently inhibit a variety of cancer cells such as P388, A549, PC3, HCT8, and SK-OV3 (Tripathi et al. 2011).

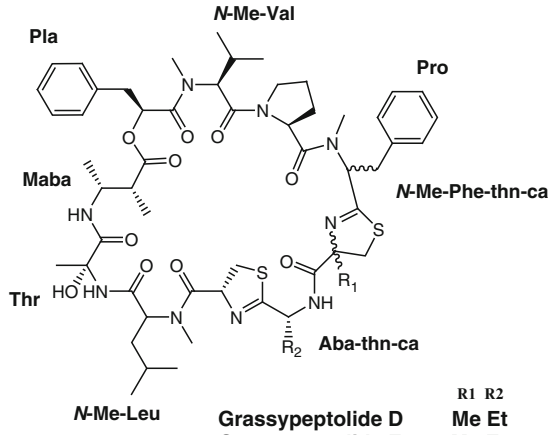
Boudreau et al. (2012) have isolated cyclodepsipeptide viequeamide A ($C_{42}H_{69}N_5O_{10}$) from marine button cyanobacterium *Rivularia* sp., collected near the island of Vieques, Puerto Rico. The stereochemistry of this cytotoxin was devised as Thr, *N*-Me-Val, Pro, 2-hydroxy-3-methylpentanoic acid (Hmpa), *N*-Me-Val, Val, 2,2-dimethyl-3-hydroxy-7-octynoic acid (Dhoya)



Apratoxin H (Thornburg et al., **2013**)

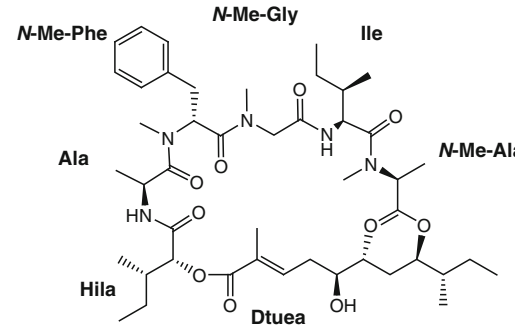


Apratoxin A sulfoxide (Thornburg et al., **2013**)

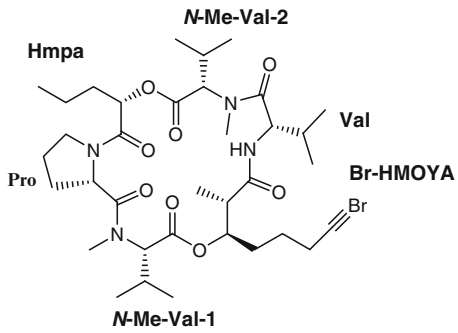


	R1	R2
Grassypeptolide D	Me	Et
Grassypeptolide E	Me	Et

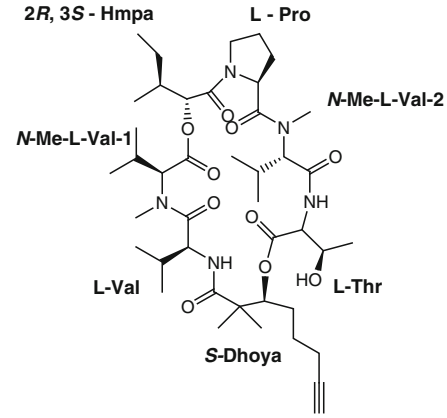
(Thornburg et al., **2011**)



Lagunamide C (Tripathi et al., **2011**)



Veraguamide A (Mevers et al., **2011**)



Viequeamide A (Boudreau et al., **2012**)

Fig. 35.2 Marine cyanobacterial metabolites

(Fig. 35.2). The presence of polar amino acid threonine may be a factor for the enhanced activity of this compound towards H460 human lung cancer cells.

Active-guided fractionation of the EtOAc:MeOH (1:1) extract of *Symploca* cf. *hydnoides* which was collected from Cetti Bay, Guam, yielded veraguamide A ($C_{37}H_{59}N_4O_8Br$). This cytotoxic cyclic hexadepsipeptide is made

up of *N*-Me-Val, Pro, Hmpa, *N*-Me-Val, Val, 8-bromo-3-hydroxy-2-methyloct-7-ynoic acid (Br-Hmoya) (Fig. 35.2). Salvador et al. (2011) have suggested that the alkynyl bromide (Br-Hmoya) moiety of veraguamide A may be responsible for its strong toxicity towards the lung cancer cell line H-460.

Anticancer peptides bisebromoamide and norbisebromoamide have been isolated by Sasaki et al. (2011) from *Lyngbya* sp., which were collected at the reef of Bise, Okinawa, Japan. The structural elements of bisebromoamide ($C_{51}H_{72}{}^{79}BrN_7O_8S$) are 2-(1-oxo-propyl) pyrrolidine (Opp), *N*-Me-Phe, Leu, 4-methyl thiazoline (4-Me-Tzn), 4-Me-Pro, *N*-methyl-3-bromotyrosine (*N*-Me-Br-Tyr), Ala, and pivalic acid (tert-butyl containing carboxylic acid). Norbisebromoamide ($C_{50}H_{70}BrN_7O_8S$) resembles bisebromoamide in the chemical composition except demethylation in thiazoline residue. Both of them are toxic to cervix cancer cells (HeLa S3). The key and unusual structural feature and elements are the fusion of thiazoline with proline, 2-Opp, and *N*-Me-Br-Tyr. Thus, the above cyclic peptides with modified amino acids, hydroxy acids, and modified fatty acids render potent cytotoxicity against human cancer cell lines.

35.2.1.4 Marine-Derived Fungal Compounds

Amazingly, marine fungi are the fluent producers of bioactive compounds with very diverse chemistry including macrocycles; heterocycles; methyl guanine with isoprene; derivatives of azole, benzoyl, benzaldehyde, and tyrosine; halogenated polyketides; pentacyclic alkaloids; cytochalasins; polyphenols; quinines; indole diketopiperazines; peptides; lactones; etc.

Boot et al. (2007) have isolated linear pentadecapeptides from marine-derived fungus *Acremonium* sp. Efrapeptin Eα ($C_{82}H_{141}N_{18}O_{16}{}^+$) is composed of 15 normal/modified amino acids such as acylated pipecolic acid (Ac Pip), aminoisobutyric acid (Aib), Pip, isovaline (Iva), Aib, Leu, β-Ala, Gly, Aib, Aib, Pip, Aib, Ala, and Leu with a cap of ($C_{13}H_{25}N_3$) bicyclic amine. Efrapeptin F ($C_{82}H_{141}N_{18}O_{16}{}^+$) is constituted with Ac-Pip, Aib, Pip, Aib, Aib, Leu, β-Ala, Gly, Aib, Pib, Aib, Ala, Leu, and Iva with cap. Likewise, efrapeptin G ($C_{83}H_{143}N_{18}O_{16}{}^+$) is made up of Ac-Pip, Aib, Pip, Iva, Aib, Leu, β-Ala, Gly, Aib, Aib, Pip, Aib, Ala, Leu, and Iva with cap. These linear peptides were found to have anticancer activity at nanomolar concentration.

Marine-derived fungus *Zygosporium masonii* was found to produce cytotoxic cyclic pentadepsipeptide zygosporamide ($C_{36}H_{50}N_4O_6$), comprised of L-Phe, L-Leu, D-Leu, L-Phe, and *O*-Leu. Presence of one D-amino acid (leucine) has significantly enhanced anticancer activity against colon cancer cell line (HT-29) (Oh et al. 2006).

Two cytotoxic indole diketopiperazines (luteoalbusins A and B) were identified from deep sea-derived fungus *Acrostalagmus luteoalbus* SCSIO F457. Luteoalbusin A ($C_{23}H_{20}N_4O_3S_2$) is composed of 2,3-disubstituted indole, 3-substituted indole, and thiodiketopiperazine (two ketones, two quaternary carbons (sulfated), and two nitrogens). Luteoalbusin B ($C_{23}H_{20}N_4O_3S_3$) additionally possesses one sulfur atom. They display significant cytotoxic activity against SF-268, MCF-7, NCI-H460, and HepG-2 cells (Wang et al. 2012).

Besides the above fungal compounds, ophiobolin K, luteoalbusins A and B, ustusorane E, trichodermamide B, vermelhotin, epoxyphomalin A and B, and aspergiolide A exhibit potent anticancerous activity against a broad spectrum of human cancer cell lines and murine cancer cells.

35.2.2 Inhibition of Enzymes/Proteins

Enzymes and proteins are employed specifically in various cellular functions including DNA replication, cell division, gene expression, metabolism, signal transduction, transport, immunity, pathogenicity, etc. By regulating the activity of specific proteins/enzymes, it will be able to control the particular cellular function. In pharmacology, various protein/enzyme inhibitors are used against specific drug targets to treat cancer, microbial infections, and neurological disorder,

regulate host immune system, etc. Such drug targets, associated problems, and its inhibitors from marine microbes are discussed below (Table 35.2).

35.2.2.1 Neutrophil Elastase

Inflammation is an immune response to protect tissues from injury/damage and it employs neutrophils (leukocytes). Activation of neutrophils releases a variety of cytotoxic products including serine proteases like elastase which digest elastin and many other proteins of invading microbes. Excessive release of elastase due to chronic inflammation additionally damages the host tissues especially the lung tissues that results in various disorders, viz., emphysema, inflammatory chronic lung diseases, chronic obstructive pulmonary disease, acute lung injury, acute respiratory distress syndrome, and cystic fibrosis. Hence, neutrophil elastase is a therapeutic target and extensive host tissue damage can be prevented by administration of elastase inhibitors (Korkmaz et al. 2010). Porcine pancreatic elastase is often used as model for human leukocyte elastase. Secondary metabolites like Symplostatins 5–10 from marine cyanobacterium *Symploca* sp. and conioimide, cereolactam, and cereoaldomine from marine fungus *Coniothyrium cereale* are potent elastase inhibitors (Elsebai et al. 2011, 2012; Salvador et al. 2013).

35.2.2.2 Protein Tyrosine Phosphatase (PTP)

Secreted *Mycobacterium tuberculosis* (MTB) PTPA binds to macrophage vacuolar-(H+)-ATPase (V-ATPase) located in the membrane. V-ATPase is involved in the acidification of the lumen and coordinates phagosome-lysosome fusion. Inhibition of V-ATPase, in turn, evades MTB from the digestion within macrophage (Wong et al. 2011). Secreted *M. tuberculosis* PTPB manipulates host signal transduction pathways and poses infection (Grundner et al. 2005). Some PTPs are positively signaling the growth factor receptors for tumor formation (oncogenic) (Östman et al. 2006). Hence, PTP is found to be a therapeutic target, and its inhibition can cure tuberculosis and certain cancers. Asperterpenoid A, sydowiols A and C, and aquastatin A were found to be inhibiting PTP (Huang et al. 2013; Liu et al. 2013; Seo et al. 2009).

35.2.2.3 p53/MDM2 Interaction

p53, a tumor-suppressor protein, arrests cell cycle at $G2_1$/S phase in response to DNA damage, oxidative stress, oncogene expression, etc., induces apoptosis, and eradicates tumor/cancer growth. But p53 is exported from the nucleus to cytosol by MDM2 (E3 ubiquitin ligase) enzyme, where p53 is conjugated with ubiquitin that, in turn, results in proteasomal degradation of p53 (Chène 2003). Inhibition of p53/MDM2 interaction with natural product would be an attractive strategy to activate p53 and check tumor/cancer growth. Hoiamide D from marine cyanobacterium *Symploca* sp. was determined to effectively inhibit p53/MDM2 interaction (Malloy et al. 2012a).

35.2.2.4 Sortase (Srt)

It is a prokaryotic signal peptidase, cleaving the carboxy terminal sorting signal (signal for protein transportation) of cell surface proteins and trafficking and covalently attaching them to the cell wall. These surface proteins are implicated in adhesion, colonization, infection, host immune evasion, etc. By inhibiting the sortase enzyme, the transportation of surface proteins will be impaired and pathogenicity of the microbe can be muted. The enzyme sortase A (SrtA) from *S. aureus* was inhibited by marine-derived fungal metabolites herqueidiketal and chrysoarticulin C (Jeon et al. 2013; Julianti et al. 2013).

35.2.2.5 Histone Deacetylase (HDAC)

Histone deacetylase is an anticancer drug target. Histone acetyl transferase acetylates the core histone of nucleosome to allow the gene expression through transcription factors. HDAC removes acetyl groups from the histones and silences the gene expression. Inhibition of HDAC leads to hyperacetylation of histones; in addition certain transcription factors are also acetylated. This, in turn, upregulates certain transfactors like tumor-suppressor p53 that arrest cell cycle and prevent cancer will get activated. Santacruzamate A from

Table 35.2 Enzyme/protein inhibitors from marine microbes

Inhibitor	Chemical nature	Source	Enzyme/protein	Concentration	Reference
Marine cyanobacterial metabolites					
Bisebromoamide	Peptide	*Lyngbya* sp.	Protein kinase	Enzyme phosphorylation activity inhibited in 10-0.1 μM range	Sasaki et al. (2011)
Credneramides A and B	Vinyl chloride fatty acids	*Trichodesmium* sp. nov.	Spontaneous calcium oscillations in murine cerebrocortical neurons	IC_{50} values of 4.0 and 3.8 μM respectively	Malloy et al. (2012b)
Ethyl tumonoate A	Tumonoic acid derivative	*Oscillatoria margaritifera*	Ca^{2+} oscillations in neocortical neurons	Complete inhibition at 10 μM	Engene et al. (2011)
Grassystatin A	Linear depsipeptide	*Lyngbya* cf. *confervoides*	Cathepsin E	IC_{50} value of 0.8 nM	Yang et al. (2012)
Hermitamides A (**1**) and B (**2**)	Lipopeptides	*Lyngbya majuscula*, Papua New Guinea	hNav (human voltage-gated sodium channel)	(**1**) Inhibits ~50 % and (**2**) inhibits ~80 % at 1 μM	De Oliveira et al. (2011)
Hoiamide D	Thiazole-containing linear peptide	*Symploca* sp., Papua New Guinea	p53/MDM2 interaction	EC_{50} value of 4.5 μM	Malloy et al. (2012a)
Janthielamide A and kimbeamide A	Vinylchlorine lipoamides	*Symploca* sp.	Sodium channel in murine neuro-2a cells	IC_{50} values of 11.5 μM and 20 μg mL^{-1} respectively	Nunnery et al. (2012)
Santacruzamate A	Amide	*Symploca* sp.	HDAC2 (histone deacetylase)	IC_{50} value of 0.119 nM	Pavlik et al. (2013)
Symplocin A	Linear peptide	*Symploca* sp.	Cathepsin E	IC_{50} value of 300 pM	Molinski et al. (2012)
Symplostatins 5–10	Cyclic depsipeptides	*Symploca* sp., Guam	(**a**) Porcine pancreatic elastase, (**b**) human neutrophil elastase, and (**c**) bovine pancreatic chymotrypsin	IC_{50} values for (**a**) 68 ± 9.7, 89 ± 11, 77 ± 5.4, 43 ± 3.2, 37 ± 3.1, and 44 ± 1.5 nM; for (**b**) 144 ± 2.9, 121 ± 12, 195 ± 28, 41 ± 9.0, 28 ± 5.8, and 21 ± 2.9 nM; and for (**c**) 322 ± 3.2, 503 ± 65, 515 ± 43, 268 ± 11, 324 ± 27, and 222 ± 5.1 nM respectively	Salvador et al. (2013)
Marine fungal metabolites					
Asperterpenoid A	Sesterterpenoid	*Aspergillus* sp. 16-5c	*Mycobacterium tuberculosis* protein tyrosine phosphatase B	IC_{50} value of 2.2 μM	Huang et al. (2013)
Aquastatin A	Glycosylated depside	*Cosmospora* sp. SF-5060	Protein tyrosine phosphatase 1B	IC_{50} value of 0.19 μM	Seo et al. (2009)

(continued)

Table 35.2 (continued)

Inhibitor	Chemical nature	Source	Enzyme/protein	Concentration	Reference
(-)-Cereolactam and (-)-cereoaldomine	Polyketide-type alkaloids	*Coniothyrium cereale*	Human leukocyte elastase	IC_{50} values of 9.28 ± 2.77 and 3.01 ± 0.23 μM, respectively	Elsebai et al. (2011)
Conioimide	Isoindole pseudoalkaloid	*Coniothyrium cereale*	Human leukocyte elastase	IC_{50} value of 0.2 μg mL^{-1}	Elsebai et al. (2012)
Chrysoarticulin C	Benzolactone	*Chrysosporium articulatum*	Sortase A	IC_{50} value of 95.1 μM	Jeon et al. (2013)
Herqueidiketal	Naphthoquinone	*Penicillium* sp.	*Staphylococcus aureus* sortase A (SrtA)	IC_{50} value of 23.6 μM	Julianti et al. (2013)
Monodictyochromes A and B	Dimeric xanthone derivatives	*Monodictys putredinis*	(**a**) Cytochrome P450 1A and (**b**) aromatase	IC_{50} values for (**a**) 5.3 and 7.5 μM, respectively and for (**b**) 24.4 and 16.5 μM respectively	Pontius et al. (2008b)
Monodictysin B, C, and monodictyxanthone	Monomeric xanthone derivatives	*Monodictys putredinis*	Cytochrome P450 1A	IC_{50} values of 23.3 ± 3.9, 3.0 ± 0.7, and 34.8 ± 7.4 μM respectively	Krick et al. (2007)
Paeciloxanthone	Xanthone	*Paecilomyces* sp. (Tree 1-7)	Acetylcholine esterase	IC_{50} value of 2.25 μg mL^{-1}	Wen et al. (2008)
Speradine A	Pentacyclic oxindole alkaloid	*Aspergillus tamarii*	Ca^{2+}-ATPase and histone deacetylase	IC_{50} values of 8 and 100 μM, respectively	Tsuda et al. (2003)
Sorbiterrin A	Sorbicillinoid	*Penicillium terrestre*	Acetylcholinesterase	IC_{50} value of 25 μg mL^{-1}	Chen et al. (2012a)
Sydowiols A and C	Pyrogallol ethers	*Aspergillus sydowii* (MF357)	*Mycobacterium tuberculosis* protein tyrosine phosphatase A	14 and 24 μg mL^{-1}, respectively	Liu et al. (2013)

Symploca sp. and speradine A from *Aspergillus tamari* displayed HDAC inhibition (Pavlik et al. 2013; Tsuda et al. 2003).

35.2.2.6 Sodium and Calcium Channels

They are transmembrane proteins maintaining the membrane potentials. They send Na^+ and Ca^{2+} ions inside the cell, respectively, to depolarize (more positive or less negative inside the cell) the membrane to achieve action potential. This is required in many excitable animal cells (neurons, myocytes, and endocrine cells) for cell-cell communication and to activate various signal transduction pathways. Clinically, sodium channel blockers are used in various treatments especially as analgesic (pain reliever) (Wood et al. 2004), and calcium channel blockers are used to cure hypertension. Hermitamides A and B, janthielamide A, and kimbeamide A are shown to block voltage-gated sodium channels (De Oliveira et al. 2011; Nunnery et al. 2012); inhibition of calcium oscillations was observed in credneramides A and B and ethyl tumonoate A-treated neurons (Engene et al. 2011; Malloy et al. 2012b).

35.2.2.7 Acetylcholine Esterase (AchE)

Acetylcholine (Ach) is a neurotransmitter transmitted from neuron to target cell across the nerve synapse, and it binds with acetylcholine receptor in nerve-nerve junction and neuromuscular junction of the signal receiver that activates signal transduction pathways in signal-receiving neuron or muscle cells. AchE located on the synaptic cleft hydrolyses acetylcholine and thereby releases choline from Ach and reduces its concentration at the synaptic cleft. This, in turn, releases Ach from Ach receptor, terminates signal transmission, and allows the Ach receptor to bind with another Ach during transmission. When AchE is inhibited, Ach accumulates at the nerve synapse and continuously transmits the signal (Pohanka 2012). Irreversible inhibitors lead to paralysis, convulsion, and death. Reversible inhibitors for short duration will be used for the treatment of various neurological diseases. AchE was inhibited by sorbiterrin A and paeciloxanthone (Chen et al. 2012a; Wen et al. 2008).

35.2.2.8 Cytochrome P450

This monooxygenase family is involved in phase I xenobiotic/drug metabolism and it detoxifies or reduces the toxicity of the drugs/xenobiotics/lipophilic compounds by oxidation. Inhibition of cytochrome P450 leads to oxidative stress that, in turn, results in cell death. Monodictyochromes A and B, monodictysin B and C, and monodictyxanthone from marine fungus *Monodictys putredinis* were found to inhibit cytochrome P450 1A (Krick et al. 2007; Pontius et al. 2008b).

35.2.3 Antimicrobial Compounds

Marine bacterial and fungal natural products that belong to carboline, quinoline, lactones, peptides, macrolactones, diketopiperazines, fatty acids, polyketides, terpenes, alkaloid, xanthones, etc., are found to have potent antimicrobial properties against human bacterial, viral, and fungal pathogens and protozoal parasites. Notably, pitipeptolide F (against *Mycobacterium tuberculosis*); brevianamide S (against *M. bovis* BCG); trichoderins A, A1, and B (against *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* H37Rv); 1-acetyl-beta-carboline (against methicillin-resistant *Staphylococcus aureus* (MRSA); bacillistatins 1 and 2 (against penicillin-resistant *Streptococcus pneumoniae* and multidrug-resistant *S. pneumoniae*); diketopiperazines (against *Vibrio anguillarum*); asperterrestide A (against influenza virus strains A/WSN/33 (H1N1) and A/Hong Kong/8/68 (H3N2)); isoaspulvinone E, aspulvinone E, and pulvic acid (against influenza A H1N1 virus); gageomacrolactins 1-3 (against pathogenic fungi *Aspergillus niger*, *Botrytis cinerea*, *Colletotrichum acutatum*, *Candida albicans*, and *Rhizoctonia solani*); coibacins A-D (against *Leishmania donovani*); lagunamide C (against *Plasmodium falciparum*); nodulisporacid A and vermelhotin (against *P. falciparum* strain 94); chaetoxanthones B and C (against *P. falciparum* and *Trypanosoma cruzi*); hypothemycin and 4-*O*-demethylhypothemycin

(against *P. falciparum* K1); *N*-(2-hydroxyphenyl)-2-phenazinamine (NHP) (against *C. albicans*); daryamides A and B (against *C. albicans*); mayamycin (benz[a]anthracene) (against *Bacillus subtilis*, *Brevibacterium epidermidis*, *Dermabacter hominis*, *Klebsiella pneumoniae*, *Propionibacterium acnes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus lentus*, *Xanthomonas campestris*, *Phytophthora infestans*); heronapyrrole A, heronapyrrole B, and heronapyrrole C (pyrroloterpenes) (against *S. aureus* and *B. subtilis*); salinosporamide A (against *P. falciparum* and *P. yoelii*); citrinin (Figure 35.1) (against methicillin-resistant *S. aureus*, rifampicin-resistant *S. aureus*, *S. aureus*, *Enterococcus faecium*, and *Cryptococcus neoformans*); etc., were some of the recently explored antimicrobial compounds with significant bioactivity (Asolkar et al. 2006; Balunas et al. 2012; Gao et al. 2012, 2013; He et al. 2013a; Lee et al. 2013; Montaser et al. 2011; Pettit et al. 2009; Prudhomme et al. 2008; Pruksakorn et al. 2010; Raju et al. 2010; Song et al. 2012; Schneemann et al. 2010; Subramani et al. 2013; Tareq et al. 2013; Tripathi et al. 2011) (Table 35.3).

35.2.4 Anti-inflammatory Compounds

Foods and nutrients in their isolated or combined forms are helpful in inhibiting the inflammatory diseases such as allergy or autoimmune diseases due to their synergistic effects in human physiology. Apart from dietary materials, the seaweeds are also used as gelling agents, stabilizers in food preparations, and pharmaceutical materials (Mohamed et al. 2012). Antioxidants often prevent inflammatory processes in the cell (Grivennikov and Karin 2010). Brown seaweeds seem to be better than red and green ones due to their higher dietary and pharmacological valuables (Kraan 2012). Seaweeds are full of hydrosoluble and liposoluble vitamins, viz., thiamine, riboflavin, β-carotene, and tocopherols, and also long-chain polyunsaturated essential fatty acids including n-3 family (n-3 LC-PUFA) such as eicosapentaenoic acid (EPA, 20:5n-3), which may act against heart diseases, thrombosis, atherosclerosis, and carcinogenesis (Johns and Nichols 1979; Khotimchenko et al. 2002). The major portion of the seaweed extracts contains essential sterols such as fucosterols and cholesterols from brown algae; green algae are containing a wide variety of sterol family than browns due to their inhabitant properties. The phenolic components, namely, sargahydroquinoic acid, sargachromanol E and D, and polyunsaturated fatty acids such as palmitic acid, palmitoleic acid, elaidic acid, linoleic acid, linolenic acid, arachidonic acid, and cis-5, 8,11,14,17-eicosanoic acid that are found in a variety of seaweeds play a significant role in anticancer and anti-inflammatory activities (Kong et al. 2010). Berge et al. (2002) reported the sulfolipids of *Porphyridium cruentum* rich in palmitic acid, arachidonic acid (C20:4 omega-6), eicosapentaenoic (C20:5 omega-3) acids, and 16:1n-9 fatty acid-inhibited DNA alpha-polymerase and with its anti-inflammatory activity and antiproliferative activities against human colon adenocarcinoma DLD1 cells. The ubiquitin-proteasome pathway (UPP) is a major nonlysosomal system responsible for intracellular protein degradation in eukaryotic cells, and inhibition of NF-κB is a crucial step in anti-inflammatory and cellular growth arrest (Chen and Dou 2010). Hwang et al. (2011) showed that the anti-inflammatory and antiproliferative activity of sulfated polysaccharide from *Sargassum hemiphyllum* could be a possible reduction in cytosol/nuclear factor-kappa B (NF-κB) p65 expression by inhibiting IL-1β, IL-6, TNF-R, NO, iNOS, and cyclooxygenase-2 (COX-2). Activated NF-κB, a well-known transcription factor, leads to the development, differentiation, and proliferation of T and B lymphocytes and the secretion of cytokines by the classical pathway. Pheophytins of *Cladophora fascicularis* are reported to be the promising drugs by inhibiting proteasome ChT-L and NF-κB for anti-inflammation and antiproliferative activity (Huang et al. 2007). Denise et al. (2011) studied the role of aquamin, a well-known nutrient sup-

Table 35.3 Antibacterial, antiviral, antifungal, and antiparasitic metabolites of marine microbes

Metabolite	Chemical nature	Source	Target	Concentration	Reference
Marine bacterial metabolites					
1-acetyl-beta-carboline	Carboline	*Pseudomonas* sp. UJ-6	MRSA (methicillin-resistant *Staphylococcus aureus*) (KCCM 40510), MRSA (KCCM 40511), MRSA DH-3, MRSA DH-4, MRSA DH-6, MRSA DH-8, MRSA DH-11, MRSA DH-12, MRSA DH-13, MRSA DH-14, MRSA DH-17, MRSA DH-18, *Staphylococcus aureus* (KCTC 1927), *S. aureus* (KCTC 1928), *Bacillus subtilis* (KCTC 1028), *Escherichia coli* (KCTC 1682), *Pseudomonas aeruginosa* (KCTC 1637), *Salmonella typhimurium* KCTC 1925), and *Klebsiella pneumonia* (KCTC 2242)	MIC values at 32, 64, 64, 128, 32, 32, 64, 128, 64, 128, 32, 64, 16, 32, 32, 32, 64, 128, and 64 μg mL^{-1} respectively	Lee et al. (2013)
1-methyl-1,4 dihydroquinoline	Quinoline	*Pseudomonas aeruginosa* isolated from eel fish of Baluchistan coast, Pakistan	MSSA (methicillin-sensitive *S. aureus*), MRSA, *S. epidermidis*, *Enterococcus faecalis*, *E. faecium*, *Micrococcus luteus*, *E. coli*, *Vibrio alginolyticus*, *Salmonella typhi*, *Shigella flexneri*, and *Proteus mirabilis*	Zone of inhibition, at 100 μg/disk, 20, 21,18, 19, 22, 24, 18, 14, 18, 17, and 19 mm respectively	Uzair et al. (2006)
24-membered macrolide macrolactins T (**1**), B (**2**), and D (**3**)	Lactones	*Bacillus marinus*, isolated from *Suaeda salsa*, Bohai Sea, China	Fungi, *Pyricularia oryzae*, *Alternaria solani*, and bacteria, *S. aureus*	MIC values for (**1**) 0.8, 2.8, and 5.5 μg mL^{-1} for (**2**) 7.5, 20.1, and 4.5 μg mL^{-1} for (**3**) >100, >100, and >100 μg mL^{-1} respectively	Xue et al. (2008)
2-aminophenoxazin-3-one (**1**), 2-amino- 6-hydroxyphenoxazin-3-one (**2**), and 2-amino-8-benzoyl-6-hydroxyphenoxazin-3-one (**3**)	Aminophenoxazin derivatives	γ-proteobacteria, *Halomonas* sp. strain GWS-BW-H8hM, East Frisian Wadden Sea	(**a**) *Bacillus subtilis*, *S. aureus*, and *C. albicans* (**b**) *B. subtilis* and *S. aureus*	Zone of inhibition, at 50 μg/disk, of (**1**) for (**a**) 20, 15, and 25 mm, respectively, of (**2**) for (**b**) 25 and 16 mm, respectively, and of (**3**) for (**b**) 16 and 10 mm respectively	Bitzer et al. (2006)

(continued)

Table 35.3 (continued)

Metabolite	Chemical nature	Source	Target	Concentration	Reference
Ariakemicins A and B (mixture)	Linear peptides	Gliding bacterium, *Rapidithrix* sp.	*Brevibacterium* sp. JCM6894, *S. aureus* IFO12732, and *B. subtilis* IFO3134	Zone of inhibition, at 2.6 µg/disk, 12, 16, and 15 mm and MIC values 83, 0.46, and 83 µg mL^{-1} respectively	Oku et al. (2008a)
Bacillistatins 1 (**a**) and 2 (**b**)	Cyclodepsipeptides	*Bacillus silvestris*	*Streptococcus pneumoniae* (ATCC 6303), penicillin-resistant *S. pneumoniae*, multidrug-resistant *S. pneumoniae* (ATCC 700673), and *S. pyogenes.*	MIC values of (**a**) 2, 1, <0. 5 and 2 µg mL^{-1} respectively and of (**b**) 1, 1, 0.00180, <0.5 and 8 µg mL^{-1} respectively	Pettit et al. (2009)
Macrolactins 1-3	24-membered macrolactones	*Bacillus* sp.	(**a**) *B. subtilis* and *E. coli* (**b**) *Saccharomyces cerevisiae*	MIC values for (**a**) 0.16 µM respectively and for (**b**) 0.16, 0.02, and 0.16 µM, respectively	Mondol et al. (2011a)
Gageomacrolactins 1-3 (**I**) and macrolactins A, B, F, and W (**II**).	Macrolactones	*Bacillus subtilis*	(**a**) Gram +ve bacteria: *S. aureus*, *B. subtilis*, and *B. cereus* and Gram –ve bacteria: *E. coli*, *S. typhi*, and *P. aeruginosa* (**b**) Pathogenic fungi: *Aspergillus niger*, *Botrytis cinerea*, *Colletotrichum acutatum*, *Candida albicans*, and *Rhizoctonia solani*	MIC values of (**I**) for (**a**) in the range of 0.02–0.05 µM and of (**I** and **II**) for (**b**) in the range of 0.04–0.3 µM	Tareq et al. (2013)
2,3,5,7-tetrabromobenzofuro [3,2-b]pyrrole and 4,4′,6-tribromo-2,2′-biphenol	Heterocyclic compounds	*Pseudoalteromonas* sp. (CMMED 290), isolated from nudibranch of Kaneohe Bay, Oahu	MRSA (ATCC 43300)	MIC values of 1.93 ± 0.05 and 2.19 ± 0.08 µM respectively	Fehér et al. (2010)
Hunanamycin A	Pyrido[1,2,3-de] quinoxaline-2,3-dione	*Bacillus hunanensis*	*Salmonella enterica*	MIC value of 12.4 µM	Hu et al. (2013)
Ieodoglucomides A (**1**) and B (**2**)	Glycolipopeptides	*Bacillus licheniformis*	Gram +ve bacteria: *S. aureus*, *B. subtilis*, and *B. cereus* Gram –ve bacteria: *E. coli*, *S. typhi*, and *P. aeruginosa* fungi: *C. albicans* and *A. niger*	MIC values of (**1**) 8, 16, 16, 16, 8, 8, 32, and 32 µg mL^{-1} respectively and of (**2**) 8, 16, 8, 16, 16, 8, 32, and 16 µg mL^{-1} respectively	Tareq et al. (2012)

Ieodomycins A–D	Fatty acids	*Bacillus* sp.	(**a**) *B. subtilis* KCTC 1021 and *E. coli* KCTC 1923 (**b**) *Saccharomyces cerevisiae* KCTC 7913	MIC values for (**a**) in the range of 32–64 µg mL^{-1} and for (**b**) 256 µg mL^{-1} respectively	Mondol et al. (2011b)
Unnarmicins C and A	Depsipeptides	*Photobacterium* sp. strain MBIC06485	(**a**) *Pseudovibrio denitrificans* JCM12308 and (**b**) *Pseudovibrio* sp. MBIC3368	Zone of inhibition, at 2.6 mg/disk, for (**a**) 7 and 10 mm respectively and for (**b**) 7 and 9 mm respectively	Oku et al. (2008b)
Marine actinobacterial metabolites					
N-(2-hydroxyphenyl)-2-phenazinamine (NHP)	Phenazines	*Nocardia dassonvillei*	*Candida albicans*	MIC value of 64 µg mL^{-1}	Gao et al. (2012)
Daryamides A and B	Daryamides	*Streptomyces* strain CNQ-085	*C. albicans*	MIC values of 62.5 and 125, respectively	Asolkar et al. (2006)
Mayamycin	Benz[a]anthracene	*Streptomyces* sp. strain HB202	*Bacillus subtilis* (DSM 347), *Brevibacterium epidermidis* (DSM 20660), *Dermabacter hominis* (DSM 7083), *Klebsiella pneumoniae* (ATCC 700603; ESBL), *Propionibacterium acnes* (DSM 1897), *Pseudomonas aeruginosa* (DSM 50071), *Staphylococcus aureus* (ATCC 12600), *Staphylococcus aureus* (ATCC 33593; MRSA), *Staphylococcus epidermidis* (DSM 20044), *Staphylococcus lentus* (DSM 6672), *Xanthomonas campestris* (DSM 2405), and *Phytophthora infestans*	IC_{50} values of 8.0, 7.45, 8.4, 2.5, 31.2, 2.5, 2.5, 1.25, 0.31, 8.0, 30.0, and 15.2 µM, respectively	Schneemann et al. (2010)
Salinosporamide A	Salinosporamides	*Salinispora tropica*	*P. falciparum*, *Plasmodium yoelii*, parasites	IC_{50} values of 11.4 nM (in vitro), 130 mg kg^{-1} (in vivo)	Prudhomme et al. (2008)
Marine cyanobacterial metabolites					
Coibacins A-D	Polyketides	*Oscillatoria* sp.	*Leishmania donovani*	IC_{50} values of 2.4, 7.2, 18.7, and 7.8 µM respectively	Balunas et al. (2012)
Lagunamide C	Cyclodepsipeptide	*Lyngbya majuscula*, Pulau Hantu Besar, Singapore	*Plasmodium falciparum*	IC_{50} value of 0.29 µM	Tripathi et al. (2011)

(continued)

Table 35.3 (continued)

Metabolite	Chemical nature	Source	Target	Concentration	Reference
Pitipeptolide F	Cyclodepsipeptide	*Lyngbya majuscula*, Guam	*Mycobacterium tuberculosis* (ATCC #25177)	Zone of inhibition 10 mm, at 10 µg/disk	Montaser et al. (2011)
Marine fungal metabolites					
Arugosin H (**1**), arugosins A and B mixture (**2**), and sterigmatocystin (**3**)	Prenylated polyketides	*Emericella nidulans* var. *acristata*, isolated as an endophyte from a Mediterranean green alga	(**a**) *Mycotypha microspora* and *Chlorella fusca* (**b**) *Bacillus megaterium*	Zones of inhibition, at 50 µg/disk, of (**1**) against (**a**) 3 and 2 mm respectively, of (**2**) against (**b**) 4 mm, and of (**3**) against (**a**) 11 and 5 mm respectively	Kralj et al. (2006)
Asperterrestide A	Cyclic tetrapeptide	*Aspergillus terreus* SCSGAF0162	Influenza virus strains A/WSN/33 (H1N1) and A/Hong Kong/8/68 (H3N2)	IC_{50} values of 15 and 8.1 µM, respectively	He et al. (2013a)
Brevianamide S	Dimeric diketopiperazine	*Aspergillus versicolor*, isolated from Bohai Sea, China	Bacillus Calmette-Guerin (BCG)	MIC value of 0.05 µg mL^{-1}	Song et al. (2012)
Chaetoxanthones B (**1**) and C (**2**)	Heterocyclic xanthones	*Chaetomium* sp.	(**a**) *Plasmodium falciparum* and (**b**) *Trypanosoma cruzi*	IC_{50} values of (**1**) against (**a**) 0.5 µgmL^{-1} and of (**2**) against (**b**) 1.5 µg mL^{-1}	Pontius et al. (2008a)
Didymellamides A (**1**) and B (**2**)	4-hydroxy-2-pyridone alkaloids	*Stagonosporopsis cucurbitacearum*	(**a**) *C. albicans* (azole resistant and azole sensitive), *C. glabrata*, and *Cryptococcus neoformans* and (**b**) *Cryptococcus neoformans*	MIC values of (**1**) against (**a**) 3.1, 3.1, 3.1, and 1.6 µg mL^{-1}, respectively and of (**2**) against (**b**) 6.3 µg mL^{-1}	Haga et al. (2013)
Emericellamides A and B	Cyclic depsipeptides	*Emericella* sp., when co-cultured with actinomycete, *Salinispora arenicola*	MRSA	MIC values of 3.8 and 6.0 µM, respectively	Oh et al. (2007)
Hypothemycin and 4-*O*-demethylhypothemycin	Polyketides	*Aigialus parvus* BCC 5311	*P. falciparum* K1	IC_{50} values of 2.8 and 3.0 µg mL^{-1}, respectively	Isaka et al. (2009)
Isoaspulvinone E, aspulvinone E, and pulvic acid	Butenolides	*Aspergillus terreus* Gwq-48	Influenza A H1N1 virus	IC_{50} values of 32.3, 56.9, and 29.1 µg mL^{-1}, respectively	Gao et al. (2013)

4-keto-clonostachydiol, clonostachydiol and two oxidized derivatives of clonostachydiol	Polyketides	*Gliocladium* sp.	*B. subtilis* and fungi *Trichophyton mentagrophytes* and *Cladosporium resinae*	Zones of inhibition, at 40 µg/disk, of 6, 2, and 1 mm, respectively	Lang et al. (2006)
Nodulisporacid A (**1**) and vermelhotin (**2**)	Heterocyclic compounds	(**1**) From *Nodulisporium* sp. CRIF1 and (**2**) from unidentified fungus CRI247-01 (order Pleosporales)	*Plasmodium falciparum* strain 94 (chloroquine resistant)	IC_{50} values of (**1**) and (**2**) 1–10 µM	Kasettrathat et al. (2008)
Paeciloxanthone	Xanthone	*Paecilomyces* sp. (Tree1-7)	*Curvularia lunata* (walker) Boedijn, *E. Coli*, and *Candida albicans*	Zones of inhibition, at 40 µg/disk, of 6, 12, and 10 mm, respectively	Wen et al. (2008)
Penicisteroide A	Polyoxygenated steroid	*Penicillium chrysogenum* QEN-24S	*Aspergillus niger* and *Alternaria brassicae*	Zones of inhibition, at 20 µg/disk, of 18 and 8 mm, respectively	Gao et al. (2011)
Sclerotides A and B	Cyclic hexapeptides	*Aspergillus sclerotiorum* PT06-1	*Candida albicans*	MIC values of 7.0 and 3.5 µM, respectively	Zheng et al. (2009)
Speradine A	Pentacyclic oxindole alkaloid	*Aspergillus tamarii*	*Micrococcus luteus*	MIC value of 16.7 µg mL^{-1}	Tsuda et al. (2003)
Sydowiol C	Pyrogallol ether	*Aspergillus sydowii* (MF357)	*S. aureus*	MIC value of 12.5 µg mL^{-1}	Liu et al. (2013)
Trichoderins A (**1**), A1 (**2**), and B (**3**)	Aminolipopeptides	*Trichoderma* sp.	*Mycobacterium smegmatis*, *M. bovis* BCG, and *M. tuberculosis* H37Rv	MIC values of (**1**) 0.1, 0.02, and 0.12 µg mL^{-1}, respectively and of (**2**) 1.56, 0.16, and 2.0 µg mL^{-1}, respectively and of (**3**) 0.63, 0.02, and 0.13 µg mL^{-1}, respectively	Pruksakorn et al. (2010)
(−)-Trypethelone	Polyketide-type alkaloid	*Coniothyrium cereale*	*Mycobacterium phlei*, *Staphylococcus aureus*, and *E. coli*	Zones of inhibition, at 20 µg/disk, of 18, 14, and 12 mm, respectively	Elsebai et al. (2011)

plement, from red algae *Lithothamnion corallioides* in activating NF-κB in RAW 264.7 cell lines. Their study revealed that aquamin inhibited the phosphorylation and degradation of IkB. Aquamin also inhibited COX-2 gene expression and thus rendered NF-κB inhibition.

C-reactive protein (CRP) is one of the acute-phase proteins involved in inflammation. The circulating CRP is synthesized and secreted predominantly by hepatocytes in response to proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1, and interleukin-6; obesity and smoking are the main factors for the activation of the above cytokines, and the natural dietary substances such as seaweeds are of great interest in inhibiting these factors (Pepys and Hirschfield 2003). Particularly, Nanri et al. (2008) found that Japanese men and women were less prone to the sustainability of the life-threatening diseases than western people due to inhibition of C-reactive protein substances by seaweed extracts that are routinely used in their food recipe. Marine-derived microbial secondary metabolites open new door for novel drug discovery. The cyclomarins are well known for their anti-inflammatory effects from marine benthic bacteria, especially from *Streptomyces* sp. with novel cyclic heptapeptides containing four unusual amino acids. Marine-derived cyclomarin A, with its naval amino acid 2-amino-3,5-dimethylhex-4-enoic acid, acts as a potent anti-inflammatory agent and displayed almost 92 % reduction in phorbol ester (PMA)-induced mouse ear edema experiment at 50 μg/ear; indomethacin showed only 72 % of such activity (Renner et al. 1999). Pyrenocine A (PA), isolated from marine *Penicillium paxilli* Ma(G)K, suppressed LPS-induced macrophage activation on both pre- and posttreatment methods. PA inhibited nitrite and PGE2 production. It also affected Mac-1 cell migration and co-stimulatory molecules on lymphocyte activation through MyD88-dependent intracellular signaling pathway (Toledo et al. 2014). They also reported that pyrenocine A inhibited NF-κB-mediated signal transduction on macrophages stimulated by LPS.

Marine cyanobacterial metabolites bearing halogenated carboxylic acid moiety depict anti-inflammatory properties. In the recent study, Choi et al. (2012) reported that honaucins A–C isolated from marine cyanobacterium *Leptolyngbya crossbyana* inhibit the LPS-induced nitric oxide (NO) production from murine macrophage RAW264.7 that, in turn, downregulates the expression of proinflammatory cytokines especially interleukin-1β. Honaucin A constitutes 3-hydroxy-γ-butyrolactone condensed with 4-chlorocrotonic acid, while honaucins B and C comprise 4-chlorocrotonic acid with 3,4-di-*O*-substituted butanoic acid. They inhibit NO production at IC_{50}s of 4.0, 4.5, and 7.8 μM respectively. Thus, marine extracts and metabolites possess noticeable anti-inflammatory activities.

35.2.5 Antioxidants

Extracellular polysaccharides from marine microbes were unveiled to have antioxidant properties. Marine fungi are also serving as one of the sources for antioxidants. Extracellular polysaccharides (EPS) designated as YSS (containing backbone (1→2)-linked α-mannopyranose, (1→6)-linked α-mannopyranose and branch β-galactofuranose, (1→6)-linked α-mannopyranose) from marine fungus *Aspergillus terreus* (Wang et al. 2013a), AVP (containing (1→6)-linked α-D-glucopyranose, (1→2)-linked α-D-mannopyranose with side chain (1→2)-linked α-D-mannopyranose trisaccharides) from coral-associated fungus *Aspergillus versicolor* LCJ-5-4 (Chen et al. 2012b), PS2-1 (containing glucuronic acid, mannose with glucose, and galactose) from *Penicillium* sp. F23-2 (Sun et al. 2009), and As1-1 isolated from mangrove endophytic fungus *Aspergillus* sp. Y16 showed antioxidant properties by quenching free radicals (Chen et al. 2011). Apart from EPS, benzamide derivative (methyl 4-(3, 4-dihydroxybenzamido) butanoate) from marine alga-derived endophytic fungus *Aspergillus wentii* EN-48 was also found to have

DPPH radical-scavenging activity (Li et al. 2014). EPS, from marine bacterium *Edwardsiella tarda*, ETW1, and ETW2 (mannan) possess antioxidant, hydroxyl, and DPPH radical-scavenging properties (Guo et al. 2010).

Besides marine microbes, seaweeds are the benevolent organisms to yield antioxidants in great quantity. Recently, sulfated polysaccharides, extracted from brown algae *Padina tetrastromatica*, *Sargassum tenerrimum*, and *S. swartzii*, were reported to have antioxidant properties. Fucoidan (sulfated polysaccharide) isolated from seaweed *Turbinaria conoides* reduces oxidative stress in myocardial-injured rat by improving cellular antioxidants (Mohankumar et al. 2012). Vijayabaskar and Shiyamala (2012) have suggested that polyphenolic contents of *Turbinaria ornata* are responsible for its radical-scavenging property. Brown seaweed *Cystoseira trinodis*, collected in Mandapam coast, India, is identified to produce antioxidants, phlorotannins by Sathya et al. (2013). Novel compound from brown alga *Ecklonia cava*, 2,7″-phloroglucinol-6,6′-bieckol (PHB), serves as antioxidant (Kang et al. 2012).

Apart from brown algae, red and green algae also produce antioxidants. Five nitrogen-containing bromophenols, 3-(2,3-dibromo-4,5-dihydroxybenzyl)pyrrolidine-2,5-dione; methyl 4-(2,3-dibromo-4,5-dihydroxybenzylamino)-4-oxobutanoate; 4-(2,3-dibromo-4,5-dihydroxybenzylamino)-4-oxobutanoic acid; 3-bromo-5-hydroxy-4-methoxybenzamide; and 2-(3-bromo-5-hydroxy-4-methoxyphenyl) acetamide, from red alga *Rhodomela confervoides* potently scavenge DPPH radicals (IC_{50}s, 5.22–23.60 μM) (Li et al. 2012). Five sesquiterpenoids (2,5,5-trimethyl-4-(4′-methyl-3′-pentenyl)-2-cyclohexen-1-ol;4-isopentyl-3,4,5,5-tetramethyl-2-cyclohexen-1-ol;4-isopentyl-3,4,5,5-tetramethyl-2-cyclohexen-1-ol;6-isopentyl-1,5,5,6-tetramethyl-1-cyclohexene;and3,4,5,5-tetramethyl-4-(3′-oxopentyl)-2-cyclohexen-1-one) from marine green alga *Ulva fasciata* are found to have antioxidant properties (Chakraborty and Paulraj 2010). Thus, these antioxidants can be used to cope up with the oxidative stress after checking for their toxicity.

35.3 Industrial Values

35.3.1 Antifouling Activity

Formation of the biofilms by microbes, macrobes, and in combination on the submerged man-made surfaces in marine environment is termed biofouling and in due course establishes large complex biological layer that causes huge economic problem worldwide every year. Though heavy metals containing inorganic biocides are used as antifoulants, they are very toxic and directed to bioaccumulation and magnification. Hence, natural sources have been searched for eco-friendly potent antifoulants. Marine organisms including bacteria, fungi, and algae are explored to produce antifouling compounds. Aspergillipeptide C, a cyclic tetrapeptide, isolated from marine Gorgonian-derived fungus *Aspergillus* sp. SCSGAF 0076, poses strong antifouling activity against the larval settlement of bryozoans *Bugula neritina* (Bao et al. 2013). Shao et al. (2011a) have isolated strong antifouling compounds, 14-membered resorcylic acid lactone (cochliomycin A) and analogs zeaenol, LL-Z1640-1, and paecilomycin F, from Gorgonian-derived fungus *Cochliobolus lunatus*. These antifoulants inhibit the larval settlement of barnacle *Balanus amphitrite* (EC_{50}s of 1.2, 5.0, 5.3, and 17.9 μg mL^{-1}, respectively). Likewise, fungus *Aspergillus* sp., isolated from gorgonian *Dichotella gemmacea*, was identified to produce aspergilone A, which is also inhibiting the settlement of *B. amphitrite* (EC_{50} of 7.68 μg mL^{-1}) (Shao et al. 2011b). A halotolerant fungus, *Penicillium* sp. OUCMDZ-776 produces antifouling alkaloid, penispirolloid A, which inhibits the larval settlement of bryozoans *Bugula neritina* (EC_{50} of 2.40 μg mL^{-1}) (He et al. 2012a). α,β-unsaturated lactones from marine *Streptomyces* sp. were found to inhibit the larval settlement of barnacle *B. amphitrite*.

Six potent antifouling compounds, chromanols S1–S6, isolated from brown alga *Sargassum horneri*, showed inhibition of various biofouling organisms including the larval settlement of hard fouling organism mussel *Mytilus*

edulis (EC_{50} of 0.11–3.34 μg mL^{-1}), spore settlement of macroalga *Ulva pertusa* zoospores (EC_{50} of 0.01–0.43 μg mL^{-1}), the diatom *Navicula annexa* (EC_{50} of 0.008–0.19 μg mL^{-1}), *Pseudomonas aeruginosa* KNP-3, and *Alteromonas* sp. KNS-8 (MICs of 1.68–36.8 and 1.02–30.4 μg mL^{-1}, respectively) (Cho 2013). Cyclic diterpenoid dictyol C, isolated from marine brown alga *Dictyota* sp., was found to have antifouling activity by inhibiting the adhesion of marine bacterial biofilm of *Pseudoalteromonas* sp. D41 with an EC_{50} of 30 μM (Viano et al. 2009).

Cho et al. (2012) have isolated two diketopiperazines, namely, (*6S*, *3S*)-6-benzyl-3-methyl-2,5-diketopiperazine and (*6S*, *3S*)-6-isobutyl-3-methyl-2,5-diketopiperazine, from marine actinomycetes *Streptomyces praecox* 291-11 (Fig. 35.1). They inhibit the settlement of diatom *Navicula annexa* and zoospores of green alga *Ulva pertusa* with the therapeutic ratio of >15 and hence, they are considered to be the promising antifoulants. In another study, Cho and Kim (2012) found that the diterpene compound lobocompactol is produced by the strain *Streptomyces cinnabarinus* PK209. Lobocompactol (Figure 35.1) is found to inhibit *Ulva pertusa* and *Navicula annexa* and showed the therapeutic ratio of 46.2 and 71.6 μg mL^{-1}, respectively and also inhibited two biofilm forming bacteria KNP-5 and KNP-8. Five diketopiperazines (DKPs), cyclo-(L-Leu-L-Pro), cyclo-(L-Phe-L-Pro), cyclo-(L-Val-L-Pro), cyclo-(L-Trp-L-Pro), and cyclo-(L-Leu-L-Val), were isolated from the deep-sea bacterium *Streptomyces fungicidicus*, showing the potential biofilm inhibitor against barnacle *B. amphitrite*. The EC_{50} value of five DKPs against barnacle larvae ranged from 0.1 to 0.28 mM, and the LC_{50} ranged from 0.47 to 0.75 mM (Li et al. 2006). Thus, any of such natural antifoulants can be tried to overcome biofouling phenomenon to save the environment.

35.3.2 Biofuel

In 2010, biofuel was fed to 2.7 % of the world's requirement which reached almost 105 billion liters as a whole. But, global demand for biofuels is continuously unabated every year. Though the land crops are used for biofuel production, the necessity for their production is dependent on the agricultural inputs (pesticides, fertilizers, farmable land, and water). An alternative source (with abundance and carbohydrate-rich source), which can be independent of above said criteria, would be beneficial for enhanced biofuel production. By concerning these, the algae, particularly seaweeds, would be expected as vital source for ethanol market due to their higher growth rate (Luning and Pang 2003), easy inhabitation, not requiring high fertilizers, low cellulose content, and devoid of lignin, a tough wall material usually found in land plants (Thomas 2002). Large-scale seaweed cultivation would be a better alternative way for reducing the environmental pollution and global warming made by various anthropogenic activities (Kraan 2013). The types of biofuel from seaweeds can be categorized into biodiesel, bio-oils, and biohydrogens. A wide variety of algal genera especially kelps have been exploited recently as a rich source of biofuel (Qin et al. 2005), Merlin et al. (2013) reported that bioethanol production from *Laminaria digitata*, a well-known brown alga, would economically be beneficial than biogas (methane) production. Biodiesel is a product of the reaction between triglycerides and mono-alcohol (most commonly methanol or ethanol) with the catalysis of alkali, acids, or enzymes. Lipid content of an algal species is an important criterion for evaluating its biodiesel-producing capacity. The lipid profiles of many marine algae resemble with that of land plants and such algae can be considered as the best source for biodiesel production (Xu et al. 2006b; Ohlrogge and Browse 1995). Cobelas and Lechado (1989) have analyzed the lipid profile of the various marine algae including cyanobacteria and reported that the fatty acid contents are as follows: C16:0 and C16:1 in the Bacillariophyceae; C16:0 and C18:1 in the Chlorophyceae; C16:0 and C18:1 in the Euglenophyceae; C16:0, C16:1, and C18:1 in the Chrysophyceae; C16:0 and C20:1 in the Cryptophyceae; C16:0 and C18:1 in the Eustigmatophyceae; C16:0 and C18:1 in the Prasinophyceae; C16:0 in the Dinophyceae;

C16:0, C16:1, and C18:1 in the Prymnesiophyceae; C16:0 in the Rhodophyceae; C14:0, C16:0, and C16:1 in the Xanthophyceae; and C16:0, C16:1, and C18:1 in Cyanobacteria.

In contrast to marine eukaryotic algae, cyanobacterial lipid content has been reduced at the laboratory conditions than in the natural environment. This made a tough challenge in using cyanophycean species for biofuel production, but by employing various genetic manipulation techniques, it is possible to increase their lipid content (Hu et al. 2008; Beer et al. 2009). Wang et al. (2013b) studied mutated *Synechocystis* sp. PCC6803 for the production of alkanes in lab condition. By suppressing the expression of enzymes acyl-acyl carrier protein reductase, aldehyde-deformylating oxygenase and overexpression of alkane biosynthetic genes in cyanobacteria significantly improved the alka(e)ne production, and the yield was increased up to 1.3 % (per DCW, dried cell weight), which was higher than 8.3-fold, comparatively to the wild-type strain (0.14 % per DCW). Recently, relevant bioenergy genes were identified in microalgae and relevant recombinant technology have been developed to engineer the pathway, resulting in higher yield of biofuel production (Beer et al. 2009).

Ahrens and Sander (2010) recently recorded that the cost for algal biomass conversion to commercial diesel product would be approximately 50 €/L. Demirbas and Demirbas (2011) have reported that the oil production from marine algae is 7–31-fold higher than palm oil from respective crop. They also added that 50 % biomass can be converted to biofuel due to high content of lipids in the plant thalli. At mean time, algal lipids were also subjected to a bunch of constraints including extraction, slurry-drying, and cost-effective biomass production and storage conditions (Hu et al. 2008). Fortunately, the average lipid content of the algal cells has been raised in stress conditions, i.e., culturing in vitro conditions. Hu et al. (2008) found that the lipid content has increased from 22.7 to 44.6 % of its dry cell weight while growing isolated diatoms at laboratory conditions. Hydrocarbons are found at higher ratio next to triacylglycerol, up to 5 % DCW (Lee and Loeblich 1971 in algae. Golueke et al. (1957) proposed an idea of anaerobic digestion of polluted biomass. Sialve et al. (2009) used these coupled techniques for the production of biogas from algal mass. On continuing this, a number of studies related to the biogas evaluation from algae have been achieved recently. El-Mashad (2013) has studied the methane production from anaerobic batch digestion between switch grass (*Panicum virgatum*) and *Spirulina platensis*. They reported that 83 % switch grass and 17 % *S. platensis* was well suited for methane (236 mL g^{-1}) production on 40 days incubation. Bruhn et al. (2011) reported that the anaerobic digestion with *Ulva lactuca*, isolated from marine environment, yielded 271 mL methane per gram of cattle manure at a cultivating facility. They also added that the dried biomass produced five- to ninefold higher yield than wet biomass during the experiment.

35.3.3 Nutraceuticals

Consumption of marine foods provides enormous health benefits such as neuroprotective agents, curing cardiovascular diseases, diabetes, etc. (Miyashita and Kim 2011). Marine foods contain natural products like polyunsaturated fatty acids (PUFAs), polysaccharides, natural pigments (NPs), essential minerals, vitamins, enzymes, and bioactive peptides which serve such benefits (Pangestuti and Kim 2011a). Soliev et al. (2011) have reviewed about marine bacterial pigments including prodiginines, carotenes, violacein, phenazines, quinones, tambjamines, melanins, scytonemin, and tryptanthrin, and they depicted a variety of biological activities like antimicrobial, anticancer, and antifouling activities. Several marine algae have been reported for acetylcholinesterase (AChE) inhibitory activity; thereby, they can be used for treating Alzheimer's disease (Pangestuti and Kim 2011b). Four marine algae, namely, *Hypnea valentiae*, *Padina gymnospora*, *Ulva reticulata*, and *Gracilaria edulis*, have been shown to inhibit AChE with the IC_{50} values of 2.6, 3.5, 10, and 3 mg mL^{-1}, respectively (Suganthy et al. 2010). The marine actinomycete

Streptomyces sp. strain LB173 produces a new phenazine natural product, geranylphenazinediol, which inhibits AChE with IC_{50} value of 2.62 μM (Ohlendorf et al. 2012). Protein tyrosine phosphatase 1B (PTP1B) is the major cause for type 2 diabetes mellitus by negative regulation of insulin signaling pathways. Yong et al. (2008) revealed the red algae *Rhodomela confervoides* shows potent inhibition against the PTP1B. Four bromophenol derivatives were derived from this red alga: 2,2′,3,3′-tetrabromo-4,4′,5,5′-tetrahydroxydiphenyl methane; 3-bormo-4,5-bis (2,3-dibromo-4,5-dihydroxybenzyl) pyrocatechol; bis(2,3-dibromo-4,5-dihydroxybenzyl) ether; and 2,2′,3-tribromo-3′,4,4′,5-tetrahydroxy-6′-ethyloxymethyldiphenylmethane, which inhibit PTP1B with IC_{50} values of 2.4, 1.7, 1.5, and 0.84 μM, respectively (Yong et al. 2008).

35.3.4 Marine Metagenomics

Marine world confers shelter for various life forms including autotrophs, heterotrophs, psychrophiles, mesophiles, thermophiles, hyperthermophiles, acidophiles, alkaliphiles, halophiles, barophiles, etc., having diverse metabolic behavior. Undoubtedly, they are adapted well to survive such extreme conditions with the help of potential biomolecules which can predominantly be of enzymes, proteins, and simple/complex compounds. Exploration of such products can be applied for various purposes that can withstand in extreme conditions. Many of such organisms are uncultivable in the laboratory mainly because of the lack of providing or mimicking their natural conditions. But, metagenomics is an important tool aiding to explore such potential products coded by a metagenome that is obtained from marine floral, faunal, and environmental samples. Metagenomics is engaged with sequential steps of DNA extraction from metagenome, library construction, library screening, target gene sequencing, and product characterization. DNA could be isolated by either direct or indirect methods, though direct methods result in higher yield. In case of lower yield, multiple displacement amplification (MDA) technique can be employed to amplify the whole genome to yield good amount of DNA. Choice of vector for library construction depends on the DNA fragment size, e.g., 10 kb fragment needs vectors used in small-size library construction, viz., high copy number plasmid pUC19; likewise, fosmids/cosmids can harbor 20–40 kb inserts, while BACs have the capacity for 100–200 kb inserts. Broad-host-range vectors can also be employed for library construction that allows the expression of inserts in different hosts. Apart from *E. coli*, hosts like *Bacillus*, *Pseudomonas*, *Streptomyces*, *and Rhizobium* (having more RNA polymerase σ factors) and certain archaebacteria are utilized for expression. To determine the bioactive clones from the constructed library, two strategies could be employed, namely, activity-based screening and sequence-based screening. The former strategy enables the finding of genes that code for novel or already known class of bioactive compounds or enzymes. It is all about the host's ability to anabolize such products and not experiencing the toxicity. The latter strategy can be accomplished with the engagement of two techniques, i.e., PCR and hybridization. It can be performed only if the knowledge of the sequence is available, and it is all about the presence of conserved domain or consensus sequence in the clones (Felczykowska et al. 2012). For the sequence-based screening of the clones for the bioactive polyketides/short peptides, biosynthetic gene clusters of modular polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), and hybrid polyketide synthase/non-ribosomal peptide synthetase can be employed. The sequence of NRPS gene clusters of target clone can be annotated into the polypeptide sequence with the help of computer packages *ClustScan* and *CompGen* (Zucko et al. 2010). He et al. (2013b) have isolated eleven cyclopeptides (Cyclo(L-Thr-L-Leu), Cyclo(L-Val-D-Pro), Cyclo(L-Ile-D-Pro), Cyclo(L-Leu-L-Pro), Cyclo(L-Val-L-Leu), Cyclo(L-Leu-L-Ile), Cyclo(L-Leu-L-Leu), Cyclo(L-Phe-L-Tyr), Cyclo(L-Trp-L-Pro), Cyclo(L-Val-L-Trp), and Cyclo(L-Ile-L-Trp)) from antibacterial (against *Bacillus cereus*) clone pDC113 of *E. coli* EPI300-T1R, by activity-based screening of

metagenomic library of Japanese marine sponge *Discodermia calyx*. Previously, red pigments Zn-coproporphyrin III, coproporphyrin III, Zn-protoporphyrin IX, and protoporphyrin IX have been isolated from the clones of pDC112 and pDC114 by functional screening of sponge *D. calyx* metagenomic library (He et al. 2012b). Donia et al. (2011) have found anticancer cyanobactin class of the compound minimide, through sequence-based screening from the ascidian sample *Didemnum molle*. Antitumor compound bryostatin, from marine bacteria *Candidatus* E. *sertula*, a symbiont of marine bryozoan *Bugula*, apratoxin A from marine cyanobacterium *Lyngbya bouillonii*, salinosporamide A from marine actinomycete *Salinispora pacifica*, and onnamide from marine sponge *Theonella swinhoei* have been discovered through sequence-based screening of NRPS/PKS gene clusters and genome mining (Nikolouli and Mossialos 2012).

Apart from bioactive compounds, marine metagenomics can also be applied for screening the oil-degrading enzymes from the microbes at the site of oil seepage from ship to control oil pollution. Enzymes like esterase, lipase, protease, DNA polymerase, cellulase, chitinase, amidase, alkaline hydroxylase, pigments, proteins, and dyes explored from extremophiles via metagenomics can be applied in various industrial and research usages (Felczykowska et al. 2012).

35.4 Molecular Applications

Among the marine organisms, the algae are found to be the ideal expression systems; this is because of higher growth rate (lower doubling time within 24 h), cost-effective biomass production, post-transcriptional and posttranslational modifications comparable with other eukaryotes, and controllable bioreactor growth (Janssen et al. 2003). Moreover, microalgae possess strong constitutive homologous promoters in their expression systems. For instance, fucoxanthin chlorophyll-binding protein (Fcp A), from marine alga *Phaeodactylum tricornutum*, has extensively been used for the expression of heterologous proteins (Walker and Collet 2005). On the other hand, cold-shock proteins (CSPs) from marine organisms are important proteins for industrial usage below 10 °C. An Antarctic psychrophilic bacterium, *Shewanella livingstonesis* Ac10, has been regarded as a better model organism in characterizing the CSP chaperons. These are with peptidyl prolyl cis-trans isomerase activity (PPIase) that catalyzes the cis-trans isomerization of proline imidic peptide bonds which is required for proper folding of protein at cold temperatures. Tig is also a cold-adapted chaperon isolated from *S. livingstonesis*, possessing PPIase activity that properly folds the nascent peptides (Heidelberg et al. 2002). An engineered fluorescent protein, phytochrome, IPF1.4, which emits light at infrared region, expressed from a marine bacterium *Deinococcus radiodurans*, has been used in the visualization of even deeper tissues in medical fields. iRFP, another phytochromic protein engineered in marine bacterium *Rhodopseudomonas palustris*, is also used in infrared imaging (Filonov et al. 2011). The marine algae *Phaeodactylum tricornutum*, *Cyclotella cryptica*, *Navicula saprophila*, *Cylindrotheca fusiformis*, and *Thalassiosira weissflogii* have been evolved as suitable expression systems for heterologous proteins with a number of selectable marker genes including ble (zeocin (bleomycin family)-binding protein), nat (nourseothricin acetyltransferase), sat-1 (streptothricin acetyltransferase), and nptII (neomycin phosphotransferase II). Zaslavskaia et al. (2001) have developed a multiple cloning site vector from diatom *P. tricornutum* by adding ble (bleomycin antibiotic) selectable marker, and multiple cloning site regions are linked with fcpA (fucoxanthin, chlorophyll a/c-binding protein) promoter. The recombinated *P. tricornutum* grew as a facultative heterotrophic organism, and this engineered organism can express various genes from heterologous species. Reporter genes including cat (chloramphenicol acetyl transferase), luc (luciferase), uidA (β glucuronidase), and gfp (green fluorescent protein) could be engineered with diatom-specific promoter (fcp) to make efficient selection of expression vectors (Montsant et al. 2004). Red alga *Porphyra yezoensis* has been genetically engineered with bacterial based nitroreductase gene (*nfsI*) to enable the organism

to detoxify the trinitrotoluene for bioremediation purpose (Bernasconi et al. 2004).

Certain animal models help the scientific communities in assessing the lethal or sublethal impacts of contaminants or mixture of contaminants for research studies. The utilization of marine organisms as biological models would be logically based on three categories, namely, filter feeders and pelagic and benthic species. Lee et al. (2009) have suggested that the presence of sema domain clusters (domain of secreted or transmembrane protein involved in the axon guidance of neural development) in the sea hare, *Aplysia californica* (mollusk), makes this organism a powerful model system for studying the functioning of the nervous system. The transparency of the sea urchin's eggs enabled them as a model for studying the fertilization phenomena, and it assists to evaluate the impacts of toxins or drugs (http://www.exploratorium.edu/imaging-station/research/urchin/story_urchin1.php).

35.5 Conclusion

This review summarizes and substantiates the broad spectrum of bioactive natural products from marine bacteria, fungi, and algae with pharmacological and industrial importance. This represents their efficient anabolic properties. Though immense of bioactive compounds with variety of pharmacological and industrial potentials have intensively been explored, a very few of them have been brought to the human applications and a few synthetic derivatives (Soblidotin (TZT1027), Plinabulin (NPI-2358), Curacin A derivative, etc.) of marine-derived natural products were tested for clinical trials in various phases. This is because of very low yield, lack of stability, difficulty in in vitro laboratory synthesis, etc. In case of microbial cultivation, parameters like aeration, growth rate, nutrients, and contaminants would influence the yield of bioactive products. Despite the vast opportunity for using marine organisms in human welfare, clear-cut knowledge is required in isolating these unique bioactive metabolites. Mass cultivation strategies using improved bioreactors, synthesizing chemical analogs of a product without affecting its functionality and improving their stability, may encourage the launching of the product. The research on this field must further be expanded and implemented for future use. Marine metagenomics is an important tool to find the potential products mainly from uncultivable and extremophilic organisms. These marine forms are the golden source for mining economically valuable products with the tool of biotechnology to aid human well-being.

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Desert Plant Biotechnology: Jojoba, Date Palm, and Acacia Species

36

Muppala P. Reddy

Abstract

Arid lands occupy nearly one third of the world's total land area, and about 13 % of the world population are living in these inhospitable areas. Agriculture production from these areas is minimal due to prevailing hostile environment, and most of the land is underutilized. Recent advances in modern agriculture and biotechnology have provided scope of utilizing those underutilized arid lands for agriculture production. Plant tissue culture is one of the applications of biotechnology by which plants can be mass propagated using vegetative and sexual tissues without losing their genetic fidelity. Somaclonal variation is common among in vitro-derived plants; however, it could broaden genetic variability and genetic improvement. Molecular marker technology can be applied to identify variability and to select useful variants. Genomic and transcriptomic data and genetic engineering through gene transfer techniques pave the way for genetic improvement of different crops and plants. This chapter discusses the efforts made to improve jojoba [*Simmondsia chinensis* (Link) C.K. Schneid], an industrial oil-yielding species; date palm (*Phoenix dactylifera L.*), one of the oldest domesticated desert trees with socioeconomic importance; and Acacia, an important fodder and energy species for semiarid and arid regions.

Keywords

Biotechnology • Micropropagation • Acacia • Jojoba • Date palm • Somatic embryos • Genomics • Molecular markers • Genetic transformation

36.1 Introduction

Survival of mankind has always depended upon the use of the Earth's resources. In the past few decades due to habitat destruction, and unsustainable utilization of natural resources, many plant

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species came under threat. Arid lands occupy nearly one third of the world's total land area, and about 13 % of the world population are living in these inhospitable areas. Agriculture production from these areas is minimal due to drought, high temperature, salinity and scarcity of water resources, and, hence, serious problem for food security. Recent advances in modern agriculture and biotechnology have provided scope of utilizing underutilized arid land resources for agriculture production and have great potential in solving many problems pertaining to agriculture, industry, environment, and health which has direct relevance to sustainable development of countries located in arid land. Tissue culture technology has the potential to multiply plants in vitro using minimum quantity of propagules without losing their genetic fidelity and facilitates large-scale production of quality planting material and in genetic improvement of species. This chapter discusses the efforts made to improve jojoba [*Simmondsia chinensis* (Link) C.K. Schneid], an industrial oil-yielding species; date palm (*Phoenix dactylifera L.*), one of the oldest domesticated desert trees with socioeconomic importance; and Acacia, an important fodder and energy species for semiarid and arid regions.

36.2 Jojoba: *Simmondsia chinensis* (Link) C.K. Schneid: Simmondsiaceae

36.2.1 Introduction

Simmondsia chinensis, commonly known as jojoba, is native to the Sonoran Desert of southwestern USA and northern Mexico (Mills et al. 1997), with a life span of 100–200 years. It is an economically important wind-pollinated, evergreen, perennial, dioecious shrub. It can tolerate extreme temperatures (−5 to 54 °C) and can be cultivated on a variety of marginal lands that are not suitable for conventional agriculture (Bhardwaj et al. 2010). The seeds contain 50–55 % oil (Chikara and Kumari 1991; Bhardwaj et al. 2010), and the oil properties are similar to the oil obtained from the sperm whale (Low and Hackett 1981), which is now listed as an endangered species. Jojoba oil and its derivatives have potential in a wide range of applications in cosmetics, lubricants, plastic industries, pharmaceuticals, and medicine and also as biofuel (Reddy and Chikara 2010; Kumar et al. 2012). Recognition of jojoba oil as an alternative to sperm whale oil and its adaptability to grow on marginal lands has led to surge of interest in agriculture production of jojoba across the world. The plant is dioecious and exhibits tremendous variability in growth and yield (Chikara and Kumari 1991). Generally male plants outnumber in seed-raised population which is not desirable in commercial plantations, and identification of sex in early seeding stages has met with limited success (Agrawal et al. 2007). Setting up a plantation with asexual propagules saves time in replanting, as well as crop production and lineage (Llorente and Apostolo 2013). Vegetatively propagation is carried out by rooting of stem cuttings or by tissue culture method. Vegetative propagation by layering (Bashir et al. 2005), grafting (Bashir et al. 2006), and stem cuttings (Bashir et al. 2007) has been established with satisfactory results using selected genotypes. The major limitations of these techniques are the availability of large quantities of desired planting material and the seasonal propagation.

36.2.2 Micropropagation

Micropropagation is an alternative method of vegetative propagation and offers advantages, a fort for commercial production in a limited space without losing its genetic nature and with a very limited starting material; the technique is also important in genetic improvement of the species. Micropropagation has reached a commercial level in many plant species in recent years (Chandra and Mishra 2003). During the past few decades, therefore, several attempts have been made to propagate jojoba in vitro on various culture media supplemented with different concentrations and combinations of plant growth regulators, with varying levels of success

(Chaturvedi and Sharma 1989; Llorente and Apostolo 1998; Roussos et al. 1999; Gao and Cao 2001; Prakash et al. 2003; Tyagi and Prakash 2004; Bashir et al. 2008; Singh et al. 2008, 2011; Kumar et al. 2009, 2010b). BAP is the most commonly used growth regulator in jojoba tissue culture (Llorente and Apostolo 1998; Tyagi and Prakash 2004; Bashir et al. 2007). According to Hassan (2003) and Kumar et al. (2009), combined effect of BAP with NAA or IAA was found to be very effective for establishment and subsequent multiplication. In most such experiments, cytokinins (BAP, kinetin, and zeatin) have been used in combination with auxins (NAA, IAA, and IBA). Bashir et al. (2008), working with different genotypes of jojoba, showed that BAP alone was better than kinetin alone or BAP + kinetin for in vitro shoot initiation, whereas Singh et al. (2008) reported BAP in combination with adenine proved to be the best for shoot induction and subsequent multiplication. Increase in KNO_3 concentration in the medium improved shoot multiplication rate and in vitro flowering in 20 % of male cultures.

Success of in vitro regeneration depends on the control of morphogenesis, which is influenced by the kind of tissue or explants, age, origin of the initial tissue, composition of media, plant growth regulators (PGRs), media additives, culture environment, etc. (Giri et al. 2004; Rai et al. 2010). In jojoba the number of shoots produced in vitro depended upon the source of explant (Gao and Cao 2001), type of explant (Hassan 2003), composition of media, nature of growth regulators and their concentrations and combination, genotype (Llorente and Apostolo 1998; Prakash et al. 2003; Tyagi and Prakash 2004), and type of vessels and cultural conditions (Mills et al. 2004). Shoot explants of male and female exhibited either differential morphogenic behavior (Prakash et al. 2003) or similar response (Singh et al. 2008) under the influence of various adjuvants. Llorente and Apostolo (2013) reported a protocol suitable for micropropagation of different genotypes of jojoba. According to Singh et al. (2011) regeneration is influenced significantly by the size of the leaf attached to the petiole and the orientation of the explant (i.e., vertical or horizontal). Petioles obtained from pruned branches of plants responded better than those from unpruned plants. However, the efficiency of regeneration of in vitro leaves was not influenced by the size of the leaf attached to the petiole, but the orientation of the explant significantly influenced regeneration (Fig. 36.1). Infection-free cultures (90–99 %) could be established when the explants were collected from the field during October to May (Singh et al. 2008), and the response of explants was better when the explants were collected between August and November (Kumar et al. 2009).

The auxin to cytokinin ratio in the medium is critical for morphogenesis (Rout et al. 2000; Pati et al. 2006). High cytokinin and low auxin concentration in the medium favored shoot induction, reverse concentrations promoted root induction, and intermediate concentration induced callus development (Harris et al. 1989). Sucrose (Kumar et al. 2009), adenine sulfate (Singh et al. 2008), and triiodobenzoic acid (Prakash et al. 2003) had a significant effect on shoot multiplication and elongation in jojoba. The superiority of BAP in shoot induction may be due to its ability to metabolize more rapidly or may induce natural hormones like zeatin synthesis within the tissues (Rai et al. 2010).

Rooting and subsequent establishment of plantlets is very important to make micropropagation successful (Pati et al. 2006). Half-strength MS medium was adequate for root induction (Singh et al. 2008; Kumar et al. 2009). Experiments involving rooting with either auxins alone or in combination with other hormones showed significant differences (Chaturvedi and Sharma 1989; Tyagi and Prakash 2004; Bashir et al. 2008). According to Singh et al. (2008) IBA alone was more effective in root induction as compared to IBA+ IAA or IBA+ IAA+ NAA. Tyagi and Prakash (2004) observed differences in the rooting behavior of male and female plants, whereas Singh et al. (2008) reported no difference in rooting response of male and female genotypes. Clonal differences in rooting and subsequent acclimatization were also recorded (Apostolo et al. 2001; Bashir et al. 2008). Twenty-four to 32 % rooting with IAA pulse treatment

Fig. 36.1 Micropropagation of jojoba using nodal segments. (**a**) Multiple shoots (primary culture), (**b**)subculture(15–20 shoots), (**c**) in vitro rooting, (**d**) ex vitro rooting, (**e**) 1-year old plants (Source: Singh et al. 2011)

and 50–60 % rooting with IAA and IBA combinations were reported (Mills et al. 1997). According to Chaturvedi and Sharma (1989), the combination of IBA and NAA was more effective in inducing rhizogenesis than either of them alone. It is an established fact that although auxins are essential for root induction, they are not required for root growth; their continued presence may even inhibit the root growth (Mills et al. 1997). Addition of activated charcoal has been reported to promote rooting (Thomas 2008). Rooting is also promoted by the addition of caffeic acid (Chaturvedi and Sharma 1989) and cyclodextrins (Apostolo et al. 2001).

Hardening of rooted plants prior to transfer in the soil increases the survival rate. Various types of substrates such as soil vermiculite mixture (Gulati and Jaiwal 1996), sterilized sand (Thakur et al.

2001), soil (Dewan et al. 1992), and soilrite and soil (Agrawal et al. 2007) have been used. Meyghani et al. (2005) observed that peat and perlite at a ratio of 1:1 or 1: 2 is the most suitable medium for transplanting and subsequent adaptation of jojoba plantlets. Singh et al. (2008) reported the best survival rate when sand alone was used as a substrate. The survival rate was higher for plantlets which developed roots in vitro in response to IBA; however, no differences were observed during field establishment (Bashir et al. 2008). Kumar et al. (2010b) observed 75 % field establishment in jojoba plants acclimatized in presterilized soil-sand (1:3), whereas Singh et al. (2008) reported 99 % field establishment when sand alone was used, as compared to soilrite alone and soilrite + sand.

36.2.3 Somatic Embryogenesis

Somatic embryos are believed to be bipolar structures that arise from individual cells and often have no vascular connection with the maternal tissue of the explant (Rai et al. 2010). Embryos may develop directly from somatic cells (direct embryogenesis) or can be preceded by numerous organized non-embryogenic mitotic cycles (indirect embryogenesis). Somatic embryogenesis has a great potential for clonal multiplication and an ideal experimental process for studying differentiation and totipotency in plant cells (Litz and Gray 1992). In addition, under controlled environmental conditions, somatic embryos germinate readily, similar to their seed embryo counterparts. Besides being useful for clonal multiplication, regeneration through somatic embryogenesis may also be useful for genetic transformation (Kim and Liu 1999) and to develop new products (Benzioni 1995). Reports on somatic embryogenesis in jojoba are sparse and focused mostly on in vitro wax production using zygotic embryos (Wang and Janick 1986). Hamama et al. (2001) developed a protocol for the induction, maturation, and germination of somatic embryos from leaf explants. Direct somatic embryogenesis was observed with some zygotic embryo explants, whereas leaf-derived embryonic calli did not mature on any of the maturation/germination media (Mohammed et al. 2008).

36.2.4 Synthetic Seeds

Due to differences in male to female ratio and long gestation period (Sharma et al. 2008; Kumar et al. 2011), jojoba may be a suitable species to develop synthetic seed, as the technology has advantages besides effective method of propagation of known sex, germplasm storage, and conservation (Rai et al. 2009). Only two reports are available on the production of synthetic seeds in jojoba (Hassan 2003; Kumar et al. 2010b), where shoot tips and axillary buds were encapsulated using sodium alginate and calcium chloride and complete plant regeneration from encapsulated shoot tips has been achieved.

36.2.5 Molecular Approaches for Identification of Genetic Fidelity and Sex Determination

While generating quality planting material using micropropagation on a commercial scale, genetic stability is the most important to maintain the advantageous characters of elite plants (Rahman and Rajora 2001). Micropropagated plants generated using shoot tips and axillary buds are reported to maintain clonal fidelity (Ostry et al. 1994), whereas Tripathi et al. (2006) reported somaclonal variation as a major constraint in commercial production of true-to-type plants. Somaclonal variations occur in response to the in vitro stresses and are manifested in the form of DNA methylation, chromosome rearrangements, and point mutations (Phillips et al. 1994). Therefore, a quality checkup for true-to-type planting material at an early stage of development is very essential. Molecular markers serve as an important tool to check the genetic uniformity and the true-to-type nature of micropropagated plants.

Among various DNA-based markers, amplified fragment length polymorphism (AFLP) and

restriction fragment length polymorphism (RFLP) are the most reliable markers for screening of genetic fidelity in tissue-cultured plants. Due to requirement of radioactive labeling and expensive enzymes, these techniques have less preference in many laboratories. Random Amplified Polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers, on the other hand, require only small amount of DNA sample, do not involve radioactive labeling, and are simple, fast, and proven to be quite efficient in detecting genetic fidelity even in closely related organism such as near isogenic lines (Reddy et al. 2002). The marker systems have been successfully used in demonstrating somaclonal variation in different plant species (Joshi and Dhawan 2007). However, such reports are very limited in jojoba. Kumar et al. (2011), using RAPD and ISSR markers, demonstrated that axillary bud multiplication is a safe method for production of true-to-type plants in jojoba. Early detection of sex (Agarwal et al. 2007, 2011; Sharma et al. 2009; Ince et al. 2010; Hossain et al. 2001) and genetic variability (Amarger and Mercier 1995) using molecular techniques is also reported in jojoba.

36.2.6 Molecular Approaches for Oil Synthesis

The liquid wax from jojoba seed has a carbon chain length of C38–C44 and is composed mainly of C20:1 fatty acids and C22:1 fatty alcohols. Oil has a multitude of important applications in medicine and cosmetics industry and as a lubricant. The high cost and limited production of jojoba waxes restrict their use in spite of high demand. The advent of genetic engineering has provided novel opportunities to increase the availability of plant-derived lipids (Kalscheuer et al. 2006). Enzymes involved in wax synthesis in developing jojoba seeds have been characterized (Metz et al. 2000) and the corresponding cDNAs cloned (Metz and Lassner 1994). Overexpression of these genes has resulted in production of jojoba wax in the seeds of transgenic model plants (Lassner et al. 1996; Lardizabal et al. 2000). Kalscheuer et al. (2006) demonstrated the possibility of de novo synthesis of wax esters in a recombinant microorganism by co-expression of a fatty alcohol-producing bifunctional acyl-coenzyme.

36.2.7 Conclusions and Future Prospects

During the recent past, significant progress has been achieved in micropropagation, marker-assisted selection, and sex detection at seedling stage. However, there is a need to exploit modern tools of biotechnology and molecular biology to develop a robust micropropagation system that can be applied to different genotypes and an efficient transformation system for genetic improvement, disease resistance, yield, etc. In addition, in vitro production of useful metabolites has not been attempted in this species and needs to be addressed in future research efforts.

36.3 Date Palm (*Phoenix dactylifera* L.)

36.3.1 Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious edible fruit-yielding tree species that grows in warm and arid climates, and its global annual production is about 5.5 million metric tons. Since ancient times this plant has been recognized as the "tree of life" because of its integration in human settlement and food security in hot and barren parts of the world, where only a few plant species can flourish. Date palm provides a major source of income for local farmers and associated industries in arid zones. The economic utility of these palms is multifold as staple food, beverages, ornamentals, and industrial materials (Balick and Beck 1990). It is one of the oldest domesticated trees with socioeconomic importance (Masmoudi-A et al. 2009). The earliest cultivation of date palm goes back to 3,700 BC, in the area between the Euphrates and the Nile River (Munier 1973). Expansion of date palm agriculture is faced with challenges stemming

from propagation and genetic improvement, a limitation that deprives the actual potential (Jain 2012). The heterozygous nature of this dioecious species hampers the use of seed which produces off-type seedlings and takes 8–10 years or more for fruiting (Jain 2012). Two to three percent of male palms are sufficient to meet the pollination requirements, but in seed-raised population, half of the progeny is composed of male trees and not possible to identify before flowering (Eke et al. 2005). The application of tissue culture techniques for date palm has many advantages for propagation of healthy selected female cultivars, large-scale multiplication, avoiding seasonal effects, production of genetically uniform plants, fast exchange of plant material, and economical reliability. Looking at the advantages, micropropagation techniques have been developed for rapid mass propagation of date palm. Plant regeneration through tissue culture is also important for genetic improvement (Parveez et al. 2000) and conservation of genetic resource (Engelmann 2004).

36.3.2 Micropropagation

Since 1970 extensive efforts were made to mass propagate date palm using tissue culture techniques (Schroeder 1970; Eeuwens 1978; Tisserat 1979; Sharma et al. 1986; Bouguedoura et al. 1990; Bhaskaran and Smith 1992; Sharon and Shankar 1998; Al-Khalifah 2000; Fki et al. 2003, 2011; Al-Khayri 2007; Al-Khateeb 2008a, b; Kumar et al. 2010a; Jain 2012).

To establish aseptic cultures, zygotic embryos (Reynolds and Murashige 1979), shoot tips (Veramendi and Navarro 1997), lateral buds (Bouguedoura et al. 1990), leaves (Fki et al. 2003), or inflorescences (Bhaskaran and Smith 1992; Fki et al. 2003) have been used. Among different types of disinfecting solutions used, mercuric chloride ($HgCl_2$) was found to be the most efficient disinfectant (Drira and Benbadis 1985). Like for many other plant species Murashige and Skoog (1962), medium has been the most preferred one, and 2,4-D is the most popular PGR for callogenesis (Fki et al. 2003). Liquid media for plant regeneration has been widely used (Fki et al. 2003; Sharma et al. 1986) for large-scale regeneration. Micropropagation via direct organogenesis has also been attempted for rapid clonal propagation of elite genetic material of date palm (Al-Khayri 2007; Khierallah and Bader 2007; Jain 2007) and different genotypes (Aaouine 2003). The major concern with this approach is somaclonal variation that is dependent on various factors including genotype, explants, and plant growth regulators (Jain 2012). Direct regeneration of vegetative buds minimizes the risk of somaclonal variation for commercial production of date palm cultivars (Jain 2012).

36.3.3 Somatic Embryogenesis

Somatic embryogenesis has tremendous potential for rapid large-scale plant production. The advantages of somatic embryogenesis are cost-effectiveness, both shoot meristem development and root meristem development taking place in a single step, quick and easy to scale up, automation, long-term storage (cryopreservation), virus-free plants, and facilitates genetic transformation (Jain 2007). Somatic embryogenesis has the high potential for mass propagation of date palm; hence, more emphasis was given to somatic embryogenesis (Reynolds and Murashige 1979; Bhaskaran and Smith 1992; El Hadrami et al. 1995; Fki et al. 2003; Al-Khateeb 2008a; Othmani et al. 2009). Somatic embryogenesis can be achieved either directly from explant (Sudhersan et al. 1993) or from embryogenic calli (Al-Khayri 2005), though the first method is the most preferred one but has not been perfected yet (Fki et al. 2011). The duration of the cultivation period in liquid medium was found to be very important for the balanced germination of somatic embryos (Jain 2007). Kumar et al. (2010a) reported an optimized protocol for somatic embryogenesis (Fig. 36.2) and regeneration from embryogenic suspension cultures of date palm for commercial production (Fki et al. 2003). Smith and Aynsley (1995) studied field performance of tissue culture-derived date palm clonally produced by somatic embryogenesis,

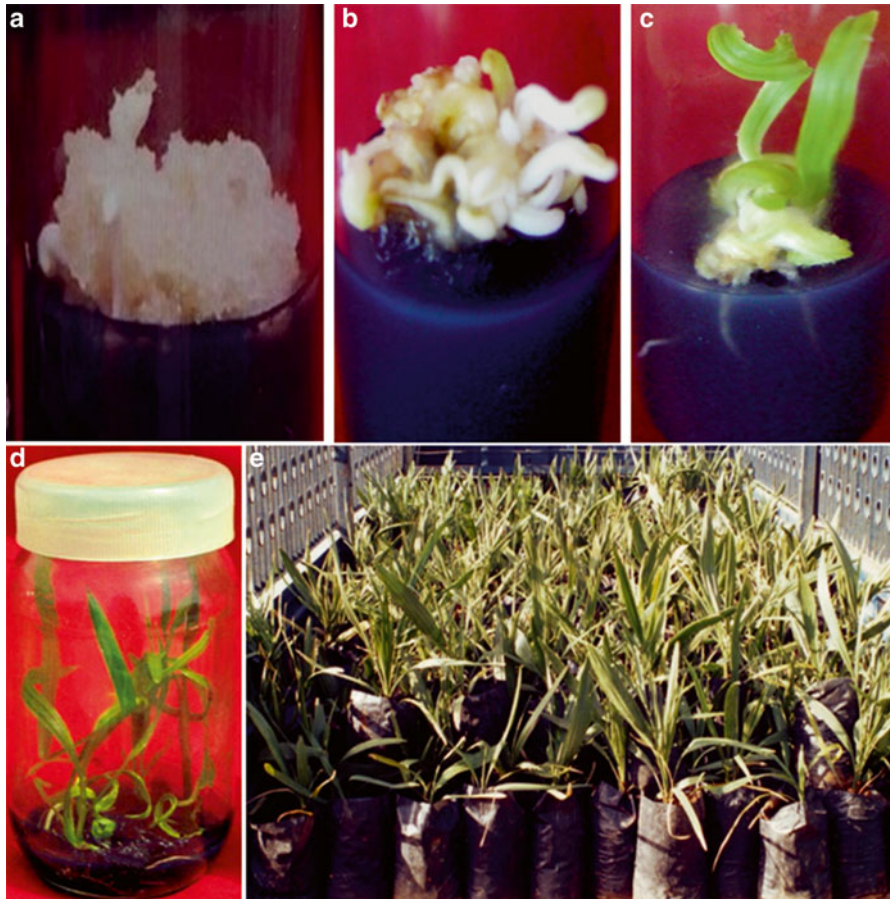

Fig. 36.2 Micropropagation of date palm through somatic embryogenesis. (**a**) Embryogenic callus, (**b**) development of somatic embryo, (**c**) germination of somatic embryo, (**d**) regenerated plantlets, (**e**) hardened plants in nursery (Source: Kumar et al. 2010a)

and the results demonstrated that these plants started bearing fruits within 4 years from field planting.

36.3.4 Embryo Rescue

Removal of a zygotic embryo from the seed and planting in a sterile nutrient culture medium is known as embryo rescue technique. Embryo culture has several potential applications in crop improvement programs and to produce hybrids (Hodel 1977). Excised embryos cultured in vitro, under suitable basal nutrient culture media, usually germinate immediately. Embryo rescue was successful in reducing the date palm height (Sudhersan et al. 2009).

36.3.5 Regeneration from Protoplasts

Ever since regeneration of fertile plants from isolated protoplasts was reported in *Nicotiana tabacum* (Nagata and Takebe 1970), plant regeneration through protoplasts has been reported for more than 400 plant species (Davey et al. 2005). There are very few reports on date palm protoplasts. Chabane et al. (2007) and Rizkalla et al. (2007) were successful in inducing callus formation

from protoplasts. However, plant regeneration from protoplast callus is yet to be accomplished.

36.3.6 Genetic Transformation

Genetic engineering helps in reducing time in developing new and improved cultivars. T-DNA delivery with *Agrobacterium tumefaciens* (Horsch et al. 1984) and direct gene transfer with particle bombardment. Klein et al. (1988) have been used successfully in genetic transformation. These developments resulted in production of the first fertile transgenic plants (McCabe et al. 1988; Fromm et al. 1990; Vasil et al. 1992; Wan and Lemaux 1994). Agrobacterium-mediated transformation has several advantages over particle bombardment method, e.g., integration of a well-defined DNA sequence, typically low copy number, and preferential integration into actively transcribed chromosomal regions (Gheysen et al. 1998), and different approaches have been pursued to improve the efficiency of Agrobacterium-mediated transformation in recalcitrant monocot plant species (Cheng et al. 2004; Kumlehn et al. 2006). Successful infection of date palm embryogenic callus with Agrobacterium, (Saker et al. 2009) and gene transfer by particle bombardment method (Habashi et al. 2008; Saker et al. 2007) has been reported. Preliminary investigations of physical factors influencing gene delivery using a biolistic gun indicated that flight distance of microprojectiles and applied pressure were the most important parameters (Saker et al. 2007).

36.3.7 Molecular Approaches for Identification of Genetic Fidelity and Sex Determination

Molecular markers are a very important resource for genetic improvement of all crops. DNA markers, especially those based on simple sequence repeats and single-nucleotide polymorphism, play an increasingly important role in plant variety identification, germplasm resource collection, and genetic improvement. Molecular marker studies are very limited in date palm. Random Amplified Polymorphic DNA (RAPD) technique has been developed to characterize date palm genotypes or cultivars and to examine their phylogenetic relationships (Trifi et al. 2000; Jain et al. 2011). Kumar et al. (2010a), using RAPD and ISSR markers, reported that the micropropagation protocol developed by them for rapid in vitro multiplication is suitable for clonal propagation of date palm and corroborated the fact that somatic embryogenesis can also be used as one of the safest modes for production of true-to-type plants (Fig. 36.3). The frequency of somaclonal variations was found to be age dependent, and RAPD analysis showed genetic variations in approximately 4 % of the analyzed plants (Saker et al. 2000).

36.3.8 Genomics

There have been a very limited number of genome-wide studies on date palm. Al-Dous et al. (2011) from Weill Cornell Medical College in Qatar tried to sequence the entire date palm genome using Solexa (illumine) sequencer based on a shotgun method and released the sequence data subsequently (http://qatar-weill.cornell.edu/research/datepalm Genome/index.html). According to their analyses, the genome assembly has a predicted genome size of ~550 Mbp, assembled 58 % of the genome (382 Mb), and predicted 25,059 genes (Al-Dous et al. 2011). Bourgis et al. (2011) have reported comparative transcriptomic study on mesocarps of both oil and date palm based on pyrosequencing data from the Roche GS FLX Titanium platform. Recently Al-Mssallem et al. (2013) report a genome assembly for an elite variety (Khalas), which is 605.4 Mb in size and covers 490 % of the genome (B671 Mb) and 496 % of its genes (B41,660 genes). Genomic sequence analysis demonstrates that *Phoenix dactylifera* experienced a clear genome-wide duplication after either ancient whole genome duplications or massive segmental duplications. Genetic diversity analysis indicates that its stress resistance and sugar metabolism-related genes tend to be enriched in the chromosomal regions, where the

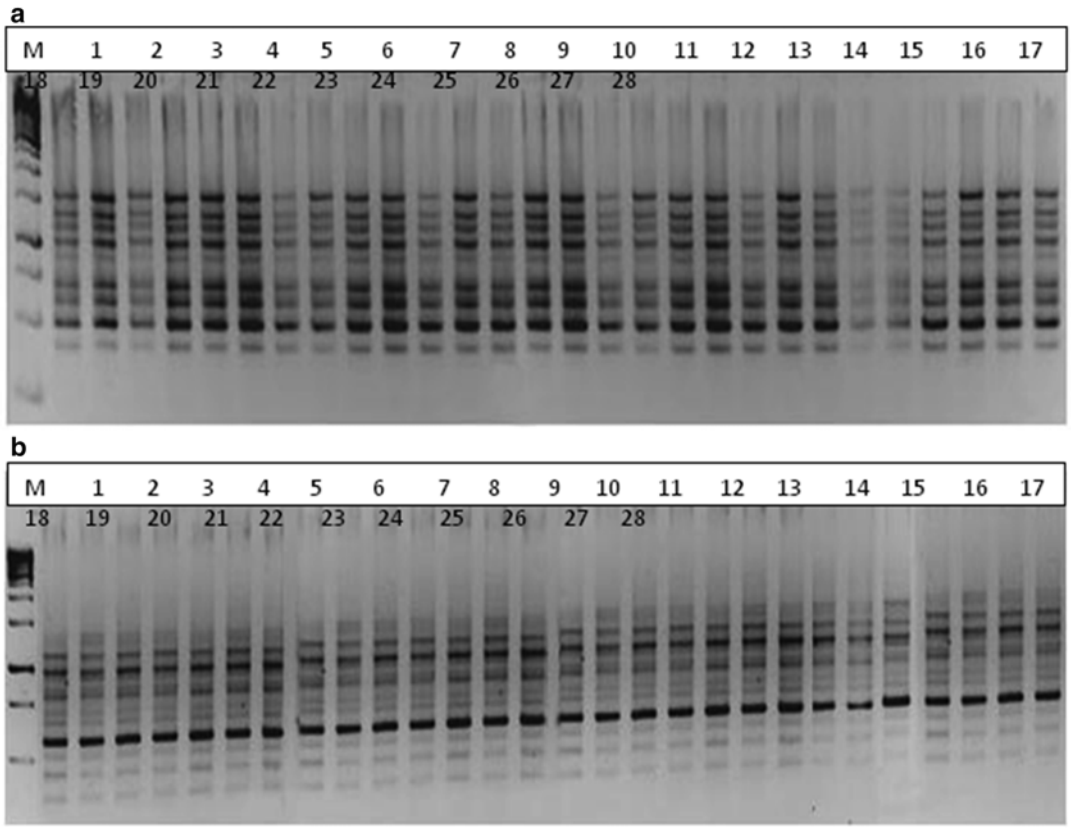

Fig. 36.3 PCR amplification products obtained with (**a**) RAPD primer OPE-15 and (**b**) ISSR primer UBC-835. Lane M represents one kb ladder; lane 1 represents mother plant; lanes 2–17 represent tissue cultured raised plants at the 42nd cycle of transfer (Source: Kumar et al. 2010a)

density of single-nucleotide polymorphisms is relatively low. Using transcriptomic data, they also illustrated the date palm's unique sugar metabolism that underlies fruit development and ripening. Besides, full-genome assemblies of plastid (Yang et al. 2010) and mitochondrion (Fang et al. 2012) and transcriptomic profiles for fruit development based on pyrosequencing data (Yin et al. 2012) are also reported. The genomic and transcriptomic data may be useful for further genomic studies not only on *P. dactylifera* but also in other Arecaceae species.

36.3.9 Conclusions and Prospects

Date palm is an important plant species in Near Eastern countries and a main source of income. Infestations of red palm weevil (RPW) have been reported in over 50 % of the date palm-growing countries (Faleiro 2006). Genetic improvement of date palm for RPW resistance seems to be the utmost priority to address. Developing a robust technology that can be applied to all the genotypes, optimization of bioreactor technology for large-scale production of propagules, and development of haploid plants are also very essential to make date palm cultivation very successful.

36.4 Acacia Species

36.4.1 Introduction

Genus *Acacia* belongs to Leguminosae family and encompasses approximately 1,300 tree and shrub species of tropical and warm temperate regions. Due to its utility in afforestation, and

agroforestry, this plant has become a key component of arid regions. The plant has a fast-growing tap root that enables it to utilize moisture stored in lower soil layers and to remain green for a long time in the dry season. The extensive root system makes it ideal for dune stabilization and preventing soil erosion. Acacia species have the natural ability to fix atmospheric nitrogen and hence can thrive in low-fertility soils (Galiana et al. 2002). They can be used for combating desertification and for restoring degraded land and for economic products, like gum, tannins, wood, and fodder (Badji et al. 1993; Quoirin et al. 2000). The fast expansion of Acacia plantations warrants the need to develop efficient methods for mass production of superior quality planting stocks (Quoirin et al. 2000). Acacia propagated through seeds exhibit poor germination, and seed-raised plants show wide genetic variability and are not true-to-mother plants. Vegetative propagation through stem cutting is not successful in Acacia species as rooting of cuttings harvested from mature tree is difficult (Beck and Dunlop 2001).

36.4.2 Micropropagation

In the last three to four decades, in vitro approaches have been efficiently utilized in large-scale propagation and genetic improvement of the tree species (Peña and Séguin 2001). Propagation of woody trees through tissue culture has many advantages over conventional propagation methods like fast multiplication, production of disease-free plants, season-independent production of plants, quick release of improved cultivars, germplasm conservation, and easy exchange (Asthana et al. 2011; Phulwaria et al. 2012). Many species of Acacia have been studied for micropropagation (Dewan et al. 1992; Nandawani 1995; Rout et al. 1995; Hossain et al. 2001; Xie and Hong 2001a, b; Al-Khalifah 2004; Abbas et al. 2010; Samake et al. 2011; Rathore et al. 2014). The results showed that *A. tortilis*, *A. ehrenbergiana*, and *A. gerrardii* responded well, whereas *A. seyal* and *A. senegal* were difficult to establish in vitro, and these differences persisted throughout in vitro and acclimatization stages (Al-Khalifah 2004). Mathur and Chandra (1983) cultured stem segments of a mature tree of *A. nilotica* that produced a few shoot buds from the nodal portion, and roots emerged from the opposite pole. Singh et al. (1993) also reported in vitro axillary bud induction from explants of a 20–25-year-old field-grown tree. However, they were not successful in inducing the roots. Recently, Rathore et al. (2014) reported a micropropagation method using liquid culture and nodal explants collected from 15- to 20-year-old *A. nilotica*. The multiplication rate in liquid cultures was ten times higher as compared to agar medium cultures. In vitro regeneration of *A. mangium* from shoot culture (Ahmad 1991; Galiana et al. 1991; Bhaskaran and Subhash 1996) and *A. mangium and A. mangium* × *A. auriculiformis*, a hybrid (Monteuuis et al. 2013), has been reported, and clonal propagation of superior plants of *A. mangium* is currently being carried out for large plantations. In vitro regeneration of *A. mangium* through somatic embryogenesis and organogenesis (Xie and Hong 2001a, b) and protoplast isolation (Toshihiro and Sonoko 1999) have also been reported.

36.4.3 Genetic Transformation

Commercial production of transgenic annual crops has become a reality in many parts of the world. Trees were considered to be recalcitrant for genetic transformation, and many researchers wonder whether genetic engineering of perennial trees will allow their eventual commercialization. Recent advances in plant molecular biology and biotechnology made it possible to develop transgenic trees for different value-added purposes (Peña and Séguin 2001; Xie and Hong 2002; Yang et al. 2008) including woody leguminous trees (Igasaki et al. 2000; Xie and Hong 2002; Yang et al. 2008). A number of different explants have been used in the development of *Agrobacterium*-mediated transformation (Archilletti et al. 1995; Franche et al. 1997; Polin et al. 2006; Li et al. 2007). More specifically, for the transformation of leguminous trees, stems from rejuvenated shoots and leaves were used as

explants (Xie and Hong 2002; Igasaki et al. 2000; Saafi and Borthakur 2002). Xie and Hong (2002) reported *Agrobacterium*-mediated genetic transformation of *A. mangium* using rejuvenated shoots as explant. High resistance to kanamycin (<300 mg L^{-1}), during initial selection of transformed plants observed, is a major limitation in legume genetic transformation (Xie and Hong 2002).

36.5 Conclusions and Future Prospects

Genetic engineering of trees entails a major investment that can generate considerable environmental and economic benefits over the long term. The research undertaken in plant molecular biology for trees is not comparable to the effort deployed for annual crops. Nevertheless, physiological and developmental processes specific to trees are being studied at the molecular level. Knowledge of the reproductive development, wood formation, and defense mechanisms is essential for the effective integration of biotechnology into tree improvement. Certainly, eliminating the requisite tissue culture step for legume transformation would be a great boost in progress toward development of high-throughput systems. Approaches for development of non-tissue culture transformation systems such as floral dip method (Desfeux et al. 2000; Li et al. 2002) or meristem culture (McCabe et al. 1988; Rohini and Rao 2000) would be a great boost in progress toward development of high-throughput systems.

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Rural Biotechnology in Transforming Agriculture and Rural Livelihood

37

Lekha Bandopadhyay and Samir Ranjan Sikdar

Abstract

The twenty-first century human society is dealing with many difficulties besides climate challenge, and most of them are though diverse but highly interconnected. Transformation in agriculture and rural development are demanding urgent attention in this scenario. Human civilization is basically an agrarian one, and rural people who practice agriculture are literally the real bread earners. Neglecting agriculture and these endogenous people has visibly started to affect our ecosystem, environment, economy, and civilization. Sustainable solutions to most of these problems can be created only by combining endogenous knowledge reserve and the modern biotechnological know-how. In order to successfully carry forward this process, we first need to ensure endogenous development. Rural development is inevitably connected to several key endeavors having global impacts like food security, protection of environment, and conservation of natural resource base including local landraces, germplasm, and biodiversity. Successful application of rurally relevant biotechnology will be a necessity in transforming agriculture besides enhancing and protecting rural livelihoods as well as rural integrity.

Keywords

Biotechnology • Rural development • Rural poverty • Food security • Genetically improved crops • Sustainable agriculture • Conservation tillage • Biological nitrogen fixation • Agroforestry • Rainwater harvesting • Deficit irrigation • Supplemental irrigation • Integrated pest management • Biopesticides • Biofertilizers • Biofuel • Integrated crop-livestock management • Fishery • Aquaculture • Algal biotechnology • Sericulture •

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Apiculture • Mushroom cultivation • Medicinal plants • Transgenic technology • Tissue culture • Micropropagation • Village seed bank • Kitchen garden • Local landraces • Germplasm • Biodiversity • Conservation

37.1 Introduction

Technology is instrumental for human development. But efficient distribution system is equally important to deliver its benefit to all (UNDP 2001). Information and communication technology have made this world literally a global village in terms of connectivity. But as evident wise use of appropriate technologies or know-how available is a great demand to give a dignified life to our resource-poor real villages (rural livelihoods where 75 % of world's poor live) over the globe. *Rural development and poverty reduction* is not anymore a charity as this is an urgent need linked to global climate change and sustainability concerns. In 2008 World Bank called for renewed emphasis on agriculture for development to reach the Millennium Development Goal of decreasing poverty by 2015. The International Assessment of Agricultural Knowledge, Science and Technology for Development (IAASTD) also stressed on the importance of transformation in the use of agricultural knowledge, science, technology, and management for social, environmental, and economic sustainability (UNEP 2008; The World Bank 2008). The field of *biotechnology* has accelerated greatly in the twentieth century following rediscovery of Gregor Mendel's laws of inheritance, discovery of DNA's (deoxyribonucleic acid) structure, and serendipitous development of recombinant DNA technology and regeneration techniques (Curtis 2008; Mirkov 2003). Biotechnological innovations and scientific rural development through responsible use of rurally relevant biotechnologies are the requirement of today. Crop productivity is declining, environment is quite degraded, and food price is on the rise. The affluent percent of the increasing world population on the other hand is demanding for diverse foods. Rural-urban integration is leading to diversification of income sources of very poor rural people whose main occupation was agriculture so far. In order to ensure *food security* and restore the environmental balance, ensuring *endogenous development*, or improving the condition of human food producers, the crucial but highly neglected human resource is of utmost importance especially in developing countries but also in some parts of the developed world. Rural development is linked to agricultural development, food security, poverty reduction, world economy, global climate, and vice versa. A successful instance in this context is Vietnam which once was a food-deficit country and is now the second largest rice exporter in world. Seventy three percent of Vietnam's population lives in rural areas, and agriculture is their main source of income. They have achieved this success largely through the development of their *smallholding endogenous farmers* (IFAD Food prices 2014a).

37.2 What Is Rural in the Modern Trend of Urbanization?

Human civilization has been an agrarian one and hence invariably rural for long. Around 10,000 years ago, the nomadic *Homo sapiens* gradually shifted to a grounded farming-based lifestyle. This is known as the Neolithic revolution or the New Stone Age in the human history that marks the beginning of a civilization based on agriculture still alive today (Gepts 2003). The *rural and urban differentiation* emerged gradually over the course of time. While urban society flourished with industrial revolution and continued to explore diverse professions. Agriculture

and related farming practices continued to be the profession of rural area. Urban lifestyle grew far more comfortable and became a coveted destination of human race. And although functionally complementing each other, rural areas remained neglected and isolated due to lower population density and poor connectivity with the urban centers of distinct economic development. In the *trending wave of urbanization* today, rural (comprised of villages) is defined as anything that is not urban. But this scenario is not static. In the developing world, rural and urban areas are becoming increasingly interconnected socially and economically blurring the line of contrasting difference of the past and creating several nonfarm income options for poor rural people. According to the UNFPA's (United Nations Population Fund) 2007 "State of World Population Report," half of the world population (3.3 billion) lives in urban areas, and it is estimated to grow up to 4.9 billion by 2030 (IFAD Rural poverty portal 2014b; UNFPA 2007). In this wave of urban-centric economic development, chronically deprived rural areas which also house half of the human population demand equal development. World food production has increased considerably in the last 50 years owing to long-term public and nonprofit investment in basic and agricultural research, but food insecurity and malnutrition are the main problem of the twenty-first century. While on the one hand increased income is putting pressure to increase food production due to increased consumption of diverse diet including more and more animal products, ironically, on the other hand, poverty is one of the main reasons behind hunger and malnutrition in many developing countries in Africa and Asia and the Pacific as well as in developed ones, and majority of the affected people live in rural areas (Cohen 2003). This loss of equilibrium is leading to outflow of more and more people from rural to urban areas in the hope of better life resulting in an overload on the urban ecosystem. However the reality is the increase in the number of poor people living in city slums having very little facilities for a decent living. The first wave of urbanization noted in Europe and North America from 1750 to 1950 fueled by industrialization increased the percent of urban population from 10 to 52. This process was gradual (spread over two centuries). But the second wave of urbanization that is now flowing over the developing world is rapid and ill prepared. The urban population is projected to increase from 18 to 56 % in a short span of 80 years (1950–2030). In order to stop this futile vicious cycle, rural development should be seen as an urgent requirement and opportunity especially by the developing countries, most of which are basically rural (UNFPA 2007).

37.3 Rural Poverty and Food Insecurity

The world poverty map points out that around 1.2 billion (one fifth of world's population) are the subject of this daily injustice (Fig. 37.1). Among them around 800 million (70 %) live in rural areas. While *rural poverty* is commonplace in developing regions of the world, it is present even in the affluent countries like North America and Europe. Thus Eastern and Southern Europe, Central America, and parts of North America suffer from rural poverty (IFAD Rural poverty portal 2014b). Rural poverty is the result of lack of assets (land, livestock, and money), limited economic opportunities, poor education, discrimination (based on gender, age, race, and ethnicity), demographic factors, violent conflicts due to regional sociopolitical situation, and environmental degradation (Cohen 2003).

Eradicate Extreme Poverty and Hunger is topping among the eight United Nations (UN) Millennium Development Goals with urgent attention. The UN is working with governments, civil societies, and other partners aiming for halving the poverty level of 1990 (from 28.3 % of all people in low- and middle-income economies to 14.2 %) by 2015. MDG 2013 report states that

> about 700 million fewer people lived in conditions of extreme poverty in 2010 than in 1990; ….however one in eight people still go to bed hungry despite major progress. (UN 2013)

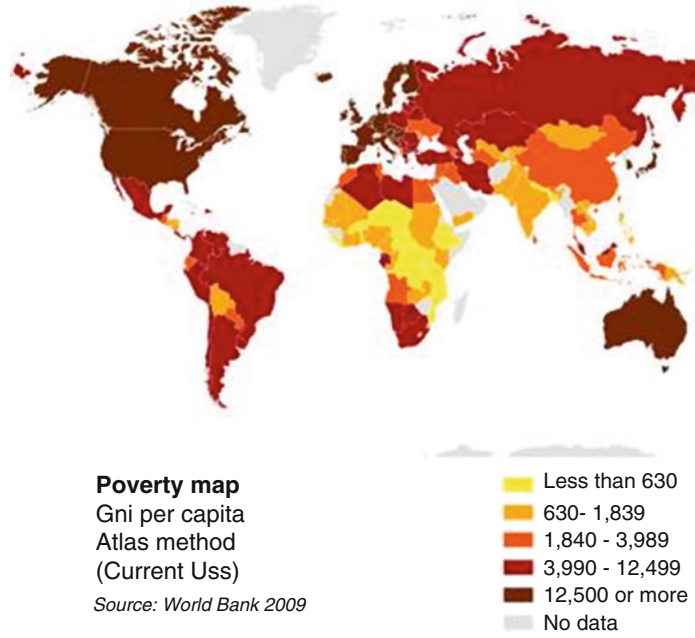

Fig. 37.1 World poverty map (Source: Rural poverty portal, International Fund for Agricultural Development 2014b)

37.4 Biotechnology Is Not a Magic Wand

The 1992 Convention on Biological Diversity (CBD) defined *biotechnology* as "any technological application that uses biological systems, living organisms or derivative thereof, to make or modify products or processes for specific uses" (The CBD 2001). Whatever be the present definition of this technology, biotechnology has always been an integral part of human development probably from prehistoric time. *Selective breeding* to raise better crop and livestock seen in the history of agriculture reflects the glorious background of biotechnology. Expansion of biotechnology in the twentieth century revolutionized farming business and will continue in doing the same in the twenty-first century. Food supply has outpaced the dreadful population growth of the twentieth century. Since 1950, international research is interested in useful research suitable in different agroecological regions around the world. *Genetically improved* high-yielding rice and maize varieties raised respectively by two CGIAR (Consultative Group on International Agricultural Research) institutes [International Rice Research Institute (IRRI), Philippines, and International Maize and Wheat Improvement Centre (CIMMYT), Mexico] and the necessary inputs like fertilizers, insecticides, and irrigation resulted in the so-called Green Revolution starting in the late 1960s worldwide but prominently visible in developing countries like Mexico and India. Since the mid-1990s, transgenic technology is offering *genetically modified* (*GM*) soybean, cotton, and canola with important traits like insect resistance and herbicide tolerance (Chrispeels and Sadava 2003; Pardey and Wright 2003). GM crops however are undergoing opposition from various sectors of the society. If there is any scientific concern to express in this matter in addition to biosafety and loss of biodiversity, it is that the concept of GM crops is based on the same principle of *purchased input* and monoculture (cultivation of selected varieties only) as was practiced in Green Revolution is a subject of unsustainability in the long term. There is looming concern of eventual loss of crop biodiversity by replacing numerous local cultivars with selected genetically uniform crops. Secondly this uniformity leads to the danger of sudden uncontrollable disease outbreaks which is unusual in conventional sustainable agriculture. In addition since it is a high-input technology and controlled by multinational biotech and seed companies, future possibilities of rendering monopoly and

increasing poverty of very poor small and medium farmers by decreasing their importance further are a large concern. However in the context of this chapter, it is important to remember that this GM debate is mostly irrelevant in rural biotechnology or development of rural livelihoods of developing countries. Most importantly, the know-how of biotechnology is not enough at this crossroad as biotechnology can't behave as a magic wand itself to solve our each and every problem. In order to utilize biotechnology effectively, implementing its sustainable and humane application is our own responsibility (IFAD 2002; Chrispeels and Sadava 2003). The twentieth century biotechnology has relied heavily upon purchased input strategy which is high input. However, intensive and high-input agricultural practices assumed so far aiming for infinite food production are now causing global concerns by deforestation (unplanned destruction of forests to create more arable land), soil degradation (intensive farming of the same land for years), water pollution (extensive use of pesticides and herbicides), and loss of crop biodiversity (using genetically uniform crops) (Chrispeels and Sadava 2003). Developing countries which economically cannot afford high-input agriculture for long should stop competing with the developed world. *Low-input farming* by combining indigenous or conventional technologies with scientific knowledge is highly suitable here and could be quite profitable if managed properly in these regions especially. This is termed as *regenerative or sustainable agriculture*. This approach takes care of preserving and restoring the natural resource base of local environment (IFAD 2002).

37.5 Promoting Sustainable Agriculture by Using Modern and Indigenous Know-How and Supporting the Resource-Poor Smallholders

Faced by striking climate changes caused by mostly anthropogenic factors, practicing sustainability is the only solution the world is hoping for (IPCC 2007). It is equally clear that *rural endogenous farmers* or *smallholders have a key role in building a sustainable livelihood*. As a true producer these farmers are also the protector of local biodiversity however being ignorant most of the time of this great fact and unappreciated as well. Poverty is the chronic burden that leaves them stuck, literally unrewarded and deprived of the due recognition (IFAD 2002). There are about 525 million smallholder farms worldwide: 388 million in Asia, 44 million in Europe and Russia, 33 million in Africa, and five million in America. These smallholder farms face varied challenges like limited access to land, water, market, credit, organization, education, or guidance to cope up with risks and that reflect in the quality and quantity of their products (IFC 2013). The 2011 Rural Poverty Report insists to support rural areas to shift from *subsistence farming to farming for business* effectively to put an end to poverty and hunger. The report has put an equal stress on preserving the natural resource base of the rural areas through intensification of sustainable agriculture (IFAD 2011). Combining the knowledge of modern biotechnology with indigenous knowledge and practices of farmers to protect the natural resource base of a region like soil, water, and local biodiversity is the key concept of sustainable agriculture. FAO's (Food and Agriculture Organization of the United Nations) "Save and Grow" guide for policymakers toward sustainable intensification of smallholder crop protection is a concise guide for everybody interested to understand this concept (FAO Save and Grow 2012a, b, c, d, e). A global transformation is highly sought after to make agriculture low input, observant, natural, locally appropriate, economic, profitable for the farmers, and environment caring. Smallholders will be instrumental in this effort for their collective experience and knowledge of local conditions. However, the majority of the smallholders are resource poor. Sufficient support in the form of fund, policy, know-how, and guidance will have to be provided to make them bring the desired transformation in farming (IFC 2013). In order to recognize the importance of endogenous small farmers in reducing poverty and ensuring food security and sustainable development, the United Nations has declared 2014 as the *International Year of Family Farming* (*IYFF*). With over 40 IYFF National Committees, the mis-

sion is to improve the situation of family farmers through communication, awareness activities, policy advocacy, and research (IFAD IYFF 2014c).

37.6 Some Sustainable Farming Techniques Practiced Successfully Around the World

37.6.1 Conservation Tillage

Soil is the base of agriculture. Conventional agriculture relies hugely upon tillage. This has adverse effects like loss of rich topsoil, soil organic matter and nutrient, and soil-water content. Intensive and extensive agriculture amplifies these losses raising serious concern. *Reduced to zero-till farming* is gradually spreading. This helps in conserving the soil, increasing drought tolerance through *water retention* and *carbon sequestration* (reducing greenhouse gas emissions), and increasing profit by *saving in labor and energy*. Weed and pest management is a problem in this practice. Applying integrated pest and weed management techniques is to be accompanied and optimized to aid this approach (FAO Soil 2012c; Shaver 2003; The World Bank 2008).

37.6.2 Biological Nitrogen Fixation

This is a form of *green manuring* to restore soil fertility. *Nitrogen-fixing legumes* (preferably inoculated with nitrogen-fixing *Rhizobium*) are utilized by intercropping with cereals to increase soil fertility. Leguminous trees and shrubs like *Sesbania sesban*, *Tephrosia vogelii*, *Crotalaria ochroleuca*, and *Faidherbia albida* which accumulate nitrogen in their leaves and roots are used in eastern and southern Africa to complement nitrogen-deficient maize farming. This is a successful application of *agroforestry*. A fernlike floating plant *Azolla pinnata* is inoculated with nitrogen-fixing bacteria (its symbiotic partner) in rice fields as green manuring in Asian wetland rice cultivation (FAO Soil 2012c; Shaver 2003; The World Bank 2008).

37.6.3 Rainwater Harvesting and Deficit Irrigation

Water is a limited but indispensable natural resource. Intensification of agriculture by high-input strategies in the twentieth century has led to depletion of surface water in many areas (Fig. 37.2). With increasing demand for water in industry, livestock, aquaculture, and domestic

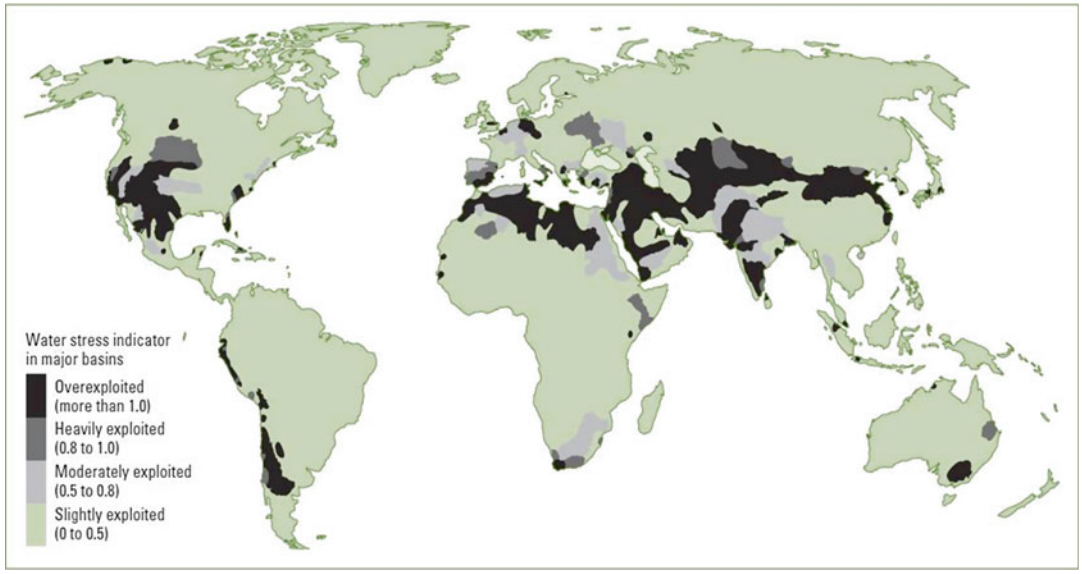

Fig. 37.2 Global water stress map noted in river basins (Source: World Development Report 2008; The World Bank 2008)

use, efficient management of water in agriculture is the key to sustainability in the twenty-first century especially in semiarid and arid zones. Using improved crop varieties and practicing good soil health management, *supplemental irrigation* by harvesting rain water, allowing *controlled water stress* (deficit) during vegetative stage, and applying water during critical growth stages like before flowering have multiple benefits like saving water and preventing soil salinization by excessive irrigation but little reduction in crop productivity. Agroforestry, conservation tillage, and integrated natural resource management approaches also have notable contribution in water management (FAO Water 2012d; UNEP 2008; The World Bank 2008).

37.6.4 Integrated Pest Management

Integrated pest management (IPM) practices of balancing on-farm pest-predator populations to achieve natural pest control without excessive use of pesticides is invaluable in maintaining agroecology as well as for sustainable agriculture as it is evident that chemical pesticides cannot be a sustainable solution for pest control besides their huge adverse environmental impacts.

Pesticides derived from natural sources such as animals, plants, microbes (bacteria, fungus, virus, or protozoan), and certain minerals are known as *biopesticides*. Biopesticides are less toxic, more specific, and a good companion of IPM. *Bt* proteins derived from strains of the bacterium *Bacillus thuringiensis* thus kill specifically one or a few related species of insect larvae (The World Bank 2008). Bentley (1983) has discussed the various aspects that are relevant to agriculture in tropics with reference to biological control and future farming practices and concluded that an integrated approach between *pure* and *applied* biologists will improve the quality of life. Likewise the role of nectaries in agriculture in relation to biological control of crop pests has been discussed by Chuabal et al. (1998), and they conclude that nectaries play a vital role in agriculture. For more details the reader may refer the original paper.

37.6.5 Integrated Crop-Livestock Management

This is the best example of sustainable agriculture in an efficient and economical way (Fig. 37.3). Thus crop residues serve as feed for livestock, while animal power is used in agriculture and other farm purposes; animal waste is used as *biofertilizer* of soil, in producing *biogas and energy* as well as *biopesticide* (Rota and Sperandini 2010).

37.6.6 Fishery and Aquaculture

Fishery and aquaculture can similarly be linked to integrated farm management for effective maintenance and management of water and other natural resources. *Fish culture in rice fields* (either in the same plot or adjacent ones) as practiced in China (world's largest fish producer and exporter) and many other Asian countries is a successful example of such integration (FAO 2012a, b, c, d, e).

37.6.7 Algal Biotechnology

Besides biofuel production, rapidly growing algae can produce oil, alcohol, protein, and biomass and have high potential of serving as a plant

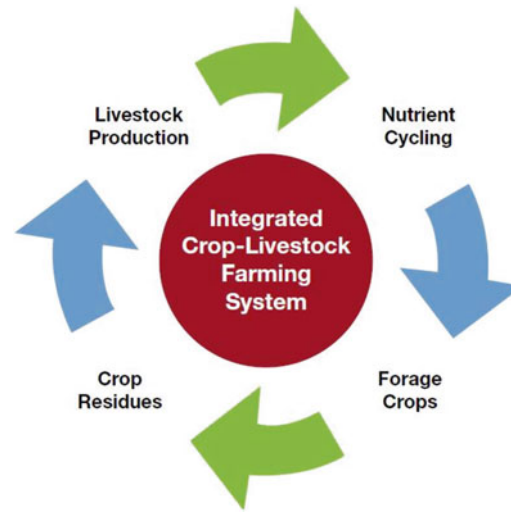

Fig. 37.3 The cyclic nature of resource management in integrated crop-livestock farming system (Source: http://www.ifad.org/lrkm/factsheet/integratedcrop.pdf, Rota and Sperandini 2010)

factory for functional foods, enzymes, drugs, and allied novel products. Algae can also be used as green fertilizer and bioinsecticide and in bioremediation. Highly potential algal biotechnology, however, still needs to overcome technological difficulties and establish itself as cost-effective.

37.6.8 Biofuel

With the ever-increasing oil prices, *first-generation liquid biofuels like ethanol and biodiesel* offer a lucrative supply of renewable energy from plants with environmental benefits. Currently ethanol is produced from maize and sugarcane, while biodiesel is produced from soybean, sunflower, palm oil, *Jatropha*, and other agricultural feedstocks. Thus substantial scientific and technological progress achieved during the last decade (2001–2011) promises successful management and domestication of *Jatropha* as a future industrial oilseed crop. As for example, genomic breeding programmes will be highly promising to develop perennial crops specially like oil palm (*Elaeis guineensis*), jatropha (*Jatropha curcas*), macaw palm (*Acrocomia aculeate*) and pongamia (*Pongamia pinnata*) dedicated for biodiesel production (Alves et al. 2015; Bahadur et al. 2013; Carels et al. 2013). The *second generations of biofuels are produced from nonedible lignocellulosic biomass* mostly derived from crop and forest residues. Converting the harmful algal blooms into biodiesel and other biofuels like bioethanol, biohydrogen, and biogas is another possibility. Production cost is a concern however so far. Many countries are producing and encouraging biofuel production (Fig. 37.4). But its economic viability (production cost and effect on food and feed prices) is still under assessment (The World Bank 2008).

37.6.9 Sericulture

Rearing silkworm on mulberry plants for production of *raw silk* is a low-input/high-output option for enhancing rural livelihoods economically as well as culturally. For example, Indian silk, famous for its diverse pattern and designs, is an ancient village craft practiced in many parts of the country. In China fish cultivation is integrated with mulberry cultivation and silkworm rearing. As a perennial plant mulberry also helps in agroforestry-based soil fertility enhancement and conservation (Bao-tong and Hua-zhu 1984; Central Silk Board 2014).

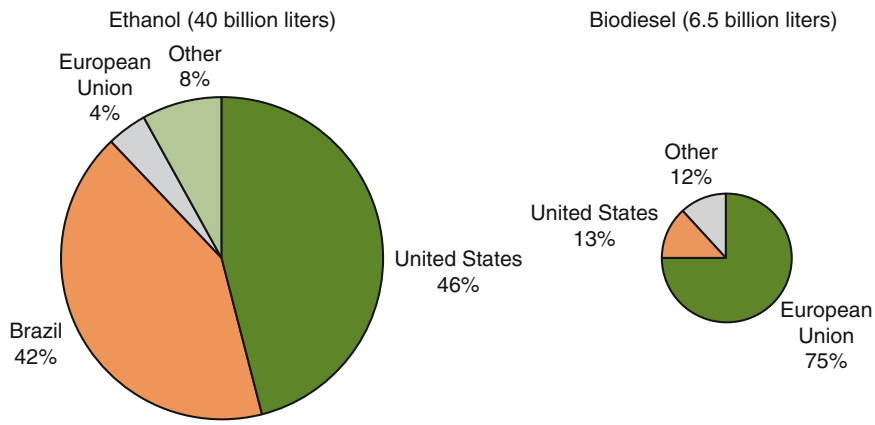

Fig. 37.4 Percentage of global production of biofuels ethanol and biodiesel in 2006 (Source: World Development Report 2008; The World Bank 2008)

37.6.10 Apiculture

Apiculture or beekeeping for production of honey and other bee products such as beeswax, royal jelly, pollen, propolis, or bee venom or queens is another option for diversifying income option for smallholders as part of farming. Colony management besides dealing with deadly honeybee pests and diseases, production and processing of honey, handling beeswax, and pollen trapping are several aspects of apiculture. Suryanaryana has reviewed the role of nectar in bee biology and beekeeping in detail and commented that modern beekeeping is still in infancy in several countries including India. Technical assistance through apiculture training for ensuring quality control of products to marketing is required at the national level for successful and truly profitable implementation of this method through the smallholders (Gegner 2003; MAAREC and USDA 2004; Suryanarayana 1998).

37.6.11 Mushroom Cultivation

As a quality food, mushroom is a good source of protein, vitamins, and minerals and has high market demand. In addition it has also many medicinal benefits including antibacterial, antiviral, and antiprotozoal activities and cytotoxicity against cancerous tumor cells. Edible mushroom cultivation using commercially viable strains and following proper culture techniques is a great option for generating food and income security for smallholders and rural people. Again, national mushroom cultivation programs supporting the technical and marketing part of the business are a necessity for the benefit of the resource-poor smallholders (Chakravarty 2011; Marshall and Nair 2009).

37.6.12 Medicinal Plants

Medicinal plants are wealth of every rural neighborhood and are greatly used by people in developing countries. Thus around 6,000–7,000 Indian plants have been documented to have usage in parallel systems of medicine like Ayurveda, Siddha, Unani, and Homeopathy in India. The increasing percentage of world population is trending toward use of plant-based medicine. Cultivation of medicinal plants is not only profitable but contributes in conservation of local and global biodiversity. Bahadur et al. have discussed in depth various aspects of medicinal plant diversity, conservation of rare/threatened plants, and biotechnological applications including micropropagation, transgenics, antibodies, vaccines from transgenics apart from antioxidants, nutraceuticals, and dermaceuticals. In addition they have provided about 60 websites where information on medicinal plants can be accessed. The World Health Organization (WHO) has also brought out a series of monographic volumes on selected medicinal plants that aims to provide scientific information on their safety, efficacy, and quality control of plants used worldwide. These provide comprehensive scientific references for drug regulatory authorities, physicians, traditional health practitioners, pharmacists, manufacturers, research scientists and the general public. Each monograph has a standard format with information on pharmacopoeial quality assurance, medicinal uses, pharmacology, safety issues, and dosage forms. Encouragement and support at the national level will be required eventually to establish the practice of cultivating, documenting, and preserving the local medicinal germplasm (Bahadur et al. 2007; Kuipers 1997; NMPB 2014; WHO 1999, 2004, 2007, 2009).

As evident most of the abovementioned farming practices have been proved to be sustainable and profitable. However, in order to inculcate these practices among the resource-poor endogenous farmers globally, much effort (in terms of training, guidance, and funding) and follow-up procedures (to ensure profitability and continuation of the practices) are still required at the national level. There are several local factors associated as well depending upon agroecology, culture, and economy of the region of interest which must be taken care of in order to be locally relevant and effective while applying these sustainable practices aiming for *endogenous development*. These efforts and investments if

successfully executed will on the way keep building the desired human resource, the core group in protecting our global environment and ensuring our every dire need sustainably utilizing the ever-increasing benefits of modern biotechnology and allied technologies.

37.7 Modern Biotechnology Can Help in Increasing Genetic Diversity to Help a Local Farmer and Ensuring Global Food Security

Revolution in biotechnology has enhanced the toolbox of modern breeder. The much-debated *transgenic technology* has a great promise in genetic modification. Genetically modified insect-resistant and herbicide-resistant crops are presently covering a notable acreage of global cultivation. *Tissue culture-based techniques* like *somatic hybridization*, *haploid breeding*, *embryo rescue*, *and culture* have aided in bringing widely additional variations from intra/interspecific to intergeneric that could be possible by conventional breeding. These methods are highly potential and free of transgenic controversies, biosafety concerns, and purchased (high) input strategies, as they are based on the same principle of genetic improvement relying upon a whole genome as practiced by conventional breeding instead of selected genes only as practiced by transgenic technology. *Mutagenesis* (induced by chemicals or radiation), *somaclonal mutagenesis*, and Targeting Induced Local Lesions in Genomes (*TILLING*: molecular biology-based technique) are the techniques for raising variations by mutation and are equally widely potential. DNA-marker-assisted selection technologies based on molecular biology techniques like *PCR* (polymerase chain reaction), *nucleic acid hybridization*, and *sequencing* have greatly complemented selection part in the wide crossing programs. Tissue culture-based *micropropagation* and *somatic embryogenesis* have been instrumental in vegetative propagation of elite or disease-free plant varieties. Biotechnology is also aiding in diagnosis of diseases, plant nutrition, and plant protection combined with IPM and other farm management techniques (FAO 2010).

Nature believes in unity in diversity. Purchased input agriculture ignores that fact. The monoculture farming has already caused notable loss in local biodiversity. Irresponsible use of pesticides has added to this by increasing threat from once manageable crop pests. Crop productivity intensification has a limit. The fact that high-input/high-output way of agriculture is not sustainable is already visible. *Endogenous development* is also an inseparable part of this. Future sustainable agricultural practices require identification and conservation of rural landraces and germplasms by maintaining *village seed banks*. Documenting rural biodiversity knowledge, raising improved varieties with agronomic traits of interest of local farmers (by breeding programs using the gene bank of wild crop relatives), and on-farm research to solve the real challenges faced by farmers using the power of biotechnological innovations are required. In addition encouraging and supporting maintenance of *kitchen garden* (for cultivation and preservation of local landraces and medicinal plants especially) by smallholders will be a real wholesome approach while aiming for both rural development and conservation of rural natural resources. CGIAR institutes including ICRISAT (International Crops Research Institute for the Semi-Arid Tropics), ICARDA (International Centre for Agricultural Research in the Dry Areas), and IRRI are working visibly in this mission. These institutes are also involved in genetic improvement in livestock and fish (CGIAR 2012; FAO 2010). However, more and more national awareness and involvement are highly desired. Creating options for rural public works is not the ultimate solution. In order to build a sustainable society endogenous development, preservation of regional natural resources and rural integrity are equally important. We need diverse schemes and projects to realize these goals at the national level throughout the globe.

Domestication of crops and livestock for the last 10,000 years has inevitably caused unavoidable loss of this precious biodiversity.

Intensification of agriculture under increasing pressure of population and consumer demand will continue to do the same as part of civilization. From 2011 to 2020, besides the Strategic Plan for Biodiversity, the "Second Global Plan of Action for Plant Genetic Resources for Food and Agriculture" will thus be supervising and monitoring the conservation of *world's heritage of plant genetic resources* across the globe. This is a supporting component of IPTGRFA (The International Treaty on Plant Genetic Resources for Food and Agriculture) (FAO 2011). *Access to genetic diversity* in crops from other regions of the world is also a necessity to find global solution for local problems. ITPGRFA and CBD are working to realize that goal through establishment of rules and regulations at the international level (FAO Crops 2012b).

37.8 Conclusion

Half of the world's population is rural, while 75 % of the world's poor live in rural areas where agriculture is the main source of income. In the current trend of urbanization however, rural people are moving toward diverse sources of income, and agriculture is becoming the less preferable choice due to its risks and challenges beyond the ability of poor rural farmers especially in developing countries to cope up with. Intensive agriculture has hugely affected global environment partly out of need and mostly out of ignorance. Transforming the way of agriculture is a necessity. Combining the benefits of conventional and modern technologies and supporting the development of endogenous farmers are a priority of today. Practicing sustainable agriculture and enhancing rural livelihood are intimately connected with overcoming the current global challenges including food security, poverty reduction, and climate changes and eventually in ensuring the sustainability of human civilization.

In this context we need to remember, however, that rural development is not an overnight event. The follow-up procedures are most important to keep the systems consistent and error-free everywhere. The helpless status of *Bt* cotton cultivation in Gujarat, India (the Navbharat 151 case), where science has got bewildered by business, politics, and ethical issues is a great reminder of this concern or need (Gupta and Chandak 2005).

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