Recent Advances in Phytochemistry 42

David R. Gang *Editor*

Phytochemicals, Plant Growth, and the Environment

Phytochemicals, Plant Growth, and the Environment

Volume 42

Recent Advances in Phytochemistry

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Phytochemicals, Plant Growth, and the Environment

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Introduction to the 42nd Volume of the Recent Advances in Phytochemistry Series

This is the second volume since the reintroduction of the *Recent Advances in Phytochemistry* (*RAP*) series, an annual journal supported by the Phytochemical Society of North America. Topics appropriate for *RAP* include the biosynthesis of natural products and regulation of metabolism, the ecology of specialized metabolites and the evolution of their pathways, and the effects of natural products or plants on human health. Research appropriate for *RAP* involves genomics, proteomics, metabolomics, natural product structural determination and new technology development, medicinal chemistry and metabolic engineering, or any of the myriad of fields that are now closely associated with what may be called "traditional phytochemistry" and plant biochemistry. The advent of post-genomics-based ways of thinking, of systems biology, of synthetic biology, of comparative genomics/proteomics/transcriptomics/metabolomics, and especially of the introduction and establishment of a mentality that leads to support of large collaborative projects has opened up many new doors to scientists interested and versed in the (bio)chemistry of plants. The goal of *RAP* is to highlight these developments.

Two main types of articles are printed in *RAP*: Perspectives and Communications. Perspectives in *RAP* are expected to synthesize results from the primary literature and perhaps from new/novel results and place these in perspective relative to the broader field. These articles not only may be similar to review articles, but also are intended to present important ideas and hypotheses, and may present proposals for interesting directions in the field. It is the hope of the Editorial Board that these articles will be of great value to a large audience. Communications are intended to represent new advances in the field that will be of interest to a large audience. Articles of both types are typically solicited from the Society membership based on the content of the annual meeting talks, but in keeping with the title "Recent Advances in Phytochemistry," the editorial board reserves the right to solicit additional perspectives and/or communications from non-attendees as well (e.g., where an editorial board member has knowledge of

an interesting recent advancement that would be of general interest to the society membership).

All submissions to *RAP* go through a rigorous peer review process, overseen by the Editorial Board, which includes external review. *RAP* is indexed with Springer-published journals. All *RAP* papers are available not only in the published volume form but also electronically through Springer's online literature services. This marks a significant change from past volumes of *RAP*, and it is the hope of the Editorial Board that this will lead to broader dissemination of the contents of and greater interest in *RAP*.

This 42nd volume of RAP includes a total of seven articles, many, but not all, based on talks presented at the 50th annual meeting of the PSNA. As was seen in *RAP* Volume 41, these seven perspectives give a very good picture of the breadth of plant (bio)chemistry research in North America, which is also indicative of the state of the field worldwide. Each of these articles describes the integration of several different approaches to ask and then answer interesting questions regarding the function of interesting plant metabolites, either in the plant itself or in interactions with the environment (natural setting or human health application).

Many of these perspectives have a strong ecological focus. McCormick et al. review the discovery of the biosynthetic pathway leading to production of trichothecene mycotoxins such as the T-2 toxin in plant pathogenic and other fungi. These compounds play very important roles in plant–pathogen interaction and are very significant from a human health perspective. In a complementary paper, Duringer et al. describe recent technological advances in monitoring mycotoxins such as ergovaline and lysergic acid in forage crops, using state-of-the-art and highly sensitive mass spectrometric means. Gross reviews the current understanding of how infochemicals mediate interactions between plants and insects and highlights how such knowledge can be used to mitigate crop losses by pests.

Two perspectives discuss how recent technological advances are making an impact on our understanding of the role of plant hormones in plant growth and development. Gouthu et al. outline highly sensitive methods for measurement of plant hormones in tissues such as developing grape berry. In contrast, McDowell and Gang outline how new transcriptional profiling techniques are shedding light on old questions, such as how rhizome development is regulated by different plant growth regulators.

The last two perspectives outline the role of biotechnology in modern plant biochemistry research. Makhzoum et al. review the long history of use of hairy roots and provide perspective on future utility of this tissue type in continuing to uncover mechanisms of plant natural product biosynthesis, among other applications. Dalton et al. outline, on the other hand, recent efforts to produce nonnative polymers of human interest in plants and outline many of the challenges associated with such investigations.

We hope that you will find these perspectives to be interesting, informative, and timely. It is our goal that *RAP* will act not only as the voice of the PSNA, but also

that it will serve as an authoritative, up-to-date resource that helps to set the gold standard for thought and research in fields related to plant biochemistry.

We welcome suggestions for future articles and comments on the new format.

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Contents

1 **Trichothecene Triangle: Toxins, Genes, and Plant Disease** 1
Susan P. McCormick, Nancy J. Alexander, and Robert H. Proctor

2 **An Analytical Method to Quantify Three Plant Hormone Families in Grape Berry Using Liquid Chromatography and Multiple Reaction Monitoring Mass Spectrometry** 19
Satyanarayana Gouthu, Jeff Morre, Claudia S. Maier, and Laurent G. Deluc

3 **Endophyte Mycotoxins in Animal Health** 37
Jennifer M. Duringer, Lia Murty, and A. Morrie Craig

4 **Production of Traditional and Novel Biopolymers in Transgenic Woody Plants** 59
David A. Dalton, Ganti Murthy, and Steven H. Strauss

5 **Drugs for Bugs: The Potential of Infochemicals Mediating Insect–Plant–Microbe Interactions for Plant Protection and Medicine** 79
Jürgen Gross

6 **Hairy Roots: An Ideal Platform for Transgenic Plant Production and Other Promising Applications** 95
Abdullah B. Makhzoum, Pooja Sharma, Mark A. Bernards, and Jocelyne Trémouillaux-Guiller

7 **A Dynamic Model for Phytohormone Control of Rhizome Growth and Development** 143
Eric T. McDowell and David R. Gang

Index 167

Chapter 1
Trichothecene Triangle: Toxins, Genes, and Plant Disease

Susan P. McCormick, Nancy J. Alexander, and Robert H. Proctor

Abstract Trichothecenes are a family of sesquiterpene epoxides that inhibit eukaryotic protein synthesis. These mycotoxins are produced in *Fusarium*-infested grains such as corn, wheat, and barley, and ingestion of contaminated grain can result in a variety of symptoms including diarrhea, hemorrhaging, and feed refusal. Biochemical and genetic investigations have characterized the genes controlling trichothecene biosynthesis. In *Fusarium*, trichothecene genes have been mapped to three loci including a 26-kb cluster of 12 genes. Production of trichothecenes by *Fusarium graminearum* has been shown to be an important virulence factor in wheat head blight. Strains of *F. graminearum* have been categorized into three different chemotypes, nivalenol (NIV), 3-acetyldeoxynivalenol (3ADON), and 15-acetyldeoxynivalenol (15ADON), based on polymorphisms observed in PCR assays. Although 15ADON-producing strains predominate in North America, there has been a recent emergence of 3ADON- and NIV-producing strains. The genetic basis for these chemotypes has been elucidated with sequence analysis, genetic engineering, and heterologous expression of trichothecene biosynthetic genes.

1.1 Introduction

Trichothecenes are a diverse family of sesquiterpenoid toxins produced by *Fusarium* and some other genera of filamentous fungi. These mycotoxins are characterized by a tricyclic 12,13-epoxytrichothec-9-ene (trichothecene) ring structure (Fig. 1.1). *Fusarium* trichothecenes have been classified as Type A or Type B based on the

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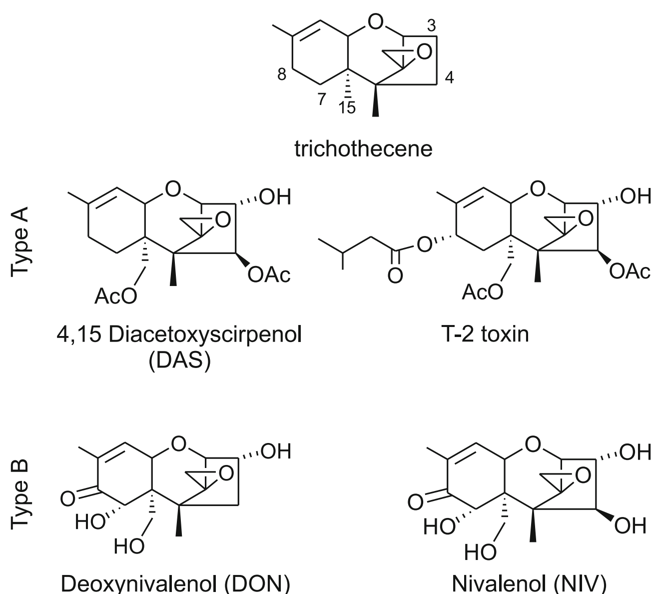

Fig. 1.1 Structures of the trichothecene skeleton with carbons numbered and representative examples of *Fusarium* Type A and Type B trichothecenes

functional group at carbon atom 8 (C-8) of the trichothecene molecule (Fig. 1.1). Type B trichothecenes have a keto function at C-8, while Type A trichothecenes have a hydrogen, hydroxyl, or ester function at C-8. Both Type A and Type B trichothecenes can occur in *Fusarium*-contaminated cereal grains. Type A trichothecenes are generally more toxic to animals, and two Type A compounds, 4,15-diacetoxyscirpenol (DAS) and T-2 toxin, are on the National Select Agent list (http://www.selectagents.gov) [1].

Trichothecenes block protein synthesis in most eukaryotes [2–4], but other cellular effects, including inhibition of mitochondrial enzymes and electrolyte loss, have also been reported [5, 6]. In animals, trichothecene exposure can cause feed refusal, immunological problems, vomiting, skin dermatitis, and hemorrhagic lesions [3, 7]. Trichothecenes are also phytotoxic and can cause chlorosis, inhibition of root elongation, and dwarfism [8, 9].

Due to the potential health hazards associated with trichothecene ingestion, the US Food and Drug Administration has set advisory levels for deoxynivalenol (a Type B trichothecene) in grain. The advisory level for finished grain products destined for human consumption is 1 ppm, and the levels for animal feed are set up to 10 ppm, depending on the animal; pigs are particularly sensitive to deoxynivalenol. At harvest, infected grain with characteristically bleached, shriveled tombstone kernels can have over 200 ppm, but only grain at 2 ppm or lower is accepted without monetary discount.

Outbreaks of *Fusarium* head blight (FHB), caused primarily by *Fusarium graminearum*, of wheat and barley have been a persistent problem in the midwestern and eastern United States and Canada. Six particularly devastating years in the Red River Valley of the Upper Midwest caused billions of dollars of losses in the 1990s [10]. Not only have grain growers suffered reduced yield and quality of grain due to FHB, they have also received lower financial reimbursement due to the presence of trichothecenes in the grain.

Research during the 1990s demonstrated that trichothecene production is a virulence factor in the *Fusarium*–wheat interaction [11–13]. Deoxynivalenol blocks the development of a heavy cell-wall protection barrier in wheat and thereby facilitates spread of *Fusarium* [14]. When wheat was infected with *Fusarium* strains that had been engineered to produce no trichothecenes, spread of the disease within the head was limited compared to plants infected with wild-type trichothecene-producing strains [13].

Here we review the biochemistry and genetics of the trichothecene biosynthetic pathway, focusing on the T-2 toxin-producing species *F. sporotrichioides*. We also review correlations between trichothecene structures produced by *Fusarium* species and variation in trichothecene biosynthetic (TRI) genes. *Fusarium* carries the genes that are necessary for trichothecene production, and the fungus must invade plant tissue for the mycotoxins contribute to plant disease.

1.2 Trichothecene Biosynthesis in *Fusarium sporotrichioides*

The proposed trichothecene biosynthetic pathway (Fig. 1.2) is based on a number of different analyses, including (1) feeding experiments in which labeled and unlabeled precursors were fed to fungal cultures [15–17], (2) feeding experiments with transgenic yeast carrying an intact trichothecene biosynthetic gene [18–20], (3) experiments with mutant strains of *Fusarium* generated by targeted, molecular genetic-induced changes to trichothecene biosynthetic genes [21–23], and (4) experiments with mutant strains of *F. sporotrichioides* with altered trichothecene production phenotypes that were induced by UV mutagenesis [24–27].

The biosynthesis of trichothecenes begins with the cyclization of farnesyl pyrophosphate to form trichodiene [28]. Trichodiene undergoes an allylic oxygenation at C-2 to form 2α-hydroxytrichodiene. Another oxygenation at C-12 adds an epoxide to form 12,13-epoxy-9,10-trichoene-2-ol, which is converted to isotrichodiol by a second allylic oxygenation at C-11. Oxygenation at C-3 leads to isotrichotriol which undergoes a nonenzymatic isomerization followed by cyclization to form isotrichodermol.

In *F. sporotrichioides*, biosynthesis of T-2 toxin proceeds from isotrichodermol with a series of acetylations and oxygenations. Isotrichodermol is acetylated at C-3 to form isotrichodermin [18], which then undergoes C-15 oxygenation to form 15-decalonectrin followed by C-15 acetylation to form calonectrin [29, 30].

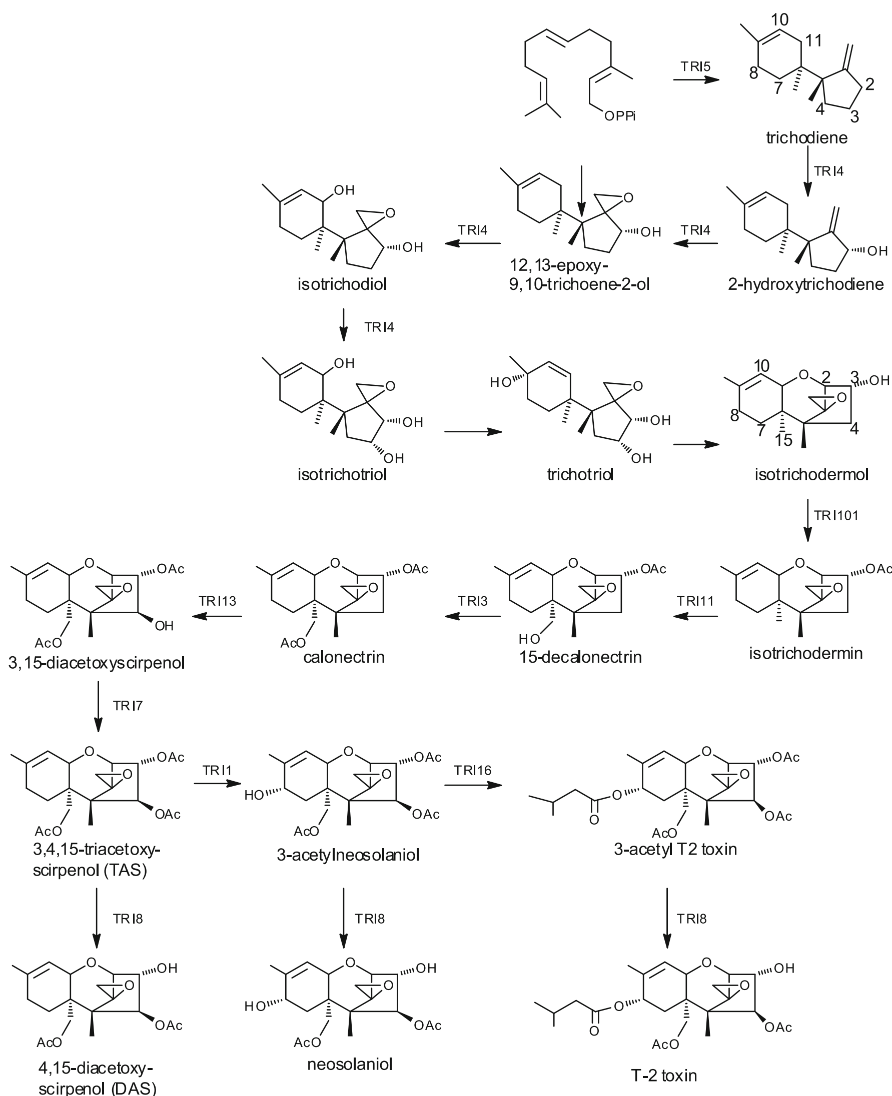

Fig. 1.2 *Fusarium sporotrichioides* trichothecene pathway leading to T-2 toxin, 4,15-diacetoxy-scirpenol (DAS), and neosolaniol (8-hydroxy-4,15-diacetoxyscirpenol)

Calonectrin undergoes C-4 oxygenation to form 3,15-diacetoxyscirpenol followed by C-4 acetylation to form 3,4,15-triacetoxyscirpenol (TAS). 3-Acetyl T-2 toxin results from oxygenation at C-8 followed by addition of an isovaleryl group derived from leucine [26]. The final step in T-2 toxin biosynthesis is removal of the C-3 acetyl group [19].

1.3 *Fusarium sporotrichioides* Trichothecene Biosynthetic Genes

Most of the known trichothecene biosynthetic (*TRI*) genes were first characterized in *Fusarium sporotrichioides* (Table 1.1). In this species, the genes occur at three genetic loci: the 12-gene core *TRI* cluster, the two-gene *TRI1/TRI16* locus, and the single-gene *TRI101* locus (Fig. 1.3). The core TRI cluster includes genes responsible for formation of the trichothecene molecule, as well as most modifications to it, while the *TRI1/TRI16* genes are responsible for modification at C-8 [21, 22], and the *TRI101* gene is responsible for the C-3 acetylation [31].

The functions of most of the trichothecene genes were determined using gene disruption, heterologous expression, as well as precursor feeding experiments with whole cells and cell-free extracts. However, two of the genes were identified by UV mutagenesis [25, 30], and one (*TRI5)* was identified by purifying the

Table 1.1 Summary of *Fusarium sporotrichioides* trichothecene biosynthetic genes and their functions

Gene	Function	Enzyme substrate
TRI1	C-8 oxygenase	3,4,15-Triacetoxyscirpenol
TRI3	C-15 acetyltransferase	15-Decalonectrin
TRI4	Trichodiene oxygenase	Trichodiene
TRI5	Trichodiene synthase	Farnesyl pyrophosphate
TRI6	Transcriptional regulator	
TRI7	C-4 acetyltransferase	3,15-Diacetoxyscirpenol
TRI8	C-3 esterase	3-Acetyl T-2 toxin
TRI9	Unknown	
TRI10	Global regulator	
TRI11	C-15 oxygenase	Isotrichodermin
TRI12	Trichothecene pump	
TRI13	C-4 oxygenase	Calonectrin
TRI14	Unknown	
TRI15	Regulatory	
TRI16	C-8 acyltransferase	3-Acetylneosolaniol
TRI101	C-3 acetyltransferase	Isotrichodermol

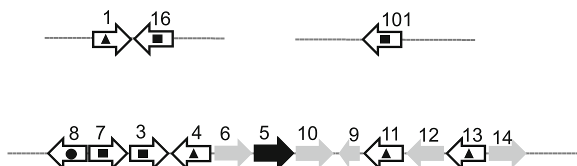

Fig. 1.3 *Fusarium sporotrichioides* loci containing genes involved in trichothecene biosynthesis: *black*, terpene cyclase; *square*, acetyl/acyltransferase; *circle*, esterase; *triangle*, P450 oxygenase; *gray*, genes for regulation, trichothecene pump, and other uncharacterized genes

corresponding enzyme, raising antibodies to the purified enzyme, and screening a library of *F. sporotrichioides* genomic DNA expressed in *E. coli* with the antibody [28, 32]. Once *TRI5* was identified, sequence analysis of adjacent regions of DNA revealed the presence of additional genes, and the role of two of these genes in trichothecene biosynthesis was determined by complementation of UV-induced mutants of *F. sporotrichioides* that were blocked in T-2 toxin production; that is, introduction of large pieces of DNA (cosmid clones) carrying *TRI5* and multiple adjacent genes into mutants restored T-2 toxin production [33].

TRI4 was identified with a UV-induced mutant [24, 33, 34] and characterized more fully by gene deletion analysis [35]. These analyses indicated that *TRI4* encodes a cytochrome P450 enzyme involved in the initial oxygenation of trichodiene at C-2. However, heterologous expression in yeast and *F. verticillioides,* organisms that do not produce trichothecenes or have trichothecene biosynthetic genes, revealed that the *TRI4* monooxygenase is multifunctional and can catalyze four oxygenation reactions that result in conversion of trichodiene to isotrichotriol [36, 37].

The next proposed step in the trichothecene biosynthetic pathway in *Fusarium sporotrichioides*, the conversion of isotrichodermol to isotrichodermin, is controlled by *TRI101* [8, 18]. This gene was initially identified from a cDNA library, prepared from *Fusarium*. When a *TRI101* cDNA was expressed in yeast, it conferred resistance to high concentrations of the trichothecenes 4,15-DAS, T-2 toxin, and deoxynivalenol by acetylating these toxins at the C-3 position [8]. Because C-3 acetylated trichothecenes are less toxic to microorganisms [8, 38], *TRI101* is considered to be a resistance gene that protects trichothecene-producing organisms from their own toxins.

The next proposed step in the pathway, the hydroxylation of isotrichodermin to form 15-decalonectrin (Fig. 1.2), is catalyzed by another cytochrome P450 encoded by *TRI11* [29, 30]. 15-Decalonectrin is then converted to calonectrin by an acetyltransferase encoded by *TRI3*. Calonectrin is then hydroxylated at the C-4 position by the *TRI13*-encoded P450 monooxygenase to form 3,15-diacetoxyscirpenol [39], which then undergoes a C-4 acetylation, catalyzed by the *TRI7*-encoded acetyltransferase [40] to form 3,4,15-triacetoxyscirpenol.

In *F. sporotrichioides*, the *TRI1*-encoded P450 monooxygenase then catalyzes C-8 hydroxylation to form 3-acetylneosolaniol [21, 41]. The *TRI16*-encoded acyltransferase catalyzes esterification of an isovaleryl moiety to the C-8 oxygen to yield 3-acetyl T-2 toxin [22, 41]. The final step in the T-2 toxin biosynthetic pathway is C-3 deacetylation of 3-acetyl T-2 toxin, a reaction catalyzed by the *TRI8*-encoded esterase [19]. This last reaction reverses the self-protecting C-3 acetylation catalyzed by the *TRI101*-encoded acetyltransferase and produces a trichothecene with increased toxicity.

Thus, T-2 toxin biosynthesis requires a sesquiterpene cyclase gene (*TRI5*), four P450 monooxygenase genes (*TRI4*, *TRI11*, *TRI13*, and *TRI1*), four acetyltransferase/acyltransferase genes (*TRI101*, *TRI3*, *TRI7*, and *TRI16*), and an esterase gene (*TRI8*). However, the core TRI cluster also includes a transport protein-encoding gene, *TRI12*, which is most likely responsible for pumping T-2 toxin out

of *Fusarium* cells into the surrounding environment [20]. The cluster also includes two genes, *TRI6* and *TRI10*, that regulate expression of other *TRI* genes inside and outside the core cluster [42, 43]. The mechanism by which *TRI10* affects *TRI* gene expression is not yet known. In contrast, *TRI6* is predicted to encode a transcription factor, a class of proteins that bind specific sequence motifs in the promoter regions of genes and induce transcription. The sequence motifs to which the Tri6 protein binds were originally demonstrated for the promoter regions of *TRI4* and *TRI5*, but the motifs were subsequently identified in the promoter regions of all known *TRI* genes within and outside the cluster [18, 19, 40, 43]. Disruption of *TRI6*, *TRI10*, or *TRI12* reduced T-2 toxin production. Transcriptional regulators are often associated with the expression of co-regulated proteins, such as those found to be in a metabolic pathway.

1.4 Variations on a Theme

The diverse number of trichothecenes produced by species in the order Hypocreales suggests that there are variations in the biosynthetic pathways and the genes that control their production. Although most of the trichothecene biosynthetic genes were first identified in *F. sporotrichioides*, homologues of some of the genes have also been identified in other trichothecene-producing organisms.

Fusarium trichothecenes have an oxygen function at the C-3 position. But trichothecenes produced by other genera, such as *Myrothecium*, *Trichothecium*, *Trichoderma*, and *Stachybotrys*, lack this C-3 oxygen. In *M. roridum*, which produces the trichothecene roridan A (Fig. 1.4), the predicted Tri4 amino acid sequence is 64% identical and 80% similar to that of *F. sporotrichioides* [44]. When the *M. roridum TRI4* was expressed in a mutant of *F. sporotrichioides* in which *TRI4* was inactivated, T-2 toxin production was not restored, but trichothecene, deoxysambucinol, and sambucinol were produced [16, 44]. This experiment, and later experiments in which *M. roridum TRI4* was expressed in *F. verticillioides* [45] and *Trichothecium roseum TRI4* [37] was expressed in yeast, indicate that in *Myrothecium* and *Trichothecium*, the *TRI4* monooxygenase catalyzes three oxygenation reactions to covert trichodiene to isotrichodiol. Therefore, the function of *TRI4* in *Myrothecium* and *Trichothecium* differs from the function in *Fusarium*, and the difference gives rise to structural variation in the trichothecenes produced by *Myrothecium*/*Trichothecium* versus *Fusarium*.

Variation in function of *TRI* gene homologues in different species of *Fusarium* has also been observed. In *F. sporotrichioides,* the *TRI1*-encoded P450 monooxygenase gene catalyzes hydroxylation of the C-8 position only [21]. In contrast, the *F. graminearum TRI1* enzyme, FgTri1, catalyzes hydroxylation of both C-7 and C-8 positions (Fig. 1.5) [23]. The predicted amino acid sequence of FgTri1 is 59% identical to the *F. sporotrichioides* Tri1 [23]. In addition, the function of *TRI16* differs in *F. sporotrichioides* and *F. graminearum*. Tri16 catalyzes esterification of

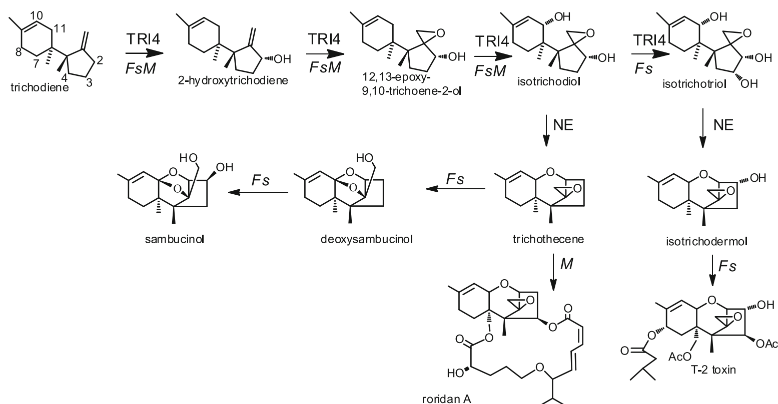

Fig. 1.4 Divergent Tri4 pathways in *Fusarium* and *Myrothecium*

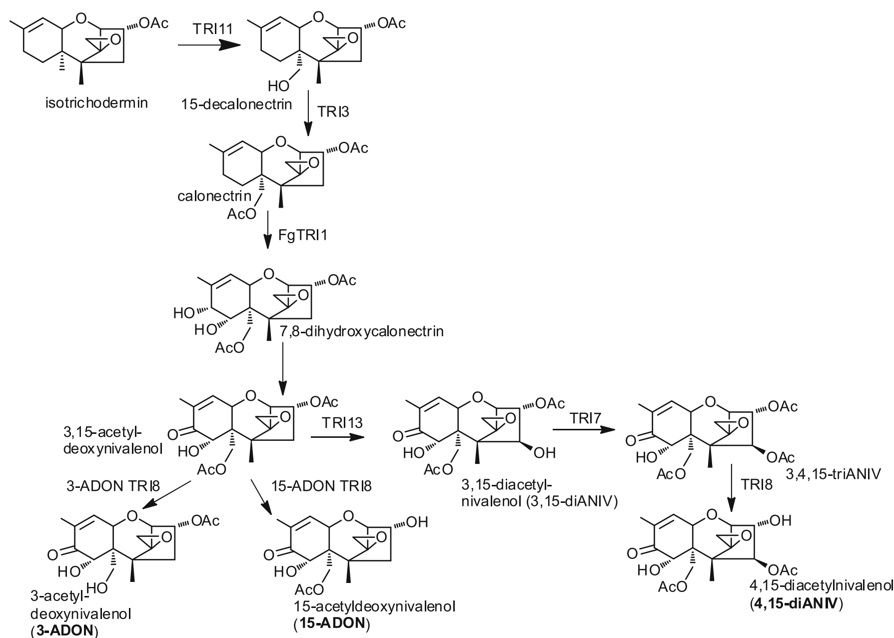

Fig. 1.5 *Fusarium graminearum* trichothecene biosynthesis from isotrichodermin leading to Type B trichothecenes 3ADON, 15ADON, and 4,15-diANIV

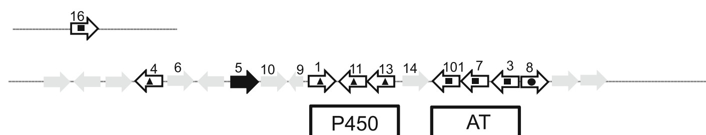

Fig. 1.6 *Fusarium equiseti* trichothecene genes: *black*, terpene cyclase; *square*, acetyl/acyltransferase; *circle*, esterase; *triangle*, P450 oxygenase; *gray*, genes for regulation, trichothecene pump, and other uncharacterized genes. *TRI1* and *TRI101* are inserted within the core TRI gene cluster, and other rearrangements result in clustering of three acetyltransferase (AT) genes and three P450 oxygenase genes

isovaleryl to the C-8 oxygen [16]. In *F. graminearum*, however, the *TRI16* has multiple insertions and deletions that prevent formation of a functional Tri16 enzyme. As a result, trichothecenes produced by *F. graminearum* do not have an isovaleryl moiety at C-8.

As noted earlier, Type B trichothecenes have a C-8 keto and a C-7 hydroxyl function, whereas Type A trichothecenes have neither. Although the enzyme responsible for the oxidation of the C-8 hydroxyl to a C-8 keto is not known, the functions (or lack of function) of *TRI1* and *TRI16* in Type A-producing species *F. sporotrichioides* and the Type B-producing species *F. graminearum* suggest that these two genes contribute to whether fusaria produce Type A (Fig. 1.2) or Type B trichothecenes (Fig. 1.5).

The organization of the *TRI* genes in *F. graminearum* is similar to that in *F. sporotrichioides* in that the genes are located at three distinct loci: the core *TRI* cluster, the *TRI1*/*TRI16* locus, and the *TRI101* locus [46]. In contrast, in the more distantly related species, *F. equiseti*, the *TRI1* and *TRI101* genes are located in the core TRI cluster (Fig. 1.6) [47]. The positions of several other *TRI* genes within the cluster are also altered in *F. equiseti* compared to *F. graminearum* and *F. sporotrichioides*: the *TRI12* gene is absent; *TRI3*, *TRI7*, and *TRI8* are located at the opposite end of the cluster; and there is a third putative regulatory gene inserted between *TRI5* and *TRI6* (Fig. 1.6) [47].

The *F. equiseti* organization of biosynthetic genes gives this fungus the potential to produce both Type A and Type B trichothecenes (Fig. 1.7). Previous work indicated that *F. graminearum* Tri1 can add a hydroxyl group at the C-7 or at the C-8 position [48]. 8-Hydroxytrichothecene intermediates formed may then be converted by a functional Tri16 acyltransferase to an isovaleryl derivative such as T-2 toxin (Type A). 7-Hydroxytrichothecene intermediates may be converted to 7,8-dihydroxytrichothecenes and then to 7-hydroxy 8-keto trichothecenes (Type B). 7-Hydroxy T-2 toxin has not been reported from *Fusarium*. Heterologous expression of *TRI1* and *TRI16* from *F. equiseti* in *tri1* or *tri16* mutant strains may be useful in determining which trichothecenes may be produced in this species.

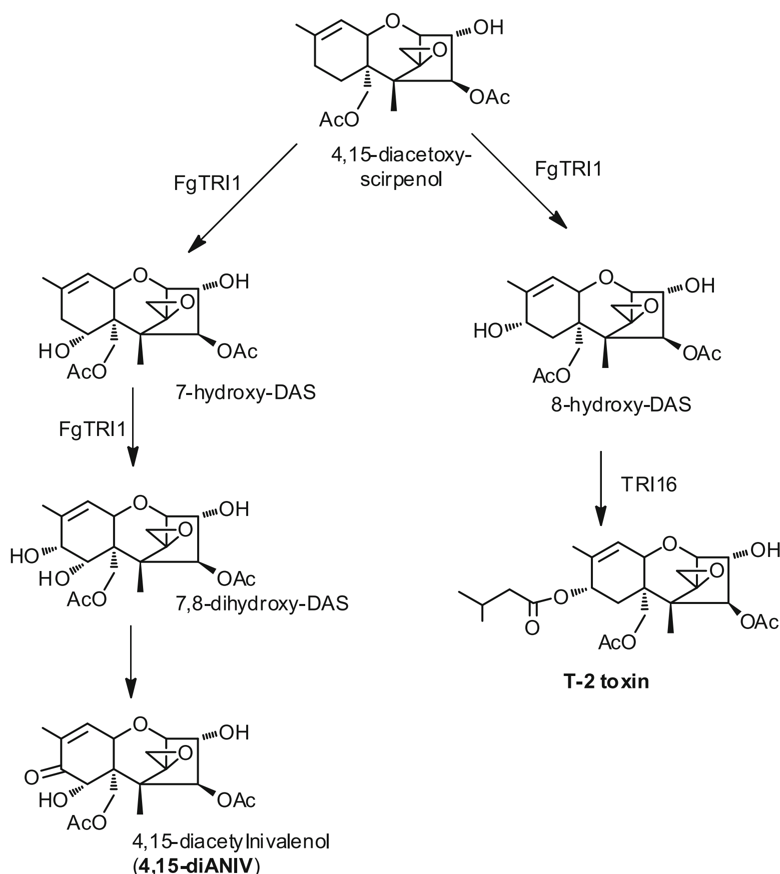

Fig. 1.7 Proposed *Fusarium equiseti* trichothecene biosynthetic pathway from 4,15-diacetoxy-scirpenol. Functional FgTri1 and FsTri16 enzymes can lead to both Type A and Type B trichothecenes

1.5 Variations in Chemotypes

While deoxynivalenol (DON, Fig. 1.1) is the most commonly detected trichothecene mycotoxin in North American wheat showing symptoms of *Fusarium* head blight (FHB), nivalenol (NIV, Fig. 1.1) has only recently been reported in North America—mainly in the southern USA [49].

The structures of DON and NIV differ from each other only by the presence (NIV) or absence (DON) of a hydroxyl function at C-4. This structural difference can be attributed to differences in a gene, *TRI13*, that controls hydroxylation at C-4 [40, 50]. *TRI13* is nonfunctional in DON-producing strains [40, 50], and as a result,

such strains cannot hydroxylate trichothecenes at C-4. Markers based on these differences have been successfully used to predict the DON and NIV chemotypes of *Fusarium* strains [51].

Within DON-producing strains, there are two trichothecene production phenotypes (chemotypes), 3ADON and 15ADON, that can be distinguished by the acetylated derivative produced in liquid culture. Although 15ADON strains have been the predominant chemotype in North America, 3ADON-producing strains have been reported from the USA and Canada and appear to be increasing in frequency [52–54]. PCR markers have been developed, based on sequence differences in the *TRI3* (C-15 acetyltransferase) region and the *TRI12* (trichothecene pump) region, that can reliably predict the 3ADON versus 15ADON chemotype of *Fusarium* strains [55]. However, the genetic basis for the different chemotypes has only recently been determined [56].

Because 3ADON and 15ADON differ at the C-3 and C-15 positions, genes that are involved in acetylation or deacetylation at these positions, *TRI3*, *TRI8*, and *TRI101*, are likely to be determinants of the 3ADON and 15ADON chemotypes. Although 15ADON lacks a C-3 acetyl group, deletion mutants of a *F. graminearum* 15ADON strain accumulate trichothecene intermediates, e.g., isotrichodermin, 15-decalonectrin, calonectrin, and 3,15-diADON (Fig. 1.5), which have a C-3 acetyl group [18, 19, 23]. In a 15ADON strain, disruption of either *TRI8* [19] or *TRI3* [57] blocked production of 15ADON, indicating that both genes are required for 15ADON production. In summary, in 15ADON strains, *TRI101*, *TRI3*, and *TRI8* have functions similar to their homologues in *F. sporotrichioides.*

Conversely, since the 3ADON structure has a C-3 acetyl group but lacks a C-15 acetyl group, a hypothesis was proposed that *TRI3* and *TRI8* are not required or are nonfunctional in 3ADON strains [31]. However, cell-free extracts of 3ADON strains could catalyze both trichothecene C-15 acetylation (conversion of 15-decalonectrin to calonectrin) and C-15 deacetylation (conversion of 3,15 diADON to 3ADON). These results indicate that *TRI3* is functional in 3ADON strains and further suggest that an additional esterase gene might be required for the removal of the C-15 acetyl group [56]. A candidate esterase gene, *ORF-G*, is located adjacent to the *TRI14* end of the core *TRI1* cluster (Fig. 1.3) [46]. However, yeast expressing *ORF-G* could not convert 3,15-diADON to 3ADON, suggesting that this esterase was not involved in trichothecene biosynthesis (McCormick & Alexander, unpublished).

To assess the role of *TRI8* in the 3ADON and 15ADON chemotypes, *TRI8* genes from 3ADON and 15ADON strains were expressed in a yeast transgenic system. Transformants with a 15ADON *TRI8* converted 3,15-diADON to 15ADON, i.e., they had trichothecene C-3 esterase activity [19], while those with *TRI8* from a 3ADON strain converted 3,15-diADON to 3ADON, i.e., they had C-15 esterase activity [56]. This clearly indicated that *TRI8* was indeed functional in 3ADON strains but that it encoded a C-15 esterase rather than a C-3 esterase [56]. Similar experiments with nivalenol chemotype strains indicated that their *TRI8* encoded a C-3 esterase, consistent with production of 4,15-diacetylnivalenol by NIV strains in liquid culture (Fig. 1.5) [56].

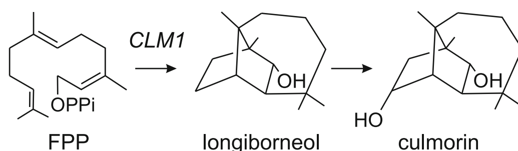

Fig. 1.8 Proposed biosynthetic pathway for culmorin in *F. graminearum*

A comparative analysis revealed significant differences in the DNA and deduced amino acid sequences of *TRI8* from 3ADON and 15ADON strains. Although the overall sequence identity was 82%, *TRI8* from 15ADON strains has 21 base pairs at the 3′-end that are absent in *TRI8* from 3ADON strains [56]. Esterases have a characteristic nucleophilic elbow with a conserved amino acid motif GXSXG [58], and the Tri8 proteins from both 15ADON and 3ADON chemotype strains have this sequence in the same approximate location [56].

From the sequence comparisons, it appears that the significant difference in the 3′-ends of the coding region might be responsible for the differences in Tri8 esterase function. In order to determine which part of the *TRI8* sequence determines whether 3ADON or 15ADON is produced, chimeric *TRI8* genes were constructed with portions of 3ADON sequence and portions of 15ADON sequence and then expressed in *F. graminearum* strains in which the native *TRI8* gene was inactivated by gene disruption (i.e., *tri8* mutants). Although there are significant differences in sequence at the 3′-end of 3ADON and 15ADON forms of *TRI8*, experiments with chimeric constructs of *TRI8* indicate that the middle portion of the gene determines the C-3 versus C-15 esterase activity and therefore whether a strain produces 15ADON or 3ADON [56]. To extend this type of analysis, similar strategies could be used to discern the key sequence changes that determine the variation in functions of *TRI4* or *TRI1* orthologues.

The role of the 3ADON and 15ADON chemotypes in plant pathogenicity of *F. graminearum* is not clear, although recent work suggests that 3ADON and 15ADON strains of the fungus exhibit the same level of virulence in *Fusarium* head blight (FHB) of wheat [53]. The availability of isogenic strains differing only in the production of 3ADON or 15ADON will facilitate an accurate assessment of how the chemotypes impact virulence. In addition to 3ADON or 15ADON, some *Fusarium* strains produce a significant amount of another sesquiterpenoid mycotoxin, culmorin (Fig. 1.8) [59–61]. Preliminary greenhouse testing of an *F. graminearum* strain that produced both culmorin and 15ADON was highly virulent (Alexander and McCormick, unpublished). Although purified culmorin did not appear to be as toxic as 15ADON in test situations [9, 62], it may act synergistically with trichothecenes as an aggressiveness factor in FHB of wheat. A gene, *CLM1*, encoding a terpene cyclase required for culmorin production, has been identified in *F. graminearum* [59]. Thus, it is now possible to generate mutants of *Fusarium* in which production of trichothecenes and/or culmorin is blocked and use these mutants to assess whether culmorin is a virulence factor in FHB.

1.6 Looking for Resistance

Since trichothecene production was demonstrated to be a virulence factor in FHB of wheat [11–13], introducing resistance to the toxins into wheat has become an attractive strategy for controlling the disease. Trichothecenes are antibiotics, and as such, their biosynthesis requires adaptations by the producing organisms for self-protection. Antibiotic-producing organisms have a number of mechanisms for self-protection. These mechanisms fall into one of three general types: alteration of target protein, metabolism to reduce toxicity, and reduction of intracellular concentration of the antibiotic with pumps.

Trichothecene-producing species of *Fusarium* have two of the three mechanisms for self-protection (or resistance): the *TRI12*-encoded trichothecene efflux pump [20] and the *TRI101*-encoded enzyme (Tri101) that modifies trichothecenes by acetylating the C-3 position of the toxins and thereby reduces their toxicity [8, 63]. Tobacco cells expressing *TRI101* from *F. sporotrichioides* were able to grow in media amended with 4,15-diacetoxyscirpenol by converting 4,15-diacetoxyscirpenol to 3,4,15-triacetoxyscirpenol which is less phytotoxic [38, 64, 65]. Both *Chlamydomonas* and *Arabidopsis* assays indicate that addition of a C-3 acetyl group of *Fusarium* trichothecenes reduces their phytotoxicity [9, 38, 65].

F. sporotrichioides TRI101 was expressed in wheat to try to improve resistance to FHB and reduce deoxynivalenol contamination. One transgenic line generated showed partial resistance to *F. graminearum* infection in greenhouse virulence assays [66]. Structural and kinetic studies later revealed that the *F. graminearum* Tri101 acetyltransferase was 70-fold better than *F. sporotrichioides* Tri101 at converting DON to 3ADON [67] and might be a better enzyme in future attempts to increase trichothecene resistance in wheat through genetic engineering. Other organisms have been reported to have trichothecene 3-*O*-acetyltransferase activity, including non-trichothecene-producing species of *Fusarium*, yeast, and *Aspergillus* [36, 48, 68, 69], and these acetyltransferases may have structural features that could improve the efficiency of DON acetylation in plants. One of the challenges of using this approach is the presence of esterases in *Fusarium* [19, 70] that can potentially reverse Tri101-catalyzed trichothecene 3-*O*-acetylation.

Other changes to the trichothecene C-3 hydroxyl group can reduce toxicity. For example, soil bacteria can convert deoxynivalenol to its C-3 keto derivative, which has a tenfold reduced immunosuppressive toxicity based on mitogen-induced and mitogen-free proliferations of mouse spleen lymphocytes [71]. A bacterial gene or enzyme responsible for the DON to 3-keto DON conversion has not been identified. Synthetic C-3 keto derivatives of isotrichodermol and 15ADON were prepared and were completely nontoxic to *Arabidopsis* leaves [9]. Preliminary experiments (McCormick unpublished) suggest that *F. graminearum* cultures can also reverse this detoxification, converting C-3 keto 15DON to 15ADON.

Although no wheat lines with strong resistance to FHB have been identified, a quantitative trait locus (QTL) for partial resistance to *Fusarium* head blight in the wheat cultivar Sumai 3 is correlated with conversion of deoxynivalenol to its

3-*O*-glucoside, which has a reduced ability to inhibit protein synthesis [72]. Recently a UDP-glucosyltransferase gene from barley has been identified and expressed in yeast where it confers resistance to DON [73]. It is unknown if *F. graminearum* glucosidases can reverse this modification. There has been some concern that trichothecene glycosides are masked mycotoxins [74], i.e., they are present in food and feed but not detected with current analytical methods used to monitor trichothecene contamination. It is not known how stable trichothecene glucosides are or if they can be converted back to the DON by glucosidases during digestion.

Although most trichothecene modifications have focused on the C-3 hydroxyl group, another promising target for preharvest detoxification is the C-13 epoxide group. Anaerobic rumen bacteria and, more recently, bacteria from catfish guts have been identified that can convert trichothecenes to their nontoxic deepoxy analogs [75, 76].

1.7 Summary

This review has covered the genetic control of the trichothecene biosynthesis in *F. sporotrichioides* and the variations in the types of trichothecenes and genes found in other trichothecene-producing fungi. *TRI* genes have undergone alterations in function and have been relocated, lost, rearranged, and rendered nonfunctional during evolution of trichothecene-producing fungi. These genetic variations can lead to different trichothecene chemotypes that may lead to differences in toxicity and pathogenicity. Trichothecenes are not only deleterious to humans and animals that consume *Fusarium*-infected grain, but their production can also act as a virulence factor in plant disease. As such, strategies that target trichothecene production or convert the trichothecenes produced to less phytotoxic products may be the key to reducing the severity of plant diseases caused by trichothecene-producing species of *Fusarium*.

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Chapter 2
An Analytical Method to Quantify Three Plant Hormone Families in Grape Berry Using Liquid Chromatography and Multiple Reaction Monitoring Mass Spectrometry

Satyanarayana Gouthu, Jeff Morre, Claudia S. Maier, and Laurent G. Deluc

Abstract Hormones play an important role during the development and ripening of grape berry. Unlike the case of ethylene in climacteric fruits, several different hormones are believed to sequentially accumulate at specific times during berry developmental stages to promote different physiological processes. To dissect this complex hormonal interaction system in a recalcitrant tissue containing several interfering compounds including sugar and phenolic compounds, an extraction protocol and an LC-MS-based analytical method that includes three hormone families have been adapted. Using this technique, we optimized a method to simultaneously detect and quantify cytokinin, auxin, and abscisic acid-related analytes in grape berries across the developmental stages and between tissues. Resulting quantifications of the analytes are consistent with the overall trend of the ABA, auxin, and cytokinin dynamics in grape and reveal new patterns not previously reported in this plant. Evolving evidence of coordinated action of several hormones during the critical phases of berry development (cell division, cell expansion, berry ripening) suggests a need to further integrate other plant growth regulator families to provide a more comprehensive picture.

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2.1 Introduction

Grape is considered the most valuable horticultural crop in the world. Among the commodity crops (http://faostat.fao.org), it is ranked second in 2008 with 66,643,404 metric tons, and its economic impact is far greater due to various uses such as fresh product, wine, liquor, and raisins. Finally, processed or freshly consumed grapes possess nutritional values and health benefits for humans making them desirable in human diet [1, 2]. But, grapes suffer from various degrees of ripeness heterogeneity at harvest that substantially contributes to inferior crop quality and productivity. To understand the cause of ripening heterogeneity, it is important first to understand the main factors controlling grape berry ripening. Such knowledge will be of great economic importance to achieve more uniform fruit having optimal concentrations of compounds with organoleptic characteristics and health-beneficial properties. Unlike in climacteric fruits, where ethylene mainly regulates the fruit ripening initiation, many hormones are thought to have a role in the grape berry ripening initiation. The interplay between these different hormones across berry development is critical in explaining the ripeness heterogeneity.

Berry development can be described as two successive sigmoid cycles with distinctive biophysical and biochemical characteristics [3] (Fig. 2.1). Among the molecular events that coordinate grape berry development, hormones play a major role [4–7]. For instance, like ethylene in climacteric fruits, abscisic acid (ABA) has long been regarded as the main regulator of the onset of grape berry ripening [4, 8–10]. However, the narrow spectrum of targeted metabolic pathways involving ABA in grape indicates that several hormones would rather act in concert to influence the berry development [6, 10–13]. For instance, auxin was found to delay ripening, and in some cases, auxin treatment is even able to "synchronize" sugar accumulation within a cluster [5, 14]. In the same way, cytokinin is suspected to delay the ripening by decreasing total soluble solids (TSS) and the anthocyanin accumulation [15]. In the context of this multihormonal role, adapting an analytical method that integrates several classes of plant hormones is important to understand their respective roles in berry ripening and ripeness heterogeneity.

2.2 ABAs, Auxins, and Cytokinins in Grape Berry

2.2.1 Metabolism of ABAs, Auxins, and Cytokinins in Plants

ABA is a sesquiterpenoid (C_{15}) (Fig. 2.2) that is built up of three isoprenoid units ($3 \times C_5$). The main regulatory step of ABA biosynthesis is the cleavage of the violaxanthin intermediate in the carotenoid pathway by nine-*cis*-epoxycarotenoid dioxygenase (NCED) to produce xanthoxin and ultimately ABA. The two main pathways of ABA catabolism are associated with oxidation and conjugation processes. Oxidation of ABA can take place at three different carbon atoms (7′, 8′, and 9′)

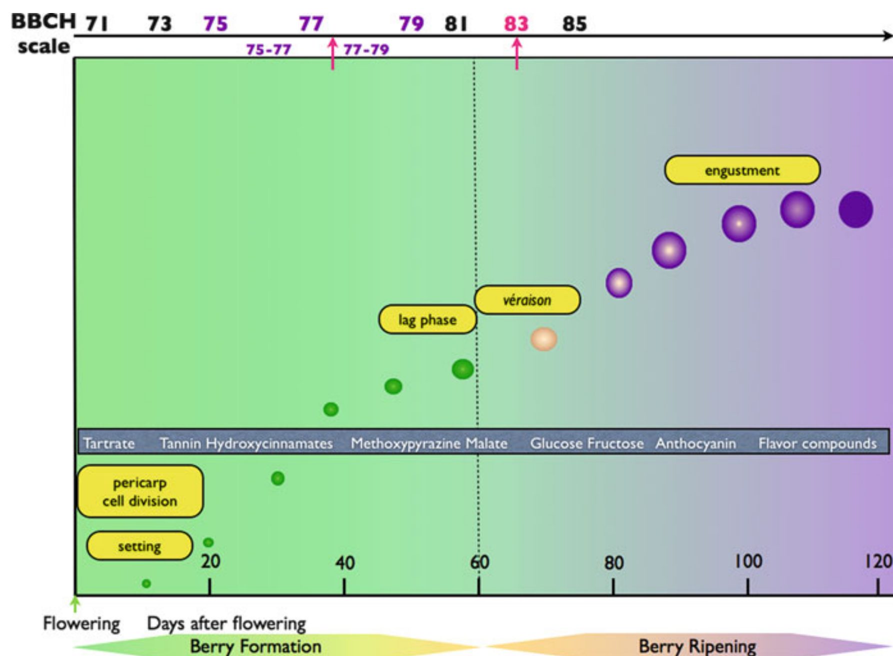

Fig. 2.1 Grape berry development chart showing major developmental stages. Redrawn from Kennedy (2005) (http://www.practicalwinery.com/julyaugust02/julaug02p14.htm). Developmental stages investigated in this chapter are identified based on BBCH-scales [46] by *purple* and *pink* numbers and *arrows*. Whole berry analyses were performed using tissue material from BBCH stages 75 to 79 (*purple numbers*). Tissue-specific analyses (seed, skin, and pulp) used samples from a green stage between 77 and 77–79 (*pink arrow*) and *véraison* stage (83)

and is generally controlled by hydroxylases of the P450 monooxygenase conserved family (CYP). Yet the 8′-position reaction appears to occur more often than the other two. The oxidation at the 8′-position yields an unstable intermediate, which is rapidly converted into phaseic acid (PA) and ultimately into dihydrophaseic acid (DPA). Conjugation of ABA and other derived compounds, such as PA and DPA, leads to the formation of glucose ester forms like ABA-glucose ester (GE) and PA-GE or glucosides like ABA-GS and DPA-GS [16, 17].

Primary metabolic steps of cytokinins (CK) are shared with the purine metabolic pathway. On the other hand, the homeostasis of the biologically active forms will depend upon the chemical structures of either the adenine moiety or the side chain [18–20]. In different plant species, different cytokinins were identified as biologically active forms (Fig. 2.3) [21]. Most bioassays, based on heterologous expression of CK receptors in yeast and in *Escherichia coli*, indicated that cytokinin nucleobases are the primary ligands for cytokinin receptors whereas the sugar conjugates are less active or inactive.

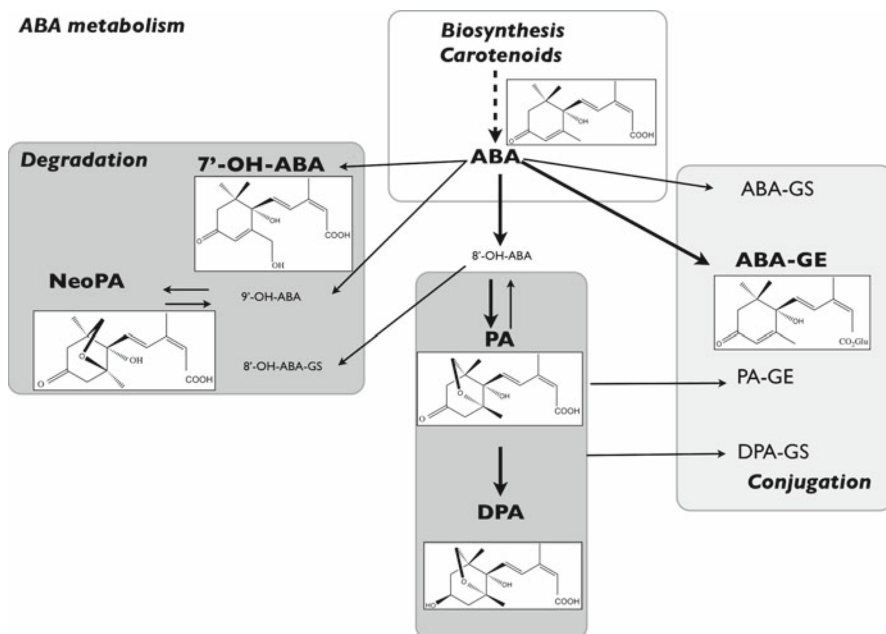

Fig. 2.2 Abscisic acid (ABA) metabolism involving biosynthetic, degradation, and conjugation pathways. Chemical structures of analytes quantified in this report are depicted

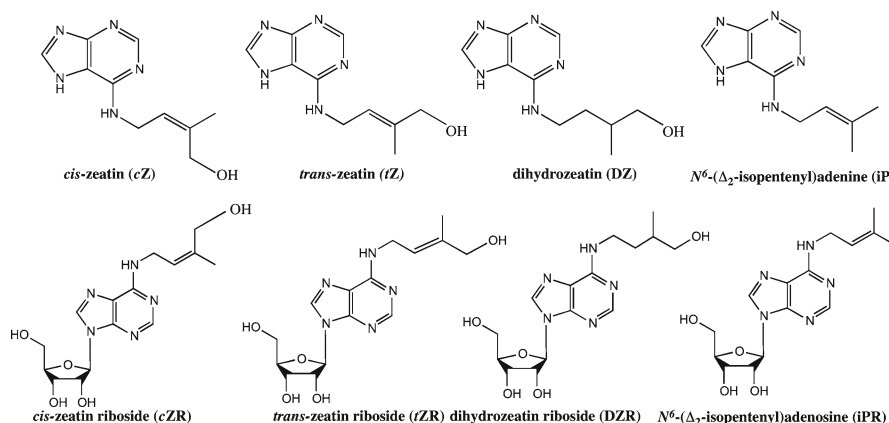

Fig. 2.3 Chemical structures of cytokinin and cytokinin-riboside conjugates analyzed in this report

Like ABAs and cytokinins, regulation of auxin homeostasis exists through various mechanisms such as biosynthesis, degradation, transport, and conjugate formation (Fig. 2.4). The major auxin found in plants is indole-3-acetic acid (IAA). IAA can be converted to ester conjugates with sugars, involving UDP-glucose transferase; to

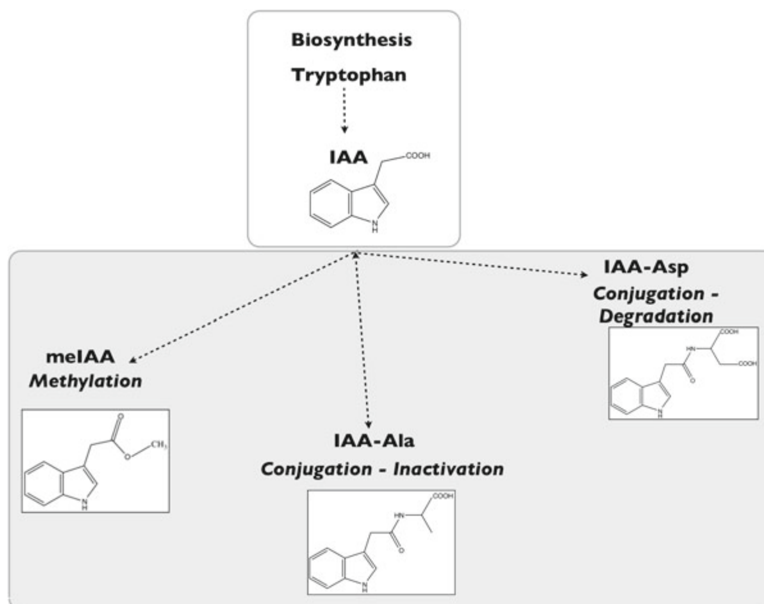

Fig. 2.4 Structures of indole-3-acetic acid (IAA) and three conjugate forms analyzed in this report

amide conjugates with amino acids by IAA amino acid-conjugate synthetases; or to methyl IAA ester by IAA carboxyl methyltransferase. Only few of them including IAA-Ala, IAA-Leu, and IAA-Phe may be hydrolyzed back to free IAA via auxin amino acid-conjugate hydrolases [22]. On the other hand, amino acid conjugates such as IAA-Asp and IAA-Glu are regarded as precursors for the degradation pathway [22]. The conversion of IAA to IAA methyl ester (meIAA) is also known to have a significant effect on auxin homeostasis and plant development [23].

2.2.2 Dynamics of Auxin, Cytokinin, and ABA During Berry Development

In grape, the first phase of berry development, also named berry formation, is characterized by the accumulation of auxins, cytokinins, and GAs that promote cell division and cell expansion [4, 24–26]. Subsequently, ABA accumulates at the onset of the ripening stage, referred to as *véraison*, and controls the accumulation of flavonoids (anthocyanins, flavonols) through the regulation of gene expression and enzyme activity associated with their biosynthetic pathways [5, 27–29]. Recent works suggest the likely role of ethylene in grape berry to promote gene expression of biosynthetic genes involved in ABA biosynthesis [13]. Apart from the control of

the pigment accumulation, there is some evidence that ABA can stimulate the uptake and storage of sugars in berries via regulation of the activity of both soluble and cell wall invertases [30]. The role of ABA in the berry ripening process has been demonstrated through external application of ABA that enhanced anthocyanin accumulation and increased transcript levels of genes in the ABA-biosynthetic pathway [31]. Auxin applications were also shown to delay the onset of ripening [32], and it was suggested to be through delaying the normal *véraison*-associated increase of ABA [5]. In the same way, the synthetic cytokinin, *N*-(2-chloro-4-pyridinyl)-*N′*-phenylurea (CPPU), has been widely used to manipulate berry development. CPPU applied before *véraison* increases berry size but results in reduced total soluble solid levels and anthocyanin accumulation [15]. The accumulation of these three classes of plant growth regulators (auxin, cytokinin, and ABA) in grape berry covers the most dramatic physiological and biochemical changes occurring during berry formation and the onset of berry ripening and seems to act in a coordinated manner to control different aspects of berry development. In this context, as it is crucial to define their relative levels at a given developmental stage, we focused our efforts to optimize an analytical method to quantify them together using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) mode. Because the biological activity of these compounds is under the influence of a subtle homeostatic balance involving biosynthesis, inactivation, and catabolism [13, 21, 33], we also included in the analysis some derived compounds of these plant growth regulators involved in their inactivation and degradation.

2.3 Method for Extraction and Quantification of ABAs, Auxins, and Cytokinins from Grape Berry Samples

As most hormones are biologically active at very low concentrations (from pg/g to ng/g of dry weight), analytical methods have to be extremely sensitive to detect these compounds in a given tissue and developmental stage. For decades, analytical measurements of auxins, cytokinins, and ABAs in grape berry relied on gas chromatography, spectroscopy, or immunoassay methods. While these methods resulted in substantial findings, they are subjected to technical limitations due to less instrumental sensitivity [4, 11]. Moreover, most of the data acquisitions were obtained separately for each hormone making any comparative analysis to understand the crosstalk between these hormones difficult [6, 10, 11, 24, 33]. Nowadays, high or ultra performance liquid chromatography (HPLC or UPLC) coupled to a tandem mass spectrometer operated in the MRM mode has become the method of choice for the analysis of plant hormones because of its high sensitivity, selectivity, and high accuracy [34, 35]. The high degree of versatility of such instrumentation also enables the analysis of many classes of hormones in a single run, which makes this technique very attractive [36]. Detection of several classes of plant growth regulators requires a multistep protocol of purification that enables to isolate every family of plant hormones through the use of differential solvents and phase extraction methods.

Solid-phase extraction (SPE) using C_{18}-bound silica has been commonly used for pre-purification of cytokinins, ABAs, and auxins [36–39]. The present method to analyze these three hormone classes in grape berry tissues has been adapted from extraction methods used by Chiwocha et al. [35] in lettuce seeds and Kojima et al. [37] in rice. Grapes, like most fruits, contain various amounts of interfering compounds including sugars, tannins, and anthocyanins, which make them a challenging experimental material. Skin samples from late stages of berry development contain high levels of pigments (anthocyanin-derived compounds) that are highly cationic in acidic conditions and may affect the recovery of these related compounds [40].

2.3.1 Extraction of ABAs, Auxins, and Cytokinins

2.3.1.1 Chemicals and Grape Berry Samples

Abscisic acid [(+/−) ABA] was purchased from Sigma-Aldrich, Oakville, ON, Canada. Other labeled and unlabeled ABA metabolites (+)-ABA-GE, d_5-ABA-GE, d_4-ABA, d_6-ABA, (−)-DPA (dihydrophaseic acid), d_3-DPA, (−)-PA (phaseic acid), d_3-PA, (−)-neoPA (neophaseic acid), d_3-neoPA, (+/−)-7′-OH-ABA, and d_4-7′-OH-ABA were obtained from NRC-PBI, Saskatchewan, Canada. Auxin and cytokinin metabolites IAA (indole-3-acetic acid), d_5-IAA, IAA-Ala (IAA-alanine), IAA-Asp (IAA-aspartate), meIAA (IAA-methyl ester), tZ (*trans*-zeatin), d_5-tZ, cZ (*cis*-zeatin), tZR (*trans*-zeatin riboside), d_5-*t*ZR, cZR (*cis*-zeatin riboside), DZ (dihydrozeatin), d_3-DZ, DZR (dihydrozeatin riboside), d_3-DZR, iP (N^6-isopentenyladenine), d_6-iP, iPR (N^6-isopentenyladenosine), and d_6-iPR were purchased from OlChemIm Ltd, Olomouc, Czech Republic.

Pinot Noir berries were sampled to represent different developmental stages. Whole berry samples were used for the set of berries representing berry formation stages (five pre-*véraison* stages: 75, 75–77, 77, 77–79, and 79). Another set of berries, representing *véraison* stage 83 and a green stage that falls between 77 and 77–79, was separated into skin, pulp, and seed tissues to estimate the proportion of the hormones in the individual berry tissues.

2.3.1.2 Solid-Phase Extraction

Extractions were performed using either whole berries or the three separate berry tissues from six different developmental stages (developmental stages highlighted in purple and pink in Fig. 2.1). Five replicate samples, containing at least 25 berries each, for each developmental stage were collected from separate vines. Fifty milligrams of homogenized and lyophilized tissues were extracted in 3 ml of extraction solvent (methanol:formic acid:water, 15:1:4). The internal standard solution, containing 20 ng of the deuterated version of each analyte (Table 2.1), was added to the extraction buffer. The homogenate was extracted at 4°C on an orbital shaker for

Table 2.1 MRM transitions and conditions used on the hybrid triple quadrupole/linear ion trap 4000 QTRAP LC-MS/MS

Analyte	Mode	Precursor m/z	Product m/z	DP	CE	EXP	RT
DPA	–	281	171	–75	–24	–13	14.25
d_3-DPA	–	284	174	–	–	–	14.21
ABA-GE	–	425	263	–80	–18	–9	16.32
d_5-ABA-GE	–	430	268	–	–	–	16.27
PA	–	279	139	–75	–18	–9	16.90
d_3-PA	–	282	142	–	–	–	16.89
7′-OH-ABA	–	279	151	–60	–22	–10	17.18
d_4-7′OH-ABA	–	283	154	–	–	–	17.16
neoPA	–	297	205	–75	–18	–11	17.17
d_3-neoPA	–	282	208	–	–	–	17.69
ABA	–	263	153	–65	–18	–13	18.46
d_4-ABA	–	267	156	–	–	–	18.43
d_6-ABA	–	269	159	–60	–18	–10	18.41
IAA	+	176	130	56	27	9	17.86
d_5-IAA	+	181	135	56	27	9	17.87
IAA-Ala	+	247	130	46	31	8	17.01
IAA-Asp	+	291	130	46	39	9	15.1
meIAA	+	190	130	46	39	6	20.72
*t*Z	+	220	136	56	23	12	8.87
d_5-*t*Z	+	225	–	–	–	–	8.76
*c*Z	+	220	136	56	23	12	9.44
*t*ZR	+	352	220	56	25	10	12.53
d_5*t*ZR	+	357	136	56	23	10	12.33
*c*ZR	+	352	220	56	25	10	13.17
iP	+	204	136	46	31	8	14.46
d_6-iP	+	210	137	46	31	8	14.43
iPR	+	336	204	56	43	8	16.02
d_6-iPR	+	342	210	56	43	8	15.92
DZ	+	222	136	56	23	9	9.2
d_3-DZ	+	225	136	56	23	10	9.13
DZR	+	354	136	66	60	9	12.52
d_3-DZR	+	357	136	56	23	10	9.13

DP declustering potential, *CE* collision energy, *EXP* collision exit potential, *RT* retention time (min)

20 h. After centrifugation at 4,415 × *g* for 15 min, supernatant was transferred to a fresh tube, and the pellet was re-extracted with 0.5-ml additional extraction solvent and then combined with the first supernatant (Fig. 2.5). To remove interfering compounds, the extract was first passed through Oasis HLB 60-mg cartridges (Waters) that were pre-equilibrated with acidified methanol (methanol:formic acid, 99:1) and acidified water (water:formic acid, 99:1) using a vacuum manifold (Visiprep DL, Supelco, USA). The eluate was evaporated overnight using a vacuum concentrator (Vacutron, VT100, Savant, USA), and the dried pellet was reconstituted with 2 ml of 1 M formic acid. This fraction was passed through Oasis MCX 60-mg cartridges (Waters, USA) pre-equilibrated with 1 M formic acid. Columns were again washed with 1 M formic acid, and then ABA and auxin analytes were eluted with 100%

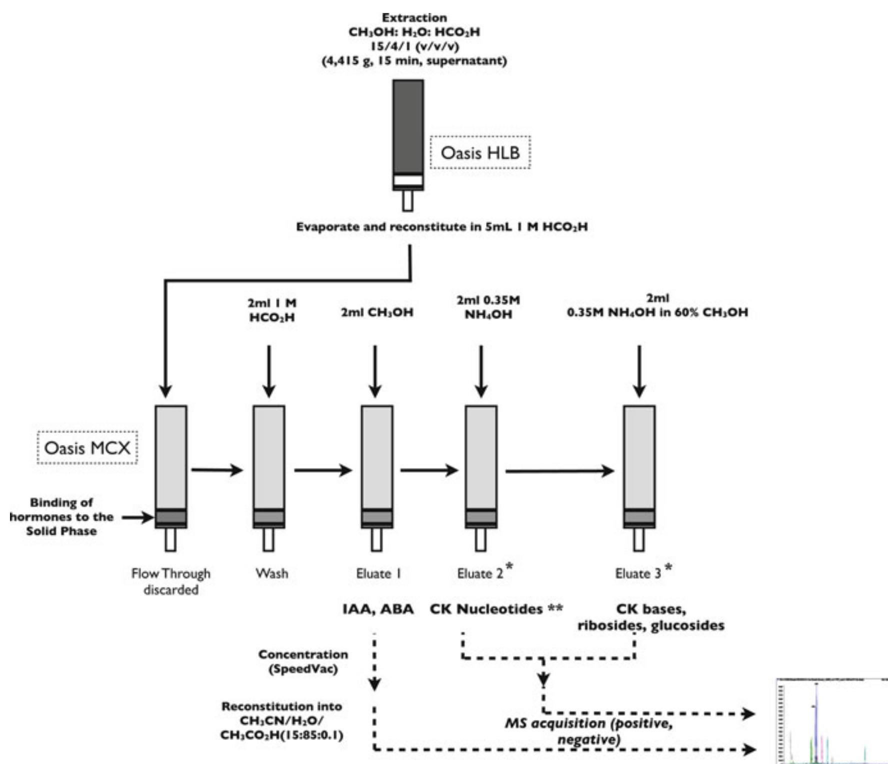

Fig. 2.5 Extraction and purification protocol for cytokinins (CK), auxins (IAA), and abscisic acid (ABA) in grape berry samples. *For late stage skin samples (stage 83), pigments may co-elute with CKs. **CK nucleotides were extracted but not quantified

methanol (eluate 1). Cytokinin nucleotides were eluted with 0.35 M ammonium hydroxide (eluate 2), and cytokinin nucleobases and glucosides were eluted with 0.35 M ammonium hydroxide in 60% methanol (eluate 3). All three fractions were combined, evaporated overnight, and reconstituted with 200 μl of reconstitution solution (acetonitrile:water:formic acid, 15:85:0.1, v/v/v) for hormone analysis.

2.3.2 Analytical Method Using LC-Tandem Mass Spectrometry in Multiple Reaction Monitoring Mode (LC-MRM)

2.3.2.1 Chromatographic Separation

Gradient conditions were based on those optimized by Chiwocha et al. (2003) [35] with slight modifications (Table 2.2). Chromatography separation was carried out using an Agilent Zorbax Extend-C_{18} column (2.1 × 150 mm; 5 μm). A binary gradient was used: solvent A was LC-MS-grade acetonitrile (J.T. Baker, Phillipsburg, NJ)

Table 2.2 HPLC gradient conditions used on the Shimadzu Prominence LC-10 AD ternary system

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Flow (ml/min)
0	2	97.9	0.1	0.2
2	2	97.9	0.1	0.2
10	10	89.9	0.1	0.2
20	60	39.9	0.1	0.2
22	90	9.9	0.1	0.2
25	90	9.9	0.1	0.2
25.5	2	97.9	0.1	0.2
40	2	97.9	0.1	0.2

Solvent A = acetonitrile, solvent B = LC-MS-grade water, and solvent C = 1% formic acid

containing 0.1% formic acid, and solvent B was HPLC-grade water (OmniSolv) containing 0.1% formic acid. The gradient elution was as follows: 2 min 2% A, 10 min 10% A, 20 min 60% A, 22 min 90% A, 25 min 90% A, and 25.5 min 2% A. The initial conditions were restored and allowed to equilibrate over the next 14.5 min, resulting in a total gradient program of 40-min duration.

2.3.2.2 LC-MS/MS Analysis and Quantification

The hormone analysis was performed on a hybrid triple quadrupole/linear ion trap 4000 QTRAP LC-MS/MS instrument equipped with a Turbo V source (Applied Biosystems, USA). Mass spectra for ABA-derived compounds were acquired in the negative mode, while the mass spectra for cytokinin- and auxin-derived compounds were acquired in the positive mode. Electrospray ionization, MRM transitions, and collision-induced dissociation (CID) conditions used on the 4000 QTRAP are described in Table 2.1.

Calibration curves were generated using eight concentrations of each analyte in 1 pg/μl to 5 ng/μl range and a constant 20 ng of corresponding deuterated internal standard. For all IAA family analytes, d_5-IAA was used as the internal standard for quantification. Likewise, for the *cis* and *trans* forms of zeatin and zeatin ribosides, d_5-*trans*-zeatin and d_5-*trans*-zeatin riboside, respectively, were used as internal standards. The standard solutions for calibration curves were processed through the same extraction protocol as described for berry samples, and 10 μl of each standard sample was analyzed by LC-MS/MS as described above. Calibration curves were generated from triplicate analysis of each standard sample using Analyst software version 1.5.1 (Applied Biosystems, USA). The concentration of the target analyte was calculated against these calibration curves, and the amount of each analyte in the sample was calculated per gram dry weight of the tissue. To determine the suppression effect of grape tissue matrix during the analysis, same concentrations of pure standards alone and with berry tissue were analyzed, and peak areas in both analyses were compared (Table 2.3). No matrix effect was observed except for cytokinin-derived compounds for which up to 20% ion suppression was observed.

Table 2.3 Ion suppression effect of berry tissue matrix

	Peak area (×10^5)	
Analyte	Matrix-free	Matrix
ABA	15	17
ABA-GE	5.8	6.1
DPA	3.9	2.9
PA	3.6	3.0
neoPA	5.1	4.7
7′-OH-ABA	1.7	1.3
IAA	0.35	0.44
IAA-Ala	9.3	14
IAA-Asp	4.1	3.0
meIAA	8.2	14
*t*Z	23	24
*c*Z	42	35
*t*ZR	36	38
*c*ZR	82	66
DZ	4.1	3.1
DZR	21	17
iP	73	58
iPR	78	58

Peak areas were compared from samples containing 1 μM of pure standards, and berry sample spiked with 1-μM standards. The concentration of spiked standards is several fold higher compared to endogenous levels of corresponding analytes

2.4 Analyte Levels in Berry Tissues

Two sets of berry samples (*Vitis vinifera* cv. Pinot Noir) were used to quantify several hormone-derived compounds of ABA, auxin, and cytokinin metabolism (Figs. 2.6, 2.7, 2.8). The first set of samples (Figs. 2.6a, 2.7a, 2.8a) consisted of whole berries from five early developmental time points (BBCH-scale, 75–79) encompassing berry formation phase [35]. The second set corresponds to berry tissue samples from *véraison* stage (seed, skin, and pulp) (BBCH-scale, 83). To compare the tissue-specific levels of the hormones of ripe and unripe berries, berries from a time point just after stage 77 were also analyzed at the tissue-specific level (Figs. 2.6b, 2.7b, 2.8b).

Six ABA-derived compounds associated with biosynthetic and degradation pathways were measured including the biologically active form (ABA), one conjugated form (ABA-GE), and four compounds of the catabolic oxidation pathway (phaseic acid, dihydrophaseic acid, neophaseic acid, and 7′-OH-ABA) (Fig. 2.6a). Recovery using this extraction protocol was estimated at 89% for ABA using d_6-ABA as

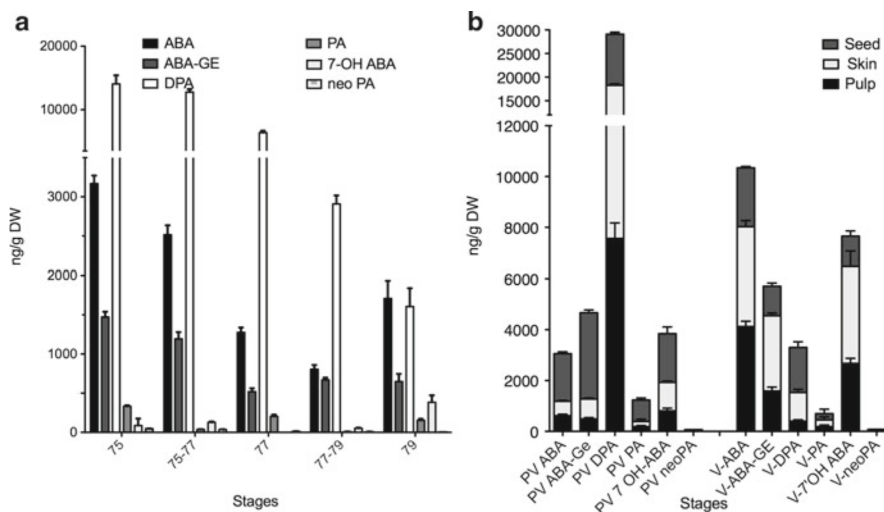

Fig. 2.6 Changes in ABA-related compounds through berry development: (**a**) Across phenological stages (BBCH-scales 75, 77, 79) and two intermediate stages (between 75–77 and 77–79). (**b**) Comparison of tissue-specific accumulation at pre-*véraison* stage (between 77 and 77–79) and *véraison* stage (BBCH 83). *PV* pre-*véraison*, *V véraison*

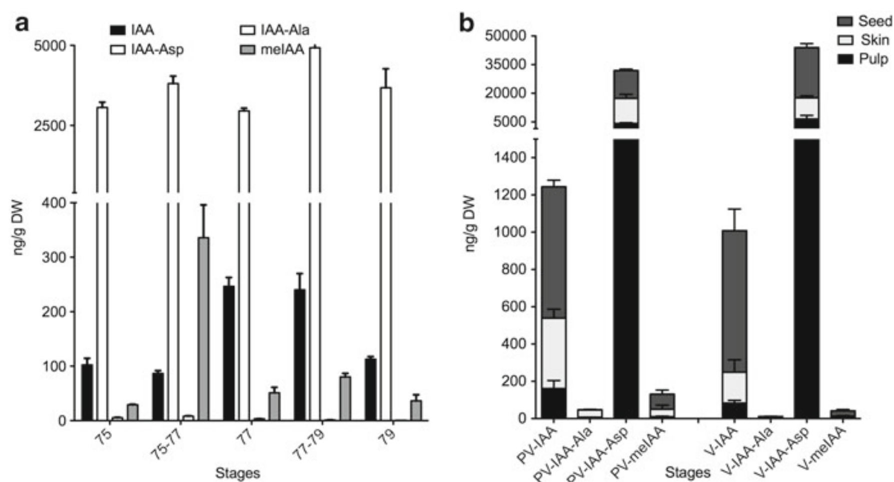

Fig. 2.7 Changes in auxin-related compounds through berry development: (**a**) Across phenological stages (BBCH-scales 75, 77, 79) and two intermediate stages (between 75–77 and 77–79). (**b**) Comparison of tissue-specific accumulation at pre-*véraison* stage (between 77 and 77–79) and *véraison* stage (BBCH 83). *PV* pre-*véraison*, *V véraison*

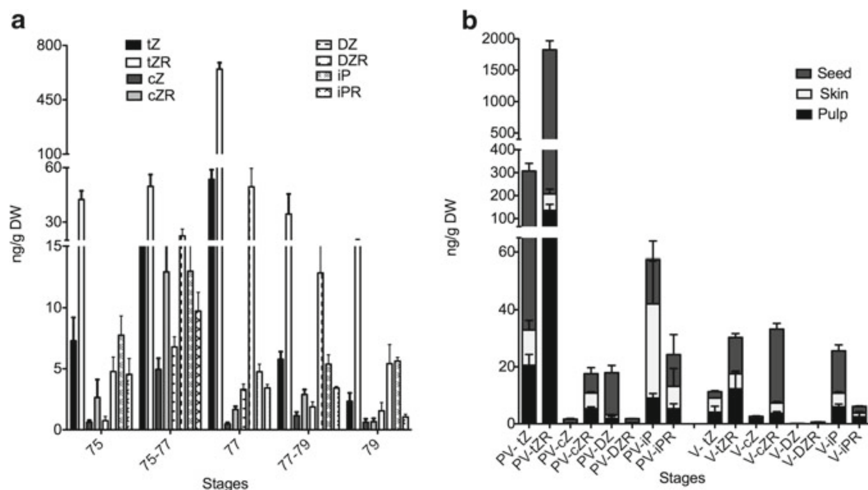

Fig. 2.8 Changes in cytokinin-related compounds through berry development: (**a**) Across phenological stages (BBCH-scales 75, 77, 79) and two intermediate stages (between 75–77 and 77–79). (**b**) Comparison of tissue-specific accumulation at pre-*véraison* stage (between 77 and 77–79) and *véraison* stage (BBCH 83). *PV* pre-*véraison*, *V véraison*

external standard. Concentrations measured in our samples are consistent with recent works reported in whole berries as well as from reports of tissue levels at the corresponding developmental stages [9, 10, 24]. Previous studies covering the complete series of developmental stages showed that ABA level is high early in berry development, after which it decreases to be low just prior to *véraison*. It increases again at the initiation of sugar and pigment accumulation in red wine grapes (approaching *véraison*) and reaches a peak two to three weeks later, after which time it declines again as the fruit approach ripeness [5, 10, 24]. In the present study, ABA showed a gradual decrease from early pre-*véraison* stages (75 to 77–79) and then started to increase, reaching 1.7 μg/g at the end of pre-*véraison* stages (Fig. 2.6a). The tissue-level analysis at *véraison* (stage 83) showed further increase of ABA (Fig. 2.6b). In Pinot Noir as pulp, skin, and seed contribute about 85%, 10%, and 5% of the berry weight, respectively, at *véraison* [41], in terms of whole berry, ABA level at *véraison* is estimated to be above 5.0 μg/g. The levels and accumulation trends of ABA and ABA-GE reported by Deluc et al. (2009) in Cabernet Sauvignon from stage 77 to 83 were similar to that of our observation in this study [9]. There are limited hormone studies done at the berry tissue level. In one recent study, accumulations of ABA metabolites in Merlot berries at *véraison* were found substantially different between skin, pulp, and seed, which were similar to our data (Fig. 2.6b) in terms of their proportions among the tissues and quantities [10]. Comparison of ABA and ABA-GE distributions among tissues in pre-*véraison* and *véraison* berries shows that seed maintains about threefold more ABA than skin

and pulp during the early stages, but at *véraison*, the pericarp tissues become the main site of accumulation with twofold more ABA than that of seed (Fig. 2.6b). Zhang et al. (2003) found a similar ABA accumulation shift between seed and pericarp tissues from early to *véraison* stages [24]. DPA, which is an ABA degradation product (through 8′-hydroxylation pathway), decreased several fold in *véraison* berries compared to pre-*véraison*, and the decrease was more pronounced in skin and pulp than in seed. Considering the role of ABA in berry ripening, the contribution of the catabolic pathway in its homeostasis at *véraison* is mostly explained by a reduction of the ABA 8′-hydroxylation pathway, which is found to be more pronounced in skin and pulp, where most changes associated with ripening occur. 7′-OH-ABA, which is at the minimum level during berry formation stages (Fig. 2.6a), showed higher levels at *véraison* suggesting that the degradation pathway of ABA shortly resumes at *véraison* stage, mainly through the 7′-hydroxylation pathway (Fig. 2.6b). The levels observed for neoPA were very low during early as well as *véraison* stages. Taken together, ABA homeostasis not only depends upon the biosynthetic pathway but also the branches of the catabolic pathway appear to play a critical role in regulating the active form of ABA. In addition, among these different branches, the 8′-hydroxylation pathway seems to be the main regulatory mechanism during pre-*véraison*, whereas 7′-hydroxylation pathway becomes the main pathway for ABA catabolism as the berry development progresses to *véraison* (Fig. 2.6a, b).

The majority of reports describe a steady decline of IAA from the berry formation to *véraison* [5, 26]. Though there was a general trend of IAA decrease from pre-*véraison* to *véraison* (Fig. 2.7a, b), we observed a clear peak in IAA level between stages 75 and 79 before declining towards *véraison* (Fig. 2.7a). This trend during the berry formation phase was also reported in Merlot cultivar by Deytieux-Belleau et al. (2007) [42]. We found the concentrations of all auxin compounds to be higher in seed tissue, especially IAA, which was tenfold higher at *véraison* (Fig. 2.7a, b). IAA-Asp was found in high levels (from 3.0 mg/g DW at stage 75 to about 10 mg/g DW at *véraison*). These results are consistent with the literature that suggests a predominant degradation pathway of IAA at *véraison* through IAA-Asp, which is a precursor of the catabolic pathway [22, 26]. IAA-Ala, known to be a storage form of IAA [43], was at very low levels during the early and *véraison* stages in the three tissues suggesting that conjugation of IAA into this form did not contribute to IAA homeostasis. Finally, meIAA accumulated in the same manner as IAA during the early stages (77–79), but its level was negligible at *véraison* (less than 4 ng/g DW). As meIAA is known to play a similar role than IAA in plants, its pattern of accumulation, high during the cell expansion phase and low at *véraison*, may suggest a likely role in complementing IAA activities in grape berry development as previously described in Arabidopsis [23].

Studies of cytokinin accumulation during grape berry development are scarce in the literature. To the best of our knowledge, the only report describing cytokinin accumulation in grape berry showed higher concentrations of zeatin and zeatin riboside during the early phase of grape berry development followed by a steady decrease to be low around *véraison* [23]. Our data showed the levels of *trans*-zeatin and

trans-zeatin riboside peak (from 53 to 647 ng/g DW, respectively) at the middle of berry formation phase (stage 77) and then decrease later on up to *véraison* stage (Fig. 2.8a, b). Levels of *trans*-zeatin and *trans*-zeatin riboside at pre-*véraison* are more than ten times higher in seed compared to pericarp tissues, which is consistent with Zhang et al. (2003) study. These high *trans*-zeatin and *trans*-zeatin riboside levels at pre-*véraison* in seed tissue could be associated with the control of embryo and endosperm development during that developmental stage as proposed in plants [44]. As in the case of auxin, tissue-specific accumulation patterns were found at stage 83 (from 12, 20, and 274 ng/g DW of *trans*-zeatin to 71, 135, and 1,650 ng/g DW of *trans*-zeatin riboside in skin, pulp, and seed, respectively, at pre-*véraison*) (Fig. 2.8b). The concentrations of the analyzed compounds in this tissue material ranged from 50 to 31 pg/g DW. Overall, trends of these compounds, seen through the developmental stages, conform to the perceived roles of these hormones [5, 32] and are in agreement with the published data derived from individual analytical methods [9, 10].

2.5 Concluding Remarks

We adapted an analytical method to quantify three classes of hormones in grape berries from the same extract. The solid-phase extraction method proves efficient in eliminating most of the interfering compounds and suppression of signal intensity during mass spectral analysis due to the complexity of grape tissue matrix. Observed levels of analytes were similar to levels reported by previous works. Using the described method, plant hormone levels were determined, which ranged from 5 ng/g to 12 mg/g for ABA-related compounds, 0.9 ng/g to 38 mg/g for IAA-related compounds, and 0.2 ng/g to 16 mg/g for cytokinin-related compounds. During the simultaneous observation of functionally interrelated hormones, we observed a concomitant increase of cytokinin and auxin levels in the middle of the berry formation phase (stage 77) and a shift in the ABA catabolic pathway from 8′ to 7′-hydroxylation pathway from pre-*véraison* to *véraison* stages, which were not previously reported. In addition, hormone data from stage 83 suggest that onset of ripening is governed by tissue-specific accumulation of hormones. Future integration of other growth regulators in the method such as gibberellin-related compounds, along with development of analytical methods for brassinosteroids and jasmonic and salicylic acids, will mainly complement and assist in the interpretation of increasingly accumulating transcriptomic data associated with berry development [45].

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Chapter 3
Endophyte Mycotoxins in Animal Health

Jennifer M. Duringer, Lia Murty, and A. Morrie Craig

Abstract Fescue toxicosis and perennial ryegrass staggers are two of the most common toxic plant diseases plaguing livestock in the United States, and result from consumption of forage containing the endophyte-produced mycotoxins ergovaline and lysergic acid (fescue toxicosis) and lolitrem B (ryegrass staggers). Our group has developed analytical assays for detecting these compounds, which serve a dual purpose (1) high-performance liquid chromatography-fluorescence assays are used to measure these compounds in feed material in order to promote "safe feed" through diagnostic testing in a service laboratory environment, and (2) highly sensitive and specific liquid chromatography-tandem mass spectrometry assays are utilized to study the fate and metabolism of these compounds in a diversity of livestock matrices so that a more refined understanding as to the etiology of the diseases these compounds cause can be achieved. A discussion applying these techniques to both current and anticipated studies is given, with an emphasis on impacts to trade and food safety regulation.

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Abbreviations

HPLC	High-performance liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
SPE	Solid phase extraction
DCM	Dichloromethane
ACN	Acetonitrile
LOD	Limit of detection
LOQ	Limit of quantitation
ELISA	Enzyme-linked immunosorbent assay
ESI(+)	Electrospray ionization in the positive ion mode
MRM	Multiple reaction monitoring
APCI(+)	Positive atmospheric pressure chemical ionization
ppb	Parts per billion

3.1 Introduction

Plant toxins are chemical defenses which likely evolved to combat herbivore predation. As such, toxic plants are responsible for a portion of the morbidity and mortality affecting profitability in livestock production, the amount of which varies by region, toxic plant exposure, and management practices. Fescue toxicosis and perennial ryegrass staggers are two of the most common toxic plant diseases plaguing livestock in the United States, and result from consumption of forage containing endophyte-produced mycotoxins. Endophyte-infected pasture and hay are nutritious food resources (6–17% protein) for ruminants and pseudoruminants and can make up a significant portion of a herd's dietary regimen. For example, tall fescue (*Festuca arundinacea*) is the most widely grown pasture grass in humid areas of the southeastern and, to a lesser extent, the northwestern United States, with greater than 140,000 km^2 in production [1]. Perennial ryegrass (*Lolium perenne*) is a valuable pasture grass in temperate regions of the world, including the northwestern United States, Australia, and New Zealand. Both grasses are also fed as hay as a component of winter rations when adequate pasture becomes unavailable. Endophytic fungi have been deliberately promoted in both tall fescue and perennial ryegrass (*Neotyphodium coenophialum* and *N. lolii*, respectively) in order to combat insect predation and to produce more vigorous, drought-resistant plants. Some endophyte strains exert these benefits through the production of ergot and lolitrem alkaloids (Fig. 3.1), which, unfortunately, also cause deleterious effects in cattle and other herbivore species when endophyte-infected grasses are grazed or fed as hay [2]. (Other endophyte strains exist which are able to transfer the beneficial characteristics of this fungal–grass symbiosis without producing the ergot and lolitrem alkaloids that are detrimental to animal health [3, 4]. As development and release of

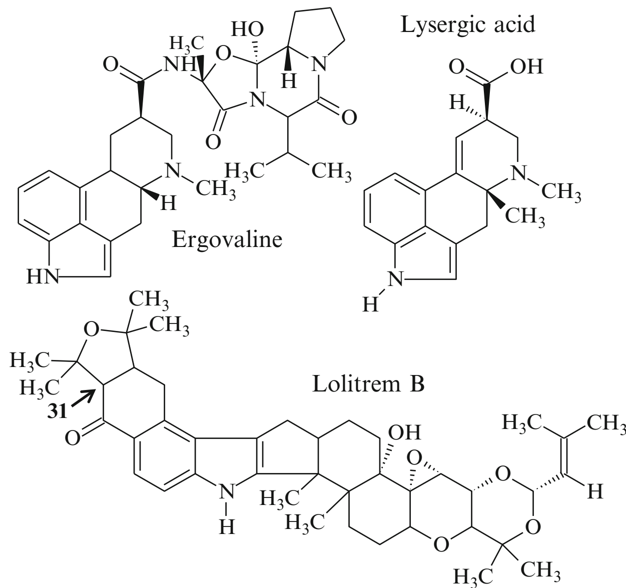

Fig. 3.1 Prominent ergot and lolitrem alkaloids found in endophyte-infected tall fescue and perennial ryegrass that are detrimental to animal health

these “novel” endophytes has taken place relatively recently, however, the economic impact of stock improvement versus cost of pasture replacement must be weighed by cattle managers to determine if this is a feasible option [5].)

It is estimated that the toxicological effects of ergot and lolitrem alkaloids cost between $0.5 billion and $1 billion in livestock losses annually in the United States alone [6], which have been categorized into three main diseases affecting animal health, namely, fescue foot, summer syndrome, and ryegrass staggers [2, 7]. “Fescue foot” typically occurs in cold environments, where tall fescue hay is fed as a significant portion of the diet, and is the result of the vasoconstrictive action of ergot alkaloids on blood vessels [8]. The alkaloids cause decreased blood flow to the extremities (hooves, ears, and tail), which become gangrenous, eventually resulting in euthanasia of the animal before it has reached full market potential. “Summer syndrome” is usually seen during hot, humid summer months when animals consume forage containing ergot alkaloids whose vasoconstrictive properties result in an inability of the animal to properly cool itself. This causes the animal to seek the coolness of shade and water, thereby spending less time grazing. Clinical signs observed in summer syndrome include reduced average daily gain, intolerance to heat, excessive salivation, rough hair coat, elevated body temperature, nervousness, lower milk production, and reduced conception rate [9]. “Ryegrass staggers” is a condition in which animals grazing on endophyte-infected perennial ryegrass pastures containing lolitrem B develop stilted gait, ataxia, tremors, and hypersensitivity

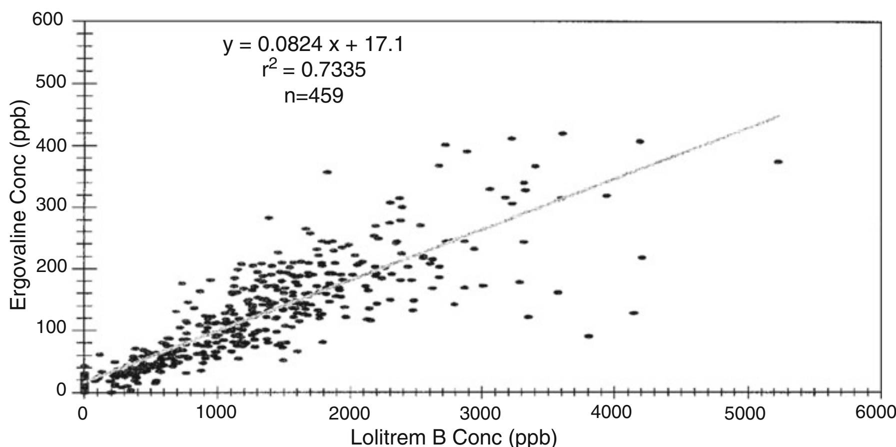

Fig. 3.2 Correlation between ergovaline and lolitrem B concentration in 459 perennial ryegrass samples (Adapted from Hovermale et al., 2001)

to external stimuli [10, 11]. Lolitrems have a long duration of action, yet their neurotoxic effects are completely reversible (Gallagher 1986); as such, livestock affected with ryegrass staggers regain normal muscle response within 3–4 days after being removed from infected feed and appear otherwise unaffected.

In order to better understand how endophyte-infected feed causes these maladies in food animals, three compounds have been identified for both monitoring in forage to promote safe feed and following in feeding studies so that the ultimate fate and disposition of these compounds can be realized. In tall fescue infected with ergot alkaloid-producing endophytes, ergovaline (Fig. 3.1) is the ergot alkaloid found in highest abundance [12, 13] and appears to be the most potent vasoconstrictor of the ergopeptides and ergolines tested to date [8, 14, 15]. Thus, it is the ergot alkaloid most often linked to fescue toxicosis (fescue foot and summer syndrome). In perennial ryegrass, lolitrem B (Fig. 3.1) is the main neurotoxin associated with ryegrass staggers [16] and acts by inhibiting large-conductance calcium-activated potassium channels [17], producing a characteristic tremoring response. For these reasons, ergovaline and lolitrem B were selected as the target toxins for establishing dietary threshold of toxicity recommendations in food animals [18] and for safe feed certification by service laboratories [19, 20]. Ergovaline is not the only ergot alkaloid present in tall fescue [12, 21], however, an examination as to the putative toxin(s) responsible for causing the maladies associated with fescue toxicosis is ongoing, with a particular focus on the breakdown product lysergic acid (Fig. 3.1) [22, 23]. Likewise, lolitrem B is one of the many end products in a complex indole-diterpene biosynthesis pathway which yields other structurally similar compounds in the plant [3]. In truth, grazing animals are potentially exposed to a myriad of alkaloids upon ingestion of endophyte-infected forage (e.g., Fig. 3.2). Future studies and discussions should therefore be modeled on exposure scenarios that more closely mimic these natural feeding conditions.

To this end, our group has developed analytical assays for detecting ergovaline, lysergic acid, and lolitrem B, which serve a dual purpose: (1) high-throughput, high-performance liquid chromatography (HPLC)-fluorescence assays are used to measure these compounds in feed material in order to promote "safe feed" through diagnostic testing in a service laboratory environment, and (2) highly sensitive and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays are utilized to study the fate and metabolism of these compounds in a diversity of livestock matrices so that a more refined understanding as to the etiology of the diseases these compounds cause can be achieved. Better definition of compartmental values will also help optimize regulation of these compounds in animal by-products; thus, the service and research pursuits both feed off and support each other.

3.2 Analytical Procedures

3.2.1 Extraction of Endophyte Mycotoxins from Plant Material

3.2.1.1 Ergovaline

We have developed a method for extraction of ergot alkaloids from plant material based on previous studies [24–26], for subsequent analysis by HPLC-fluorescence or LC-MS/MS. Seed and straw samples are ground in a Cyclotec 1093 sample mill and passed through a 0.5-mm screen. One gram of the ground plant material is weighed into a glass screw-top tube. To each tube of sample, control, or reference material (as neat standard is expensive and difficult to synthesize, ground seed or straw is mixed in large batches at four target concentrations to generate material for use in a standard curve which is validated using >98% pure ergovaline (Forrest Smith, Auburn University)), 10 mL of chloroform plus 1 mL internal standard (1 μg/mL ergotamine in chloroform) and 1 mL of 0.001 N NaOH are added to deprotonate ergot alkaloids that may have been protonated in acidic conditions. The tubes are capped and mixed for 18–24 h in the dark, then centrifuged at 1,700 × *g*. Five milliliters of organic supernatant from the centrifugation step is applied to 500 mg/6 mL solid phase extraction (SPE) columns (United Chemical Technologies, Bristol, PA) containing Ergosil® (Analtech, Newark, DE) and anhydrous sodium sulfate (EMD Chemicals, Darmstadt, Germany). Ergovaline is extracted by conditioning with chloroform, followed by a 3:1 chloroform:acetone (v/v) wash and elution with 2.5 mL methanol. The eluent is dried under nitrogen at 50°C, then reconstituted in 0.5 mL methanol. Samples are mixed for 10 s, sonicated for 10 s, and centrifuged at 913 × *g* for 5 min. Samples are transferred to amber HPLC vials and sealed for analysis. The percent recovery for this method is 91% for seed and plant material. Inter-assay and intra-assay variations are 5.7 and 3.7%, respectively [27].

3.2.1.2 Lysergic Acid

In addition to ergovaline, lysergic acid (Fig. 3.1) has been proposed as a causative agent in fescue toxicosis [22] and is present in tall fescue [28–30]. Lysergic acid is extracted from plant material by weighing 1 g of sample (dried, ground to 0.5-mm particle size) into glass screw cap tubes, to which 10 mL of 1:1 water:acetonitrile (v/v) is added and rotated for 16 h in the dark at room temperature. Samples are subsequently centrifuged for 10 min at 2,000 × *g*; then 5 mL of the liquid layer are adjusted to pH 5.0–5.5 with 10% acetic acid. Cation-exchange SPE cartridges (Discovery DSC-SCX, Sigma-Aldrich, St. Louis, MO) are preconditioned with 3 mL of methanol, followed by 3 mL of 0.1-M HCl and two 3-mL portions of pure water. Acidified extracts are loaded onto the preconditioned columns, and columns are washed with duplicate 3-mL aliquots of water. Lysergic acid is eluted with 3 mL of 95:5 methanol:ammonium hydroxide (v/v), the solvent removed using a centrifugal evaporator at room temperature, and the dried residue reconstituted in 200 μL of 50:50 methanol:0.05-M phosphate (pH 9.5) (v/v). Sample extracts are then sonicated for 30 s and transferred to a centrifuge filter and centrifuged at 10,000 × *g* for 5 min. Filtrates are transferred to HPLC vials and sealed for analysis. This extraction method resulted in recoveries of 64% and 79% for 30- and 150-ng/g spikes into endophyte-free seed, respectively, and recoveries of 61 and 77% for 30- and 150-ng/g spikes into endophyte-free straw, respectively [29].

3.2.1.3 Lolitrem B

Initially, lolitrem B was extracted and further purified using column chromatography with silica gel, reversed-phase thin-layer chromatography, and HPLC [16]. The first large-scale isolation used ground perennial ryegrass seed and solvent extraction with petroleum ether, then purification with liquid-liquid partitioning and flash chromatography [31]. Another method for the purpose of lolitrem B quantitation involves solvent extraction and filtering before HPLC analysis [27]. Plant material is ground as described above for the ergovaline and lysergic acid extractions. Three milliliters of a 2:1 chloroform:methanol (v/v) mixture is added to 0.2 g of sample, control, or reference material, capped and rotated for 18–24 h in the dark. (Due to the same cost and availability circumstances described above for the ergovaline standard, our lab uses straw or seed reference material mixed in large batches at four concentrations that is validated using highly purified lolitrem B (AgResearch, Ltd, New Zealand) to establish a calibration curve.) Next, the samples are centrifuged at 2,000 × *g* for 10 min, and 1.6 mL of supernatant is pulled off and dried under nitrogen at ambient temperature. One milliliter of dichloromethane (DCM) is added to the evaporated supernatant, capped and sonicated for 10 s, followed by mixing for 10 s. An additional 1 mL of DCM is added, and the sample is again sonicated and vortexed for 20 s to ensure the entire sample is dissolved. CUSIL 500-mg/6-mL SPE cartridges (United Chemical Technologies, Bristol, PA) are loaded onto a positive pressure manifold and preconditioned with

2 mL DCM. The samples are loaded onto the SPE, followed by a 2-mL DCM wash. A 0.5-mL wash of elution solution (4:1 DCM:acetonitrile (ACN) (v/v)) is added to the cartridges, and positive pressure is applied after dripping is no longer observed. The sample is then eluted with 3.0 mL of elution solution, and the eluent is collected in glass culture tubes. The SPE columns are allowed to stop dripping and then dried to force remaining liquid out of the columns. These tubes are capped and mixed, and 1.5 mL is transferred to amber HPLC vials and sealed for analysis by HPLC-fluorescence. The percent recovery for this method is 91.5% for plant material. Inter- and intra-assay variations are 14.3/9.3% and 8.3/5.9% for straw/seed, respectively [27].

3.2.2 Extraction of Endophyte Mycotoxins from Animal Matrices

3.2.2.1 Ergovaline

The best method for extracting ergovaline from animal matrices (blood, feces, urine, and ruminal fluid) involves cleanup with silica-based C18 SPE columns. When extracting for ergovaline, tissue matrices are typically pretreated with a dilute base to ensure optimum recovery, as treatment with strong acids or bases will completely hydrolyze the amide bond. Jaussaud et al. (1998) reported recovery rates from 90 to 102% for ergovaline extracted out of ovine plasma using a sodium hydroxide pretreatment and a liquid–liquid extraction with diethyl oxide. Ergovaline can be extracted from dried feces following the same methodology used for plant material [24, 28]. Extraction of ergovaline from urine and ruminal fluid can be performed by placing it in chloroform buffered with K_3PO_4 and adding ergotamine as an internal standard, then rotating for 5 h in the dark. The supernatant is then added to an SPE-containing Ergosil® and anhydrous sodium sulfate and extracted as described above for plant material. The final ruminal fluid extract is dried under nitrogen and reconstituted in methanol for analysis by HPLC [28].

3.2.2.2 Lysergic Acid

The presence of lysergic acid (Fig. 3.1) in body matrices is a good indicator that the grass animals are consuming contains lysergic acid and/or ergot alkaloids, as the lysergic acid moiety is common to all ergot alkaloids and has been found as a breakdown product in ruminal fluid and urine in feeding studies. For instance, lysergic acid was present in ruminal fluid, urine, and endophyte-infected tall fescue in feeding experiments with cattle [29] and sheep [28] and in urine and endophyte-infected tall fescue fed to horses [30]. Lysergic acid can be extracted by acidifying the matrix, taking it through an SPE (Discovery DSC-SCX, Sigma-Aldrich) extraction procedure involving preconditioning with methanol and 0.1-M HCl, washing with pure water, and elution with a methanol:ammonium hydroxide (95:5 v/v)

solution [29]. Acidifying the matrix before SPE cleanup helps to extract the compound from complex matrices by protonating the carboxylic acid group at pH lower than 3.44 while selecting for deprotonation at pH above 9. (The pK_a of the carboxylic group is 3.44, while the –NH group has a pK_b of 7.68 and is protonated at pH less than 2.) Percent recoveries for high-spiked (150 ng/g) samples of ruminal fluid, urine, and feces were 81, 88, and 87%, respectively. For a low spike of 30 ng/g, the percent recoveries of ruminal fluid, urine, and feces were 80, 85, and 81%, respectively.

3.2.2.3 Lolitrem B

While chlorinated solvents are best for extracting lolitrem B from grass and seed, Miyazaki et al. (2004) were able to use a 9:1 (v/v) hexane:ethyl acetate solvent mixture for extracting lolitrem B from bovine fat and other tissues, followed by a 9:1 (v/v) hexane:ethyl acetate prewash and wash on Sep-Pak Plus Silica SPE columns (Waters, Milford, MA), with elution using a 7:3 (v/v) hexane:ethyl acetate solution. The eluent was dried under nitrogen and reconstituted in 85:15 DCM:ACN before analysis via HPLC-fluorescence and HPLC-MS. Our group has successfully used this extraction procedure for bovine feces and a similar method for extracting lolitrem B and its metabolites from bovine urine. Alternatively, lolitrem B quantification in bovine feces is also possible using the same extraction method described above for quantitating lolitrem B in plant material [27].

With the extraction methods detailed above for ergovaline and lysergic acid in blood, feces, urine, and ruminal fluid and lolitrem B in fat, tissues, feces, and urine, detection of endophyte mycotoxins in animal matrices is possible and can be used as a diagnostic tool to confirm cases of endophyte toxicosis. The feeding trials conducted in sheep, cattle, and horses found fecal ergovaline and urinary lysergic acid to be the primary excretory products formed [28–30]. From these studies, we can conclude that a fecal sample extracted for ergovaline and a urine sample extracted for lysergic acid would be the best tools for clinical diagnosis of fescue toxicity. Studies like these are still needed for lolitrem B in order to determine the best matrix and extraction method to use as a tool for diagnosis of ryegrass staggers.

3.2.3 HPLC-Fluorescence Analysis of Endophyte Mycotoxins

HPLC-fluorescence is currently the most frequently used platform for quantification of ergovaline and lolitrem B in diagnostic laboratories which certify “safe feed” [20] or provide data to aid in the diagnosis of endophyte toxicosis in clinical cases [27], as it remains a cost-effective and robust tool with the capacity for high-throughput applications.

3.2.3.1 Ergovaline

The current protocol in our laboratory for HPLC analysis of ergovaline involves reversed-phase chromatography with fluorescence detection (excitation and emission wavelengths of 250 nm and 420 nm, respectively) and a gradient pump program run at a flow rate of 1.0 mL/min with 30% ACN and 2 mM ammonium carbonate in purified water as mobile phase A and ACN as mobile phase B, as follows: 0–5.5 min at 99% A, decreased linearly from 5.5 to 7.5 min to 35% A, held at 35% A from 7.5 to 9.5 min, then raised linearly to 99% A from 9.5 to 10.5 min and held for another 1.5 min before cycling to the next sample. A Gemini C18 3-μ column (Phenomenex, Torrance, CA) is used in conjunction with a guard column cartridge (Phenomenex) of similar packing. The retention time for ergovaline is 8 min, while that for ergotamine is 9 min. In addition, the introduction of Kinetex core-shell columns (Phenomenex) has recently provided an alternative in column selection, giving better peak separation, resolution, and shorter run times. We are currently using this technology in our lab to analyze for ergovaline with a 4.6 × 100-mm, 2.6-μ, 100 Å C18 column run at a flow rate of 1.8 mL/min and an injection volume of 10 μL. The pump program is the same as for the Gemini C18 column, except it is scaled to a total run time of 4.5 min.

Using the TotalChrom data system (Perkin Elmer, Waltham, MA), a standard curve is constructed from reference material of concentrations around 100; 400–500; 900–1,000; and 2,000 ng/g of plant material. A linear regression fit of the peak area versus the amount of analyte injected is used to determine the amount of ergovaline in unknown samples. The limit of detection (LOD) is 31 ng/mL, and the limit of quantitation (LOQ) is 100 ng/mL for forage samples. The LOD for ergovaline in rumen fluid is 10 ng/mL. While this method may be sufficient for analysis of plant material for regulatory purposes, it is not sufficient as a research tool to determine total distribution and metabolism of ergovaline in a feed study with livestock where analysis of body matrices requires a much lower LOD/LOQ. For example, ergovaline was previously extracted from plasma by two groups using a liquid diethyl oxide extraction and subsequently quantitated by HPLC-fluorescence; they determined their LOQ to be 3.5 ng/mL [32] and LOD to be 1.2 ng/mL [33]. Both extractions required a large amount of sample (4 mL plasma), and while they were good for determining the kinetic properties of ergovaline after a single intravenous dose, the actual amount of ergovaline ingested by livestock on a daily basis in typical feeding experiments would not be detected or quantified, based on data which found actual serum levels of ergovaline to be 0.7–3.8 pg/mL (pregnant mares grazing on endophyte-infected tall fescue pastures with a daily dose of approximately 1 mg/day ergovaline) [34]. Instead, Lehner et al. (2008) assayed their sera by LC-MS/MS which had an LOQ of 1 pg/mL. We review the usefulness of LC-MS/MS for these types of samples below. Based on these studies, we also suggest that better data may come from analyzing serum, instead of plasma, as there will likely be less interference since the blood has already been allowed to clot, allowing any unnecessary components to be removed.

Additionally, HPLC-fluorescence can be used where separation and purification of mixtures of ergot alkaloids and their metabolites are needed, particularly before use of instrumentation such as high-resolution mass spectrometry. For example, this technique was used to isolate metabolic products from ergotamine incubations in mice [35]. Isolation of ergotamine, its epimer, and seven transformation products was accomplished by manual peak collection via monitoring on a photodiode array detector (254 nm), cleanup of the incubation matrix on C18 SPE cartridges, dry-down under nitrogen, and reconstitution in methanol before analysis by mass spectrometry.

3.2.3.2 Lysergic Acid

Lysergic acid can be analyzed by HPLC-fluorescence with the same parameters used for ergovaline analysis, with a few exceptions. Better detection and quantification can be accomplished by using a 0.05-M phosphate-buffered mobile phase and a 5-μ C18 column, rather than the 3-μ column size used for ergovaline. Lodge-Ivey et al. (2006) reported limits of quantitation and detection for multiple matrices for lysergic acid. Seed, straw, and feces had LOQ/LOD values of 24.2/7.26, 14.5/4.34, and 36.0/10.80 ng/g, respectively. Ruminal fluid and urine LOQ/LODs were 5.5/1.64 and 18.4/5.52 ng/mL, respectively. Alternatively, ergoline molecules like lysergic acid can be measured using an enzyme-linked immunosorbent assay (ELISA) which was developed with antibodies against lysergol [36]. However, this ELISA has some limitations when the goal is to detect all ergot alkaloids, since it has variable specificity to individual alkaloids. In particular, it exhibits low binding affinity for the ergopeptides ergotamine, ergocryptine, ergocornine, and ergocristine [23].

3.2.3.3 Lolitrem B

Our group performs lolitrem B quantitation by HPLC-fluorescence detection using normal phase separation and an isocratic mobile phase (DCM:ACN:H_2O 4:1:0.02 (v/v)) run at 0.5 mL/min for 15 min [16, 37, 38]. Lolitrem B is detected using a fluorescence detector set with an excitation wavelength of 268 nm and an emission wavelength of 440 nm. A Zorbax Rx-SIL, 5-μ, 4.6×250-mm analytical column (Agilent Technologies, Santa Clara, CA) is used in conjunction with a hand-packed silica guard column. The retention time of lolitrem B is 8.3 min. Using the TotalChrom data system, a standard curve is constructed from reference material of concentrations around 500, 900, 2,000, and 4,000 ng/g of plant material. A linear regression fit of peak height versus the amount of analyte injected is used to determine the amount of lolitrem B in unknown samples. The LOD is 30 ng/mL and LOQ is 100 ng/mL for plant material. HPLC-fluorescence is sufficient for regulatory purposes, but the metabolism of lolitrem B is still largely unknown, and expensive analytical standards are necessary for quantifying lolitrem B in a variety of matrices. Like ergovaline, this will require the sensitivity and specificity of analytical tools like LC-MS/MS.

Table 3.1 Precursor and product ions used in MRM analysis of commonly tested ergot alkaloids[a]

Analyte	Precursor ion (*m/z*)[b]	Product ion (*m/z*)
Lysergol	255.1	240.2, 197.2
Ergine	268.1	223.2, 208.2
Lysergic acid	269.3	223, 167, 44
Ergometrine	326.2	223.2, 208.2
Ergovaline	534.2	223.2, 208.2
Ergosine	548.4	223.2, 208.2
Ergocornine	562.2	223.2, 208.2
α-Ergocryptine	576.4	223.2, 208.2
Ergotamine	582.2	223.2, 208.2
Ergocristine	610.4	592.4, 223.2

[a]Adapted from Lehner et al. (2004) and Sulyok et al. (2007)
[b]All precursor ions are given as the $[M+H]^+$ ion

3.2.4 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis of Endophyte Mycotoxins

3.2.4.1 Ergovaline

A need exists for a fast, highly specific, highly sensitive method of detection for ergot alkaloids which can be easily utilized in a variety of matrices. In recent years, LC-MS/MS has become a prominent tool for identifying and quantifying ergot alkaloids as it capitalizes on these characteristics, specifically electrospray ionization in the positive ion mode (ESI (+)) using multiple reaction monitoring (MRM). Detection of ergovaline is best done by MRM because the fragmentation pattern of the product ions is similar for most of the commonly tested ergot alkaloids (Table 3.1). The most common product ions produced are *m/z* 223 and *m/z* 208 (Fig. 3.3), representing the lysergic acid and demethylated lysergic acid moieties, respectively [39]. Representative spectra of the two main compounds associated with fescue toxicosis (ergovaline and lysergic acid) and LC-MS/MS conditions used in our laboratory are presented in Fig. 3.4. Analysis of ergot alkaloids in food products by LC-MS/MS has LOQs of 0.17–2.78 ng/g and LODs of 0.02–1.2 ng/g [40], up to three orders of magnitude below those of HPLC-fluorescence.

When using LC-MS/MS to detect and quantify ergot alkaloids, two major variables must be considered: (1) pKa values of 4.0 to 6.2 dictate that amines of rings 2 and 4 (Fig. 3.3a) be charged in acidic solution and neutral at alkaline pH. Thus, it is common practice to use weak volatile bases in the mobile phase to enhance detection [40]. Mobile phases similar to those described for HPLC-fluorescence are sufficient for mass spectrometry analysis. (2) Ergot alkaloids form epimers that do not necessarily fragment consistently (Table 3.2) [41]. In particular, some epimers may favor a different fragment, such as ergometrinine, which favors the *m/z* 208.2 over the *m/z* 223.2 fragment. Epimers also increase in concentration the longer they are suspended in organic solvents or held at room temperature. For instance, ergovaline was shown to

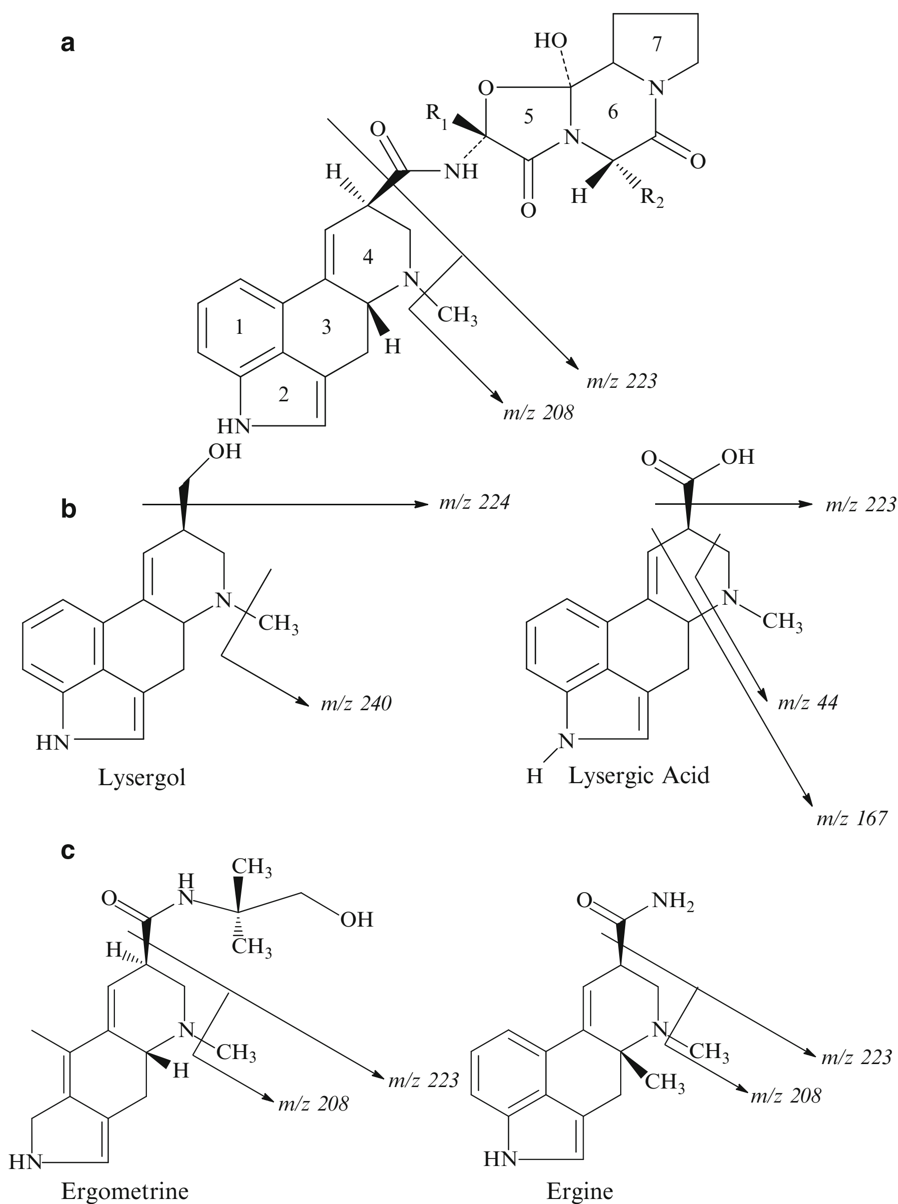

Fig. 3.3 (**a**) Typical fragmentation pattern for ergot alkaloids that contain the general peptide structure (ergovaline, ergotamine, ergocornine, ergocryptine, ergocristine and ergonovine). (**b**) Lysergol and lysergic acid give slightly different fragmentation patterns. (**c**) Ergometrine and ergine show the same fragmentation pattern of *m/z* 223 and *m/z* 208 but do not have the same general peptide structure as represented in (**a**) (Adapted from Lehner et al. 2004)

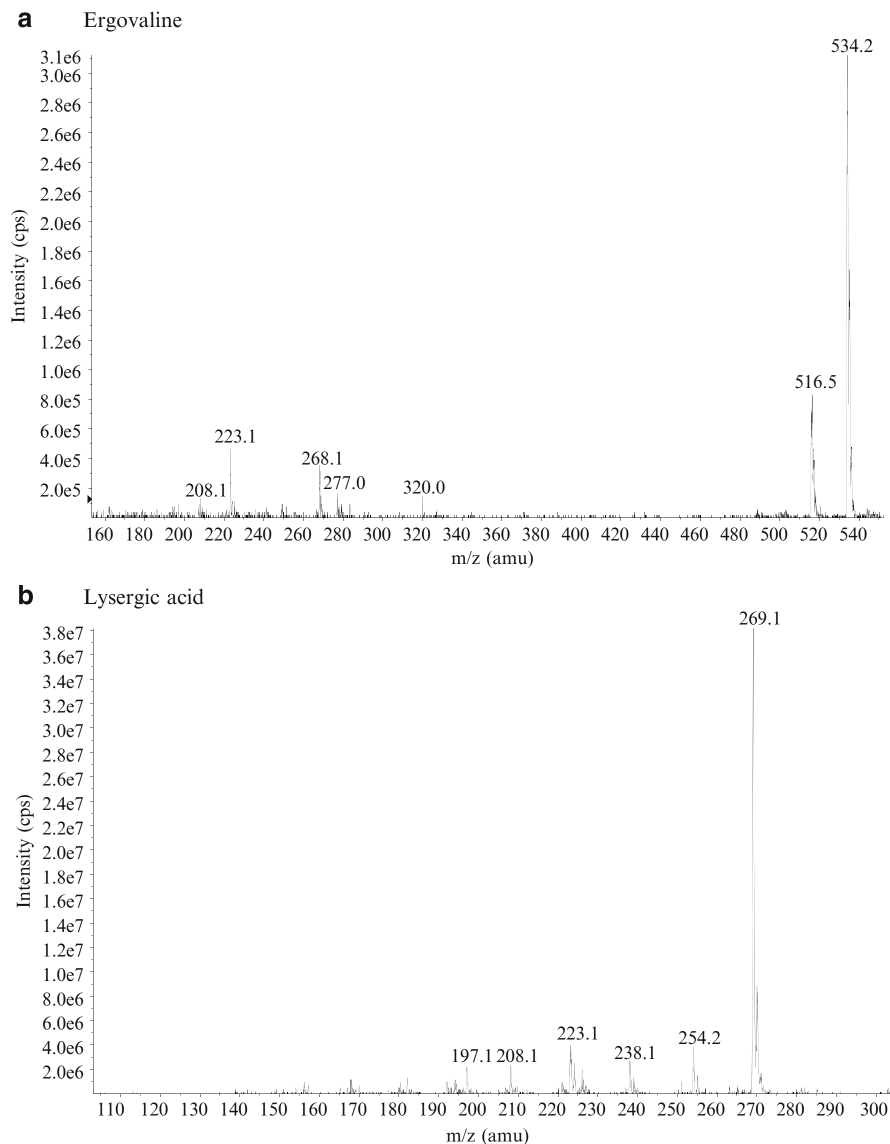

Fig. 3.4 ESI(+)-enhanced mass spectrometry spectra of ergovaline prepared in a 50:50 (v/v) mix of 2 mM ammonium carbonate and acetonitrile (**a**) and D-lysergic acid prepared in acetonitrile (**b**). Methods: A positive enhanced mass spectra scan was performed using a 3,200-QTRAP hybrid triple-quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA) with a Turbo V electrospray ionization source operated at 600°C, ion spray voltage of 5.5 kV, declustering potential of 61 V, and a collision energy of 10 eV. A linear gradient (0–100% B over 30 min, holding for 10 min at 100% B, and equilibrating for 10 min at 100% A) of 30% acetonitrile, 2 mM ammonium carbonate in water (mobile phase A), and acetonitrile (mobile phase B) was run on a 2.6-μ, C18, 100 Å, 100×4.60-mm Kinetex column using a flow rate of 1.4 mL/min. For (**a**), the common ergot fragments of *m*/*z* 149, 208, 223, 249, 269, 277, 320, 488, and 516 are visible, as seen in Lehner et al. (2004 and 2005). For (**b**), the common ergot fragment *m*/*z* 208 is visible, as is the parent *m*/*z* of 268

Table 3.2 Ergot alkaloid epimer precursor and product ions used in MRM analysis[a]

Analyte	Precursor ion (*m/z*)[b]	Product ion (*m/z*)
Ergometrinine	326.2	208.2, 223.2
Ergosinine	548.4	223.2, 208.2
Ergocoroninine	562.2	544.2, 223.2
α-Ergocryptinine	576.4	558.4, 223.2
Ergotaminine	582.2	223.2, 208.2
Ergocristinine	610.4	592.5, 223.2

[a]Adapted from Sulyok et al. (2007)
[b]All precursor ions are given as the $[M+H]^+$ ion

epimerize at 37°C in 0.1-M phosphate-buffered 9% aqueous fetal bovine serum solutions and in water, methanol, and acetonitrile, reaching an epimerization equilibrium between 1 and 19 hours [42]. Further, ergot alkaloid epimers generally chromatograph separately from their non-epimerized counterparts [35, 43], thereby eliminating part of the total concentration in the original sample to be measured, resulting in concentrations lower than what the sample truly contains. To this end, epimer formation can cause major variability in quantitation by affecting peak areas and intensity as well as retention times if analyzing standards or samples that have been suspended in solvents for an extended period of time. Therefore, it is of paramount importance to keep this in mind when handling standards and samples and in performing experiments where animals are dosed using a liquid solution of these toxins.

3.2.4.2 Lolitrem Compounds

Currently, mass spectrometry by positive atmospheric pressure chemical ionization (APCI (+)) is the ionization mode of choice for analysis of lolitrem B [44]; it has also been used to identify the lolitrem B biosynthesis pathway in plants and new lolitrem-like compounds [3]. A Phenomenex Prodigy ODS (30), 5-μ, 150×4.6-mm column was used with mobile phases consisting of 40% aqueous ACN 0.1% acetic acid (A) and ACN and 0.1% acetic acid (B) run at 1 mL/min using a gradient beginning with 20% B, rising linearly to 50% B at 20 min, then to 100% B at 40 min and recycling after 60 min [3]. The mass spectrometer was operated with nitrogen sheath and auxiliary gas set to 40 and 10 psi, respectively. Source voltage was 6 kV, capillary temperature was 200°C, and vaporizer temperature was set to 450°C.

Our lab has adapted this mass spectrometry method for the purpose of quantifying lolitrem B in bovine matrices such as urine, serum, fat, and feces, using a shorter run time. Quantitation is accomplished on an LC-MS/MS system via APCI (+) and MRM detection using the transitions 686.4/237.9, 686.4/196.3, 686.4/628.4, and 686.4/238.3. The transition 686.4/237.9 gives the best R^2 value (e.g., $R^2=0.9994$ for lolitrem B-spiked urine) when running a calibration curve; therefore it is used for quantitation, while the other transitions are used for qualitative confirmation. Additional quality control parameters (LOD, LOQ, intra- and inter-assay variation) are currently being defined. MRM analyses are conducted using an autosampler

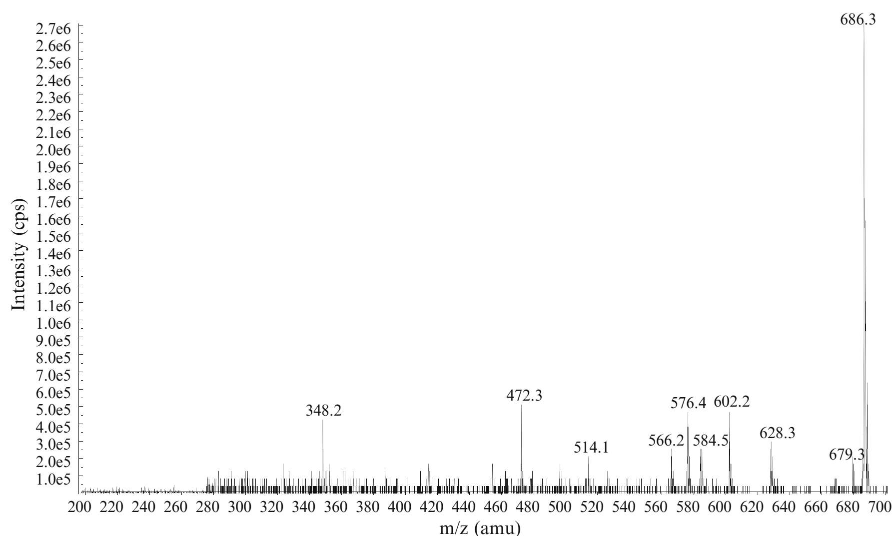

Fig. 3.5 APCI(+)-enhanced mass spectrometry spectrum of lolitrem B standard prepared in acetonitrile. Methods: A positive enhanced mass spectrometry scan was used to acquire this spectra on a 3,200-QTRAP hybrid triple-quadrupole/linear ion trap mass spectrometer with a Turbo V atmospheric pressure chemical ionization (APCI) source operated at 450°C and nebulizer current of 6 μA, using a declustering potential of 20 V and a collision energy of 10 eV. LC conditions from Young et al. (2009) were used with the exception of flow rate, which was 0.5 mL/min. Common fragments specific to lolitrem B are *m/z* 576, 602, and 628 (Nielsen and Smedsgaard 2003; Young et al. 2006; Young et al. 2009)

cooling tray at 4°C, a flow rate of 0.5 mL/min, and a linear gradient of 20–100% B over 20 min, using the same mobile phases described above in Young et al. (2009). Lolitrem B has a retention time of 14.5 min using this method. Mass spectrometer conditions are as follows: declustering potential = 61 V, entrance potential = 7 V, collision cell exit potential = 4.0 V, collision energy = 51 V, ion spray voltage = 5,500 V, temperature = 450°C, nebulizer gas = 65 psi, turbo gas = 0 psi, curtain gas = 30 psi, and nebulizer current = 6 μA. For metabolite mining, an EMS scan is performed using the full-length, 60-min gradient described in Young et al. (2009) (Fig. 3.5).

3.3 Impacts of Endophyte Mycotoxins on Animal Health

3.3.1 Physiological Impacts and Metabolic Endpoints

3.3.1.1 Ergot Alkaloids

Ergot alkaloids act as α-adrenergic and serotonergic agonists which stimulates contraction of smooth muscle cells, resulting in peripheral vasoconstriction [7, 45]. In cold temperatures in which peripheral vasoconstriction is already occurring,

additional vasoconstriction induced by ergot alkaloids can result in tissue ischemia, necrosis, and sloughing of extremities (fescue foot). In hot climes, peripheral vasoconstriction causes general malaise and reduced average daily weight gains (summer slump). The ergopeptides can also alter prolactin secretion by binding to and blocking dopamine receptors [7]; suppressed serum prolactin concentration is, therefore, often used as an indicator of fescue toxicosis in livestock. Depressed serum prolactin in prepartum cattle can result in reduced milk yield but has negligible effects once lactogenesis occurs. Cows grazing endophyte-infected fescue are also reported to have reduced pregnancy rates [46] and increased rates of early embryonic death [47], leading to reproductive efficiency problems. Mares consuming endophyte-infected fescue exhibit increased gestation lengths, agalactia, foal and mare mortality, and weak and poorly developed foals [48]. These late gestational effects are the reasoning behind the zero tolerance level for ergot alkaloids advised for mares in the last 2–3 months of pregnancy. When combined, these performance and reproductive impacts on animal health cause significant monetary losses for livestock producers, estimated to be upwards of $1 billion in the United States alone [6].

In order to determine the toxic moiety/moieties responsible for these maladies, metabolism of ergot alkaloids has been studied in a variety of animal models, using a myriad of analytical tools. The use of radiolabeled compounds has shown biliary (fecal) excretion to be the primary route of elimination in monogastric models and humans, with a small amount detected in the urine [49, 50]. More recent work has shown lysergic acid to be a metabolic breakdown product of ergot alkaloids in the ruminal fluid and urine of sheep and cattle [22, 28], with the possibility of conjugated parent molecules being excreted in the urine as well. A feeding study conducted in sheep with endophyte-infected tall fescue containing toxic ergovaline levels (0.610 mg/kg) fed over 28 days examined feed, ruminal fluid, urine, and feces for ergovaline and lysergic acid [28]. The authors recovered 35% of the dietary ergovaline in the feces and 248% of the dietary lysergic acid in the urine and feces. They also observed an increase of lysergic acid in the ruminal fluid over time. These data indicate that ergovaline is being metabolized to lysergic acid by ruminal microbes, which is then passed into the urine for excretion. In another study where horses were fed endophyte-infected tall fescue seed containing 0.5 mg/kg ergovaline and 0.3 mg/kg lysergic acid over 21 days, a similar pattern was observed whereby urinary excretion of lysergic acid was the primary route of elimination, with fecal excretion of ergovaline playing a more minor role [30].

For the proportion of ergot alkaloids that do enter systemic circulation and pass through the liver, CYP3A is the main cytochrome P450 subfamily responsible for metabolism, with N-dealkylation and mono- and dihydroxylation being the main oxidative reactions carried out by these enzymes [35, 43, 51–53]. An equine study examined the effects of a single IV dose of 15 μg/kg bwt ergovaline in plasma and found the elimination half-life and total clearance of ergovaline to be 57 min and 0.020 L/min kg, respectively [32]. While the study of Bony et al. (2001) and that of DeLorme et al. (2007) provide pieces to begin putting together a model for the absorption, distribution, metabolism, and excretion of ergot alkaloids (Fig. 3.6), a

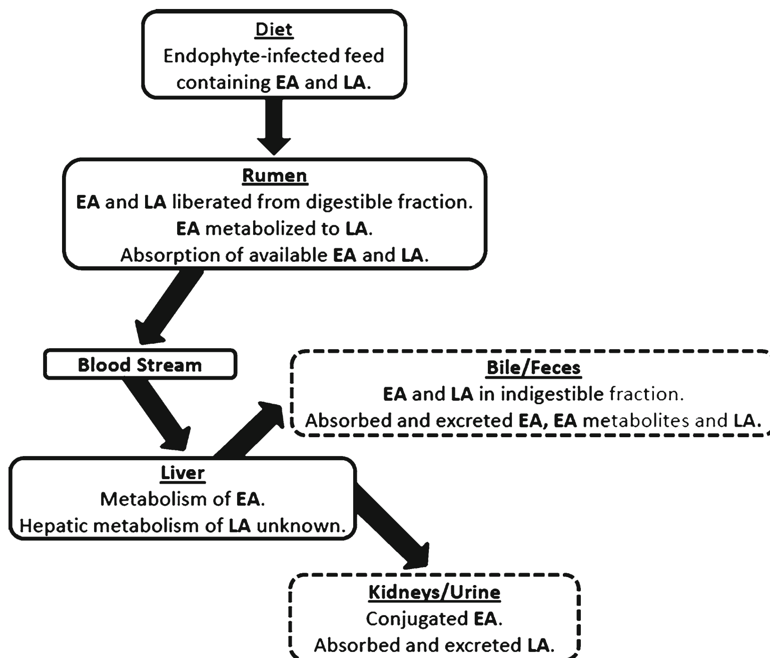

Fig. 3.6 Proposed model for the absorption, distribution, metabolism, and excretion of ergot alkaloids and lysergic acid. Compartments with broken lines signify routes of excretion. *EA* ergot alkaloids, *LA* lysergic acid (Adapted from DeLorme et al., 2007)

multicompartmental toxicokinetic study utilizing the specificity and sensitivity of LC-MS/MS for metabolite mining is needed for a more detailed understanding of the metabolic fate of these compounds in livestock.

3.3.1.2 Lolitrem B

The molecular site of action of lolitrem B is on large-conductance calcium-activated potassium channels, which was first shown in human embryonic kidney cells [17] and later in mice deficient for this ion channel (*Kcnma1*$^{-/-}$) [54]. Specifically, the β-4 subunit is responsible for modulating motor control and is associated with the ataxia observed upon ingestion of this toxin [54]. Seven other members of the lolitrem family of compounds have been examined in a similar manner and have also been shown to inhibit this channel to varying degrees [55]. Lolitrem B was found to be one of the most potent inhibitors; the presence of an isoprene unit appears to be a determinant of an individual molecule's potency, as is an α-oriented hydrogen atom at position 31 (indicated in Fig. 3.1). However, to date, no detailed toxicokinetic study on the fate and metabolism of lolitrem B once ingested has been carried out.

In addition, public health concerns over the safety of meat and other by-products from food animals which have consumed endophyte mycotoxins in imported ryegrass straw have been raised [37]. Specifically, the presence of lolitrem B in fat of exposed animals has been questioned since lolitrem B is somewhat lipophilic. Based on pilot studies, 3–10% of the ingested lolitrem B dose is sequestered in the fat (with no detectable lolitrem B found in skeletal muscle, liver, kidney, heart, or cerebrum) [37, 56]. Thus, a chronic toxicokinetic study mimicking extended lolitrem B feed intake would be valuable to establish definitive compartmental values. Utilizing LC-MS/MS capabilities, such a study could determine (1) whether lolitrem B accumulates in adipose tissue and if it reaches steady-state concentrations; (2) if lolitrem B is retained in adipose tissue until metabolic liberation (similar to polychlorinated biphenyls) or if it is depleted once dietary exposure to lolitrem B is removed; (3) the relationship of level of dietary lolitrem B and lolitrem B concentrations found in adipose and other tissues; and (4) the metabolic pathway(s) of lolitrem B, all of which would be useful parameters to define in the interest of food safety regulation.

3.3.2 Dose Response Studies: Impacting the Forage Trade

Feeding trials have been conducted to establish the threshold of toxicity for ergovaline and lolitrem B in feed material for cattle and sheep [18, 57]; evidence from clinical cases provides an estimate for threshold values in horses [19] (Table 3.3). No threshold levels have been determined for camelids; however, anecdotal evidence of poor milk production (ergot alkaloids) and intention tremors and hypermetria (lolitrem B) have been reported and associated with consumption of endophyte-infected feed (personal communications, A. Morrie Craig and Linda Blythe, DVM). Ergovaline dietary levels of 400–750 parts per billion (ppb) and 500–800 ppb for cattle and sheep, respectively, and 1,800–2,000 ppb lolitrem B for both species are approximated threshold values for the disease syndromes caused by these mycotoxins. Environmental variables, including summertime heat and wintertime cold, could influence the development of fescue toxicoses at fixed dietary concentrations. Such uncertainty surrounding the environment-dose interaction is the rationale behind the broad range of threshold dose values listed for ergovaline. Additionally, investigations as to the bioaccumulation potential of these compounds are warranted and could shift the parameters by which these compounds are regulated in food products from dietary exposure to residue analyses.

Regarding variables in the plant, the crown and seed heads of the plant concentrate both toxins relative to the stem, so it is advised to avoid overgrazing pastures and to harvest grass hay above the crown, before seed head production has occurred or after seed has been harvested. It takes one to three weeks for clinical signs of these diseases to appear in livestock, so a history of the animal's diet, including whether or not the straw was fed as 100% or only part of the ration and the duration feed was consumed, is a key component to making a diagnosis. If the suspected feed material is available, testing is recommended at a certified testing laboratory (e.g.,

Table 3.3 Threshold values for fescue toxicosis (ergovaline) and ryegrass staggers (lolitrem B) in livestock

Livestock species	Ergovaline (ppb)	Lolitrem B (ppb)
Cattle	400–750	1,800–2,000
Sheep	500–800	1,800–2,000
Horses	300–500[a]	ND
Camelids	ND	ND

[a]Except in the last 60–90 days of pregnancy when the threshold is 0 ppb
ND not determined

http://oregonstate.edu/endophyte-lab/). A review of typical cases and interactions between livestock producers/veterinarians (both domestic and international) and testing laboratories is described in [56]. In addition, hay producers/exporters are encouraged to test any tall fescue and perennial ryegrass forage products which may contain endophyte in order to ensure the material being distributed is safe for livestock to consume.

In conclusion, the methods described herein are useful both for the promotion of safe livestock feed through service laboratories and as clinical tools for diagnosing cases of endophyte toxicoses. Additionally, they are a research asset which will allow for a better understanding of the diseases caused by these mycotoxins so that development of more effective preventative and/or therapeutic measures can be realized. Lastly, it is worth noting that animals are often exposed to a mixture of ergovaline, other ergot alkaloids, lolitrem B, and a myriad of other plant toxins in endophyte-infected feed materials [27], yet experimental investigation as to the additive or multiplicative effects of these toxins on the development of disease has not been undertaken. Further, the possible impact of bioaccumulation of these toxins under varying exposure conditions on both toxicity and as residues in food products has not been thoroughly investigated and could affect the manner by which these compounds are regulated.

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Chapter 4
Production of Traditional and Novel Biopolymers in Transgenic Woody Plants

David A. Dalton, Ganti Murthy, and Steven H. Strauss

Abstract Recent advances in plant biotechnology are expanding the potential for woody plants to provide industrially useful biopolymers. Transgenic approaches can enable plants to produce novel compounds that are not normally present (e.g., bioplastics such as polyhydroxybutyrate). This chapter summarizes the strategies that have been used to produce biopolymers in plants, with emphasis on bioplastics from transgenic poplar. So far, the yields of bioplastic in plants have been accompanied by unfavorable metabolic expenses associated with the diversion of carbon resources, but it may be possible to obtain improvements through careful control of expression of the three genes for biosynthesis of polyhydroxybutyrate. This chapter also discusses the potential for transgenic technology to improve the yields and qualities of traditional biopolymers including cellulose (wood), latex, and oil. A major emphasis with wood has been the modification of lignin content and structure to facilitate pulp and biofuel production. Other ongoing projects involving biopolymers may lead to improved production of latex from guayule (*Parthenium argentatum*) and Russian dandelion (*Taraxacum kok-saghyz*) and of fuel oil from *Jatropha* (*Jatropha curcas*). We believe that substantial improvements in these traditional plant products are likely with additional research on control of gene expression and if regulatory concerns about field research and commercial deployment can be adequately addressed.

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4.1 Introduction

Woody plants have long been vital sources of industrial biopolymers, most notably wood and rubber. Indeed, the importance of these materials as socioeconomic driving forces can scarcely be understated. The advent of genetic engineering is currently providing opportunities to enhance the production of these compounds as well as to develop new woody plant-based production systems for novel compounds. The potential advantages of such innovations are substantial and include the reduction of petroleum use, CO_2 emissions, and pressure on natural resources. Bioplastics and biofuels have the potential to be biodegradable, close to carbon neutral, and thus truly sustainable on a long-term basis. Furthermore, transgenics offers great potential for improving traditional biopolymers from woody plants, such as wood, rubber, and oils. Numerous research efforts have recently been initiated to fulfill the promise of engineering of transgenes in woody plants, but no new products or systems have yet reached commercial feasibility. This review will cover these developments with respect to biopolymers in transgenic woody plants. We will not cover improvements specifically in nonwoody plants, even though some of those concepts (e.g., pharmaceuticals, proteins, vitamins) are certainly applicable to woody plants. Nor will we cover biopolymers that have been produced only in nonwoody transgenic plants (e.g., cyanophycin; [1]). Research into transgenic woody plants is very much an emerging field. Since actual applications have yet to appear, we will also discuss likely scenarios for future development as well as impediments towards their fulfillment.

4.2 The Advantages of Woody Plants

Woody plants offer many advantages over nonwoody plants with regard to the production of compounds for human use. Perhaps the most compelling of these advantages is simply the issue of scale. Most plant-made industrial products would be needed in large amounts, and trees have sufficient biomass to provide larger yields. For instance, poplar (*Populus* spp.) and eucalyptus (*Eucalyptus* spp.) can accumulate biomass at rates over 25 Mg ha^{-1} yr^{-1}, which is roughly twice the biomass yield expected for switchgrass (*Panicum virgatum*) [2, 3]. Even if the target compounds are present at low concentrations, the recoverable yields per hectare could still be substantial, and the residual biomass could serve a beneficial unrelated use. Indeed, one of the most compelling arguments for engineering these products in woody plants is that the compound would be a secondary or coproduct, with the bulk of the plant serving as biofuel or a more traditional product such as timber or fiber for paper. Metabolic pathways for synthesis of phenylpropanoids (e.g., lignin), terpenoids, and cellulose are well characterized and generally highly active in woody plants, thus providing opportunities for production of many novel compounds.

Poplar (*Populus* spp.) is by far the leading model for genetic studies in woody plants. The moniker of poplar as "*Arabidopsis* for forestry" is a valid reflection of its dominance in the field [4]. Poplar shares many of the advantages of *Arabidopsis*

including ease of *Agrobacterium*-based transformation, reliable regeneration from tissue culture, availability of the complete genome physical map and sequence (http://www.upsc.se/Technology/Populus-Genome-Programme/index.html), a large EST database, cDNA microarrays, and corresponding expression analyses that can be accessed on the World Wide Web (http://popgenie.org/tool/efp-browser). Furthermore, substantial genetic resources have been developed over many years in the form of hybrids consisting of interspecific crosses and backcrosses. These hybrids complement the natural genetic and ecological diversity of poplar that arises from the large number of species (~35) that are widely distributed throughout a range of habitats in the northern hemisphere [5, 6].

Unlike *Arabidopsis*, poplar allows for genetic studies related to secondary growth (wood), mycorrhizal associations, and seasonality (dormancy). Poplars are among the fastest growing of any tree species, and silvicultural practices are well developed. Poplars have substantial commercial value, and this has led to a corresponding influx of resources towards its further development as a crop.

Other tree species besides poplar have proven tractable with respect to transgenic technology. Some of these species are important conventional providers of fiber and timber such as eucalypts (*Eucalyptus*), ash (*Fraxinus*), birch (*Betula*), black locust (*Robinia*), chestnut (*Castanea*), conifers (especially *Pinus*), elm (*Ulmus*), European oaks (*Quercus* spp.), sweetgum (*Liquidambar*), yellow poplar (*Liriodendron*), and walnut (*Juglans*) [7–9]. Techniques have also been developed to genetically transform other tree species with specialized uses, such as latex from the rubber tree (*Hevea*) and oil from *Jatropha* (*Jatropha*; see later sections); however, the transgenic technology for these other species lags behind that of poplar, though genomic resources are rapidly being developed, especially for *Eucalyptus* and *Jatropha* [10–12].

Despite these success stories, not all woody plants are amenable to GE (genetic engineering) technology. For instance, American oaks and alder (*Alnus rubra*) can be transformed with standard *Agrobacterium*-based techniques but are difficult to regenerate into plants [9, 13]. Douglas-fir (*Pseudotsuga*), one of the top timber-producing species in the world, has not been a target for GE technology in part because it is difficult and costly to transform and there is no obvious need for transgenic manipulation. Many other tree species, especially those from the tropics, have simply not been investigated for their capacity for GE technology. Future technological improvements have great promise for extending the range of tree species amenable to GE technology and for the production of unique or enhanced products.

4.3 Bioplastics

Biotechnology offers the potential to develop transgenic plants that produce bioplastics, especially poly(3-hydroxyalkanoates) or PHAs. These are structurally simple natural polymers synthesized by most genera of eubacteria to serve as carbon and energy storage compounds that are osmotically inert and easily reclaimed for

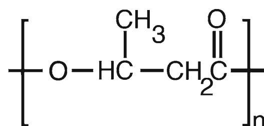

Fig. 4.1 Chemical structure of (PHB) polyhydroxybutyrate. Other PHAs of commercial interest contain longer chains at the methyl position of PHB

metabolism [14, 15]. PHAs are typically synthesized during periods of stress and can make up to 90% of the bacterial cell dry weight. The simplest PHA is polyhydroxybutyrate (PHB, Fig. 4.1). PHAs have the potential to be truly transformative products since they are a biodegradable plastic that is not based on petroleum.

The biosynthesis of PHB involves three enzymes (phbA, phbB, and phbC) that convert acetyl CoA into PHB in a pathway that has been well described in other reviews [16–20]. The production of PHB through bacterial fermentation is routine and already a commercial success. Although no plants produce PHAs naturally, there are at least 12 plant species that have been engineered to do so through the introduction of bacterial transgenes. Private industry is actively pursuing the development of PHB production in various crop plants, especially switchgrass [21]. Other types of PHAs with longer side chains (compared to the methyl group of PHB) are also being developed in transgenic plants [22].

The recent report of PHB production in transgenic poplar is the first example of PHB production in a woody plant (Fig. 4.2) [23]. Yields of up to 2% (w/w leaf dry weight) were reported following application of an ecdysone-based inducing agent. A number of critical issues need to be resolved before such ventures become practical.

4.4 Subcellular Location

Early work with PHB production in *Arabidopsis* established that localization to chloroplasts led to higher yields and lower impact on plant health [24]. The generally accepted explanation for this is that chloroplasts have a high flux of acetyl CoA, which is used for biosynthesis of fatty acids and is also the precursor for biosynthesis of PHB. Consequently, most subsequent work with transgenic PHB-producing plants has incorporated a plastid-targeting sequence into the appropriate genes for PHB biosynthesis (*phbA*, *phbB*, and *phbC*; Fig. 4.3). The presence of PHB granules in chloroplasts could lead to a physical disruption of the internal organization of chloroplasts and a subsequent decrease in photosynthesis. In poplar, the granules nearly fill the entire volume of chloroplasts, suggesting that this might be a barrier to achieving high yields [23]. There has been some interest in targeting the PHB biosynthetic enzymes to peroxisomes. This strategy has proven successful in yeast, *Arabidopsis*, and sugarcane with plant yields up to 1.6–1.8% dry weight [25–27].

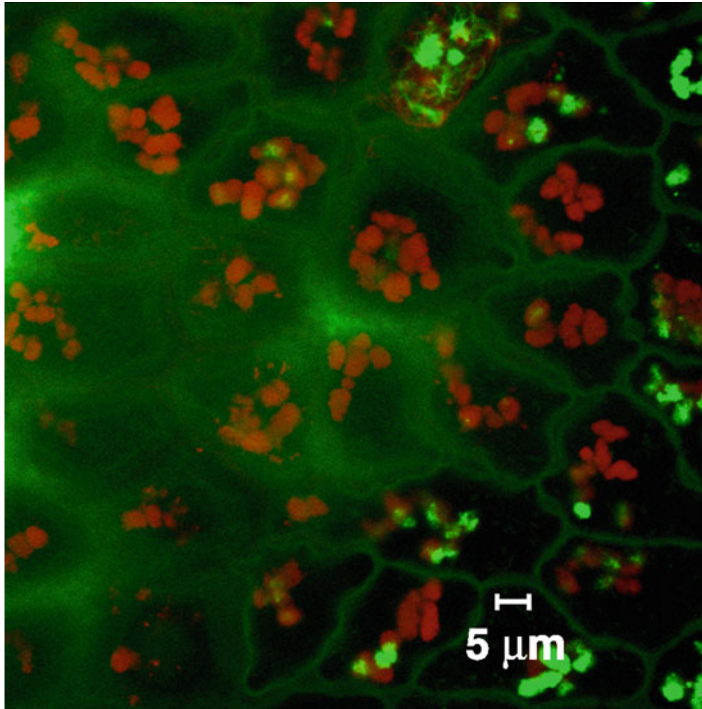

Fig. 4.2 Confocal microscopy showing *red* and *green* fluorescent agglomerations of the bioplastic polyhydroxybutyrate (PHB) in leaf palisade mesophyll cells of transgenic poplar. Separate images were merged such that the *red* color shows a section through a plane that is 8 mm below the *green* section (Photo by Peter Kitin)

Fig. 4.3 T-DNA region of PHB expression cassette used to transform poplar. *Pnos* promoter for nopaline synthase, *NPTII* kanamycin resistance gene, *NT* terminator for nopaline synthase gene, *35S* promoter for 35 S cauliflower mosaic virus, *GRvH* glucocorticoid response element, *greMP* minimal promoter with glucocorticoid response element binding site, *TS* plastid-targeting sequence, *phb ABC* genes for biosynthesis of PHB

Woody plants offer possibilities for locating PHB biosynthesis outside of chloroplasts or even outside of leaves. For instance, production could be directed to the vascular cambium or ray parenchyma using appropriate promoters such as that for the bark storage protein (BSP) [28]. It might even be possible to incorporate bioplastic production into the cell walls of secondary xylem, perhaps by indirect association of PHB with cell walls from cells in which PHB has been synthesized intracellularly and then released following cell death. This could create unique

materials that could be used directly without complicated extraction or processing. Such concepts are highly speculative at this time, although similar successful modifications have been achieved with fibers of cotton and flax [29, 30].

4.5 The Metabolic Cost (Trade-Off) of PHB Production

A key problem with PHB production in plants is controlling expression in such a manner that the overall growth of the plant is not impacted too heavily. In extreme cases, it has been possible to achieve PHB yields as high as 40% (dry weight) in *Arabidopsis*, but the concomitant diversion of carbon resources to PHB results in severe stunting or other gross defects [31]. Metabolic profiling of PHB-producing plants has indicated substantial decreases in isocitrate and fumarate, suggesting an impairment of the tricarboxylic acid cycle due to diversion of acetyl CoA [31]. Surprisingly, no changes were detected in fatty acids, and increases were observed in proline, mannitol, and several sugars. In terms of growth and overall plant health, the most thorough examination of the metabolic trade-off costs of PHB production in any plant was recently reported for poplar [23]. This study included a chemically inducible promoter based on an analog of ecdysone in order to minimize negative impacts. After induction, leaves contained variable amounts of PHB (between 0 and 2% of leaf dry weight; Fig. 4.4). A greenhouse study indicated that there were no negative consequences of PHB production on growth unless the PHB content exceeded 1%. At PHB levels above 1%, significant decreases were observed in all growth parameters except for wood density (Table 4.1, Fig. 4.5). Chlorophyll fluorescence (Fv/Fm) also declined significantly, indicating that the plants were under stress.

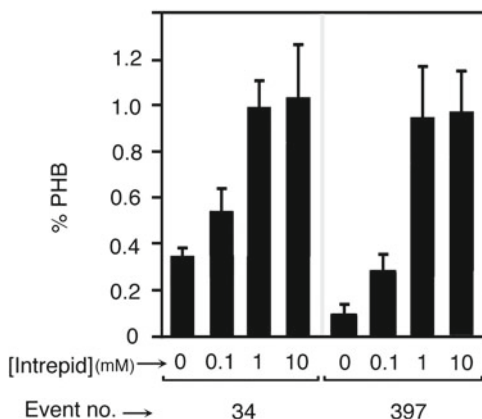

Fig. 4.4 PHB concentrations (% by dry weight) in leaves of transgenic poplar following 6 weeks of biweekly application of the inducing chemical Intrepid at concentrations from 0 to 10 mM (From [23])

Table 4.1 Effects of PHB content on growth parameters in transgenic poplar. Data are based on 144 6-month old plants that received various concentrations (0–10 mM) of the inducing chemical Intrepid. Intrepid alone had no significant effect on plant health (Modified from [23])

Parameter	Mean for all plants	Mean for plants with <1% PHB	Mean for plants with >1% PHB	% decrease if PHB > 1%
Height (cm)	62.51	63.84	57.38	10.1*
Diameter (mm)	6.69	6.87	6.06	11.8*
Volume index (mm^3)	30459	32635	24147	26.0*
Leaf mass (g)	6.44	6.71	5.35	20.3*
Stem mass (g)	4.57	4.88	3.35	31.4*
Root mass (g)	3.07	3.29	2.16	34.2*
Shoot (leaf + stem) mass (g)	9.679	10.893	8.089	25.7*
Total mass (g)	14.11	15.22	10.42	31.5*
Chlorophyll fluorescence	0.733	0.736	0.713	3.1**
Wood density (g/cm^3)	0.327	0.328	0.321	2.1
PHB %	0.659	0.394	1.495	–

*P value < 0.05
**P value < 0.001

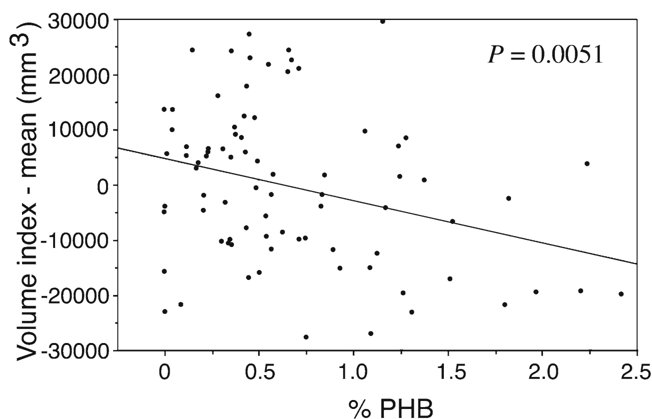

Fig. 4.5 Decreases in volume index (height × diameter squared) as a function of PHB content in leaves of transgenic poplar. Values are for individual plants and are corrected for the means of separate transformation events (From [23])

4.6 Regulation of Expression of the Genes for PHB Production

Most transgenic PHB-producing plants have been based on the common, constitutive 35 S cauliflower mosaic virus (CaMV) or an enhanced version. Some studies have attempted to use genetic constructs incorporating inducible promoters in an attempt to increase PHB yield while minimizing impacts on plant health. The goal is to allow plants to reach maturity before resources are diverted to PHB production.

The first such inducing study was the use of salicylic acid to control expression of *phbA*. In this case, the transformation efficiency was improved, but the yield of PHB was still rather low [32]. Lössl et al. used a transplastomic *phb* operon that was inducible with ethanol, which led to slightly higher yields [33]. The use of the *cab-m5* light-inducible promoter in switchgrass was successful with respectable though highly variable yields of up to 3.7% [21]. The most successful inducible system developed to date was based on conventional nuclear transformation of *Arabidopsis* and a commercially available nonsteroidal ecdysone analog [34]. PHB yields of up to 14% were observed. When used in transgenic poplar, this same inducible system allowed for yields of up to ~2%, although plant health was impaired at levels above 1% as described above [23]. The advantages of this ecdysone-analog-based system include the availability of commercial inducing chemicals such as Intrepid® and Mimec® that are already licensed for field use, the absence of negative effects on nontarget organisms (including the plant to which it is applied), efficient transport throughout the plant, and full induction at low concentrations (1 mM for poplar). The study of inducible promoters in plants is an emerging field, and the development of improved systems holds considerable promise (reviewed by [35]).

Most recently, a transplastomic approach has been used in tobacco to include a polycistronic synthetic operon that was driven by the native *psbA* promoter (the *psbA* gene codes for the DI subunit of photosystem II [36]). The transgenes for PHB biosynthesis were thus inserted into the plastome as an extension of the *psbA* operon in such a manner that no foreign promoter was required. This system had the double advantage of providing some of the highest yields (18.8%) of PHB yet observed as well as a high level of gene containment due to the maternal inheritance of plastid DNA. This development is highly encouraging and may in fact be the breakthrough that leads to a viable commercial system, especially considering that the goal for such feasibility has been set at a PHB yield of >7.5% by Metabolix [21].

4.7 Extraction and Recovery

Historically, most of the research and commercial efforts for PHB production and extraction have been focused on the microbial fermentation, which generally yields PHB concentrations much higher (up to 90% of cell dry weight) than those obtained in transgenic plants [37, 38]. Several extraction methods for PHA recovery have been developed. These typically involve centrifugation, filtration, extraction with organic solvents chloroform and methanol, bleaching with sodium hypochlorite, and digestion with enzymes [37–40]. These methods are suitable for extraction of PHB in bacterial cells; they are not suitable for plant biomass. In particular, chlorophyll in the plant biomass interferes with the organic solvent extraction; sodium hypochlorite and enzymatic digestion are not severe enough to degrade the recalcitrant plant cell walls. Recently, a modified solvent sequential extraction method was used to extract the PHB from genetically modified hybrid poplar leaves [41]. There is a need for additional research into extraction of PHB, especially from the higher

plants, as none of the current extraction processes meet all the requirements of economics, safety, and scalability.

Primary challenges in economically viable commercial production of microbial PHB are in the high feedstock and the extraction costs. Presently many companies produce PHA (primarily PHB) via the fermentation route in the price range of $2.15–$5.31/kg, and current world production capacity of PHA is estimated to be 100,000 tons/year [42]. While the technical feasibility of PHB production and extraction from higher plants has been demonstrated, there is little information on the techno-economic feasibility of PHB extraction from higher plants. However, based on preliminary techno-economic analysis, it is known that the PHB content of the plant biomass is a significant factor in the commercial feasibility of PHB production from plants [41].

Life cycle assessments (LCA) have demonstrated the advantages of microbially produced PHAs over petrochemical plastics [18, 43–45]. While one of the advantages attributed to production of PHB in higher plants is the direct conversion of sunlight and CO_2 into biodegradable plastics, relatively few LCA studies exist for the production of PHA from higher plants. In one such study, Kurdiker et al. demonstrated that life cycle reductions in greenhouse gas profiles of plant-derived PHA are better than those of polyethylene produced from fossil resources [46]. However, the LCA studies for higher plants need to be performed in conjunction with techno-economic feasibility of the PHA production in higher plants. Given the present state of research and development, as well as the significant technical challenges to be addressed, it may be reasonable to expect large-scale commercial production of PHA from plants in about 10 years.

4.8 Biopolymers in Wood

Wood is the most prominent plant-produced biopolymer and has long been the target of scientific improvements based on conventional genetic breeding and selection. Efforts based on transgenics have focused so far primarily on reducing the content of lignin so as to facilitate the production of paper. Lignin biosynthesis proceeds via the shikimate and phenylpropanoid pathways and is thus complex with a large range of potential targets for genetic manipulation. Furthermore, many essential compounds besides lignin are produced in these pathways, so any metabolic tinkering has the potential to introduce unintended disruptions. One early example involved the antisense inhibition of the lignin biosynthetic pathway gene *Pt4CL1* encoding 4-coumarate:coenzyme A ligase (4CL) in aspen (*Populus tremuloides*) [47]. This resulted in up to a 45% reduction in lignin and a surprising 15% increase in cellulose content. Leaf, root, and stem growth were all enhanced. Co-downregulation of *4CL* and *CAld5H* (another gene in required for lignin biosynthesis) resulted in an additive effect with even further reductions in lignin and increases in cellulose [48]. Additional successes in reducing lignin content and/or increasing the potential efficiency of pulping have involved transgenes that alter the

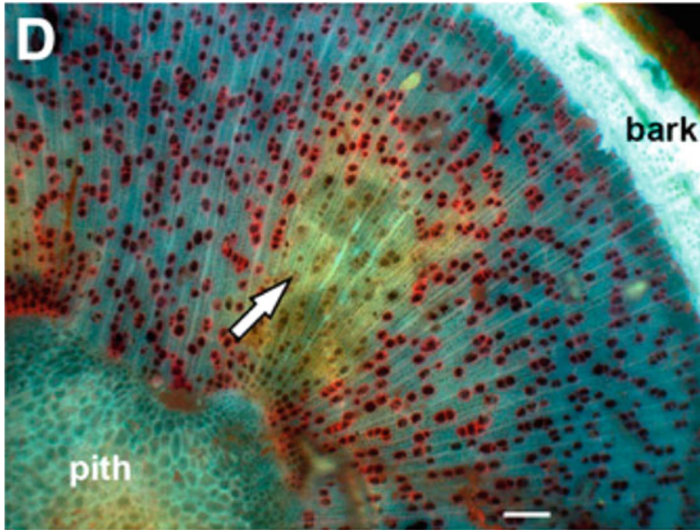

Fig. 4.6 Cross section of low-lignin, transgenic poplar stem visualized by cryofluorescence. Conducting vessels are stained red. The *arrow* indicates nonconductive tissue that is a result of phenolic deposition within a low-lignin zone. *Bar* = 200 μm (From [51])

relative ratios of the components of lignin. These studies have been at least partially successful and have recently been reviewed by Li et al. [49]. It is also possible to downregulate lignin production in poplar by transforming with the transcription factor EgMYB1 from *Eucalyptus* [50].

Further studies involving downregulation of *4CL* in transgenic poplar have revealed cautionary flaws in the general strategy. The decrease in lignin impairs wood structure and strength, xylem conductivity, growth efficiency, saccharification potential, and survival [51–53]. Adequate lignification is required for mechanical support of the stem, water transport, and general health of the tree (Fig. 4.6), so it appears that the goal of reduced lignin content has unintended, possibly unavoidable, negative consequences. Reports of the apparent lack of negative stem effects in reduced-lignin, nonwoody plants (specifically *Arabidopsis*) appear to be of limited relevance to woody plants [54]. An alternative approach to modifying woody cell walls for enhanced properties involves engineering of pathways for synthesis of hemicelluloses or pectins [55, 56]. These studies are still in the very early stages but offer considerable promise.

4.9 Latex

Rubber ranks behind only wood in terms of plant industrial products with commercial value. Rubber tree (*Hevea brasiliensis*) is readily transformed with *Agrobacterium*-based techniques [57], but there are no reports of transgenic

modifications in which the products are modified. *Hevea* does have potential in this regard, as the pathway by which latex is produced (the isoprene or terpenoid pathway) naturally leads to hundreds of valuable compounds such as essential oils, carotenoids, sterols, phytohormones, miscellaneous pharmaceuticals (e.g., artemisinin and taxol), and many others. Since latex from *Hevea* naturally contains proteins such as heveins that are dangerously allergenic to some humans, it might prove feasible to eliminate these problematic by-products using transgenics. A hevein promoter that restricts gene expression to the latex-producing cells (laticifers) has recently been used successfully to allow for expression of a GUS reporter in *Hevea* [58].

There is currently considerable interest in the USA in developing latex-producing crops that can be grown in temperate regions. Two plant species from the Asteraceae are the focus of these efforts: guayule (*Parthenium argentatum*) and Russian dandelion (*Taraxacum kok-saghyz*) [59–61]. Guayule, a shrub that is native to the southwestern USA and northern Mexico, was used successfully during World War II to provide a domestic source of rubber, but the practice was abandoned at the conclusion of the war. The revival of guayule is being led by various companies such as Yulex (Maricopa, AZ) and Mendel Biotechnology (Hayward, CA), the Agricultural Research Service of the US Department of Agriculture, and consortia such as PENRA (http://oardc.osu.edu/penra/) and EU-PEARLS (http://www.eu-pearls.eu/UK/). Conventional breeding and selection of guayule has been used to improve rubber yield by as much as 250% [61]. Guayule can be transformed with *Agrobacterium* [62], but attempts to improve the yield or quality by introducing genes for allylic diphosphate synthases led to inconsistent results [63]. A further advantage of latex from guayule is that it lacks the allergens that are problematic with latex from *Hevea*.

Russian dandelion is a perennial plant that is native to Uzbekistan and Kazakhstan. The history of Russian dandelion parallels that of guayule in that it was cultivated extensively and successively in the Soviet Union (and, to a lesser extent, in the USA) during World War II as an emergency source of high-quality rubber, but the practice was mostly abandoned after the war. As with guayule, Russian dandelion is readily amenable to transformation with *Agrobacterium*. In a recent attempt to improve yields and quality, RNAi gene silencing of a gene for polyphenol oxidase led to decreased coagulation of latex and a 4–5-fold increase in latex expulsion, but it is uncertain if such an approach would be useful in other species [64].

Future improvements through transgenics of latex-bearing plants will likely require the development of molecular tools and genomic resources that are currently not available. Such progress may be especially critical for *Hevea*, which has an extremely low genetic diversity and is threatened by a fungal pathogen (South American leaf blight, *Microcyclus ulei*) that is endemic to the Amazon basin. Despite repeated attempts, breeders have not been able to develop blight-resistant lines of *Hevea* using conventional methods [59, 65]. Due to this fungal pathogen, it is no longer possible to grow *Hevea* on a plantation scale in Central or South America, even in the Amazon basin where *Hevea* is native. World rubber supplies would be decimated if the fungus were to become established in Southeast Asia where 80% of global rubber production occurs. Transgenics might provide a means

for saving *Hevea* from future epidemics and perhaps even allow for its reintroduction into South America, a scenario that would have huge social and economic benefits for that region.

Finally, we note that the synthesis of latex through the mevalonate pathway begins with the same precursor (acetyl CoA) as does the synthesis of PHB. Latex-producing plants have a naturally high flux of acetyl CoA and a proven ability to provide products on a long-term nondestructive basis. Would these plants make good candidates for genetic engineering of bioplastic production? Specifically, could the expression of *phb* transgenes be controlled by the laticifer-specific hevein promoters mentioned above? These questions are, to our knowledge, beyond the realm of current research and will likely remain unanswered in the near term. Major resources would be required, and these are unlikely to be committed unless other GE tree programs currently in development prove successful and provide compelling incentives.

4.10 Oils for Biofuels

Woody plants also have great potential to provide oils suitable for use as biodiesel (reviewed by [12, 66]). Despite the recent, huge influx of resources into biofuels, little attention has yet been paid to genetic engineering of woody crops for oil production. Gressel has argued that transgenics are imperative for the development of these crops [12]. Most current crops are poorly suited for this purpose in part because they have been domesticated for millennia for another goal—food production. At present, biodiesel is produced from oil from palm, soybean, and rape, but several other underexploited oilseed-bearing shrubs offer potential for future development. These shrubs include most notably *Jatropha* (*Jatropha curcas*), but also castor bean (*Ricinus communis*), Jojoba (*Simmondsia chinensis*), *Pongamia pinnata*, and *Calophyllum inophyllum* [12].

Of these potential new oil crops, *Jatropha*, a nonfood shrub that requires low agricultural input, has recently risen to the center of attention. Seeds of *Jatropha* contain 30–40% oil, and the resultant biodiesel has properties comparable to petroleum-derived diesel [67]. This plant, which is native to tropical America, is cultivated in tropical and subtropical regions around the world. Numerous countries, most prominently India, are pushing hard for its development as a means of energy independence and income for impoverished, rural populations. Several NGOs and private companies are vigorously promoting *Jatropha* as a source of biofuels that are considerably cheaper than petroleum-based fuels. The resources being applied are substantial. For example, the Syngenta Foundation is developing a germplasm bank and conducting field trials (http://www.syngentafoundation.org/index.cfm?pageID=554), and Synthetic Genomics Inc., in collaboration with the Asiatic Centre for Genome Technology, recently completed the full genome sequence for *Jatropha* (http://www.syntheticgenomics.com/media/press/52009.html). Similar efforts are also being directed to develop genetic resources for palm (*Elaeis guineensis*) oil [12].

The situation as outlined above seems highly encouraging, but a deeper examination reveals some serious impediments to the successful development of oilseed crops such as *Jatropha*. These hindrances are often overlooked in the glow of the eco-friendly appeal of biodiesel. The most pressing restraint has to do with the extreme toxicity of *Jatropha* [12]. The oil (commonly called "hell oil") and the seed proteins are extremely poisonous to humans, either by contact, ingestion, or inhalation. Seeds contain toxic alkaloids and, more importantly, a type of toxalbumin (curcin) that is similar in structure and effect to ricin—the notorious deadly protein from castor bean that has long been feared for its potential as a bioweapon. Furthermore, the oil contains phorbol esters (diterpenoids) that are a strong irritant and a potential carcinogen [68].

Existing cultivars vary widely with respect to content of toxins [69]. Conventional breeding and selection has resulted in some varieties with reduced toxicity but progress has been slow. Transgenics may be the best option to accelerate the development of novel crops such as *Jatropha*. Techniques for transformation of *Jatropha* based on *Agrobacterium* and biolistics have recently been developed [70, 71]. These techniques could be used to eliminate toxins, increase yield, and design crops with traits better suited to standard agricultural practices. Vega-Sánchez and Ronald have suggested a number of specific genes for enzymes and transcription factors that could be targeted to improve either the quality or quantity of plant oils, though none of these have yet been used in transgenic studies of a woody species [66]. Yields in *Arabidopsis* and rape have been increased up to 40% with such strategies.

4.11 Regulatory and Market Obstacles to Developmental Research and Commercial Applications

Transgenic trees modified for changes in feedstock quality or engineered to produce new industrial products face substantial regulatory and market hurdles. We have recently described in detail the applicable laws and derived regulations that pertain to trees and other perennial crops in the USA [72, 73] and the importance of field trials for study of transgenic traits [74], and earlier described the restrictions to research and commercial use of transgenic trees in forestry programs that are "green certified" by the Forest Stewardship Certification (FSC) system in the USA and elsewhere in the world [75, 76]. In brief, transgenic modifications to wood quality, such as modifications to lignin, will in most cases have significant impacts on tree physiology, adaptation, and productivity that are often invisible, or much less expressed, in greenhouse or laboratory environments. For example, Voelker et al., in a recent series of papers [52, 53, 77], showed how the much heralded fast-growth, low-lignin trees described a decade earlier [47, 48] completely failed to display improved growth or desirable changes to wood characteristics in a field environment. Thus, it is essential that new kinds of transgenic genotypes are field tested very early in their development. As discussed above, it is well known that PHB, when expressed to high levels, has deleterious effects on plant health and

biomass yields. Thus, field trials are required to establish whether PHB and other biopolymers can be produced to commercial levels without excessive impact on general plant health.

Unfortunately, field trials are intensively regulated in the USA and most other countries in the world, making them costly and risky to undertake [78]. As a result, very few academic research laboratories and companies are engaged in transgenic field research. Any modifications to wood quality, even when using native genes (often called cisgenes [79]), are regulated as though they are hazards to the environment. Thus, serious federal penalties would be imposed for the same small releases to the environment as commonly occur during conventional tree-breeding research. Transgenic trees that produce bioindustrial compounds such as PHB are regulated to an even higher level of scrutiny, requiring separate harvesting equipment, special treatment of biomass, multiple inspections per year, and other costly management procedures [78]. Because companies that are FSC certified are not allowed to plant any transgenic trees, even for short-term, contained research trials [76], poplar-growing companies such as GreenWood Resources and Potlatch in the USA, who once collaborated extensively with transgenic biotechnology researchers, can no longer host any transgenic research on their lands. Thus, all the costs of field trials must be borne by public sector researchers, who often do not have the land, funds, or means to do so. In addition, even if the technology was successful in providing economic and environmental benefits, such companies could not grow or market products from the trees commercially without losing their certification. This constellation of obstacles provides a strong disincentive to companies and to public sector granting agencies, to invest in research and development.

Despite these impediments, some field trials with GM trees have been accomplished with an encouraging track record of safety. A recent survey of publicly accessible databases worldwide tallied >700 field trials with GM trees with not a single incidence of harm to biodiversity, human health, or the environment [80].

4.12 Prospects

The only transgenic trees for any products currently in commercial use in the world are transgenic papaya in Hawaii and Bt poplar in China [80]. GE papayas are a special case because there was strong support from growers and almost no risk of gene flow because of the cleistogamous flowers (i.e., little to no pollen released) and lack of wild relatives [81]. Without GE technology, papayas would have disappeared as a viable commercial crop in Hawaii due to an uncontrollable epidemic of ringspot virus. Conversely, Bt poplars have benefited from the much more lenient regulatory policies in China where over one million Bt poplars have been planted in the field [80, 82]. Many other transgenic tree projects are currently under development in China and will likely see field applications within the next decade. Thus, it seems likely that China may provide the testing grounds that ultimately establish the

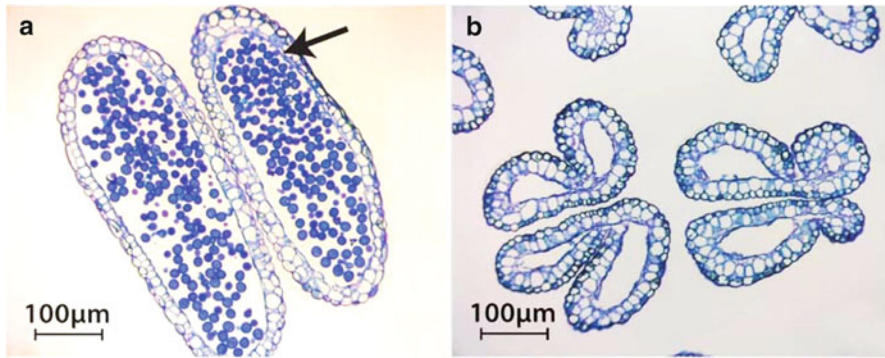

Fig. 4.7 Transverse sections of nearly mature anthers showing pollen grains (*arrow*) in nontransgenic control (**a**) and lack of pollen grains in male sterile anthers (**b**) of field-grown poplar. Sterility was produced by action of an RNase (barnase) whose expression was driven by a tapetal-specific promoter. Interruption of tapetal function prevented formation of normal pollen grains (From [83])

balance of safety and risks, as well as the economic incentives, for transgenic trees upon which western countries could ultimately base future policies.

The most pressing issue of true environmental concern regarding transgenic trees is the spread of transgenes into native populations, an issue particularly relevant to poplar with its many native species and long-distance pollen and seed movement. Thus, it is unlikely that any flowering transgenic poplars will be allowed for field use in the USA until absolute sterility can be assured, or there is substantive evidence from many years of careful study of an absence of significant environmental harms. For biofuels, coppice systems where harvest precedes flowering would obviate this issue. At least five strategies are currently being explored towards sterility technologies for trees grown through flowering (reviewed by [83]). Some of these techniques have been at least partly successful (Fig. 4.7), but not absolutely infallible. Consequently, they may fall short of meeting the "precautionary principle" (PP) in the judgment of some. The PP is interpreted by some environmental groups as the guiding rule that no policy or action should be undertaken unless the scientific proof is completely convincing that there is zero risk of harm to the public or environment from any action or policy. However, for many transgenes, the benefits may outweigh risks, such as for biofuel applications, and these risks may be lower than for use of exotic poplars, as are commonly used with no gene flow restriction. A case in point is the familiar Lombardy poplar, a bud sport mutant of *Populus nigra* that originated in Italy and has been widely planted in the USA for over two centuries with no adverse consequences. Thus, in addition to the market and regulatory factors discussed above, political and legal fights are likely to play a significant role in commercial prospects for transgenic trees—as they have for recently commercialized transgenic crops.

4.13 Conclusions

Transgenic woody plants are emerging as a potential source of numerous biopolymers, both novel and traditional, that are of great economic value to humans. Their development could greatly advance broad goals towards sustainability and independence from fossil fuels. Prominent in this regard are bioplastics, fuel oils, latex, and enhanced wood products. Many of the applications that have been proposed for other GE crops, such as enhanced stress tolerance, improved nutritional qualities, and production of pharmaceuticals and proteins (e.g., vaccines and spider silk polymers), could also apply to woody plants with the added advantage that the perennial nature and general size of trees suggest larger yields as well as substantial secondary uses, especially as biofuels. Technological and policy-based restrictions have slowed their development but are probably surmountable as long as policies are guided by sound science and not political rhetoric.

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Chapter 5
Drugs for Bugs: The Potential of Infochemicals Mediating Insect–Plant–Microbe Interactions for Plant Protection and Medicine

Jürgen Gross

Abstract The results obtained by the analysis of functions and structures of plant, insect, and microbe metabolites in interactions among each other and with their environment in natural settings hold a strong potential for developing new applications in plant protection or even human medicine. By identification and synthesis of chemical compounds responsible, e.g., for the regulation of migration between insects' different host plants, we can gain access to important natural sources for the development of effective strategies using attractive and/or repellent molecules for biotechnical control of plant pests in the context of sustainable agricultural production. In addition, newly detected insect-born infochemicals, which have antifungal or antibacterial activity, bear a potential for the development of new active ingredients for medical purpose.

5.1 General Introduction

Secondary metabolites are produced by many organisms like plants, protozoans, fungi, and bacteria in response to external stimuli. They are often used in pharmacognosy as sources for developing new drugs, and today one- to two-third of the top-selling drugs in the world are derived from such natural products [1, 2]. But also insects produce secondary metabolites which possess an enormous potential for the discovery of new natural products with a value for plant protection and medicine. Today there are over one million described insect species, and some scientists estimate the number of still unnamed species between 3 and 30 million [3]. This means that indeed more than 80 % of the world's biodiversity may be contributed by insects

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[4], but up to now, only a few insect species have been analyzed for molecules relevant for plant protection and medicine. A recently published review describes some examples of specific insect-derived compounds with medically relevant properties and summarizes these topics to emphasize the value of insects and other arthropods as reservoirs of potentially useful new natural products [5]. Furthermore, transgenic expression of antimicrobial peptides in crops has become a novel approach among the strategies to combat phytopathogens in modern plant protection measures. Especially the potential of insect antimicrobial peptides as transgenes for the production of disease-resistant crops has just started to be explored and may provide tools to be ahead of the evolutionary adaptability of phytopathogens [6]. Thus, there is a need of innovative approaches using methods and techniques from chemical ecology, behavioral ecology, and phytochemistry to assess the potential of infochemicals derived from insects, plants, and microbes for their value as new agents for plant protection and medicine.

5.2 What Are Infochemicals?

The communication within and between organisms in multitrophic systems is mainly mediated by infochemicals (Fig. 5.1). These chemical compounds are the words in a chemical language, which is used for communication between the different partners in an ecosystem such as that consisting of herbivorous insects, their host plants, and associated microorganisms. Infochemicals convey information in an interaction between two individuals, leading in the receiver to a behavioral or physiological response. We distinguish between pheromones, which mediate interactions between organisms of the same species, and allelochemicals, which mediate interactions between two individuals that belong to different species [7]. Pheromones can be divided into releaser (behavioral) and primer (physiological) pheromones, depending on their effect on the receiving individual. Examples for releaser pheromones are

Infochemicals				
intraspecific		**interspecific**		
Pheromones		**Allelochemicals**		
Primer-Pheromone	Physiological response		**Releaser**	**Receiver**
Releaser-Pheromone	Behavioral response	**Allomone**	+	-
		Kairomone	-	+
		Synomone	+	+
		Apneumone	o	+

Fig. 5.1 Overview of infochemical terminology (after [7])

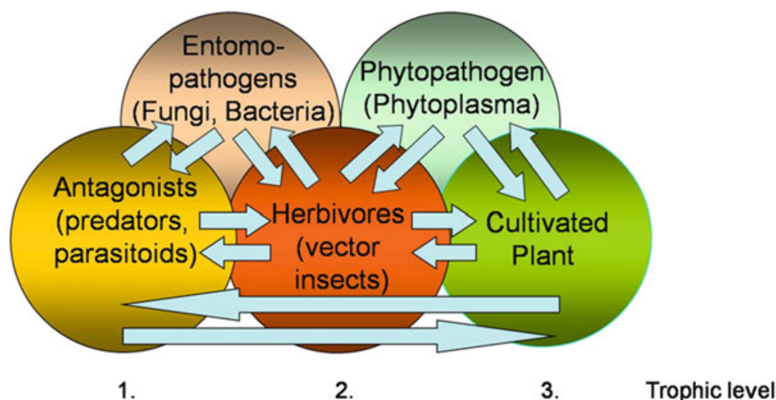

Fig. 5.2 Chemically mediated interactions (arrows) in a terrestrial multitrophic system including plants, insect herbivores, insect antagonists (predators and parasitoids), and pathogens (entomopathogens and phytoplasmas)

sexual pheromones, foraging pheromones, host-marking pheromones, and many more. A well-known example of a pheromone that collates both effects is the queen substance of honeybees (9-oxo-trans-2-decenoic acid), which is produced in the mandibular glands of the queen and which inhibits the development of ovaries in worker bees in the colony (primer effect). It also affects the behavior of the workers, preventing them from maintaining special brood cells for larvae that develop into new queens (releaser effect). Allelochemicals are further divided in allomones (favorable to producer, detrimental to receiver), kairomones (favorable to receiver, detrimental to producer), and synomones (favorable to producer and receiver), depending on whether the transmitted information is favorable or not for the sender or the receiver, respectively [7]. Further examples for the different types of allelochemicals are given in the following sections.

In complex ecosystems, many specific infochemicals are involved in the communication within and between the different partners. Partners in typical agriculture ecosystems are cultivated plants (first trophic level), herbivorous animals like insects (second trophic level), and predators like birds and predaceous insects or parasitoids (animal antagonists: third trophic level). By damaging leaves and buds or sucking on phloem tissue of cultivated plants, herbivorous insects may additionally vector plant pathogens like fungi, viruses, bacteria, and phytoplasmas. The insects may get infected also by pathogens. All of these different interactions are at least in part chemically mediated by infochemicals (Fig. 5.2).

5.3 Applications of Infochemicals in Plant Protection

There are many possibilities for the integration of infochemical use in sustainable plant protection strategies. But in almost any case, only pheromones are used, due to their high species specificity. Sexual pheromones especially are used for monitoring

of pest insects and for disturbing mating behavior (mating disruption technique). Aggregation pheromones can be used for mass trapping, but this type of pheromone occurs only in a few known species. For the so-called attract-and-kill systems, an attractive compound (a pheromone or a kairomone) is combined with an insecticide. Finally, the potential of infochemicals in plant protection applications can be used in complex push-and-pull strategies.

5.3.1 Monitoring

Delta traps consisting of sticky foils or funnel traps filled with toxic fluids are often equipped with artificial dispensers emitting synthetic sexual pheromones. They are widely used for monitoring the population dynamics of insect pests, which is the basis for decisions regarding chemical control and for calculating the optimal date for spraying insecticides. Many insect pheromones were identified in the past and are today commercially available for trapping moths, beetles, flies, and other pest insects. The internet database "Pherobase" lists hundreds of sexual pheromones for monitoring purposes [8], and many of them are commercially available. These species-specific traps have brought applied pest management strategies a long way forward. However, they also do have some weaknesses: the amount of caught specimen is not only influenced by population densities but also by weather conditions and dispenser specifications, and finally they have to compete against the natural sources of pheromones, the females themselves.

5.3.2 Mating Disruption

Another environmentally friendly application method for pheromones in plant protection is the mating disruption technique. Especially in the taxon Lepidoptera, females emit a trail of sex pheromones, the so-called pheromone plume, which is used by the males to locate them. This technique exploits the male insects' natural response to follow the corresponding plume by introducing artificial dispensers emitting synthetic pheromones into their habitat. The synthetic pheromone is a volatile organic chemical designed to mimic the species-specific sex pheromone produced by the female insect. The general effect of mating disruption is to confuse the male insects by masking the natural pheromone plumes and affecting the males' ability to respond to calling females. Consequently, the male population experiences a reduced probability of successfully locating and mating with females, which can lead to termination of breeding followed by the collapse of insect infestation. The internet database "Pherobase" lists currently 149 species for which mating disruption techniques have been proven, and 133 of these are Lepidoptera [8]. But only for the control of a small number of species does this technique have of broader commercial impact (e.g., codling moth *Cydia pomonella*, European grapevine moth *Lobesia botrana*, and grape-berry moth *Eupocoelia ambiguella*).

5.3.3 Mass Trapping

For the biological control of species that produce aggregation pheromones, mass-trapping systems can be developed. Although there are only a few examples of this technique with a satisfactory effectiveness, two prominent examples are known from the control of the bark beetle *Ips typographus* in Europe or the mountain pine beetle *Dendroctonus ponderosae* in North America. Excellent results of the mass-trapping technique were obtained in Central and South America by using sophisticated pheromone traps emitting male-produced aggregation pheromones of different weevil species: e.g., the West Indian sugarcane weevil, the banana weevil, and the American palm weevil [9]. Allelochemicals like kairomones are less used in mass trapping as pheromones but sometimes in combination with pheromones. A well-known example for an effective kairomone is the pear ester (ethyl (*E*,*Z*)-2,4-decadienoate), a characteristic volatile component of ripe pear. This kairomone is an attractant for adult and larval stages of codling moth *Cydia pomonella*. Its identification has allowed the development of several new approaches to successful monitoring and mass trapping of this pest [10–12]. In total, 111 compounds are listed in "Pherobase," which have the potential for mass-trapping applications [8], but only a few are used in today's pest control strategies.

Recently, new findings have been reported for psyllids, which could be used for the development of new chemically lured traps for monitoring and also mass trapping [13–16]. This will be described in more detail below.

5.3.4 Attract and Kill (Lure and Kill)

Another approach using sex pheromones or other attractive compounds is the attract-and-kill or lure-and-kill method. A viscous paste or a spray containing an attractant mixed with an insecticide or granulosis virus can be distributed as small droplets or a film on twigs or leaves of cultivated plants. When a female sex pheromone was used as attractant and a contact insecticide as toxin, males are lured to the droplet, try to mate with it, and finally get killed [17]. In other cases, an insect could be lured by a plant kairomone like pear ester and killed by an insecticide or granulosis virus after feeding on the droplet [18].

5.3.5 Push and Pull

More complex approaches for using the potential of allelochemicals in plant protection are the so-called push-and-pull strategies [19]. They consist of cropping systems in which specifically chosen companion plants are grown in between and around the main crop. Some of these companion plants (intercrop) release

infochemicals that repel insect pests from the main crop ("push" component). Furthermore, crops which attract insect pests more strongly than the main crop are planted in its surroundings ("pull" component) [20]. Future directions for improving existing push-and-pull strategies or the development of new techniques may also include biotechnical applications consisting of artificial dispensers emitting synthetic repellent compounds and traps supplied with synthetic attractants.

Finally, the use of infochemicals in pest control, like most pest management strategies, can be a useful technique but should not be considered a stand-alone treatment program. The likeliness of successful pest control can be improved by a combination of different techniques like push–pull strategies together with a controlled application of natural or synthetic pesticides.

5.4 Research on Chemically Mediated Multitrophic Interactions

When more than two trophic levels are involved in the communication between the individuals of an ecosystem (e.g., cultivated plants, herbivorous insects, predators, and their microbial antagonists), this is described as multitrophic interaction. The main focus of research on chemically mediated multitrophic interactions is to identify the factors that regulate the population dynamics of the different involved species, as was recently shown in a plant–phytoplasma–vector system [13, 16]. Both plant morphology and chemistry, i.e., their primary and secondary (specialized) metabolites, including emitted volatile organic compounds (VOCs), determine the interactions between the first, second, and third trophic level [21, 22]. The ability of herbivores to adapt to plant-produced infochemicals like allomones and kairomones and to develop strategies to cope with them, along with the interactions with antagonists such as predators, parasitoids, or pathogenic microorganisms, has large effects on community composition, ecosystem processes, and finally speciation [22–25].

Fundamental research on the chemical composition and ecological role of infochemicals mediating insect–plant–microbe interactions is eminent for the understanding of multitrophic interactions. Of special relevance for the effective development of new control strategies or detecting new active molecules are those plant–insect systems in which a vector species and a transmitted microorganism are additionally involved. If a pathogen infects both its host and vector, as may occur when some parasites are vectored by insects [26] and also in plant–phytoplasma–insect interactions [27], pathogens and their hosts (plant and vector) have to avoid detrimental effects from each other. In the following sections, I will report on recent research results on the chemical composition and the ecological roles of infochemicals mediating insect–plant–microbe interactions and their potential for plant protection or medical purposes.

5.5 Vectors of Phytoplasma Diseases

Plant diseases caused by small bacteria (0.3–0.5 μm) called phytoplasmas are responsible for more than 700 different plant diseases worldwide that have large economic impact [27]. One of them, *Candidatus* Phytoplasma mali, the agent of the apple proliferation disease, caused a loss of about €25 million in Germany and €100 million in Italy in the year 2001 due to the induction of "witches' brooms" and tasteless undersized fruit in apples (*Malus domestica*) [28]. Phytoplasmas diverged from gram-positive eubacteria but lack a cell wall and have a strongly reduced genome (580–2,200 kb) as well as a limited metabolism. They are transmitted by insects (primarily leafhoppers, planthoppers, and psyllids) and need to infect both their host plants and vectoring insects. While their distribution in plants is limited to the phloem tissue, in animals they can invade many different tissues.

Most of the known vectors of phytoplasmas are phloem feeders that show a nondestructive feeding, but also some true bugs are confirmed vectors, which show a more destructive feeding pattern [28]. The three phytoplasma species belonging to the apple proliferation group causing pear decline, European stone fruit yellows, and apple proliferation are the economically most important fruit tree phytoplasmas (see example above) and are widespread in the temperate regions of Europe [29, 30]. In recent years, the univoltine psyllid species *Cacopsylla picta* was identified as vector for "*Ca.* P. mali" in northeastern Italy [31] and Germany [13, 32]. Additionally, the hawthorn psyllid *C. melanoneura* was identified as another vector of this disease in northwestern Italy [33]. These two psyllid species move during their life cycle between two groups of host plants, one reproduction host (apple or also hawthorn, providing mating area for the adults and food for the offspring; only in spring) and several species of conifers as overwintering host (providing food and shelter during summer, fall, and especially overwintering for the adults) [16]. Both psyllid species use chemical cues for the identification of their alternate host plants during migration [34].

Analysis for the first time of the complex chemically mediated interactions between the apple proliferation phytoplasma (*C.* P. mali), its vectors *Cacopsylla picta* and *C. melanoneura* and their host plants (reproduction host and overwintering host), showed that this phytoplasma lures the highly adapted vector *C. picta* [13] to infected apple plants by changing the odor of the tree. The phytoplasma prods apple trees to produce more ß-caryophyllene that preferentially attracts newly hatched adults of *C. picta* (emigrants) just before emigrating for their overwintering host [14, 15]. By feeding on infected plants, the probability of an acquisition of the phytoplasma increases. After overwintering, the psyllids return to apple plants (remigrants) but now prefer to lay their eggs on uninfected plants, which increases the opportunity to transmit the phytoplasma [16]. Which infochemicals regulate this egg-laying behavior still remains unknown. By developing on apple plants infected by *Ca.* P. mali, the nymphs suffer higher mortality and lower weights compared to development on uninfected plants [16]. In contrast, infection by *Ca.* P. mali is tolerated by adults and seems to have no detrimental effect. Thus, *C. picta*

evolved mechanisms to minimize harmful effects for its offspring emanated by the phytoplasma. Finally, this behavior ensures the development of a new, vital vector generation [16].

We have started to exploit our findings by creating insect traps containing attractive components like ß-caryophyllene for the capture of psyllids for monitoring purposes [14]. While psyllids are very tiny insects and different species are morphologically similar and very difficult to identify, we will reduce the amount of applied chemical insecticides by determining the correct date for spraying with such infochemical traps. Because the infochemical produced by infected plants is attractive to both genders of vectoring psyllids, it could also be possible to develop mass-trapping systems for a more sustainable control of these insects. We have initiated further fundamental research projects on some other phytoplasma–vector–plant systems with the aim to develop species-specific traps for monitoring and mass trapping of several vector species.

5.6 From Pest to Pesticide: New Active Compounds and Their Potential for Plant Protection

Larvae of some insect species, like any leaf beetle or sawfly larvae, possess exocrine glands, which are inserted in the body surface and contain reservoirs of glandular secretions [35–38]. Other species produce allomones, which are stored in their hemolymph and could be released by so far two known mechanisms of autohemorrhage: the so-called reflex bleeding (ladybird beetles) [39] or easy bleeding (some sawflies) [35]. The secreted toxins vary in structure and biosynthetic origin and bear a potential to be exploited for plant protection purposes.

5.6.1 Leaf Beetles (Coleoptera: Chrysomelidae)

The major components secreted by leaf beetle larvae belonging to the taxa *Phaedon*, *Gastrophysa*, *Linaeidea*, and most *Phratora* species are iridoid monoterpenes, which are produced either de novo via the acetate–mevalonate pathway or acquired by sequestration of secondary metabolites from their host plants [40–42], while larvae of *Chrysomela* spp. and the brassy willow leaf beetle *Phratora vitellinae* emit secretions, in which salicylaldehyde is the major component [43]. When feeding upon willows (Salicaceae), larvae of the latter species sequester phenolic glycosides (e.g., salicin) from their host plants as precursors to produce salicylaldehyde. Different biological functions have been reported for larval glandular secretions from beetles: some prevent intraspecific competition [44], while others show insecticidal activities or act as allomones in defense against predators. The repellent activity of leaf beetle allomones acts only against some generalist predators [24, 45],

while it failed in defense against many predatory bugs or the multicolored Asian lady beetle *Harmonia axyridis* (unpublished results). Additionally, in the secretions of many species, an antimicrobial activity was measured [46, 47].

The volatile secretions of some leaf beetle larvae have an additional function: *P. vitellinae* constitutively release volatile components of its glandular secretions to combat pathogens on its body surface [25]. We could identify salicylaldehyde as the major component of their enveloping perfume cloud, which was emitted by furrow-shaped openings of larval glandular reservoirs and which inhibited in vitro the growth of the bacterial entomopathogen *Bacillus thuringiensis*. The suggested role of salicylaldehyde for external disinfection of the microenvironment of the larvae was confirmed in vivo by its removal from glandular reservoirs. This resulted in an enhanced susceptibility of the larvae to infection with the fungal entomopathogens *Beauveria bassiana* and *Metarhizium anisopliae* [25]. The larvae of further leaf beetle species also possess openings for emitting antimicrobial active VOCs [38]. Moreover, the volatile toxins epichrysomelidial and chrysomelidial, which are produced by the mustard leaf beetle *Phaedon cochleariae*, are released in the larval headspace and inhibited the growth of *B. thuringiensis* [38, 48]. Hence, it can be concluded that this recently identified mechanism is more widespread in the taxon Chrysomelini but not solely restricted to this group.

5.6.2 Sawflies (Hymenoptera: Tenthredinidae)

Larvae of the sawfly species *Hoplocampa flava* and *H. testudinea* (Hymenoptera: Tenthredinidae), which feed inside the fruits of plums and apples, respectively, contain in their ventral glands about 20 different acetogenins. These aliphatic chains are represented by aldehydes, acids, alcohols, and esters in both species, while the aromatic benzaldehyde is only abundant in *H. flava* [49]. Fourteen of these compounds, which were available as synthetic molecules, were tested for their antimicrobial activity, and it was shown that 13 of them inhibited the growth of the entomopathogenous bacterium *B. thuringiensis tenebrionis* (Fig. 5.3 [50]). Further, another volatile component known from a leaf-mining sawfly larvae, 8-oxocitral, was proven to be a potent fungicide [51]. Interestingly, all of these sawfly species producing antimicrobially active compounds live endophytically and therefore already possess good protection against microbial agents. An explanation for this may be that the full-grown larvae need to leave the fruit or the leaf mine and have to crawl into the soil in order to pupate, where they may be subject to microbial infestation.

As antimicrobial active compounds emitted by insect larvae are able to inhibit the growth of entomopathogenic microorganisms, they should bear the potential to inhibit also phytopathogenic microbes. Indeed, some of the VOCs emitted by sawfly larvae inhibit the entomopathogenous bacterium *Erwinia amylovora*, the microbial agent of the plant disease called "fire blight." This disease causes annual crop losses of several million Euros worldwide. The growth inhibition caused by synthetic VOCs was similar to the common antibiotic streptomycin, which is regularly used for the

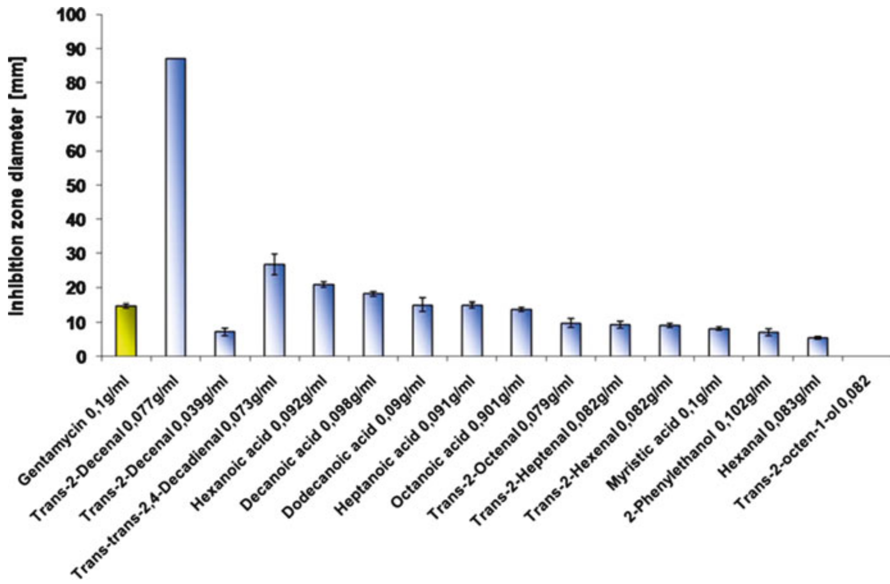

Fig. 5.3 Antibacterial effects of synthetic compounds originating from sawfly species *Hoplocampa flava* and *H. testudinea* (Boevé et al. 1997) compared to antibiotic gentamicin. Inhibition zone test against *Bacillus thuringiensis tenebrionis* (after [50])

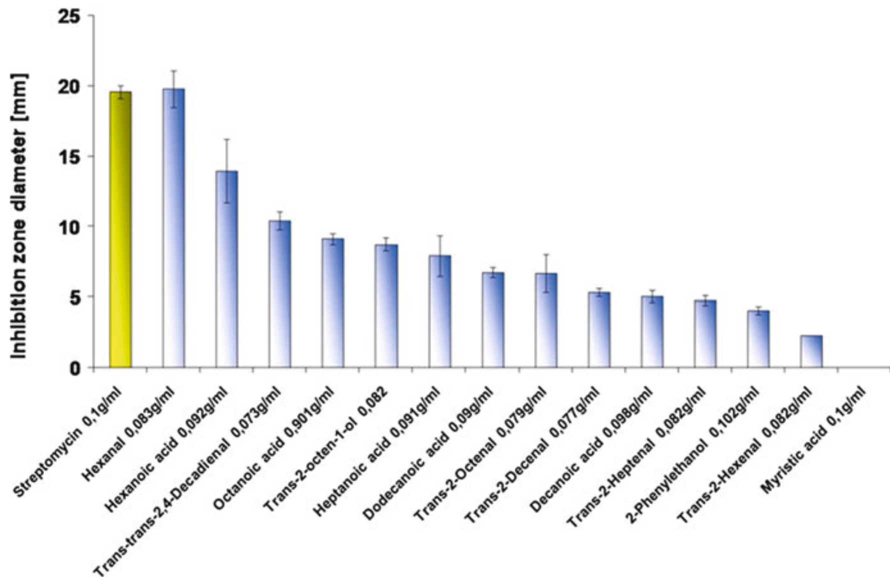

Fig. 5.4 Antibacterial effects of synthetic compounds originating from sawflies [49] compared to the antibiotic streptomycin. Inhibition zone test against *Erwinia amylovora*, the microbial agent of fire blight disease (after [50])

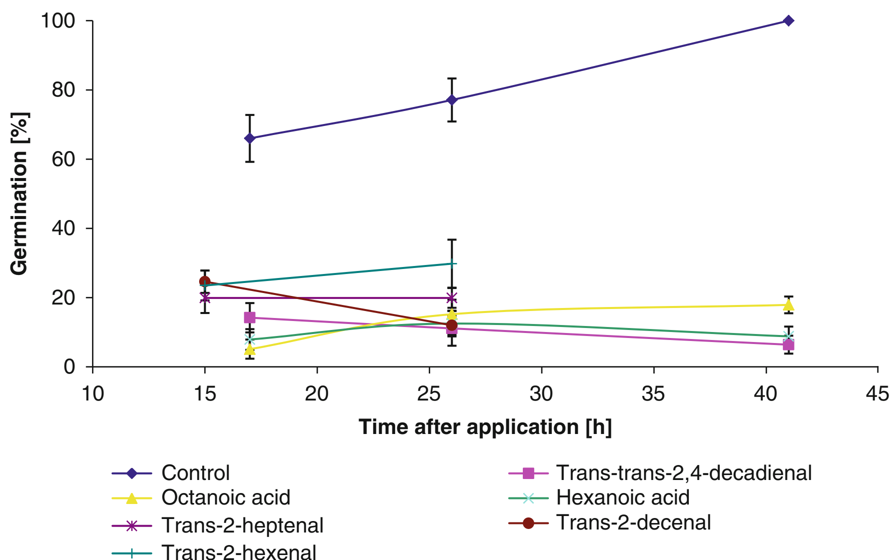

Fig. 5.5 Antifungal effects of synthetic compounds originating from sawflies and leaf beetles in vitro: germination [%] of *Venturia inaequalis* (apple scab)

control of this disease in many countries (Fig. 5.4 [50]). Additionally, some of these compounds inhibit also the germination of the phytopathogenic fungus *Venturia inaequalis*, the microbial agent of the top fruit disease "apple scab" (Fig. 5.5).

Besides such laboratory studies, it is very important to test these effects in further investigations under field conditions. Due to their chemical structures, some of the beetle and sawfly components have also phytotoxic effects. In the light of changing production processes, reduction of pesticide use, increasing resistance against many pesticides, and severe admission restrictions in authorization processes, new directions for the development of innovative and sustainable control of plant diseases need to be established. Especially the control of bacterial plant diseases like fire blight remains very difficult because antibiotics should be reserved to cure human diseases and not be used for large-scale plant protection. As the risk of increasing resistance against such rare medicaments is very high, the development of multidrug-resistant microbes, the so-called superbugs, could be amplified. Thus, the study of chemical–ecological relations of insect–plant–microbes interactions could open a new door to a sustainable, environment-friendly, and ecologically safe plant protection in the twenty-first century.

5.6.3 *Harmonia axyridis (Coleoptera: Coccinellidae)*

The multicolored ladybird *H. axyridis* (Coleoptera: Coccinellidae) is a polyphagous predatory beetle native to Central and Eastern Asia [52]. It was introduced to

America and Europe for biological control of pest insects. Since its establishment in the 1980s, it has become the dominant ladybird in North America [53], and within recent years, it has become established all over Central Europe [54]. *H. axyridis* has attained status as a potential pest in fruit production and a pest in viticulture, as in late summer and fall adults aggregate and feed on soft fruit and grapes. During processing of grapes, the beetles often get crushed or release their hemolymph by reflex bleeding into the must. This causes a specific off-flavor in wine called "ladybird taint." In 2001, US winegrowers suffered from enormous economic losses after processing millions of beetles together with grapes [55, 56].

Recently, it was shown that *H. axyridis* was less susceptible to the entomopathogenic fungus *Beauveria bassiana* than two of the native European species, *Adalia bipunctata* and *Coccinella septempunctata* [57]. In order to elucidate which defense strategy is responsible for its high resistance to diseases, the hemolymph of *H. axyridis* was tested for antimicrobial activity against several gram-positive and gram-negative bacteria, as well as the yeast *Saccharomyces cerevisiae* [58]. The results were outstanding: the hemolymph of both adults and larvae strongly inhibited the growth of all tested bacteria as well as the yeast. Antimicrobial activity in *H. axyridis* was between 100 and 10,000 times higher compared to the activity in the hemolymph from *C. septempunctata*. In contrast to most other insect species where the immune system needs an induction, *H. axyridis* possess a constitutive permanent defense [58].

The chemical agent that is responsible for these remarkable effects is so far unknown. While the activity of a muramidase, which dissolves the murein of bacterial cell walls in *H. axyridis*' hemolymph, was much higher than in *C. septempunctata*, it does not explain the outstanding activity against gram-negative bacteria and yeast. The contents of several methoxypyrazines in *H. axyridis* are also not responsible for its strong immune defense due to their weak antimicrobial activity [59]. Thus, for detection and identification of the responsible active compound(s) and their modes of action, further studies will be necessary.

5.7 Drugs from Bugs: New Active Compounds and Their Potential for Human Medicine

In addition to efforts with plants, marine organisms, and microorganisms, the identification of new active compounds from insects and assessment of their potential for medical purposes should be a focus of pharmacognosy in the future. In case phenomena like the disinfection of the microenvironment by insect volatiles are widely spread in nature, a targeted screening for antimicrobial active volatile secretions from other insect species may result in identification of novel fumigants that could be used as a source of innovative antibiotics or fungicides, or at least for decontamination in hospitals. Furthermore, antimicrobial agents from the hemolymph of strongly immune-defended insects like the multicolored ladybird bear also a potential as leads for developing new antibiotics to combat multidrug-resistant superbugs.

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Chapter 6
Hairy Roots: An Ideal Platform for Transgenic Plant Production and Other Promising Applications

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Abstract The infection of plants by *Agrobacterium rhizogenes* results in a "hairy root" phenotype characterized by rapid growth in hormone-free medium, an unusual ageotropism and extensive lateral branching. The pathological rhizogenicity of *A. rhizogenes* arises from the stable insertion of a region of the *A. rhizogenes* Ri (root-inducing) plasmid into the plant nuclear genome. This plasmid can be engineered to contain foreign genes, which can also be stably inserted into the host genome. As such, *A. rhizogenes* represents a viable alternative for the genetic transformation of plant tissue not readily transformed by *A. tumefaciens*. However, to be effective as a genetic transformation system, the routine regeneration of full plants from hairy root cultures is essential. In this chapter, we report on some important features of hairy roots, describe recent progress in the regeneration of plants from *A. rhizogenes*-derived hairy roots and provide a summary of selected applications. These include the use of *A. rhizogenes* as an efficient system to boost rhizogenesis in recalcitrant plant species and to create new plant varieties and the use of hairy root cultures and *A. rhizogenes*-engineered plants for secondary metabolite production, in phytoremediation and for the production of recombinant proteins (i.e. molecular farming) for the healthcare industry.

6.1 Introduction

The etiological *Agrobacterium rhizogenes* agent, a gram-negative soil bacterium, is responsible for neoplastic disorders [1] such as hairy root syndrome, which is characterized by the emergence of adventitious roots at the wound site of infected plants [2].

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The pathological rhizogenicity of *A. rhizogenes* is a consequence of the stable insertion of the bacterial DNA segment (so-called T-DNA) encompassing a region of the large and extrachromosomal *A. rhizogenes* Ri (i.e. root-inducing) plasmid (200 kb) [3] into the nuclear genome of higher plants [2, 4] including dicotyledons, gymnosperms and some monocotyledon species [5] (Table 6.1). Fundamental

Table 6.1 Selected source plants and *A. rhizogenes* strains used to generate hairy roots. For each species, the tissue source (explant) and *A. rhizogenes* strain used to generate a stable hairy root culture is listed

Plant species	Infected explants	*A. rhizogenes* strain(s)	References
Arabidopsis thaliana	Stems	R1000	[6]
Armorica rusticana	Leaves	pRi 15834	[7]
Artemisia annua	Shoot tips of meristems	LBA9402, 9365, 9340, K599, 15834	[8]
	Stems	ATCC15834	[9]
Asimina tribola	Seedling cuttings, shoots of rooted seedlings	MT232 (TR105 mutant), MSU-1 (A4 wild type)	[10]
Atropa belladonna	Shoots	T37	[11]
Beta vulgaris	Leaf stalks	LBA 4402	[12]
	Plant	LMG-150	[13]
Brassica juncea	Stems	A4	[14]
	Seedlings	15834	[15]
Brassica napus	Radicles	ARqua1	[16]
		AR 25	[17]
Brugmansia candida		LBA9402	[18]
Camptotheca acuminate	Cotyledons, hypocotyls, leaves	ATCC15834, R1000	[19]
Cassia obtusifolia	Cotyledons	LBA9402	[20]
Casuarina glauca	Hypocotyls	A4RS	[21]
Catharanthus roseus	Seedlings	15834	[22–24]
	Leaves	A4	[25]
Chenopodium amaranticolor	Stems	A4	[14]
Cichorium intybus	Seedlings	15834	[26]
Coffea Arabica	Hypocotyls	A4RS	[27, 28]
Cucumis sativus	Cotyledons	ATCC15834	[29]
	Plantlet	K599	[30]
Cucurbita pepo	Cotyledons	8196	[31]
		8196 and 15384	[32]
Daucus carota,	Roots, leaves and stems	1855, 2659, 8196	[33]
		15834	[2]
Duboisia spp.	Shoots	T37	[11]
Echinacea purpurea	Leaves	43057	[34]
Eschschoizia californica	Leaves	R1000	[35]
Fagopyrum esculentum	Leaves	R1000	[36]

(continued)

Table 6.1 (continued)

Plant species	Infected explants	*A. rhizogenes* strain(s)	References
Gentiana macrophylla	Leaves and stems	A4, R1000, 11325, LBA9402	[37]
Glycine max	Cotyledons	K599	[38–43]
	Meristem with one fully expanded leaf,		[44]
	Plantlet		[45]
	Hypocotyls	R1000 and K599	[46]
Glycyrrhiza uralensis	Hypocotyls	LBA9402	[47]
	Cotyledons	R1601	[47]
	Cotyledonary nodes	A4	[48]
Gynostemma pentaphyllum	Leaves	ATCC15834	[49]
Kalanchoe blossfeldiana	Leaves	ATCC15834	[50]
Levisticum officinale	Seedlings	A4	[51]
Linum album	Cotyledon	LBA9402	[52]
Lotus japonicas	Hypocotyls	ATCC15384	[53]
Lycopersicon spp.	Hypocotyls	8196	[54]
Medicago truncatula	Radicles	ARqua1	[55]
Mitragyna speciosa	Stems and leaves	ATCC15834	[56]
Nicotiana benthamiana	Plant	R1000	[57]
	Seedlings	A4	[58]
Nicotiana tabacum	Roots, leaves and stems	1855, 2659, 8196	[33]
Panax ginseng	Roots	A4	[59, 60]
Papaver somnifereum	Leaves	R1000	[35]
	Hypocotyls	LBA 9402, 15834	[61]
Phaseolus vulgaris	Roots, leaves and stems	1855, 2659, 8196	[33]
	Cotyledon axes	K599	[62]
Pinus halepensis	Embryos, seedlings, shoots	LBA9402	[63]
Pisum sativum	Stems	R1000	[64]
Plumbago rosea	Shoots induced from young nodes		[65]
Populus tremuloides	Hypocotyls	ARqua1-pTSC5	[66]
Rauvolfia micrantha	Cotyledons and leaves	ATCC15834, LMG-150, A 2/83 and A 20/83	[67]
Rhodiola sachalinensis	Roots, stem, leaves and cotyledons	A4	[68]
Solanum tuberosum	Tuber	15834	[2]
Thlaspi caerulescens	Seedlings	15834, A4, TR105	[69]
Vigna aconitifolia	Seedlings	A4	[70]
Withania somnifera	Cotyledons and leaves	ATCC15834, LMG-150, A 2/83 and A 20/83	[71]
	Roots, stems, hypocotyls, cotyledonary nodal segments, cotyledons and young leaves	R1601	[72]

research has strengthened our understanding of the different stages underlying the molecular mechanisms leading to the transfer of the *A. rhizogenes* T-DNA to the nuclear plant genome (e.g., [73–76]). Nevertheless, details of the molecular events, such as the involvement of host factors during the insertion of the T-DNA into the plant genome, are still lacking. After its insertion, the T-DNA, comprising loci found between the specific T_R and T_L border sequences (25-bp repeats), confers a high genetic and biochemical stability to the transformed roots. Different strains of *A. rhizogenes* vary in their capacity for transferring T-DNA from bacteria into plant genomes, and the hairy roots resulting from these strains can exhibit different morphologies [8]. Agropine-type *A. rhizogenes* strains, termed hypervirulent, are characterized by the Ri plasmid *pRi*A4, *pRi*1855 or *pRi*LBA9402 [77] and can independently integrate two separate T-DNA regions into the plant genome. One region carries the root loci (*rol*) genes (T_L-DNA) responsible for the hairy root phenotype [33], while the other carries the *aux*1–2 genes (T_R-DNA) [78] encoding enzymes controlling auxin biosynthesis. T_R-DNA, which plays an ancillary role in hairy root initiation because of its *aux1–2* genes, also carries the genes coding for opine synthesis [79]. Indeed, transformed roots produce specific opines, derivatives of amino acids and sugars [80–82], which are used as a nutritive source by the agrobacteria. Sequencing of the agropine-type T_L-DNA made possible the identification of 18 open reading frames, including the 10, 11, 12 and 15 orfs [83] corresponding to the *A*, *B*, *C* and *D rol* genes [84]. Other *A. rhizogenes* strains, harbouring the Ri plasmids *pRi*8196, *pRi*2659 and *pRi*1724 encoding mannopine, cucumopine [85] and mikimopine, respectively, possess one single T-DNA (i.e. T_L-DNA) capable of inducing transformed roots from the *rol* genes.

Synergistic action of the *rol A, B, C* and *D* loci leads to the formation of adventitious roots in wounded plants. But it has been shown that each *rol* gene is able on its own to promote root formation in *Nicotiana tabacum.* Nevertheless, each gene differs in its efficiency; for example, *rolB* is stronger than *rolA* and *rolC* [86–88]. In host tissues, the endogenous *rol* gene promoter directs expression in the parenchyma and phloem ray cells (*rolB* promoter) and in the phloem companion cells (for the *rolC* promoter). Sucrose and indole acetic acid (IAA) have been reported as necessary for the induction of adventitious roots because the *rolC* promoter is regulated by sucrose and the *rolB* promoter is regulated by IAA [89, 90]. The proteins expressed from the *A, B and C rol* genes are located in the nucleus, plasma membrane and cytoplasm, respectively, of competent plant cells [89, 90]. *rolB* and *rolC* genes code for specific and different β-glucosidases. However, while the *rolB*–β-glucosidase has been shown to be able to hydrolyse indole–β-glucosides and the *rolC*–β-glucosidase can release cytokinins from their glucoside conjugates [91, 92], chemically induced *rolC* gene expression did not lead to cytokinin hydrolysis in *N. tabacum* transgenic plants [93]. Thus, the in vivo role of *rol* gene products remains unclear. Gorpenchenko et al. [94] emphasized the surprising capacity of the bacterial *rolC* gene to initiate somatic embryogenesis after its integration into the genome of higher plants, whereas the action of the *rolC* gene is conventionally known to induce hairy roots. No similar effect has been described in other plant species.

Overall, the involvement of *rol* genes in oncogenesis is not well understood [95]. Nevertheless, it is known that *rol* genes affect plant metabolism and, in particular, levels of polyamines and phytohormones. For example, the levels of putrescine, spermidine and spermine in tobacco plants expressing *rolA* were approximately double those of control plants [96], while those transformed with either *rolB*, *rolC* or *rolABC* showed little change in polyamine content. Consistent with this was a 165% increase in arginine decarboxylase activity in *rolA*-transformed roots. Similarly, incorporation of the *rolC* gene into *Solanum lycopersicum* L. affected IAA but not ABA levels. In addition, the *rol* genes are apparently involved in the stimulation of secondary metabolism with remarkable stability, over long-term cultivation [95]. For example, the impact of the absence of T-DNA orfs on monoterpenoid indole alkaloids (MIA) biosynthetic pathway gene regulation and alkaloid accumulation in *Catharanthus roseus* hairy roots has been demonstrated, wherein T_R-DNA insertion appeared to be an important factor in gene regulation and alkaloid accumulation [97].

The capacity of *A. rhizogenes* to infect plants and induce hairy roots has been widely used for establishing in vitro cultures of transgenic roots [82]. Many methods of bacterial infection have been successfully employed, and several factors, including the bacterial concentration, time of co-culture, temperature, light and sugar content, have been found to influence the transformation efficiency of the wounded plant cells [98]. Plant species also influence the success of the *Agrobacterium*-mediated transformation. To date, over a hundred dicotyledonous species, belonging to 26 families, have been reported as being amenable to the generation of hairy root cultures [99–101] (Table 6.1). Typically, inoculation is done on surface-sterilized wounded explants such as stem segments, cotyledons, leaves, roots, petioles and/or leaf discs. After 1–4 weeks, primary adventitious roots emerge from the wound site of the plant tissues, with each primary root likely deriving from the differentiation of a single plant cell transformed by *A. rhizogenes*. After excision, each root placed onto solid medium gives rise to a hairy root clone, which can be cultured on solid medium or in liquid medium [102] (Fig. 6.1).

Anatomically, the roots generated by *A. rhizogenes* infection are similar in their structure to wild-type roots with some notable exceptions. For example, the root anatomy of California (*Eschscholzia californica* Cham.) and opium (*Papaver somniferum* L.) poppy differed in cell arrangement and structure between wild-type and transgenic hairy roots [35]. More specifically, root epidermal cells from transgenic (hairy) roots were loosely packed and gave rise to many root hairs. In contrast, wild-type roots showed a more tight packing of epidermal cells with few giving rise to root hair extensions. Both wild-type and transgenic roots possessed a central stele surrounded by cortical cells. Peres et al. [54] observed that cortical cells and vascular bundles were highly proliferated in tomato (*Lycopersicon* spp.) hairy roots and the cortical cells were loosely packed, suggesting that hairy roots of different species can have different anatomies. Some of these differences arise from the typical hairy root syndrome characterized by a rapid growth in hormone-free medium, an unusual ageotropism and extensive lateral branching [104, 105]. Furthermore, in the hairy roots of some species, amyloplasts can be distributed in lower amounts than in their corresponding wild-type roots, thus possibly leading to a weak gravitropism

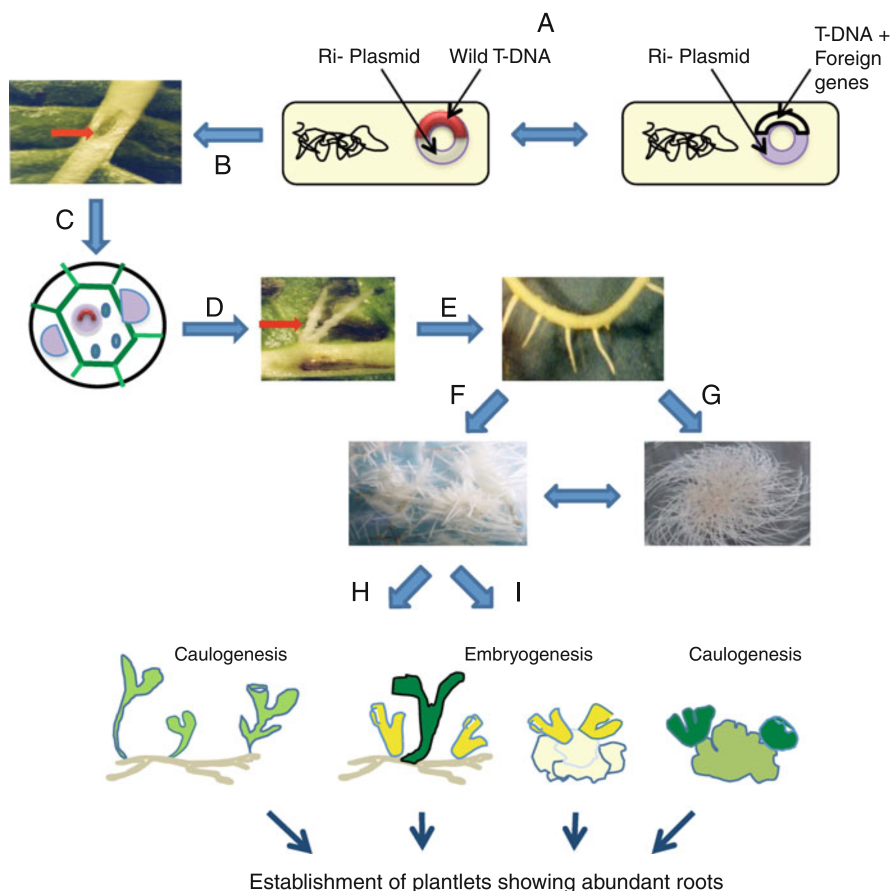

Fig. 6.1 *Stages of hairy root formation*. *Agrobacterium rhizogenes* harbouring either wild-type T-DNA or a foreign gene inserted into the Ri plasmid (**a**) is used to infect a wounded plant or plant organ (*red arrow*) (**b**). As part of the infection process, the T-DNA portion of the Ri plasmid is inserted randomly into the host plant genome (**c**). After a short period of time (usually 2–3 weeks), roots begin to emerge from the wound site (*red arrow*) (**d**), from which individual clones can be selected (**e**). Once established, hairy roots can be cultured indefinitely on either solid (**f**) or liquid (**g**) medium and can be manipulated to generate shoots either directly (**h**) or via an intervening callus or somatic embryo stage (**i**). Adapted from [103]

[17, 106]. While hairy roots have been established in more than 100 species, hairy root anatomy has not been fully explored, especially at the cell wall chemistry level. Otherwise, where hairy root morphology is described, it has been found to be generally similar to that of wild-type roots [16, 17, 107]. Hairy roots are sensitive to endogenous auxin, the treatment with which can lead to callus and root proliferation, leading to a different morphology in hairy roots than in wild-type roots [31, 52, 54, 59, 108–110].

The generation of hairy roots via *Agrobacterium*-mediated transformation opens up new avenues for numerous fundamental and practical applications. In this chapter, we

highlight some characteristics of hairy roots that make them amenable to be used as tools for genetic engineering processes, produce secondary metabolites, induce rooting in recalcitrant species, create new plant varieties in horticulture as well as have potential usefulness in phytoremediation and molecular farming.

6.2 Hairy Roots: A Genetic Engineering Platform

Hairy roots represent a true engineering platform based on the genetic horizontal transfer of wild or recombined Ri T-DNAs (Fig. 6.2) from *A. rhizogenes* into host plant tissue. To date, various plant species have been transformed by integrating T-DNAs into their genome (Table 6.1). The first transgenic plants engineered by

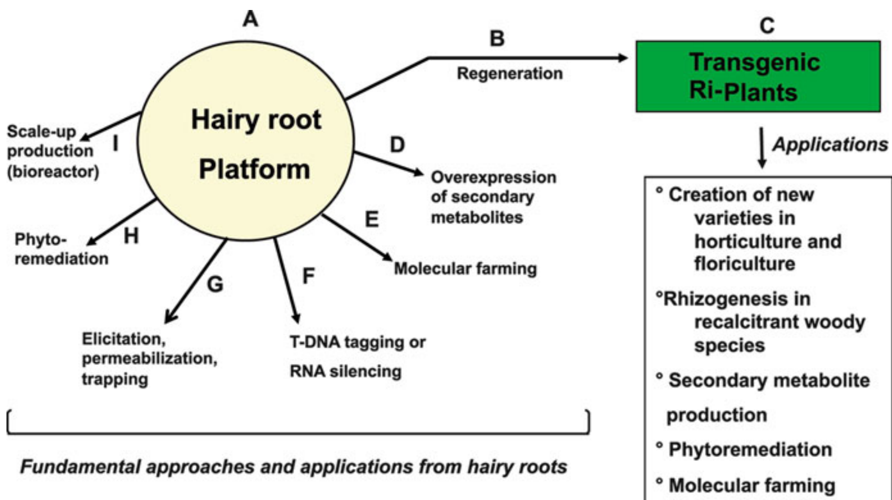

Fig. 6.2 *Hairy roots: a true bioengineering platform.* (**a**) Formation of hairy roots is coincident with the transfer of the wild-type or recombined T-DNA of *Agrobacterium rhizogenes* into the plant genome (see also Fig. 6.1). (**b**) Transgenic plants can be regenerated from buds, embryos and callus developed from hairy root cultures, with the resulting transgenic Ri plants (**c**) used for multiple applications, similar or complementary to those described for hairy root cultures. For example, hairy root cultures can be used directly as a metabolic engineering platform for overexpression of valuable secondary metabolites and recombinant proteins from transformed roots cultured in vivo (**d**, **e**). Similarly, T-DNA activation tagging and RNA silencing followed by transcriptome analyses can be applied to hairy root cultures (**f**) to facilitate the discovery of new metabolic genes via gain-of-function (T-DNA activation tagging) or of a loss-of-function (RNA silencing) mutation. (**g**) Elicitation treatment, including the addition of signal molecules (e.g., MeJA) to the hairy root culture medium, can help to optimize secondary metabolite production. Also, permeability agents added to the culture medium can facilitate the release of metabolites that accumulate in hairy root cells. Trapping systems, such as hydrophobic polymeric resins, can be used for the absorption and harvest of secondary metabolites secreted in the culture medium. (**h**) Phytoremediation can be achieved with hairy root cultures, providing a mechanism to sequester, degrade and rend harmless soil, sediment and water surface pollutants. (**i**) Hairy root cultures are amenable to the scale-up of production in bioreactors, opening the possibility to accumulate significant amounts of secondary metabolites or recombinant proteins on a large scale. See text for further details and examples

A. rhizogenes transfer of foreign genes were described in 1984 (see [111]). The use of *A. rhizogenes* as a gene transfer system makes possible the development of different or new strategies, for example, enhanced accumulation of valuable metabolites in transgenic roots [103, 112], producing recombinant proteins, and discovering new genes using T-DNA activation tagging [113] or RNA silencing processes followed by transcriptome analyses, in species that are not readily transformed using *A. tumefaciens*. The T-DNA activation tagging approach permits the creation of gain-of-function mutations, while gene silencing allows loss-of-function analyses [114].

6.2.1 Hairy Root-Activation Tagging System

Activation tagging, resulting from the random insertion of either promoter or enhancer sequences into host genomic DNA, can lead to novel, dominant (or semi-dominant) mutations through the overexpression of genes adjacent to the T-DNA [115]. Such mutations are observed in primary transformants [116]. A dominant mutation can arise through the random insertion of T-DNA constructs carrying a series of strong enhancer sequences positioned next to the right- or left-hand T-DNA border and can generate overexpression of endogenous genes [102, 116].

For example, tagged "hairy roots" from *Arabidopsis thaliana*, *Solanum tuberosum* and *Nicotiana tabacum* resulted from transformation by *A. rhizogenes* harbouring the binary vector pHR-AT (hairy root-activation tagging) or its derivative pHR-AT-GFP. Each binary vector contained, within the same T-DNA, a cluster of *rol* (*A, B, C*) genes together with the right border facing four tandem repeats of the cauliflower mosaic virus 35 S (CaMV) enhancer element. Molecular analyses of the pHR-AT-GFP-transformed *Arabidopsis* lines revealed that expression of genes adjacent to the T-DNA insertion site was significantly increased [113]. Such vectors provide unique tools that can be applied to understanding distinctive features of root biology, such as identifying genes involved in root-specific secondary metabolism or in regulating induced secondary metabolism in response to various environmental stresses [113]. Busov and collaborators [116] demonstrated the power of this dominant gene-tagging approach in *Populus*, wherein the ability to identify genes controlling development of perennial plants through phenotypic analysis in field environments was demonstrated.

Another recent strategy, based upon a methyl jasmonate (MeJA) inducible system, was applied to cell suspensions of *Catharanthus roseus* in order to identify new genes involved in the metabolic pathways of terpenoid indole alkaloids [117]. Likewise, addition of MeJA to the *Panax ginseng* hairy root cultures revealed genes involved in the biosynthesis of secondary metabolites through the analysis of 3,134 expressed sequence tags (ESTs) [118]. Among the *P. ginseng* transcripts identified, several genes encoding enzymes involved in ginsenoside biosynthesis, for example, squalene epoxidase, cytochrome P450s and glycosyltransferases, were found. Among them, a novel oxidosqualene cyclase (OSC) gene was identified by analysis of the transcripts. However, such a gain of information is only possible from the genes responding to the MeJA treatment [114].

6.2.2 RNA Silencing Using Hairy Root Transformation Mediated by *A. rhizogenes*

RNA silencing is a natural genetic control mechanism involved in virus resistance, genome maintenance and developmental control in plants and hairy root systems provide a powerful tool for loss-of-function analyses of genes using this approach [102]. Kumagai and Kouchi [119] described the first successful RNA silencing in roots using hairpin RNAs (hpRNAs) in *Lotus japonicus* roots and root nodules. Transgenic plant lines of *L. japonicus,* expressing β-glucuronidase (GUS) under the control of constitutive or nodule-specific promoters, were "supertransformed" with *A. rhizogenes* harbouring molecular constructs for the expression of hpRNAs possessing sequences complementary to the *GUS* coding region. GUS enzyme activity was either decreased or silenced in more than 60% of the hairy root lines generated. Likewise, silencing of the *GUS* gene was also observed in symbiotic nodules developed from hairy roots of *L. japonicus* [119]. Such a transient RNA silencing system using hairy root transformation makes possible experiments designed to understand the function of genes expressed in roots. Indeed, more recently, *A. rhizogenes*-based RNAi approaches have been used to study the role of lipoxygenase genes in soybean root nodule development [46] and GmMYB176 in the regulation of soybean root isoflavonoid biosynthesis [120]. Importantly, these studies were not possible using *A. tumefaciens*-based transformation systems, due in part to the poor transformation efficiency and long regeneration time of soybean. In tobacco, *A. rhizogenes*-mediated RNAi downregulation of ornithine decarboxylase in hairy roots resulted in reduced levels of pyridine alkaloids [121] and an inability of transformed tissue to produce alkaloids in response to wounding.

6.3 Update of Ri Plant Regeneration

In order to be useful as a genetic transformation system, there has to be a process to generate viable whole plants from hairy roots, a feat that has proven to be more difficult than expected in several species. Indeed, there is an absolute need to regenerate whole plants from hairy roots for their use in applications in horticulture/floriculture (e.g., creating new varieties), in metabolic engineering to overexpress genes responsible for the production of secondary metabolite synthesized in aerial parts of plants, and/or in phytoremediation and molecular farming applications (Fig. 6.2).

In the mid-1980s, development of the *A. tumefaciens*-mediated transformation provided the basis for modern plant biotechnology (reviewed in [122]) and gave rise to a technology of choice for developing transgenic plants through the use of disarmed versions of the *A. tumefaciens* Ti plasmid [77]. *Agrobacterium rhizogenes*, a closely related species to *A. tumefaciens* [4], represents a viable alternative for the genetic transformation of plant tissue not readily transformed by *A. tumefaciens* [80, 123].

6.3.1 Similarities and Differences Between *Agrobacterium* Species

Agrobacterium tumefaciens and *A. rhizogenes* both belong to the *Rhizobiaceae* and are gram-negative bacteria, ubiquitous in the rhizosphere. The pathogenicity of each species depends on a large plasmid called Ti (for tumour-inducing) in *A. tumefaciens* and Ri (for root-inducing) in *A. rhizogenes*. Both *Agrobacterium* species transform plant cells in a similar way through horizontal T-DNA transfer, causing development of plant tumours and opines in the target plant tissues [123]. T_R-DNA of the agropine-type Ri plasmid contains the regions *aux1–2* that are highly homologous to those called *tms1–2* carried by the *A. tumefaciens* Ti plasmid. The *tms1–2* genes code for enzymes involved in biosynthesis of IAA [124]. Moreover, numerous homologies between Ti and Ri plasmids have been reported by Jouanin [125] from studies on the restriction map of agropine-type Ri plasmid. Both neoplastic disorder types are responsible for producing specific opines necessary for *Agrobacterium* growth [81, 82].

Ti and Ri plasmids, carrying a set of oncogenes and opine catabolism genes, include the vir region, which plays a crucial role in the T-DNA transfer to the plant genome. A common secretion system to the two bacteria, involving these *vir* genes, begins with bacterium-plant attachment, followed by induction of the expression of the VirA1/VirG proteins by specific host signals [74, 126, 127]. A single-stranded (ss) T-DNA molecule, termed T-strand, i.e. one mobile copy of the T-DNA, is produced by the combined action of the VirD1/VirD2 protein complex. In the bacterial cell, the VirD2 protein nicks the border sequences in the T-DNA and covalently attaches the T-strand to the 5′ end [74]. A secretion signal in VirD2 shuttles the immature T-complex into plant cells along with other Vir molecules (VirE2/VirF) [128, 129]. This T-complex is translocated into the host cell by a VirB (B1 to B11)/VirD4 type IV secretion system [129–131] which requires interaction of a bacterial T-pilus [132]. Afterwards, the T-strand is coated with numerous (more than 600 monomers) VirE2 proteins that form a mature T-complex and assure the protection of the T-strand for its travel through the host cytoplasm/nucleus and make possible its integration to the plant nuclear genome.

Most *vir* genes carried by Ti/Ri plasmids and involved in the T-DNA secretion system are similar. Nevertheless, the *virE2* and *virE1* genes (a secretory chaperone) are critical for pathogenesis by *A. tumefaciens*, but missing from the Ri plasmid (and genome) of some *A. rhizogenes* strains [73, 133]. Instead of *virE2*/*virE1*, the Ri plasmid *GALLS* genes encode two full-length GALLS (GALLS-FL, GALLS-CT) proteins [134, 135], which are essential for promoting the nuclear import of the T-strands [128]. Both GALLS proteins can be required to substitute for VirE2; sometimes, only GALLS-FL is necessary in some plant hosts [129]. *A. rhizogenes*, in order to palliate the loss of *vir*E1 and *vir*E2, appropriated an unusual conjugation gene (GALLS) for restoring its ability to import T-DNA to plant genomes [129]. The GALLS genes are able to restore pathogenicity to *virE2* mutant *A. tumefaciens* [73, 128, 135]. Similarly, GALLS and VirE2 interact with host proteins such as

importin a4 [128, 129]. VirE2 seems to only interact with VIP1 and VIP2 host proteins [129, 136, 137]. This selective interaction with host proteins, which may influence the transformation efficiency [129], can partly explain why the GALLS amino acid sequence possesses different functional domains and modes of action when compared with the VirE2 proteins [133].

GALLS-FL contains domains for ATP binding and helicase motifs similar to TraA (a strand transferase involved in bacterial conjugation), nuclear localization and C-terminal signals for translocation mediated by the VirB/D4 IV secretion [129, 133, 135]. By contrast, binding of VirE2 to T-strands makes possible the movement ssDNA without ATP hydrolysis [129].

Gene replacements by homologous recombination are precious events in genetic engineering: “the Holy Grail of plant science and biotechnology”, wrote W. Ream [129]. The GALLS protein, in contrast to the VirE2, is not abundant enough to coat the total length of T-strands; thus, the DNA associated with it can remain available for homologous recombination [129, 138].

Despite large similarities, *A. tumefaciens* and *A. rhizogenes* differ in several important points. In fact, the tumour (a crown gall tumour) induced by *A. tumefaciens* [139] corresponds to a mixture of undifferentiated cells, a true mosaic of transformed and some non-transformed cells, whereas the root proliferation resulting from the incorporation of *rol* genes from the Ri T-DNA into the host genome results in transformed cells organized into organs [4, 33, 86, 140]. Another fundamental difference between wild-type *A. rhizogenes*-derived hairy roots, compared with virulent strain *A. tumefaciens*-derived transformants, is that hairy roots can naturally evolve into plantlets with high regeneration rates, whereas only disarmed versions of the *A. tumefaciens* Ti plasmid can lead to transgenic plants [123].

When *A. tumefaciens*-mediated transformation attempts led to low transformation efficiencies with *Lotus corniculatus,* Jian et al. [141] obtained transgenic superroot-derived *L. corniculatus* plants after a fast and highly efficient (up to 92%) transformation with *A. rhizogenes*. They concluded that such a system, coupling transformation and regeneration, provides a powerful tool for gene function testing in *L. corniculatus.*

6.3.2 Overall View of the Plants Transformed by A. rhizogenes

Pioneer work on *Brassica napus* regeneration has shown that fertile Ri plants were able to sexually transmit the hairy root-phenotype trait to their descendants [104, 142, 143]. Recent analyses on the Mendelian heredity of *A. rhizogenes*-transformed *Medicago truncatula* plants confirmed that the Ri T-DNA genes segregated out in the progeny [144]. Genetically stable, fertile plants have been regenerated from hairy roots of several plant species [104], which opens up the possibility for fundamental studies and multiple potential applications by coupling the advantages of the transgenic roots and entire plants.

Indeed, from 1984 to 2001, *A. rhizogenes*-mediated transformation allowed the establishment of hairy root cultures of 116 plant species [101] (Table 6.1) and the

regeneration of whole transgenic plants belonging to 79 plant species [130] (Table 6.2). Plant species greatly differ in their susceptibility to *A. rhizogenes* strains [178], and depending on the *A. rhizogenes* strain used, transgenic plants spontaneously develop from hairy roots, as exemplified by *Allocasuarina verticillata*, an atmospheric nitrogen-fixing tree [105]. Different *A. rhizogenes* strains have been tested with numerous plant species and tissues in order to determine the best transformation efficiency leading to hairy roots (Table 6.1). In addition, the successful development of transgenic shoots depends on the in vitro culture conditions (Table 6.2) and several physiological parameters, such as the explant juvenility [179]. Hairy roots maintained as organ cultures for a prolonged time were still able to regenerate cytologically normal shoots [180]. *Agrobacterium rhizogenes* has proven to be as efficient a tool for plant genetic engineering as its relative *A. tumefaciens* and thereby extends the host range of plants amenable to genetic modification [123]. And compared with *A. tumefaciens*, hairy roots can often naturally lead to the plantlets. In Japan, such plants, when derived from "wild" *A. rhizogenes* strains, are free from the legal controls of GMOs [181, 182].

A survey of the literature reveals that plant regeneration from hairy roots can take several routes. After *A. rhizogenes*-mediated transformation, shoots/plantlets can develop from adventitious roots either spontaneously or directly, i.e. without or with exogenous hormone (Table 6.2), via the formation of adventitious buds or somatic embryos [77] (Fig. 6.1). On the contrary, some hairy roots, after transfer to hormone-containing media, require a callusing phase before developing entire plantlets [180] (Table 6.2, Fig. 6.1). These routes to Ri plant regeneration are discussed more fully in the following sections.

6.3.3 Spontaneous or Direct Plant Regeneration from Buds

The regeneration of plants from hairy roots can be achieved in the absence of hormonal treatments or may require a specific hormonal regime (Table 6.2). Fast shoot regeneration, without the use of selective agents, prevents the development of chimerical plants and limits the risk of somaclonal variation [183].

6.3.3.1 Shoot Regeneration from Hairy Roots

Adventitious shoots spontaneously initiated on transformed root segments of *Centaurium erythraea* [150, 151, 184], *Aesculus hippocastanum* [145] and *Duboisia hybrid* [155] hairy roots placed on medium devoid of hormonal supplement. Up to 100% of hairy roots of *D. hybrid* cultured with IAA (0.1 mg/l) and BAP (2 mg/l) produced shoots, whereas only 33% of roots showed visible shoots on medium containing 5 mg/l BAP [155], suggesting that an appropriate hormone regime can facilitate shoot formation. By contrast, shoot regeneration capacity was lacking in

Table 6.2 Regeneration of plants from *A. rhizogenes*-transformed tissues. For each entry, the regeneration conditions used to generate shoots and/or plantlets from *A. rhizogenes* transformed tissue is summarized. Entries are organized according to whether the shoots were regenerated directly from hairy roots, callus or embryos

Species	Explant	*A. rhizogenes* strain(s)	Regeneration conditions[a]	References
Plants/Shoots regenerated from hairy roots directly				
Aesculus hippocastanum	Androgenic embryos	A4GUS	Liquid MS with 6.75 mg/l BAP and after solid MS with 2.25 mg/l BA	[145]
Allocasuarina verticillata	Epicotyl, cotyledon and hypocotyls	A4, 2659	For first strain: MH medium with 0.25 mg/l NAA and 0.1 mg/l BAP for second strain: without hormones	[105]
Angelonia alicariifolia	Leaves	A13 and D6	Half strength MS (basal salts medium) 20% sucrose and 2% gellan gum, NAA and BAP	[146]
Apocynum venetum	Roots	LBA9402, R1000	Half strength MS without hormones	[147]
Astralagus sinicus	Seedlings cut into two pieces in the hypocotyl	DC-AR2	Highest N of shoots: MS 5–7.5 mg/l BAP somatic. Embryos developed from transformed roots: 7.5–10.0 mg/l 2,4-D	[148]
Brassica napus	Stems	A4	3 mg/l 2,4-D liquid medium followed by transfer to solid medium 1 mg/l NAA, 0.5 mg/l IPA, 0.5 mg/l BAP, 10% glucose, 10% sucrose and 10% mannitol	[143]
Catharanthus roseus	Hypocotyls	R1000	MS with 0.225 mg/l BAP and 1 mg/l NAA	[149]
Centaurium erythraea	Stems, internodes of seedlings	A4M70GUS	½ MS solid medium culture and after on MS without growth regulators	[150]
	Leaves and leaves pieces of 0.5 cm	LBA 9402	Woody plant medium in dark and without growth regulators	[151]
Citrus aurantifolia	Inter-nodal segments	A4	7.5 mg/l BA and 1.0 mg/l NAA	[152]
Crotalaria juncea	Cotyledon segments 1 cm^2	A4, A13(MAFF02-10266)	B5 medium with 3.0 mg/l BAP	[153]
Daucus carota	Discs	A4	Macro and micro elements of Monnier (1976), vitamins of Morel and Whitmore (1951), 5 ml/l of 0.0056 mg/l of $FeSO_4$-7 H_2O, 0.0075 mg/l of NaEDTA and 0.03 mg/l sucrose	[104]

(continued)

Table 6.2 (continued)

Species	Explant	*A. rhizogenes* strain(s)	Regeneration conditions[a]	References
	Root	pRi15834	Solidified Monnier's medium, 0.08 mg/l 2,4-D and 0.15 mg/l KT	[109]
Duboisia myoporoides	Leaves	ATCC15834	BAP 5 mg/l	[154]
Duboisia myoporoides X D. leichhardtii	Leaf discs approximately 1 cm	A4	Spontaneous regeneration of shoots from hairy root lines	[155]
Hypericum perforatum	Seedlings	A4M70GUS	Liquid and agar solidified medium with increasing concentrations of sucrose (0.5 –8% mg/l)	[156]
Isatis indigotica	Plant	R1601, ATCC15834 and A4	MS media without growth regulators had highest hairy root proliferation	[157]
Nicotiana tabacum	Seedlings	A4	Macro and micro elements of Monnier (1976), vitamins of Morel and Whitmore (1951), 5 ml/l of 0.0056 mg/l of $FeSO_4$-7 H_2O, 0.0075 mg/l of NaEDTA and 0.03 mg/l sucrose	[104]
Pelargonium spp.	Leaves and inter-nodal segments	A4 and LBA9402	Leaves: MS media 5.0 mg/l KT and 1.0 mg/l NAA Internodal segments: MS media 8.0 mg/l and 1.0 mg/l NAA	[158]
Plumbago rosea,	In vitro raised shoots	A4	MS supplemented with 2 mg/L of BAP	[159]
Saussurea involucrata	Root segments	R1601, R1000 and LBA9402	Shoots formed on calli derived from hairy roots on 0.5 mg/l IBA	[160]
Solanum nigrum	Hypocotyls or leaves	ATCC 15834	Solid MS medium, 30% sucrose and 0.2% Phytagel	[161]
Solanum tuberosum	Internodal segments	ATCC 15834	Spontaneous regeneration of shoots from hairy roots on B5 liquid medium	[162]
Taraxacum platycarpum	Cotyledons, hypocotyls and roots	15834	MS with 1.0 mg/l BAP	[110]
Plants/shoots regenerated through a callus stage				
Alhagi pseudoalhagi	Cotyledon and hypocotyls	A4	Acetosyringone: Vital MS and 3.0 mg/l BAP	[163]
	Shoot segments	A4	3.0 mg/l BAP and 0.5 mg/l NAA	[164]

Crotalaria juncea	Cotyledon segments 1 cm^2	A4 and A13 (MAFF02-10266)	B5 medium with 3.0 mg/l BAP	[153]
Lycopersicon esculentum	Hypocotyls	DCAR-2	1.0 mg/l GA3 and 0.5 mg/l NAA	[165]
Medicago truncatula	Seedlings were inoculated at the radicle sections	ARqua1	Fahraeus medium, later MTR-2 medium and for bud formation MTR-3	[144]
Mentha piperita	Leaves	IFO14554	½ B5 medium with 0.18 mg/l NAA, 2.5 mg CPPU, 50 mg/l Km, 10% sucrose, 5% gellan gum at pH 5.8	[166]
Nicotiana tabacum	Leaves	LBA4404	MS media with 1.0 mg/l BAP, 0.1 mg/l NAA and 300×10^{-3} mg/l Km, 500×10^{-3} mg/l Cb	[167]
Onobrychis viciaefolia	Hypocotyl segments	LBA9402	Callus induction from hairy root segments: MS with 0–2 mg/l 2,4-D and 0-0.5 mg/l BAP. Shoots from calli on MS0 medium	[168]
Panax ginseng	Stems	A4	Half strength macrosalts, 15% sucrose, 4 mg/l BAP, 0.5 mg/l GA3	[94]
Pelargonium spp.	Internodal segments	A4 and LBA9402	Callus from intermodal segments on MS media with 10 mg/l KT and 1 mg/l NAA Shoots from callus on MS media with 0.5 mg/l BAP and 0.1 mg/l NAA	[158]
Rehmannia glutinosa	Leaves, stems and petioles	15834	½ MS media, 0.2 mg/l KT and 3 mg/l BAP	[169]
Tylophora indica	Leaves and stems	LBA9402 and A4	Hormone free basal media	[170]
Plants/shoots regenerated through somatic embryos				
Aralia elata	Pieces of petioles, roots 1 cm and leaves 1 cm^2	ATCC 15834	Somatic embryos from hairy root in dark on ½ MS with 1.0 mg/l 2,4-D. Plantlets from somatic embryos on ½ MS, hormone free	[171]
Astralagus sinicus	Seedlings cut into two pieces in the hypocotyl	DC-AR2	Highest N of shoots: MS 5–7.5 mg/l BAP. Somatic embryos developed from transformed roots : 7.5–10.0 mg/l 2,4-D	[172]
Coffea canephora	Somatic embryos derived from hypocotyls	A4 with vector having borders of *A. tumefaciens*	½ MS salts and B5 vitamins, 0.45 mg/l IAA, 0.25 mg/l BAP, 2% sucrose	[173]

(continued)

Table 6.2 (continued)

Species	Explant	*A. rhizogenes* strain(s)	Regeneration conditions[a]	References
Coronilla varia	Segments of cotyledons and hypocotyls of 15d-old seedlings	15834	Embryogenic calluses on MS medium containing 0.2 mg/l 2,4-D, 0.5 mg/l NAA and 0.5 mg/l KT. Plants from embryogenic calluses on MS with 0.5 mg/l KT and 0.2 mg/l IBA	[174]
Cucurbita pepo	Embryogenic tissues	Wild strain 8196	Spontaneous	[175]
Medicago sativa	Cotyledons	LBA9402	MS0	[176]
Zea mays	Infected callus	R1601, A4 and ATCC 15834	MS with 1.6 mg/l ZT and 0.4 mg/l NAA	[177]

[a] *2,4-D* 2, 4-Dichlorophenoxy acetic acid, *BAP* 6-benzylaminopurine, *Cb* carbenicillin, *CPPU N*-(2-Chloro-4-pyridyl)-*N'*-phenylurea, *GA3* gibberelic acid, *IBA* indole-3-butryic acid, *IPA* β-indole propionic acid, *Km* kanamycin, *KT* kinetin, *MH* Mueller-Hinton, *MS* Murashige and Skoog, *MS0* Murashige and Skoog medium without hormone, MTR-2, MS basal medium supplemented with 5.0 mg/l 2,4-D, 0.5 mg/l BA, 3% (w/v) sucrose, 5.0 mg/l phosphinothricin, 250 mg/l cefotaxime and solidified with 0.8% w/v agar; MTR-3: MS basal medium supplemented with 2% (w/v) sucrose, 2.5 mg/l phophinothricin, 250 mg/l cefotaxime and solidified with 0.8% (w/v) agar; *NAA* naphthalene acetic acid, *ZT* zeatin

some *D. hybrid* species [154]. *Solanum nigrum* hairy roots, placed in the same cultural conditions, spontaneously generated plantlets able to produce in vitro flowers and fruits, in contrast to the untransformed plants [161]. No buds were observed at the apical meristems; instead, shoots emerged unequally in the central portions of these roots [161].

Nevertheless, the shoot regenerative capacity of the hairy roots from some plant species can be improved or expressed in media containing combinations of growth regulators. In *Taraxacum platycarpum*, shoots from hairy roots and non-transformed roots developed without hormone addition, but when the culture medium was supplemented with 1 mg/l BAP, the highest frequencies of bud formation were found for the transgenic roots (88.5%) compared with controls (31.7%) [110]. In *Aesculus hippocastanum*, the bud number was significantly increased by adding exogenous cytokinin, for which BAP generated less vitrified shoots than kinetin and thidiazuron (TDZ) [145]. Hairy root-derived shoots of *Catharanthus roseus* were directly regenerated, with efficiency up to 80%, but in the presence of 13.3 or 31.1 μM BAP and 5.4 or 10.7 μM naphthalene acetic acid (NAA) in combination [149].

6.3.3.2 Rooting from the Regenerated Shoots

Shoots of *Centaurium erythraea*, which needed to be individually cultured in the presence of 0.1 mg/l IAA and 1.0 mg/l BAP, were subsequently rooted at a high frequency (68–97%) on MS without any growth regulators before their transfer to greenhouse conditions [184]. In *A. hippocastanum*, rooting was improved with 10 mM auxin and occurred more readily in the presence of indole butyric acid (IBA) (80% of explants) than IAA (40% of explants) [145]. Sometimes, as observed in *Alhagi pseudoalhagi*, successive transfers onto the nutritive media are necessary to generate roots on shoots derived from *A. rhizogenes*-generated callus. Such plants, which exhibited intensive rooting, could overcome environmental constraints such as the lack of water [163].

6.3.4 Transgenic Embryos Derived from Hairy Roots

Whole plants can be regenerated from hairy roots or hairy root-derived callus, coming from the *A. rhizogenes* transformation of various explants through the spontaneous or indirect development of somatic embryos (Fig. 6.1, Table 6.2). Somatic embryos resulting from one cell (direct embryogenesis) or only a few cells (indirect embryogenesis) are widely used for large-scale clonal propagation of plants with genetic conformity to the mother plants, and multiplication of F1 hybrids [185]. It is also an effective method for the regeneration of genetically stable single cell-derived plants [186], and otherwise recalcitrant species, including trees [187]. Moreover, direct somatic embryogenesis is also an interesting strategy for genetic transformation of plant species [188] of economic or medicinal value [71].

Embryos of *Astragalus sinicus* were obtained from hairy root segments cultured with 2,4-D (7.5–10 mg/l) [172], a key growth factor known for inducing somatic embryogenesis. Likewise, spontaneous regeneration of plants from hairy root-derived shoots was commonly associated with embryogenic callus formation in *Panax ginseng* [189] and *Cucurbita pepo* [175]. After transfer to light, these embryogenic calluses gave rise to somatic embryos, which developed into shoots [170]. Interestingly, embryogenic calluses of *C. pepo* spontaneously appeared from 3-year-old hairy root cultures initially derived from *A. rhizogenes* wild strain 8196 infection [32]. Root segments and petioles of *Aralia elata*, producing medicinal compounds, gave rise to hairy roots from which two transgenic lines cultured with 2,4-D (1.0 mg/l) led to embryogenic calluses. At the cotyledon-shaped stage, such embryos could germinate into plantlets, and 100% of those were successfully acclimatized under greenhouse conditions [171]. Gorpenchenko et al. [94] reported that *A. rhizogenes rolC*-gene expression alone was sufficient to induce calluses of *P. ginseng*-containing tumour clusters to develop into roots or embryogenic cell lines, from which embryos and shoots emerged on the medium devoid of growth regulators.

6.3.5 Callusing Phase Before Shoot Regeneration

A third, albeit indirect, path leading to whole plants from hairy roots makes use of an intermediate callus phase. In *Panax ginseng*, *Cucurbita pepo* and *Tylophora indica,* for example, shoots and yellow calluses simultaneously appeared from transformed root clones placed in medium devoid of hormones and stored in the dark [170, 175, 189]. In other cases, hairy root segments derived from *A. rhizogenes* transformation require an exogenous auxin and/or cytokinin treatment to induce callus, from which shoots and plantlets can be obtained (Fig. 6.1, Table 6.2).

The formation of hairy root-derived callus from *Mentha piperita* was superior on B5 medium supplemented with 1 μM NAA, 10 μM 4-*N*-(2-chloro-4-pyridyl)-*N′*-phenylurea and 10% coconut powder, compared with the absence of hormones. Shoots and roots evolved from callus pieces placed onto medium without added hormone, and the regenerated plantlets were transferred to greenhouse conditions [166]. Hairy roots of *Crotalaria juncea* L., placed onto BAP-containing media for 3 months, produced callus with green spots from which adventitious shoots developed into plantlets [153].

6.3.6 Plant Regeneration Without Any Intervening Hairy Root Stage

Plants transformed with wild-type Ri T-DNA strains of *A. rhizogenes* often display abnormalities inherent to the hairy root phenotype [87, 90, 104]. As such, this particular aspect of *A. rhizogenes*-based genetic transformation has not been encouraging

for the use of such regenerants in programmes of applied research, notably in agronomical fields. An alternative approach to producing *A. rhizogenes*-derived plants, without any aberrant phenotype expression, would be to create disarmed versions of the wild-type Ri T-DNA genes, as has been done with *A. tumefaciens* [77]. Such an approach implies that the oncogenes responsible for the hairy root phenotype would be deleted from the wild-type Ri T-DNA. This deletion should be without consequence for T-DNA insertion, since with the exception of the right and left borders, none of the other T-DNA regions are required for the horizontal transfer of foreign genes into the plant genome [190]. Therefore, the plants could be transformed with *Agrobacterium rhizogenes,* as with *A. tumefaciens*, making use of its recombined plasmid-carrying foreign genes. Indeed, it is simultaneously possible to delete the *rol* genes and to replace the T-DNA loci by additional genes conferring resistance to antibiotics, herbicides, parasites and insects or overexpressing recombined proteins or secondary metabolites before integration into the plant genome [191]. For example, expression of a synthetic gene encoding cryptogein, able to mimic a pathogen attack, increased calystegine's and certain flavonoids' levels in whole Ri plants of *Convolvulus arvensis* and *Arabidopsis thaliana,* respectively [192].

An alternative strategy to overcome potentially undesirable phenotypes associated with *A. rhizogenes* transformation is to use binary plasmids constructed for use in *A. rhizogenes* with *A. tumefaciens* as the vector, obviating the need of novel vector sets [193], or alternative transformation approaches. Two types of vectors can be used for genetic engineering processes. The first type, a co-integrative vector (i.e. as a carrier of additional genes), is introduced into *Agrobacterium* by conjugation or electroporation. Then, it will be directly inserted into the disarmed Ri or Ti plasmid [194]. The second type, termed a binary vector, contains two plasmids. The main Ti plasmid, for example, carries only virulence genes because its T-DNA has been removed, and the second plasmid carries the foreign gene inserted between the border sequences [195]. Therefore, using binary vectors, the problem of aberrant phenotype can potentially be bypassed. For example, transgenic *C. canephora* plantlets, resulting from the genetic transfer of disarmed Ri T-DNA, were normal in appearance, i.e. without the typical root proliferation frequently associated with the hairy root syndrome [173]. More specifically, one plasmid carries only the virulence functions and the wild T-DNA, whereas the second vector contains the gene of interest flanked between the T_R and T_L border sequences. In this way, wild-type Ri T-DNA and the gene of interest are integrated in different regions of the host genome. The first generation of Ri plants coming from root-derived adventitious buds will present the hairy root syndrome. However, segregation of the Ri T-DNA and gene of interest among the transgenic plants of the second generation allows the elimination of all Ri plants displaying the hairy root syndrome [173]. Similarly, transgenic plants derived from leaf explants of *Centaurium erythraea* have been generated through co-culture with an *A. rhizogenes* strain carrying a binary vector [184]. Using binary vectors and sonication-mediated transformation process has made possible the development of transgenic plants of *Coffea canephora* [173]. That is, establishing hairy roots of *C. canephora* by direct co-culture of hypocotyl segments with the wild-type *A. rhizogenes* strain A4 was not possible. So, to develop transgenic

plants more readily for large-scale propagation, primary and secondary somatic embryos of *C. canephora* were transformed using a sonication-based method in the presence of *A. rhizogenes* strain A4 harbouring only the binary vector pCAMBIA 1301. From this, the development of transgenic plants without a hairy root phenotype was observed from these embryos due to the integration of the *A. rhizogenes* T-DNA devoid of the *rol* genes [173]. Consequently, the deletion of oncogenes and/or additional genes of interest inserted on the T-DNA opens up opportunities to utilize *Agrobacterium* vectors for a wide range of genetic transformation.

6.4 Promising Applications of Hairy Roots and/or Ri Plants

The generation of genetically stable hairy root cultures and/or fertile hairy root-derived plants from many plant species has opened up the possibilities for fundamental studies and multiple potential applications of biotechnology to otherwise recalcitrant plant species. Significant progress has been made in various biotechnological fields, including horticulture/floriculture, rhizogenesis of recalcitrant plants, production of secondary metabolites, phytoremediation and molecular farming (Fig. 6.2); these are discussed below.

6.4.1 Morphological Phenotype Expressed in Hairy Root-Derived Plants Used in Horticulture/Floriculture

6.4.1.1 Morphological Ri Phenotype of Regenerated Plants

Introducing new varieties of plants based on the selection of phenotypes arising from *A. rhizogenes*-mediated transformation is a novel biotechnological approach for improvement of plant species in the horticulture/floriculture industry [181]. Ri plants, compared to wild-type plants, often show morphological differences resulting from expression of the *rol A, B, C* and/or *D* gene located on the T-DNA inserted into the host genome [104, 170, 196]. Plants of *Nicotiana tabacum, Brassica napus* and other species transformed by *A. rhizogenes* exhibited an abnormal phenotype characterized by traits such as wrinkled leaves, short internodes, dwarfism, an increase in the number of the lateral shoots, flower size, number of flowers and abundant plagiotropic roots with extensive branching [87, 104, 142, 143, 178]. Similarly, before their transfer to soil in a greenhouse, transgenic, somatic embryo-derived plantlets of *Aralia elata* displayed distinct morphological features compared to the mother plants [171]. Likewise, these Ri plants can also show specific, characteristic traits due to the expression of one single *rol* gene, such as short internodes and wrinkled leaves (for *rol A*) or reduced length of stamens and a reduced apical dominance due to abundant and plagiotropic roots (for *rol B*) or short internodes and increased flowering (for *rol C*) [180].

However, not all plants derived from hairy roots show an altered phenotype. For example, half of the *Catharanthus roseus* plants, regenerated from hairy roots by Choi et al. [149], showed a normal morpho-type. Likewise, Satheeshkumar et al. [159] observed only a few morphological variants among 38 hairy root-derived plants of *Plumbago rosea* after their transfer to the field. These authors confirmed the presence of T-DNA in these "normal" regenerants by Southern blot analysis. Both plants of *Alhagi pseudoalhagi*, arising from hairy root-derived calluses [163] and Ri plants of *Hypericum perforatum* [156], were characterized by normal leaf morphology and good stem elongation. Nevertheless, these plants had a more extensive root system than non-transformed plants.

6.4.1.2 Plant Improvement and Selection of the Ri Phenotype for Creating Original Varieties

Classical breeding, particularly in floriculture, makes possible the creation of new plant varieties. However, the available gene pool for expressing new characteristics is genetically limited to the parental background [196]. In this context, the integration of *rol* genes into the plant genome may be a suitable approach in plant breeding programmes to introduce novel agronomic or aesthetic characteristics [197]. Cytological abnormalities can lead to the creation of novel plant and flower shapes and sizes as well as flower colours advantageous in horticulture or floriculture [196]. For example, *Kalanchoe blossfeldiana* is an economically important potted plant in Europe; but to be marketed, must have a compact growth habit. Yet, *K. blossfeldiana* naturally has an elongated growth habit. Usually, the desired compact growth can be obtained through the application of chemical growth retardants, compounds that are more or less hazardous to human health and the environment. An alternative approach for producing compact genotypes has been achieved by inserting *rol* genes into the plants. That is, Ri lines of *K. Blossfeldiana* plants, differing in their degree of Ri phenotype, displayed internodes clearly shorter than wild type and resulted in a compact growth compared to control plants [50]. A dwarf growth habit is a particularly desirable trait for some ornamental plants. Indeed, plants from *A. rhizogenes*-derived hairy roots of the interspecific hybrid *G. triflora* × *G. scabra* show numerous variant lines of dwarf plants, with blue, pink or white flowers. In Japan, these dwarf variants are widely used as ornamental plants and cut flowers [181]. Likewise, sterile *Angelonia salicariifolia* leaves were co-cultured with two mikimopine-type *A. rhizogenes* strains to introduce a dwarf trait into this ornamental plant. A transformed root segment-derived plants exhibited phenotypic alterations such as dwarfness and smaller leaves [146].

6.4.2 Hairy Roots: An Efficient System to Boost Rhizogenesis in Recalcitrant Plant Species

Vegetative propagation can be limiting for some economically important tree species grown for fruit production or as ornamentals because of the laborious process required for root initiation. While rooting of woody plants can be improved by

auxin treatments, such as with IBA, or the use of polyamines, *A. rhizogenes* has also been used to improve rhizogenesis in recalcitrant species, including forest trees (reviewed in [180, 198, 199]). In *Asimina triloba* L. cuttings, no rooting resulted from any hormonal or other applications, while transformation with *A. rhizogenes* (strains MSU-1 and MT-232) led to approximately 33% of cuttings from seedlings forming roots [10]. Genetic transformation of tree explants and their subsequent regeneration have been achieved from some genera. Previously, high-frequency transformation (17–92%) of many species of poplar, such as cottonwoods, using wild-type *Agrobacterium rhizogenes* has been achieved, although the *Populus* species remain particularly recalcitrant to genetic transformation (132). *A. rhizogenes*-mediated transformation has been used to obtain transgenic plants from 55 genera. A diverse range of dicotyledon plant families is represented, including one gymnosperm family (*Pinus* genera) (133). Subsequently, other woody plants have been genetically transformed by *A. rhizogenes*, such as *Aralia elata* (134).

6.4.3 Hairy Roots: A Promising Strategy for Secondary Metabolite Production

6.4.3.1 Tremendous Medicinal Potential in Plants

Increased secondary metabolite production in hairy roots cultured in vitro, over their wild-type counterparts, may be seen as one of the most exciting spin-offs of biotechnology [102]. Due to their great richness in secondary products, such as indole alkaloids, terpenoids and phenolics, plants represent an immense source of therapeutic and/or industrial compounds. For example, plant-derived biomolecules, such as vinblastine (*Catharanthus roseus*), morphine and codeine (*Papaver somniferum*), digoxin (*Digitalis lanata*), quinine and quinidine (*Cinchona* spp.), artemisinin (*Artemisia annua*) and taxol (*Taxus baccata* or *Taxus brevifolia*), are efficient in the treatment of different pathology types relating to cancer, or cardiovascular and metabolic disorders, and/or other infectious diseases [200]. Many plant metabolites are commercially available as drugs, flavours, food additives, cosmetics, fragrances and insecticides [201]. In spite of this, it is generally acknowledged that less than half of the estimated 200,000+ natural products produced by plants have been identified, and even fewer still have been examined for their biological properties (e.g., ecological, pharmacological) [202].

6.4.3.2 Biomolecule Production in Whole Plants or In Vitro Cell Cultures

Natural production/accumulation of phytochemicals in plants is frequently low and depends on the physiological and developmental stage of the plant [203], as well as cultural conditions, climatic changes or geopolitical constraints. Very high quantities

of plants are required to extract sufficient amounts of target secondary metabolites, but a large-scale extraction can represent serious economic and ecological problems [99]. In the worst case scenario, an over-exploitation of wild plants for commercial purposes can lead to their near extinct or their being listed as protected, endemic or rare wild species, for example, *Saussurea involucrata* in China [204]. Likewise, the intensive collection of the aerial parts of *Centaurium erythraea*, which produces secoiridoid glycosides possessing pharmacological activity for the treatment of the gastrointestinal disorders, endangers this species [184].

In view of the increased demand for secondary metabolites, particularly as drug precursors, there is a need to develop new efficient approaches for production. The production of natural products in in vitro plant cell cultures seemed to be a promising alternative to their extraction from wild or cultured plants. Indeed, natural products such as shikonin, berberine, ginsenosides and other compounds of interest have been successfully produced by in vitro cell cultures [205]. Indeed, numerous natural products can readily accumulate in vitro in callus or cell suspension cultures [206]. For example, cell suspensions of *Ginkgo biloba*, newly initiated from female prothallus, produced two specific types of terpene trilactones: bilobalide and ginkgolides, possessing a strong anti-PAF (platelet activating factor) activity [207]. However, the major disadvantage to using undifferentiated cell cultures, limiting their extension to the full-scale production of these metabolites, is their genetic instability that can lead to a rapid loss of accumulation potential [208]. Indeed, the levels of the secondary products often remain low in undifferentiated cultures [99, 209], and most often, they decrease after several subculture cycles [210].

6.4.3.3 Hairy Root Cultures: An Alternative Way for the Enhanced Accumulation of Secondary Metabolites in Culture

Cultures of differentiated tissues, such as roots or shoots, offer a viable alternative for solving the progressive decrease in quantity of secondary metabolites often seen in cell suspension cultures maintained in vitro [211]. Indeed, numerous secondary compounds with valuable therapeutic properties accumulate in "normal" plant roots. But root harvesting is destructive, resulting in loss of the tissues. Due to their prolific growth and suitability for continuous culture, hairy roots are a potential source for the continuous production of desirable phytochemicals [179] including terpenoids, alkaloids and phenolics [212].

Agrobacterium rhizogenes-derived root cultures have been widely investigated for their in vitro production of plant metabolites [7, 82, 104], at similar [19, 67, 213–216] or higher levels [112, 217–220] than those present in the wild-type roots or plants. The genetic stability of hairy roots is in part responsible for the stable production of secondary metabolites in them [221]. Likewise, the wild-type profile of secondary metabolites is often conserved in hairy roots [222]. However, sometimes, the amount of specific biomolecules is altered (positively/negatively) by transformation with armed *A. rhizogenes* strains [35]. Jung and Tepfer [103] reported that

genetic transformation by the Ri T-DNA was able to stimulate, at once, biomass and tropane alkaloid production in *Atropa belladonna* and *Calystegia sepium* roots grown in vitro. While the creation of hairy root clones from various explants can lead to the production of desirable bioactive compounds comparable to the "normal" roots, considerable variation in biomass exists from one clone to another. However, metabolic production and root biomass can be optimized by modifications to several culture parameters, such as the medium composition, growth conditions or elicitation treatment, for example, with methyl jasmonate (MeJA) [49]. Jasmonic acid, an important signalling molecule in plants that is synthesized from the octadecanoid pathway, controls the production of numerous secondary metabolites [68, 223]. In this way, the accumulation of biomass and production of salidroside were enhanced in the *Rhodiola sachalinensis* hairy roots after addition of an elicitor and a metabolic precursor to the culture medium [224]. Precursor feeding and elicitation, cell permeabilization, trapping molecules and stress conditions have been investigated in attempts to raise the secretion of secondary metabolites in culture [224–226], as has been demonstrated for betalaine from red beet hairy roots [13]. Likewise, Medina-Bolivar et al. [227] reported that hairy roots of *Arachis hypogaea* offer a valuable production platform for the production of naturally occurring stilbenes such as resveratrol and its associated derivatives, which possess antioxidant and antitumour activities [228]. After a single 24-h sodium acetate elicitation, *trans*-resveratrol was induced and secreted at superior (60-fold) levels, compared with untransformed plants [227]. As a cautionary note, however, while elicitation may be suitable to enhance the production of several bioactive molecules, there remains a genetic limit to the amount of product that can be produced [202].

6.4.3.4 Scale-Up of Hairy Root Cultures

A major problem with organ cultures as natural product factories remains the difficulty in growing them on a large scale. With respect to hairy root cultures, this is further exacerbated by the need for specific bioreactors to ensure homogenization of the transgenic roots [229]. In addition, the maintenance of sterility remains a significant constraint in scaling-up for the commercial exploitation of hairy root cultures, leading to very high production costs [180]. However, recent progress in the scaling-up of hairy root cultures makes this approach a promising tool for industrial processes [114]. Two scale-up systems are worth mentioning. The first, based on an airlift concept, has been used for producing artemisinin from *Artemisia annua* hairy root cultures in a bubble column bioreactor [226] and for accumulating phytoestrogens (for treating menopause and different oestrogen-dependent tumours) through an efficient co-culture process of shoots and hairy roots of *Genista tinctoria* in a basket bubble reactor [230]. The second, based on an airlift mesh-draught (in mist reactor), has been adopted by the emergent German ROOTec Company (http://www.rootec.com) for producing camptothecin, an anti-cancer drug, which accumulated as 10-hydroxycamptothecin (a more powerful and less toxic natural derivative), and was secreted into the medium by the *Camptotheca acuminata* hairy roots [19].

6.4.3.5 Necessity for Ri Plant Regeneration in the Production of Secondary Metabolites

Although a large diversity of secondary metabolites is synthesized in hairy roots, some final products, targeted for their great pharmaceutical value, are stored or biosynthesized in the leaves or aerial parts, as exemplified by the two bisindole alkaloids, vinblastine and vincristine. The final steps in the biosynthesis of these terpenoid indole alkaloids (TIAs) are carried out in leaves, whereas the precursors are produced in the roots [226, 231, 232]. The possibility of regenerating *C. roseus* plants from hairy roots may allow a higher production of TIAs through genetic engineering, particularly both vinblastine and vincristine [203]. Likewise, the production of essential oils of mint, normally produced in the leaves and stems, was successful in plants regenerated from hairy root cultures [166], notably at higher levels [180].

Regenerated plants from *A. rhizogenes*-derived hairy roots of *Centaurium erythraea* provide a source of secoiridoids (which accumulate in vitro in the aerial parts and roots) equivalent to the total spectrum of compounds found in non-transformed plants, but with eightfold higher levels than commercially available herbs [184]. Similarly, plants of *Ophiorrhiza pumila,* regenerated from hairy roots at high percentages (up to 83%), produced camptothecin at 66–111% of levels found in the wild-type plants. Derivatives of this antitumour monoterpenoid indole alkaloid are used clinically for cancer chemotherapy [233].

Most hairy root-derived rose-scented geranium (*Perlagonium* sp.) plants synthesized a panel of essential oils similar to those of wild-type parents, except for two Ri plants that produced two oils having better olfactory value, representing an interesting improvement from a commercial point of view [158, 234].

Nevertheless, high levels of natural product production in the hairy root-derived plants can be the result of biomass increase because of the concomitant prolific root growth. For example, after *A. rhizogenes*-mediated Ri T-DNA insertion, the spontaneous regeneration of *Tylophora indica* shoots placed in liquid medium without any additional growth regulators seemed to be associated with an important increase of tylophorine accumulation. Indeed, this phenanthroindolizidine alkaloid was synthesized in transgenic plants at 160–280% of levels found in untransformed plants. However, this apparent hyper-productivity was due in part to the significant biomass increase of the hairy root cultures [170].

In some cases, metabolic production in Ri plants can be lower compared to untransformed control plants. For example, based on IR spectrophotometer analyses, the solasodine profiles of normal and transgenic plants of *Solanum nigrum* were similar; however, total solasodine levels, as well as the distribution of solasodine in different parts (roots, stems, leaves and fruits) of the hairy root-derived plants, were inferior compared to those of untransformed control plants. That is, in untransformed plants, solasodine levels reached an average of 265.7 mg/plant, with 78% occurring in stems, whereas in transgenic plants, had an average of 84.4 mg/plant with the highest solasodine level (51.6% of total) detected in leaves [235]. Likewise, Celma et al. [155] reported that while plantlets of *Duboisia myoporoides* × *D. leichhardtii* with the strongest hairy root morphology syndrome produced the highest amounts of the tropane alkaloid scopolamine, the overall accumulation of scopolamine and

hyoscyamine (a related tropane alkaloid) was less than in non-transformed plants. These authors concluded that *A. rhizogenes*-transformed plants did not provide a viable alternative to agriculture production of the *Duboisia* hybrid clones obtained by conventional breeding. Consequently, the application of *A. rhizogenes*-based plant transformation is not always associated with higher levels of desired product accumulation. We infer from both previous examples that the hairy root platform, as well as hairy root-derived plants, are not always efficient routes to boost the secondary metabolite accumulation.

Alternatively, natural product production can be increased by altering culture conditions. For example, *Camptotheca acuminata,* a tree native to central China, produces camptothecin, a potent inhibitor of topoisomerase I (an enzyme required during DNA replication) [211], used for the treatment of colorectal and ovarian carcinomas. In callus or cell cultures of *C. acuminata,* the accumulation of this natural product was very disappointing; nevertheless, the establishment of differentiated tissues (roots or shoots) presents an obvious alternative in vitro production system. Thus, hairy roots of *C. acuminata* were cultured in bioreactor systems for the large-scale production of camptothecin, with the expected compound partly released into the culture medium. Production was increased by adding biogenic elicitors to the culture medium [211]. Similarly, *Solanum khasianum* hairy root cultures made possible the increased production of solasodine, an anti-neoplastic agent [236].

6.4.3.6 Secondary Metabolite Accumulation Correlated with the Insertion of *rol* Genes

Some authors have reported that changes in secondary metabolite production in hairy roots and Ri plants correlate with changes in the phenotype induced by the insertion of *rol* genes and with the quantity of the polypeptide encoded by the *rolC* gene [237]. Interestingly, both the capacity to grow and produce nicotine in hairy roots and Ri plants of *Nicotiana tabacum* cv. Xanthi were higher after integration of the three *rol* genes (*A*, *B*, *C*) together than with *rolC* alone. In addition, the level of nicotine accumulation was positively correlated with the levels of the polypeptide encoded by the *rolC* gene, as detected by immunoassays [237]. The *rolA* gene appears to be an activator of growth and secondary metabolism. Although the *rolB* gene has emerged as the most powerful stimulator, its use is presently disputed owing to its growth-suppressing effect. More positively, the self-activation of *rolC* gene seems to be promising [95].

6.4.4 Phytoremediation: Hairy Roots and Ri Plants Offer Potential Applications for Cleaning Up Polluted Environments

Phytoremediation is recognized as one of the top ten more important technologies for detoxifying inorganic (radionuclides and toxic metal pollutants, such as chromium,

lead, mercury and nickel) and organic contaminants (such as trichloroethylene (TCE), chloroform and bisphenol A) present in ground water and surface waters or in soils [238]. Phytoremediation also makes possible the removal of pharmaceuticals from the environment, for example, antibiotics, ibuprofen (anti-inflammatory drug) or acetaminophen (paracetamol) (analgesic), that have been continuously discharged into the environment in various amounts since the beginning of the twentieth century [239]. Likewise, dyes found in the effluents of textile and dyeing industries, many of which are either toxic to flora and fauna or mutagenic and carcinogenic, can be decolorized into harmless compounds by plants [240]. In this way, phytoremediation can be defined as an emerging low-cost technology that utilizes green plants to detoxify biotic and xenobiotic pollutants for environmental restoration of polluted sites [241, 242]. This biotechnological process represents a potentially environmentally responsible alternative to the physical or physicochemical remediation techniques (such as incineration [243], soil washing, excavation, immobilization or extraction) used to clean up contaminated sites, for which the cost is often rather expensive.

6.4.4.1 Remediation Processes for Decontamination of Polluted Soils

Rhizoremediation is described as one specific aspect of phytoremediation that involves a symbiotic association between plant roots and rhizosphere microbes, i.e. yeast, fungi and bacteria [244] (Fig. 6.3). More specifically, plant roots provide an environmental niche and essential nutrients to rhizosphere microorganisms that are capable of degrading pollutants into harmless products [245]. Once absorbed by the plant, contaminants can be translocated to aerial plant parts before being compartmentalized, further metabolized or volatilized [246]. Phytovolatilization is a mechanism by which contaminants are taken up and eliminated from the plant via the transpiration stream into the atmosphere (Fig. 6.3). Another aspect of remediation is rhizofiltration, which makes possible the uptake of contaminants from surface or ground water. Plants at sites polluted with toxic metals absorb, trap and remove the metals from soil or ground water by phytoextraction or rhizofiltration (Fig. 6.3).

6.4.4.2 Certain Plant Species and Transgenic Plants Appear as Being Good Phytoremediation Candidates

Many plants, whether wild-type, transgenic and/or associated with rhizosphere microorganisms, are excellent candidates for phytoremediation-based removal and immobilization of pollutants [247]. For example, poplar, rye grass, Indian mustard and rice have been found to be well suited for phytoremediation because of their inherent capacity to metabolize a large variety of environmental pollutants [248]. More than 400 species of natural hyperaccumulators of environmental pollutants have been identified [245], and among these, *Brassicaceae* have been demonstrated to be especially effective hyperaccumulators of toxic metals [249]. Alternatively,

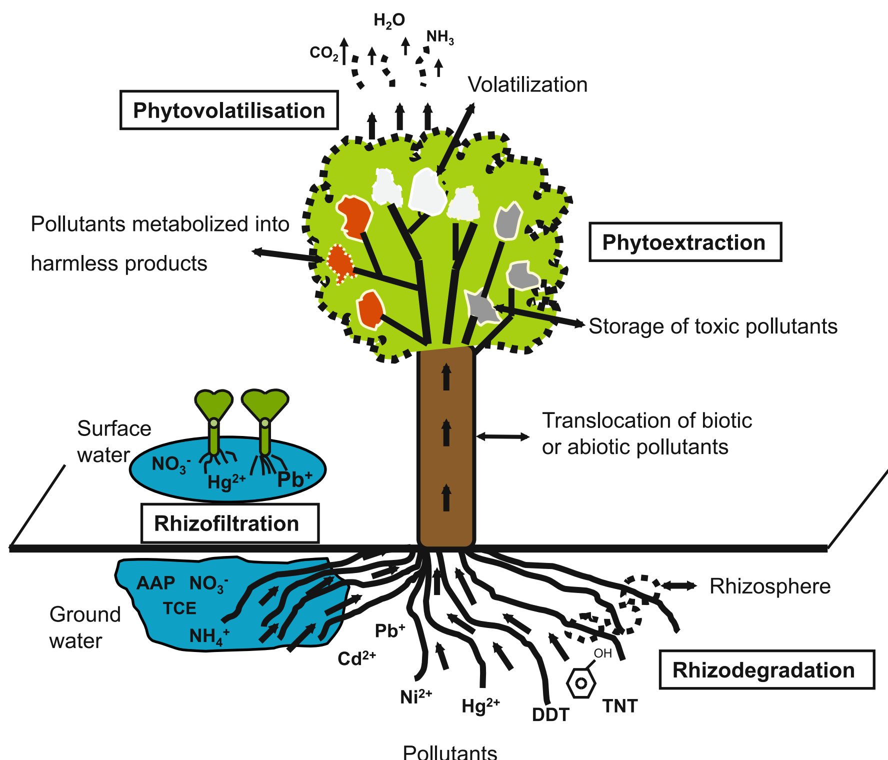

Fig. 6.3 *Phytoremediation: green approaches to clean up contaminated environments*. Ri Plants can be used to filter toxic metal species and pollutants from either surface or ground water (or both). Once inside the plant, these ions/molecules can be stored in the root tissue or translocated to the above-ground parts, where a number of possible fates exist. For example, toxic pollutants can be stored and/or rendered harmless through metabolism or volatilization. Alternatively, the rhizosphere surrounding the roots of Ri plants can facilitate the microbial degradation of toxins through a rhizodegradation process resulting from a quasi symbiosis between soil microorganisms (including bacteria, yeast and fungi) and plant roots. See text for further details and examples

some plants possess enzymes capable of degrading pollutants, such as *Typhonium flagelliforme*, which can decolorize and degrade dyes [239], making it suitable for cleaning up dyes found in the effluents of textile and dyeing industries.

Transgenic plants used in phytoremediation have usually been generated by genetic modification using *Agrobacterium tumefaciens* [250]; however, hairy roots derived from *A. rhizogenes*-based plant transformation displaying a typical root proliferation phenotype offer a promising system to exploit the capacity of plant species to absorb and eliminate contaminants arising from industrial effluents [114]. Hairy root cultures, possessing a metabolic potential similar to that of normal roots, are widely used as laboratory models to study the fate of plants treated with contaminants under in vitro conditions, for example, C*atharanthus roseus* [251, 252] and *Brassica juncea* [26]. Likewise, hairy roots of *Armoracia rusticana* were capable of the total removal of acetaminophen from applied treatments [238].

Hairy roots derived from hyperaccumulator plant species, such as *Thlaspi caerulescens*, *Brassica juncea* or *Chenopodium amaranticolor*, have been shown to uptake specific toxic metals (lead, uranium or nickel) from a polluted environment [14, 15, 69]. There is a particular interest in *A. rhizogenes*-mediated genetic transformation of popular trees (*Populus* sp.) due to its extensive root system, rapid growth, water uptake and important biomass production.

Plants do not normally use exogenous organic molecules (such as herbicides) as an energy or carbon source. The complete elimination of many organic contaminants is problematic because plant catabolic pathways lack the necessary enzymatic machinery for the complete degradation of many organic contaminants, such as 2,4,6-trinitrotoluene or 2,4,6-trinitrophenol, which ultimately accumulate in planta as a result. By contrast, bacteria and mammals, being heterotrophic organisms, often possess the enzymatic machinery necessary to achieve a complete mineralization of organic molecules and can be used as an enzyme source to complement the metabolic capabilities of plants. Hence, the idea to enhance plant biodegradation of organics by genetic transformation has been developed, corresponding to a strategy similar to that used to develop transgenic crops [242]. As noted above, genetic transformation has the potential to increase the natural remediation capacities of plants [249]. After transferring specific foreign genes into the plant genome, the detoxification potential of transgenic regenerants can be improved [246]. For example, transgenic potatoes and rice plants expressing the mammalian cytochrome P450 genes have been used to detoxify herbicides (reviewed in [253]).

6.4.4.3 Potential Approaches and Future Challenges

One objective of phytoremediation is to completely mineralize organic pollutants, including polychlorinated biphenyls (dioxin, polycyclic aromatic hydrocarbons) and other pesticides, into relatively nontoxic molecules (carbon dioxide, nitrate and ammonia) [241]. However, transgenic plants exhibiting new or improved phenotypes constitute a valuable model for alternative approaches to phytoremediation, for example, transgenic plants expressing antibodies capable of neutralizing and/or accumulating toxic molecules present in the rhizosphere. To this end, Drake et al. [249] have used a model system composed of two hydroponically grown transgenic plant types of *Nicotiana tabacum*, both expressing a murine antibody (Guy's 13 monoclonal antibody). This elegant approach consists of rhizosecretion-mediated antibody neutralization and *in planta* antigen sequestration achieved by the expression of IgG and mIgG in plants, respectively. In IgG transformants, functional antibody is secreted from the roots and subsequently binds with antigen (SA I/II) in the aqueous medium surrounding these roots. In the mIgG plants, antibody is expressed in leaves and retained in the plant plasma membrane by means of a transmembrane sequence. These mIgG plants are able to bind SA I/II to membrane-associated antibody after uptake of antigen through the roots and its transport through the whole plant. The immune complex was subsequently degraded in planta.

Several experimental processes have demonstrated the potential for transgenic plants and trees to degrade environmental pollutants recalcitrant to or incompletely

degraded by native plant enzymatic systems [237]. However, in spite of our understanding of the mechanisms of phytoremediation, as well as the successful studies achieved in the laboratories and greenhouse, much more research effort is necessary to translate remediation research to the field [244].

6.4.5 Medical Molecular Farming: Recombinant Proteins in Transgenic Plants

Over the past 17 years, plants have emerged as promising "bio-farming" protein expression systems based on the large-scale production of recombinant proteins [254] compared with traditional systems based on microbial or animal cells or transgenic animals [255]. Genetically engineered plants possess the potential to biosynthesize almost unlimited pharmacologically active recombinant proteins such as interferon, proteases, blood substitutes, antibodies, vaccines and other therapeutic entities [256] for use in disease diagnostics, prevention or therapy [257]. Consequently, a tremendous effort has been made to integrate foreign genes into plants in order to provide high quantities of recombinant proteins. Basic methods to transfer genes into plants, notably, by using *A. tumefaciens* (or *A. rhizogenes)* as vector, are well established. With this stable transgenic approach, future plant generations heritably retain the ability to express the product of the integrated gene [258]. Even if the plants are not a universal solution for producing all recombinant proteins, plant expression systems remain a good choice, depending on the type of protein and its applications [259, 260].

6.4.5.1 Transgenic Plants as Ideal Bioreactors for Producing Therapeutic Proteins

The biotechnological process of molecular farming consists of the transformation of plant tissue (i.e. plant cell, explant tissue, whole plant cultures) by integrating gene(s) that encode, for example, an immunogenic protein capable of stopping infection by pathogenic microorganisms [101], and regenerating plants that can be grown on a large scale to recover the recombinant protein in active form, in quantities that make the process economically viable. The choice of host species as production system is largely drawn from model plants (tobacco, *Arabidopsis*), cereal crops (wheat, maize, rice) and/or fruit crops (banana, tomato) [261]. Several plant parts, such as fruits, leaves and seeds, can be used to recover the expected therapeutic products [258]. In addition, the amount of biomass that whole plants can generate, often in almost any soil or climate in the world, can be significant [258]. This makes the use of transgenic plants for the production of therapeutics more tractable in more places, including developing countries.

In 1990, human serum albumin produced in tobacco and potato leaves and cell suspensions was the first potentially therapeutic plant-derived protein [262, 263],

and human growth hormone was the first recombinant protein with a therapeutic efficiency to be successfully expressed in transgenic plants (reviewed in [264]). Afterwards, more complex proteins, such as IgG monoclonal antibodies (diagnostic reagents) [265], particularly, the IgG1 antibody, were produced in plants by academic or commercial groups in view of applications in humans or animals [264]. Owing to a strong demand, several other recombinant proteins have been produced, such as the gastric lipase used for treating pancreas insufficiency (cystic fibrosis disorder) and tumour-specific antibodies for non-Hodgkin's lymphoma [260]. Another group reported the creation of transgenic plants capable of producing recombinant human acetylcholinesterase (ACH $-R_{ER}$) to commercially viable levels [57]. Demands for existing therapeutic proteins, for example, erythropoietin and insulin for treating anaemia and diabetes, respectively, are expected to rise considerably. For example, human insulin is the recombinant biopharmaceutical with the largest annual production, near to 13 tons of protein [266]. One transgenic plant-derived biopharmaceutical, hirudin (a native enzyme with strong anticoagulant property that is naturally secreted by leeches), is now being commercially produced in Canada for the first time [267].

Theoretically, any protein, including complex antibodies, can be expressed in cereal crops, especially in maize, which has many advantageous properties. Indeed the mature maize seed, as other cereals, is desiccated, lacks active proteases and possesses a rich panel of molecular chaperones and disulphide isomerases capable of facilitating the folding of recombinant proteins [268]. Maize, as described recently for the production of HIV-neutralizing monoclonal antibodies 2 G12, effects post-translational modifications required for optimal biological activity [268].

6.4.5.2 Recombinant Proteins Need Post-Translational Modifications

Since plants are higher eukaryotic organisms and they possess an endomembrane system, they are able to assemble and fold recombinant proteins using signal peptides and chaperones that are homologous to those of mammalian cells [260, 268, 269]. Therapeutic proteins are generally glycoproteins and require post-translational glycosylation for stability, folding and biological activity [270]. Plants, as in all higher eukaryotic organisms, can modify proteins via glycosylation leading to N-linked and O-linked glycans [260]. The effective biological activity of these complex recombinant proteins requires specific post-translational modifications or assembly steps that bacteria cannot perform correctly. Together, these attributes make plant-based recombinant protein production more favourable than bacterial-based systems [258].

Antibodies are glycoproteins with a common basic structure composed of two identical heavy (H) chain polypeptides and two identical light (L) chain polypeptides linked together by disulphide bridges and non-covalent bonds [260]. Plants expressing recombinant antibodies can form these disulphide bonds in the chloroplasts and/or endoplasmic reticulum (ER) [271], forming functional antibodies. For example, individual cDNAs for immunoglobulin gamma (g) and kappa (k) chains

were separately expressed in two tobacco plant types. To lead to functional antibodies, the assembly of both immunoglobulin chains was achieved through a sexual cross between the two transgenic plants. Potentially, this useful alternative method could be applied to the assembly of oligomers other than antibodies [272]. Likewise, Koprowski [258] reports the production of a tobacco plant expressing the heavy and light chains of human rabies antibody. Both chains were recombined in these plants producing complete anti-rabies antibodies, as efficient as the animal antibodies, after exposure to rabies.

In spite of the efficiency of the plant cell glycosylation machinery over bacterial systems, the resultant N-glycan structures present in plants and animals differ [270]. Thereby, plant antibodies lack *N*-acetyl neuraminic acid, which is the predominant terminal sugar residue in mammalian complex glycans [269]. Consequently, the differences in glycan structures inherent to both kingdoms represent one of the major drawbacks affecting the use of the plant-derived monoclonal antibodies (mAb) [270]. To overcome this imperfect glycosylation, strategies that include the humanization of plant *N*-glycans by expression of b(1,4)-galactosyltransferase and sialyltransferase in plants have been adopted [270, 273]. It has been suggested that, owing to these two different glycosylation modes, plant-derived recombinant proteins may be responsible for an immunogenicity and allergenicity increase. But, in their human study of mucosal application of plant secretory antibody, Ma et al. [260] did not observe any immune response to the plant recombinant glycoprotein after six applications of the antibody.

In spite of the potential glycosylation problem, plant cells are efficient bioreactors for protein synthesis, folding and disulphide bond formation, assembly of multimeric proteins, post-translational changes, processing and secretion [274]. Since the stability of recombinant proteins is without doubt, the most important parameter limiting production in molecular farming, the use of plants as bioreactors to form stable, properly processed proteins, is a significant advantage. However, the ability of host plants to biosynthesize recombinant proteins is in part dependent on its spare metabolic capacity [261], leading to limitations in the capacity for overall production.

6.4.5.3 Antigen Production and Plant-Made Vaccines from Transgenic Plants

The worldwide threat of pandemic diseases provides reason to examine the effectiveness and suitability of vaccine production and vaccination programmes. Vaccination remains the most efficient and cost-effective means for providing protection against infectious pathologies [271]. Traditionally, vaccines have been made from killed or attenuated organisms; however, subunit vaccines composed of one or more antigenic epitopes have often replaced these. Most vaccines are produced by mammalian, yeast and/or insect cell cultures, owing to their ability to express recombinant proteins similarly to that of the native organism [101].

Another emerging area of molecular farming focuses on the production of vaccines and a large range of immunogenic antigens in plants [264]. The concept of

vaccine production in transgenic plants first appeared in 1992 [275], using hepatitis B as a model. However, the plant system used to produce a plant-made vaccine is as important as the antigen itself. The plant production system can influence antigen content, stability and authentic conformation and ease of production scale-up, harvest and processing [101]. Antigens can be produced from hairy roots, and an elegant process leading to hairy root lines of potato able to express the hepatitis B surface antigen (HBsAg) has been described [162]. Initially, internodal stem segments, taken from in vitro grown shoot cultures of potato, were infected and transformed with *A. tumefaciens*, harbouring two plant expression vectors, pEFEHBS and pEFEHER, containing the hepatitis B gene encoding the surface antigen of this virus. Then, internodal stem segments derived from positive transgenic plants were co-cultured with the *A. rhizogenes* strain ATCC 15834 to initiate hairy root formation. Transgenic plantlets emerged spontaneously from these transformed roots and expressed the hepatitis B surface antigen (HBsAg) [162].

Vaccines are generally administrated by injection, i.e. by parenteral application, but it is not without dangers due to the possibility of contamination of the needles. A non-invasive alternative to this form of immunization is the use edible vaccines that can be administrated orally, such as the transgenic plant-based vaccine for hepatitis B [276]. For example, the HBS (hepatitis B virus), expressed in transgenic lupin and lettuce plants, led to a specific antibody response that appeared when mice and human volunteers ate the plants containing antigens [264, 275].

One of the biggest disappointments after 20 years of "molecular farming" is a notable lack of enthusiasm in the take-up of this new technology by the pharmaceutical industry [259, 260]. Nevertheless, several biotechnology companies are now actively developing, field testing and patenting plant expression systems, while clinical trials are proceeding on the first biopharmaceuticals derived from the recombinant proteins [267]. Possible plant-based production systems offer opportunities to obtain a large panel of antigens and drugs against diverse pathologies and for various means of delivery to be explored.

6.4.5.4 Advantages and Safety of the Recombinant Proteins Expressed in Plants

One obvious advantage of plant expression systems, compared with other vaccine production systems, is the reduced manufacturing cost [271]. While fermenters and bioreactors can be replaced with plant growth rooms or greenhouses, transgenic plants can also be grown in the field [271]. The production cost of recombinant proteins in plants has been estimated to be 10- to 50-fold lower than the same protein produced in *Escherichia coli* or mammalian cells [277]. Another major advantage is that the plant host systems are not known to harbour human or zoonotic pathogenic microorganism compared with traditional systems using animal cells, microorganisms or transgenic animals [264]. Plant-derived products, purified or not purified, are less likely to be infected by human pathogens, because the plants are not the hosts of human infectious agents [271]. Additionally, vaccines coming from

plants overcome the risk of contamination with mammalian pathogens and decrease the cost of immunization programmes [101, 278]. Moreover, when plants are used as bioreactors for producing therapeutic proteins, ethical considerations involving the use of the animals are also avoided [259, 260]. Future research needs to focus on making plant-made vaccines safely, consistently and as efficiently as pharmaceuticals produced by other sources [101].

6.4.5.5 Extraction and Purification of Recombinant Proteins

Optimal downstream processing of recombinant proteins remains a challenge for using plants as efficient bioreactor systems. Nevertheless, Farinas et al. [279] have reported a highly efficient method for the extraction of human proinsulin (rHProinsulin) from the endosperm of corn seeds produced at 0.42% of the total soluble protein. Depending on the intended use, recombinant proteins extracted from plants need greater or lesser degrees of purification. At one extreme, plant-made drugs intended for intravenous use in human therapy must be purified to the greatest standards [261], while at the other, oral antigens require little or no processing.

After harvest, processing and storage, the stability, potency and efficacy of therapeutic proteins must be determined. The current focus for most groups is to provide purified antigens (or other therapeutic proteins) produced from plants grown in contained conditions [271]. However, low recombinant protein content and the expense of extraction and purification limit large-scale manufacturing of recombinant proteins in plants.

6.4.5.6 Hairy Roots: A Powerful System for Expressing and Secreting Recombinant Proteins

Hairy root cultures have been used in recent years for the synthesis of recombinant therapeutic proteins, especially those that have been challenging to express in bacteria, yeast and mammalian expression systems [212]. For example, Woods et al. [57] demonstrated that the recombinant human acetylcholinesterase (ACH–R_{ER}) accumulated in hairy root cultures derived from transgenic plants. Likewise, of two transgenic lines of *Nicotiana tabacum* (Ac2 and AcK6) engineered to express 14D9 murine monoclonal antibodies (IgG1 type), only, the AcK6 line produced the 14D9-C-terminal KDEL (Lys-Asp-Glu-Leu) fusion protein, allowing for protein retention in the endoplasmic reticulum (ER) [280, 281]. Hairy roots established from the AcK6 line stably expressed and accumulated 14D9, and the addition of DMSO or gelatine to these cultures increased the antibody contents in biomass or the total 14D9 amounts, respectively, in the medium culture [281].

The rhizosecretion of the model recombinant protein, human-secreted alkaline phosphatase, from hairy roots initiated from the stems of transgenic tobacco plants and cultured under non-sterile hydroponic conditions was 5–7 times higher than that from adventitious transgenic roots placed in the same conditions [274]. Such a

high accumulation of pharmacologically relevant compounds is potentiated by the ease of plant transformation and harvesting phytochemical compounds, especially when they are produced in a sterile, secure and controlled environment. Indeed, hairy roots are normally cultured under aseptic growth conditions avoiding pollution of the recombinant protein [114].

6.4.5.7 Potential Drawbacks to the Production of Foreign Proteins in Plants

Recombinant proteins have a natural tendency towards structural heterogeneity derived in part from the instability of polypeptide chains expressed in heterologous environments [282]. In response to this, major advances have been achieved to optimize transgene transcription and translation in planta [283] and to improve the post-translational stability and accumulation of heterologous proteins in plant systems [12]. The specific tissue or organ chosen for foreign protein production has a strong influence on the integrity and quality of the final product. For example, the same antibody expressed in tobacco plants or cell lines had different glycosylation profiles and localized in different subcellular structures [284].

One source of product loss in the production of antibodies or other heterologous proteins in plant systems is proteolytic degradation [12, 285]. The activity of plant proteases can dramatically affect the structural integrity and activity of recombinant proteins in the plant expression systems [282]. Even heterologous proteins bearing an N-terminal signal peptide targeting them to the secretory pathway may undergo in planta proteolytic alteration during the transit between the ER and Golgi apparatus, before being secreted into the intercellular spaces (apoplast) or directed into vacuoles. Likewise, antibodies with a KDEL ER retention sequence or even those without any signal peptide are often subject to degradation by proteases [12]. Similarly, protease activity may be observed ex planta during plant tissue homogenization necessary for extraction of the recombinant proteins. However, antibodies, secreted by suspension-cultured cells or roots into culture medium, can be purified directly from the liquid culture medium, without grinding, limiting the proteolytic degradation [12].

The parameters that lead to an optimal extraction (i.e. lowest cost, highest recovery of intact proteins) of, for example, recombinant immunoglobulin G antibodies have a direct impact on the initial choice of expression strategy and, therefore, must be considered at an early stage [6]. One strategy to simplify recombinant protein purification and minimize proteolysis of intact recombinant proteins produced in hairy root cultures is to recover the recombinant protein continuously in small amounts [212].

The addition of gelatin as a substitute substrate for the protease activity during extraction can be used to minimize target protein degradation and increase the total levels of recovered antibodies from tobacco hairy roots by 68% [282]. Other approaches are the coexpression of recombinant proteins with peptidase inhibitors [9] or the use of transgenic hosts with reduced proteolytic capacities, gene knockout or silencing of plant peptidases. But these processes are only applicable if the target peptidases are not crucial for plant growth [12, 282, 283]. RNA interference is an

easy and efficient possibility to determine how silencing peptidases might prevent proteolysis of recombinant proteins [9].

Another potential disadvantage to the production of recombinant proteins in plants on a large scale, for example, in the field, is the potential risk of transgene leakage into non-transgenic crops or wild plant species. While this can be avoided by scale-up process restricted to greenhouses [284], one benefit to the use of hairy roots is the ability to culture them in large (e.g. 1,000 L) airlift bioreactors (see Sect. 6.4.3.4).

6.4.5.8 Advances in Molecular Farming: Plant-Made Pharmaceutical and Vaccine Applications

The medicinal and commercial interest in plant-derived vaccines and drugs for human and animal use depends on the performance in clinical trials. Spök et al. [11] have presented a detailed list focused on the advanced development stages of vaccines or plant-made pharmaceuticals for treating human and animal disorders and for use as nutraceuticals. Three additional applications of transgenic plant-derived vaccines and pharmaceuticals for human use are worth mentioning since these have reached phase II or III clinical trials. Among them, an antibody-based cancer vaccine (phase II testing) using tobacco as the plant host is being developed by Large Scale Biology, a USA-based company. Another is the insulin production for diabetes treatment, with safflower as the plant host (phase III testing). This product, which has been accepted by the FDA, with commercialization expected for 2010, is being developed by SemBioSys in Canada [11]. The third application, having reached phase II clinical trials, is a gastric lipase produced from transgenic maize for the treatment of the pathologic disorders of cystic fibrosis or mucoviscidosis (i.e. an inherited disease). The commercialization of this product, by Meristem Therapeutics Company in France, was expected for 2009/2010 [11].

Likewise, Karg and Kallio [264] have reported several examples of entire-plant-derived pharmaceuticals that are in preclinical and clinical development. Several vaccines, including oral vaccines, are in clinical trials or have completed clinical trials by October 2007 [264]. Before obtaining market authorization, however, vaccines and plant-made pharmaceuticals will be subject to the regulations of the FDA (US) and the European Agency (EMEA) for the Evaluation of Medicinal Products [276]. It is anticipated that these success stories represent the beginning of a new era in plant-derived oral vaccines and plant-made pharmaceuticals being produced in hairy root-mediated culture.

6.5 Conclusion

Hairy roots are well-differentiated organs with a high genetic stability and potentially unlimited propagation in vitro. They provide a potential model for many otherwise recalcitrant experimental systems and, as a research tool, open new routes to

gene function and transcriptome analyses, large-scale production of valuable secondary metabolites and animal protein overexpression in confined and controlled in vitro conditions, enzymological studies, phytoremediation and root-biotic interactions. *Agrobacterium rhizogenes*-transformed roots represent an invaluable system that allows gene function investigation and make possible the creation of metabolic routes by integrating bacterial genes or a better understanding of the genes involved in the biosynthesis pathways as well as the enzymes limiting the accumulation of secondary metabolites of interest. However, to be most useful as a genetic transformation system, there has to be a process to generate viable whole plants from hairy roots, a feat that has proven to be more difficult than expected. In the preceding sections, we have summarized progress towards routine plant regeneration from hairy root cultures derived from *A. rhizogenes*-infected plant tissue. This progress has opened up the possibility for multiple potential applications in industrial and agronomic fields by coupling the advantages of the transgenic roots and entire plants.

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Chapter 7
A Dynamic Model for Phytohormone Control of Rhizome Growth and Development

Eric T. McDowell and David R. Gang

Abstract Despite the economic and medicinal importance of plant rhizomes, the biology of rhizomes has received only cursory attention in recent years. We review the existing literature on rhizome growth, development, and function and discuss outstanding questions that may benefit from application of new technologies such as next-generation sequencing, detailed metabolite profiling, and proteomics. In addition, we outline a new model of the environmental and phytohormone control of rhizome apical dominance and shooting and discuss how this model is followed in different rhizomatous species. The relationship between source carbon availability and specific phytohormones with regard to control of apical dominance is discussed.

7.1 Introduction

The underground plant stem, or rhizome, is one of the least understood parts of plant anatomy. As evidenced by the chronic mislabeling by grocers of the ginger rhizome as a root, the underappreciation by the general public of this vegetative organ's biological significance is pervasive.

The rhizome's function as a site of energy or metabolite storage and as a propagative tissue is central to its biological and evolutionary importance, permitting many plants, such as sea grasses [1] and many members of the mint family [2–6], to compete in their native environments. As a site of storage of starch and other nutrients, the rhizome ensures that resources are available for early environmental colonization following catastrophic environmental changes [7–10] or overwintering.

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The ability of the rhizome to store starch also enables the hardiness and invasiveness of many of world's most noxious weeds, such as purple nutsedge (*Cyperus rotundus* L.), Bermuda grass (*Cynodon dactylon* (L.) Pers.), Johnson grass (*Sorghum halepense* (L.) Pers.), quack grass (*Elymus repens* (L.) Gould = *Agropyron repens* (L.) P. Beauv. = *Elytrigia repens* (L.) Nevski), and cogongrass (*Imperata cylindrica* (L.) Beauv.) to name just a few [11–17].

Specialized metabolites have also evolved presumably to preserve the rhizome from herbivore and pathogen attack. Examples include the gingerols and curcuminoids from ginger (*Zingiber officinale* Rosc.) and turmeric (*Curcuma longa* L.), compounds known to interact with pain receptors or possess antimicrobial properties [18–23]. However, in humans, many of these rhizome-derived or rhizome-stored compounds are medicinally or economically important [19, 24–29]. Thus, increasing our understanding of how rhizomes function, of rhizome biology, may have a significant impact on our understanding of how important medicinal compounds are produced, how plants are able to survive severe drought and cold stress, how upright stems evolved, and how to control important weedy species.

Despite the economic and medical importance of the rhizome, much of our current knowledge of rhizome biology is decades old. Application of new technologies [30–37], such as advances in sequencing and bioinformatics [38, 39], now enables comparative functional genomic analyses to be applied to rhizome biology. We review what is known regarding control of rhizome growth, with special attention paid to development and interactions with the environment.

7.2 Rhizome Origin and Evolution

Evolution of plant morphology has received considerable attention in recent years [40–42]. The limited information that is available on the historical relationship between the rhizome and the plant kingdom highlights a role initially as the first stem of vascular plants and then in adaptation to selective pressures. Rhizomes were present in the earliest known bryophytes (*Pallavicinites devonicus* (Huber) Schuster, upper Devonian, 350 mya) and likely also present earlier in Cooksonia [43–47]. Because animals had not yet invaded land (this was to occur around 80 million years after Cooksonia), there was no immediate selection pressure to protect the site of cell division, the meristem, from grazing animals. Under such conditions, growth by an apical meristem would most likely have been favored because growth from the stem apex would not be restricted by the firm attachments to the underlying substrate that result from rhizomatous growth and that would restrict intercalary or basal meristematic growth. Thus, the establishment of the apical meristem as the predominant growth habit is probably directly attributable to the rhizomatous growth form adopted by the initial land colonizing plants. The ability to grow from an apical meristem eventually led to the great diversity in terrestrial plant forms that we witness today, from the smallest herb to the tallest tree.

Despite its evolutionary and functional importance, however, the rhizome is not a common anatomical feature across the plant kingdom. Many lineages lost and

then others later rediscovered rhizomatous growth. We have hypothesized that as selection pressure against the apical meristem began to mount because of great diversification of herbivorous insects and grazing animals, intercalary and basal meristem growth habits began to be adopted. These meristem types are better able to protect the developing plant tissue from grazers. Thus, in many "advanced" angiosperm lineages, such as in the *Poaceae*, *Govenia*, and *Rhodocoma*, we see the apparent reemergence of these alternative meristems [48]. However, even these plant families have not given up the apical meristem. Instead, the same plants that produce aboveground shoots from basal meristems often produce belowground rhizomes that grow from apical meristems, just as their earliest land-dwelling progenitors had done. Throughout evolutionary history and still today, rhizomatous growth is an important growth habit adopted by numerous and diverse plant species across the plant kingdom (with the exception of the gymnosperms), including the ferns.

The physiological mechanisms likely responsible for the loss and readoption of the rhizome in the plant kingdom can be predicted by comparing the fossil record to tissues of extant plants. Despite the fact that fossils of the earliest land plants show roots approximately 410–395 mya [49], the stele structure of current rhizomes is much more similar to those of upright stems than to roots, either primitive or modern. The close similarities between the rhizome stele and that of the upright stem indicate that the rhizome was truly the progenitor of the upright stem. Moreover, the reemergence of the rhizome in advanced plants, with concomitant retention of roots, supports this hypothesis.

The transformation of the rhizome into the upright stem occurred as plants colonized land, encountering steadily drier environmental conditions and increased competition for light. The new environmental conditions resulted in adaptations such as the vascularization of the rhizome, the evolution of lignins, and suberization of the hypodermis of the rhizome [50], features that became important as foundational elements of the upright stem. Together, vascularization and lignification permitted further mechanical support, allowing both increased aerial growth to attain access to sunlight and the ability to transport water, nutrients, and photosynthates to and from all plant extremities. Moreover, suberization of the rhizome hypodermis and stele decreased the likelihood of catastrophic water loss through dehydration.

Besides providing better mechanical support and protection from dehydration, the adaptations of vascularization, lignification, and suberization provided an additional benefit: a protected area for storage of starch reserves. In fact, the rhizome as a storage organ is likely one reason that many current rhizomatous species are found in extreme environments that experience periodic, catastrophic changes, cyclical deficiencies in nutrient accessibly, or intense competition [51–54]. Thus, the prevalence of the rhizome in such extreme environments suggests that the rhizome has played an important role in responding to selective pressures by conferring species with accessible energy reserves, enabling speedy recolonization and monopolization of local resources despite the maintenance of inefficient nutrient uptake mechanisms [51–55]. Notwithstanding the selective advantages of the rhizome in some environments, there are potentially significant drawbacks to rhizome-mediated reproduction. Most importantly is the fact that successive cycles of asexual reproduction can result in loss of genetic diversity and eventual extinction of the rhizomatous species

in the event of additional, severe environmental changes. For rhizomatous species that reproduce both sexually and asexually, the problem of reduced genetic diversity is alleviated. Such species can undergo the much more energy efficient method of clonal reproduction compared to sexual reproduction to maintain selective advantage under "normal" conditions, although sexual reproduction may still occur following stress [56–58].

Sexual reproduction and consequent interspecies hybridization is another mechanism that may account for the gain and loss of the rhizome. Loss or gain of degrees of rhizomatousness is often observed following hybridization with domesticated cereals and their wild progenitors, indicating that the genes responsible for the rhizome are preserved in select genera and are often additive [48, 51, 59, 60]. Moreover, QTL experiments examining rhizomatous traits have also identified genes or markers influencing aspects of rhizome growth and development [48, 60] including a number of MADS box, bHLH, and MYB transcription factors. Other gene candidates indicate the importance of phytohormones in rhizome biology [48, 59–64]. As many of the previous studies have identified genes with regulatory effects, it is not surprising that introduction or modulation of regulatory elements via hybridization or mutation can affect the presence or absence of a rhizomatous phenotype.

Several recent studies have provided evidence demonstrating that hybridization resulting in the addition of few regulatory genes can result in rhizomatous progeny [48, 65–68]. For instance, Jang et al. (2009) [60, 69] both quantified and traced the origins and evolutionary history of the expressed sequence tags (ESTs) expressed preferentially in the rhizomes of *Sorghum halepenses*, the allotetraploid progeny of nonrhizomatous *Sorghum bicolor* and rhizomatous *Sorghum propinquum*, and determined that a large majority of the preferentially expressed rhizome ESTs originated in *S. propinquum* rather than *S. bicolor*. The same study also revealed that a comparison of orthologous genes with rhizome tip preferential expression in *S. propinquum* and both parental species showed little or no differences in coding region, their mutation rate, and relatively similar numbers of paralogs. Interestingly, the Ka/Ks ratio comparisons for both *S. bicolor* and *S. propinquum* demonstrated that the genes with rhizome-enriched expression in *S. propinquum* are undergoing purifying selection since their evolutionary divergence. Jang et al. hypothesized that the loss of the rhizome phenotype over evolutionary time is likely the result of loss or mutation of one or a few regulatory genes rather than the loss of many over time. Conversely, reintroduction of a rhizomatous phenotype can be accomplished by the addition of the appropriate regulatory element by hybridization.

7.3 Environmental Control of Rhizome Apical Dominance and Rhizome Rooting

Plant rhizomes come in two general morphologies: ipsilateral and contralateral. Ipsilateral rhizomes, largely uncharacterized but typified by *Zingiber officinale,* exhibit a growth pattern with the rhizome apex located on the same end of the plant

as the shoot. All new rhizome and shoot growth occurs immediately next to the existing parent shoot. New shoots replace the old shoots that die off, extending the rhizome. The alternative rhizome morphology is the better understood contralateral rhizome, typified by *Elymus repens*, where the rhizome apex is at one end of the plant and the aerial shoot at the other. Interspersed between the rhizome apex and the aerial shoot are a series of nodes, each separated by internodes. At each node, lateral buds or lateral rhizomes are attached and culminate in an apical bud on the distal end. New shoots or lateral rhizomes are derived from the lateral or apical buds.

Control of rhizome lateral buds and apices is determined by the environment, the parent shoot, and the rhizome apex. Specifically, the environment modulates signals emanating from both the shoot and the rhizome apex that control apical dominance in the rhizome. To best understand the complex relationships underpinning rhizome apical dominance, it is essential to examine rhizome responses to both environmental and physical changes like removal of the parent shoot and/or the rhizome apex.

Surgical experiments with contralateral rhizomes have determined that signals emanating from both the parent shoot and rhizome apex repress lateral bud growth. For instance, isolated rhizome fragments lacking both the parent shoots and the rhizome apex show increased incidence of nodes being converted into shoots. The proportion of rhizome fragments and the conversion of nodes into shoots is length dependent: the smaller the rhizome fragments, the greater the proportion of shooting nodes. Smaller rhizome fragments experience quicker reestablishment of a dominant node in shooting rhizome fragments—usually the second node from the rhizome apex stump [70]. Other studies involving denuded rhizomes [71–73] showed that removal of the rhizome outer layers stimulates growth or shooting. Robertson et al. demonstrated the importance of roots on shooting as isolated rhizomes lacking their apices experience lowered growth or shooting rates and removal of roots results in complete abolition of shooting [72].

Experiments with *E. repens* rhizomes have revealed that nitrogen, water, temperature, and light conditions regulate rhizome shooting. Under low nitrogen or drought conditions, the development of additional shoots from the lateral buds is suppressed, whereas high nitrogen or abundant water conditions transform lateral buds into shoots and rhizome growth is curtailed [33, 74–76]. Other environmental conditions modify lateral buds as well. Increased temperature or low intensity light can promote the development of shoots at lateral buds nearest to the apex, while high intensity light under the same environmental conditions promotes rhizome growth [34, 77, 78]. Suppression of growth at lateral buds nearest to the rhizome apex and the modification of rhizome apical dominance in response to the environment illustrate that lateral buds are influenced by the distance from either the rhizome apex or the shoot.

Additional experiments involving simultaneous changes in environmental conditions with removal of the shoot and/or rhizome apex point to the fact that environmental factors determine the strength of rhizome apical dominance. For instance, under low nitrogen conditions, removal of the shoot results in the middle two nodes of four-node rhizome segments being transformed into shoots [79]. If similar *E. repens* rhizomes grown on high nitrogen have their shoots removed, an increase

in the repressive effect of the rhizome apex, or apical dominance, is observed. Only the node furthest from the apex becomes a shoot, while the remaining lateral buds are maintained as rhizomes [80]. Comparable trends are observed if only the rhizome apex is removed in low and high nitrogen rhizomes: removal of the rhizome apex of low nitrogen results in rhizomatous growth at all lateral rhizomes [79], while high nitrogen induces shoot growth in nodes closest to the rhizome apical stump [78]. Removal of the shoot from rhizomes grown in the dark with low nitrogen results in loss of apical dominance near the apical end of the rhizome. The loss of apical dominance results in the establishment of a new dominant shoot(s) over time at 1 or 2 nodes (depending on rhizome segment size) immediately basal to the rhizome apex. The new dominant shoots then act to repress shoot growth at nearby nodes.

Thus, rhizomes possess considerable flexibility to sense and react to their environment. Much of this flexibility is via modulation of rhizome apical dominance by either the rhizome apex or the shoot. Responses to loss of either the rhizome apex or the shoot depend on environmental conditions and result in differential changes in the growth patterns of adjoining lateral buds, indicating that the nature of the control signals is different. Thus, there must be additional signaling pathways involved in the communication of environmental cues to the rhizome.

7.4 Phytohormone Control of Rhizome Morphology

Investigations into the role of phytohormones and rhizome growth patterns implicate sugars, auxin, cytokinin, gibberellic acid (GA), ethylene, and abscisic acid (ABA) in controlling differentiation of rhizome meristems into rhizomes or shoots (Fig. 7.1). More specifically, interactions between rhizome rooting, the rhizome outer layer, and distance from the center of rhizome apical dominance (the rhizome apex) are of paramount importance.

Rhizome rooting is a prerequisite for shooting to occur [72] and is dependent on the relative concentrations of auxin and other phytohormones present. For example, shooting rhizome cultures exposed to increasing concentrations of auxin exhibited increased rooting, an effect inhibited by GA [81] or ABA [82]. Auxin is likely a principal agent of apical dominance in plant rhizomes [83] as the synthetic auxin 1-naphthalene acetic acid (NAA) can replace the apical dominance of surgically

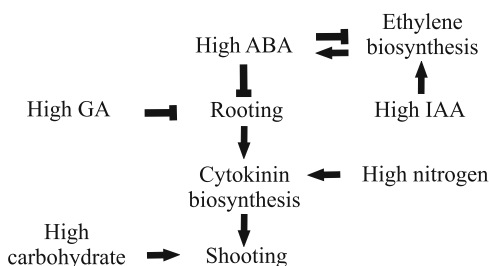

Fig. 7.1 Summary of rhizome responses and predicted routes of reaction to environmental conditions or exposure to phytohormones in *Elymus repens* rhizomes. *Black arrows* denote an inducing action, while *blocks* indicate an inhibitory action

removed rhizome apex [84]. However, indole-acetic acid (IAA) cannot inhibit shooting when directly applied to axillary buds [85]. Examination of endogenous auxin concentrations in rhizomes revealed concentration maxima nearest to the rhizome apex and along the lower regions where roots originate, specifically within the lower immature secondary vascular, cambial, and cortical tissues [86]. Additional girdling experiments involving radiolabeled IAA revealed auxin travels basipetally toward the shoot from the rhizome apex [86], while removal of either the rhizome apex or outer layer results in an increase in auxin concentration at nodes nearest the apex. Inversely, removal of the parent plant results in the dramatic decrease of auxin at all nodes [71].

ABA is also a candidate for the repressive signal for rhizome apical dominance. Concentrated primarily in the same areas as auxin, ABA can replace the apical dominance of the rhizome apex in decapitated rhizome fragments [73], but only if the shoot is not attached [85]. ABA can also inhibit shooting at axillary nodes when directly applied [85]. Removal of the rhizome apex results in lowered ABA concentration at the closest nodes, and a similar phenomenon is observed in denuded rhizomes [71]. Water availability also profoundly affects rhizome apical dominance and shooting. A principle messenger of water stress in *Viridaeplantae*, rhizome ABA (and ethylene) is largely absent under abundant water conditions and correlates with an increased incidence of shooting. Inversely, rhizome growth occurs under drought conditions, as do ABA and ethylene biosyntheses. Addition of either ethylene or ABA under abundant water conditions mimics drought conditions, resulting in decreased shooting and increased rhizome growth [87]. Repression of shooting in stressed rhizomes by ABA is also likely through the repression of rooting even if shoots were removed, which usually stimulates root growth [82].

ABA's suppressive effect on root bud formation and apical dominance (with auxin) has been characterized in a wide variety of non-rhizomatous plants [88–91]. Briefly, auxin, ethylene, and ABA biosyntheses exist in a feedback loop: high concentrations of auxin induce ethylene, which in turn inhibits ABA biosynthesis [92–98]. While the exact molecular mechanisms of how auxin, ethylene, and ABA affect rhizome growth response to environmental and physical stimuli are unknown, other studies in *Arabidopsis thaliana* may help explain the inability of an apically applied auxin/cytokinin mix to substitute for the rhizome tip in *E. repens* rhizomes [84] during anthesis. For example, DELLA proteins, which are known to be involved both in flowering time and lateral root induction/growth, may play a role in this process [99, 100]. If true, hypothetical DELLA involvement or other candidates suggested by Hu et al. [66] could spell out potential avenues of research to more definitively tie auxin, ethylene, ABA, and GA together in rhizome growth and development. Thus, Leakey's observation that apically applied auxin can maintain correlative inhibition of lateral buds in rhizomes with surgically removed rhizome apexes, but also requires simultaneous supply of factors from the parent plant, indirectly implicates phytohormones like ABA and GA in playing some role in rhizome apical dominance [84].

The relationship of ethylene, ABA, and auxin explains similar effects of ethylene and ABA in rhizomes [87]. Like ABA, ethylene is synthesized under drought conditions and has the effect of promoting rhizome growth [87]. Alternatively, ethylene

evolution is restrained under conditions of abundant water or low nitrogen availability [87, 101, 102].

Unlike ethylene or ABA, cytokinin promotes shooting in rhizomatous species and inhibits rhizome branching and ethylene evolution [101, 102]. Experiments in *E. repens* demonstrated that endogenous cytokinins are concentrated near the apical half of the rhizome [73], but the ultimate origin of cytokinins, their direction of transport, and the distribution of likely source tissue (roots) on the rhizome remain unknown. In particular, we do not know if the distribution of cytokinin in rhizome roots mirror observed cytokinin concentrations across the rhizome. Studies in other, nonrhizomatous plants indicate that cytokinins are primarily produced in the root [103, 104], and roots are necessary for rhizomes to produce shoots [72]. Cytokinin supply is limited by auxin in addition to environmental factors like nitrogen [104–109]. Nitrogen levels sensed by the roots modulate the amount of cytokinin produced by the root, matching earlier observations by McIntyre concerning rhizome shooting responses under high and low nitrogen [33, 75, 77, 78]. Studies using transgenic potatoes harboring nonendogenous isopentenyltransferase (IPT) transgenes also showed that cytokinins play additional roles besides control of shooting. Specifically, transgenic IPT potato clones demonstrated pleiotropic phenotypes including production of only shoots at nodes, a greater ability to form stolons and tubers, or lower sucrose content [110]. Thus, cytokinins likely play important roles in starch storage or mobilization, in addition to shooting in rhizomatous plants. Similar observations concerning nodal production, branching, and biomass have been observed in cytokinin-supplemented cultures of the aquatic rhizomatous *Ruppia maritima* [111].

The role of cytokinin in starch storage or mobilization is similar to that of other substances known to effect rhizome biology: sugars, gibberellic acid (GA), and light. While sugar, GA, and light have been examined for their roles in the rhizome, their examination is cursory compared to auxin, ABA, and cytokinin. Consequently, the exact orientation and magnitude of likely sucrose and GA gradients within the rhizome is currently unknown (Fig. 7.2). Additionally, the full extent of sugar, GA, and light's role in rhizome biology as well as the exact nature of their interactions with other factors is also illusive. For example, sugar, GA, and light likely play an important role in breaking dormancy: a largely undescribed and temperature-dependent process that occurs when the rhizome has been exposed to select temperatures for a period of time, stimulating shoots later [37, 82, 112, 113].

Previous work has demonstrated that GA biosynthesis is temperature sensitive [114, 115] and thus may be involved in breaking of dormancy [112, 113]. Work on the invasive *Euphorbia esula* revealed that root bud growth on isolated roots is inhibited by the independent addition of either high carbohydrate or GA conditions but is alleviated upon addition of either GA or sucrose, respectively [82]. The main source of GA appears to be shoot-derived, but additional experiments indicate GA biosynthesis likely also occurs in root buds following shoot removal [82, 116]. Likewise, GA applied basally to isolated (shootless) *E. repens* rhizome fragments inhibited shooting at lateral buds and encouraged rhizome growth [66, 85], while the basal addition of sucrose to shootless high nitrogen rhizome fragments released lateral rhizomes from the influence of the still attached rhizome apex [80, 117].

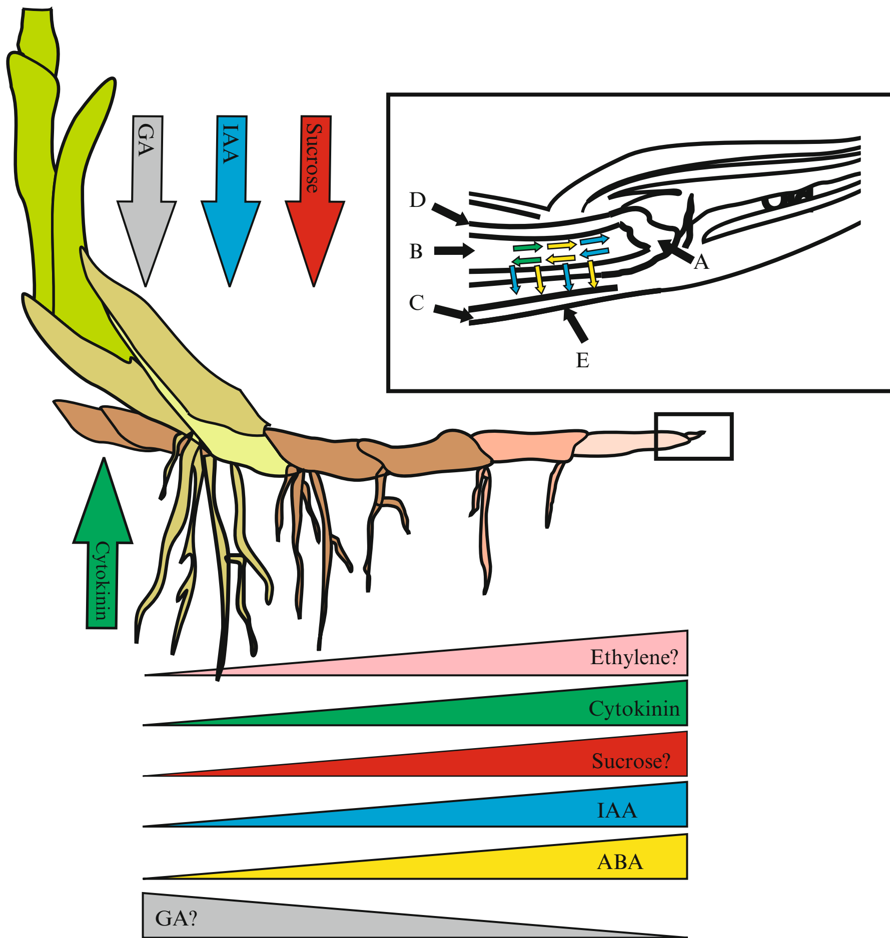

Fig. 7.2 Approximate and predicted phytohormone concentration gradients in *E. repens* rhizomes under normal growing conditions. Identity and predicted direction of signaling molecule flow is indicated by color-coded *arrows*, while *triangles* indicate location and orientation of estimated phytohormone concentration gradients. *Question marks* denote predicted approximate phytohormone concentration gradients not presently measured in the literature. *Inset*: enlargement of rhizome apex and predicted direction of phytohormone transport. (**a**) rhizome apical meristem, (**b**) stele, (**c**) endodermis, (**d**) cortex, (**e**) epidermis

Thus, while GA appears to work similarly to inhibit rooting in species with and without rhizomes, the same cannot be said for sucrose and its influence on rooting in rhizomatous plants.

Additional antagonistic effects of sugar and GA on the curvature of new shoots from the rhizome have also been observed: addition of sucrose to *Cynodon dactylon* rhizomes reduced the upward curvature of rhizomes exposed to light, an effect that

is canceled if GA is added [118]. The effects of GA and sugar on shooting are also likely involved in the response of rhizome buds exposed to light. Rhizome buds exposed to light transform into shoots [34, 119], an effect mitigated if the light is in the far red spectrum. The effect of the far red spectrum on rhizome shooting is further mitigated if the 690- and 720-nm wavelengths are removed [119]. These observations point to conditional and tissue-specific roles of sugar and GA in rhizomes in rhizome dormancy, apical dominance, and the probable origins of these signals.

7.5 Recent Rhizome Datasets

Spurred by the economic and medicinal importance of rhizome species, research groups have identified genes putatively involved in rhizome biochemistry and biology using large expressed sequence tag (EST) or quantitative trait loci (QTL) datasets. Of interest are the rhizome EST datasets (*Glycyrrhiza uralensis*, *Leymus cinereus x Leymus triticoides*, *Elymus wawawaiensis/Elymus lanceolatus*, *Zingiber officinal*e, *Curcuma longa*, *Sorghum halepense*, and *Sorghum propinquum)*, as these provide potential for future comparisons.

One comparison between the *Zingiber officinale*, *Curcuma longa*, *Sorghum halepense,* and *Sorghum propinquum* EST libraries has identified genes putatively involved in regulating rhizome morphology [60, 120]. The resulting conserved transcripts with enriched rhizome expression in both *Zingiber officinale* and *Curcuma longa* include a number of putative transcription factors annotated to be involved in ethylene or auxin response. Also identified are putative transcriptional regulators annotated as MYB and MADS box proteins. Subsequent comparisons of the ginger/turmeric/sorghum MADS box sequences with rice revealed that many are orthologous to MADS box proteins associated with rhizome QTLs from *Oryza sativa* x *Oryza longistaminata* hybrids [48].

Additional transcriptome comparisons have identified ESTs annotated as being involved in auxin response or transport as well as GA-associated *cis*-acting sequences with highly expressed rhizome genes in *Sorghum halepense* and *Sorghum propinquum* [60, 69]. Comparable approaches identified rhizome habit QTLs from *Leymus cinereus x Leymus triticoides* or *Elymus wawawaiensis/Elymus lanceolatus* hybrids [63, 121, 122]. A *Leymus* SCARECROW-like GRAS family transcription factor was identified that aligned to both a Leymus chromosome group 3 growth habit QTL and a rice chromosome 1 region containing the *wheat tiller inhibition* gene (*tin3*) [63]. The relevance of these findings is that GRAS family proteins are known to be involved in GA signaling in *Arabidopsis thaliana* [123, 124] and therefore are likely to play a role in rhizome development. Additionally, it is likely that the rhizome itself may contribute to its own phytohormone pools. Unfortunately, there is little direct evidence for this besides the rhizome-enriched expression of several ESTs annotated to be involved in GA biosynthesis in *O. longistaminata* [Hu et al. 2011].

7.6 A New Model for Control of Rhizome Development

Ratios of auxin, cytokinin, GA, and ABA to each other determine the developmental fate of the rhizome and mediate its response to the environment [31, 71, 73, 81, 82, 84, 86, 87, 101, 102, 111, 118, 125–130]. Thus, the theory of water/nutrient competition and phytohormone control of apical dominance in rhizomes is one and the same. Detailed over several papers, McIntyre theorizes that the rhizome apex's ability to repress lateral buds is partially through the monopolization of nitrogen and carbohydrate resources by either the parent plant or the rhizome apex [33–35, 74–80, 131]. While this theory does explain some aspects of how changes in aboveground conditions or physical integrity of the parent shoot is sensed by the rhizome below, it fails to explain the mechanisms determining rhizome or shoot growth. To address this question, we have integrated an analysis of past rhizome experiments with recent knowledge of plant genomics and phytohormone signaling/responses. The result is a new model that explains past observations in rhizome biology.

In this model, the parent shoots of intact contralateral rhizomes contribute sugars, GA, and auxin to the growing rhizome (Fig. 7.2). Once in the rhizome, shoot-derived sugars are transported to sustain growth of the rhizome apex [35, 80]. Additionally, GA is likely concentrated nearest the shoot, preventing rooting and shoot growth at the node nearest to the parent shoot [85]. Auxin from the shoot is also transported towards the rhizome apex via the stele, analogous to the shoot apical meristem in *Arabidopsis thaliana* [132], creating a rhizome apex-centered auxin distribution [71, 73]. Once at the rhizome apex, an auxin concentration maximum is created and auxin is transported back towards the shoot via the lower immature secondary vascular, cambial, and cortical regions [86]. High local concentrations of auxin stimulate expression of 1-aminocyclopropane-1-carboxylic acid synthase and ethylene, in turn stimulating ABA biosynthesis [94, 95]. Biosynthesis of ABA continues unabated due to the continued presence of sequestered ethylene due to the underground nature of the rhizome [93, 98]. Once synthesized, ABA is transported back towards the shoot via the apoplast and the transpiration stream [133], resulting in observed distributions of rhizome ABA [71, 73]. ABA produced in this manner counteracts the root-promoting concentrations of auxin nearest to the rhizome apex [82, 89] and limits the availability of root-derived cytokinins near the rhizome apex. The lack of roots near the rhizome apex is important as root formation precedes the formation of new shoots in *Zingiber officinale*, and high levels of cytokinins are heavily associated with induction of shoots in rhizome cultures and release from apical dominance [83, 101, 102].

Perturbations, both physical and environmental, alter the delicate balance of phytohormone and sugar ratios within the rhizome and result in changed behaviors at the various nodes comprising the contralateral rhizome. Modulation of cytokinin content is likely via isopentenyltransferase (IPT) activity, as nitrate addition in *A. thaliana* upregulates AtIPT3 and AtIPT5 in roots [134, 135]. High nitrogen conditions also result in higher concentrations of shoot-inducing cytokinins and lower sucrose

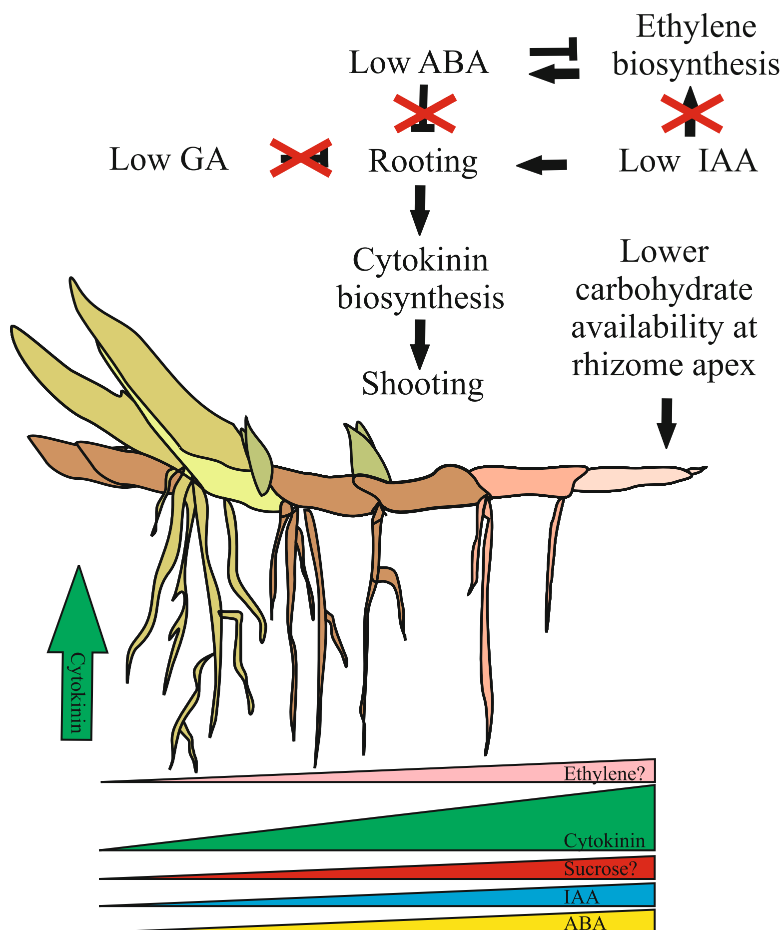

Fig. 7.3 Predicted relative phytohormone concentration gradients in *E. repens* rhizomes lacking their parent shoot. *Arrows*, *blocks*, and *triangles* as described for Fig. 7.2. Removal of the shoot reduces the quantity of incoming signaling molecules responsible for inhibiting root initiation and growth (sucrose, IAA, and by extension: ethylene and ABA), while concentrations of shoot-inducing cytokinins relative to root inhibitors have increased. Remaining IAA concentrations are sufficient to induce root initiation and growth

content in the rhizome, while low nitrogen reduces ethylene biosynthesis [101]. Together, these results indicate that nitrogen availability can influence (via ethylene) the rhizome's ability to produce shoots and store and distribute sucrose, allowing rhizomes under high nitrogen conditions to devote most of their fixed carbon towards aggressive growth.

Removal of either the shoot or the rhizome apex alters the existing fates of the rhizome nodes [79]. Removal of the parent shoot from four-node, low nitrogen grown *E. repens* rhizomes results in a slight reduction of rhizome apical dominance and transformation of the middle two nodes into shoots (Fig. 7.3). Alternatively,

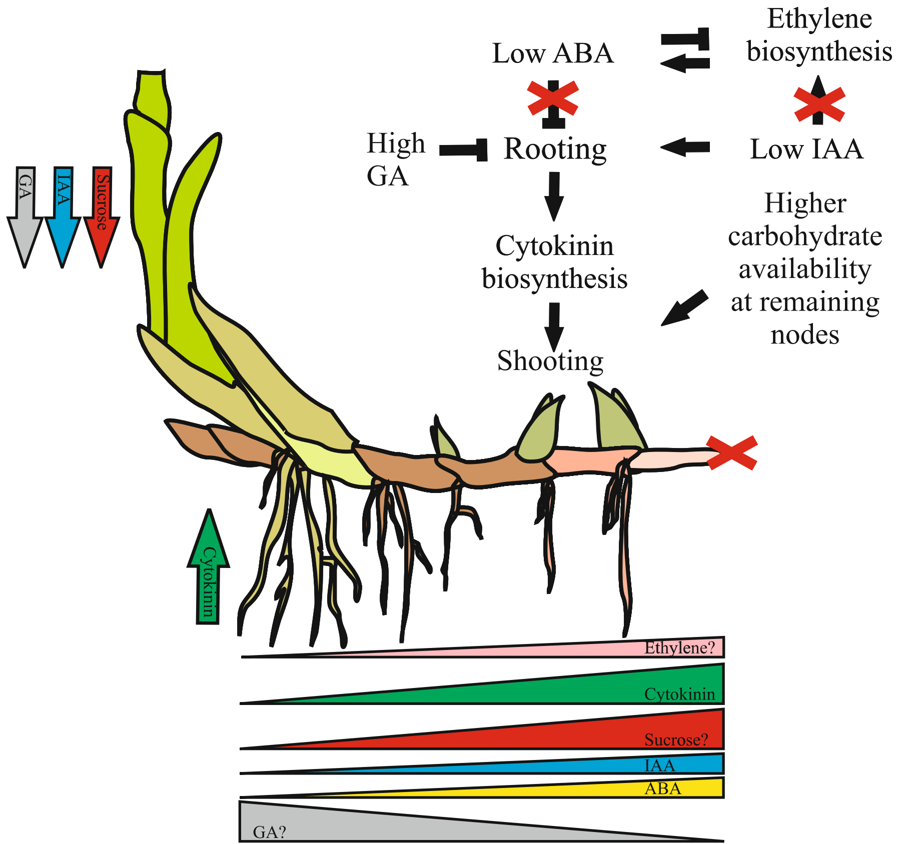

Fig. 7.4 Predicted phytohormone concentration gradients in *E. repens* rhizomes lacking their rhizome apex. *Arrows*, *blocks*, and *triangles* as described for Fig. 7.2. Lowered concentrations of molecules inhibitory to root initiation (IAA and ABA) on the rhizome apex side result in reduced rhizome apical dominance. Remaining IAA concentrations are sufficient to induce root initiation and growth. Loss of the rhizome apex also results in redistribution of shoot-derived sucrose at remaining nodes in sufficient quantity to overcome the inhibitory effect of shoot-derived GA on rooting, thereby inducing increasing root production and consequent shoot development

removal of just the rhizome apex results in a much more dramatic reduction in rhizome apical dominance and transformation of all nodes into rhizomes (Fig. 7.4). Removal of both the shoot and the rhizome apex results in transformation of all nodes into shoots (Fig. 7.5) [79], the most dominant shoot is the node nearest to the rhizome apex [70]. All of these behaviors can be explained by the model presented above.

In instances when just the shoot is removed, the rhizome's ultimate source of auxin and shoot-inhibiting GA are removed (Fig. 7.3). Reduced amounts of auxin are transported towards the rhizome apex and diminish ABA stimulated via ethylene. Lowered ABA no longer inhibits rooting and root-derived cytokinin production. Increased cytokinin stimulates nodes to the shoot fate, an effect tempered by the remaining presence of rhizome apical-derived IAA.

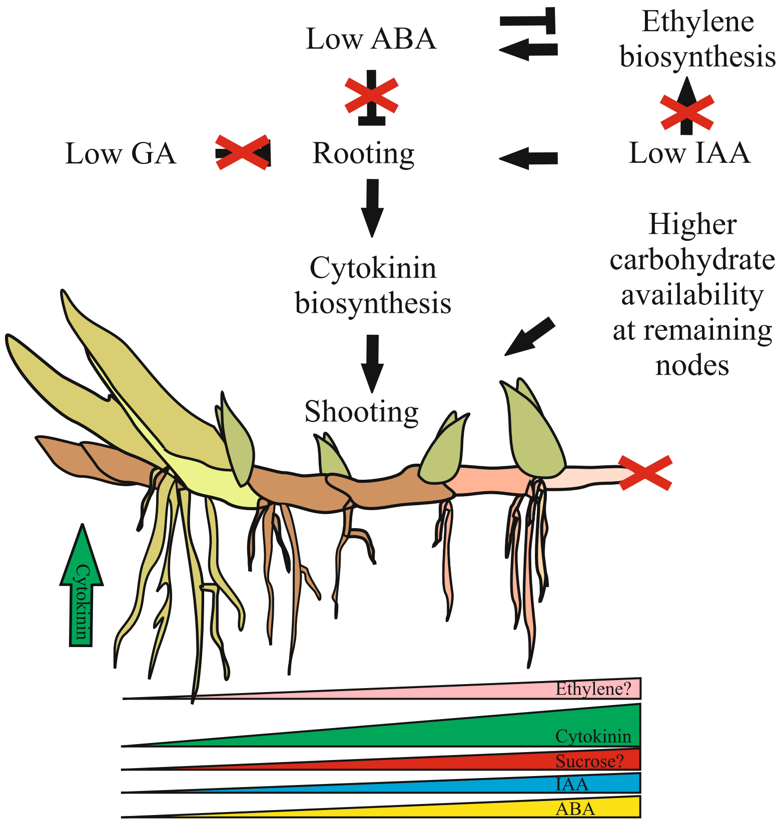

Fig. 7.5 Predicted phytohormone concentration gradients in *E. repens* rhizomes lacking both the shoot and rhizome apex. *Arrows, blocks, and triangles* as described for Fig. 7.2. Signaling molecules responsible for inhibiting root initiation and growth on both sides of the rhizome have been reduced (shoot: GA; apex: ABA, IAA), while concentrations of shoot-inducing cytokinins relative to root inhibitors have increased. Remaining IAA concentrations are sufficient to induce root initiation and growth

Alternative situations involving removal of the rhizome apex while the parent shoot remains result in loss of rhizome apical dominance and rhizome growth at all remaining nodes [79]. Thus, loss of the rhizome apex while still attached to the parent shoot results in sugar, GA, and auxin still being contributed to the rhizome, while the main centers responsible for rhizome apical dominance have been removed (Fig. 7.4). Loss of the rhizome apex allows shoot-derived sucrose to be used for growth at all remaining nodes, overriding the inhibitory effect of GA. As the parent shoot still contributes inhibitory GA to the rhizome, reduced rooting can occur on the shoot side of the rhizome despite the reduced amounts of shoot-derived auxin [81] and ABA near the apical stump. Together, all nodes produce rhizome growth, and a new rhizome apex is reestablished at the node furthest from the shoot [70].

The final circumstance of altered rhizome growth patterns is when both the parental shoot and the rhizome apex are removed (Fig. 7.5). Removal of both the rhizome apex and the shoots results in shoots being produced at all rhizome lateral buds [79]. Rhizomes lacking both the rhizome apex and parent shoot lack input of new sugar and GA from the shoot, in addition to lowered concentrations of ABA and auxin at the rhizome apex. Insufficient concentrations of GA and ABA fail to inhibit root growth on both ends of the rhizome, while the residual auxin pools in the lower regions of the rhizome fragment promote rooting. Root production increases the endogenous cytokinin within the rhizome, resulting in new growth as shoots that utilize the existing stores of sugar once monopolized by the rhizome apex. Eventually, all new shoot growth ceases and a new dominant shoot is established [70].

7.7 Concluding Remarks and Future Directions

Despite the large body of work concerning phytohormonal control of rhizome apical dominance, there are few studies applying new technologies to this topic. For instance, one aspect of rhizome apical dominance not mentioned in our model concerns the recently discovered strigolactones involved in ABA biosynthesis, control of shoot branching, and root growth [136–140]. Strigolactones likely play similar roles in rhizomes, and their involvement in rhizome apical dominance is also likely as the functions are conserved over evolutionary time. To this end, additional studies are required to determine if similar root and shoot responses are evident in rhizomatous species. Also worth considering would be studies to further elucidate the interplay between cytokinin, auxin, sugar, and GA in rhizome apical dominance and environmental response. Specifically, the actual sources of rhizome auxin, cytokinin, and GA need to be experimentally determined as should the direction of transport of these phytohormones within the rhizome. For instance, recent evidence presented by Hu et al. (2011) points to the possibility that at least some of the GA may be actually synthesized within the rhizome itself, a fact that should be tested experimentally.

The exact mechanism of auxin origin and transport in contralateral rhizomes is also unknown. Two studies [71, 73] have determined that a rhizome auxin maximum is located at or near the rhizome apex. Is this a site of auxin origin or the point of accumulation? If it is a site of auxin accumulation, does the majority of the auxin found at this point originate from the shoot or is it derived elsewhere in the rhizome? Studies in *A. thaliana* have revealed that directional transport of auxin takes place via auxin influx and efflux proteins [141–145], and we assume without evidence that a similar mechanism is at work in the rhizome.

Other aspects of this model require further elucidation: specifically, additional determination of the concentrations of auxin, GA, ABA, cytokinin, and sucrose. Our model postulates that cyclical changes occur in phytohormone or nutrient concentrations, which determine activity and fate of specific nodes. These assertions require validation. New advances in "omics" technologies, mass spectrometry (for quantification of phytohormone levels across the rhizome), and bioinformatics

should play invaluable roles in helping further flesh out and improve our knowledge in rhizome development and differentiation, particularly with regard to GA. Prior work in *Sorghum* suggested that promoter elements and transcripts associated with GA response are preferentially represented in rhizome apex EST collections [60]. With regard to severity of rhizome apical dominance or dormancy, other studies have also noted the sensitivity of rhizomatous species to temperature or exposure of rhizome tissue to light [34, 112, 118, 119]. Possible culprits in these temperature and light-sensitive processes are GA-related, as GA biosynthesis is both temperature and light sensitive [114, 115]. However, the concentration and distribution of GA within the rhizome and its changes in response to the environment or physical changes remain uncertain, and the distribution and site of biosynthesis of GA destined for the rhizome need to be ascertained. Prior studies indicate GA is mainly synthesized in actively growing tissues like leaves/aerial internodes and possibly root buds [146, 147]. Is this the case in species with rhizomes? Is GA primarily located and produced on the shoot side of the rhizome? What are the seasonal distributions of GA, and how do these change when the rhizome is exposed to light?

Finally, attention should be paid to investigating the control of rhizome apical dominance in species with ipsilateral rhizome symmetries. At present, little knowledge exists concerning how ipsilateral rhizomes function in response to physical or environment changes. Similar mechanisms may or may not be at work in ipsilateral rhizomes as in contralateral rhizomes.

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Index

A
Abscisic acids (ABAs)
in grape berry
analyte levels, 29–32
chromatographic separation, 27–28
dynamics, 23–24
LC-MS/MS analysis and quantification, 28, 29
metabolism, 20, 22
solid-phase extraction, 25–27
rhizomes, 149–150
Aesculus hippocastanum, 107
Aggregation pheromones, 82, 83
Agrobacterium rhizogenes
and *A. tumefaciens*, 104–105
plant regeneration, 105–106
strains and source plants, 96–98
Alhagi pseudoalhagi, 108
Allelochemicals, 81
Allocasuarina verticillata, 107
Alnus rubra, 61
Angelonia alicariifolia, 107
Antimicrobial activity, *Harmonia axyridis*, 90
Apical dominance, 146–148
Apocynum venetum, 107
Apple scab, 89
Arabidopsis thaliana, 96
Aralia elata, 109, 114
Armorica rusticana, 96
Artemisia annua, 96
Asimina tribola, 96
Astralagus sinicus, 107, 109
Atropa belladonna, 96, 118
Attract-and-kill method, 83
Auxins, in grape berry
analyte levels, 30, 32
chromatographic separation, 27–28
dynamics, 24
LC-MS/MS analysis and quantification, 28, 29
metabolism, 22–23
solid-phase extraction, 25–27

B
Beta vulgaris, 96
Bioplastics, 61–62
Brassica
B. juncea, 96, 123
B. napus, 96, 107
Brugmansia candida, 96

C
Cacopsylla
C. melanoneura, 85
C. picta, 85
Camptotheca acuminata, 96, 120
Cassia obtusifolia, 96
Casuarina glauca, 96
Catharanthus roseus, 96, 107
Centaurium erythraea, 107
Chemically mediated multitrophic interactions, 81, 84
Chenopodium amaranticolor, 96
Cichorium intybus, 96
Citrus aurantifolia, 107
Coffea
C. arabica, 96
C. canephora, 109
Contralateral rhizomes, 147
Coronilla varia, 110
Crotalaria juncea, 107, 109
Cucumis sativus, 96

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Cucurbita pepo, 96, 110
Cytokinins
  in grape berry
    analyte levels, 31–33
    chromatographic separation, 27–28
    dynamics, 24
    LC-MS/MS analysis and quantification, 28, 29
    metabolism, 21, 22
    solid-phase extraction, 25–27
  rhizomes, 150

D
Daucus carota, 96, 107
Deoxynivalenol, 2, 3
4,15-Diacetoxyscirpenol (DAS), 2
Duboisia
  D. myoporoides, 108
  D. spp., 96

E
Echinacea purpurea, 96
Elymus repens, 147
  phytohormone concentration gradients, 151, 154–156
Endophyte mycotoxins
  extraction from animal matrices
    ergovaline, 43
    lolitrem B, 44
    lysergic acid, 43–44
  extraction from plant material
    ergovaline, 41
    lolitrem B, 42–43
    lysergic acid, 42
  HPLC-fluorescence, 44–46
  impacts on animal health
    dose response studies, 54–55
    physiological impacts, 51–54
  liquid chromatography-tandem mass spectrometry, 47–51
Enzyme-linked immunosorbent assay (ELISA), lysergic acid, 46
  Ergot alkaloids. *See also* Ergovalineabsorption, distribution, metabolism and excretion, model for, 52–53
  fragmentation pattern, 47, 48
  ions in multiple reaction monitoring analysis, 47
  production of, 38–39
  toxicological effects, 39
Ergovaline
  dose response studies, 54–55
  extraction
    from animal matrices, 43
    from plant material, 41
  HPLC-fluorescence, 45–46
  liquid chromatography-tandem mass spectrometry, 47–50
  physiological impacts, in animal health, 51–53
Erwinia amylovora, 87
Eschschoizia californica, 96
Expressed sequence tags (ESTs), 146, 152
Extraction method
  ABAs, 25–26
  cytokinins, 27
  PHB, 66–67

F
Fagopyrum esculentum, 96
Fescue foot, 39
Festuca arundinacea, 38
Fire blight, 87
Fusarium
  F. graminearum
    biosythetic pathway for culmorin, 12
    head blight, 3
    trichothecene biosynthesis, 8, 9
  F. sporotrichioides
    trichothecene biosynthesis, 3–4
    trichothecene biosynthetic genes, 5–7
  head blight, 3, 12

G
Genetic engineering platform, hairy roots, 101
  activation tagging, 102
  RNA silencing, 103
Gentiana macrophylla, 97
Gibberellic acid (GA), 150–152
Glycine max, 97
Glycyrrhiza uralensis, 97
Grape berry
  ABAs, auxins and cytokinins
    analytical method, 27–29
    dynamics, 23–24
    extraction method, 25–27
    metabolism, 20–23
  analyte levels, 29–33
  development chart, 20, 21
Greenhouse study, PHB production, 64
Guayule. *See Parthenium argentatum*
Gynostemma pentaphyllum, 97

H
Hairy roots
  applications
    horticulture/floriculture, 114–115
    medical molecular farming, 124–130
    phytoremediation, 120–124
    rhizogenesis, 115–116
    secondary metabolite production,
      116–120
  generation of, 96–97
  genetic engineering platform, 101
    activation tagging, 102
    RNA silencing, 103
  plant regeneration
    Agrobacterium rhizogenes, 104–106
    callusing phase, 112
    rooting from regenerated shoots, 111
    shoot regeneration, 106, 111
    transgenic embryos, 111–112
    without intervening root, 112–114
  stages in formation, 99, 100
Harmonia axyridis, 89–90
Hell oil, 71
Hevea brasiliensis, 68–69
Hirudin, 125
Honeybees, 81
Hoplocampa flava, 87, 88
Horticulture/floriculture, hairy root, 114–115
Hypericum perforatum, 108
Hypervirulent, 98

I
Indole-3-acetic acid (IAA)
  decline in berry formation, 30, 32
  rhizomes, 149
  structures, 22–23
Infochemicals
  applications
    attract-and-kill, 83
    mass trapping, 83
    mating disruption, 82
    monitoring, 82
    push-and-pull, 83–84
  chemically mediated interactions, 81
  description, 80–81
Insect–plant–microbes interactions.
      See Chemically mediated
      multitrophic interactions
Ion suppression effect, berry tissue matrix,
      28, 29
Ipsilateral rhizomes, 146–147
Isatis indigotica, 108

J
Jatropha curcas, 70–71

K
Kairomone, 83
Kalanchoe blossfeldiana, 97, 115

L
Ladybird taint, 90
Latex, 68–70
Leaf beetles, 86–87
Levisticum officinale, 97
Life cycle assessments, poly
      (3-hydroxyalkanoates), 67
Lignin biosynthesis, 67–68
Linum album, 97
  Lolitrem alkaloids. *See also*
      Lolitrem Bproduction of,
      38–39
  toxicological effects, 39
Lolitrem B
  dose response studies, 54–55
  extraction
    from animal matrices, 44
    from plant material, 42–43
  HPLC-fluorescence, 46
  liquid chromatography-tandem mass
      spectrometry, 50
  physiological impacts, in animal health,
      53–54
Lolium perenne, 38
Lotus japonicas, 97
Lure-and-kill method.. *See* Attract-and-kill
      method
Lycopersicon
  L. esculentum, 109
  L. spp., 97
Lysergic acid
  extraction
    from animal matrices, 43–44
    from plant material, 42
  HPLC-fluorescence, 46
  liquid chromatography-tandem
      mass spectrometry,
      47–49

M
Malus domestica, 85
Mass trapping technique, 83
Mating disruption technique, 82

Medicago
  M. sativa, 110
  M. truncatula, 97, 109
  Medical molecular farming. *See also* Recombinant proteinsplant-made pharmaceutical and vaccine applications, 130
  transgenic plants, 124–125
Mentha piperita, 109
Mitragyna speciosa, 97

N
Neotyphodium
  N. coenophialum, 38
  N. lolii, 38
Nicotiana
  N. benthamiana, 97
  N. tabacum, 97, 108, 109

O
Onobrychis viciaefolia, 109
9-Oxo-trans-2-decenoic acid, 81

P
Panax ginseng, 97, 109
Papaver somnifereum, 97
Parthenium argentatum, 69
Pelargonium spp., 108, 109
Phaedon cochleariae, 87
Phaseolus vulgaris, 97
Pherobase, 82
Pheromones, 80–81
  aggregation, 82, 83
  sexual, 81–82
Phratora vitellinae, 86–87
Phytoplasmas, 85–86
Phytoremediation
  definition, 121
  plant species and transgenic plants, 121–123
  potential approaches, 123–124
  rhizoremediation, 121, 122
Phytovolatilization, 121
Pinot Noir berries, 25
Pinus halepensis, 97
Pisum sativum, 97
Plant protection
  Harmonia axyridis, 89–90
  leaf beetles, 86–87
  sawflies, 87–89
Plumbago rosea, 97, 108
Poly(3-hydroxyalkanoates), 61–62
Polyhydroxybutyrate (PHB)
  chemical structure, 62
  production
    extraction and recovery, 66–67
    gene expression, regulation of, 65–66
    metabolic cost, 64, 65
    in transgenic poplar, 62, 63
  regulatory and market obstacles, 71–72
  subcellular location, 62–64
Poplar
  advantages, 60–61
  PHB
    concentrations, 64
    growth parameters, 65
    production, 62, 63
  stem, 68
Populus
  P. nigra, 73
  P. tremuloides, 97
Precautionary principle, 73
Primer effect, 81
Push-and-pull method, 83–84

R
Rauvolfia micrantha, 97
Recombinant proteins
  extraction and purification, 128
  secretion, 128–129
Reflex bleeding, 86
Rehmannia glutinosa, 109
Releaser effect, 81
Rhizofiltration, 121
Rhizome apex.. *See* Apical dominance
Rhizomes
  apical dominance, 146–148
  control model for development, 153–157
  datasets, 152
  functions, 143–144
  metabolites for preservation, 144
  origin and evolution, 144–146
  phytohormone control
    ABA, 149–150
    auxin, 148–149
    cytokinin, 150
    gibberellic acid, 150–152
  rooting, 146–148
Rhodiola sachalinensis, 97
Russian dandelion. *See Taraxacum kok-saghyz*
  Ryegrass staggers. *See also* Lolitrem Bdescription, 39–40

ergovaline and lolitrem B concentration in, 40
threshold values, in livestock, 55

S

Salicylaldehyde, 86, 87
Saussurea involucrata, 108
Sawflies, 87–89
Secondary metabolites, 79
accumulation, 120
production, 119–120
biomolecule production, 116–117
in culture, 117–118
medicinal potential, plants, 116
Sesquiterpenoid toxins, 1
Sexual pheromones, 81–82
Shoot regeneration, hairy root, 106, 111
Solanum
S. nigrum, 108
S. tuberosum, 97, 108
Sorghum propinquum, 146
Summer syndrome, 39
Superbugs, 89
drugs from, 90
Syngenta foundation, 70
Synthetic pheromone, 82

T

Taraxacum kok-saghyz, 69
Thlaspi caerulescens, 97
Trichothecenes
biosynthesis, 3–4
biosynthetic genes and functions, 5–7
cellular effects, 2
resistance, 13–14
structure, 1–2
variations
in chemotypes, 10–12
on theme, 7–9
T-2 toxin, 2
biosynthesis, 6
Fusarium sporotrichioides, 3–4
Tylophora indica, 109

V

Véraison, 23–24
Vigna aconitifolia, 97
Vitis vinifera, 29

W

Withania somnifera, 97
Woody plants
advantages, 60–61
biopolymers, 67–68
latex, 68–70
oils for biofuels, 67–68

Z

Zea mays, 110
Zingiber officinale, 146–147