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Epigenetics in Plants of Agronomic Importance: Fundamentals and Applications

Transcriptional Regulation and Chromatin Remodelling in Plants

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Preface

The first Agricultural Revolution, that is, the initial transition from hunting and gathering to settled agriculture, is considered by many to have begun around 12,000 years ago. Since then, humans have domesticated hundreds of plant species and it is considered that the evolution of crop plants took place as human behavioral ecology changed from food gathering to farming. Domestication of wild species of plants comprises a variety of evolutionary changes (phenotypic and genetic divergence amongst domesticated populations) that may diminish the fitness of a plant in the wild but increase it under human exploitation. Since then, the selection of populations with desirable alleles, the meticulous breeding of high yielding genotypes, ease of farming and quality, and numerous technological advances have allowed crop production to increase and in this way supply the nutritional requirements of an ever-increasing human population.

During the last decades, and in particular as a part of the Green Revolution, modern breeding methods, novel research, development, and technology transfer initiatives have increased dramatically agriculture production worldwide. Many beneficial traits in crop species include, for example, increased yield, enhanced abiotic/biotic stress tolerance, improved nutritional quality, delayed ripening, increased post-harvest quality, delayed senescence, etc. However, it is now patent that if agriculture is to support human population for years to come, additional sustainable strategies for crop production must be developed (e.g., exploiting the positive associations with soil organisms while avoiding the negative ones), in concert with a profound understanding of the relationship between crop genotype and environment. Thus, it is opportune to evaluate the mechanisms that plants may have evolved to adapt to sudden changes in the environment. Furthermore, we need to comprehend the mechanisms by which (epi)genetic variation may modify plant gene regulation and phenotype, and we should concentrate on how the (epi)genome acts as a potent new source of diversity for agronomical important traits and its potential for exploitation in crop improvement programs. Epigenetic phenomena influence gene expression at the chromatin structure and organization level thereby modulating the access of regulatory complexes to the genome. Current research on epigenetic mechanisms suggests they are involved in almost every aspect of plant

life including agronomically important traits such as flowering time, fruit development, responses to environmental factors, and plant immunity. Hence, epigenetics is emerging as a very important field in plant genetic improvement. Although fundamental epigenetic mechanisms in crops are beginning to be elucidated, we anticipate they will be extensively employed in the future for crop improvement.

The idea of publishing this book has arisen from the fact that Epigenetics has become an important player in the study of gene regulation not only in mammals but also in plants. The inception of research in epigenetics came from the desire to understand how it affects plant development and behavior. Plants are vital in our life because they provide us with oxygen, food, clothing, and medicines. This book gives us comprehensive knowledge about the fundaments and applications of epigenetics in plants of agronomic importance. A total of eight chapters describe the importance of epigenetics in agriculture and highlight the applications of this field in crop plants, such as coffee, maize, tomato, wheat, sugarbeet, beans, and others. Topics cover from general mechanisms of epigenetic regulation, such as DNA methylation and posttranslational modifications of histones, to the smallest player with the biggest role in gene regulation, small RNAs. We believe the information contained in this book will enhance the knowledge to develop, in the future, novel approaches to manipulate and selectively activate and/or inhibit proteins and metabolic pathways to counter plant pathogens, to better cope with environmental stresses and to increase crop productivity. In the foreseeable future there would be a strong presence of epigenetics in food production, plant fitness, and crop improvement. We hope that readers of this book will find a first glance of the many contributions the field of epigenetics may bring to the table in order to help cover the food demand in the world. Finally, we would like to thank all colleagues who agreed to provide outstanding chapter contributions.

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Chapter 1
The Role of Germinally Inherited Epialleles in Plant Breeding

Megan House and Lewis Lukens

Abstract Plant breeding focuses on repeated selection of individuals with desired traits from phenotypically variable populations. Breeders may be able to explain the broad sense heritability for a trait, the proportion of the total trait variance between genetically distinct lines compared to within a line, or the narrow sense heritability, the proportion of the trait variation that is due to the additive effects of genes. However, breeders rarely know the underlying causes of the observed genetic variation. In this chapter, we take a trait-focused approach to review the degree to which plant variation is due to epigenetic variation and to what degree epigenetic factors are suitable for selection in plant breeding. We suggest that the amount of trait variation that is due to heritable differences in chromatin states is far lower than variation due to changes in the primary sequence of DNA. In addition, epigenetic states are often unstable, and selection on only a small number of epigenetic states could lead to consistent plant improvement.

Keywords Epigenetics • Plant breeding • Epialleles • Epimutagens • Trait variation

1.1 Introduction

The term "epigenetics" has a number of definitions. Waddington (1942) used the term to explain how one genome gives rise to multiple cell lineages that follow diverse developmental trajectories. In other words, epigenetics referred to mechanisms that enable the developmentally appropriate expression of genes. In Waddington's conception, epigenetic information laid down in development is erased during gametogenesis, consistent with the Mendelian principle that genes

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passed across generations are unaltered by developmental or environmental stimuli. More recently, epigenetics can refer to meiotically heritable changes in gene function that are not due to differences in nucleotide sequence, and we use this definition in our work. Here, an organism imposes chemical changes to DNA or chromatin within a germ cell, and these changes are transmitted to the subsequent generation. Epimutation is a process that generates an epiallele, and the term epiallele refers to a gene with distinct biochemical modifications. Thus, a trait that both varies within a population because of polymorphic nucleotide sequence (s) and is correlated between parent/offspring pairs because of shared nucleotide sequence(s) exhibits genetic inheritance. A trait that both varies within a population because of variable chromatin structures and is correlated between parent/offspring pairs because of these structures exhibits complete epigenetic inheritance.

In this chapter, we first review how the inheritance of variable chromatin states—induced chemically, genetically, or by the environment—can contribute to phenotypic variation. We then address the stability of epialleles across generations. Finally, we highlight the role of epigenetic variation in plant breeding.

1.2 Meiotically Inherited Epigenetic Differences Can Cause Phenotypic Variation

Many epialleles characterized to date are marked by DNA methylation differences. Treatment of plants with DNA methylation inhibitors, such as 5-azacytidine (5azaC) and 5-azadeoxycytidine (azadC), can induce heritable, phenotypic changes (Fig. 1.1, Table 1.1). For example, Fieldes (1994) induced heritable phenotypic changes in flax by 5azaC treatment. Relative to untreated plants, plants growing from treated seeds were often shorter, had fewer leaves on the main stem, and had a reduced flowering times (Fieldes 1994; Amyot 1997; House 2010). From first generation progeny of treated plants, Fieldes et al. selected six lines of flax that were short and early-flowering (Fieldes 1994). Flax is self-pollinating, and these traits were stably transmitted to the next generation. Flowering time variation in populations derived from crossing the early-flowering line to the wild type indicated that at least three independent epialleles contribute to early flowering (Fieldes and Amyot 1999). In Triticale, a wheat x rye hybrid plant, plants from seeds treated with 5azaC have a number of heritable, phenotypic differences relative to plants from untreated seeds (Heslop-Harrison 1990). The 5azaC treatment resulted in plants that are taller than controls, have increased tillering and an increased time to maturity, and these novel traits persisted through two subsequent generations, at which point the study concluded (Heslop-Harrison 1990). Akimoto et al. (2007) noted that two plants grown from a population of 100 rice seeds (*Oryza sativa* spp. *japonica*, 'Yamada-nishiki') treated with azadC differed from plants grown from untreated seeds. Most remarkable was a line that was dwarf and flowered 10–14 days early. Similar, chemically induced heritable variation has been observed in

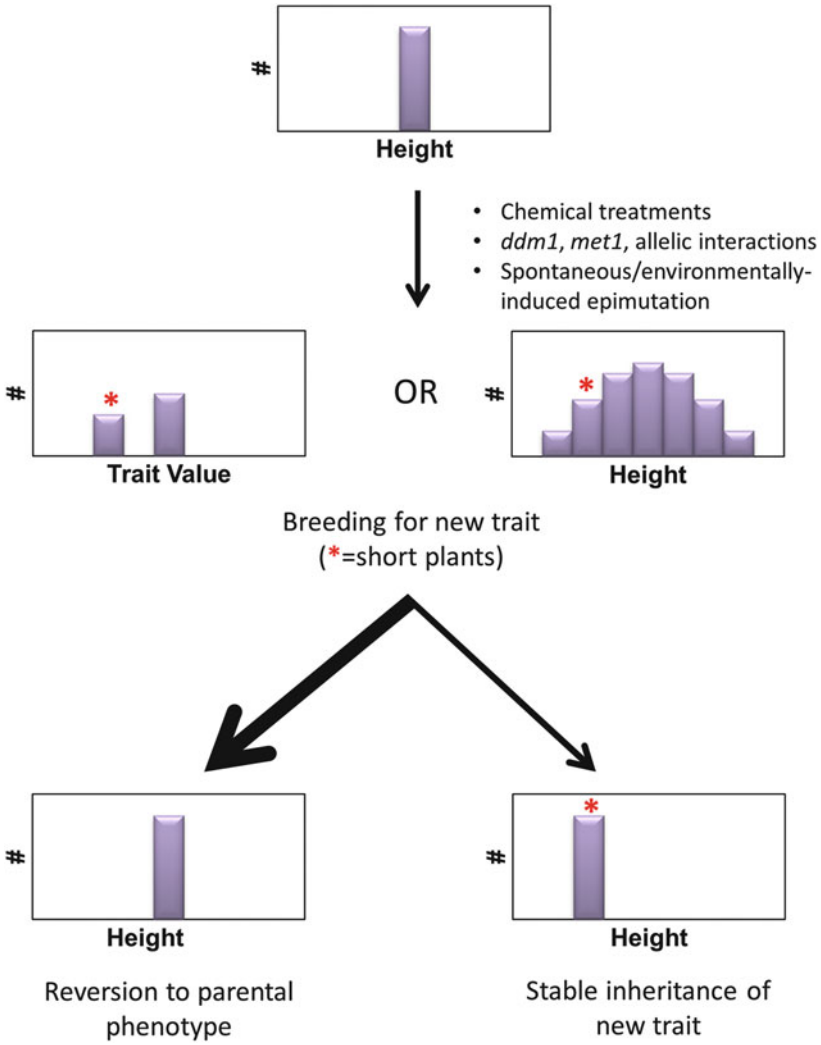

Fig. 1.1 Epimutations induced by several phenomena can generate phenotypic novelty that is in some cases stably inherited. Within the histograms above, the X axis represents a trait value for a plant, for example plant height. The Y axis represents the number of individuals within a population with that trait value. New discrete or continuous trait values arise because of epimutation. The *asterisk* represents a new, favorable trait value. The *arrows* represent the relative frequency of outcomes of selection for the *asterisk* plants. On the *left*, selection was not successful. The trait has reverted to its ancestral value. On the *right*, selection successfully shifted the trait value of the population

other crops, including Brassica (altered leaf morphology, reduced number of anthers, altered phyllotaxy, deformed flowers and change in the time to flowering) (King 1995), rice (dwarfism and delayed ear emergence) (Sano et al. 1990), and

Table 1.1 Examples of epialleles described in this chapter

Species	Locus	Nature of change	Trait affected	References
L. vulgaris	*Lcyc*	Spontaneous	Floral architecture	Gustafsson (1979), Cubas et al. (1999)
Tomato	*Cnr*	Spontaneous	Skin pigmentation and fruit ripening	Thompson et al. (1999), Manning et al. (2006)
Zea mays	*B1*	Spontaneous (Paramutation)	Pigmentation	Coe (1966), Patterson et al. (1993, 1995), Stam et al. (2002)
Flax	?	Induced (5azaC)	Height, flowering time and leaf number	Fieldes (1994), Fieldes et al. (2005)
Rice	?	Induced (azadC)	Height and pathogen resistance	Akimoto et al. (2007)
Triticale	?	Induced (5azaC)	Height, tillering and flowering time	Heslop-Harrison (1990)
Maize	*Spm*	Spontaneous	Anthocyanin production	McClintock (1957, 1965), Peterson (1966), Fowler and Peterson (1978), Banks et al. (1988)
Arabidopsis	*SUP*	Mutagen	Floral morphology	Jacobsen and Meyerowitz (1997), Ito et al. (2003)
Arabidopsis	*FWA*	Mutagen	Flowering time	Soppe et al. (2000)

Melandrium (appearance of bisexual flowers on a normally dioecious plant) (Janoušek et al. 1996).

The concept that all variation generated by DNA demethylating agents is caused by epigenetic variation is attractive, but some heritable traits among treated populations may have a genetic basis. 5azaC and azadC demethylating agents can act as weak mutagens (Zimmermann and Scheel 1984). In addition, demethylation can activate quiescent transposons leading to transposon mutagenesis (Scortecci et al. 1997).

Mutations within genes important for maintaining DNA methylation also act as epimutagens and generate heritable epialleles. For example, the *Arabidopsis thaliana* gene *DDM1* (*Deficient in DNA Methylation 1*) encodes an ATPase chromatin remodeller that is involved in the maintenance of DNA methylation in both CG and non-CG sequence contexts (Jeddeloh et al. 1999) and in the silencing of repeat elements such as transposons (Hirochika et al. 2000; Miura et al. 2001; Singer et al. 2001). Genomic DNA of the *ddm1* mutant is hypomethylated throughout the genome (Vongs et al. 1993). *ddm1* plants have weak phenotypes, but after several generations of selfing, novel traits including leaf structure, flowering time, flower structure, both increased and decreased apical dominance, and reduced internode length arise at high frequency within mutant lines (Kakutani

et al. 1996). Some epialleles that appear within the *ddm1* mutant background, such as the ball phenotype, are stably inherited (Kakutani et al. 1996; Soppe et al. 2000; Saze and Kakutani 2007). METHYLTRANSFERASE1 (MET1) is also required for propagating ^{m}CG methylation during DNA replication, and *Arabidopsis* (ecotype C24) *MET1* antisense lines show the heritable effects of aberrant DNA methylation patterns through the gradual loss of CG methylation (Finnegan et al. 1996). A number of traits arise in *met1* lines including reduced apical dominance, altered flowering time, altered floral morphology, decreased plant size, and altered leaf shape and size (Finnegan et al. 1996). As with *ddm1* mutants, floral traits persist in individuals without the silencing transgene (Finnegan et al. 1996).

As with chemical treatments, mutations that reduce DNA methylation can have secondary effects on DNA sequence through novel transpositions. The clam phenotype is a severe, heritable phenotype induced by *ddm1*. Plant growth is severely inhibited. Miura et al. (2001) discovered the trait was due to insertion of a CACTA family transposon released in the low methylation genome. Nonetheless, the rate of transposition is low in part because of RNA dependent DNA methylation (Miura et al. 2001; Singer et al. 2001; Teixeira et al. 2009). Transposons have unlikely generated the large amount of trait variation amongst mutant lines. For example, a locus controlling late flowering traits was genetically mapped to *FWA* (Soppe et al. 2000). *FWA* has tandem repeats within its promoter. While the repeats are methylated in diploid tissues of wild-type plants, exposure to *ddm1* has caused low DNA methylation levels and thus activated alleles to repress flowering (Soppe et al. 2000).

Some trait variation is also caused by allelic interactions between homologous alleles. Studies of maize pigmentation inheritance have revealed a number of these scenarios. Brink (1956) noted that the effect of R^r male gametes from R^rR^{st} plants with stippled aleurone differed from the effect of R^r male gametes from R^rR^r sibling plants. When crossed to a tester strain with colorless aleurone (*rr*), the latter gave the expected dark mottled kernels. However, the former produced testcross progeny with stippled seed (Brink 1956). Brink termed *R* alleles from the former cross as R' alleles. These R' epialleles are transmitted across generations. While kernels from $rr \times Rr$ test crosses show the expected dark mottling, kernels from the $rr \times R'r$ test crosses are weakly pigmented. The *booster1*(*b1*) locus in maize (Coe 1966) also regulates production of anthocyanin pigments. Plants homozygous for the *B-I* (*B-Intense*) allele at the *b1* gene have dark purple pigmentation and high levels of gene expression, whereas plants homozygous for the B' allele are lightly pigmented (Coe 1966) and have low levels of transcription at the *b1* gene (10- to 20-fold lower than *B-I* homozygotes) (Patterson et al. 1993). In heterozygotes that carry both the *B-I* allele and the B' allele, *B-I* is converted (paramutated) to B' with 100 % frequency (Coe 1966). The new B' allele is designated B', and is able to paramutate a *B-I* allele to B' in the following generation (Coe 1966). A region of tandem repeats ~6 kb in length and ~100 kb upstream of the *b1* gene is crucial for the paramutagenicity and the paramutability of the B' and *B-I* alleles (Stam et al. 2002). Double stranded RNA is very likely the key factor that changes paramutable alleles to paramutagenic alleles (Alleman et al. 2006). RNA-dependent RNA polymerase,

mediator of paramutation1 (*mop1*) is necessary for paramutation to occur (Alleman et al. 2006).

Heritable epialleles may also arise spontaneously through known or unknown environmental triggers and can be observed within plant populations. In maize, some variation is caused by chromatin modifications of TEs that are associated with pigment regulatory loci. McClintock described *Supressor-mutator* (*Spm*) transposable elements (also known as *Enhancer* elements) that have variable effects on anthocyanin pigmentation (McClintock 1957; McClintock 1965). Phases of *Spm* activity are heritable through meiosis. Active *Spm* elements have low cytosine methylation, or none at all. Intermediate elements are partially methylated, and inactive forms are fully methylated (Banks et al. 1988). A toadflax (*Linaria vulgaris*) mutant, originally described by Linnaeus, has radially symmetric flowers rather than the wild-type bilaterally symmetric flowers (Gustafsson 1979). Cubas et al. (1999) mapped the floral shape difference to a *cycloidea* type gene (*Lcyc*). The mutant and wild-type alleles differ at a single nucleotide that does not explain the phenotypic difference (Cubas et al. 1999). Chromatin state seems to be the key factor distinguishing wild type and mutant alleles. Among an F2 population derived from a cross of wild type and mutant plants, the radially symmetrical floral trait correlates perfectly with the cytosine methylation status of Sau3A restriction enzyme recognition sites. Plants with radially symmetrical flowers have high cytosine methylation upstream and within the coding sequences of *Lcyc*. In tomato, one dominant locus *Colourless non-ripening* (*Cnr*) causes plants to generate fruit with a colourless pericarp, inhibited softening, and reduced ethylene production (Thompson et al. 1999). The mutation was mapped to a 95 kb interval, but the nucleotide sequences of mutant and wild-type alleles were identical (Manning et al. 2006). An open reading frame with reduced expression in the mutant fruit compared to the wild-type fruit was identified as a *SQUAMOSA promoter binding-like* gene (*SPL*) transcription factor. A 286-bp region located 2.4 kb upstream of the gene is hypermethylated in mutant plants relative to the wild type (Manning et al. 2006). Similarly, plants homozygous for *clark kent* (*clk*) alleles of the *A. thaliana SUPERMAN* gene have a higher number of stamens and carpels than do wild-type plants (Bowman et al. 1992). *clk* and wild-type alleles have no sequence polymorphisms but the *clk* allele is extensively methylated relative to the wild-type allele (Jacobsen and Meyerowitz 1997).

1.3 The Stability of Epigenetic Effects Across Generations

Epialleles such as those described above have two attributes that suggest utility in plant breeding. The epialleles have effects on traits, and these effects are heritable. Many epialleles are also remarkably stable across generations. Fieldes et al. (2005) demonstrated that seed from self-pollinated, early-flowering flax lines generated by 5azaC treatment did not revert and continued to flower early for over eight generations. The level of total genomic cytosine methylation within early flowering

plants was also stably inherited. Fieldes et al. (2005) estimated that 5–8 % of cytosines were methylated in the early flowering lines; while 14 % of cytosines were methylated within the control lines. Akimoto et al. (2007) reported that the dwarf rice line generated by azadC treatment was stably inherited over nine generations. The same line had higher resistance to infection by a *Xanthomonas oryzae* strain than did the wild-type line (Akimoto et al. 2007). The *Cnr* epiallele described above also has high stability. Between the years of 1993 and 2006, more than 3,000 mutant plants with the colourless phenotype were grown, and of those plants a revertant 'ripening sector' containing wild-type pigmentation was observed on only three fruits on three separate plants (Manning et al. 2006). The B' epi-allele in maize is extremely stable once formed (Coe 1966; Stam et al. 2002). Patterson et al. reported scoring over 20,000 progeny of B'/B' plants and seeing no revertants to *B-I* (Coe 1966; Patterson et al. 1993).

Two experiments in *Arabidopsis thaliana* also suggest traits due to *ddm1*- and *met1*-induced epigenetic variation can be stably inherited through many generations. Reinders et al. (2009) generated RILs derived from a cross between a wild-type plant and homozygous *met1-3* mutant. Plants homozygous for the wild-type *met1* allele in the F2 were selected for six generations of inbreeding. Reinders et al. (2009) reported that flowering time, plant growth (biomass), and salt stress tolerances appeared stable in particular epi-RILs. Similarly, Johannes et al. (2009) studied the effects of inherited hypomethylated epialleles created in a *ddm1* mutant background on plant height and flowering time variation. Johannes et al. did not estimate narrow sense heritability, but genetic differences among RIL lineages are surprisingly high for flowering time ($H^2 = 0.26$) and plant height ($H^2 = 0.32$). The trait variance explained among the RILs is about 1/10 to 1/3 the variance explained across a diverse set of natural accessions (Roux et al. 2011). These experiments are designed such that variation among the RILs should be attributed to epigenetic differences. Nonetheless, at least some trait variation may be genetic. Parental lines, despite having a recent, shared common ancestor, likely have some polymorphic DNA sites. Mutations could also occur during inbreeding. For example, Reinders et al. (2009) found CACTA transposition in a significant number of RILs, despite the fact that the element does not transpose within the parental lines.

Despite these examples, stable inheritance of traits caused by epialleles seems to be the exception rather than the rule. Among the epialleles generated by chemical treatment that have phenotypic effects, many lose their effect over generations (Fieldes 1994). In maize, R' can readily revert to R. Male gametes from the RR offspring of RR^{st} plants when used in test crosses with rr resulted in plants with darkly mottled kernels (Brink 1956). The anthocyanin traits conditional on *Spm* activity, as described by McClintock, are reversible and highly changeable. For instance, she observed that elements can remain silent for multiple generations after which they return spontaneously, and at a low frequency, to an active state (McClintock 1957, 1965; Fedoroff 1999). The radially symmetrical form of *L. vulgaris* is widespread (Gustafsson 1979). However, from a segregating population derived from intermating five F1 individuals from a cross between a radially flowered mutant and bilaterally flowered wild-type plant, only 5 of 39 plants (13 %)

had radially symmetric flowers (Cubas et al. 1999). In addition, among the five plants with radially symmetrical flowers, four had partial reversions to the wild-type phenotype (Cubas et al. 1999).

Finally, epialleles that are consistent in some contexts appear inconsistent in others. For example, as noted above *FWA* epialleles have sufficient stability of expression to enable map-based cloning (Kakutani 1997; Soppe et al. 2000) in which *FWA* is hypomethylated. However, among the RIL population developed from the *ddm1* mutant, Johannes et al. (2009) found that among 22 epi-RILs, including 12 late-flowering lines, *FWA* had wild-type levels of DNA methylation.

1.4 Conclusion: The Importance of Epigenetics for Past and Future Crop Breeding

Judging from published research, the molecular basis of most heritable trait variation within and between breeding populations is overwhelmingly genetic. This fact suggests to us that past plant breeding has likely minimally utilized epigenetic variation. Researchers may have *a priori* examined traits that were more likely under genetic than epigenetic control. For example, chromatin variation may be a more common regulatory mechanism for genes with limited or low activity (Gemma et al. 2013). Researchers may have also not reported cases in which trait variation cannot be attributed to a DNA polymorphism. Although such scenarios are possible, they unlikely explain the predominance of DNA polymorphisms as causative factors. Instead, many genes may have chromatin structures that have evolved to be resistant to epimutation. Given the importance of chromatin structure regulation throughout development, a mutation that causes stable inheritance of an epigenetic state on an allele may well be deleterious (Jorgensen 1993). In addition, we suggest that epigenetic variation in plant populations under strong artificial selection is lower than epigenetic variation within natural populations. Because epialleles obtain novel epiallelic states at high frequency and cause traits to vary, these alleles may be selected against.

Nonetheless, plant breeding requires significant traits to vary and for selection on those traits to be effective. As noted above, epigenetic differences can cause meaningful trait variation that is heritable. In addition, novel variation is generated far more quickly from epimutation than from DNA mutation. Thus, for traits controlled by genes whose mis-regulation is not deleterious, epialleles may be promising sources of new trait variation. Novel epialleles would especially play a role in breeding populations where there is little genetic variation. Finally, much is known about the molecular basis of chromatin state transmission (Chandler 2010; Daxinger and Whitelaw 2012). It would be intriguing to modify some of these molecular components to target and stabilize epigenetic states.

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Chapter 2
Epigenetics and Heterosis in Crop Plants

Peter Ryder, Peter C. McKeown, Antoine Fort, and Charles Spillane

Abstract Heterosis refers to improved or altered performance observed in F1 hybrid organisms when compared to their parents. Heterosis has revolutionized agriculture by improving key agronomic traits in crop plants. However, even after decades of research in this area a unifying molecular theory of heterosis remains somewhat elusive. For many years it has been observed that the *dominant*, *over-dominant* and *epistasis* models have prevailed for explaining multigenic heterosis. The use of whole transcriptome, proteome, metabolome and epigenome profiling approaches can further generate and inform hypotheses regarding heterosis. This chapter reviews the models that have been used to explain heterosis. We also review the mechanistic basis of epigenetic pathways in plants and describe how they may also be considered in relation to understanding heterosis. There are a number of findings that support potential links between epigenetic regulation and heterosis in model and crop plants, including the potential for DNA methylation, histone modification and small RNAs to influence heterotic effects in F1 hybrids. Overall, we assess some opportunities and challenges for epigenetic research to advance the molecular understanding of heterosis.

Keywords Heterosis • Epigenetics • DNA methylation • sRNA • Transchromosomal methylation • Hybrid vigor • Parent-of-origin

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2.1 Importance of Heterosis for Crop Improvement

Heterosis is the phenomenon observed when the F1 progeny of a cross exhibit improved or transgressive values for growth or other traits when compared to their parents. The discovery of heterosis was recorded as early as the 1700s when the botanist Joseph Koelreuter observed that F1 hybrid tobacco plants exceeded the height of their parents (Reed 1942). The first characterization of heterosis was accomplished in a pioneering study performed by Darwin in 1876. By comparing the self-fertilized and cross-fertilized progeny of pairs of inbred parents of 60 plant species he observed that the F1 hybrids from crossed plants were typically taller and more vigorous than self-fertilized crosses (Darwin 1876). This phenomenon was later verified independently by George Shull (1908) and East and Jones (1919) in breeding programs of maize (*Zea mays* L.), with Shull being the first to coin the term "heterosis" in a lecture given in 1914.

The exploitation of heterosis has had revolutionary effects on global agriculture and has led to increased yields in a range of crop species (Mendoza and Haynes 1974; Duvick 2001; Schnable and Springer 2013). Heterosis has been applied with particular success in maize (Crow 1998; Duvick 2001), but has also been deployed in other crops such as wheat (Wang et al. 2006; Qi et al. 2012), tomatoes (Williams and Gilbert 1960; Krieger et al. 2010) and rice (Yu et al. 1997). Heterosis has also been harnessed in livestock including cattle (Neufeld Arce 2006) and observed in other mammals such as mice (Leamy and Thorpe 1984; Han et al. 2008). The phenomenon of heterosis is assumed to be widespread amongst eukaryotes (Goff 2011; Baranwal et al. 2012).

In plants, heterosis is often considered to be a complex and multigenic trait, involving alterations to numerous quantitative traits such as vegetative growth rate and plant stature, accumulation of metabolites, flowering time, biomass, seed size, and tolerance to biotic and abiotic stresses (Baranwal et al. 2012). Such changes can lead to heterotic phenotypes leading to increased yield of a crop. Notably, heterosis can occur in either 'direction', either increasing the trait value of interest relative to the parents, or decreasing it. Depending on the trait in question, either may be of potential interest in crop breeding programs (for example, so-called 'negative' heterosis for seed size may be of value for fruit crops). Heterosis can be classified in two ways: (1) heterosis that exceeds the mean of the parental values (termed mid-parent heterosis) or (2) heterosis which exceeds the values of both parents (termed best-parent heterosis).

Adoption of hybrid maize became more widespread in the USA in the 1930s. Maize yields increased by approximately 2 % year on year through the use of heterotic F1 hybrids in the period 1930–1940. Heterosis research improvements occurred in parallel to agronomic improvements, including advances in farm machinery and fertilizers. Heterosis breeding systems have also been subject to ongoing improvements (e.g. through the establishment of double haploid approaches to create inbred lines more rapidly than conventional methods like single seed descent). The success of hybrid crops relies upon the willingness of farmers to purchase F1 hybrids each year from breeding companies, because heterosis is largely restricted to the F1 generation (Hufford and Mazer 2003).

A range of genetic models have been advanced to explain the occurrence of heterosis in the offspring of certain crosses, whether in plants or other organisms. However, it is recognized that these models may not be able to wholly explain all aspects of heterosis (Groszmann et al. 2013). These models are described below.

2.2 Genetic Models for Explaining Heterosis: Successes and Limitations

Although the underlying mechanisms of heterosis are still not fully understood, increased heterozygosity is often positively correlated with increased fitness in many species (Darwin 1876). When genetically distinct genomes hybridize for the first time they may encounter genetic shock and asynchrony effects (Gernand et al. 2005). If the genomes are genetically incompatible, post-fertilization aberrations and seed abortion may occur, preventing the production of viable F1 progeny. This is termed hybrid incompatibility (Burke and Arnold 2001), which is observed in some inter-specific hybridizations (Burkart-Waco et al. 2013). However if two genetically distinct genomes hybridize and overcome the post-fertilization barriers and produce viable offspring, heterosis may be observed in some instances (Birchler et al. 2010; Chen 2010).

Inbreeding depression is commonly considered the conceptual opposite of heterosis. In maize it has been predicted that heterosis can occur by reversing inbreeding depression on self-fertilized lines (Good and Hallauer 1977). Inbreeding depression is defined as "the reduced survival and fertility of offspring of related individuals" (Charlesworth and Willis 2009). Outcrossing organisms including plants and animals which undergo multiple rounds of inbreeding generally display slower growth, lower fertility and increased disease susceptibility (Charlesworth and Charlesworth 1987). Most genetic models for explaining heterosis rely upon considerations of the impact of heterozygosity and homozygosity at particular loci in inbred and outbred individuals. The most widely considered genetic models for explaining heterosis are the *dominance*, *overdominance* and *epistasis* models (Lewontin 1964).

These three models have been developed to allow better scientific understanding of the biological phenomenon of heterosis. The development of accurate models is a prerequisite for rational exploitation of the potential value of heterosis in agriculture and other applied biology areas. However, despite consistent research in this field for over 70 years, a clear unifying molecular or genetic model remains elusive. It is likely that no one model can fully explain either hybrid vigor or heterosis. It is important to note that these theories are not mutually exclusive, and that it is likely that different mechanisms can explain heterosis observed under different combinations of crosses in different species, or affecting different phenotypes (Chen 2013; Schnable and Springer 2013).

2.2.1 Dominance Model of Heterosis

The *dominance model* of heterosis proposes that following hybridization between genetically distant genomes, the F1 generation display heterotic characteristics as a result of the complementation of multiple slightly deleterious alleles from the genome of one parent line by superior, dominant alleles from the other (Birchler et al. 2003). This can lead to F1 offspring that exceed the trait values observed in either parent. In Fig. 2.1a, slightly deleterious alleles ("a" and "b") are present in the genomes of parental lines P1 and P2, which have genotypes aa,BB and AA,bb respectively. Although alleles significantly reducing the fitness of the organism are expected to be purged by natural selection (Schnable and Springer 2013), mildly deleterious alleles may persist in a population due to linkage with beneficial or essential alleles. Upon hybridization, the F1 offspring will be heterozygous at both loci i.e. genotype Aa,Bb. The deleterious alleles at both loci can thus be complemented, leading to increased fitness or enhanced values of other traits observed. The heterosis effect observed in the F1 progeny is not stably inherited in subsequent generations due to independent segregation. The dominance model is also applicable in the case of crosses in which one parent contains advantageous genes which are entirely missing or non-functional in another (Fu and Dooner 2002; Birchler et al. 2010). In both cases, the dominance model (masking of deleterious recessive alleles) presents heterosis as a simple reversal of inbreeding depression (unmasking of deleterious recessive alleles).

2.2.2 Over-Dominance Model of Heterosis

Since its development in the early part of the twentieth century, the dominance model has explained significant aspects of heterosis (Davenport 1908; Jones 1917; Troyer 2006). However, the dominance model also suffers from certain limitations which suggest that it is only a partial explanation for the phenomenon of heterosis. A key criticism of this model is that if complementation of deleterious alleles is causal for heterosis then the potential to generate heterosis by crossing commercially-available inbred lines should decrease over time (Springer and Stupar 2007). Elite maize germplasm has been exploited in breeding programs for nearly 90 years, and during this period the majority of slightly deleterious alleles would be expected to have been purged (Duvick 2001). Models of heterosis relying entirely on the concept of dominance would predict that the potential for heterosis should also have decreased over the same time period (Birchler et al. 2003). However, the extent of heterosis generated in breeding programs has not reduced over time, and may even have increased somewhat (Duvick 1999), suggesting that heterosis is more than a simple complementation of deleterious alleles by dominant ones.

The extent of heterosis and inbreeding depression in polyploid plants when compared with their diploid counterparts also suggests that dominance models of heterosis are incomplete. Since polyploids have the potential to possess higher allelic

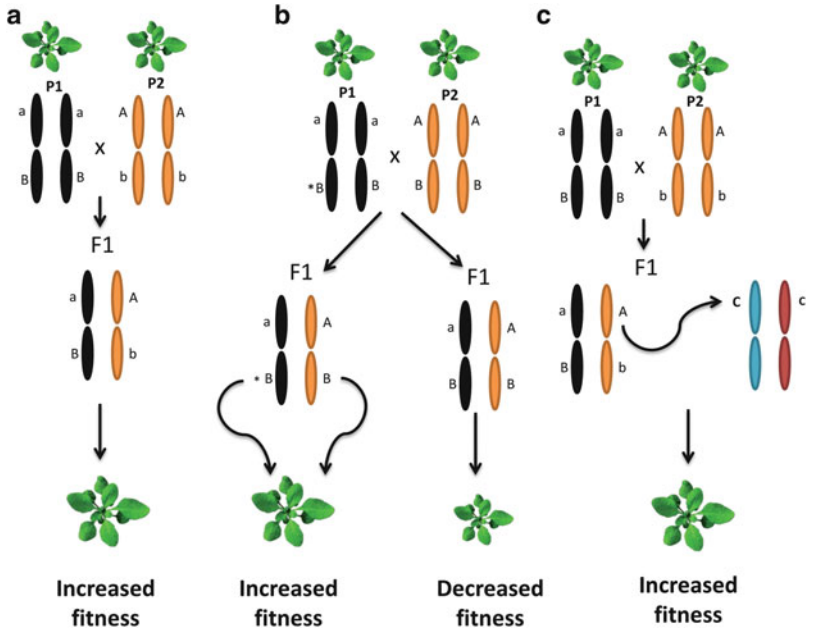

Fig. 2.1 Schematic of genetic models for explaining heterosis. (**a**) Dominance model; (**b**) Over-dominance model; (**c**) Epistasis. For full descriptions, see text

diversity than their diploid counterparts, the onset of inbreeding depression in polyploids should occur more slowly during the self-fertilization of polyploids than in diploid progenitors, as homozygous offspring are produced less frequently. However, it has been shown that inbreeding depression rates are similar in diploids (2×) and tetraploids (4×) of various plant species (Rice and Dudley 1974; Birchler et al. 2005). Furthermore, the levels of heterosis observed when inbreeding depression is reversed continue to increase with increasing heterozygosity (Birchler et al. 2005), which would not be the case if heterosis depended upon the masking of slightly deleterious alleles. In the case of polyploid plants, it is likely that complementation of deleterious alleles by dominance therefore plays only a limited role in heterosis.

Limitations in genetic models of heterosis based on dominance led to the development of alternative models based on transgressive (or over-dominant) interactions between alleles rather than simple complementation, or based on allelic dosage effects (the onset and reversal of inbreeding depression in polyploids has been explained with reference to allelic dosage effects (Birchler et al. 2005)). The *over-dominance model* proposes that synergistic allelic interaction at particular heterozygous loci leads to superior performance in the F1 progeny. In Fig. 2.1b, *B is an allele variant of B (irrespective of dominance in this case). F1 hybrids inherit both alleles and act synergistically to cause a heterotic effect. If *B is not inherited, the F1 progeny exhibit no heterotic effect.

One of the most exciting developments in our understanding of over-dominant heterosis is the identification of cases of "single locus over-dominance" (Mckeown

et al. 2013a) such as that involving the *SINGLE FLOWER TRUSS* (*SFT*) locus in tomato. *SFT* is a *FLOWERING TIME* (*FT*) related gene that when present in a heterozygous state increases tomato yields by up to 60 % (Krieger et al. 2010). Other cases of single locus heterosis have been observed in the model plant organism *Arabidopsis thaliana* (Meyer et al. 2010; Smith et al. 2011), as well as in other agronomic crops including wheat (Li et al. 2013), rice (Hua et al. 2003; Goff and Zhang 2013) and maize (Schnable and Springer 2013). This could lead to the production of heterodimeric protein complexes with greater activity than either homodimeric complex, for example.

The identification of over-dominant loci could potentially lead to easier and faster deployment of heterosis. The conventional method of generating hybrids (crossing inbred lines in different combinations to identify non-additive traits in F1 progeny (Duvick 2001) is time consuming, laborious and expensive. With the aid of denser genetic maps for agronomic crops, quantitative trait loci (QTL) maps relevant for the study of heterosis are being generated (Basunanda et al. 2010; Schön et al. 2010; Mckeown et al. 2013b; Wallace et al. 2014). Such methods still face potential pitfalls such as false positives arising as a result of pseudo-overdominance. This is defined as a phenomenon where two or more tightly linked dominant alleles in a repulsion phase can induce heterosis in F1 offspring which mimics over-dominance effects (Crow 1952; Schnable and Springer 2013). Heterosis due to epistatic interactions can also mimic over-dominance (see below). Accurate identification of individual loci that can induce heterosis when in a heterozygotic state could be extremely useful for crop breeding programs as it would allow better prediction of heterotic crosses, and, potentially, direct manipulation of the loci concerned. The advent of genome editing techniques using Transcription Activator-like Effector Nucleases (TALENs) or Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) could potentially be used to efficiently generate over-dominant alleles to induce artificial overdominant heterosis as previously proposed (Mckeown et al. 2013a).

2.2.3 Heterosis, Epistasis and Complexity

Whereas the over-dominance model proposes that interactions at individual loci can induce heterosis, the *epistasis* model posits that heterosis can arise from epistatic interactions between alleles at different loci. Many heterotic epistatic relationships could in principle occur in F1 hybrids when one allele is complemented and its gene product affects the function of one or more products of other genes. For example, in Fig. 2.1c the gene product of dominant allele "A" has an epistatic interaction with the gene product of "C", an unlinked locus. In some instances, this interaction can cause heterotic effects in the F1 progeny. An allele having an epistatic relationships with the allele of another locus *in trans* can mimic an over-dominant heterotic QTL.

QTL's associated with heterosis suggest that in most crosses the molecular basis of heterosis is likely to be complex, and likely multigenic (Meyer et al. 2010; Riedelsheimer et al. 2012). It is likely that heterosis cannot be entirely explained by

any single unifying mechanism. Instead, heterosis is likely to be a complex, multifactorial trait that can involve allelic interactions at one or several loci. Microarray-based transcriptome profiling of maize inbred lines B73 and Mo17 and their resulting F1 hybrids, has identified many different types of effects on gene expression including additive, high- and low-parent dominance, overdominance and underdominance (Swanson-Wagner et al. 2006). Some researchers have proposed that terms such as dominance, over-dominance, and epistasis should be abandoned in the context of heterosis models as they may be imposing artificial distinctions which do not easily correspond with the biology of the system (Birchler et al. 2010).

2.3 Is There an Epigenetic Component to Heterosis?

Despite the successes of the *dominant*, *over-dominant* and *epistatic* models, a comprehensive framework for understanding heterosis still remains elusive. This has led to the suggestion that even the sum-total of all genetic interactions in a hybrid F1 genome cannot fully explain every aspect of heterosis (Baranwal et al. 2012; Groszmann et al. 2013; Schnable and Springer 2013). Indeed, consideration can be given as to whether non-genetic mechanisms underlying heterosis might exist. Such cases of heterosis could fall into the category of 'epigenetic' effects, of the kind which have been shown to regulate gene expression, cell fate and non-Mendelian inheritance (Mckeown and Spillane 2014). Here we review evidence that suggests that there may be epigenetic components to heterosis in at least some cases, beginning with a summary of what epigenetic effects are, and how they could be contributing to heterosis effects.

Epigenetics is broadly defined as the study of heritable changes in gene activity that cannot be attributed to DNA sequence changes (Mckeown and Spillane 2014). It has been said that "epigenetics emphasizes heritable changes in gene expression that cannot be tied to genetic variation" (Richards 2006). A critical consequence of epigenetic effects is that the same genotype can display diverse phenotypes due to differential modification of the epigenetic state. For example, epialleles are alleles of a locus which have identical DNA sequences but display different epigenetic states, and which have been proposed to influence a variety of phenotypes in plants and animals (Richards 2006). The inheritance of epigenetic marks can deviate from the rules of Mendelian inheritance. The transmission of epigenetic marks through generations (as opposed to cell lineages) is a hotly investigated area of biology due to its implications for the inheritance of acquired characteristics.

Some of the most studied epigenetic mechanisms are DNA methylation, histone modifications and chromatin remodeling, and the RNAi pathway (including RNA directed DNA methylation, RdDM). Such epigenetic regulatory mechanisms can target and epigenetically modify DNA sequences (Kooter et al. 1999). Epigenetic variation at the level of DNA and chromatin can cause gene expression to spatio-temporally change throughout development of an organism, and during

gametogenesis and sexual reproduction in mammals and plants (Hsieh et al. 2009; Slotkin et al. 2009; Feng et al. 2010; Calarco et al. 2012). The following section of this chapter describes three well-known epigenetic pathways, and presents some studies that suggest that epigenetic mechanisms may contribute to heterosis effects.

2.3.1 DNA Methylation and Heterosis

DNA methylation refers to the covalent addition of methyl groups to the bases of a DNA molecule, usually at the 5′ positions of cytosine residues as catalyzed by DNA methyltransferases (He et al. 2013). DNA methylation occurs in many taxa. The function and control of DNA methylation has been deeply investigated in the model plant *Arabidopsis thaliana*. Whereas cytosine methylation (^{m}C) in animal genomes is often restricted to CpG contexts, in plant genomes it occurs more widely (Fig. 2.2A). In all sequence contexts the DOMAINS REARRANGED METHYLATION 2 (DRM2) gene-product plays a major role in establishment of ^{m}C (Cao and Jacobsen 2002). Symmetric methylation in CpG contexts is maintained by the methyltransferase METHYLTRANSFERASE 1 (MET1). Cytosine methylation in CpHpG contexts (where H = A, C or T) is maintained by a feedback loop involving CHROMOMETHYLASE 3 (CMT3) and the H3K9me2 methyltransferase, KRYPTONITE (KYP) (Cao and Jacobsen 2002). In contrast, asymmetric cytosine methylation (in a CpHpH context) is maintained by *de novo* methylation through a pathway known as RNA directed DNA methylation (RdDM) in which the methyltransferase DRM2 methylates CpHpH motifs. Active demethylation can also occur through the action of DNA glycosylase-ligases such as DEMETER (DME) (Penterman et al. 2007; Zhu 2009). DNA methylation is known to be important for the silencing of active transposons, genetic repeat elements found in pericentromeric regions of chromosomes and promoter regions of genes (Lippman et al. 2004).

A number of correlative studies have suggested that epigenetic effects, including cytosine methylation (^{m}C) of DNA, may be involved in pathways contributing to heterosis. Several studies have identified differences in ^{m}C patterns in heterotic F1 hybrids when compared to their respective parents (in maize, for example (Zhao et al. 2007). Similarly, in rice, differences in ^{m}C patterns are observed between inbred lines and are correlated with transcript level changes at some of the differentially methylated regions (DMRs) in the F1 hybrids (He et al. 2010).

Two studies analyzed crosses between *A. thaliana* accessions in which the F1 offspring display heterosis for biomass. Shen et al. (2012) performed genome-wide methylation profiling by constructing methyl-seq libraries of *A. thaliana* accessions Landsberg *erecta* (L*er*-0) and C24 parental inbred lines and their reciprocal hybrid lines, L*er*-0 × C24 and C24 × L*er*-0. Through this approach it was possible to analyse global methylation patterns in the parental and F1 genotypes. It was found that the overall level of DNA methylation was higher in the F1 hybrids compared to the parents. In a similar approach Greaves et al. (2012) performed

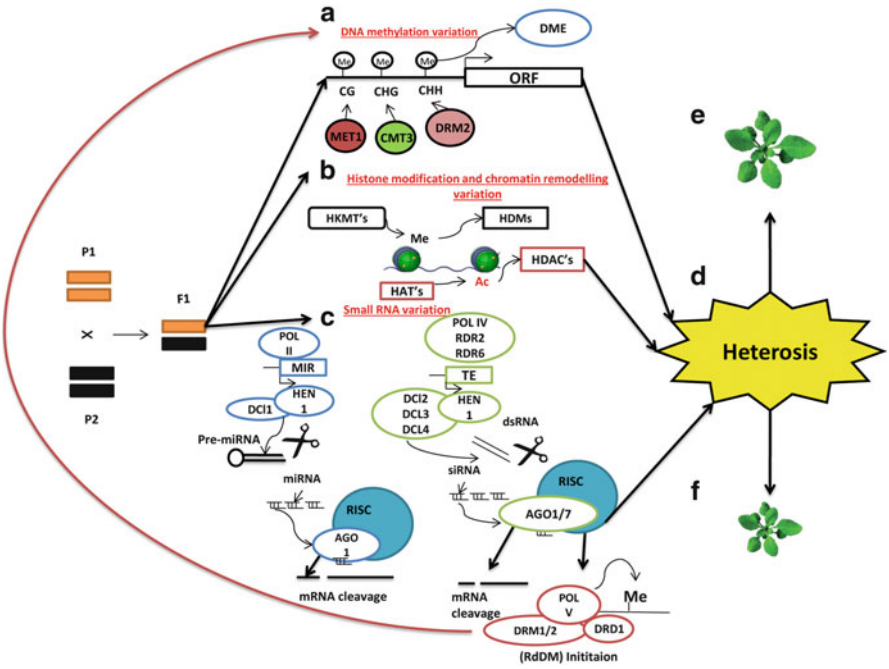

Fig. 2.2 A possible model linking epigenetics to the alteration of biological networks. Two distinct genomes hybridize to create a heterotic F1 hybrid: (*A*) Differential methylation patterns can occur in F1 hybrids where there is allelic variation at particular loci. Such methylation patterns are established and maintained symmetrically (CpG, CpHpG) by METHYTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) respectively and asymmetrically (CpHpH) by the *de novo* methyltransferase DOMAINS REARRANGED METHYLATION 2 (DRM2). *De novo* methylation can be established by RdDM (*red arrow*). (*B*) Histone lysine methyltransferases (HKMT's), demethylases (HDM's), histone acetylases (HAT's) and deacetylases (HDAC's) can produce unique histone modification patterns in F1 hybrids to activate (H3K4me) or repress transcription (H3K27me3). (*C*) sRNAs can accumulate at different levels in hybrids. miRNAs are established by POL II mediated *MIR* transcription to create precursor miRNA (Pre-miRNA) which is diced by DICER LIKE 1 (DCL1) in collaboration with HUA ENHANCER 1 (HEN1). Mature miRNA are loaded into the RNA-ASSOCIATED SILENCING COMPLEX (RISC) associated with ARGONUATE 1 (AGO1) and mediate post-transcriptional gene silencing (PTGS). sRNAs are derived primarily from transposons in heterochromatic regions or by endogenous MIR genes. They are diced by DCL2, 3 or 4 and loaded into RISC accompanied by AGO and either mediate PTGS or initiate *de novo* methylation by RdDM (*red arrow*). Such epigenetic pathways have the potential to either independently or synergistically establish heterosis (*D*), and either improve (*E*) or deteriorate (*F*) vigor in F1 hybrids

whole methylome profiling on L*er-0* and C24 parental lines and their reciprocal F1 hybrids. By using a methylation clustering approach the differences in total ^{m}C between the parents was determined to be 23 % (Greaves et al. 2012). Of this, CpHpH methylation showed the greatest variation. In addition, regions with differential methylation in a CpHpH context were enriched in gene bodies and their flanking regions. When assessing the methylome of F1 hybrids, both additive and

non-additive methylation differences were observed, with CpHpH methylation being predominantly lower than the mid parent value in hybrids. Non-additive methylation clusters were enriched in genic regions, in a similar pattern to their parental lines. This could suggest a possible link between differential ^{m}CpHpH in parental plants and the occurrence of non-additive methylation in this context in their F1 hybrid offspring, at least in *A. thaliana* (Greaves et al. 2012).

2.3.2 Heterosis and Histone Modifications

DNA methylation frequently interacts with covalent modifications of the histone octamers which 'package' the DNA into nucleosomes and into chromatin. Histone modification refers to the covalent modification of histone proteins, usually on their N-terminal tails, which causes nucleosome rearrangement, chromatin remodeling and altered transcriptional potential. A multitude of histone modification marks have been documented in plants and other eukaryotes (Berger 2007). Key histone modifications include methylation and acetylation, especially of lysine (Lys, K) residues (which are abundant on histone N-terminal tails). Such modifications are orchestrated by complexes of histone lysine methyltransferases and demethylases (HKMT's and HDM's), and acetylases and deacetylases (HAT's and HDAC's) (Fig. 2.2B) (Cao and Jacobsen 2002; Chandler and Stam 2004; Gendrel et al. 2005; Fuchs et al. 2006; Pfluger and Wagner 2007). Histone modification marks can act as binding sites for different chromatin remodeling enzyme complexes, as in the case of KYP mentioned above, and can lead to the formation of stable epigenetic loops involving feedback between DNA methylation and histone modification.

A possible link between histone modifications and heterosis has been suggested (Ni et al. 2008). This study demonstrated that genes involved in the circadian clock of *A. thaliana* underwent transcriptional changes in both diploid and allotetraploid F1 hybrids which were associated with altered histone modifications. The circadian clock, which is an intracellular biochemical mechanism that synchronizes biological events between day and night cycles, operates by matching daily changes in gene or protein activity (defined by their periods and amplitudes) to aspects of the external environment, such as daylight (Dodd et al. 2005). In plants, the circadian clock is known to control many biological processes, which include starch biosynthesis and growth rate. Plants that are exposed to environments that match its internal circadian rhythm are more vigorous than plants that are not. By using antibodies against the H3-Lys-9 acetylation (H3K9ac) and H3-Lys4 dimethylation (H3K4me2) marks which commonly correlate with gene activation in *A. thaliana* (Jenuwein and Allis 2001), Ni and colleagues found both modifications to occur at key clock regulatory genes in F1 hybrids. Functional alterations of the internal clock by histone-mediated control of the *CCA1* and *LHY* genes may lead to the differential biomass accumulation observed in hybrids and polyploids (Miller et al. 2012; Shen et al. 2012; Chen 2013).

Studies in rice have shown that overexpressing or knocking out histone deacetylase genes can lead to non-additive gene expression in hybrids at some loci, which could in principle lead to over-dominance for a trait controlled by the locus. By using high-throughput ChIP-Seq with three histone marks (H3K4me3, H3K9ac, and H3K27me3) global histone mark patterns could be compared between two rice subspecies and their resulting F1 hybrid (He et al. 2010). Correlations were found between the transcriptional activation mark, H3K4me3, and the transcriptional repression mark, H3K27me3, linked to dynamic expression patterns between hybrids and parents. Independent studies on 6 days after pollination (DAP) F1 hybrid maize endosperm transcriptomes identified significant expression variations in the key histone variant HTA112, when compared to parental inbred lines (Jahnke et al. 2010). These studies raise the possibility that features of heterosis could be associated with alterations of epigenetic histone modifications.

2.3.3 sRNAs: Roles in Epigenetic Regulation and Heterosis

In plants, epigenetic regulatory loops may also involve small RNA molecules, i.e. short (20–27 nucleotide, nt) non-coding RNA's (Simon and Meyers 2011). Such sRNA can regulate gene expression and also act as an RNA-based immune system to counteract against foreign viral RNA or transposons which are deleterious to genome integrity (Vaucheret 2006). These sRNA-mediated processes include transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (Vance and Vaucheret 2001; Waterhouse et al. 2001; Boutet et al. 2003; Lippman et al. 2004).

Plant sRNAs include two major classes, the microRNAs (miRNA) and small interfering RNAs (siRNA) (Fig. 2.2C). miRNA precursors are endogenously transcribed from endogenous *MIR* genes by RNA POLYMERASE II (RNA Pol II) and are then cleaved ("diced") to a length of 20–27 nt by DICER LIKE 1 (DCL1). The mature miRNAs are then loaded into the RNA Induced Silencing Complex (RISC) complex, accompanied by the ARGONAUTE 1 (AGO1) endonuclease (Bartel 2004). The loaded complex is then guided to messenger RNAs with sequence similarity to the mature miRNAs in order to cleave the mRNA transcripts and/or inhibit translation. Small interfering RNA (siRNA) biogenesis pathways are mostly stimulated by the presence of aberrant double stranded RNAs produced from transposons in heterochromatic regions or by invading viral RNA. They act to maintain genome stability by silencing transposons and help to protect against viral RNA invasion (Baulcombe 2004; Slotkin and Martienssen 2007). Although there is some uncertainty regarding how the biogenesis of plant siRNAs is regulated, it is considered that RNA is transcribed by RNA POLYMERASE IV (Pol IV) and reverse transcribed into double stranded RNA (dsRNA) by RNA DEPENDANT RNA POLYMERASE 6 (RDR6) or RNA DEPENDANT RNA POLYMERASE 2 (RDR2). dsRNAs are subsequently diced by either DCL2, 3 or 4 to generate mature 20–24 nt siRNAs which are loaded into RISC (accompanied by AGO

proteins) to catalyze either mRNA cleavage or stimulation of the RdDM pathway for *de novo* DNA methylation and/or histone modifications (Vaucheret 2006; Castel and Martienssen 2013). It should be noted that this model is based upon *Arabidopsis thaliana* and could vary between species.

As RdDM can direct DNA methylation and heterochromatin formation (Feng et al. 2010), it has been speculated that sRNAs could also regulate epigenetic changes associated with heterosis. Indeed, sRNA levels show substantial variation between parental inbred lines and their F1 hybrid or allopolyploid offspring in several taxa e.g. the *Arabidopsis* genus (Ha et al. 2009; Groszmann et al. 2011; Li et al. 2012; Shen et al. 2012), and the monocot cereals such as wheat (Kenan-Eichler et al. 2011), maize (Barber et al. 2012; Ding et al. 2012) and rice (Chen et al. 2010; He et al. 2010; Chodavarapu et al. 2012).

A number of studies have provided evidence to support the hypothesis that such non-additive changes might be involved in heterosis. For example, crosses between the *A. thaliana* accessions Col-0 and L*er*-0 demonstrated a decrease in the accumulation of 24-nt siRNA in the hybrids compared to the parents, concomitant with altered patterns of CpHpH methylation (Groszmann et al. 2011). Potentially, heterosis could be induced by the hybridization of epigenetically divergent parents as a result of increased epiallelic variation within the offspring (Chen 2013). When differences in DNA methylation between parental and heterotic F1 hybrid *A. thaliana* lines were mapped at single base-pair resolution across the genome, the hybrids displayed elevated methylation levels, especially in transposable elements (Shen et al. 2012). A parallel genome-wide sRNA-seq experiment demonstrated that production of sRNA differed between the parental lines and hybrids. In addition, sites of sRNA synthesis were significantly associated with loci undergoing increased DNA methylation (Shen et al. 2012). This study suggests a link between sRNA and ^{m}C accumulation with altered expression in F1 hybrids at selective loci.

To date, most studies of the possible links between sRNAs, DNA methylation and heterosis have been based upon inference and correlation. However, similar to the use of histone modification mutants in rice, some studies have functionally tested the possibility that sRNA-mediated pathways might be necessary for heterosis. HUA ENHANCER 1 (HEN1) is an *A. thaliana* methyltransferase that methylates mature sRNAs of both siRNA and miRNA classes to increase their stability (Vilkaitis et al. 2010). When a *hen1* mutant was crossed to the L*er*-0 background to generate F1 hybrids (*hen1* × L*er*-0) it was found that the resulting F1 hybrids showed reduced size, and that plant vigor was compromised. These results indicate that the association between sRNAs and some heterotic traits might indeed be causal. However, contrasting results were presented by studies using mutants for the maize *MODIFIER OF PARAMUTATION 1* (*MOP1*) gene, which is considered to be the homologue of *A. thaliana RDR2* and is essential for the biogenesis of heterochromatic 24-nt siRNAs in maize (Lisch et al. 2002; Barber et al. 2012). The maize functional study found that heterosis was not disturbed in *mop1* hybrids (Barber et al. 2012). Such differences may be because HEN1 is important not only for the stability of 24-nt siRNAs but also other classes of sRNA's including miRNA's, while the role of MOP1 is restricted to the generation of 24-nt siRNA.

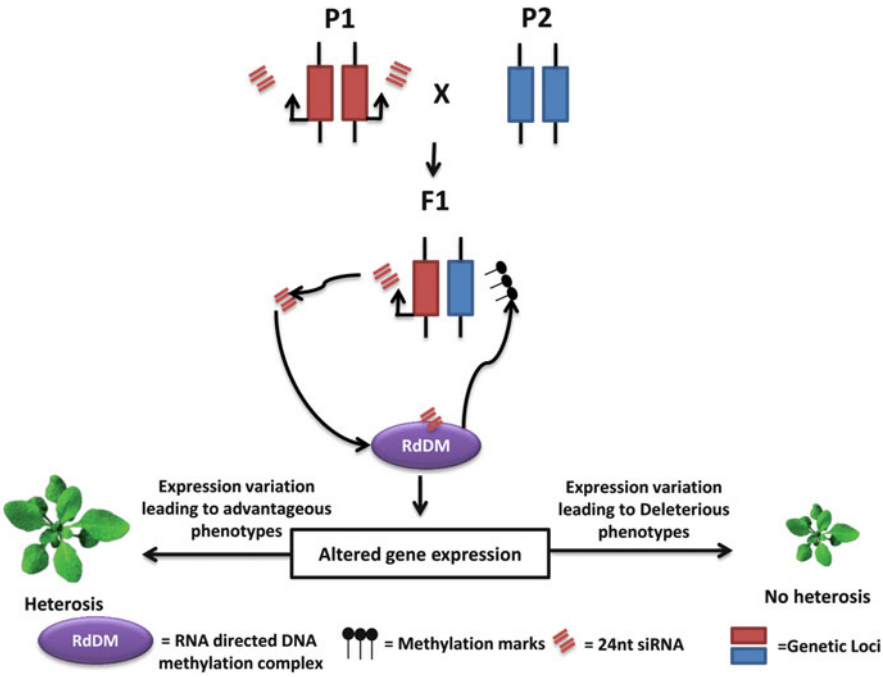

Fig. 2.3 Possible roles for methylation, siRNA and RdDM in heterosis. (A) Two distinct genomes (P1, P2) with various levels of siRNA accumulation hybridize to create a heterotic F1 hybrid (F1). (B) Upon hybridization siRNAs can interact in cis or trans with genetic elements containing their complementary sequence. siRNAs can interact with RdDM pathways to silence genes via trans chromosomal methylation. (B) Methylation marks may be removed allowing expression of both alleles in the F1 hybrids. (C) siRNA may be generated via the allele inherited by P1 but does not methylate its homologous allele leading to allele specific expression. Such types of epigenetic amendments may lead to altered expression levels in F1 hybrids which could potentially lead to heterotic effects which either improve (G) or deteriorate (F) vigor in F1 hybrids

2.3.4 Genome Wide Epigenetic Networks as a Component of Heterosis?

Allelic methylation differences in F1 hybrids have been shown to occur through *trans*-acting phenomena termed Trans-Chromosomal Methylation (TCM) (Fig. 2.3) and Trans-Chromosomal Demethylation (TCDM) (Greaves et al. 2012). Such methylation events predominantly occur in F1 hybrids at allelic sites where differentially methylated regions exist between the genomes of the parents. In such cases, it is sometimes found that the methylation of one allele will be increased or decreased such that it matches the methylation status of the homologous allele derived from the other parent. Between them, TCM and TCDM events accounted for 86 % of the total non-additive methylation differences observed in F1 hybrids (Greaves et al. 2012). Comparative analysis of methylation and siRNA distribution

in parental (C24, L*er*-0) and F1 hybrid lines (C24 x L*er*-0, L*er*-0 x C24) indicated that there was also a positive correlation between siRNA abundance and such non-additive methylation. These changes were in some cases also found to correlate with gene expression changes that departed from the mid-parent value at these loci. These studies suggest that RdDM may play a role in modulating DNA methylation levels between the alleles at hybrid loci, leading to non-additive methylation and heterotic gene expression in hybrid plants.

A recent study investigated the inheritance pattern of TCM and TCDM at specific loci in the *A. thaliana* genome (Greaves et al. 2014). By assessing total methylation levels at loci previously shown to undergo TCM and TCDM in reciprocal L*er*-0 × C24 F1 hybrids, it was determined that altered methylation patterns were stably inherited into the F2 generation. Interestingly, however, ^{m}C patterns were transmitted to the F1 offspring outcrosses or backcrosses by the C24 genomic segment only. When L*er*-0 segments that were newly methylated were backcrossed to unmethylated L*er*-0 segments, a paramutation-like phenomenon occurred and this phenomenon appeared to direct *de novo* methylation via TCM.

2.4 Parent-of-Origin Genome Dosage Effects and Their Links to Heterosis

To test for evidence of parent-of-origin effects on heterosis in phenotypic traits, our lab investigated the effects of polyploidization and hybridization on the phenotypes of triploid plants produced from inter-ploidy crosses. The phenotypes measured were the reproductive traits of ovule number and fertility (Duszynska et al. 2013). These were determined in *A. thaliana* F1 hybrid triploids generated by crossing 89 diploid accessions using tetraploid L*er*-0 plants, again using a reciprocal design to allow parent-of-origin effects to be identified. All traits showed dramatic alterations in certain F1 hybrid lines, which were in many cases found to be heterotic. Strikingly, a strong parent-of-origin-effect was displayed between maternal excess 3×(M) and paternal excess 3×(P) F1 hybrid triploids with respect to both total ovule number per silique, and their fertility (Duszynska et al. 2013). Our study suggests that parent-of-origin effects (argued to be *sensu latu* epigenetic in nature) can determine whether the F1 progeny display heterosis for certain traits. Regardless of its mechanistic basis, some of the modulation of parental effects on heterosis by natural variation are manifested in diploid–diploid crosses while other elements can be 'cryptic', and are only manifested in inter-ploidy crosses.

Are such effects a peculiarity of *A. thaliana*, or other plants consisting of highly-inbred homozygous populations, or are they of broader relevance? The effect of genome dosage on heterosis in *Z. mays* has been investigated using inbred diploid lines (B73, Mo17) and their reciprocal F1 hybrids, when compared to matched triploid derivatives (Yao et al. 2013). It was observed that reciprocal F1 triploid hybrids varied in the extent of heterosis. Such studies contradict the predictions of a

strict dominance model of heterosis as it is predicted that complementation of recessive mutations would occur equally in both triploid hybrids. Such studies demonstrate that parent-of-origin effects can influence heterosis in both monocots and dicots.

2.5 Future Directions

The search for a unifying biological mechanism for heterosis still remains elusive even after over 100 years of research in this area. The key models of *dominance, overdominance* and *epistasis* are still in use for describing multigenic heterosis. However, investigations of epigenetic processes including DNA methylation, histone modification and sRNA expression and accumulation provide some new perspectives in relation to heterosis. Early studies suggesting links between non-additive DNA methylation with heterosis in F1 hybrids (Zhao et al. 2007) have been complemented with additional studies correlating sRNA, DNA methylation and histone modification with heterosis (Ni et al. 2008; He et al. 2013). Global siRNA differences have been observed between F1 hybrids and parents in *Arabidopsis thaliana* (Groszmann et al. 2011). An increased understanding and prediction of TCM and TCDM events in plant epigenomes in both *Arabidopsis thaliana* and crops has the potential to contribute to further unraveling of the molecular basis of heterosis. To date, the bulk of epigenetic heterosis research has been conducted in the model crop *Arabidopsis thaliana* and *Zea mays*. Expanding epigenetic research into other crops that display heterosis effects will contribute to advancing of understanding regarding the molecular basis of heterosis. Clearly, while there is evidence that epigenetic variation may be linked to heterosis, the functional studies to test whether epigenetic regulation is causally central to heterosis are currently lacking. The ongoing rapid advances in functional genomics and epigenomics now pave the way for a deeper mechanistic understanding of both the genetic and epigenetic contributions to heterosis effects.

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Chapter 3
Epigenetic Variation Amongst Polyploidy Crop Species

Andrew Bottley

Abstract Many agronomically important crop species such as wheat are (or were once) polyploid, with at least one round of whole genome duplication occurring before domestication. This genetic buffering or redundancy allows for sequence divergence, and in turn the development of functional variations between duplicated genes (homoeologues). Homoeologues may encode proteins with different properties and plant breeders have successfully used this genetic resource to introduce new genetic diversity into breeding populations. However duplicated genes are also subject to extensive epigenetic control and are therefore not always equally expressed. The preferential bias in the expression or the silencing of a specific homoeologue may be heritable and can be stable across many generations. There is also mounting evidence to suggest that selective homoeologue expression occurs in response to stresses such as salinity and may be specific to individual pathways or processes. Importantly, this type of epigenetic variation may segregate within a breeding population and is readily observed in newly synthesised polyploid hybrids.

It is now known that heritable phenotypic characteristics are determined by a combination of both genotype and epigenotype. Therefore the epigenome of polyploid crop species such as wheat and cotton represents a potent new source of diversity for agronomically important traits such as those linked to abiotic stress, secondary metabolite synthesis and fibre development. This text describes the characterisation of epigenetic variation in polyploidy crop species and its potential for exploitation by breeders for crop improvement.

Keywords Homoeologues • Polyploidy • Gene duplication • Wheat • Gene silencing

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3.1 Background and Context

With an ever increasing global population, the need to provide a secure food supply has never been greater. It is therefore a grand challenge to crop breeders and agronomic scientists to maximise yields and make best use of agricultural resources available. Although substantial gains in productivity have been achieved in the years since the beginning of the last century, yields of a number of important crop species have plateaued in recent decades (see Grassini et al. 2013). During the 1800s, average UK wheat yields were in the order of approximately 1 tonne per hectare, this figure now stands at 9 tonnes per hectare today (*Source*: Rothemsted Research). Improvements in agronomic technologies such as mechanised cultivation and the development of new and better fertilizers all contributed to a year on year rise in yields; however advances in the science of crop genetics and marker assisted breeding have contributed to the dramatic increase in the quantity and quality of wheat.

It has been suggested that a regional increase of just by 2 tonnes per hectare for African farmland would tangibly impact on global food security (Professor Martin Parry, Rothamsted Research) and although UK production levels remain significantly higher than the global average; it is an aspiration to double output within the next 20 years (*Source*: Biotechnology and Biological Sciences Research Council). To achieve these ambitious aims a number of issues will need to be resolved; the need to identify and capture new sources of diversity within wheat breeding populations is one such challenge. Although thus far a successful strategy, the breeding and interbreeding of a narrow panel of elite wheat's has resulted in a 'genetic bottle-neck', resulting in a breeding population with limited potential for new desirable traits. This chapter discusses a potentially valuable new source of tractable diversity; a facet of biology that underpins developmental growth and abiotic stress responses. Although epigenetics is more widely studied in model organisms or human disease biology, this area of research may be productive for the improvement of polyploidy crop species.

3.2 Wheat as a Crop and Evolutionary History

The evolution of hexaploid wheat *Triticum aestivum* (genome formula AABBDD) can be traced to three diploid species; *T. urartu* (A genome), a species closely related to *Aegilops speltoides* (B genome) and *Ae. tauschii* (D genome) (Kihara 1944; McFadden and Sears 1946; Sarker and Stebbins 1956; Dvorak et al. 1993). Molecular clock-based studies have indicated that T. urartu and *Ae. speltoides* hybridised to form alloploid *T. turgidum* (AB) approximately 0.5 million years ago, while the integration of the *Ae. tauschii* to form *T. aestivum* occurred approximately 8,000 years ago (Huang et al. 2002). Archaeological evidence suggests that tetraploid (emmer) was the predominant dietary grain pre 9500 BC in this region,

while the consumption of hexaploid grains began approximately 9,500–7,500 years ago (Harris 1998; Kislev 1984). As no wild forms of hexaploid wheat have yet been identified, it is likely that hexaploid hybrids naturally occurred at the margins of cultivated emmer and was then selected by early agriculturalists; presumably as this hybrid possessed superior traits compared to tetraploid emmer.

3.3 Wheat Polyploidy

Commercially cultivated wheat is predominantly either tetraploid or hexaploid, although the diploid *T. monococcum* is still sporadically cultivated in some parts of the Middle East (Salimi et al. 2005; Vallega 1995). Tetraploid durum wheat has two complete groups of seven chromosomes and its grain is typically suited to the manufacture of pasta. Hexaploid wheat has three groups of seven chromosomes, and as it is commonly used for bread making. Hence it is often referred to as bread wheat.

Allopolyploidy is genetically unstable and over evolutionary time, most polyploidy species eventually revert to diploidy through various processes of genomic re-arrangements or deletions. Wheat is able to maintain three intact diploid genomes largely due to the action of genes such as Ph1; a gene which maintains diploid-like chromosome pairing (Riley and Chapman 1958). *T. aestivum* is just one of many species to undergo speciation through polyploidy and as many as 80 % of all known angiosperms are thought to have experienced a ploidy event(s) at some stage of their evolutionary history (Masterson 1994). Although it is difficult to precisely determine when and how many rounds of duplication and reorganization may have occurred within the evolutionary history of a species, through the use of comparative mapping etc. it is well established that polyploidy is a common and ancient phenomenon in plants (Brubaker et al. 1999; Gaut and Doebley 1997).

As the different parent genome donor species of hexaploid wheat probably descend from a common progenitor (Zohary and Feldman 1962), their constituent genomes although differing in size and structure are highly homologous in content. Therefore a functional consequence of an increase in ploidy is multiple copies of genes with near identical sequence. Over time, the accumulation of random mutations led to a divergence in sequence between duplicates derived from a single 'ancestor' gene (Feldman et al. 1997); in turn this allows for a functional divergence of the gene product (see Blanc and Wolfe 2004).

3.4 Gene Duplication and Fate of Duplicated Genes

The homology between the three genomes (A, B and D) has been subjected to sequence analysis using a range of techniques. These approaches include *in-silico* sequence alignment, EST mapping and most recently whole genome sequence

alignment (Gill et al. 1991, 2004; Somers et al. 2003; Qi et al. 2004; Brenchley et al. 2012). Historical approaches used to comparatively asses the structural relationship between each homoloeogous chromosome included meiotic chromosome pairing (Chapman and Riley 1970), mapping (Erayman et al. 2004) and aneuploid analysis (Sears 1954), and fluorescent in-situ hybridisation. The level of single nucleotide polymorphisms between homoeologous coding regions is estimated to occur at 1 in every 24 bases (Somers et al. 2003); however the consequence to the transcriptome or ultimately the proteome of this sequence variation remains essentially unexplored.

In addition to mutation, sequence deletion has also shaped the diversity that exists between homoeologous gene sequences. Cryptic polyploids, such as maize, are thought to have evolved from ancient polyploids by a process of pseudogene formation followed by sequence loss. In a study investigating the fate of duplicated maize genes, Lai et al. (2004) suggested that within as little as five million years, approximately 50 % of duplicated genes were lost through deletion. Deletions are also a common occurrence in established polyploids and may impact on important agronomic traits e.g. A polymorphism for a puroindoline A deletion (or for a point mutation in puroindoline B) in the hexaploid wheat D genome dramatically affects grain hardness (Giroux and Morris 1998). Research investigating gene deletions in the D genome of *T. aestivum* suggest that at little as 0.17 % of the D genome has been deleted during the past 8,500 years and that deletions in established wheats occur at low frequencies (Dvorak et al. 2004). Surprisingly some loci were deleted from all three genomes, indicating a predisposition for the deletion of specific sequences (Dvorak et al. 2004). This research suggests deletions occur gradually in established polyploids rather than as a rapid loss of sequence following hybridisation (Dvorak et al. 2004). Homoeologue deletion may negatively impact on the potential for each remaining homoeologues to become co-opted for a specific function or recruited into a specific pathway.

Homoeologous genes are by nature near identical in sequence and it is therefore logical to assume that homoeologes should be expressed at relatively similar levels (Gottlieb 2003). Early techniques such as enzymatic staining suggested however this assumption may not be correct for all genes. Using this technique to profile protein levels for a group of wheat isoenzymes, researchers unexpectedly found that of 54 sets of genes for which a genetic profile had been elucidated, 42 showed co-expression of all three homoeoalleles, but for 12 sets the product of only one homoeoallele could be identified (data extracted from Mcintosh et al. 1998). Similar variation in expression has also been reported among the Glu-1 homoeologues, a set of genes encoding an important class of seed storage protein (Flavell and O'Dell 1990). This work suggests that although homoeologues may possess near identical sequence homology, they are not always equally expressed (see review by Doyle et al. 2008).

3.5 Silencing in Crop Polyploidy Species

Early studies investigating epigenetic regulation or gene silencing in hexaploid wheat suggested that a bias in the expression or the silencing of individual homoeologues was a fairly rare occurrence. With little evidence to suggest that silencing was widespread, it was not considered an important factor in the organisation and regulation of genes within the genome of polyploidy species (Hart 1996). However, as gene expression in wheat and other polyploids have been more extensively researched, estimates of the levels of silencing have been revised upwards. Kashkush et al. (2002) estimated that between 1 and 5 % of genes in newly synthesised wheat hexaploids are silenced. This is comparable with the work by He et al. (2003), which estimated by cDNA-AFLP analysis that about 7–8 % of genes are silenced in established wheats. He et al. (2003) suggested that genes located on the D genome may be silenced at a higher frequency than equivalents located on either the B or the A genomes. This may be due to the evolutionary history of wheat in which the D genome progenitor species hybridised with an established AB polyploidy species. The hypothesis would therefore be that silencing is directed at the 'invading' sequence. An alternative hypothesis suggests that any bias in the frequency of silencing may be due to an as yet unknown structural characteristic of the D genome itself (He et al. 2003).

Exploiting large collections of EST data, Mochida et al. (2004) concluded that silencing affected 11 of 90 sets of homoeoalleles tested (12 %). Using an SSCP platform, Adams et al. (2003) suggested that about 25 % of genes may be silenced in established tetraploid cotton. The authors (2004) also identified a similar difference between *de novo* and established cotton hybrids; using cDNA-AFLP they were able to demonstrate that about 5 % of all genes are silenced in a newly synthesised cotton allotetraploid. In our study using SSCP and seedling leaf tissue of 'CS', at least one homoeolocus was silenced for 27 % of the genes expressed (Bottley et al. 2006). This represents 9 % of the total number of homoeologues (52 homoeologues of a total of 582) present. The frequency of silencing was numerically greatest in the D genome, although this was not statistically significantly as assessed by a chi-squared test in our experiments. Collectively, this work suggests that not all silencing is imposed immediately after hybridisation but that some silencing may gradually accumulate over evolutionary time.

In addition to the discovery that at least some homoeologues may be silenced after polyploidisation, Kashkush et al. (2002) amongst others also described a phenomenon whereby homoeologue activation occurred in newly synthesised polyploids. Transcriptionally silent sequences in diploid/tetraploid parent lines can become active in the polyploid progeny, occurring at a frequency of ~0.2 % of all genes (Kashkush et al. 2002). It should be noted that two thirds of activated transcripts showed a high degree of sequence homology to transposable elements (Kashkush et al. 2003).

Genes identified as possessing silent homoeologues in hexaploid wheat have a diverse range of functions e.g. ABC transporter genes to Rubisco subunits

(He et al. 2003; Kashkush et al. 2002). The absence of a link between function and silencing, particularly in newly synthesised polyploids is consistent with the theory of "genomic shock" as opposed to a functionally controlled regulatory process. This model however contradicts data which suggests that silencing accumulates gradually. The most likely hypothesis is that some silencing or a bias in the expression occurs immediately after hybridisation and then new layers of regulation and complexity accumulate over many generations.

3.6 Frequency of Polyploidy Associated Silencing in Model Species

Silencing associated with polyploidy is widespread and not limited to cereal and fibre crops. Experiments using polyploids lines derived from model species, such as hybrids synthesised from *Arabidopsis thaliana* and *Cardaminopsis arenosa*, demonstrate this phenomenon is a common feature associated with a change in ploidy. However although silencing occurs in *Arabidopsis* polyploids, the patterns and frequencies of silencing are markedly different to those identified for hexaploid wheats or tetraploid cotton. Comai et al. (2000) showed that contrary to the preferential silencing of the wheat D genome (He et al. 2003), silenced transcripts in the *Arabidopsis thaliana* × *Cardaminopsis arenosa* hybrid map at an equal frequency to both the *Arabidopsis* and *Cardaminopsis* genomes. Also the frequency of silencing is estimated to be in the region of 0.4 %, differing from hexaploid wheat by ~10-fold (Comai et al. 2000). Differences in frequencies of silencing identified between polyploids generated artificially in the lab using Arabidopsis spp. and those hybrids originating from the hybridisation of diverse progenitor wheat spp. may relate to the level of homology present in the sequences of merging genomes. *Arabidopsis* and *Cardaminopsis* are highly similar, only divergent in sequence for 5 % of coding regions (Comai et al. 2000). Both size and genome homology are therefore likely to be important factors governing the overall frequency of silencing and will likely impact on the ability to derive new sources of epigenetic variation through the formation of synthetic hybrids.

3.7 Patterns of Silencing

Where tested, a significant proportion of cotton homoeologues appear to be differentially transcribed/silenced, importantly however this bias in expression may be linked to discrete organs or tissues (Adams et al. 2004). Further that in some instances, silencing may be associated with a specific process such as the preferential expression of the A genome in cotton fibre filament production (Yang et al. 2006). *In-silico* analysis of pistil wheat EST libraries identified that of

54 genes tested, over half showed a bias or silencing of expression, however this figure was substantially lower in equivalent data sets obtained from emerging spike tissue (Mochida et al. 2003). Using an SSCP approach we were able to demonstrate that tissue specific silencing is widespread in hexaploid wheat (Bottley et al. 2006). In some instances silencing could be detected in only one tissue, conversely in other examples homoeologues were silenced in both root and leaf tissue. More unusually, in the instance of the gene FtsZ which encodes a plastid division protein, the A genome homoeologue was silenced in the leaf and the D homoeologue was silenced in the root. This may represent the subfunctionalisation of these homoeologues i.e. the A genome homoeologue is in the process of being recruited as a root specific gene.

Differences in the expression of homoeologues amongst different tissues are informative. If the A genome homoeologue is silenced in leaf tissue but expressed in the root tissue of the same plant, this absence of expression cannot be explained by homoeologue deletion or inactivation by transposition or mutation. In most instances where a homoeolocus is silenced in leaf tissue but expressed in root tissue, this is likely due to tissue specific regulation. Research by authors such as Yang et al. (2006) also further suggests that this process is not merely a random consequence of gene duplication; rather an evolutionary process which serves to recruit duplicates into different functions or pathways as described above.

3.8 Consequences to Pathways and Enzymes

The consequence of bias or the selective expression of only one homoeologue is not necessarily trivial. Nomura et al. (2005) showed that the enzymatic properties of the homoeologous biosynthetic TaBx isozymes were specific to each homoeologue. To summarise, the enzymatic activity of each homoeologue protein differs by twofold between the A and B genome copies and a difference of up to 13-fold between the A and D genome copies. Thus the properties of TaBx enzymes which populate the proteome can be significantly affected by the identity or relative levels of the homoeologous transcripts that are transcribed; it is unlikely therefore that each homoeologue contributes equally to a pathway or process.

3.9 Silencing as a Stress Response

The experiments described above established the prevalence of silencing in a number of different agronomically important crop species. These data are also suggestive that homoeologue specific regulation plays a substantive role in specific pathways and processes (e.g. Yang et al. 2006). In 2007 Liu and Adams demonstrated that a bias or silencing of different homoeologues formed part of an abiotic stress response for one gene. It had already been well established that diploid

species initiate stress responses which result in rapid and genome wide changes in gene expression (e.g. Ouyang et al. 2007), and polyploidy species respond in a similar manner (Kawaura et al. 2008). It had also been established that genes may be differentially regulated between sensitive and tolerant varieties in response to different stresses (Gulick et al. 2005), although a genetic explanation seemed the most likely cause. The data first published in 2007 then subsequent work published by Dong and Adams (2011), and by Chaudhary et al. (2009) etc. all suggest that a bias or the silencing of individual homoeologs in tetraploid cotton is a common feature of the polyploid cotton stress response e.g. the relative levels of up to 70 % of all homoeologue transcripts may be altered by some stresses.

A similar pattern of selective expression has been observed in polyploidy wheat. Where tested, the expression of the individual RAD50 DNA damage repair homoeologues is not equal; the B genome copy accounts for ~70 % of the transcript pool in tetraploid wheat and ~60 % in hexaploid wheat (Pérez et al. 2011). Stresses such as drought can elicit variation in the relative transcription of homoeologues of the cell wall invertase gene family (Webster et al. 2012), while we observed stress specific silencing for a broad range of different genes (8.9 % of 112 genes tested) could be induced by salt stress (Bottley 2013). In our study an identical silencing response was observed in more than one cultivar tested and in some instances the same silencing profile could be obtained through the exposure of seedlings to a second distinct stress e.g. cold. Cumulatively this data suggests that this a bias in expression of these homoeologues represents a generic stress response across a range of polyploidy species. Work by researchers such as Shoeva et al. (2014) are beginning to characterise these types of stress responses through the dissection of the relative expression of homoeologues encoding stress-linked proteins or metabolites e.g. the expression of different homoeologue transcripts linked to the Chalcone pathway.

It is possible that the selective expression of homoeologues located to one genome as opposed to another is reflective of the relative stress tolerant properties of the progenitor species. In a simple model this may fit with the proposed mechanism of homoeologue specific regulation proposed by Udall and Wendel (2006) e.g. in a simplified model, a stress specific transcription factor has a greater affinity for the promoter of homoeologue A compared to homoeologue B. This promoter sequence of homoeologue A may have evolved under a greater selection pressure of stress exposure due to the environment experienced by the plant A. It is possible that this type of epigenetic response differs among varieties of wheat, however further research is required to establish how variations in the epigenome can be exploited to develop polyploidy crop species with greater stress resistance properties.

3.10 Segregation and Differences Between Varieties and Transgenerational Stability

Patterns of gene expression amongst different wheat varieties are not uniform. Using a microarray platform, Gulick et al. (2005) demonstrated that for two wheat varieties 65 of 947 genes tested are differentially regulated. Although this study was unable to differentiate between the relative levels of each homoeologue transcript, it demonstrates variations in the expression among varieties of the same species are not uncommon. Intriguingly research investigating the distribution of methylation using methylation sensitive enzyme experiments suggests that methylation is more frequently polymorphic amongst 20 accessions of the cotton polyploidy *Gossypium hirsutum* than equivalent genetic diversity (Keyte et al. 2006). This suggests a candidate mechanism which underpins differences in expression between varieties and it is worth mentioning in this section that methylation can be both stable and heritable.

Where tested, profiles of silencing differed among a panel of 16 different wheat varieties, cultivars commonly used to generate most commercially grown crop lines (see Bottley and Koebner 2008). Plants were profiled to identify silencing in both leaf and root tissue and no variety showed the same homologous expression profile when each were tested for the expression of 15 genes. Although overall frequencies of silencing were similar in each cultivar, each line possessed a unique pattern of silencing. Some homoeologues were silenced rarely, whereas other homoeologues were silenced frequently and silenced in more than one variety.

In order to understand the heritability of this silencing, the expression of a homoeolog identified as silenced in only one parent line was profiled in the progeny of a cross between the varieties Avalon and Cadenza. The same homoeologue was identified as silent in a number of offspring, although the trend favours a ratio where expression was more common than silencing. Interestingly a small but significant variation in the percentage of silenced homoelogues has been identified between two replicates of the same variety of tetraploid cotton (Adams et al. 2003). Although initially attributed by the authors to be an artefact of the cDNA-AFLP technique employed, it is possible this represents a layer of intra-species variation not yet fully appreciated.

Although in some instances silencing is stochastic, research investigating hexaploid wheat, tetraploid cotton and artificially generated Arabidopsis hybrids, has proven that silencing may be stable and heritable across many generations (Bottley et al. 2006; Adams et al. 2003; Wang et al. 2004). It should be noted that where silencing has previously been documented to be unstable or random, this may reflect unrecorded changes in abiotic stress or subtle variations in growth conditions which are then reflected in profiles of transcription (discussed above). Conversely it may be suggested that a heritable pattern of expression merely reflects the same response by the same genotype to the same conditions, rather than heritable transgenerational silencing.

To summarise, patterns of silencing are not always identical among cultivars or varieties of the same species, may be heritable and can segregate within breeding populations (Bottley and Koebner 2008). With this in mind, it is likely that within the panel of elite wheat's there exists a substantial amount of 'untapped' epigenetic variability. This is also likely to be true for other polyploidy species such as cotton. As described above the consequence of this type of epigenetic control is not without consequence and it is likely that silencing or a bias in the expression of different homoeologues forms an intrinsic part of a polyploidy specific stress response. Therefore it is not unreasonable to suggest that each variety possesses a unique epigenetic-type in addition to genotype, and that this layer of epigenetics may segregate differently within breading populations.

3.11 Newly Synthesised Polyploids

The rates of silencing identified in newly synthesised polyploidy plants differ markedly from the frequencies observed for established polyploid equivalents. "Genomic shock" has been proposed as a possible driver for polyploidy decay (McClintock 1984) and may in-part explain the phenomenon of homoeologue specific silencing; in this model, genomic instability occurs immediately upon hybridisation, and is followed by a period of stabilization (reviewed by Chen and Ni 2006). Intriguingly polyploidy may also lead to the re-activation of previously silenced genes; this phenomenon, although not as frequent as silencing, has been documented in wheat, cotton and Arabidopsis polyploids (Kashkush et al. 2002; Adams et al. 2003; Wang et al. 2004).

Using a cDNA-AFLP platform to assay the frequency of silencing in newly synthesised cotton polyploids, approximately 5 % of 2,000 transcripts were identified as silent (Adams et al. 2004). A similar figure was observed for newly synthesised wheat hexaploids polyploids using the same technique—an estimate of between 1 and 5 % of genes were silenced in these lines (Kashkush et al. 2002). The frequency of silencing for tetraploid *Arabidopsis* hybrids was substantially lower (0.4 %) than tetraploid cotton equivalents, which likely reflects the importance of the composition of the relative genomes rather than a consequence of mere duplication (Comai et al. 2000).

Using an SSCP platform, we profiled the expression of 36 genes amongst a panel of number of newly synthesised polyploidy wheats (data unpublished). Genes were tested for expression in hybrid root and leaf tissue and equivalent material obtained from six parental lines each with differing backgrounds (diploid e.g. *Aegilops tauschii* spp. *strangulata* and *tetraploid T. turgidum* spp. durum cv. *carthlicum*). We identified rates of silencing in these newly synthesised wheat hexaploid lines which ranged from ~5 to 10 %. Interestingly, in some instances silencing was maintained i.e. present in both the parent and the hybrid, however in other examples silencing was only observed in the newly synthesised line. One possible explanation is that this variation in the rate of silencing which is observed amongst newly

synthesised plants is reflective of the degree of homology which exists between the different parental lines. This data, together with the data recorded for other polyploidy crop species suggests that the process of forming new hybrids may introduce epigenetic variation; new diversity within the epigenome distinct from the originating progenitor plants.

3.12 Exploiting Epigenetics as an Agronomic Tool

Epigenetic variation may shape phenotype. A few important examples of this have been described in the literature for diploid species e.g. the colourless non-ripening phenotype tomato epimutant described by Manning et al. (2006); a dramatic example where an epi-polymorphism alone determines an alternate ripening process. It is therefore not controversial to suggest that selecting for epigenetic variation or the incorporation of techniques such as epimarkers may have a role in exploiting the epigenetic diversity which already exists within breeding populations of polyploid crop species. It is likely that epigenetic variation may determine agronomically important traits such as fibre production in cotton or drought stress in wheat. It is possible that some epigenetic modifiers are stochastic and therefore not amenable for use as a breeding resource; however it is equally likely that patterns of silencing represent a valuable resource if they can be exploited. Although further research is required to fully understand the mechanisms which determine and regulate homoeologue specific silencing, it is becoming clear that in polyploidy species the blend in the expression of different genomes may represent an important resource for crop breeders.

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Chapter 4
Histone H3 Phosphorylation in Plants and Other Organisms

Izabel Moraes and Juan Armando Casas-Mollano

Abstract Post-translational histone modifications, such as methylation and phosphorylation, play an important role in determining chromatin states associated with gene activation or repression. Histone H3 phosphorylation in particular has been linked to a variety of cellular processes during the cell cycle. H3 phosphorylation is involved in chromosome condensation and segregation during mitosis and meiosis in plants and animals. During interphase, H3 phosphorylation has been implicated in transcriptional regulation, DNA replication and apoptosis. Phosphorylation also occurs in the histone variants, H3.3 and CENH3, during cell division. The diverse and sometimes contrasting processes in which H3 phosphorylation participates have made difficult to completely understand its function. In addition, functional differences on H3 phosphorylation have been observed in diverse organisms despite the conservation of the modified residue. Here we discuss the most recent findings about the roles of histone H3 phosphorylation, the proteins involved in phosphorylating particular residues and the mechanisms by which this modification results in a particular gene expression state. The differences and similarities between plants and other model systems are emphasized.

Keywords Histone phosphorylation • Kinase • Histone variant • Cell cycle • Chromatin • Gene silencing • Transcription

4.1 Introduction

The correct packing of DNA into chromosomes is essential for a successful cell division and transmission of genetic information into daughter cells. On the other hand, processes such as DNA replication, repair, recombination and transcription

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require relaxation of chromatin. Within the chromatin, DNA is wrapped in 147 base pair units around an octamer of four core histones, H2A, H2B, H3 and H4, linked by histone H1, to form the nucleosomes. In the nucleosomes, histones are subject to a wide variety of post-translational modifications including acetylation (ac), methylation (me), ubiquitylation (ub) and phosphorylation (ph). The transition between the decondensed euchromatin and the highly compact heterochromatin is accompanied of changes in the post-translational modifications of histone proteins, which mediate the accessibility to the genome. Different mechanisms were proposed to affect chromatin states via histone modification. For instance, some modifications may cause a structural change in histones affecting their affinity for DNA and/or intranucleosomal interactions. Histone modifications may also serve as a binding site for protein recognition modules, such as the chromodomains that recognize methylated lysine, that form part of complexes able to modify the chromatin structure (Jenuwein and Allis 2001).

While histone methylation at different sites recruits either silencing or activating proteins, acetylation has been linked to the creation of a permissive transcriptional environment within the chromatin. Histone phosphorylation, on the other hand, is a key regulator of a wide range of cellular processes including mitosis, meiosis, DNA repair, replication and recombination.

Many of the histone modifications and their function are conserved between eukaryotes. For example, methylation of histone H3 lysine 4 (H3K4) seems to be a universal mark for transcriptional activity in most eukaryotes (reviewed in Ong and Corces 2011). However, other modifications appear to have different and even opposing biological readouts in different organisms. For instance, cell-cycle dependent phosphorylation of H3T3, H3T11, H3S10 and H3S28 is conserved, but the significance of these modifications apparently has been reversed in animals and plants (Houben et al. 2005). Unlike mammal H3T3ph and H3T11ph, which localize mostly in the centromeric regions of chromosomes (Dai et al. 2005; Preuss et al. 2003), H3T3ph and H3T11ph are distributed in the entire length of the plant chromosome (Houben et al. 2005; Caperta et al. 2008). Furthermore, phosphorylation of H3 during mitosis and meiosis is involved in chromosome condensation and cohesion, whereas the same modification regulates transcription in interphase cells. Due to these contrasting observations a proper function for H3 phosphorylation remains controversial and requires further investigation.

Phosphorylation of the canonical histone H3 and of the variants H3.3 and Centromeric Histone H3 (CENH3) occurs at Serine (S), Threonine (T) and Tyrosine (Y) (Fig. 4.1). The addition of a phosphate group to the amino acid residues on the histone H3 is carried out by several kinase proteins (Table 4.1) whereas the removal is performed by phosphatases (see Sect. 4.2). Several phosphorylated residues have been identified in the histone H3. Threonine 3, 6, 11, 32 and 45, serine 10 and 28, and tyrosine 41 are phosphorylated in the histone H3 of metazoans (Table 4.1). Similarly in plants, mainly by using modification-specific antibodies, histone H3 has also been shown to be phosphorylated at several conserved residues (Table 4.1).

In this chapter the latest advances in investigating H3 phosphorylation function will be reviewed, focusing on the most recent findings on plants. We will emphasize on the roles that phosphorylation of canonical H3 plays throughout cell cycle.

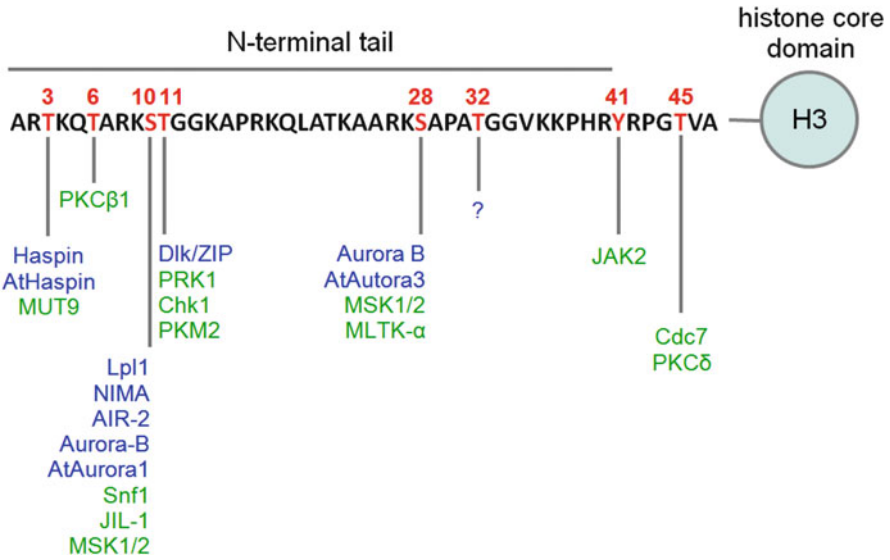

Fig. 4.1 Phosphorylation sites of canonical histone H3 (*red*) and the responsible kinases in mitosis (*blue*) and interphase (*green*). Due to space constrains only MSK1/2 is shown as a representative H3S10 kinase in mammals

The function of phosphorylated residues in the histone H3 variants, H3.3 and CENH3 will also be explored.

4.2 Cell Cycle and H3 Phosphorylation

Early studies showed that H3 phosphorylation displays a fluctuating pattern during the cell cycle. Levels of H3 phosphorylation are low during interphase, increase in the beginning of prophase, and decrease again during telophase. This cell-cycle dependent phosphorylation, demonstrated in mammalian cells (Hendzel et al. 1997) and plants (Houben et al. 1999) first suggested a role in chromatin condensation during cell division. In contrast, induction of H3 phosphorylation has been associated to transcriptional activation in response to different stimuli in interphase. Thus, histone H3 phosphorylation is associated with two opposed chromatin states: chromatin condensation during mitosis and transcriptionally permissive chromatin at interphase (Prigent and Dimitrov 2003). Furthermore, whereas chromatin condensation implies global H3 phosphorylation, during interphase H3 phosphorylation seems to be restricted to a subset of nucleosomes, targeted mainly to regulatory regions of target genes (Mahadevan et al. 1991; Barratt et al. 1994). These contrasting observations suggest that the specific biological read out of H3 phosphorylation may depend on the chromatin environment and is influenced by other histone modifications (Pérez-Cadahía et al. 2009).

Table 4.1 Phosphorylation sites of canonical and variant histone H3 in different organisms, and the kinase proteins currently known to phosphorylate these residues

Histone	Site	Kinase	Organism	Reference
Mitosis				
H3	T3	Haspin	Mammals	Dai et al. (2005)
	T3	AtHaspin	Arabidopsis	Ashtiyani et al. (2011)
	T6	?	Arabidopsis	Karimi-Ashtiyani and Houben (2013)
	S10	Lpl1	*S. cerevisiae*	Hsu et al. (2000)
		NIMA	*A. nidulans*	de Souza et al. (2000)
		AIR-2	*C. elegans*	Rogers et al. (2002)
		Aurora B	Mammals	Richie and Golden (2005)
		AtAurora	Arabidopsis	Demidov et al. (2005)
	S28	Aurora B	Mammals	Goto et al. (2002)
	S28	AtAurora1	Arabidopsis	Demidov et al. (2005), Kawabe et al. (2005)
	T11	Dlk/ZIP	Mammals	Preuss et al. (2003)
	T11	?	Arabidopsis	Houben et al. (2005)
H3.3	S31	?	Mammals	Hake et al. (2005)
CENH3	S7	Aurora A/B	Mammals	Zeitlin et al. (2001a, b), Kunitoku et al. (2003)
	S50	?	*Z. mays*	Zhang et al. (2005)
Interphase				
H3	T3	MUT9	*Chlamydomonas*	Casas-Mollano et al. (2008)
		?	*Tobacco*	Houben et al. (2007)
	T6	PKCβ1	Mammals	Metzger et al. (2010)
	S10	Snf1	*S. cerevisiae*	Lo et al. (2001)
		JIL-1	*D. melanogaster*	Wang et al. (2001)
		MSK1/2, IKK-α, JNK, PKA, Akt1, Cot, PIM1, CDK8	Mammals	DeManno et al. (1999), Thomson et al. (1999), Anest et al. (2003), He et al. (2003), Soloaga et al. (2003), Yamamoto et al. (2003), Zippo et al. (2007), Choi et al. (2008), Meyer et al. (2008), Tiwari et al. (2011)
		?	Arabidopsis, tobacco	Houben et al. (2007), Sokol et al. (2007)
	T11	PRK1, Chk1, PKM2	Mammals	Metzger et al. (2008), Shimada et al. (2008), Yang et al. (2012)
	T45	Cdc7	*S. cerevisiae*	Baker et al. (2010)
		PKCδ	Mammals	Hurd et al. (2009)
	S28	MSK1/2, MLTK-α	Mammals	Zhong et al. (2001), Soloaga et al. (2003), Choi et al. (2005)
	Y41	JAK2	Mammals	Dawson et al. (2009)

4.2.1 Histone H3 Phosphorylation in Mitosis and Meiosis

The generation of a specific polyclonal antibody against a H3 peptide phosphorylated at serine 10 allowed to demonstrate that chromosome condensation is

accompanied of H3S10 phosphorylation (Hendzel et al. 1997). Since this first H3 phosphorylated residue was identified, phosphorylation sites within the N-terminal tail of histone H3 such as T3, T11, and S28 have been extensively characterized. These marks are present during cell division and play a role in chromosome condensation and segregation as well.

4.2.1.1 Serine Phosphorylation

H3S10ph is the most studied and best characterized modification. H3S10ph has been associated with chromosome condensation in eukaryotes as divergent as *Tetrahymena thermophila*, *Aspergillus nidulans*, *Caenorhabditis elegans*, plants and vertebrates (see Table 4.1). In mammals, phosphorylation of H3S10 starts in late G2 at pericentromeric heterochromatin, spreading to the entire chromosome as mitosis proceeds, with the higher peak on metaphase (Goto et al. 1999). H3S10 dephosphorylation begins in anaphase and ends in telophase, revealing a strong correlation between chromosome condensation and phosphorylation (Hendzel et al. 1997). Indeed, it has been shown that H3S10 phosphorylation is required for chromosome condensation but not for its maintenance during mitosis. Mammalian cells treated with hypotonic solution to achieve dephosphorylation of H3 did not result in a loss of chromosome compactation (Van Hooser et al. 1998). Also, a *Tetrahymena thermophila* strain carrying a mutation at H3S10ph displayed problems with chromosome condensation and abnormal segregation, revealing that phosphorylation at this residue is essential for cell division (Wei et al. 1999).

The distribution pattern of H3S10 phosphorylation in plant cells differs of that described in mammals (Fig. 4.2). In *Arabidopsis thaliana*, *Hordeum vulgare* and *Triticum aestivum*, H3S10ph is restricted to pericentromeric regions at metaphase (Houben et al. 2007), being visible from early prophase until telophase. In maize, H3S10ph distribution has been studied in mitosis and meiosis with the use of a specific antibody that recognizes H3S10ph (Kaszas and Cande 2000). While phosphorylation in mitosis is restricted to pericentromeric regions and starts in prophase, in meiotic chromosomes phosphorylation starts in prometaphase, and dephosphorylation starts with maize chromosome separation. This observation points towards a role of H3 phosphorylation in maintenance of sister chromatid cohesion at different stages rather than chromosome condensation (Kaszas and Cande 2000). In monocentric chromosomes, newly replicated sister chromatids are held together from the S phase, the time of their synthesis, until anaphase, when the cohesion is dissolved in order to allow the segregation to opposite poles of the spindle. In contrast, chromosomes displaying the kinetochore distributed over its entire length, referred to as holocentric, cohere along the entire chromatid (Fig. 4.2). In agreement with the hypothesis that H3S10 is involved in cohesion, in plants harboring holocentric chromosomes such as *Luzula*, H3S10ph is distributed over the entire length of the chromosome (Gernand et al. 2003) (Fig. 4.2).

Despite metaphase chromosomes being heavily phosphorylated at H3S10 in most organisms analyzed so far, *S. cerevisiae* represents an exception: a yeast

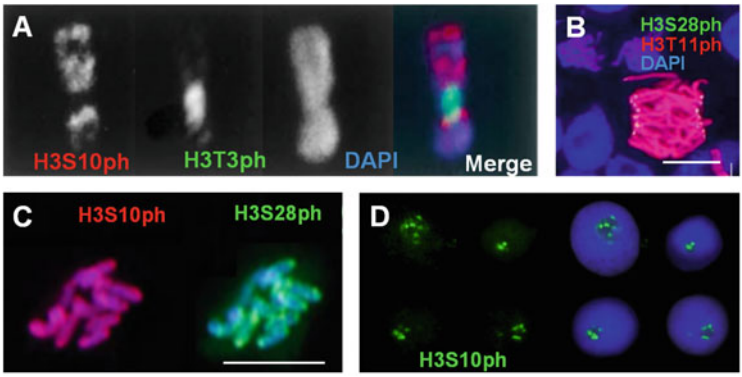

Fig. 4.2 Subcellular localization of H3 phosphorylation. (**a**) In human cells, H3S10ph and H3S28ph (not shown) are distributed over the chromosome length while H3T3ph and H3T11ph (not shown) signals are restricted to the pericentromeric regions (Reprinted from Dai et al. (2005) with permission from Cold Spring Harbor Laboratory Press). (**b**) In plant chromosomes H3 phosphorylation distribution pattern is the opposite of that found in mammalians: H3S10ph (not shown) and H3S28ph are found at the centromeric region whereas H3T3ph (not shown) and H3T11ph signals occupy the whole chromosome (Reprinted from Fuchs et al. (2006) with permission from Elsevier), except for (**c**) plants carrying holocentric chromosomes such as Luzula, in which H3S10ph and H3S28ph signals are distributed over the entire chromosomes (Reprinted from Houben et al. (2013) with permission from Springer). (**d**) Distribution of H3S10ph during interphase of differentiated tobacco mesophyll cells (Reprinted from Granot et al. (2009) with permission from Elsevier)

strain carrying a mutation at histone H3 at serine 10 exhibited identical cell cycle progression to the wild-type, indicating that H3S10 phosphorylation is not required for cell-cycle progression (Hsu et al. 2000). Similarly, in Xenopus egg extracts, the N-terminus part of histone H2B and not of histone H3 is required for chromosome condensation (de la Barre et al. 2001). Thus, H3S10ph is not universally required for chromosome condensation and H2B may fulfill the role of H3 phosphorylation.

In mammals, phosphorylation of H3S10 and H3S28 occur in mitosis but also during transcriptional activation in interphase cells (see Sect. 4.2.2.1). The protein kinases responsible for H3 phosphorylation are different at these two opposite states: Ipl1/Aurora B kinase (Goto et al. 1999) mediates phosphorylation in mitosis, and MAPK kinases (mitogen-activated protein kinase) phosphorylate H3 in interphase cells (Hsu et al. 2000).

In Arabidopsis, three Aurora kinases subdivided in two major groups have been identified: AtAurora1, AtAurora2 and AtAurora3. The first two kinases display very similar structure and expression patterns, suggesting that these proteins were originated by gene duplication. In contrast, AtAurora3 is more closely related to animal Auroras and yeast lpl1. All three AtAuroras phosphorylate H3S10 in vitro and AtAurora3 also phosphorylates H3S28 (Demidov et al. 2005; Kawabe et al. 2005; Kurihara et al. 2006). Additionally, the AtAurora kinases are expressed mostly in dividing cells indicating that they may participate in H3 phosphorylation during cell division (Demidov et al. 2005; Kawabe et al. 2005). However, only

AtAurora3 showed a distribution pattern similar to that of phosphorylated H3S10 and H3S28 (Kawabe et al. 2005). Furthermore, the mitotic phosphorylation of H3S10 is maintained in the *aur1-2 aur2-2* double mutant (Van Damme et al. 2011). These observations suggest that AtAurora3 is likely to be the main kinase responsible for H3S10ph during mitosis.

The distribution of H3S28ph is similar to that of H3S10ph (Fig. 4.2). In mammalian cells, H3S10 phosphorylation begins before mitosis, whereas H3S28 phosphorylation starts in prophase. The fact that chromosome condensation also starts in prophase suggests that H3S28 phosphorylation participates on the initiation of mitotic chromosome condensation in mammals (Gernand et al. 2003). The dynamic of H3S28 phosphorylation is similar between plants and metazoans except that the distribution of H3S28ph in plants correlates with the position of centromeres in monocentric and holocentric species. In plants, H3S10ph is first detected in early prophase, while H3S28ph seems diffuse at this stage (Gernand et al. 2003). Dephosphorylation proceeds with the decondensation of chromosomes but in H3S28 the process is faster than for H3S10.

In animals, treatment with the phosphatase inhibitor calyculin resulted in the presence of H3S28ph in G2 cells. Gernand et al. (2003) did not observe the same effect in plants after treatment with the phosphatase inhibitor cantharidin. Nevertheless, cantharidin resulted in high levels of H3 phosphorylation on the whole chromosome of barley (Manzanero et al. 2002). This could be attributed to the use of different phosphatase inhibitors or to the differences between the species analyzed.

4.2.1.2 Threonine Phosphorylation

In comparison to serine phosphorylation, there are considerably fewer studies focusing on phosphorylation at threonine residues. Both H3T3 and H3T11 phosphorylation occur at the centromere in mammals (Preuss et al. 2003; Dai et al. 2005). H3T3ph levels are high within the inner centromere during prometaphase and decrease in anaphase (Polioudaki et al. 2004). In plants, on the other hand, both modifications occur over the entire chromosome (Fig. 4.2). Chromosome condensation in plants is accompanied of H3T3 phosphorylation in mitosis and meiosis (Houben et al. 2005). H3T3 phosphorylation in mitosis and meiosis II starts in prophase and is maintained until anaphase. Similarly to phosphorylation of H3S10 and H3S28, during meiosis II H3T3ph is restricted to pericentromeric regions. Thus, H3T3ph and H3T11ph, seem to be related to sister chromatid cohesion, as they begin to be phosphorylated as chromosome condensation proceeds (Houben et al. 2005; Caperta et al. 2008).

Haspin has been shown to be the mitotic kinase that phosphorylates H3T3 in mammals (Dai et al. 2005) and in *A. thaliana* (Ashtiyani et al. 2011; Kurihara et al. 2011). In agreement with its function in cell division, Haspin localizes at the chromosomes during mitosis (Dai et al. 2005; Ashtiyani et al. 2011; Kurihara et al. 2011). Dai et al. (2005) demonstrated that reduced levels of H3T3

phosphorylation induced by RNA interference-mediated depletion of the Haspin kinase, results in late-prometaphase configurations in mammalian cells. Overexpression of Haspin, on the other hand, causes a deficit in cell proliferation and a delay in the transition from G2 to mitosis. Taken together, these observations indicate that H3T3ph is important for correct chromosome segregation. H3T3ph also seems to play a role on kinetochore assembly, because phosphorylation of H3T3 by Haspin is required for recruitment of transient components of the kinetochore such as Aurora B and Survivin (Kelly et al. 2010; Wang et al. 2010). Indeed, H3T3ph serves a docking site for the binding of the BIR domain of Survivin and, consequently, of the chromosomal passenger complex to the inner centromere. H3T3ph also participates on the kinetochore-microtubule attachment for proper chromosome segregation (Yamagishi et al. 2010).

In Arabidopsis, RNAi downregulation of AtHaspin leads to reduction of H3T3ph in mitotic and meiotic chromosomes and affects chromatin condensation but not sister chromatid cohesion (Ashtiyani et al. 2011). These findings further suggest that, in contrast to animals, H3T3ph in plants is associated to chromosome condensation rather than to segregation. Furthermore, Survivin, the protein which recognizes H3T3ph and is necessary for recruitment of Aurora B does not have a homolog in plants. Thus, whether AtHaspin and AtAurora act in coordination to establish the H3 phosphorylation patterns during cell division remains to be explored.

In mammals, H3T11ph, similarly to H3T3ph, localizes at the centromeres during mitosis and meiosis (Fig. 4.2). Because phosphorylation of H3T11 occurs after the establishment of centromere complexes, Preuss et al. (2003) suggested that H3T11ph might have a function on kinetochore assembly in mammalian chromosomes, possibly serving as a recognition site for other kinetochore proteins.

In plants, H3T11 phosphorylation has a very similar kinetics to that of H3T3ph. Immunolabelling experiments in *Vicia faba* and *A. thaliana* demonstrated that H3T11ph signals are distributed along the entire chromosome from metaphase until anaphase (Houben et al. 2005) (Fig. 4.2). With the progression to telophase, the signal faded. In contrast, the timing of H3T3 and H3T11 phosphorylation differ in mitosis (Kurihara et al. 2011). While H3T3ph starts in early prophase and is dephosphorylated in anaphase, H3T11 begins in late prophase and ends in telophase. This distinct timing may be explained by the action of two different kinases and phosphatases (Kurihara et al. 2011).

H3T6ph has also been associated to both interphase (see Sect. 4.2.2.2) and mitotic cells. Immunostaining in murine C2C12 cells with an H3T6ph specific antibody indicates that this residue is phosphorylated in mitotic cells (Ali et al. 2013). However, the timing and distribution of this modification have not been further examined. H3T6ph has also been detected in mitotic cells of Arabidopsis and *Secale cereale*. During mitosis H3T6ph first appears as diffuse signals at early prophase but become highly phosphorylated along the entire length of the chromosomes during metaphase and anaphase. Finally dephosphorylation begins in anaphase. The timing and distribution of H3T6ph indicates that phosphorylation at this site might be related to chromosome condensation (Karimi-Ashtiyani and Houben 2013).

In plants, H3T32 is also phosphorylated, displaying a distribution pattern similar to H3T3ph and H3T11ph during mitosis (Caperta et al. 2008). The kinase that phosphorylates this site is still unknown, and even less is known about it in animals. Indeed, only one study reported the presence of H3T32ph in mammals so far but its distribution throughout the cell cycle or involvement in mitosis has not been reported (Tamada et al. 2006).

Despite our knowledge of kinase proteins and signaling pathways underlying histone modifications has been increasing considerably, much of the mechanisms resulting in cellular events due to such modifications still require further investigation.

4.2.2 Histone H3 Phosphorylation During Interphase

Although histone phosphorylation was first shown to have a role in mitosis, it also participates in multiple pathways of the cell cycle. Histone phosphorylation plays a role in transcriptional regulation in interphase cells, in replication during the S-phase and also in apoptosis. Unlike other modifications such as methylation and acetylation, histone phosphorylation is usually the result of activation of signaling cascades (reviewed by Baek 2011). Many of the upstream kinases that participate in pathways regulating transcription via activation of phosphorylation-dependent signaling cascades will also have an effect on gene expression through direct phosphorylation of histone residues (Baek 2011). Phosphorylation of several histone H3 residues during interphase has been described in different organisms. For some of these modifications, the kinases responsible, their mechanisms of action and interplay with other histones modifications has just started to be elucidated.

4.2.2.1 Serine Phosphorylation

Phosphorylation of H3S10 has been implicated in transcriptional activation in response to several external stimuli including stress, cytokines and growth factors. Phosphorylation of H3S10 in this case is rapid and transient, and correlated with the activation of the target genes (review by Healy et al. 2012; Sawicka and Seiser 2012). Several protein kinases responsible for in vivo H3S10ph in interphase cells of metazoan and yeast cells have been already identified (Table 4.1).

H3S10ph is tightly associated to acetylation of H3K14 and the double modification H3S10phK14ac is indeed necessary for transcriptional activation of the target genes (Clayton et al. 2000; Cheung et al. 2000). In yeast, a mutation on Serine 10 leads to downregulation of some genes activated by the histone acetyltransferase Gcn5 (Lo et al. 2000). Similarly, activation of the phospholipid biosynthetic gene *INO1* requires H3S10 phosphorylation by the Snf1 kinase, followed by H3K14 acetylation by Gcn5 (Lo et al. 2001, 2005). H3S10ph appears

to facilitate acetylation of H3K14 by increasing the affinity of Gcn5 and other histone acetyltransferases for the histone H3 tail (Cheung et al. 2000; Lo et al. 2000). However, as shown in yeast and mammalian systems, the dependence of H3K14ac for H3S10ph is not universal but rather occurs in a promoter specific fashion (Lo et al. 2001, 2005; Soloaga et al. 2003).

The mechanisms by which H3S10ph mediates transcriptional activation are under extensive investigation. H3S10ph may facilitate gene activation by establishing a "methyl/phos switch" with the adjacent H3K9me3. The methyl/phos switch hypothesis proposes that the more dynamic H3S10ph will modulate the binding of effector proteins to the more stable methylated H3K9. In agreement with this hypothesis, during mitosis H3S10 phosphorylation by the Aurora kinase leads to impaired binding of Heterochromatin Protein 1 (HP1) to H3K9me3 (Fischle et al. 2005; Hirota et al. 2005).

Several mechanisms mediate histone modification functions, among them, recruitment of readers for histone marks, the so-called effector proteins (Yun et al. 2011). H3S10ph, in combination with other modifications, may also modulate transcriptional activity by generating a binding platform for effectors proteins. For instance, the 14-3-3 family includes phospho-histone binding proteins linked to transcriptional regulation. In mammalian and yeast cells, 14-3-3 proteins are able to bind specifically to H3S10ph and H3S28ph, with even higher affinity when the H3K14 residue is acetylated (Macdonald et al. 2005; Winter et al. 2008; Walter et al. 2008). In mammalian cells, 14-3-3 proteins are recruited to the chromatin of *c-fos* and *c-jun* upon gene activation (Macdonald et al. 2005). Interestingly, 14-3-3 proteins seem to be required for gene activation but are not necessary for H3S10 phosphorylation or H3K14 acetylation (Winter et al. 2008). These observations indicate that 14-3-3 proteins may be the reader of effector complexes that mediates transcriptional activation of genes marked with H3S10phK14ac. However, the downstream mechanism of gene activation remains unidentified. Induction of some genes, like the histone deacetylase 1 (*hdac1*) gene, is accompanied by a rapid and transient increase in H3S10phK14ac, recruitment of 14-3-3ζ and loss of HP1γ from the gene promoter (Winter et al. 2008). Thus, displacement of HP1γ from H3K9me2 by H3S10phK14ac and binding of 14-3-3 to the latter may allow the establishment of a transcriptionally permissive chromatin environment without erasure of the repressive H3K9 methyl mark.

H3S10ph has also been linked to R-loops in metazoans. R-loops consist of a three stranded nucleic acid structure in which an RNA:DNA hybrid is form in the template strand leaving a displaced single-stranded DNA. R-loops are form as key intermediates during plasmid and mitochondrial replication, and also during immunoglobulin class switching in humans. However, formation of R-loops at high frequencies can cause genome instability (reviewed by Aguilera and García-Muse 2012). H3S10ph and chromatin condensation were found associated with R-loop formation in yeast, nematodes and humans, suggesting that they are functionally linked. The proposed model suggests that the accumulation of R-loops induces phosphorylation of H3S10 in the surrounding chromatin. H3S10ph then leads to a condensed chromatin structure that may interfere with replication and/or

transcription and cause DNA damage, which finally could lead to genome instability (Castellano-Pozo et al. 2013).

Among plants, H3S10ph was found in interphase cells of tobacco (Fig. 4.2). Immunolabeling experiments on nuclei extracted from tobacco leaves revealed that H3S10ph is localized in punctuated regions within the nucleolus, whereas in tobacco BY-2 cells H3S10ph could not be detected during interphase and its occurrence was restricted to mitotic chromosomes. In addition, H3S10ph associates to the 26S and 18S rDNA transcriptional units but is excluded from the non-transcribed intergenetic space (Granot et al. 2009). A similar punctuate distribution of H3S10ph was observed in interphase nuclei of root apexes from maize. In this case, an association between H3S10ph and 26S and 18S rDNA was also observed (Rossi et al. 2007). The similar distribution of H3S10ph in tobacco and maize interphase and its association to rDNA suggests a function for H3S10ph in rDNA gene regulation in plants.

Sokol et al. (2007) observed a global increase in H3S10ph and H3S10phK14ac in tobacco BY-2 and Arabidopsis T87 cells lines when subjected to cold and salt stress, or when treated with abscisic acid (ABA). The increased phosphorylation observed was correlated with transcriptional activation of stress specific genes (Sokol et al. 2007). Furthermore, induction of H3S10ph and H3T3ph was detected when tobacco BY-2 cells were treated with different concentrations of sucrose or sodium chloride (Houben et al. 2007). A similar response of H3S10ph was observed when leaves from tobacco and Arabidopsis where subjected to salt stress (Sokol et al. 2007). The increase in H3S10ph appears to occur in interphase cells since it was observed independently of the rate of cell division in BY-2 cells and in leaves, which are form mainly by differentiated and un-dividing cells (Houben et al. 2007). Thus, like in metazoans, H3S10ph and H3K14ac possibly participate in the transcriptional activation in response to external stimuli during interphase. To date, a direct association of H3S10ph with the promoter of the induced genes has not been demonstrated in plants. As it has been previously discussed, many of the metazoan kinases participating in H3S10ph are not conserved in plants (Cerutti and Casas-Mollano 2009). Thus, the role of H3S10ph and its kinases remain to be explored in detail, as mechanistic differences are likely to exist between H3S10ph in plants and animals.

Similarly to H3S10ph, H3S28ph has also been associated with two opposed chromatin states. Both residues are quickly and transiently phosphorylated in response to stress or growth factor stimulation during interphase, but also play a role in mitosis (Zhong et al. 2001; Soloaga et al. 2003; Choi et al. 2005). Although both residues are phosphorylated by the same signaling kinases (i.e. MSK1 and MSK2 in mammals) they appear to be independently distributed to different genomic regions, where they may activate a different subset of genes. Upon stimulation of H3S10ph and H3S28ph, both modifications were visualized, using immunofluorescence, as foci that localize away from regions densely stained with DAPI. More important, these foci did not co-localize, indicating that these two modifications occur at different chromosomal regions (Dunn and Davie 2005). Further evidence for the independent distribution of H3S10ph and H3S28ph

comes from sequential immunoprecipitation studies demonstrating that both modifications are targeted to different nucleosomes (Dyson et al. 2005).

Only a few studies have focused on the mechanism of gene activation mediated by H3S28ph. Gehani et al. (2010) demonstrated that MSK1/2-mediated phosphorylation of H3S28 in response to stress, mitogen activation and Retinoic acid-induced neuronal differentiation, leads to polycomb repressive complex (PRC) displacement and gene activation of polycomb-group (PcG) targets. In a similar way, targeting of the MSK1 kinase to the α-globin gene lead to gene reactivation, increased H3S28ph and displacement of PRC proteins from the promoter. Furthermore, replacement of H3K27me3 by H3K27ac was also observed at the α-globin promoter (Lau and Cheung 2011). These observations suggest that H3S28ph contributes to gene activation by causing the displacement of PRC complexes.

Following a characteristic pattern of phosphorylation observed in other residues, H3S28ph was shown to be increased during cell division and reduced in other stages of the plant cell cycle. Indeed, immunostaining with an anti-H3S28ph antibody could not detect this modification in interphase nuclei of all the plants studied (Gernand et al. 2003; Zhang et al. 2005). However, the possibility still remains that level of H3S28ph is very low and limited to particular genomic regions in interphase cells, or that it may be induced under certain external stimuli.

4.2.2.2 Threonine Phosphorylation

In contrast to the well documented role for H3S10ph and H3S28ph, to our knowledge there are not reports involving H3T3ph in any pathways during interphase cells in metazoans. Indeed, immunoblot analysis failed to detect H3T3ph in synchronized interphase mammalian cells (Zhou et al. 2006).

In the plant kingdom, H3T3ph has been shown to be involved in transcriptional regulation. In the green algae *Chlamydomonas reinhardtii*, a search for mutants defective in the silencing of transgenes identified a novel serine/threonine protein kinase, MUT9p, with similarity to casein kinase I, to be involved in this process (Jeong et al. 2002). Later on, it was demonstrated that MUT9p phosphorylates H3T3 and that this modification is enriched at the chromatin of silent transgenes and endogenous transposons. Furthermore, a mutation in *MUT9* resulted not only in reduced levels of H3T3ph but also of H3K4me1, a mark associated with gene silencing of transgenes and transposons (Van Dijk et al. 2005; Casas-Mollano et al. 2008). Similarly, a mutant lacking a H3K4 methyltransferase subunit, with severely reduced levels of H3K4me1, showed a partial loss of H3T3ph (Van Dijk et al. 2005; Casas-Mollano et al. 2008). These observations indicate that H3T3ph and H3K4me1 are two interdependent histone modifications associated with transcriptional repression in Chlamydomonas (Casas-Mollano et al. 2008).

The functional mechanism of H3T3ph in gene silencing has not been examined in Chlamydomonas. H3T3ph appears to operate antagonistically to H3K4me2 and H3K4me3, with H3T3ph associated to silent genes and the last two marks associated predominantly to transcriptionally active genes. Because H3T3 is adjacent to

the H3K4 residue, it has been proposed that this pair will form a methyl/phos switch (Fischle et al. 2003). Possibly, H3T3ph induces gene silencing by interfering with the binding of effector proteins to H3K4me2/me3 marks (Casas-Mollano et al. 2008). In fact, it has been shown in metazoans that H3T3 phosphorylation almost always results in severely reduced affinity of H3K4me3-binding proteins to the N-terminal tail of the histone H3 (Garske et al. 2010). However, whether H3T3ph might interfere with the binding of effectors complexes to H3K4me3 has not been investigated in plants.

Phylogenetic analysis demonstrates that MUT9p kinase true orthologs are restricted to the plant kingdom. Additionally, intra- and inter-specific gene duplications, some preceding the divergence between monocots and dicots, had led to an increased number of MUT9 homologs in higher plants. Thus, the MUT9 protein has evolved into a small gene family of plant specific kinases whose role in higher plants awaits further exploration (Casas-Mollano et al. 2008; Cerutti and Casas-Mollano 2009).

In higher plants, induction of H3T3ph and H3S10ph was detected in BY-2 tobacco cells treated with different concentrations of sucrose and sodium chloride. The increase in both modifications occurred independently of any increase in cell division indicating that it happened on interphase rather than due to an increase in the number of mitotic cells (Houben et al. 2007). These observations suggest that H3T3ph and H3S10ph may participate in the modulation of the chromatin environment of genes involved in process of energy and carbohydrate metabolism and osmotic stress responses (Houben et al. 2007).

Phosphorylation of H3T6 is an interphase modification that participates in androgen receptor-activated gene expression in mammalian cells. Ligand dependent expression of androgen receptor (AR) target genes is characterized by the phosphorylation of H3T6 and H3T11, removal of H3K9 methylation and acetylation of H3K9 and H3K14 (Metzger et al. 2005, 2008, 2010; Wissmann et al. 2007). Removal of the repressive marks from H3K9 is carried out by the cooperative activity of the lysine specific demethylase 1 (LSD1) and the Jumonji C domain-containing protein JMJ2C (Metzger et al. 2005; Wissmann et al. 2007). During AR-response, activation of Protein kinase C (PKCβ1) leads to phosphorylation of H3T6 at AR-target genes. In the presence of H3T6ph, LSD1 is prevented from demethylating H3K4me2, but allowed to demethylate H3K9me2. Therefore, H3T6ph regulates gene expression by modulating the activity of the LSD1 demethylase (Metzger et al. 2010).

In plants, immunostaining experiments revealed that H3T6ph occurs mainly in mitosis, but it was also detected at very low levels in interphase cells in Arabidopsis and *Secale cereale* (Karimi-Ashtiyani and Houben 2013). However, a genome wide comparison of kinases between yeast and Arabidopsis indicates that the PKC family of kinases, including PKCβ1, are absent in the genome of higher plants (Wang et al. 2003). Currently, it remains unknown whether other kinases evolved to phosphorylate H3T6 or if this modification is actually present during interphase chromatin of plant cells.

Like H3T6ph, phosphorylation of H3T11 is also involved in regulating AR-dependent gene expression in mammals. Upon androgen stimulation, the PKC-related kinase (PRK1) phosphorylates H3T11 at the androgen response elements (AREs) located in the promoter of the target genes. Phosphorylation of H3T11 leads to an enhanced activity of the H3K9 demethylase, JMJD2C, which removes the methyl groups from H3K9me3, a repressive methyl mark. H3T11ph is also necessary for the acetylation of H3K9 and H3K14, possibly by facilitating the binding of histone acetyltransferases to the AREs of androgen receptor target genes (Metzger et al. 2008). Taken together, these findings indicate that H3T11ph and H3T6ph are early activation marks that promote the removal of a repressive histone modification and contribute to the establishment of a chromatin environment, conducing to transcription of the AR target genes.

H3T11ph also participates in the regulation of a number of cell-cycle progression genes. In mouse cells, the Chromatin-associated checkpoint kinase 1 (Chk1) phosphorylates H3T11 at the *cyclin B1* and *cdk1* promoters. H3T11ph then enhances the association of the GCN5 histone acetyltransferase to the chromatin of the target genes thereby allowing transcriptional activation. When DNA damage occurs, phosphorylation of Chk1 leads to its rapid dissociation from chromatin. Then, dephosphorylation of H3T11 by an unknown phosphatase leads to dissociation of GCN5 from the promoter of the target genes, resulting in decreased H3K9ac and repression of *cyclin B1* and *cdk1* genes (Shimada et al. 2008). Phosphorylation of H3T11 also mediates gene target activation during epidermal growth factor receptor (EGFR) activation. During EGFR activation, phosphorylation of H3T11 by the tumor-specific piruvate kinase M2 (PKM2) leads to dissociation of the histone deacetylase 3 and increased H3K9ac in the *cyclin D1* and *c-Myc* target genes (Yang et al. 2012). These observations suggest that regulation of H3 acetylation is a common feature of transcriptional activation mediated by H3T11ph.

Similar mechanisms of transcriptional activation by H3T11ph may occur in other eukaryotes. Enhanced binding of the GCN5 histone acetyltransferase to the histone H3 tail phosphorylated at T11 has been predicted by a structural analysis in *Tetrahymena* (Clements et al. 2003). Yeast GCN5 has enhanced affinity for the H3 tail in the presence of phosphorylated H3T11 or H3S10 (Shimada et al. 2008). Furthermore, in vivo mutagenesis of H3T11 and H3S10 in yeast indicates that the H3T11 residue is necessary for transcription of genes regulated by GCN5 and H3S10ph (Clements et al. 2003). Thus, the recruitment of GCN5 mediated by H3T11ph appears to be a conserved mechanism of transcriptional regulation in several eukaryotes.

In plants, immunostaining with an antibody specific for H3T11ph did not detect any signal during interphase. However, upon treatment with the phosphatase inhibitor cantharidin, immunoflurescence signals co-localizing with pericentromeres were observed. It was postulated that increased levels of H3T11ph may occur due to a change in the balance of phosphorylation/dephosphorylation either on the H3T11 residue or in the kinase(s) responsible for this modification (Houben et al. 2005). However, whether H3T11ph plays a role in

transcriptional regulation during interphase in plants, as it does in other eukaryotes, awaits further examination.

Phosphorylation of H3T45 has been linked to several processes in different organisms. H3T45 phosphorylation is induced in DMSO-treated HL60 cells and neutrophils undergoing apoptosis, indicating an association of this modification with latter stages of the apoptotic process. Furthermore, the ability of PKCδ to phosphorylate H3T45 in vitro, and the close correlation between the kinetic of PKCδ activation and H3T45ph pointed to PKCδ as the kinase phosphorylating H3T45 in apoptotic neutrophils (Hurd et al. 2009). In yeast cells, H3T45 phosphorylation by the S-phase kinase cdc7 was linked to replication. H3T45 phosphorylation occurs during S-phase and is dependent on the activity of a complex containing the cdc7 kinase. Furthermore, loss of H3T45ph resulted in sensitivity to replication stress and slow growth, both phenotypes indicative of replication defects (Baker et al. 2010). Interestingly, induction of apoptosis does not lead to increased H3T45ph suggesting that, in contrast to human neutrophils, in yeast this modification does not participate in this pathway (Baker et al. 2010).

The mechanism by which H3T45ph participates in replication and apoptosis has not been elucidated. H3T45 residue forms part of the H3 αN helix, a highly conserved region critical for the interaction between DNA and the histone octamer. Thus, it was postulated that phosphorylation of H3T45 may help to disrupt DNA-histone contacts during the replication process and to facilitate DNA nicking and/or fragmentation during apoptosis (Hurd et al. 2009; Baker et al. 2010).

Phosphorylation of H3T45 has not been explored at all in plants and from the two H3T45 kinases identified in other organisms, PKCδ and cdc7, only cdc7 has a homolog in plants (Jouannic et al. 2001). However, considering how critical this position is for nucleosome stability and that modification of this residue is a critical step in different pathways, it is likely that H3T45ph may also be a crucial event in plant processes requiring structural changes in the nucleosomes such as apoptosis and replication.

4.2.2.3 Tyrosine Phosphorylation

In contrast to threonine and serine phosphorylation, only one tyrosine residue on the histone H3, H3Y41, has been found to be phosphorylated so far (Fig. 4.1). Phosphorylation of H3Y41 has been implicated in gene activation in mammalian cells. During haematopoiesis, Janus kinase 2 (JAK2), a tyrosine kinase involved in cytoplasmic signaling cascades, is localized in the nucleus, where it phosphorylates H3Y41. Phosphorylation of H3Y41 in haemetopoietic cells leads to displacement of HP1α from the promoter of JAK2 target genes such as the leukemogenic gene *lmo2*. Although it is well established that HP1 can bind to H3K9me3 through their chromodomain, HP1 also binds the H3Y41 region through its chromo-shadow domain (CDS). The displacement mechanism is based on the ability of H3Y41ph to interfere with the binding to the CSD of HP1α to the histone H3 (Dawson et al. 2009).

To our knowledge, the presence of H3Y41 phosphorylation has not been reported in plants. In addition, reciprocal BLAST searches indicate that homologs of the JAK2 kinases are unlikely to exist in plants (unpublished results). However, a single homolog of HP1 Protein, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) has been identified in Arabidopsis (Gaudin et al. 2001). Like their mammalian homologs, this protein contains conserved chromo- and CSD domains separated by a "hinge" region. LHP1 chromodomain was shown to bind H3K27me3 instead of H3K9me3, and to participate in the silencing of euchromatic genes (Turck et al. 2007; Zhang et al. 2007). The LHP1 CSD domain, on the other hand, is necessary for dimerization and for interaction with other proteins such as the MADS box transcription factor SHORT VEGETATIVE PHASE (Gaudin et al. 2001; Liu et al. 2009). Considering the relative conserved function and domain structure of LHP1, it is possible that its CSD domain may also bind histone H3. Whether this interaction really occurs in plants and if phosphorylation, or any other post-translational modification, regulates this interaction, remains to be investigated.

4.3 Phosphorylation of H3 Histone Variants

Expression of canonical histones is regulated during cell cycle, and is restricted to S phase during DNA replication. Histone variants, on the other hand, have been identified for all core histones and are assembled into chromatin in a replication-independent manner. Several studies have focused on H3 variants because they participate in many cell functions.

The H3 variants H3.2 and H3.3 are evolutionary conserved. Histone H3.2 only differs from canonical H3 in a cysteine-serine substitution at position 96 whereas H3.3 differs from H3 in five amino acids and is expressed throughout the cell cycle (Hake and Allis 2006). Like their canonical counterpart, histone variants are also subjected to covalent modifications. H3.3 is associated with sites of active transcription and is particularly enriched in modifications that mark active chromatin such as H3K4me3, H3K9ac and H3K14ac among others (Ahmad and Henikoff 2002; McKittrick et al. 2004). Phosphorylation of H3.3 has been observed during mitosis in a serine which occupies the position 31 (Hake et al. 2005). Unlike H3S10ph and H3S28ph, that during mitosis first become phosphorylated in prophase, H3.3S31 phosphorylation occurs in late prometaphase, while dephosphorylation starts in anaphase. In mitotic chromosomes, H3.3S31ph localizes at regions immediately adjacent to centromeres, even though H3.3 is distributed along the entire chromosome (Hake et al. 2005). The contrasting timing and distribution of H3.3S31 and of H3S10ph and H3S28ph weight against H3.3S31 being involved in initial chromosome condensation. H3.3S31 is conserved from yeast to human, and in Arabidopsis it is replaced for another phosphorylatable residue, threonine. Yet, whether phosphorylation of this residue actually occurs, or if it has a function during mitosis and/or meiosis in plants remains to be studied.

The Centromeric Histone H3 (CENH3) constitutes another class of H3 variant that substitutes the canonical H3 in the centromeric chromatin where it functions as an epigenetic mark for the centromere and kinetochore (Van Hooser et al. 2001). CENH3 proteins have been identified in humans (CENP-A), *S. cerevisiae* (Cse4), Drosophila (CID) and plants (HTR12). In some groups of plants and animals, CenH3 has been found under adaptive selection possibly as a response to rapid changes in centromeric DNA (Malik and Henikoff 2001; Talbert et al. 2002). Unlike H3.2 and H3.3, CENH3 has a conserved C-terminal histone-fold domain but is highly variable between species at the N-terminus, likely as a result of adaptive selection (Malik and Henikoff 2003).

Phosphorylation and other post-translational modifications have been identified in human, yeast and plant CENH3 (Table 4.1). In humans, phosphorylation of S7 of CENP-A by the Aurora kinase (Table 4.1) has been shown to contribute to kinetochore function, cytokinesis and proper chromosome alignment (Zeitlin et al. 2001a, b; Kunitoku et al. 2003). Additionally, immunolocalization with anti-CENP-A-S7ph revealed similar phosphorylation pattern to that of H3S10ph, except that CENP-A is dephosphorylated faster at anaphase (Zeitlin et al. 2001b).

Recently, it has been demonstrated that neither amino acid composition nor length, but rather serine phosphorylation at the N-terminus is necessary for mitotic progression in HeLa cells (Goutte-Gattat et al. 2013). This study also showed that phosphorylation of the N-terminus recruits 14-3-3 proteins, which helps to stabilize the interaction between CENP-A-containing nucleosomes and the centromeric protein CENP-C. Stable binding of CENP-C to the inner centromere will then initiate the assembly of a functional kinetochore. Similarly, yeast Cse4 was shown to be phosphorylated in vivo at S22, S33 and S40, all sites localized at the N-terminal domain. Like in mammalian CENP-A, the main contributor for this phosphorylation is the Aurora kinase homologue Ipl1. Furthermore, by using non-modifiable and phosphomimetic mutants, Boeckmann et al. (2013) demonstrated that phosphorylation of Cse4 facilitates chromosome bi-orientation, presumably by destabilizing defective kinetochores, thereby ensuring correct chromosome segregation. Thus, phosphorylation of the highly variable N-terminus at CENH3 appears to be critical for chromosome alignment and segregation in yeast and mammals.

In Arabidopsis, the C-terminal and not the N-terminal domain of CENH3 seems to be required for its targeting to the centromere. Arabidopsis CENH3 carrying only the N-terminal domain failed to target the centromeres and displayed diffuse localization throughout the nuclei, while the C-terminal domain presented normal centromere targeting (Lermontova et al. 2006). Further experiments using a CENH3 null mutant, *cenh3-1*, indicate that although the C-terminus alone is necessary for centromere targeting, an N-terminal domain is still required to nucleate a functional kinetochore (Ravi et al. 2010). Replacement of the CENH3 N-terminus by the histone H3.3 tail rescued the embryo-lethal phenotype of *cenh3-1*, but produced sterile plants. This observation suggests that the heterologous H3.3 N-terminal tail can replace the function of the CENH3 tail, however, the sterility observed indicates that it can function in mitosis but not in meiosis

(Ravi et al. 2010). Yet, it remains to be explored whether phosphorylation or any other modifications in the C- and N-terminal domains of Arabidopsis CENH3 are required for its loading into centromeres and to initiate kinetochore assembly.

CENH3 phosphorylation at the N-terminal domain has been reported in the monocotyledonous plant *Zea mays*. Using a specific antibody, Zhang et al. (2005) demonstrated that the S50 residue of ZmCENH3 is phosphorylated during mitosis and meiosis. The kinetic of ZmCENH3-S50 phosphorylation is similar to that of canonical H3S28ph. However, the signals only overlap at cohesive regions between centromeres. Interestingly, the timing of phosphorylation in maize CENH3 is similar to that reported for human CENP-A-S7ph. Furthermore, both maize CENH3-S50 and CENP-A-S7 residues are located at the variable N-terminus (Zhang et al. 2005). Possibly, as it happens with CENP-A in mammalians and Cse4 in yeast, phosphorylation of ZmCenH3 is required for recruitment of proteins that mediate kinetochore assembly and chromosome segregation. However, further experiments will be necessary to determine whether phosphorylation of CENH3 contributes to this function in maize.

4.4 Future Perspectives

Although in the past couple of years there has been an increased number of studies aiming to comprehend the roles histone H3 phosphorylation plays in cellular processes, many questions still remain open and a complete understanding of the mechanisms of chromatin regulation by histone phosphorylation is still far away. One of the main unanswered questions is: how phosphorylation and other histone modifications results in a specific biological read out? Answering this question is a challenging task mostly because the meaning of histone modifications may depend on the signaling context, chromatin environment and their crosstalk with other modifications (Berger 2007; Kouzarides 2007). An added challenge to the study of histone phosphorylation in plants is that the meaning of the histone modifications may not be universal but rather lineage specific (Loidl 2004; Fuchs et al. 2006; Cerutti and Casas-Mollano 2009). Additionally, several of the kinases involved in phosphorylating H3 residues likely evolved after the divergence between plants and animals (Cerutti and Casas-Mollano 2009). Therefore, a correct understanding of the functional significance of histone phosphorylation has to be determined in a plant-specific context.

Despite of its importance, the study of epigenetic mechanisms in crops is still in its infancy. Recent studies characterized H3 phosphorylation distribution and dynamics in plants, but only two of the responsible kinases have been identified so far. In fact, the mechanisms and functions of H3 phosphorylation are far less understood in plants than in metazoans. Additionally, even though histone phosphorylation in some important crops has been demonstrated, most studies on the kinases responsible and mechanisms of action have been mainly carried out in the model plant Arabidopsis. As a consequence, there is a gap of information in crops

plants that needs to be filled in order to better understand the importance of H3 phosphorylation for plant development and to take advantage of this information for the breeding of new crops.

Deep knowledge of how histone phosphorylation events are orquestrated during cell division and the mechanisms by which they lead to chromosome condensation and segregation may help to develop new biotechnological approaches for crop breeding. For instance, manipulation of the right kinase(s) and/or phosphorylation event(s) could provide tools to regulate chromosome segregation during mitosis, allowing the formation of unreduced gametes that breeders can use to generate new lines with different ploidy levels.

Phosphorylation is also an important regulator of the response to environmental stimuli in plants, and H3 phosphorylation levels have been shown to increase as a response to different stress treatments. Therefore, the study of histone phosphorylation may provide novel target genes for improvement of abiotic stress tolerance in crop plants, currently a key issue for climate-proofing of crop plants to ensure that food supply demands are met over the coming decades. To reach this goal, understanding how histone phosphorylation helps the plant to cope and recover from environmental stress will be essential.

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Chapter 5
Tomato Epigenetics: Deciphering the "Beyond" Genetic Information in a Vegetable Fleshy-Fruited Crop

Fabio T.S. Nogueira

Abstract The first natural plant mutant for which the molecular basis was determined to be an epimutation rather than a change in DNA sequence was a peloric variant of toadflax, *Linaria vulgaris*. Remarkably, the second example of a natural epimutant came from the vegetable fleshy-fruited crop tomato (*Solanum lycopersicum*). The discovery of the molecular basis for the *Colorless nonripening* (*Cnr*) epimutation was a landmark for plant epigenetics and, importantly, linked epigenetic mechanisms with an important agronomical trait. More recently, several studies on tomato have contributed to our better understanding of epigenetic mechanisms underlying important heritable crop traits, such as ripening and stress response. Epigenetic mechanisms have also been associated with transgressive segregation in hybrids generated from crosses between cultivated tomato and close wild relatives. Therefore, we can only envision that tomato will became a model for studying the epigenetic basis of economically important phenotypes, allowing for their more efficient exploitation in plant breeding.

Keywords Tomato • Small RNAs • DNA methylation • Epiallele

5.1 Introduction

Tomato (*Solanum lycopersicum*) is a major vegetable fleshy-fruited crop, accounting for 14 % of the world vegetable production. Over 100 million metric tons/year, a $1.6 billion market, were produced in 2010 (FAO 2013). Tomato is a rich source of micronutrients for human diet and its fruits can be used either for fresh consumption or for processing. It is also an important model species for research on fruit development and metabolite accumulation.

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Tomato belongs to the large and diverse *Solanaceae* family also called Nightshades, which includes more than three thousand species from several habitats. Among them, major crops arose from the "Old World" (Eggplant from Asia) and the "New World" (pepper, potato, tobacco, and tomato). The *Lycopersicon* clade contains the domesticated tomato and its 12 closest wild relatives (Peralta and Spooner 2005). Tomato originated in the Andean region of the Americas, and its domestication is thought to have taken place in Central America (Bai and Lindhout 2007). Domesticated tomato has been bred to improve productivity, fruit quality, and resistance to biotic and abiotic stresses, most of which are agronomically key traits for several crops. Modern cultivars are commercialized as hybrids with high performance in the field.

In spite of its importance as a crop and as a model plant for research, only recently the genome of domesticated tomato was sequenced (The Tomato Genome Consortium 2012). Tomato chromosomes contain pericentric heterochromatin and distal euchromatin, with repeats concentrated within and around centromeres, in chromomeres and telomeres (The Tomato Genome Consortium 2012). Interestingly, tomato has fewer high-copy, full-length long terminal repeat (LTR) retrotransposons when compared with *Arabidopsis thaliana* and *Sorghum bicolor* (The Arabidopsis Genome Initiative 2000; Paterson et al. 2009). This data supports previous findings that tomato genome is largely comprised of fast-evolving, low-copy DNA (Zamir and Tanksley 1988). This unique feature is likely to play an important role in tomato breeding.

A new step for understanding how the tomato genome "behaves" and evolves and its implication in tomato breeding and genetic control of agronomical traits is coming from next generation sequencing techniques. Such techniques allow the identification of not only genetic but also epigenetic "players". As an example of the latter, information from high throughput sequencing of tomato small RNA (sRNA) populations suggests that most sRNAs map preferentially to the euchromatin portion of its genome, which is contrasting to what is generally observed in Arabidopsis. Differential expression of tomato sRNAs was observed during fruit development and they apparently mapped to a number of gene promoters, including those of genes associated with cell-wall biogenesis (The Tomato Genome Consortium 2012). These sRNAs may function as "triggers" to generate epigenetic modifications that likely affect gene regulation and genome stability. Indeed, it is well established in model plants, such as Arabidopsis, that epigenetic modifications of the DNA and histones serve as heritable marks that can influence gene expression states. Therefore, deciphering the tomato epigenome and its function may help to identify candidate genes for tomato improvement, should epigenetic variants be discovered.

In this chapter I will first highlight the main findings on tomato epigenetics until today. I will then discuss how we may combine valuable information regarding epigenetic and genetic natural variation to help to improve the future of tomato breeding.

5.2 Epigenetic Studies on Tomato

5.2.1 DNA Methylation and Histone Modifications

Given that only a few spontaneous epimutations have been described in plants (Cubas et al. 1999; Kalisz and Purugganan 2004), the finding that tomato natural mutant *Colorless non-ripening* (*Cnr*) is due to an epimutation was unexpected (Thompson et al. 1999). Although the dominant pleiotropic mutation *Cnr* was described in tomato more than a decade ago, only recently its epigenetic "nature" was revealed (Thompson et al. 1999; Manning et al. 2006). *Cnr* epiallele inhibits normal ripening and produces a severe phenotype by which fruits develop a colorless, mealy pericarp. Such phenotype is due to an absence of ripening-related carotenoid biosynthesis and modifications in the cell wall structure of the pericarp (Eriksson et al. 2004). *Cnr* epiallele corresponds to the *SBP3*-like (*SQUAMOSA promoter binding protein3-like*) gene (Solyc02g077920), a tomato SBP-box family member (Salinas et al. 2012). The SBP-box family of transcription factors is unique to plants and their members are characterized by a highly conserved SBP domain of approximately 76 amino acid residues, involved in DNA binding and nuclear localization (Preston and Hileman 2013).

In *Cnr* mutant, the epigenetic allele of *SBP3*-like/*CNR* gene is heavily methylated mostly in a 300 bp region located approximately 2 kb upstream of the ATG (Fig. 5.1), while its wild-type counterpart is not. Given that hypermethylation in upstream sequences is generally associated with gene silencing (Seymour et al. 2008), modifications in the methylation status likely explain the reduced

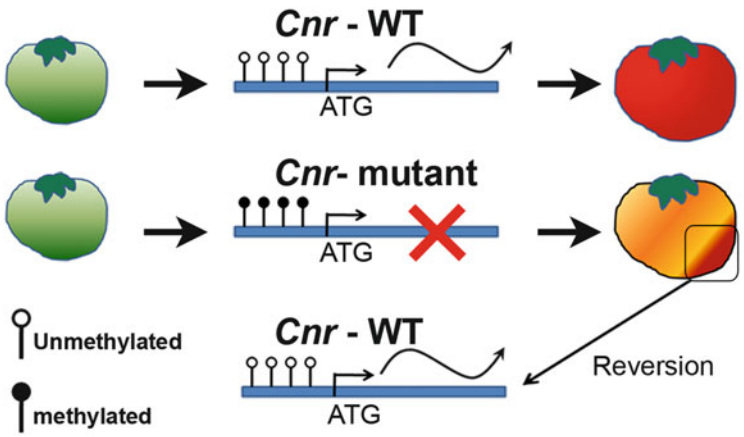

Fig. 5.1 Graphic representation showing how the natural epiallele *Cnr* prevents ripening, resulting in yellow fruits. Such epiallele is the result of changes in methylation status on CpG and CpHpG regions within the promoter and 5′-UTR of *SBP3*-like/*CNR* gene. Interestingly, some occasional revertant 'ripening' sectors that have a wild-type ripening phenotype are observed in mutant fruits

SBP3-like/*CNR* expression in *Cnr* fruits. Moreover, in non-mutant or wild-type plants, the promoter of *SBP3*-like/*CNR* appears to be demethylated just prior to the onset of ripening. Such observation led to the hypothesis that DNA methylation contribute to the regulation of fruit ripening (Seymour et al. 2008). *Cnr* epimutation is stable over generations as few revertants were observed (Manning et al. 2006), implying that epigenetic modifications were inherited in a Mendelian fashion and resulted in the suppression of *SBP3*-like/*CNR* transcription during fruit development. While the nature of the epimutation in the *Cnr* mutant is well established, the possible causes for the appearance of this epialelle are less understood. Interestingly, in the mutant, most of the methylated cytosines are in a symmetrical sequence context (CpG, CpHpG, where H is A, C or T), which is generally maintained by METHYLTRANSFERASE1 (MET1) and CHROMOMETHYLASE3 (CMT3) methyltransferases in Arabidopsis, respectively (Martienssen and Colot 2001; Lindroth et al. 2001).

In silico survey in Sol Genomics (http://solgenomics.net) suggests that tomato has one *MET1* homolog, which is located at chromosome 11. Two possible homologs of *CMT3* in the tomato genome are located at chromosomes 1 and 12 (Table 5.1). Expression profiles retrieved from RNA-seq data of the Tomato eFP Browser (http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi) showed that *MET1* and *CMT3* homologs are lowly expressed in "Breaker fruit" stages while *SBP3*-like/*CNR* is highly expressed (Fig. 5.2). Future studies are needed to address whether tomato MET1 and CMT3 enzymes are indeed involved in the generation of the natural *Cnr* epialelle.

Some clues regarding possible causes of the epimutation in the *Cnr* mutant allele may come from evaluating *CNR*, *MET1*, and *CMT3* loci in different genetic backgrounds. For example, *Cnr* epialelle arose in tomato Liberto background, in which the DNA in the *SBP3*-like/*CNR* genomic region showed an increased predisposition for methylation in comparison with that from Ailsa Craig background (Thompson et al. 1999; Manning et al. 2006). Therefore, one can speculate that the Liberto cultivar is more likely to give rise to *Cnr* mutant plants than the Alisa Craig cultivar. Additionally, Liberto cultivar is more similar in this respect to fruits from *Lycopersicon cheesmanii* (Manning et al. 2006). *L. cheesmanii* is one of the wild tomato species endemic to the Galapagos archipelago and exhibits a range of peculiar phenotypes when compared with cultivated tomato (Arkive 2013). Particularly, *L. cheesmanii* '*long*' displays bright orange-yellow fruits (Nuez et al. 2004). It will be fascinating to evaluate whether fruit phenotype in this wild relative is a result of *SBP3*-like/*CNR* genomic region being more prone to changes in methylation status during fruit development and ripening than cultivated tomato. It is feasible that the fruit phenotype in this species may be a result of epigenetic-driven modifications in the expression of *SBP3*-like/*CNR* locus. Assuming that such modifications can be confirmed, they must be the product of Darwinian evolution, which would have produced the (epi)genetic mechanisms that underlie these effects on DNA methylation status in specific loci.

Is it possible that other tomato loci are also prone to changes in methylation status during fruit development? In other words, could we identify novel epialelles

Table 5.1 Tomato cytosine-5 DNA methyltransferases

Protein name	Putative function	Locus no.	Chromosome
MET1	Maintenance of CpG methylation	Solyc11g030600	11
CMT3-like	CpHpG methylation in repetitive DNA and transposons in heterochromatin	Solyc12g100330	12
		Solyc01g006100	1
DRM-like[a]	De novo: CpG, CpHpG, CpHpH	Solyc02g062740	2
	Maintenance: CpHpG, CpHpH	Solyc10g078190	10

[a]Domains-rearranged methyltransferases-like proteins

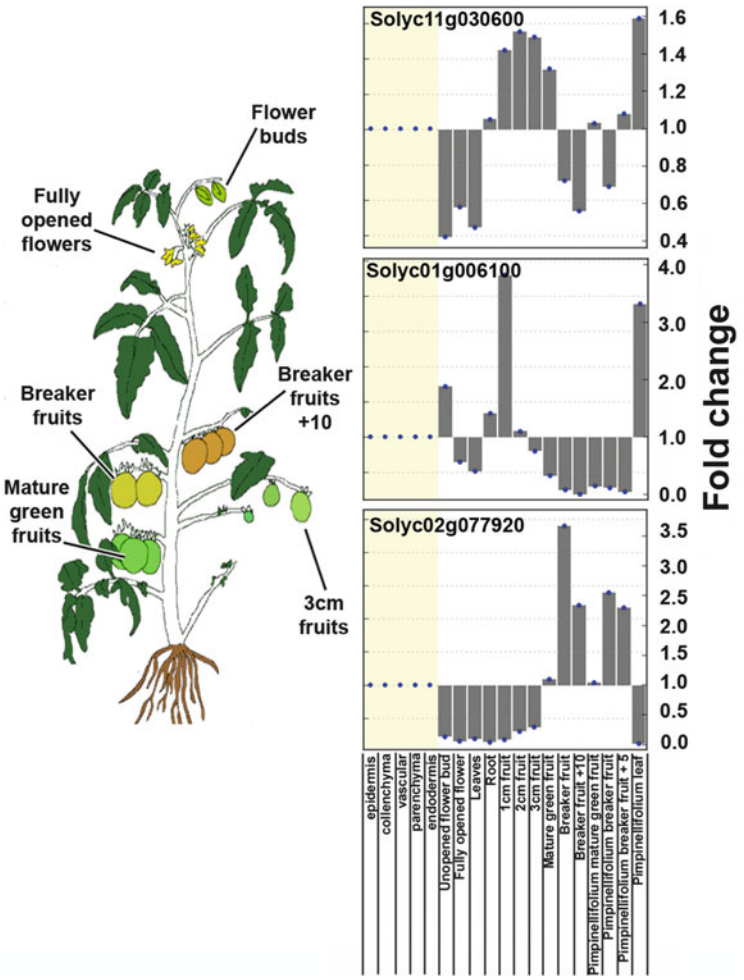

Fig. 5.2 Expression profiles of *SBP3*-like/*CNR* (Solyc02g077920), *MET1* (Solyc11g030600), and *CMT3-like* (Solyc01g006100) genes in different tissues and organs. The figure was generated using RNA-seq data from Tomato eFP Browser (http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi). Adult tomato plant showing tissues/organs analyzed is shown in the *left panel*

associated with natural changes in fruit development and ripening? A promising answer for this important biological and agronomical question may come from genome-wide analyzes of the DNA methylation status during fruit development and ripening. Recently, Zhong et al. (2013) provided the first insights into the link between the fruit ripening genetic program and DNA methylation state. After injecting a chemical inhibitor of cytosine methylation, 5-azacytidine, the authors performed whole-genome bisulfite sequencing in four stages of fruit development, from immature to ripe, identifying more than 50,000 differentially methylated regions (representing 1 % of the tomato genome). The sequencing of these epigenomes provided, among others, one crucial finding: in wild-type fruits, the degree of methylation of promoter regions decreased progressively along fruit development (Zhong et al. 2013). Several of these promoters belong to typical ripening-related genes, implying that potential epialleles associated with ripening and fruit quality might arise during breeding programs that use distinct genetic backgrounds and growing conditions.

Evidence so far suggests a key role of the epigenome structure and developmental dynamics in coordinating tomato fruit ripening. Such evidence include data showing that binding of the MADS-box transcription factor RIPENING INHIBITOR (RIN)—a key regulator of ripening (Vrebalov et al. 2002)—to a set of promoters was inhibited in the *Cnr* background, suggesting that promoter hypermethylation blocks RIN binding (Martel et al. 2011). Progressive demethylation of ripening-related gene promoters seems to be necessary for binding of transcriptional regulators (such as RIN), thus triggering the accumulation of ripening-related transcripts (Martel et al. 2011). Intriguingly, Zhong et al. (2013) observed that binding sites for the RIN transcription factor are hypermethylated in the *rin* loss-of-function mutant, which suggest that promoter methylation status of some genes may be altered by the binding of the transcription factors themselves. Similar results were observed for the mouse epigenome (Stadler et al. 2011). Nonetheless, the mechanism(s) underlying demethylation of gene promoters during wild-type fruit development remain(s) unclear and further efforts are needed to unravel additional endogenous and/or exogenous cues that contribute to this epigenetic modification. In summary, it seems that tomato fruit cells take advantage of epigenome reprogramming along with fruit-specific transcription factors to regulate the fruit transition into a ripening-competent state when the seeds become viable.

Among the three main phases that precede tomato fruit ripening (Gillpasy et al. 1993), phase III corresponds to the developmental stage in which fruit grows basically due to cell expansion concomitant with a dramatic increase in nuclear ploidy level, a process termed endoreduplication (Joubès et al. 1999). Endoreduplication could lead to variation in DNA methylation in specific fruit tissues. To evaluate the possible correlation between endoreduplication and methylation status in fruit tissues, Teyssier et al. (2008) employed Southern experiments with methyl-sensitive restriction enzymes along with HPLC analysis to demonstrate tissue-specific variation in DNA methylation levels. The authors observed an increase in CpG and/or CpHpG methylation at specific loci (mostly repetitive sequences and retransposons) in pericarp genomic DNA during fruit development.

Interestingly, a sharp decrease of the global DNA methylation level was also observed in pericarp during the onset of the fruit ripening, which is consistent with the methylome data from Zhong et al. (2013). Conversely, no major variation of DNA methylation either global or locus-specific was observed in locular tissue, which could reflect tissue-specific variations of DNA methylation during fruit development and ripening (Teyssier et al. 2008). The reasons for tissue-specific differences in DNA methylation are still obscure, but it is unlikely to be triggered by the induction of endoreduplication in fruit tissues. For instance, cytosine methylation did not increase significantly in locular tissue at the loci analyzed by the authors, although their nuclei were highly endoreduplicated (Teyssier et al. 2008). Therefore, it seems that an increase in endoreduplication is not necessarily followed by an increase in DNA methylation in all tomato fruit tissues, though the authors did not verify this fact by using whole-genome bisulfite sequencing. As mentioned before, the mechanisms underlying the differential DNA methylation in developing fruits are still not elucidated. However, it is possible that differential and tissue-enriched expression of specific DNA methyltransferases (Table 5.1) during fruit development (Fig. 5.2) may be partially responsible for the DNA methylation patterns observed (Teyssier et al. 2008).

An appealing connection between plant epigenetics and stress was hypothesized by the Kovalchuk group in Arabidopsis and experimentally supported in rice, in which at least some stress-induced phenotypes depend upon altered DNA methylation (Boyko and Kovalchuk 2008; Wang et al. 2011). Recent findings in tomato are consistent with such conjectures. González et al. (2011) investigated DNA methylation within gene bodies by evaluating the distribution of cytosine methylation in *Abcisic acid stress and ripening1* (*Asr1*), a tomato water stress-inducible gene of the *LEA* (*late embryogenesis abundant*) superfamily. Similarly to data from Arabidopsis, it was found in tomato that DNA methylation at CpG sites within plant gene bodies is not necessarily associated with silencing as it is in animals (Zhang et al. 2006; González et al. 2011). Indeed, dehydration stress incited higher CpG methylation levels in the first exon of the *Asr1* gene, concomitant with enhanced gene expression. However, tomato plants under drought stress displayed removal of methyl marks at approximately 70 % of asymmetric CpHpH (where H is A, C or T) sites and a decrease of the repressive histone H3K27me3 epigenetic mark and an induction of expression of the same gene. Interestingly, most demethylated sites were present in intronic regions of the *Asr1* gene (González et al. 2011). These sites may be targets for RNA-directed DNA methylation (RdDM) as it has been demonstrated that intron-derived siRNAs mediate DNA methylation of their host genes (Chen et al. 2011). Although the authors did not check whether intronic regions of the *Asr1* gene have potential to form internal hairpin structures, these structures—if present—could produce siRNAs to mediate RdDM of *Asr1* in *cis*.

The same research group has recently published a related study on the *Asr1* paralog, *Asr2*, which has been a target for positive selection during the evolution of the *Solanum* genus in arid environments (González et al. 2013). Similarly to *Asr1*, loss of DNA methylation and the repressive histone H3K27me3 epigenetic mark were observed in the gene body and regulatory regions of *Asr2* under stress

conditions. Taken together, these two studies suggest that rapidly acquired novel epialleles of stress-related genes due to desiccation might be an alternative mechanism for plant adaptation to environmental drought conditions, not only in Arabidopsis but also in species with larger and more complex genomes such as tomato.

The finding that CpHpH methylation in tomato can occur in the body of stress-associated genes lacking repeated sequences, may represent an alternative mechanism for the stress-driven gain or loss of epigenetic marks that regulate gene expression in plants. DNA methylation within gene bodies in plants is emerging as an important epigenetic modification, as it regulates gene expression and plant development in some cases, though how those mechanisms operate remains elusive (Teixeira and Colot 2009).

How epigenetic states of gene activity are maintained steadfastly throughout consecutive rounds of cell division is one of the central questions in developmental biology. Investigations in metazoans, plants and microorganisms suggest an important and conserved role of the DDB1-CUL4-based ubiquitin E3 ligase complex in perpetuating epigenetic marks on chromatin, most likely via regulating histone modification or/and DNA methylation (Higa et al. 2006). This complex contains the adapter protein DDB1 (UV-damaged DNA binding protein 1) that binds to UV-damaged DNA and participates in DNA repair pathways at the stage of binding and recognition (Chu and Chang 1988). Recently, a study on tomato DDB1 suggested that this protein plays an important role in regulating the epigenetic state of genes controlling organ size, growth habit, and photosynthesis (Liu et al. 2012; Tang et al. 2012). Transgenic plants overexpressing an alternatively spliced tomato *DDB1* transcript, $DDB1^F$, displayed reduced organ size and a decrease in DNA methylation level at the *SlWEE1* gene (*Solanum lycopersicum WEE1*), a negative regulator of cell division. Reduced DNA methylation in the *SlWEE1* promoter was shown to be correlated with high expression levels of this gene in the transgenic plants, likely leading to growth arrest of the fruits (Liu et al. 2012; Tang et al. 2012).

Another interesting finding was that some of the phenotypes (reduced organ size and high shoot branching) observed in transgenic tomato plants overexpressing $DDB1^F$ are independent of the presence of the transgene in subsequent generations. For example, plants of the T2 and T3 generations containing no $DDB1^F$ transgene showed reduced organ size and higher axillary branching, similarly to phenotypes present in T1 plants containing the transgene (Liu et al. 2012; Tang et al. 2012). However, at later generations (T4 plants), fruit weight and shoot branching phenotypes reverted to wild-type phenotypes (Tang et al. 2012). Based upon these observations, the authors concluded that both phenotypes are epigenetically controlled and can be transmitted over three generations (Liu et al. 2012; Tang et al. 2012).

Although the results on tomato DDB1 are exciting, the mechanism(s) leading to such heritable epigenetic changes in specific loci remain(s) to be determined. In Arabidopsis, DDB1-CUL4-based ubiquitin E3 ligase interacts with components of the Polycomb Repressive Complex 2 (PRC2), required for epigenetic silencing of

chromatin, thus indicating a novel role of ubiquitylation in epigenetic regulation of gene expression (Dumbliauskas et al. 2011). Assuming a conserved role of DDB1 in tomato, one can speculate that overexpression of $DDB1^F$ may lead to degradation of epigenetic regulators, such as DNA methyltransferases, consequently reducing methylation levels of target genes. As observed by Liu et al. (2012), $DDB1^F$ transgene seems to be responsible for the initiation of the decreased methylation of the *SlWEE1* gene, but not for its maintenance across generations. This observation implies the action of additional epigenetic "players" on the maintenance of the methylation levels of *SlWEE1* and likely other genes encoding negative regulators of cell division, which could have an impact in multiple traits of agronomic importance in tomato (Tang et al. 2012).

Grafting is a significant technique to improve performance of horticultural plants including several agronomically important woody fruit trees and vegetables. This method is generally performed by grafting the shoot part of a plant (scion) onto a root part of another plant (rootstock), often with distinct genetic backgrounds, even different species or genera (Burge et al. 2002). The recently documented mobility of various genetic components including DNAs and RNAs between the scion and stock (Haroldsen et al. 2012) have risen the question whether phenotypic traits altered in the grafted products have a heritable basis as a result of the exchanging of genetic information. Although DNA exchange has been documented, it only occurred at very low frequencies (Thyssen et al. 2012; Stegemann et al. 2012). Small RNAs of 21–24 nucleotide (nt) in size were also reported to be able to move across the graft union via plasmodesmata and phloem. Significantly, movement of 24-nt siRNAs was capable of directing DNA methylation in the genome of the recipient cells (Molnar et al. 2010), tantalizingly suggesting that epigenetic modifications may take place in the grafted products, probably resulting in heritable new characteristics passing to the next generation of non-grafted plants.

To test this hypothesis, Wu et al. (2013) analyzed relative DNA methylation levels by using methylation-sensitive amplified polymorphism (MSAP) and locus-specific bisulfite-sequencing in seed plants, self- and hetero-grafted scions/rootstocks, selfed progenies of scions and their seed-plant controls of pure-line cultivars of tomato, eggplant (*Solanum melongena* L.), and pepper (*Capsicum annuum* L.). Extensive alterations in two DNA methylation contexts (CpG and CpHpG) were observed in all independent samples of multiple interspecific graftings tested involving these three *Solanaceae* species. Importantly, such alterations seem to be heritable for some loci, which is surprising if taken into consideration that the induced epigenetic modifications would have to affect primordial cells that are destined to form gametal cells. Based on gene expression analyzes, the authors suggested that methylation pattern alterations and their inheritance induced by grafting were at least in part due to perturbed expression of the cellular machinery required for DNA methylation. Therefore, it seems that, at least in *Solanaceae* species, inter-species hetero-grafting produces heritable alteration in DNA methylation patterns that may produce functional developmental consequences in the graft hybrids. Such functional consequences could help to generate hetero-grafted scions/rootstocks with agronomic relevance. Moreover, we can hypothesize that

these alterations in DNA methylation constitute an important genetic component underlying the Darwinian concepts of graft hybridization and graft hybrid, concepts of which were put forward by Charles Darwin more than two centuries ago (Darwin 1868).

In addition to DNA methylation, nucleosome remodeling and histone posttranslational modifications contribute to modulate different chromatin states that control transcription and other chromatin-based nuclear processes (Sadeh and Allis 2011; Kouzarides 2007). While DNA methylation status and its modifications have been fairly documented in tomato, studies on histone modifications are missing for this crop. To initiate these studies, Aiese Cigliano et al. (2013) identified and performed expression profiling analyzes of *histone modifier* genes (*HMs*) in tomato. This in silico study identified over 100 *HMs* loci including 32 histone acetylases, 14 histone deacetylases, 52 histone methylases, and 26 histone demethylases. Putative roles of these genes in tomato development were addressed by analyzing the expression data of all the *HMs* identified in distinct organs and developmental stages. Differential expression of members of the distinct classes of *HMs* suggests a complex regulatory network of histone modifications and likely transcriptional control during tomato development. By taking advantage of the existing *Solanum pennellii* introgression lines (ILs), in near future it will be possible to integrate the map position of *HMs*, their expression profiles and the phenotypes of ILs in order to select candidate *HM* genes involved in the process of interest to be used in tomato breeding programs.

5.2.2 Small RNAs

Small RNAs and enzymes involved in their biogenesis and function are also important components of the plant epigenetic machinery. Plant sRNAs are produced either by double- or single-strand RNA precursors (dsRNAs or ssRNAs, respectively). Depending on the nature of the precursor RNA, sRNAs are classified into microRNAs (miRNAs) that are produced from stable ssRNA hairpin structures and small interfering RNAs (siRNAs) that are processed from long dsRNAs (Brodersen and Voinnet 2006). Formation of long dsRNAs requires the activity of RNA-dependent RNA polymerases (RDRs), while their processing depends upon the activity of distinct members of Dicer-like (DCL) family. In the case of miRNA precursors, their processing is generally initiated by the DCL1 enzyme. The 19–25 mer imperfect duplexes produced by DCL are unwound and one of the strands binds to Argonaute (AGO) proteins. The AGO-containing complexes (sometimes referred to as "silencing complexes") are then guided by the incorporated sRNAs to target RNA or DNA that are recognized by sequence complementarity (Brodersen and Voinnet 2006). Multiple copies of *DCL*, *AGO* and *RDR* genes are found in plants. For instance, the Arabidopsis genome contains 4 *DCL*, 10 *AGO* and 6 *RDR* genes, whereas a total of 32 and 28 genes (including *DCLs*, *AGOs* and *RDRs*) in rice and maize, respectively, have been identified thus far (Kapoor

et al. 2008; Qian et al. 2011). Functional analyzes of these genes revealed that different sRNA-associated enzymes play multiple roles in regulating growth and development as well as in response to abiotic and biotic stresses.

In tomato, 7 *SlDCL*, 15 *SlAGO*, and 6 *SlRDR* genes have been identified so far (Bai et al. 2012). One recent study conducted by Xian et al. (2013) analyzed in details the localization and expression patterns of all tomato *AGOs*, showing that some *SlAGOs* have unique expression patterns during fruit development. For instance, *SlAGO7* expressed extremely high in −2 dpa (2 days before anthesis) fruits but was downregulated in 8 dpa to red fruits. This observation suggests that *SlAGO7*, which is a homolog of Arabidopsis *AGO7*, might regulate early stages of fruit formation, presumably through regulating synthesis of 21-mer trans-acting siRNAs (tasiRNAs) to maintain proper expression of the *AUXIN RESPONSE FACTOR* (*ARF*) genes (Montgomery et al. 2008). Such hypothesis is supported by the fact that *ARF3* and *ARF4* mediate reproductive organ asymmetry as shown by mutations in both genes that led to strong flower phenotypes in Arabidopsis, likely due to alterations in auxin signaling (Pekker et al. 2005). Interestingly, one of the mutants of the tomato wiry leaf syndrome (*w2*) was identified as having mutations in the *SlAGO7* locus, therefore renamed as *w2-ago7*. *w2-ago7* mutant plants fail to produce tasiRNAs, resulting in misregulation of *SlARF3* and *SlARF4* genes and leading to the formation of shoestring leaves that lack leaf blade expansion (Yifhar et al. 2012). An interesting finding in this study was that, unlike Arabidopsis AGO7, *SlAGO7* is not only dedicated to generate tasiRNAs but also is required for the biogenesis of numerous tomato small RNAs. The source and functions of the sRNAs requiring AGO7 are presently unknown. However, this phenomenon illustrates the complexity of tomato small RNA biogenesis and our limited appreciation of its significance. Notably, *w2-ago7* plants display flowers with narrow organs that are fused at their base, while wild-type tomato flowers have five sepals, five yellow fused petals and stamens, and two to three fused carpels (Yifhar et al. 2012). Although the authors did not analyze reproductive phenotypes in this particular study, it would be of economical importance to evaluate the effect of tomato wiry leaf syndrome and tasiRNAs on flower and fruit development.

As expected, tomato small RNA population is vast and complex and, although a subset of sRNAs is conserved across different families, several sRNAs are family and species-specific (Moxon et al. 2008; Mohorianu et al. 2011). The most conserved class of tomato sRNAs is the miRNA class, but even miRNAs are not well conserved. Moxon et al. (2008) cloned quite a few novel miRNAs that seems to be tomato-specific. However, the authors failed to validate most predicted targets for these novel miRNAs. One possible explanation is that some of the newly identified sRNAs were mistakenly classified as miRNAs. Many putative nonconserved miRNAs, which are not supported by biogenesis data (demonstration of DCL1 dependency or cloning of perfect miRNA* sequences, which represent the opposite strand of the mature miRNA forming the imperfect small RNA duplex), could be siRNAs rather than miRNAs. In fact, current computational approaches to predict non-conserved miRNAs and targets from RNA-seq data produce a considerable quantity of false positive and an unknown amount of false negative results, and thus

the need for better prediction algorithms is evident (Moxon et al. 2008; Hamzeiy et al. 2014).

Transposon-specific sRNAs are usually abundant in small RNA libraries. A particular class of transposons, miniature inverted-repeat transposable elements (MITEs), has been shown to be able of generating sRNAs and regulating gene expression in a genome-wide fashion (Lu et al. 2012). Moreover, MITE-derived sRNAs may represent the evolutionary link between miRNAs and siRNAs in humans and plants (Piriyapongsa et al. 2007; Zanca et al. 2010; Ortiz-Morea et al. 2013). In the *Solanaceae*, including tomato, a number of MITE families were identified and some are capable of affecting gene function and regulation potentially through physical genome changes and by generating small RNAs that are primarily 24-mer in length (Kuang et al. 2009). In *Solanaceae* species, Kuang et al. (2009) showed that these MITE-associated 24-mer sRNAs are generated by RDR2, DCL3, and possibly DCL4. This study and others proposed that the amplification and diversification of MITEs and other transposable elements (TEs) in plant genomes may contribute to evolution of networks of coordinately regulated genes via insertion and subsequent selection of homologous elements in many protein-coding genes. These homologous mobile elements may became target sites for co-regulation by silencing complexes loaded with target-specific MITEs and other TE-associated small RNAs.

By evaluating the accumulation patterns of sRNA populations during tomato fruit development, it was possible to determine that there are various genomic regions that give rise to differentially expressed sRNAs during this process and only a small fraction of these sRNAs are miRNAs (Mohorianu et al. 2011). Furthermore, it was also found that, in contrast to Arabidopsis, most tomato sRNAs that are not strand biased (e.g., heterochromatin siRNAs) have perfect matches with protein-coding genes or regions annotated as protein-coding genes (Mohorianu et al. 2011). Along with data from tomato genome and methylomes, sRNA profiles in fruits point out a scenario in which several ripening-related genes or loci may be co-opted for using sRNA-based regulation (The Tomato Genome Consortium 2012; Zhong et al. 2013). One such example are three loci that show homology to the ethylene-responsive factors, *EIN3* and *EIN4*. sRNAs matching these loci were mainly 22-mer and showed no strand bias, suggesting that they were produced by DCL2 from RDR-generated dsRNAs (Mohorianu et al. 2011). Although it is currently unknown how sRNAs are produced from these loci, it is possible that they regulate their genomic region of origin in *cis* or even other mRNAs in *trans*, thus contributing to complex regulatory networks during fruit development and ripening. Nonetheless, the final proof that ripening-associated genes are either sources of these sRNAs or their targets can only come from experiments using *DCL*-deficient tomato mutants.

Similarly to other species, several families of conserved miRNAs and targets were identified in tomato by using bioinformatic and cloning techniques (Moxon et al. 2008; Mohorianu et al. 2011; Zhang et al. 2008; Karlova et al. 2013). Some miRNA families showed differential accumulation during fruit development, suggesting a particular role in this developmental process in tomato. For instance, miR159, miR162 and miR165/166 were abundantly expressed during early fruit

development and the expression of miR156, miR164 and miR396 was shown to increase during ripening (Mohorianu et al. 2011). My research group has recently generated transgenic tomato plants ectopically expressing miR156 and miR164 (Ferreira e Silva et al. 2014). Both miRNAs seem to affect early stages of flower and fruit development, as their overexpression in transgenic plants led to disorganization of floral organs and therefore to the formation of fruits with odd shape and less seeds. By using degradome-coupled to deep sequencing analysis, Karlova et al. (2013) identified known ripening regulators, such as *CNR* and *APETALA2a* (*SlAP2a*), with developmentally regulated degradation patterns. The levels of the intact messenger of both *CNR* and *SlAP2a* seem to be actively modulated during ripening, by miR156/157 and miR172, respectively. microRNA modulation of these two central regulators of tomato ripening adds another layer of complexity to the regulatory networks taking place during this developmental process. According to our data and others, the function of miR156/157 in fruit ripening is still unclear as fruits of miR156/157-overexpressing plants still ripe normally (Zhang et al. 2011; Ferreira e Silva et al. 2014). However, one can speculate that the main function of miR156/157 and likely miR172 in wild-type plants is to fine-tune the expression of *CNR* and *SlAP2a* to appropriate levels in particular stages of fruit ripening. Along with DNA methylation levels, miRNA regulation may contribute to the proper balance of gene expression during tomato fruit development and ripening.

Although functional studies are still necessary to precisely determine the roles of conserved and non-conserved miRNAs during fruit development, their functions in tomato leaf development are well documented. By cloning the miR319-insensitive version of *LANCEOLATE* (*LA*) gene from the partially dominant mutant *Lanceolate* (*La*), Ori and coworkers (2007) demonstrated that regulation of *LA* by miR319 defines a flexible window of morphogenetic competence along the developing leaf margin that is required for the elaboration of compound leaves. In another study, Berger et al. (2009) analyzed *goblet* (*gob*) loss-of-function mutants, in which primary leaflets are often fused, and secondary leaflets and marginal serrations are absent. *GOB* encodes a NAC-domain transcription factor that is negatively regulated by miR164. Accordingly, leaf-specific overexpression of the miR164 also led to loss of secondary-leaflet initiation and to smooth leaflet margins in transgenic plants. Along with phenotypic and molecular analyzes of the dominant mutant *Gob*, which contains a miR164-insensitive version of the *GOB* gene, the above mentioned observations indicate that the miR164/*GOB* module is crucial for the proper development of leaflet boundaries in tomato. Considering the discoveries presented thus far, the future surely holds novel and exciting breakthroughs regarding the roles of miRNAs and targets in tomato development. Such knowledge may become crucial for breeding programs aimed at modifying developmental parameters in tomato, such as leaf patterning and ripening.

5.3 How Knowledge on Epigenetics Can Contribute to Tomato Breeding?

The crossing between genetically distinct parents provides the mixing of genomes in the resulting hybrids that is essential for the generation of new, favorable genetic combinations, known as breeding. Together with genetic natural variation, epigenetic regulation may be a genome-wide phenomenon that contributes to increasing the yield in many hybrids commercialized today. For example, epigenetic mechanisms can account, at least in part, for the extreme phenotypes found in hybrids when comparing with their parents. Such phenotypes are sometimes heritable and go beyond the F1 generation. The heritability of these phenotypes indicates they are different from those associated with heterosis or hybrid necrosis (Bomblies and Weigel 2007; Birchler et al. 2010). The expression "transgressive segregation" was coined to describe the phenotypic novelty of these hybrid lineages that transgress the parental range. Many eukaryotes exhibit transgressive segregation, though it is more frequent in plants than animals (Rieseberg et al. 1999).

Shivaprasad et al. (2012) investigated the possibility that stable transgressive phenotypes in the progeny of crosses between cultivated tomato and a wild relative (*Solanum pennellii*) were associated with genome-wide epigenetic modifications. The initial hypothesis was that transgressive segregation in the progeny would be affected by epistatic interactions between small RNAs and their targets from the opposite parent. To support this hypothesis, siRNAs corresponding to *S. pennellii phenylalanine ammonia-lyase* (*PAL*) mRNAs were highly represented in some hybrids relatively to the parents. The presumption was that these siRNAs acted in *trans* (perhaps like tasiRNAs) and led to the observed increase in DNA methylation on *PAL* loci in late generations. As neither siRNA accumulation nor DNA methylation alterations were evident in the F1 progeny but rather in subsequent generations (Shivaprasad et al. 2012), the authors suggested that the epigenetic effects observed in late generations were initiated by interactions occurring during gametogenesis of the F1 progeny and that they were subsequently reinforced by RNA-directed DNA methylation (Fig. 5.3).

In addition to changes in siRNAs and DNA methylation, Shivaprasad et al. (2012) observed that transgressive phenotypes in the progeny can also be mediated by alterations in the expression of specific miRNAs. miR395 was highly expressed in some of the hybrid progeny, suggesting that one of the parents contributes an allele at a trans-regulatory locus that can specifically increase the abundance of the miRNA generated from the miR395 allele contributed by one or both parents. A possible explanation could be this trans-regulatory locus encodes a transcription factor that regulates expression of the miR395 precursor, being present or more efficiently expressed only in one of the parents (Fig. 5.3). This microRNA has been shown to be induced by salt stress in different species (Ding et al. 2009; Jia et al. 2009). Accordingly, there was a positive correlation between elevated accumulation of miR395 in particular tomato progenies and their higher tolerance to salinity stress (Shivaprasad et al. 2012).

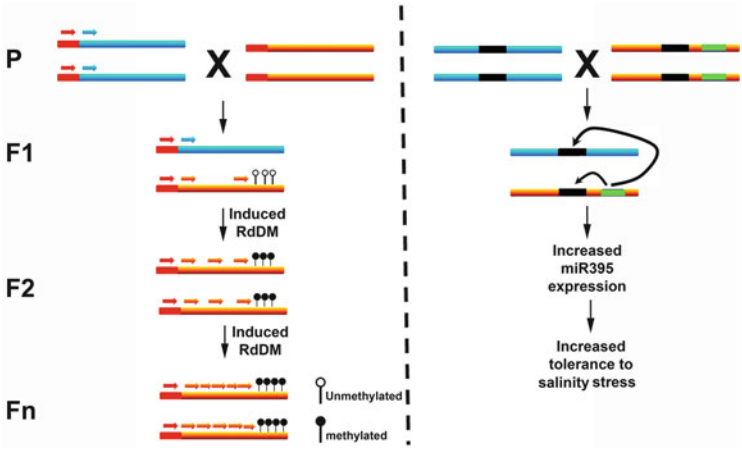

Fig. 5.3 Possible scenarios for epigenetic-based transgressive segregation. *Left panel*: interaction between allelic or non-allelic loci that share only limited sequence identity (*red box*) in F1 can lead to generation and spreading of siRNAs (*small arrows*), perhaps through a phenomenon called transitivity. As a result, this sRNA production may direct gradual small RNA amplification and RNA-dependent DNA methylation (RdDM) over several generations. *Right panel*: introduction of an allele at a trans-regulatory locus (*light green box*) in F1 leads to the enhancing of transcription of *MIR395* locus (*black box*) and possibly increases salt tolerance in particular hybrids. *P* parents

This study in tomato provides some of the first concrete evidence for epigenetic phenomena generating entirely new allelic states not easily explained by Mendelian laws. However, these findings are just a flavor of what kind of genetic and epigenetic variations we may achieve by combining the genomes of cultivated tomato and wild relatives, creating not only the classical ILs but also "epigenetic inbred lines" or epi-ILs. Based on the wide variety of close wild relatives and easy crossing, tomato will probably became a model for studying epigenetic basis of transgressive segregation, allowing for its more efficient utilization in plant breeding.

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Chapter 6
Epigenetic Advances on Somatic Embryogenesis of Agronomical and Important Crops

Geovanny I. Nic-Can and Clelia De la Peña

Abstract Under *in vitro* conditions, differentiated plant cells can be induced to generate organs, shoots, or somatic embryos, which can regenerate a new functional plant. Somatic embryogenesis (SE) has been relevant for clonal propagation for a wide range of important agronomical and economical crops. In addition, SE provides an interesting model to study epigenetic changes during plant development. For instance, during cellular differentiation, sophisticated epigenetics mechanisms, such as DNA methylation, histone modifications and microRNAs can modulate the chromatin structure and change the expression of several genes. In this chapter, we describe the epigenetics events that modulate the embryogenic response in agronomical and important crops. Therefore, the knowledge about epigenetic mechanisms during the SE process could help to increase the embryogenic capacity of different plants improving new strategies to increase agronomical traits of crops.

Keywords Epigenetics • DNA methylation • Histone modifications • miRNAs • Somatic embryogenesis

6.1 Introduction

Plants are very important in our daily life in many aspects, such as providing oxygen, fibers, medicines and food. Now more than ever, plants as food suppliers are becoming less accessible due to the increasing human population. This fact has allowed to establish biotechnological strategies that provide a better understanding

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in plant biology in order to have more available food. For instance, plant tissue cell cultures (PTC) have important applications in plant massive propagation, germplasm conservation and biodiversity, and induction of genetic and epigenetic variation (Miguel and Marum 2011; Smulders and de Klerk 2011; Loyola-Vargas and Ochoa-Alejo 2012).

Plants *in vitro* propagation, such as somatic embryogenesis (SE), has been possible because plant cells have the capacity to regenerate a whole organism from differentiated somatic cells (Zimmerman 1993; Ikeuchi et al. 2013). Although the molecular events by which somatic cells become an embryo are largely unknown, advances in the genetics and genome-wide transcripts data begin to unravel the complex network of early molecular mechanisms of plant cell totipotency (Tsuwamoto et al. 2010; Chupeau et al. 2013; Florentin et al. 2013).

Plant cell totipotency has been described as the onset of a new way of development in which cells can change their cellular program. This is possible due to global chromatin reorganization, which modify gene expression (Verdeil et al. 2007; Zeng et al. 2007). Chromatin organization is carried out by epigenetic modifications inside the cell, which can be heritable through successive cell division without changing DNA sequence (Grant-Downton and Dickinson 2005; Bird 2007). Epigenetics is mediated by transient changes in DNA methylation, posttranslational modifications (PTMs) of the core nucleosome histones and small and microRNAs (miRNAs), which altogether determine whether the chromatin state is actively or silently transcribed (Feng et al. 2010; Bannister and Kouzarides 2011).

DNA methylation as well as histone modifications, such as dimethylation of histone H3 at lysine 9 (H3K9me2) or H3K27me3 are engaged in gene silencing (Jackson et al. 2004; Zhang et al. 2006, 2007), whereas H3K4me2/3 and H3K36me2/3 are associated with active transcription (Xu et al. 2008; Zhang et al. 2009). On the other hand, miRNAs can guide the degradation or repression of the messenger RNA (Mosher and Melnyk 2010). In recently years, these epigenetics mechanisms have emerged as critical factors in the differentiation of plant cells and SE (Nodine and Bartel 2010; Grafi et al. 2011; Bobadilla et al. 2013).

6.2 Genes Involved During Somatic Embryogenesis

In plants, zygotic embryogenesis (ZE) is a biological process that involves the transition from a fertilized egg to a mature embryo generation (Capron et al. 2009). Unlike mammalian cells, plant cells can form an embryo without the conventional fertilization events through of process known as SE (Zimmerman 1993). This complex process is the product of molecular regulation carried out in certain responsive cells that acquires the totipotency to produce embryogenic cells, which give rise to somatic embryos and regenerated functional plants (Fig. 6.1). The development of somatic embryos from an explant starts from a cellular dedifferentiated tissue [also know proembryogenic mass (PM)] then a series of developmental stages generate from this tissue, such as globular (G), heart (H), torpedo (T) and cotyledonary (C) (Fig. 6.1).

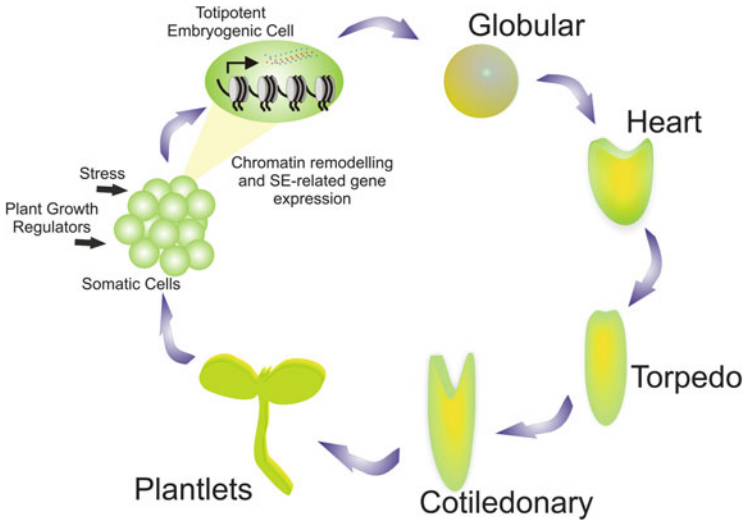

Fig. 6.1 Morphological events during the somatic embryogenesis (SE) process. SE induction requires of certain conditions that exerts a considerable stress in the somatic cells. Under these conditions, responsive cells are able to reprogram the molecular and epigenetic conditions to acquire the totipotent embryogenic cell status. The embryogenic process proceeds through the dedifferentiation and proliferation of the cells in order to generate the first embryogenic stages, globular. Then, different embryogenic stages are developed, such as heart, torpedo and cotyledonary until the maturation of a complete and functional plant

Early phases of the SE are characterized by a complex regulation of gene expression patterns in order to initiate the transition among the different developmental stages of the embryo. The genes more studied in SE are *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1* (*SERK1*) gene, which was found in somatic cells that acquire embryogenic competence in *Daucus carota* (Schmidt et al. 1997), *LEAFY COTYLEDON1-2* (*LEC1* and *LEC2*) genes, which encode transcription factors that has been proposed as key regulators for embryogenic identity in *Arabidopsis thaliana*, and *AGAMOUS-LIKE15* (*AGL15*) that is also a transcription factor specifically expressed in embryogenic cells of *Arabidopsis* (Thakare et al. 2008). Also, it has been investigated *BABY BOOM* (*BBM*), which encode a transcription factor belonging to AP2/ERF family that activates a complex network of developmental pathways related with proliferation and growth (Boutilier et al. 2002) and *WUSCHEL* (*WUS*), which is a homeobox gene critical for stem cell determination of the shoot meristem (Zuo et al. 2002). All these genes represent a spatial and temporal regulation during somatic embryo generation and development. For instance, when *LEC1* and *LEC2* are expressed ectopically, the generation of somatic embryo are promoted on the vegetative tissues of the plant (Lotan et al. 1998; Braybrook et al. 2006). *AGL15*, constitutively expressed, shows an increase in the production of somatic embryos (Thakare et al. 2008). On the other hand, *BBM*, ectopically expressed in both *Brasica napus* and *Arabidopsis,* generates somatic embryos even in absence of plant

hormones (Boutilier et al. 2002). In the case of WUS, it has been reported that this transcription factor promotes vegetative-to-embryogenic transition in *Arabidopsis*, suggesting that *WUS* plays a key role during embryogenesis (Zuo et al. 2002).

6.3 Epigenetic Events in Somatic Embryogenesis

SE induction requires a serial of experimental steps that exerts a considerable stress in the explant such as salt concentration, culture conditions and plant hormones, such as auxin and cytokinin. These stress involve chromatin remodeling at specific sites that lead to gene activation/repression probably involved in the acquisition or maintenance of pluripotency (Avivi et al. 2004; Costas et al. 2011; Florentin et al. 2013). There are reports that indicate that plant hormones, especially the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), promote an increase in the levels of DNA methylation during the formation of embryogenic cells (LoSchiavo et al. 1989; Levanic et al. 2004). In contrast, treatments of embryogenic cultures with 5-azacytidine (5-AzaC, a demethylating drug) disrupts the generation of somatic embryos (Yamamoto et al. 2005; Fraga et al. 2012).

Experimental data has revealed that DNA methylation is involved in the regulation of *WUS* expression and auxin signaling components. It was found that the mutant *METHYLATRANSFERASE1* (*MET1*) leads to an increase in *WUS* expression improving shoot regeneration (Li et al. 2011). This suggests that the decrease in DNA methylation levels could be an important step during the vegetative-embryogenic transition. In addition, recent studies revealed that genome reprogramming through the Polycomb Repressive Complex 2 (PRC2)-mediated H3K27me3 and H3K27 demethylation pathways are an essential step for cell fate transition in plants (Bouyer et al. 2011; Zheng and Chen 2011). For instance, double mutants of PRC2 components such as *CURLY LEAF* (*CLF*), *SWINGER* (*SWN*) or *EMBRYOGENIC FLOWER* (*EMF2*) and *VERNALIZATION2* (*VRN2*) (Chanvivattana et al. 2004; Schubert et al. 2005) cause cell dedifferentiation, callus development and embryogenic structures generation, whereas the loss of *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) function, another essential component of PRC2, generate high amount of somatic embryos (Bouyer et al. 2011). These results suggest that the loss of H3K27me3 could be a key element in the acquisition of cellular totipotency. The loss of function in the PRC2 components mentioned above are correlated with the increase in the expression of some SE-related genes such as *BBM1*, *LEC1*, *LEC2*, or *AGL15* (Zhang et al. 2007, 2008; Aichinger et al. 2010; Berger et al. 2011). In addition, the chromatin remodeling factor PICKLE (PKL), are also involved in the regulation of *LEC1* and *LEC2* expression (Zhang et al. 2012a). For instance, the loss of *PKL* function induces the expression of *LEC* genes promoting embryogenic characteristics (Ogas et al. 1999). However, under normal conditions, PKL protein is present at the promoter region of multiple enriched H3K27me3-loci, such as *LEC1* and *LEC2* (Zhang et al. 2012a). This indicates that PKL facilities the chromatin remodeling by promoting the expression of *PRC2* (Aichinger et al. 2010; Zhang et al. 2012a).

On the other hand, new lines of evidence suggest that the miRNAs are also involved in the repression of early maturation in embryogenesis because of the lacking of *DICER-LIKE1*, a component required for miRNAs biogenesis. These suppress the early embryos development, suggesting that miRNAs could prevent the precocious expression of dedifferentiation-related genes, avoiding the correct pattern of embryogenic formation of plant embryos (Nodine and Bartel 2010; Willmann et al. 2011).

6.4 Epigenetics Studies on Somatic Embryogenesis of Agronomically Important Crops

SE is considered as the most used tool for massive propagation of economically and agronomically important crops (Loyola-Vargas et al. 2008; Yang and Zhang 2010). However, in some crops, the SE efficiency has been compromised by the apparition of phenotypic variations, which in some cases is a major problem to be resolved. A great effort has been focusing on determining the genetic and epigenetic variability among the produced plants in order to establish a method to detect plants with higher response during *in vitro* culture. In order to establish efficient embryogenic cultures, stable and homogeneous, it is necessary to obtain information about the epigenetic mechanisms involved during the dedifferentiation, redifferentiation and morphogenesis; key developmental processes of the SE. Here, we described the epigenetics studies performed during SE in important crops and their contribution to plant development.

6.4.1 Chesnut

Chestnut (*Castanea sativa* M.) is an important agricultural and ecological crop. An important aspect to study this plant is seeds production. Seed quality depends on the degree of polyembryony (the presence of 16 ovules in the ovary) and in this specie the monoembryony is the principal goal for chesnut improvement, in which SE has been used as important tool. SE embryos, induced from ovules and immature embryos, in this species has been characterized (Sauer and Wilhelm 2005); however, the molecular events that control this process are still unknown. Besides SE, epigenetics studies has also been applied to understand bud dormancy of this tree (Santamaría et al. 2009). For instance, it has been found that there are an opposite relationship between DNA methylation and histone H4 acetylation in bud burst. In addition, the transcriptomic data analysis of both dormant apical bud and bud dormancy released showed evidence about some genes epigenetically regulated (Santamaría et al. 2011).

The role of DNA methylation and the embryogenic capacity in this species has been evaluated recently (Viejo et al. 2010). It was found that fertilized ovules became dominant and go through a DNA demethylation before the initiation of zygotic embryo development (Viejo et al. 2010). Therefore, a new embryogenic

competence could be established under *in vitro* conditions during this time and somatic embryos can be obtained from this tissue. In contrast, the unfertilized ovules experience an increase on DNA methylation levels leading to the degeneration and blocking of the SE (Viejo et al. 2010).

6.4.2 Sugarbeet

Epigenetic studies in sugarbeet (*Beta vulgaris* L.) have been few; however, the generated information has helped to understand the relationship between morphogenesis and epigenetics. The morphogenetic state of three lines derived from the same mother plant displays an important change in DNA methylation content and this is correlated with the cell plasticity capacity and cell differentiation state (Causevic et al. 2005). Cell wall formation is an important step to allow cell differentiation, in this fact, the morphogenetic properties of organogenic lines are related with accumulation of peroxidase, incorporation of cell wall-bound compounds and low levels of DNA methylation (Causevic et al. 2005). In contrast, decrease of both peroxidase and cell wall-bound compounds and increase of DNA methylation levels lead to the loss of the organogenic potential. Also, histone modifications have been studied in this plant. For instance, a comparison between an organogenic line with a dedifferentiated line showed that there are higher levels of H3 acetylation levels in the organogenic line than in the dedifferentiated line. In addition, high activity of DNA methyltransferase and histone deacetylase was found in the organogenic line and a decrease of DNA methylation and hyperacetylated histone H3 was found in the dedifferentiated line. Therefore, this indicates that the organogenic capacity of sugarbeet is under epigenetic control (Causevic et al. 2006).

6.4.3 Coffee

Coffee is an important crop that is widely consumed around the world. *Coffea arabica* and *C. canephora* are responsible for 70 and 30 %, respectively, of the world coffee production (Mondengo et al. 2011). In addition, among the perennial species, coffee is one of the few examples where the SE has been used for commercial applications (Etienne et al. 2013). This has lead to the advance of the genetic manipulation and generation of transcriptomic data on *Coffea* spp., (Privat et al. 2011; Mondengo et al. 2011). In addition, it was recently reported the importance of epigenetic mechanism in the SE regulation on this species (Nic-Can et al. 2013) (Nic-Can et al. unpublished data).

In *C. canephora,* the somatic cells from leaf explants present dynamic changes between DNA methylation and PMTs of histone H3, which promote the regulation of SE-related genes (Nic-Can et al. 2013). For instance, it was observed that the decrease on DNA methylation levels and histone repressive marks (H3K9me2 and

H3K27me3) are related with the onset of dedifferentiation and cellular proliferation before the generation of the PM and the G stage. In addition, it was also found that the transition from the H stage to the C stage was accompanied by a gradual increase of DNA methylation, H3K9me2 and H3K27me3.

Furthermore, it has been showed that the overexpression of *LEC1* and *BBM* as well the loss of function of some histone methyltransferases of H3K27me3 can produce embryogenic structures (Lotan et al. 1998; Boutilier et al. 2002; Chanvivattana et al. 2004; Schubert et al. 2005). We reported that the modulation of *LEC1* and *BBM* expression were regulated by this repressive mark (Nic-Can et al. 2013). For instance, a reduction or loss of H3K27me3 was observed in the locus of both transcriptional factors at early events of the SE, but a gradual accumulation of the same repressive mark was detected during the later embryogenic stages. It has been also observed that a decrease on DNA methylation levels at early SE process was related with the increase of *SERK* expression, while its low expression was induced by an increase of DNA methylation during somatic maturation. On the other hand, in *C. canephora* certain levels of DNA methylation are also required to sustain the embryogenic potential because when the DNA methylation is inhibited with 5-AzaC, an important downfall in DNA methylation levels was observed (Nic-Can et al. 2013). The use of this demethylating agent produced a high expression of *SERK*; however, it repressed *LEC1* and *BBM* expression and the SE in this plant was disrupted. Therefore, the proper regulation of two different epigenetic mechanisms could be crucial to acquire the embryogenic competence.

On the other hand, we found that phenolic compounds, such as caffeine, which has been reported to induce DNA hypomethylation (Barrès et al. 2012), hydroxybenzoic acid and trans-cinnamic acid affect DNA methylation and inhibit the SE process in *C. canephora* at different levels (Nic-Can et al. unpublished data). These results were consistent with reports that indicate that phenolic compounds interfere with the SE process (Umehara et al. 2007; Kouakou et al. 2007), and they also inhibit the DNA methyltransferases activity (Causevic et al. 2005; Lee and Zhu 2006). These findings suggest that the phenolic compounds secreted during the SE process can affected the activity of DNA methyltransferases in *C. arabica* and; therefore, disrupt the SE process.

6.4.4 Carrot

The important role of DNA methylation and its relation with cell plasticity during somatic embryos development has also been described in carrot (*Daucus carota*) (LoSchiavo et al. 1989; Yamamoto et al. 2005). It was found that the presence of gradual concentrations of 2,4-D induced an increase on DNA methylation levels and the SE was blocked. However, in the absence of this auxin the SE proceeds. During the PM formation, the DNA methylation slightly decreased, but during the transition from G stage to the generation of plantlets DNA methylation showed a gradual increase (LoSchiavo et al. 1989). However, carrot suspension cells contain

two thermostable inhibitors of low molecular weight, which that affect the activity of DNA methyltransferases, and their addition into the embryogenic cultures induce a dramatic disruption of the SE process. Treatments with 5-AzaC also induce the suppression of the SE at the H stage and downregulate the *LEC1* expression, particularly if this drug is applied at early moments of the SE induction (LoSchiavo et al. 1989; Yamamoto et al. 2005). Recently, Shibukawa et al. (2009) showed that *LEC1* expression in carrot is influencing by DNA methylation changes, since a loss of DNA methylation in *LEC1* promoter at early SE process promoted a high *LEC1* expression, while the reduced level of its expression was induced by an increase on DNA methylation levels.

On the other hand, carrot ARGONAUTE1 (*AGO1*) has specific expression during the earliest embryogenic stages (Takahata 2008). This finding agrees with miRNAs accumulation in order to regulate the somatic embryo development in other species (Wu et al. 2011; Zhang et al. 2012b).

6.4.5 Valencia Sweet Orange

Citrus sinensis L. Osbeck, commonly named valencia sweet orange, is one of the most important woody fruit crops in the world. However, the improvement of this species through conventional genetic breeding is difficult because of its long juvenile time, polyembryony as well as male and female sterility (Pan et al. 2009). Therefore, SE has been applied in this specie to propagate plants, germplasm conservation and genetic improvement (Wu et al. 2008; Singh and Rajam 2009). There are few molecular studies associated with the SE development of this specie among them proteomic analysis (Pan et al. 2009), miRNAs expression (Wu et al. 2011) and global transcription profiles have been carried out during embryo development (Ge et al. 2012). Although studies on DNA and histone methylation patterns in the SE of sweet orange have not been performed, the studies of Marques et al. (2012), who characterized the distribution of DNA methylation as well the mono-, di- and trimethylation of H3K4, H3K9 and H3K27 in the chromosomes of *C. sinensis*, could help to explore the gene regulation through chromatin modification during the begin of SE.

In the case of miRNAs, recently it was shown the important role that they play in the differentiation from somatic cells to somatic embryos in this plant (Wu et al. 2011). It was evaluated ten conserved miRNAs during the SE development and the differential expression was observed through the embryo differentiation process. It was found that miR156, miR168 and miR171 were expressed during the embryogenic calli (EC) induction. Four miRNAs (miR159, 164, 390, and 397) were highly expressed at G stage whereas miR166, 167 and 398 were expressed in the C stage. However, a highly increase of miR390, 397 and 398 were related with non-embryogenic calli (NEC). The same authors determined that some members of the *SQUAMOSA PROMOTER BINDING-LIKE* gene family (*SPL2*, *SPL4*, *SPL5*, *SPL9*) and *SQUAMOSA PROMOTER BINDING* (SPB), which

are involved in the inducing the floral transition earlier and shortening vegetative phase (Lal et al. 2011), showed higher expression in NEC than in EC and were target by miR156. This was consistent with the accumulation of this miRNA during the SE process suggesting that miR156 could be required for the formation of the stages G and C.

6.4.6 Longan

Longan (*Dimocarpus longan* Lour.) is a tropical fruit, which embryo development influences the production and quality of the fruit. Although the advances in the improvement of the fruit are limited by the heterozygous nature of the plant, the SE in this specie has been used as a model to answer some molecular aspects of the embryogenic process (Lai et al. 2010; Lin and Lai 2010). There are no reports associated with the DNA methylation or PTM of histones in this plant; however, the regulatory role of miRNAs during its SE has been recently described (Lin and Lai 2013).

Many miRNAs (29 novel and 643 conserved divided in 169 families) were identified during the SE of longan by Lin and Lai (2013) and some of them regulate more than one target. Some of these miRNAs have been involved in plant metabolism, signal transduction, apoptosis, abiotic stress and developmental (Lin and Lai 2013). It is worth noting that 20 conserved and four novel miRNAs display a specific expression pattern depending on the embryogenic stage. This indicates that different miRNAs can modulate the morphogenesis at different developmental stages (Table 6.1). For instance, during the generation of EC and PM at the beginning of the SE, the miR17 was lower expressed than the miR156c. miR166c* (*, indicates complementary strand of the mature functional miRNA) showed high expression in the same developmental stages, but moderate expression during the G stage. In addition, the transition from the stage H to T was modulated by several miRNAs among them highlights the members of the family miR159 and miR390 and two novel miRNAs, such as miR4a and miR26. Furthermore, different miRNAs families were required for the formation of the C stage and mature embryos (Table 6.1) whereas that miR2089*-1 was expressed elsewhere (Lin and Lai 2013).

Moreover, it has been also identified that miR168* repressed the *AGO1* expression particularly in the C developmental stage of longan embryo, suggesting that a decrease in miRNAs could be necessary for embryo maturation (Lin and Lai 2013). These results imply that several miRNAs families regulate the morphogenesis in each embryogenic stage. However, even several miRNAs are conserved among species and they can modulate differentially the SE among different species.

Table 6.1 Distribution of microRNAs among embryogenic stages of longan

Embryogenic stages				
EC	PM	G	H and T	C and ME
miR17	miR156c	miR166c*	miR4a	miR24
miR156c	miR166c*		miR26	miR156a
miR166c*			miR159a.1	miR167a
			miR159a.2	miR168a*
			miR159b*	miR397a
			miR159c	miR398b.1
			miR159f	miR398b.2
			miR160a	miR808
			miR390a.1	miR5077
			miR390a.1*	

Novel microRNAs identified in longan SE are underline

6.4.7 Rice

Rice (*Oriza sativa*) is one of the most important cereal in the world, which has been considered as a plant model for genomic and epigenetic research (Li et al. 2008; He et al. 2010). For instance, it has been generated a high resolution maps of genomic distributions of DNA methylation and histone modifications as well as the transcriptome of messenger RNA and small RNA of two subspecies of rice (He et al. 2010). In addition, DNA methylation studies on seed genome have revealed that DNA hypomethylation in rice endosperm could be crucial to transposon silencing (through small RNAs) in the embryo (Zemach et al. 2010). Unfortunately, it is unknown how these epigenetics mechanism are involved in the SE process.

There are reports that indicate that miRNAs could be involved in the cell plasticity of rice by modulating the transition of rice embryogenic calli from undifferentiated to differentiated stage (Luo et al. 2006; Chen et al. 2011). These studies have found that miR166, miR397, miR408 and miR528 are highly expressed in undifferentiated embryogenic calli, suggesting that these miRNAs could be required to maintain the cells as meristematic state. This is consistent with reports that indicate that miR166 indirectly regulate *WUS* expression, whereas miR397 target laccase genes, which are involved in lignification of cell wall (Luo et al. 2006). On the other hand, miR156 is consistent with its role in the transition of undifferentiated to dedifferentiated thereby contributing to morphogenesis (Luo et al. 2006).

6.5 Species with Epigenetic Instability and Somaclonal Variation

The use of suspension cells for SE induction involves extensive cells division (generally for the use of 2,4-D to maintain the dedifferentiated state of the cells), which could increase the risk of genetic and epigenetic instability generating

somaclonal variation (SV) in the obtained plantlets (Smulders and de Klerk 2011). SV can modify the phenotype of regenerated plants and their agronomical traits affecting large scale clonal micropropagation (Loyola-Vargas et al. 2008).

6.5.1 Coffee

A recently study showed that the massive propagation of F1 hybrids of *C. arabica* from secondary embryogenesis and embryogenic suspensions (both obtained firstly from embryogenic calli) ensured the high proliferation of somatic embryos with a very low frequency of phenotypic variants (Bobadilla et al. 2013). Despite of low SV, the phenotypic variants such as angustifolia and variegate were the most frequent in the plants generated from SE. However, these phenotypes are also produced among *C. arabica*-seed plants. Molecular analysis through amplified fragment length polymorphism (AFLP) and methylation-sensitive amplified polymorphism (MSAP) were carried out to determine genetic and epigenetic variations in the tissue culture. However, no AFLP polymorphism between SE-plantlets and the mother plant was found, whereas that DNA methylation polymorphism from MSAP analysis was low. This is because SE process did not caused additional changes in the DNA methylation levels, indicating that the plant derived from secondary embryogenesis and embryogenic suspensions maintain high genetic and epigenetic stability. Interestingly, the main change in the phenotypic variant was the loss in the chromosome number indicating that mitotic aberrations play a major role in the SV in coffee.

6.5.2 Oil Palm

Oil palm (*Elaeis guineensis Jaq*) has been large-scale propagated through SE. However, most of the plants obtained in this way present aberrations in their flowers and this phenotype is called "mantled", in which the fruit does not develop properly. The quality of the regenerates obtained depends on the embryogenic calli line used in the micropropagation. The fast growing calli generates a high percentage of mantled palms than the nodular compact calli and this differential response is found in the content of DNA methylation, (Jaligot et al. 2000). Hypomethylation has been related to mantled phenotypes; however, in some cases DNA methylation reduction during tissue culture among regenerated palms does not correlate with abnormalities (Matthes et al. 2001). In addition, DNA hypomethylation in the fast growing calli is not related with the lack of expression of DNA methyltransferases because the expression of *MET1* and *CMT1* show an increase. Therefore, the molecular events that lead to the mantled phenotype are still unknown (Rival et al. 2008).

6.5.3 Cacao

Theobroma cacao L. presents changes in its content of DNA methylation, which has been related with the loss of embryogenic potential. This specie present a high heterogeneity between regenerated individuals from the same progeny, and also it is difficult to obtain plants through cuttings from elite material (Alemanno et al. 2003). These problems increase the interest to study SE in this species; however, cocoa plants obtained via SE present elevated genetic mutations and significant epigenetic variation (determined by single sequence repeat and MSAP, respectively) among tissues from the same plant (Rodríguez-López et al. 2010). Interestingly, it was observed that both genetic and epigenetic variations decreased after 10 weeks of culture and the calli only produce few somatic embryos. Therefore, it was suggested that the cell lineages with high rate of mutations progressively loss totipotency, but those totipotent cells, free of mutations, can predominate and generate late somatic embryos. These late embryos contained few genetic abnormalities as well as an epigenetic profile similar to the mother tissue suggesting a link between stability of DNA methylation and repression of *de novo* mutations.

6.5.4 Grapevine

Grapevines (*Vitis vinifera* L.) fruits are required to wine production (Coutos-Thevenot et al. 1992). Generally, the clones of grapevine is propagated in *vitro* are genetically identical to each other. However, their limited natural variability has been considered as a problem to grapevine improvement (Baránek et al. 2010). Therefore, the application of the SE in this plant was used to evaluate the genetic and phenotypic variations in two species of grapevine, Chardonnay clone 96 and Syrah clone 174 (Schellenbaum et al. 2008). The molecular analysis carried out by AFLP revealed a low genetic variation among the somaclones obtained in comparison to their mother clones. However, the degree of DNA methylation changes determined by MSAP showed a major difference compared to the mother clones suggesting an extensive epigenetic diversification in the somatic embryos of *V. vinifera* particularly by demethylation events induced by the SE process. This indicates that the high variation could be a useful tool for selection of new phenotypes to improve the cultivars.

6.6 Conclusions

Plant somatic cells can retain their plasticity and have the capacity to dedifferentiate and change their developmental fate to reach cell totipotency. Currently, it is accepted that a wide of epigenetic regulation is involved in key developmental processes of the SE (Fig. 6.2). Increase of DNA methylation and high levels of both

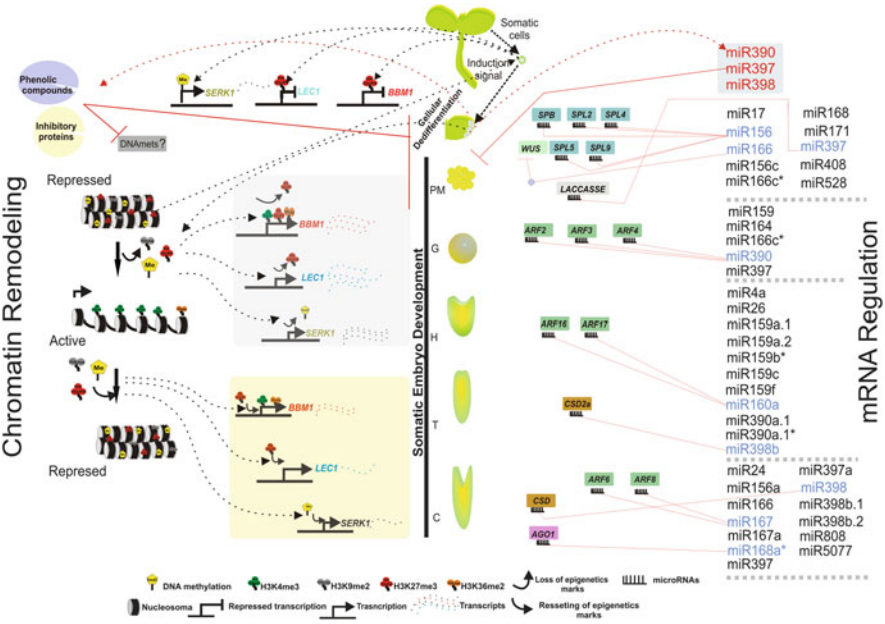

Fig. 6.2 Epigenetic regulation of somatic embryogenesis in plants. Differentiated somatic cells placing in SE induction media experiment a molecular reprogramming, which is related with changes in the chromatin structure. These changes are due to DNA methylation and histone modifications leading to trigger the SE-related gene expression. In order to reach the embryogenic status, the repressed chromatin of the somatic cells loss the epigenetics marks related with transcriptional repression such as DNA methylation, H3K9me2 and H3K27me3 activating gene transcription. These marks are related with the onset of cellular dedifferentiation and the establishment of the proembryogenic mass (PM). DNA demethylation promotes the expression of *SERK*, whereas the removal of H3K27me3 promotes the expression of *LEC1* and *BBM1* even in globular (G) and heart (H) embryogenic stages. In addition, expression of miR156, 166 or 397 contributes to promote the meristematic state of cells at the beginning of the transition from dedifferentiated stage to differentiation stage regulating *SPB*, *SPL* or laccase genes. Moreover, miR390 expression is required to regulate the embryo pattern formation at early SE stages. During the differentiation and embryo maturation of torpedo (T) and cotyledonary (C) stages, the resetting of DNA methylation, H3K9me2 and H3K27me3 lead to the heterochromatin formation downloading the expression of *BBM1*, *LEC1* and *SERK*. At the same time, T and C stages demand an increase of miRNAs that regulate the transcripts levels of the AUXIN RESPONSE FACTORS (*ARFs*) in response to auxin signal, stress (*CSD*) or for the same regulation of miRNAs abundance (*AGO1*). On the other hand, somatic cells can also release inhibitory factors, such as phenolic compounds or proteins that are able to inhibit DNA methyltransferases (DNAmets) activity that induces the SE disruption. The up-regulation of miR390, 397 and 398 leads the dedifferentiated cells disturbing the embryogenic capacity. Red-ended lines represent repression activity. Red circle at the line miR166 indicates an indirectly regulation of *WUSCHEL* (*WUS*). miRNAs highlighted in blue show that they are targeting some messenger RNA (mRNA). miRNAs highlighted in *red* show that they are involved in loss of embryogenic capacity. miRNAs* indicate complementary strand of mature functional miRNA. *ARGONAUTE1* (*AGO1*); *AUXIN RESPONSE FACTOR* (*ARF2-4*, *6*, *8*, *16* and *17*); *BABY BOOM1* (*BBM1*); *CU/ZN-SUPEROXIDE DISMUTASE* (*CSD, CSD2a*); *EMBRYOGENESIS RECEPTOR-LIKE KINASE 1* (*SERK1*); *LEAFY COTYLEDON1* (*LEC1*); *SOMATIC SQUAMOSA PROMOTER BINDING-LIKE* gene family (*SPL2*, *SPL4*, *SPL5*, *SPL9*) and SPB

H3K9me2 and H3K27me3 as well as high expression of specific miRNAs are important during the embryo maturation (Fig. 6.2). However, DNA demethylation, reduction of both H3K9me2 and H3K27me3, and presence of miRNAs such as miR156 and miR166 are important to induce cell dedifferentiation, cellular reprogramming and totipotency acquisition in some species.

Somatic cells can also release inhibitory compounds that affect DNA methyltransferases activity or induce high expression of certain miRNAs impairing the SE process. Coming evidence shows that epigenetic mechanisms are the heart that controls the genome transcription and these determine the embryogenic capacity of somatic cells. Decoding the complex interactions among the different epigenetics mechanisms involved during the early events of plant development would be useful to improve our understanding about gene regulation in plants. Furthermore, these studies would open the opportunity to create new strategies to increase the embryogenic response in different plant species, particularly in those species that show recalcitrancy to SE, but especially to increase the quality and production of important crops.

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Chapter 7
MicroRNA Expression and Regulation During Plant Somatic Embryogenesis

Tzvetanka D. Dinkova and Naholi D. Alejandri-Ramirez

Abstract Small RNAs play important regulatory roles in gene expression during development, stress response and phytohormone signaling. Two major classes of sRNAs are found: microRNAs (miRNAs), and small interferent RNAs (siRNA). These molecules are usually 20–24 bases long, present high complementarity to their mRNAs targets and regulate the transcription or translation processes. In spite of the substantial amount of experimental work with plant small RNAs, little is known about their expression pattern and function during somatic embryogenesis, a process commonly used for genetic transformation, plant propagation and artificial seed production. In this chapter, an overview of the studies involving microRNAs in plant somatic embryogenesis is approached with a particular emphasis in maize embryogenic callus induction, subculture and regeneration.

Keywords Embryogenic callus • Gene regulation • Maize • MicroRNA • Somatic embryogenesis

7.1 Introduction

Somatic embryogenesis was originally described for carrot cells cultured under low auxin levels (Steward 1958). Upon removing the auxin, somatic embryos are able to regenerate plants revealing the cell totipotency in plant cells. Totipotency is the ability to undergo a series of genetic switches to first produce dedifferentiated pro-embryogenic tissues called embryogenic callus (EC) and then, when desired, to regenerate a complete plant from a single somatic cell (Nomura and Komamine 1986).

A genetic reprogramming takes place as a consequence of the auxin presence and depletion and its regulation has been poorly elucidated (Goldberg et al. 1989).

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In addition, the use of 2,4-dichlorophenoxiacetic acid (2,4-D) and/or a prolonged subculture of the EC are prone to induce genetic rearrangements and epigenetic variations in SE (Goldberg et al. 1989; Jiménez and Bangerth 2001).

Recently, genes involved in the non-coding RNA regulatory pathways have shown important changes in their expression patterns during in vitro tissue culture, such as callus and cell suspensions (Tandurzic et al. 2008; Chiu et al. 2010). Hence, the regulatory function of small RNAs involved in gene expression regulation at either transcriptional or post-transcriptional level is expected to have a central role in both, dedifferentiation and plant regeneration (Rodriguez-Enriquez et al. 2011).

In this chapter, first a global overview of somatic embryogenesis, microRNAs and their relationship is described. Then, a particular miRNA expression and their target regulation is approached during maize embryogenic callus induction, long-term subculture and plant regeneration.

7.2 Somatic Embryogenesis

Usually, the first step in somatic embryogenesis consists in callus induction. This process could be defined as the re-organization and re-structuring of somatic cells to enable the formation of highly dividing cell groups termed embryogenic callus. The process includes a number of characteristic events: dedifferentiation of cells, activation of cell division and reprogramming of cell physiology, metabolism and gene expression patterns (Zimmerman 1993).

The second step of somatic embryogenesis consists in plant regeneration. The ability to regenerate plant is called "embryogenic potential" of the callus culture. It is usually highest during the first year of subculture and resides primarily within a subpopulation of the culture termed "proembryogenic masses" (PEMs; Halperln 1966) or "State 1" cell clusters (Nomura and Komamine 1985). Somatic embryo development and differentiation initiates from a single cell and highly resembles its zygotic counterpart suggesting that the genetic program underlying the whole process is conserved in the somatic cell and does not depend on the maternal environment.

The EC has been advantageously used in many plant species for clonal propagation, transformation and genetic improvement (Stasolla and Yeung 2003). In addition the EC subculture and further promotion of complete somatic embryo differentiation offers a unique model to study the physiological and genetic program of the plant embryogenesis process.

Theoretically, any explant could be used to induce plant somatic embryogenesis, but only a few cells from a given explant show embryogenic competence. This could be due to variable cell sensitivity to the auxin, the developmental stage of the explant and the ability to perform accelerated cell division. Normally, the EC or PEMs are formed in the presence of auxin, commonly 2,4-dichlorophenoxiacetic acid (2,4-D) in a concentration between 1 and 10 μM. However in some cases it could be necessary to combine different phytohormone (Duncan et al. 1985; Jiménez and Bangerth 2001).

Once the EC is formed, the culture will continuously proliferate forming PEMs, but the somatic embryos will not fully develop in the presence of auxin. Since the auxin is depleted from the medium after few weeks, EC should be subcultured every 3–4 weeks. The transition from EC and PEMs to somatic embryo is allowed upon the gradual remove of the auxin and in the presence of light. Nevertheless, sometimes the EC is unable to achieve successful differentiation and a healthy mature somatic embryo, compromising the plant regeneration.

7.3 Maize Somatic Embryogenesis

Plant regeneration through maize somatic embryogenesis was reported first by Green and Philips (1975), using immature embryos as the initial explant. Despite the multiple improvements of induction, maintenance and regeneration media over the years, the ability to regenerate plants from maize EC is known to be highly dependent on the genotype and explant (Obert et al. 2009). Of utmost importance is the developmental stage and size of immature embryo, usually between 12 and 18 days after pollination, to generate the EC type II (Armstrong and Green 1985), which is associated with high frequency of plant regeneration over long periods of time. Regarding the genotype, several inbred lines have been reported with good EC induction and plant regeneration (Armstrong et al. 1991; Jakubekova et al. 2011; Shen et al. 2012), while others, such as the B73 line are recalcitrant to EC induction and plant regeneration (Hodges et al. 1986).

The Mexican landrace Tuxpeño has been shown to produce EC type II from 15 days after pollination immature embryos and high plant regeneration frequency for over 2 years of subculture (Garrocho-Villegas et al. 2012). Although the conservation of the regeneration capacity through a long period of callus subculture might be advantageous for purposes such as plant transformation and propagation, it could also cause somaclonal variation at high frequency due to genetic and epigenetic modifications (Larkin and Scowcroft 1988: Kaeppler et al. 2000).

7.4 Gene Regulation During Somatic Embryogenesis

Regarding gene expression, a general characteristic of plant SE is that specific embryonic mRNAs appear and decay during the process probably according to particular signals, such as: phytohormone response, accelerated growth and division, differentiation. The mechanisms underlying gene activation/repression in particular SE stages are still poorly characterized. A dramatic transition from unorganized cell growth in the callus to the full differentiation of a somatic embryo strongly suggests a gene expression reprogramming, presumably at transcriptional level (Dodeman et al. 1998).

7.5 Plant Small RNA Pathway

Small RNAs (sRNAs) are non-coding RNAs with very low molecular weight (Tang 2005). Plant sRNAs can be classified in two main groups: small interfering RNAs (siRNAs) and microRNAs (miRNAs). Their genetic origin is different, as is their final outcome of regulation. However, several components of their biogenesis pathway and function are commonly shared between the two sRNAs classes (Vazquez 2006).

The sRNA generation in plants involves a Dicer-like (DCL) RNase type III enzyme to produce a 19–27 nt long RNA from a double-stranded RNA (dsRNA) precursor. This intermediary has a 2 nt 3′-end overhangs in the duplex and is protected from degradation through 2′-O-methylation in the 3′-end riboses by the methyltransferase HEN1 (Huang et al. 2009). The sRNA duplex is taken by an Argonaute (AGO) enzyme in the company of with other proteins within an RNA-induced silencing complex (RISC), where it exerts a regulatory function through a target mRNA binding (Tang 2005). Other enzymes involved in plant sRNAs biogenesis are the RNA-dependent RNA polymerases (RDRs), dsRNA-binding proteins and chaperones (Chen 2009).

Plant microRNAs (miRNAs) have been intensively studied during the past decade. They play important regulatory roles in several processes, by guiding target mRNA repression, either through degradation or translation inhibition. In the past few years, their role has been also approached in the process of SE (Wu et al. 2011; Shen et al. 2012).

miRNAs are initially transcribed by RNA pol II from MIR genes, to generate a primary precursor (pri-miRNA) with 5′-end cap and 3′-end polyadenylation modifications. Such precursors are characterized by internal stem-loop structures (Fig. 7.1) stabilized by the RNA-binding protein DAWDLE (DDL; Voinnet 2009). The pri-miRNAs are subject of DCL1 processing, HEN1-mediated methylation of the duplex, export to the cytoplasm and active miRNA strand selection by AGO1 or other AGO protein in a particular RISC (Tang 2005; Vaucheret 2008). A miRNA-charged RISC is able to recognize perfect or imperfect complementary sequences' in target mRNAs and mediate their degradation or translational inhibition (Lanet et al. 2009). An interesting miRNA property in plants is their ability to perform cell-to-cell movement, possibly through plasmodesmata, being capable to exert a regulatory role in cells different from where they were synthesized (Dunoyer and Voinnet 2009).

Even though diverse miRNA families have been studied in *Arabidopsis*, maize and rice, there are still many economically or evolutively important plant species where this kind of molecules has not been explored in detail. Probably, even for those widely studied species, new miRNAs await to be discovered since their expression could depend on certain environmental conditions or development.

Plant miRNAs display high complementarity between their sequence and target mRNAs. This has allowed a better prediction of miRNA targets in plants than in animals, where the complementarity between a miRNA and its target mRNA is

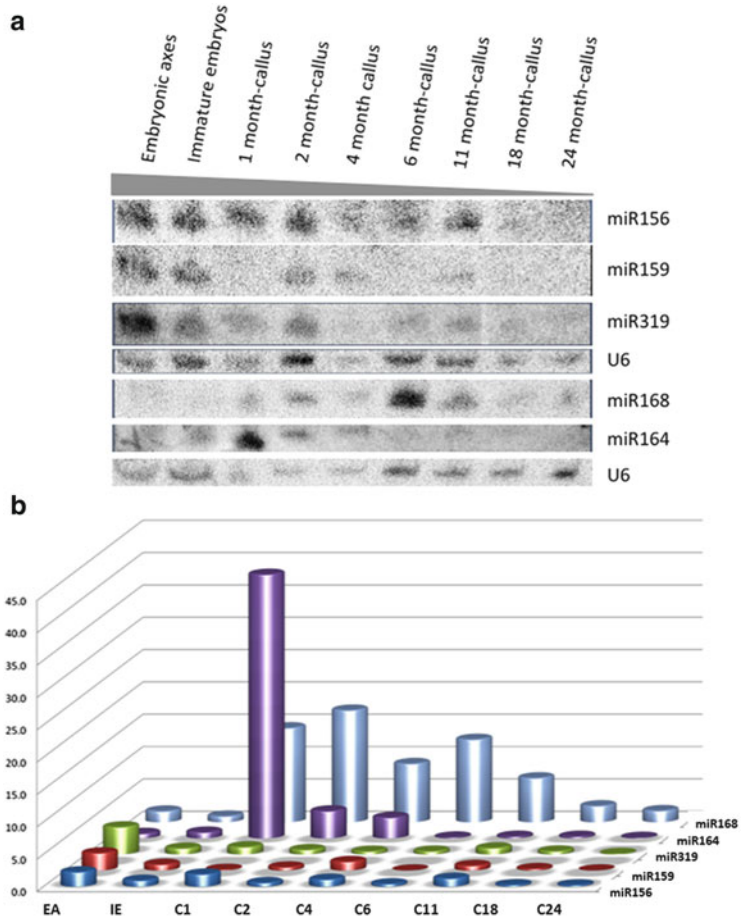

Fig. 7.1 Expression of conserved miRNAs during maize embryogenic callus induction and long-term subculture. Total RNA isolated from embryonic axes of mature seed (EA), immature embryo at 15 days after pollination used as the explant for callus induction (IE) and embryogenic callus subcultured on proliferation medium for 1–24 months (C1–C24) was blotted and probed with end-labeled oligonucleotides complementary to the miRNA. The snRNA U6 was used as control for loading in stripped and re-probed blots. (**a**) Northern blots. (**b**) Densitometry analysis of replicated experiments normalized by U6

generally limited to the 5′ region of the miRNA (German et al. 2008). Accordingly, it was assumed that plant miRNAs function exclusively through target mRNA slicing in the center region of pairing, as opposed to animals where regulation is exerted by translation inhibition and degradation through deadenylation. As a consequence, most of the studies aiming to experimentally prove miRNA targets searched for the presence of sliced cleavage products of mRNAs predicted as target of specific miRNA (German et al. 2008). However, there is genetic and biochemical

evidence that several miRNAs regulate their targets by repressing translation (Bordersen et al. 2008; Lanet et al. 2009).

Specific miRNAs have been implied in developmental processes in plants and animals. Curiously, many of their characterized targets are mRNAs corresponding to transcription factors. miRNA function is crucial in plant differentiation, vegetative to reproductive phase transition, organ morphogenesis, phytohormone stimulation, stress response and the control of their own biogenesis pathway (Mallory and Vaucheret 2006; Chen 2009).

7.6 miRNAs in Somatic Embryogenesis

First studies involving the small RNA biogenesis pathway in SE consisted in the comparison between undifferentiated and differentiated tissues, i.e. before and after 2,4-D depletion (Luo et al. 2006; Takahata 2008). These studies revealed that some miRNAs are specific to the undifferentiated callus (miR398), whereas others are induced upon differentiation (miR156). According to their proved mRNA targets, laccase-domain proteins for miR398 and the Squamosa Binding Protein domain-transcription factors for miR156, it was proposed that miRNAs could participate in regulating the maintenance of highly proliferating undifferentiated cells as opposed to differentiation towards a fully developed somatic embryo (Luo et al. 2006). In addition, hours upon 2,4-D depletion, carrot AGO1 mRNA displays a striking induction profile accompanied with a decrease to almost undetectable levels in fully differentiated somatic embryos (Takahata 2008) suggesting that the miRNA pathway is tightly regulated during the SE process.

During the last 3 years, several works were published on the miRNA presence/regulation during somatic embryogenesis of different plant species: *Larix leptolepsis* (Zhang et al. 2010; Zhang et al. 2012) *Oryza sativa* (Chen et al. 2011), *Citrus sinensis* (Wu et al. 2011), *Pinus taeda* (Li et al. 2012), *Gossipum hirsutum* (Yang et al. 2013), *Dimocarpus longan* (Lin and Lai 2013). Most of these references used a high-throughput sequencing technology to compare the presence of conserved and species-specific miRNAs in the embryogenic callus before and during different stages of somatic embryogenesis. In at least two of them embryogenic callus was also compared with non-embryogenic callus (Yang et al. 2013). Overall, the findings pointed out towards an up-regulation of several miRNAs during the particular stages of somatic embryo differentiation. For instance, several family members of miR156, miR159 and miR167 are highly expressed in the stages from globular to cotiledonary embryo.

The embryogenic callus is characterized by low expression of many miRNAs related to flowering and leaf development, while several miRNAs related to stress are increased with respect to the original explant. However, the particular pattern of miRNA observed upon callus induction, between embryogenic and non-embryogenic callus, as well as during somatic embryogenesis and differentiation is dependent on the plant species. For example, while for rice embryogenic calli miR171,

Table 7.1 Conserved miRNA families analyzed during maize SE

miRNA	Target mRNA	Physiological process
miR156	SPL genes	Flowering time
miR159	MYB transcription factor family	Flowering time
miR319	TCP transcription factor family	Flowering time, leaf development
miR164	NAC transcription factor family	Auxin response, leaf development
miR167	ARF transcription factor family	Auxin response
miR168	AGO1	miRNA regulation

miR390 and miR398 are preferentially expressed before induction of plant differentiation (Luo et al. 2006), in citrus these miRNA species are increased during the differentiation process (Wu et al. 2011). A recent study on *Larix leptolepsis* SE revealed by quantitative RT-PCR that different miRNAs from the same family, i.e. miR156a and miR156b, display differential expression patterns during the differentiation process (Zhang et al. 2012). This suggests that fine regulation by miRNAs differing in few nucleotides on selected mRNA targets is probably taking place during SE.

7.7 miRNA Patterns During Induction and Long-Term Subculture of Maize Embryogenic Callus

The multiple sequencing projects for miRNA species in different plants have revealed that there are a few families conserved in all plants that perform particular and important functions in plant development, stress response and phytohormone signaling (Mallory and Vaucheret 2006). On the other hand, there are also species-specific miRNAs, which may show much lower expression and are stimulated under specific conditions. In our laboratory, the question regarding miRNA function in Somatic Embryogenesis has been approached during maize embryogenic callus induction, subculture end plant regeneration. Particularly, we tested whether the subculturing time length might influence the expression pattern of conserved miRNAs during the subculture and upon the stimulation of plant regeneration.

For this purpose we evaluated the expression of some conserved miRNAs whose known functional relevance is indicated in Table 7.1. As shown in Fig. 7.1, the induced maize embryogenic callus had different miRNA pattern when compared with the original explant (immature embryo). Particularly, miR159 levels diminished, while miR164 and miR168 increased after induction. miR156 and miR319 did not show important changes. During short-term callus subculture (2–11 months) miR156, miR159 and miR319 maintained similar levels to that observed after the dedifferentiation process.

However, miR164 decreased the initial expression peak found in 1 month-callus to gradually reach levels lower than the present in immature embryos. Interestingly, miR168 whose known target is the mRNA of AGO1 had a peak of expression in the

6 month of callus subculture followed by decrease in later subcultures. Since AGO1 is at the center of the miRNA biogenesis pathway and is required for RISC to exert the silencing function of most miRNAs, the fluctuation of miR168 in the embryogenic callus subculture, could be important for the regulation of many miRNA targets. The long-term subcultured callus (11–24 months) are characterized by very low levels for all of the analyzed miRNAs. miR168 is the only one that showed detectable levels in the 24 month-callus.

It is worth to mention that miR168 and miR159 have been reported as highly sensitive to the presence of AGO1 in Arabidopsis (Vaucheret et al. 2006). Apparently, a higher presence of AGO1 is associated with higher levels of miR168 and lower levels of miR159. In regard with miR164, it has been related with auxin response and embryogenesis also for Arabidopsis. Hence, it could be important the regulation exerted by miR164 on NAC transcription factors for maize immature embryo dedifferentiation and the embryogenic callus formation.

7.8 miRNA Target mRNA Levels During Induction and Long-Term Subculture of Maize Embryogenic Callus

Most of the targets of known conserved miRNAs encode transcription factors. The transcript levels of known targets for each miRNA analyzed during maize embryogenic callus induction and subculture was tested to evaluate whether there was an inverse correlation between the miRNA and its target as expected. Figure 7.2 represents the analysis of targets indicated in Table 7.1 by semi-quantitative RT-PCR. During induction and short-term subculture of the embryogenic callus, the inverse correlation is clearly appreciated: i.e. NAM1, a member of the NAC transcription factor family, which is target of miR164, is clearly depleted upon callus induction coincident with the highest expression of miR164. Interestingly, in the 1-month callus a sharp decrease is observed for all analyzed targets, except for PP2A, a miR319 target.

Since the transcript level is directed by transcription versus degradation, further increase in the presence of miRNAs might be due to reactivation in transcription in the 2-month sample. The AGO1 transcript (targeted by miR168) is almost undetectable in the young callus, suggesting that its expression is tightly controlled during the establishment of undifferentiated highly proliferating cells. In long-term embryogenic callus subcultures, most of the analyzed miRNA targets appear refractory to changes in the levels of their corresponding miRNA. For instance, the 11 month sample displays high level of AGO1 in the presence of miR168, or SPL5 and SPL6 (targets of miR156) have similar levels as those in the 6 month-callus, while miR156 is slightly increased between these two time points.

These observations suggest that for some miRNAs, like miR164, target degradation is probably the mechanism of silencing used, whilst for miR168 and

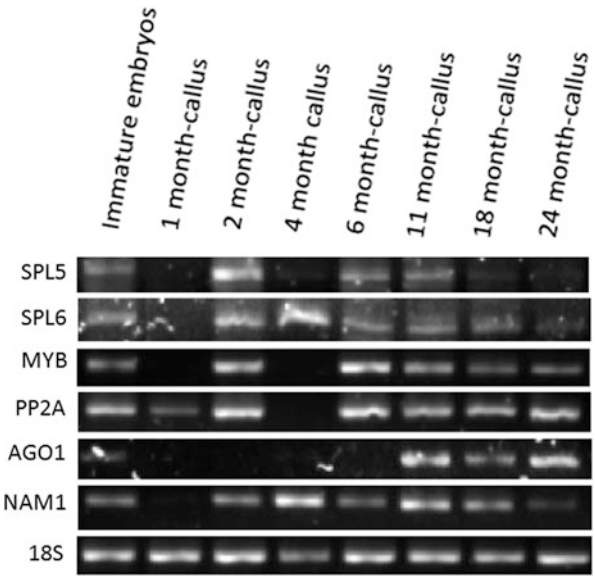

Fig. 7.2 Evaluation of miRNA target mRNA level during maize embryogenic callus induction and long term-subculture. Final point RT-PCR expression analysis was done for experimentally proved target genes of selected miRNAs. The 18S rRNA was used as internal control. The correspondence between miRNA and its target is according to Table 7.1

miR156, translation inhibition could be operating. In the future an important goal would be to differentiate between these two mechanisms in regard with the time of subculture.

7.9 miRNA Expression Upon Differentiation of Short- and Long-Term Subcultured Maize Embryogenic Callus

Since there is a progressive decrease of miRNAs in long-term maize subculture, we hypothesized that the expression of specific miRNAs which are required during the process of somatic embryogenesis and plant regeneration (Zhang et al. 2012; Lin and Lai 2013; Yang et al. 2013) could be affected as the time spent by the embryogenic callus under non-differentiated, proliferative stimulus increases. Therefore, we tested the presence of miR156, miR159, miR164, miR167, miR168 and miR319 after the gradual remove of 2,4-D and light exposure, for short-term subcultured (6 months) and long-term subcultured (18 months) embryogenic callus (Fig. 7.3, panels a and b, respectively). For each subculture period and 2,4-D concentration, differentiated and non-differentiated callus were sampled. Finally, a plantlet fully differentiated from embryogenic callus either short term- or long term-subcultured was also included.

Interestingly, the miRNA expression stimulation response in differentiated long-term subcultured callus was much higher than that observed for the short-term subcultured ones (Fig. 7.3, panel b versus a, first lane), particularly during the first

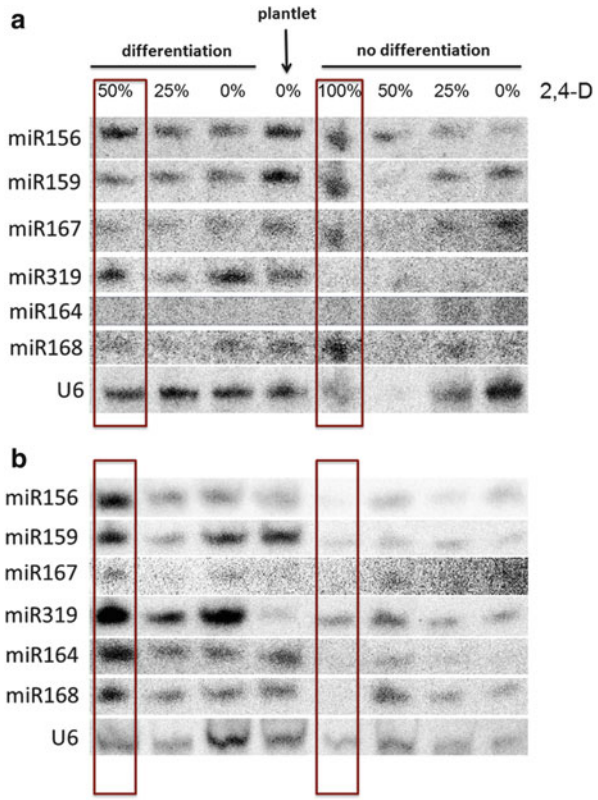

Fig. 7.3 Expression of conserved miRNAs during maize somatic embryogenesis of 6 month-callus (**a**) and 18 month-callus (**b**). After the indicated times of subculture, embryogenic callus was placed under light in maturation medium with half the concentration of 2,4-D used in the proliferation medium (Garrocho-Villegas et al. 2013) and sampled after 1 week in callus with evidence or not of differentiation. Every 3 weeks the differentiating callus was transferred to maturation medium with decreasing 2,4-D concentration until none 2,4-D was added (0 %). For undifferentiated callus, the 100 % of 2,4-D refers to the starting level of miRNAs before differentiation induction according to the time of subculture. The RNA analysis was performed as indicated in Fig. 7.1. *Rectangles* refer to stages were important differences between short-term and long-term subcultured callus were observed

half reduction of 2,4-D concentration (50 %). For the non-differentiated embryogenic callus such increase was not observed for either short-term or long-term subculture. However, a higher concentration of miRNAs was detected at 100 % 2,4-D in the short-term subcultures with respect to the long-term ones as expected. Regarding the number of regenerated plants for each subculture, there were no appreciable differences (not shown). However, contrary to what expected, differentiation was sooner appreciated for the long-term subculture.

Two main conclusions could be drawn from these experiments: (1) specific miRNA expression is required in order to achieve complete somatic embryogenesis and further plant regeneration; (2) The miRNA response of long-term subcultured

embryogenic callus to the differentiation-promoting conditions requires a higher expression of specific miRNAs. Regarding the second conclusion, one cause could be that the tested miRNAs showed much lower levels in long-term subcultured callus (Fig. 7.1) requiring then greater induction to regulate their targets. Indeed, as shown in Fig. 7.2, several of the mRNA targets of the tested miRNAs had increased levels while the subculture period was prolonged. On the other hand, the 6 month-subcultured callus is characterized by high levels of miR168, which negatively regulates AGO1. Since AGO 1 is required for the biogenesis and stabilization of most 21 nt mature miRNA, the expected increase of miRNAs in the differentiation condition might be affected by miR168-mediated repression of AGO1. To prove any of these hypotheses, the levels of AGO1 should be measured and quantitative qRT-PCR could help to determine the actual levels of mature miRNAs and their precursors.

7.10 Concluding Remarks

Research concerned plant miRNAs involved in Somatic Embryogenesis is just arising. This model of plant development offers unique features to explore both, miRNA and siRNA mediated gene silencing. The process could be dissected in different stages of differentiation or embryogenic potential and the amount of tissue is not a bottleneck as in the zygotic counterpart. One caveat could be the heterogeneity of callus tissue present during the subculture. Hence, the reproducibility of observed changes is an important issue. The overall scenario suggests that many miRNAs, most of them conserved in the plant kingdom, that are present in the initial explant tend to decrease along the embryogenic callus induction and subculture. Curiously, miR168, which down-regulates the central enzyme for the RNA-mediated silencing pathway, AGO1, is induced during dedifferentiation and after several subcultures decreases. Such behavior could be required for the establishment of highly proliferating embryogenic callus. During differentiation, a burst of newly synthesized miRNAs is crucial, since their expression pattern highly differs between differentiated and undifferentiated tissues under the same growth conditions. Therefore, the presence of AGO1 would be of utmost relevance during this process. An important perspective will be evaluate the expression of components involved in the small RNA biogenesis pathways in the model of Somatic Embryogenesis. In addition, the regulatory mechanisms guiding selected miRNA expression remain unexplored. The inclusion of siRNA analysis, whose regulatory function differs from that of miRNAs, will also shed a light on the other side of Somatic Embryogenesis, the somaclonal variation and epigenetic changes generated during the extended exposure to the auxin 2,4-D.

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Chapter 8
Can Epigenetics Help Forest Plants to Adapt to Climate Change?

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Abstract Forest trees, as long-lived sessile organisms, have to rapidly and reversibly adapt to different unfavorable environments (seasons, periods of extreme weather, etc.) in order to maintain their growth and dispersion capacities. In this context, epigenetic regulation and its underlying mechanisms seem to have a crucial role as a linker between the environment and the genome, being involved in the regulation of leaf development, floral transition, dormancy, and the responses to several abiotic stresses. Environmental stresses can also induce epigenetic marks that can be inherited as a pre adaption by subsequent generations as a form of maternal effect also called epigenetic memory. This memory, together with the natural epigenetic variation, is responsible for some phenotype variation and adaptation capacity to new environmental niches that recently became to be explored as a very promising way to obtain progenies pre-adapted to different environmental conditions. In this chapter, we provide an overview of the epigenetic mechanisms related to abiotic stress adaption in forest trees, considering their possible role as a new tool for plant biotechnology and ecosystem conservation.

Keywords Forest trees • Epigenetic memory • Environmental stress • Abiotic stress response • Memory stress • Conifers

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8.1 Introduction

Plant development is plastic and strongly influenced by biotic and abiotic factors. Unlike animals, plants cannot avoid unfavourable situations such as climate changes or environmental stress like freezing, drought, or pollutants by moving over long distances to more suitable environments. In consequence, they have to be able to adapt rapidly to new conditions to ensure their survival, being required specific interactions between developmental programmes and signalling pathways triggered from external stimuli that must be coordinated at the level of chromatin organization (Reyes et al. 2002).

By definition, stress is any external factor that exerts a disadvantageous influence on a plant (Grativol et al. 2012). With evidence of climate changes and biological responses to those alterations accumulating rapidly, growing attention has turned to the fate of trees and forests under stressful environmental conditions (Aitken et al. 2008). Adverse environmental cues strongly influence forest distribution and survival, by distorting the growth, development and productivity of plants (Hamanishi and Campbell 2011; Sahu et al. 2013).

In the current context of global change, it becomes mandatory to analyze and understand the adaptive mechanisms of the plant species, especially those that maintain our ecosystems and forests. Some of the physiological changes as a consequence of the influence of environmental factors such as low and high temperature, drought, radiation, or salinity have been described; however, the regulation of these processes at chromatin level and those mechanisms that are implied in long term stress responses and acclimation, are still poorly described in most cases.

Among the mechanisms that mediate these responses, epigenetics is one of the most determinant regulatory systems (Grant-Downton and Dickinson 2005, 2006; Zhang et al. 2010). DNA methylation and histone post-translational modifications (PTMs) have been revealed as key mechanisms for controlling chromatin structure and function (Kouzarides 2007) and regulating cell growth and differentiation (Valledor et al. 2007; De Carvalho et al. 2010; Feng et al. 2010; Bräutigam et al. 2013; Lafon-Placette et al. 2013). Furthermore, these mechanisms are dynamic and can consequently be reverted or adapted to particular environmental situations, constituting a link between genotype and phenotype (Schmitz and Ecker 2012). The study of global changes in DNA methylation or specific histone PTMs has enabled the characterisation and monitoring of several processes such as flower development (Zluvova et al. 2001; Meijon et al. 2010), or stress response (Chinnusamy and Zhu 2009; Correia et al. 2013). The recent characterization of some environmental signals that influence on epigenetic marks to control, i.e. flowering, and on the resultant changes in phenotype as a consequence of gene expression, had raised a significant interest in stress-responsive epigenetic mechanisms. This knowledge will provide important information about how natural populations will survive in the current climate change context (Madlung and Comai 2004).

Epigenetic regulation of stress response is a fascinating field itself, but it is closely linked to another two processes: the epigenetic natural variation and the epigenetic memory. Environmentally generated epigenetic variation has gained

increasing attention over the last years, as one of the main sources of quick phenotypic variation and evolutionary change. However, the underlying mechanisms in natural populations are poorly understood because of the difficulty of characterizing epigenetics in genetically and environmentally heterogeneous *populations*. In this chapter, we provide an introduction to these mechanisms describing recent results mainly on forest species, aiming not only to describe the mechanistic interpretation of epigenetics, but also its implications as a potential new tool for plant breeding and conservation as a way to select or induce new epi-varieties adapted to changing ecosystems.

8.2 Epigenetic Regulation in Plant Environmental Responses

8.2.1 Epigenetic Regulation of Development

Trees, during their long lifespan, must adjust their growth and development to predominant environmental conditions to increase their life expectancy and fitness. The regulation of key stages of plant development (dormancy, flowering, and organ maturation and development) are regulated epigenetically (Grant-Downton and Dickinson 2005, 2006; Valledor et al. 2007; Bräutigam et al. 2013).

8.2.1.1 Epigenetic Regulation of Leaf Development

Tree aging and maturation are characterized by different growth patterns in terms of organ differentiation (i.e. heteroblasty), cell differentiation, and the gain of physiological competence. The role of DNA methylation and histone post translational modifications during these processes has been extensively studied in some tree species (Fraga et al. 2002; Hasbún et al. 2007; Valledor et al. 2007; Monteuuis et al. 2009). In *Pinus radiata* it has been shown that plant ageing and phase change courses along with an increase in DNA methylation level of apical buds (Fraga et al. 2002). Similar effect occurs during needle development, in which primordial needles showed lower DNA methylation level compared to mature needles. At histone level the younger tissues show higher abundance of marks related to euchromatin-gene expression such as AcH3 or H4K4me3, these marks are progressively lost during development, and are replaced by repressive marks such as H4K9me3 (Valledor et al. 2010) (Fig. 8.1). In Acacia, an heteroblastic species, different leaf morphologies had been related to different DNA methylation levels (Monteuuis et al. 2009): microshoots with juvenile or mature morphology were observed, being the degree of DNA methylation higher in juvenile buds. Epigenetic regulation is not only related to organ shape or growth, but also to the regulation of the primary and secondary metabolism, being some genes related to organ function i.e. photosynthesis, secondary metabolism regulated by these mechanisms (Charron et al. 2009).

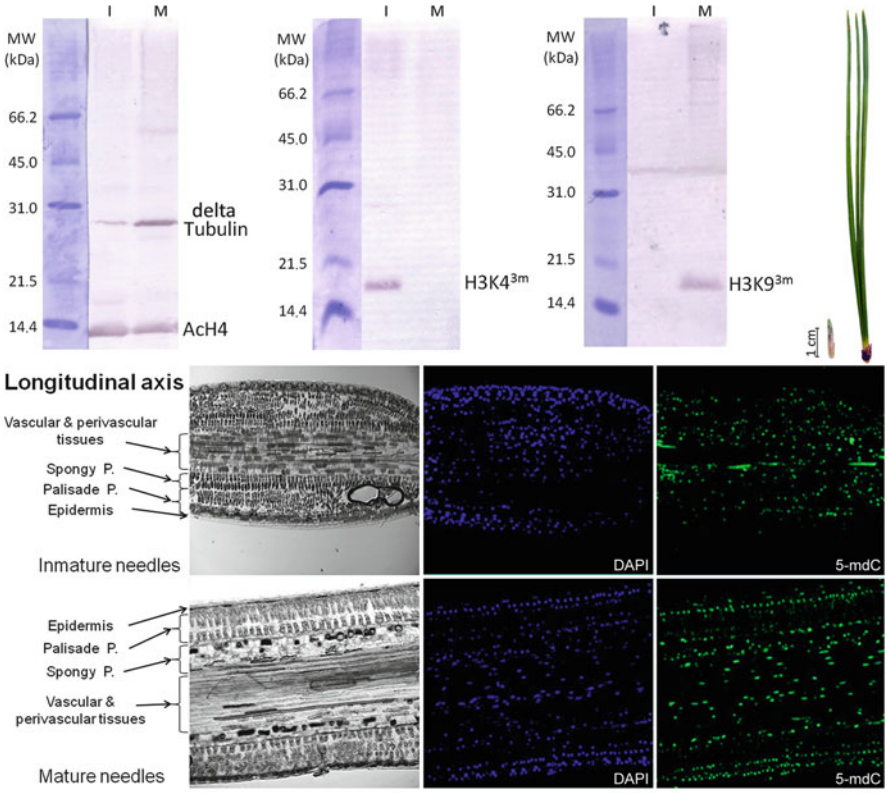

Fig. 8.1 Changes in main epigenetic marks in mature and inmmature needle fascicles (*top left*). Blots showing the identification and quantification of AcH4, H3K4^{3m} and H3K9^{3m} by Western Blotting on protein extracts from immature (I) and mature (M) scions. Immature needles show permissive histone marks (AcH4, H3K4me3), which are lost during development. Mature needles are characterized by histone repressive marks such as H3K9me3. *Lower panel* show the different layout of 5-mdC in immature and mature needles. Needles structure was characterized by DIC (*left*), nuclei are shown in *blue* (DAPI stain) and 5-mdC is shown in *green*. Immature needles show 5-mdC only in its vascular and perivascular tissues while this mark is increased to almost all tissue layers in mature needles

Despite the availability of genome-wide mapping of DNA methylation and histone PTMs in Arabidopsis, Oryza, and Populus (Zhang et al. 2006, 2007; Li et al. 2008; Lafon-Placette et al. 2013) and the common consensus of the permissive (H4ac, H3K4me3) and repressive (H3K9me3 and H3K27me3) effects of histone PTMs at the transcriptional level (Berger 2007; Kouzarides 2007), in-depth studies of the gene-specific epigenetic mechanisms involved in the control of plant organ differentiation and maturation are still limited with almost any information concerning the specific regulation of enzymes related to primary and secondary metabolism at any developmental stage or environmental situation. One of the few studies regarding physiological implications of epigenetics was conducted by Charron et al. (2009) and analysed the landscape of H3K9ac, H3K9me3,

H3K27ac, and H3K27me3 during the de-etiolation process in *Arabidopsis* giving an epigenomic overview of this process.

8.2.1.2 Floral Transition

A striking example of how epigenome reacts to environment involves the induction of flowering by exposure to low winter temperatures in *Arabidopsis thaliana* and many other flowering plants. Floral transition is achieved through a complex genetic network and regulated by multiple environmental and endogenous cues. Histone methylation participates in repression of expression of an inhibitor of flowering during cold. In annual species such as *Arabidopsis* this histone methylation is stably inherited through mitosis after return from cold to warm temperatures allowing the plant to flower continuously during spring and summer until it senesces. However, in perennial, histone modifications rapidly disappears when temperatures rise, allowing expression of the floral inhibitor to increase and limiting flowering to a short interval. In this case, epigenetic histone modifications control a key adaptive trait, and their pattern changes rapidly during evolution associated with life-history strategy (Turck and Coupland 2014).

Dynamic changes between chromatin states facilitating or inhibiting DNA transcription regulate the expression of floral induction pathways in response to environmental and developmental signals. The regulation of the *FLOWERING LOCUS C* (*FLC*) in *Arabidopsis thaliana* provides a plant model of how chromatin-modifying systems have emerged as important components in the control of transition to flowering. Genetic and molecular studies have revealed three systems of *FLC* regulation: vernalization, the autonomous pathway, and *FRIGIDA* (*FRI*). All these involve changes in the state of *FLC* chromatin by DNA methylation and/or histone modification (Dennis and Peacock 2007; Farrona et al. 2008). The results of Meijon et al. (2010) showed that epigenetic mechanisms such as DNA methylation (Fig. 8.2) and histone H4 acetylation have opposite and particular dynamics during the transition from vegetative to reproductive development in the apical shoots of azalea (*Rhododendron* sp). Global levels of DNA methylation and histone H4 acetylation as well as immunodetection of 5-mdC and acetylated H4, in addition to a morphological study have permitted the delimitation of four basic phases in the development of the azalea bud and allowed the identification of a stage of epigenetic reprogramming which showed a sharp decrease of whole DNA methylation (Fig. 8.2). DNA methylation and histone modification have been revealed as hallmarks that establish the functional status of chromatin domains (Tessadori et al. 2004) and confer the flexibility of transcriptional regulation necessary for plant development and adaptive responses to the environment (Vaillant and Paszkowski 2007).

8.2.1.3 Dormancy

Bud dormancy is the phenological event that best describes the seasonal adaptation capacity of perennial plants, being bud formation and growth cessation the main

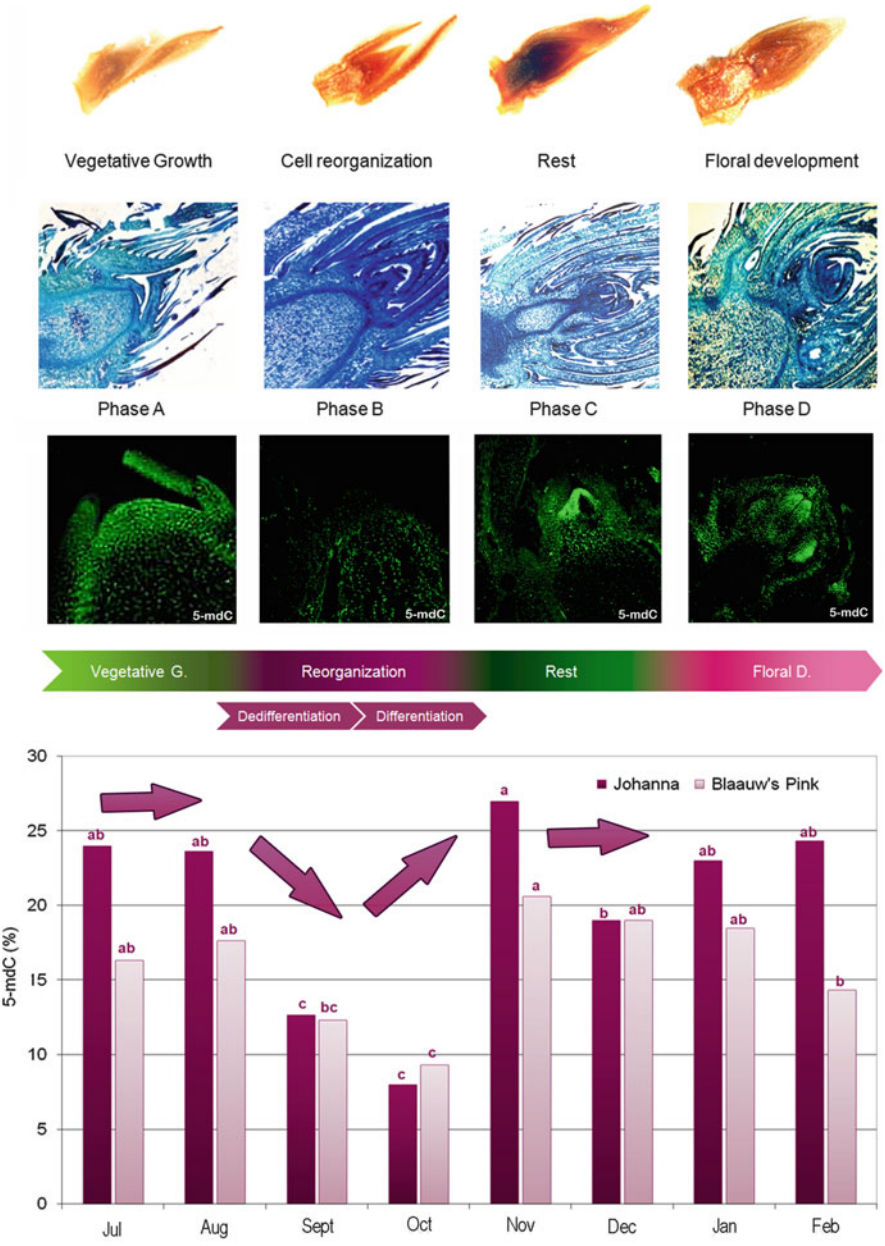

Fig. 8.2 Adapted from Meijon et al. (2010). Immunolocalization and global profile of DNA methylation (5-mdC %) from July to February in buds of Blaauw's Pink and Johanna cultivars of azalea and relationship with histological study of floral bud development. Within each cultivar, *different letters* indicate significant differences between dates (Two-way ANOVA; $p \leq 0.001$; $n = 4$)

variables that characterized the ecological adaptability, the distribution and the reproductive success (Chuine and Beaubien 2001). As to bud dormancy imposition and release, they are two complex and gradual processes induced, imposed and maintained by exogenous as well as exogenous factors such as temperature, nutritional state, photoperiod conditions, phytohormones and others (Arora et al. 2003). Among them, hormonal and environmental signals are important inducing factors of dormancy imposition exerting their effect by the activation/inactivation of specific gene expression programs for para-, eco- and/or endodormancy (Lang et al. 1987). Epigenetics is also playing a key role in these processes, serving as a link between environment, physiological status, and gene regulation. In Arabidopsis, the WD40 family protein HOS15 confers cold tolerance mediated by deacetylation of histone H4 and, therefore, inactivation of the transcription of downstream genes like *RD29A* (Zhu et al. 2008). In *Castanea sativa* trees cold tolerance is acquired at the end of the growth season, when temperature drops and coinciding with buds development and the induction of the dormancy. There is growing evidence that chromatin remodeling is involved in dormancy progression (Horvath et al. 2003; Druart et al. 2007; Mazzitelli et al. 2007; Ruttink et al. 2007). For example, histone H4 acetylation increases during dormancy break in potato tubers, while DNA methylation decreases (David Law and Suttle 2004). In *C. sativa*, a relationship between global DNA methylation levels, acetylated H4 histone and bud dormancy have been demonstrated (Santamaria et al. 2009). Moreover, increased and decreased methylation levels have been observed in bud set and burst, respectively. Meanwhile, intermediate buds with paradormancy were characterized by reduced fluctuations in DNA methylation, especially during bud burst. Furthermore, acetylation levels of the histone H4 from terminal buds were observed to be higher during bud burst than during bud set using immunodetection. Hence, global levels of DNA methylation and histone H4 acetylation show opposite patterns, which, at the same time, coincide with changes in bud dormancy in *C. sativa*. As a result of these epigenetic changes, differences at transcription level were observed. Thus, while in the transcriptome of non-dormant apical buds of *C. sativa* genes in the functional groups for energy, protein with binding function or cofactor requirement, protein synthesis, biogenesis of cellular components, cell cycling and DNA processing were found, the dormancy transcriptome is mainly characterized by stress-related, cell rescue, defense and virulence genes as well as genes related to the interaction with the environment. A comparison of *C. sativa* bud dormancy transcriptome with data for seed dormancy in Arabidopsis suggests that there is a core set of genes that play a principal role in the process (Santamaría et al. 2011), including a one gene conferring an epigenetic mark (Cadman et al. 2006).

8.2.2 Abiotic Stress Response

When plants are exposed to stressful conditions, they resort to various sophisticated mechanisms to respond and acclimatize by prompt and harmonized changes at

transcriptional and post-transcriptional levels of whole gene complexes (Golldack et al. 2011). Recently, there has been considerable interest in whether environmental factors modulate the establishment and maintenance of epigenetic modifications, and could thereby influence gene expression and phenotype (Sahu et al. 2013) and numerous studies have provided new insights into the epigenetic control of stress adaptation in trees. This is an interesting issue to address in trees especially due to the longevity and size of these plants, which enable a greater opportunity for transposon activation and epiallele formation caused by stresses or other factors (Brunner et al. 2004). The epigenetic modifications reported as critical to overcome stress include both DNA and histone methylation, histone acetylation, modifications in chromatin, generation of small RNAs.

In 2003, Kovalchuk et al. reported the first evidence of the involvement of epigenetic changes in the adaptation of Scots pine (*Pinus silvestris*) to chronic radiation exposure. These authors evaluated global genome methylation of control and radiation-exposed pine trees using a method based on cleavage by a methylation-sensitive *Hpa*II restriction endonuclease. They found that genomic DNA of exposed pine trees was considerably hypermethylated and that hypermethylation appeared to be dependent upon the radiation dose absorbed by the trees. Moreover, these authors argued that hypermethylation should be considered a defence strategy that prevents plant genome instability and reshuffling of the hereditary material, enabling survival in an extreme environment.

Lu et al. (2008) reported the cloning of small RNAs from abiotic stressed tissues of *Populus trichocarpa* and the identification of 68 putative miRNA sequences (Lu et al. 2008). The expression of a majority of the novel miRNAs was altered in response to cold, heat, salt, dehydration, and mechanical stresses and the individual miRNAs of a family responded differentially to stress, so that the authors suggested that the members of a family might have different functions. From these results, Lu et al. (2008) revealed possible roles for miRNAs in the regulatory networks associated with the long-term growth of tree species and provided useful information for developing trees with a greater level of stress resistance.

In order to test a possible role of epigenetic mechanisms in the plasticity of hybrid poplars in response to water deficit, six genotypes of *Populus deltoides* × *P. nigra* were submitted to a moderate water deficit and levels of DNA methylation and histone acetylation were measured at the shoot apex, as well as various morphological traits such as the height of the plants, their biomass and the total leaf area to characterize the productivity (Gourcilleau et al. 2010a). The main results included genotypic variation observed for the morphological traits and the epigenetic variables. Gourcilleau et al. (2010a) established correlations among the morphological and epigenetic variables and detected genotypic variation for DNA methylation, suggesting that this variation could mean different strategies among hybrids. Also, they demonstrated a positive correlation between DNA methylation percentage and productivity under well watered conditions.

In a different drought study with *Populus*, Raj et al. (2011) tested the hypothesis that current responses to water deficit were contingent on environmental history and analysed the transcriptome level drought responses and parallel differences in

genome-wide DNA methylation of three economically important hybrid genotypes derived from two different locations. The authors found differences in transcript abundance patterns in response to drought based on differences in geographic origin and a more pronounced response for the genotypes with the longest time since establishment and last common propagation. Besides, the differences in genome-wide DNA methylation followed the transcriptome level trends, suggesting an epigenomic basis for the clone history-dependent transcriptome divergence and providing insights into the interplay between genotype and environment (Raj et al. 2011).

In keeping with this, Lira-Medeiros et al. (2010) aimed to unravel how CpG-methylation variation was distributed among mangrove plants occurring in nearby contrasting natural environment, subjected daily to salinity and nutrient variations, at a riverside (RS) or near a salt marsh (SM), and how this variation was correlated with observed morphological variations. These authors used methyl-sensitive amplified polymorphism (MSAP) to assess genetic and epigenetic (CpG-methylation) variation in *Laguncularia racemosa* genomes. The results showed that SM plants were hypomethylated in comparison to RS and that within-population diversity was significantly greater for epigenetic than genetic data in both locations, but SM also had less epigenetic diversity than RS. Co-Inertia analysis, exploring jointly the genetic and epigenetic data, showed that individuals with similar genetic profiles presented divergent epigenetic profiles that were characteristic of the population in a particular environment, suggesting that CpG-methylation changes may be associated with environmental heterogeneity. This work defended an important role of epigenetic variation in helping individuals to cope with different environments (Lira-Medeiros et al. 2010).

Recently, Rico et al. (2014) assessed the capacity of trees in a natural forest to produce rapid acclimation responses based on epigenetic modifications. They analysed natural populations of *Quercus ilex*, using MSAP technique to assess patterns and levels of methylation in individuals from unstressed forest plots and from plots experimentally exposed to drought for 12 years at levels projected for the coming decades. Their work showed that the percentage of hypermethylated loci increased and the percentage of fully methylated loci decreased in plants exposed to drought. Besides, multivariate analyses exploring the status of methylation at MSAP loci showed clear differentiation depending on stress. As argued by the authors, the observed changes in DNA methylation have highlighted the large capacity of plants to rapidly acclimate to changing environmental conditions, including trees with long life spans (Rico et al. 2014).

With respect to temperature stress, cold and heat are also documented as inducing specific epigenetic modifications in trees (Uthup et al. 2011; Correia et al. 2013). Uthup et al. (2011) reported the identification of DNA methylation patterns and their putative relationship with cold stress in the tree crop *Hevea brasiliensis* by analysing the presence of methylation on regulatory sequences of four major genes involved in the rubber biosynthesis pathway and one general defence-related gene of three high-yielding popular rubber clones grown at two different agroclimatic conditions. The authors found several significant variations

in the methylation pattern at core DNA binding motifs within all the five genes and identified several consistent clone-specific and location-specific methylation patterns. Uthup et al. (2011) defended that the differences in methylation pattern observed indicated the direct impact of stress on the genome and supported the hypothesis of site-specific stress-induced DNA methylation. Moreover, it was assumed that some of the methylation patterns observed might be involved in the stress-responsive mechanism in plants by which they adapt to extreme conditions (Uthup et al. 2011).

Correia et al. (2013) monitored DNA methylation and histone H3 acetylation in *Quercus suber* plants, after subjecting this species to a cumulative temperature increase from 25 to 55 °C under laboratory conditions in order to test whether epigenetic code was related to heat stress tolerance. Global DNA methylation increased at 55 °C, a dynamic methylation-demethylation pattern over stress was found and the abundance index of acetylated H3 decreased from 25 to 45 °C. The authors stated that epigenetic mechanisms, such as DNA methylation and histone H3 acetylation, have opposite and particular dynamics (Fig. 8.3) that could be crucial for the stepwise establishment of this species into such high stress, enabling its acclimation and survival (Correia et al. 2013).

The documented epigenetic studies highlight the large capacity of plants to rapidly acclimate to changing environmental conditions, including trees with long life spans. As already stated by, the possible role of forest tree epigenetics as a new source of adaptive traits in plant breeding, biotechnology, and ecosystem conservation under rapid climate change is an essential tool that should be explored. In addition to abiotic stress, forest trees face other challenges, namely pests and pathogens, which combined with the current environmental change may result in unprecedented effects (Atkinson and Urwin 2012). Although this is a matter out of the scope of this review, this is an important issue because epigenetic variation can also play multiple roles in disease development and need to be better understood to manage plant diseases better.

8.3 Epigenetics and Natural Variation: New Ways to Adapt to Specific Niches

8.3.1 Epigenetic Control of Natural Variation

To date hundreds of genes and functional polymorphisms underlying natural variation in plant development and physiology have been identified. In crop plants, these include genes involved in domestication traits, such as those related to plant architecture, fruit and seed structure and morphology, as well as yield and quality traits improved by subsequent crop breeding. The analysis of natural variation in wild species has begun to elucidate the molecular bases of phenotypic differences related to plant adaptation to distinct natural environments and to determine the

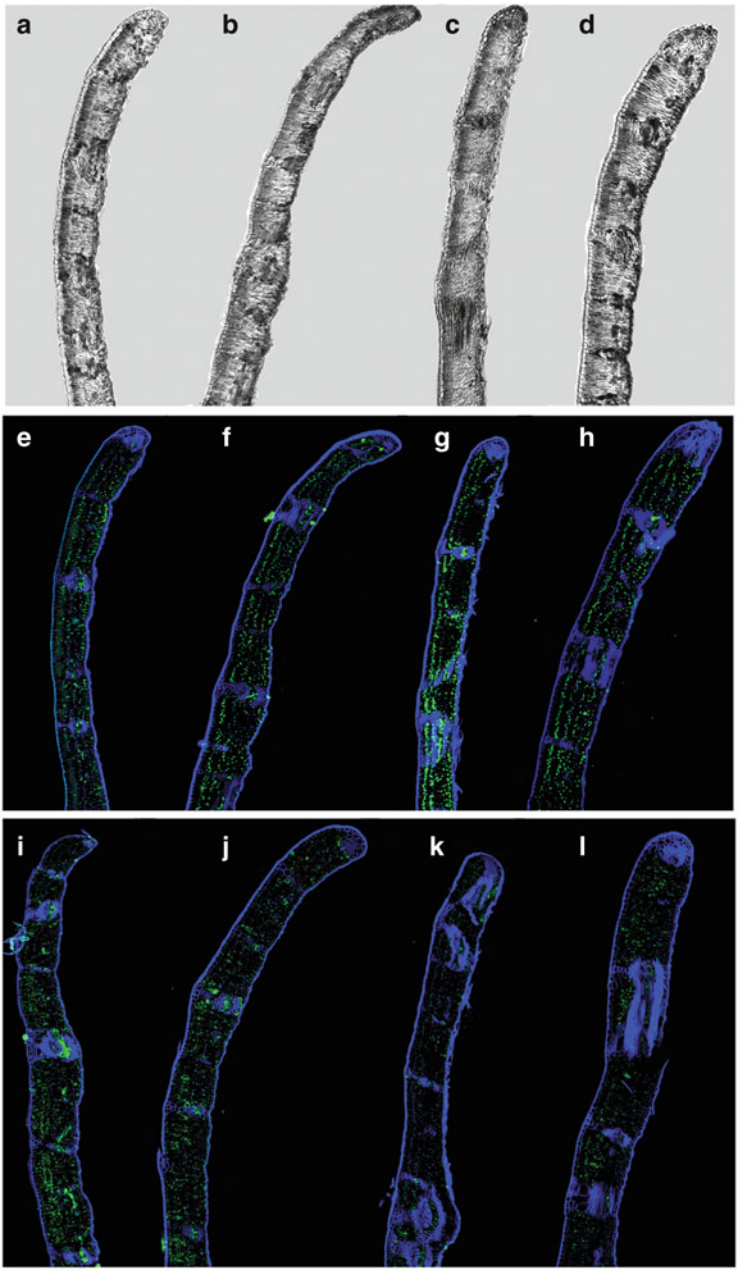

Fig. 8.3 Effect of cultivation temperature over DNA methylation and AcH3 epigenetic marks in Quercus suber leaves. Plants were grown at different temperatures and landscapes of DNA methylation and AcH3 were determined by using confocal microscope and labelled specific antibodies. Differential Interference Contrast (DIC) of a Quercus leaf section excised from plants grown at (**a**) 25 °C; (**b**) 35 °C; (**c**) 45 °C; (**d**) 55 °C. 5-mdC and AcH3 labelling: DAPI (*blue* signals) superposition and 5-mdC (*green* signals) of leaf section at (**e**) 25 °C; (**f**) 35 °C; (**g**) 45 °C; (**h**) 55 °C or AcH3 (*green* signals) of leaf section at (**i**) 25 °C; (**j**) 35 °C; (**k**) 45 °C; (**l**) 55 °C

ecological and evolutionary processes that maintain this variation. The molecular approach to these questions has been applied mainly to the wild annual crucifer *Arabidopsis thaliana*, which has become a model plant for the study of natural variation. Overall, functional polymorphisms appear in all types of species and can be caused by mutational gene alteration, or also by epigenetic alteration. Epigenetics regulation refers to changes in gene expression that occur through changes in DNA methylation, histone modification, small or micro-RNAs, or most inclusively, other mechanisms that alter how DNA sequences are translated into functional gene products. With the discovery that epigenetic modifications to gene expression can be inherited across cell lineages or even across organismal generations, enormous interest has been generated in the potential evolutionary consequences of epigenetic inheritance.

Various environmental signals and stresses can induce persistent changes in epigenetic modifications, thereby creating a flexible memory system for short or prolonged periods of time (Whittle et al. 2009; Yakovlev et al. 2010). In this context of environmental challenges, such epigenetic modifications may be thought of as relatively plastic yet heritable marks that allow for rapid responses and adaptations and, at the same time, might avoid excessive genetic diversification (Boyko and Kovalchuk 2008; Lira-Medeiros et al. 2010).

8.3.1.1 Natural Epigenetic Variation and Adaptation to Specific Niches

Epigenetic variation is likely to contribute to phenotypic plasticity and adaptive capacity of plant species, and may be especially important for long-lived organisms with complex life cycles such as forest trees (Bräutigam et al. 2013). The bibliography describes several examples in which changes in gene expression caused by variation in DNA methylation lead to alterations in plant development. In these examples, the presence of repeated sequences, or transposons, within the promoters of the affected genes are associated with DNA methylation and gene inactivation. Small interfering RNAs expressed from these sequences recruit DNA methylation to the gene. Some of these methylated alleles are unstable giving rise to reverting during mitosis and to progeny in which the methylated state is lost. However, others are stable for many generations and persist through speciation. These examples indicate that although DNA methylation influences gene expression, this is frequently dependent on classical changes to DNA sequence such as transposon insertions. By contrast, forms of histone methylation cause repression of gene expression that is stably inherited through mitosis but that can also be erased over time or during meiosis.

The relation between genetic variation, epigenetic, phenotypic variation and adaptation to environmental factors has been studied in violet (Herrera and Bazaga 2010), orchid (Paun et al. 2010, 2011) and recently in Betula (Wu et al. 2013). These studies have revealed an adaptive phenotypic variation mediated by coordination of genetic and epigenetic mechanisms.

The link between epigenetic natural variation and phenotypic variation that affects floral morphology and flowering time has been described in genes that control the expression of these traits in *Arabidopsis thaliana* at individual and population level (Bastow et al. 2004; Johannes et al. 2009). In *Arabidopsis*, one of the most advanced studies in epigenetic regulation is the expression of *FLOWERING LOCUS C* (*FLC*), *FRIGIDA* (*FRI*) gene controlling flowering time. In the last years, has been described associations between these genes and variation of latitude, temperature and precipitation suggest that that allelic variation in FLC and FRI is involved in climatic adaptation (Mendez-Vigo et al. 2011). In these cases has been shown a correlation between the pattern of DNA methylation and the expression of flowering traits without variation in the nucleotide sequence, which may indicate that the control of the expression of these traits could have an independent epigenetic component of genetic variation. This epigenetic component is mediated by the presence of epialleles in the genome, that is, identical alleles of a gene at the level of nucleotide sequence, but which differ in their epigenetic modifications. Epialleles presence is often associated with differences in the expression of these genes, flowering time regulators.

Another source of natural epigenetic variation arises from polyploidization processes. Polyploidy is a common mode of evolution in flowering. The profound effects of polyploidy on gene expression appear to be caused more by hybridity that have been described that induce epigenetic alterations (Doyle et al. 2008). In addition, new variants caused by epigenetic genomic duplication contribute to potential phenotypic and ecological divergence between polyploids and their parental taxa (Doyle et al. 2008). Thus, comparisons of the level of DNA methylation of three allopolyploid species of orchids from the same parental taxa showed a wide variation in methylation profiles that were correlated with the growth environment (Paun et al. 2010).

Environmental conditions can also induce significant changes in epigenetic marks that play a key role in the adaptation responses of the plant (Mirouze and Paszkowski 2011). Persistent changes in epigenetic modifications can even create a "memory stress" which in some cases is inherited by the offspring not subjected to stress conditions but presumably prepares the next generation to find these new conditions (Richards 2011). Analysis of epigenetic recombinant inbred lines (epiRILs) of Arabidopsis grown in different environmental conditions showed that the variation of DNA methylation status can cause significant heritable variation in traits of ecological interest, such as drought tolerance, or nutrient limitation (Zhang et al. 2013).

Knowledge of the regulatory mechanisms involved in adaptive epigenetic responses may help to guide management of genetic resources and plant breeding, especially in long-lived forest trees where changes in allele frequency are expected to occur very slowly.

8.3.2 Epigenetic and Phenotypic Variation in Forest Species

Trees are long-lived organisms that have to endure the variable environment over their ling lifetime. This long generation times impose limits on natural selection under rapidly changing climate conditions. Consequently, phenotypic plasticity is crucial to the survival of the forest species. Despite to important role that epigenetic code has been shown play in adaptation process and plasticity of plant, natural variation in epigenetic marks and the relation to phenotypic traits is still an underexplored area. However a few studies in higher plants (and even less in tree species) have investigated the extend of natural epigenetic variation and its relationship to phenotypic variation and adaptation potential (Cervera et al. 2002; Bossdorf et al. 2008; Jablonka and Raz 2009; Marfil et al. 2009; Herrera and Bazaga 2010; Lira-Medeiros et al. 2010; Paun et al. 2010). The studies in violet and orchids (Herrera and Bazaga 2010; Paun et al. 2010) showed coordinated genetic-epigenetic adaptive differentiation, indicating the involvement of epigenetic processes in adaptation and evolution by influencing primary phenotypic diversity.

In trees, epigenetic variation in natural populations plays an important role in long-term adaptation to different environments. Analysis of DNA nucleotide sequences and methylation patterns in white mangrove (*Laguncularia racemosa*) detected greater epigenetic than genetic variation within and between populations in contrasting environments (Lira-Medeiros et al. 2010). On the other hand, in *Populus*, Raj et al. (2011) studied same genotypes of poplar obtained from different geographic locations under the same environmental conditions and drought. They could observe that transcriptome-level responses to water withholding are influenced by geographic origin for two of the three genotypes, and are paralleled by differences in total DNA methylation. These results underline the importance of epigenetic mechanisms related to the adaptation of long-lived species like poplar trees to the local environment (Raj et al. 2011). Furthermore in poplars, genotypic variation for both DNA methylation and traits related to biomass productivity was observed in hybrids (*Populus deltoids* × *P. nigra*), and a positive correlation was established among these variables in well-watered conditions (Gourcilleau et al. 2010b). Similarly, in Betula (*Betula ermanii*), Wu et al. (2013) assessed the genetic and epigenetic population structure in selected populations from two contrasting habitats. In this study, populations from the alpine tundra showed significantly greater epigenetic diversity and differentiation than the subalpine forest populations.

However, despite of prominent observations in these studies, the knowledge to understanding of the adaptive capability of long-lived forest trees is still a long way to go.

8.4 Epigenetic Memory as a Way to Improve the Environmental Adaption of the Progenies

As long-lived organisms, forest trees must deal with variable environmental conditions throughout their its lifetimes. Under rapidly changing climate conditions of last centuries, trees species must be highly adaptable, displaying a wide range of phenotypes as a function of their environments. The speed of these adaptive processes point to the need of a quick evolutionary systems that can act alternatively or complementary to microevolution (which requires several generations to achieve adaption). Epigenetics can work alongside with microevolution to provide an additional mechanism for trees, and other organisms, to rapidly adapt to their environment. Epigenetic regulation will by definition translate the genotype into phenotype and thus also potentially induce transgenerational changes in the epigenome in response to environmental conditions. As the climate changes, developing seeds receive environmental cues that allow them to make adjustments to improve their ability to grow in a novel climate. The evolutionary impact of such regulation would rely on an "epigenetic memory" of stressful conditions faced by the ancestor leading to a better adaptation of the progeny. But probably at some point, our climate may change too drastically for even epigenetics to overcome. It is clear that gene conservation and understand the molecular basis of the epigenetic memory can open new alternatives for sustainable use of forest plants (Walter et al. 2013).

It was widely believed that the inheritance of traits was only governed by genetics, since the epigenetic modifications that an organism acquires during their lifespan are largely reset between generations through gametogenesis and imprinting. However, recent discoveries demonstrated the transgenerational epigenetic inheritance. This has emerged as a rapidly growing field, providing evidence suggesting that some epigenetic changes result in persistent phenotypes across generations (Whittle et al. 2009; Hauser et al. 2011; Yakovlev et al. 2012; Lim and Brunet 2013).

Conifers from the temperate and boreal regions, such as Norway spruce and Scots pine, have developed systems to modify their performance (phenotype) to tolerate seasonal changes in climatic conditions. They are able to acclimate from active growth to frost-tolerant winter dormancy and deacclimate back to active growth in a cyclic manner, synchronized with seasonal changes in temperature and day length. Microevolutionary adaptation is vital for development of tolerance, resistance and avoidance of environmental constraints (Alberto et al. 2013). However, there are studies indicating that adaptive phenomena cannot be explained only by traditional Mendelian genetics, but are likely influenced by Non-Genetic Inheritance (NGH) or epigenetic mechanisms (Kvaalen and Johnsen 2008; Rohde and Junttila 2008; Bräutigam et al. 2013; Salinas et al. 2013; Vivas et al. 2013).

Epigenetics have effect on gene activity without change genetic code and can be transferred from one cell generation to other (mitosis) or from one individual to the progeny (meiosis). Such changes may persist over rounds of cell generations and

also across generations (Saze 2008; Youngson and Whitelaw 2008). They may play a role in short-term adaptation to climate change. Epigenetic mechanisms in memory formation have been well described in plants with short generation times (Molinier et al. 2006; Lang-Mladek et al. 2010; Becker and Weigel 2012; Zhong et al. 2013) or other organisms (Levenson and Sweatt 2005), but few were known for more long-lived species such as trees (Yakovlev et al. 2010, 2011). For surprise to classical geneticists, adaptive traits displaying clear clinal variation among Norway spruce populations, are adjusted by an epigenetic mechanism. Yakovlev et al. (2012) present results from genetic research with Norway spruce providing evidence that the species can adjust its performance in adaptive traits by a rapid and likely epigenetic mechanism, through a type of long-term memory from the time of its embryo development.

Epigenetic memory effects on phenotypic plasticity and inheritance of phenotypic variation need further investigation. Epigenetic variation can be partly inherited from one generation to the next while being still sensitive to environmental variation in plants and animals (Harper 2005; Daxinger and Whitelaw 2010; Grossniklaus et al. 2013; Mirbahai and Chipman 2014). Maternal epigenetic effects are known in Arabidopsis (Johannes et al. 2009; Lang-Mladek et al. 2010), but so far their nature has not been studied much in trees (Bräutigam et al. 2013; Vivas et al. 2013). Epigenetic effects can also occur during seed maturation. Temperature differences during embryogenesis caused differences in phenology in *Picea abies* (Kvaalen and Johnsen 2008; Rohde and Junttila 2008). The "memory" effects acting on phenological traits lasted for more than 20 years after germination and affected long-term growth under field conditions (Skrøppa et al. 2007). The existence of different levels of epigenetic response among genotypes (epitypes), as observed in *P. abies* (Kvaalen and Johnsen 2008), might also indicate a genetic component of the epigenetic memory. Thus, distinct epitypes can be produced from the same genotype in *P. abies*, a process not well documented in other tree species so far. In view of rapid climate change, strategies to increase diversity for selection might be of prime importance for survival of species within their current geographic distribution, and therefore this epigenetic "memory" mechanism is likely of evolutionary significance and has obvious practical implications.

The molecular mechanisms involved in adaptive epigenetic memory in Norway spruce are starting to be unraveled (Yakovlev et al. 2010, 2011). The very large genome sizes of conifers indicate the presence of large amounts of non-coding DNA. This seemingly excess DNA might also demand a higher extent of epigenetic regulation. Also, much of this extra DNA might code for, or be involved in, gene regulation by sRNAs and in shaping of the chromatin structure and DNA availability. These are already known to play a direct role in modulating epigenetic modifications and transposon silencing in plants. DNA methylation, histone modification, ncRNAs and TE are the main components of epigenetic modifications. ncRNAs might turn out to be the most important epigenetic memory determinants because they are mobile within and between cells and can act if DNA methylation, histone and other DNA-binding protein modifications are lost during repeated cell divisions.

The epigenetic memory effect has practical implications for forest tree breeding and conservation. About tree breeding, care must be taken that family seed lots generated for progeny testing and for selection of the next generation are produced under similar temperature and day length conditions. This phenomenon is not only of important for breeding but has evolutionary significance for conservation of forest genetic resources. Epigenetic memory can advance or delay phonological processes of high adaptive value (Yakovlev et al. 2012).

8.5 Concluding Remarks and Future Perspectives

Evidences suggesting the role of epigenetics as a key level of regulation of the gene expression acting as a linker between the environment and the genome, and therefore as an important player in adaptation to environmental changes, are clearly increasing. At the same time, our knowledge about the actual epigenetic mechanisms underlying that plasticity is constantly increasing. However, many questions remain about the mechanisms and the roles of epigenetic processes in enabling rapid adaptation of plants to their environment, especially in forest trees as consequence of their inherent difficulties as study species.

All the generated knowledge regarding this topic is and will be potentially applicable and will concomitantly bring with it the development of new biotechnological tools that will be very useful for breeding genetics and the designing of management programs and strategies that will enable the improvement of the efficiency and productivity of our forests.

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