Raúl Alvarez-Venegas
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Epigenetics in Plants of Agronomic Importance: Fundamentals and Applications

Transcriptional Regulation and Chromatin Remodelling in Plants

Second Edition

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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 among domesticated populations) that may diminish the fitness of a plant in the wild but increase it under human exploitation. Thereafter, 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, and delayed senescence. However, it is still 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 appropriate 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 epigenetic variation may modify plant gene regulation and phenotype, and we should concentrate on how the epigenome acts as a potent new source of diversity for agronomically important traits and its potential for exploitation in crop improvement programs.

The word "epigenetics" was originally formulated by Conrad Waddington to incorporate "epi" ("above" or "on top") with the word "genetics." He took the Greek word "epigenesis," a theory of development, and changed it to epigenetics. However,

Waddington did not use a precise definition for epigenetics. It was not until 1994 that Robin Holliday broadly re-defined epigenetics as "the study of the changes in gene expression which occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression." Nonetheless, the most familiar definition of epigenetics refers to changes in gene expression that do not involve changes in the DNA sequence, but that are inherited after cell divisions, even in the absence of the signal or event that initiated the change.

Nowadays, it is widely accepted that 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 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 new edition has arisen from the fact that epigenetics is an important player in the study of gene regulation not only in animals but also in fungi, protists, prokaryotic organisms, and plants. The inception of research in epigenetics came from the desire to understand how it affects plant development and behavior.

This book gives us comprehensive knowledge about the fundaments and applications of epigenetics in plants of agronomic importance. A total of 15 chapters (12 new and updated chapters) describe the importance of epigenetics in agriculture and highlight the applications of this field in crop plants. 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 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 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.

This second edition was built over the contents of the first one and has been expanded to include novel research fields on plant epigenetics. New chapters on the epigenetic regulation of biotic and abiotic stresses in plants, epigenetics of light signaling, RNA epigenetics, epigenetic reprogramming of the germline, and on the function of small RNAs in establishing cell dedifferentiation and further plant regeneration in crops have been included. Novel findings on germinally inherited

epialleles and epigenetics in forest trees have been updated. The expanded content and updated chapters reflect the rapid pace at which new discoveries in plant epigenetics are being made not only in model plants but also in agronomically important plants.

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Chapter 1
Epigenetic Mechanisms of Abiotic Stress Response and Memory in Plants

Iva Mozgova, Pawel Mikulski, Ales Pecinka, and Sara Farrona

Abstract Being sessile organisms, plants are exposed to multiple stimuli without possibility for escape. Therefore, plants have evolved to be able to adapt their developmental and physiological responses to the surrounding environment. Some environmental stresses will rarely occur during the life of the plant, but others, such as seasonal drought or heat, can be recurrent. Therefore, plant responses to these stresses can be transient to provide plants with the required tools to acclimate and survive, whereas others may promote a state that we will refer to as "memory" throughout the chapter, which predisposes the plant for a more efficient stress response upon next encounter of stress. The possibility of transferring this memory to the next generation has been also proposed, which implies a lack of resetting of the priming memory during sexual reproduction. Different epigenetic and chromatin-related modifications such as DNA methylation, histone modifications, and chromatin remodeling have been associated with the memory to both biotic and abiotic stresses. This chapter reviews how and which epigenetic processes are involved in

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remembering a past abiotic stress event and also forgetting it. Contradictory arguments concerning transgenerational memory and its implications in phenotypic variation are critically discussed. In addition, the stability of epigenetic modifications during asexual propagation and its impact on clonally propagated plants is addressed. Finally, we mention possible agricultural implications of the epigenetic mechanisms involved in plant memory and propose future applications for breeding of epigenetically modified crops considering new challenges arising from climate change.

1.1 Introduction

Crop production is deeply affected by the environmental conditions and current models for climate change indicate that future conditions will become even more challenging. Climate trends show that the Earth tends to be less cold with an increase in temperatures in every season, especially for minimal temperatures, in most of the crop producing regions, which is coupled to a major increase in the frequency of temperature extremes (Alexander et al. 2006; Lobell et al. 2011; Lobell and Gourdji 2012). Although more difficult to predict, the numbers of drought periods have shown a tendency to increase over the last 50 years in some parts of the world (i.e., Africa, southern Europe, east and south Asia, and eastern Australia) and will become much more frequent by the end of the twenty-first century, while the wet regions will become even wetter (Skliris et al. 2016). There is, therefore, a complex interconnection between climate change and food security, which is at a risk due to the effects of increasing temperatures, water-cycle changes, and higher CO_2 levels on plant yields. Indeed, a decline in the production and a subsequent price increase of important crops (i.e., wheat—*Triticum aestivum*, maize—*Zea mays*, and barley—*Hordeum vulgare*) has already been linked to global warming (Lobell et al. 2011; Lobell and Gourdji 2012; Moore and Lobell 2015). Thus, understanding the phenotypic variation of plants and how food and feed production can be secured has taken a central position in crop science.

Plants can efficiently respond to abiotic or biotic environmental conditions and modify their development and physiology accordingly. In this review, we focus on the response and memory of abiotic stresses such as extreme temperatures, drought, and salinity. Stress can be considered as any situation that can alter plant fitness and cause a substantial loss in yield. Abiotic stresses are major cause of food scarcity being responsible for estimated 50% loss in staple crops (reviewed in Boyer 1982; Bray et al. 2000). One of the main abiotic stresses that plants face are extreme temperatures, both high and low. Heat will most probably increase in the future affecting many countries, including developing countries where hunger is already an issue (reviewed in Lobell and Gourdji 2012). Increase in temperature is particularly dramatic during plant reproduction and seed filling, having a significant impact on yield (reviewed in Kosina et al. 2007). As part of global warming, heat stress usually comes in combination with water scarcity, which according to predictions will become more acute, and with higher CO_2 and UV radiation (reviewed in Williamson

et al. 2014). On the other hand, floods, which will be more recurrent in other regions of the globe, present also major agronomic constraints especially affecting yield and grazing land and, in more extreme situation, causing plant death due to hypoxia (reviewed in Jackson and Colmer 2005). Soil water content is directly linked to other main stresses including salinity and nutrient availability. Soil salinization has a strong impact on plant growth affecting the photosynthetic rate, absorbance of nutrients, and increasing senescence (reviewed in Hanin et al. 2016). Chilling temperatures also impair plant metabolism, germination, and reproduction, whereas freezing temperatures additionally cause tissue and membranes damage and cell dehydration (reviewed in Xin and Browse 2000).

An intricate network of processes involved in sensing and responding to the environment, which implies massive changes in gene expression and nuclear organization, aids the plant to cope with the stress (reviewed in Probst and Mittelsten Scheid 2015; Asensi-Fabado et al. 2017). However, plants will seldom be affected by individual conditions and, hence, they usually respond to multiple stresses at the same time. However, the challenge of simultaneously applying different stresses and analyzing their overlapping action still limits our understanding of the complexity of plant responses to abiotic stresses. Therefore, for the breeding of new crop varieties better adapted to future more severe climate conditions, multidimensional experimental approaches more closely mimicking on-field conditions will be required (reviewed in Mittler 2006; Ahuja et al. 2010; Qin et al. 2011).

Whereas some stresses occur occasionally, generating a temporal stress response in the plant, many of the abiotic changes occur as daily (e.g., day and night changes) or seasonal fluctuations (e.g., summer and winter seasons in temperate climates or dry and humid seasons in tropical areas). Recurrent stresses can therefore induce a cellular memory that poses or primes the plant for a faster and stronger response upon repeated stress exposure. This stress memory is also known as priming or, in the case of abiotic stress, as acclimation or hardening (reviewed in Bruce et al. 2007). Therefore, the priming of plants implies: (1) the action of a first stress condition that, in addition to inducing a stress response in the plant, may trigger the formation of a molecular memory, (2) the end of this first stress condition, (3) a lapse of time during which the memory can perdure in the absence of the stress that generated it, and (4) the occurrence of a second stress that will activate the recovery of the stress memory to induce a new enhanced plant response. Furthermore, an additional (5) step entails resetting the memory or maintaining it for transfer to the next generation(s) through a process usually known as inter-/transgenerational memory (Fig. 1.1 and reviewed in Bruce et al. 2007; Pecinka and Mittelsten Scheid 2012; Chen and Arora 2013; Kinoshita and Seki 2014; Avramova 2015; Crisp et al. 2016; Hilker et al. 2016; Bäurle 2017; He and Li 2018). The second stress that retrieves the memory can be of the same nature as the first one, but it seems that a different abiotic stress, or even a biotic one, can activate the priming memory, indicating a complex crosstalk between different types of stresses (reviewed in Hilker et al. 2016; Asensi-Fabado et al. 2017; Lämke and Bäurle 2017; Friedrich et al. 2018). Eventually, primed plants will be readier to respond to a second stress showing an

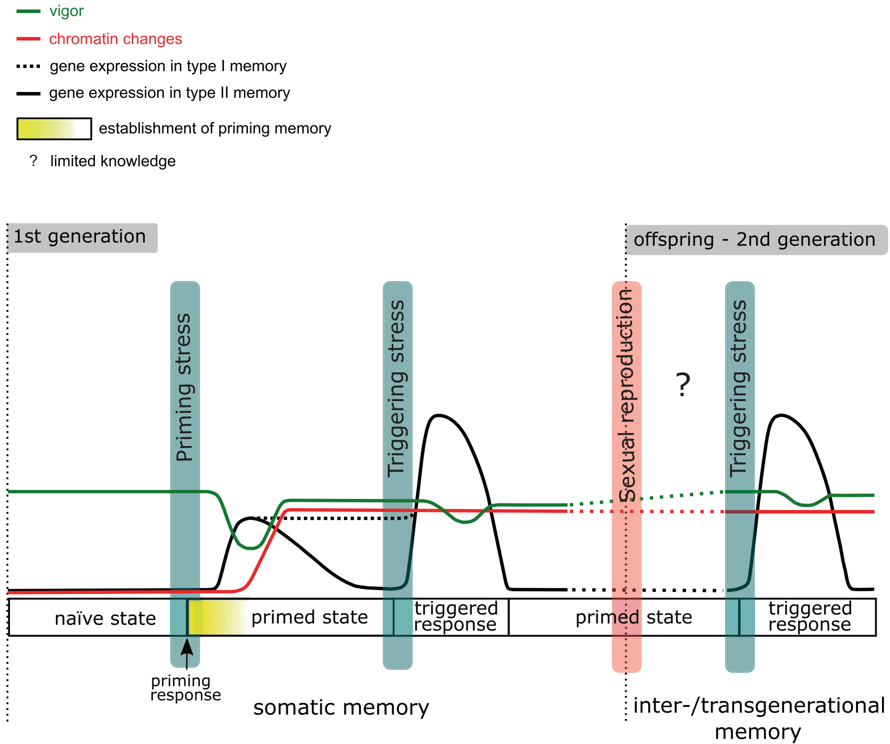

Fig. 1.1 Somatic and transgenerational memory induced by environmental stresses. Plants growing under naïve conditions can experience a first environmental stress that will promote transcriptional changes correlated with chromatin changes (i.e., DNA methylation, histone PTMs, DNA-dependent chromatin remodeling, deposition of new histone variants) of stress-responsive genes. This can result in sustained (type I) or temporal (type II) activation and/or repression of genes (Bäurle 2017) and addition and/or removal of specific chromatin modifications. However, for simplification, the figure focuses on transcriptional activation and addition of new chromatin modifications. Encountering the stress may also impair plant vigor. After the stress, the plant enters in a primed state in which transcription of stress-responsive genes may recover to original expression levels. Amplitude of the recovering phase varies depending on the environmental cue and on memory genes. Plant vigor also recovers, although a phenotypic cost may be applied. However, the new chromatin state of memory genes will be stably maintained. When the plant perceives a second stress, this triggers the response of memory genes. The triggered response can be faster, stronger, more sensitive, and/or different to the first one (Lämke and Bäurle 2017). Intensity and amplitude of the response also differs depending on experimental conditions. Although most of our current knowledge indicates that the primed state perdures for a finite period within the same generation (somatic memory) and resetting of the primed state occurs during sexual reproduction, in some cases the chromatin state linked to the stress memory may be inherited by the offspring (inter-/transgenerational memory). Although much less is known of this possibility (?), inheritance of the memory could provide the new plant generation with molecular tools to better cope with recurrent stresses

improved phenotypic adaptation with minor fitness cost and, hence, survival and yield. On the other hand, the priming stage increases plant sensitivity, affects development and growth, and can be more cost-effective to reset than to maintain; therefore, plants may employ mechanisms to elucidate whether to memorize or to forget (reviewed in Avramova 2015; Crisp et al. 2016; Bäurle 2017).

Transcriptional reprogramming is a common feature of the primed state. Genes that show a memory will modify their expression in response to both the first and the second stress, but expression levels will be significantly different in the second response. Considering that the primed state between the two stresses can last from days to months (as in the case of somatic memory—see Sect. 1.2), or stress can even recur in the subsequent generation(s) (as in intergenerational or transgenerational memory—see Sect. 1.2, Fig. 1.1), the transcriptional memory and molecular mechanisms that underlie it need to have the potential to be maintained and transmitted through cell division and even sexual reproduction. These criteria are met by genetic and epigenetic mechanisms. In fact, different epigenetic processes and chromatin-related mechanisms have been involved in setting memory of passed environmental events (Fig. 1.1 and reviewed in Bruce et al. 2007; Chen and Arora 2013; Kinoshita and Seki 2014; Avramova 2015; Crisp et al. 2016; Hilker et al. 2016; Bäurle 2017; He and Li 2018). Other processes, such as stability and modification of proteins, have also been involved in the priming memory (reviewed in Pastor et al. 2013).

Chromatin, the molecular complex containing DNA and nuclear proteins, mainly histones, plays an essential role in transcriptional regulation. DNA and histones can be modified by the addition of chemical groups, methyl group being by far the most common in the case of DNA and variable chemical post-translational modifications (PTMs) in case of histones (e.g., methyl, acetyl, phosphate, and ubiquitin groups being most common). The presence of these chromatin marks or their combinations acts to regulate gene expression by modifying the accessibility of DNA or the recruitment of specific proteins to chromatin. Furthermore, chromatin marks present on a gene may be stably transmitted through cell division contributing to the maintenance of its transcriptional status. In addition to primary DNA sequence, this adds a new layer of information that can be mitotically and/or meiotically transmitted and underlies epigenetic inheritance (reviewed in Zentner and Henikoff 2013; Du et al. 2015). Different pieces of evidence demonstrate that in the presence of a stress that triggers transcriptional changes, epigenetic modifications will be added/removed to/from specific key stress-response genes and create a stable chromatin environment that will perdure even once the stress that induced it has passed. This environment-triggered epigenetic memory will contribute to the phenotypic plasticity of the plant in the event of a new stress. The implication of this long-lasting chromatin-related memory has been subject of lively discussion due to the obvious potential for improving crop adaptation and its relationship to Lamarck's vision of genetic inheritance (Pecinka and Mittelsten Scheid 2012).

1.2 Somatic, Inter- and Transgenerational Memory

Memory of stress experienced by plants can be somatic (or intra-generational), lasting for a varied period of time within the exposed plant generation after the immediate stress response. Intergenerational memory persists into the next generation of progeny of the exposed plants and transgenerational memory is transmitted into further generation(s) in the absence of stress (Fig. 1.1; reviewed in Heard and Martienssen 2014; Lämke and Bäurle 2017). We will focus on the molecular mechanisms underlying stress memory and in particular on mechanisms connected to modification of chromatin structure (chromatin-based memory) in model and crop plants. At present, strong experimental support exists for somatic memory that persists in the range of days to weeks following the initial stress treatment, while less and often contradictory examples of intergenerational or transgenerational memory are available.

1.2.1 Somatic Memory

Several molecular mechanisms that contribute to somatic memory of abiotic stress have been identified (reviewed in Conrath et al. 2015; Avramova 2015; Crisp et al. 2016; Bäurle 2017; Lämke and Bäurle 2017). Somatic memory has been connected to the persistence of stress-induced metabolites (Pastor et al. 2014; Balmer et al. 2015; Hu et al. 2016), to sustained expression of genes after the stress response ends (Charng et al. 2006b; Stief et al. 2014), to stalling of RNA polymerase II that potentiates transcription (Ding et al. 2012), to the accumulation of proteins (e.g., mitogen-activated protein kinases—MPKs, Beckers et al. 2009), or to mitotic stability of stress-induced chromatin changes (Ding et al. 2012; Sani et al. 2013; Singh et al. 2014; Weng et al. 2014; Lämke et al. 2016; Brzezinka et al. 2016, 2018; Feng et al. 2016; Liu et al. 2018b). Based on the transcription level of the stress-response genes, chromatin-based transcriptional memory can be separated into type I, during which transcriptional activity of stress-responsive genes persists, and type II, during which the initial stress-induced transcription ceases but a second exposure to stress can induce a modified response in comparison with the response of naïve plants (reviewed in Bäurle 2017) (Fig. 1.1). Somatic memory of abiotic stresses seems limited to several days or weeks (Bäurle 2017; Lämke and Bäurle 2017). Several chromatin-based mechanisms have been shown to contribute to somatic memory. These include nucleosome occupancy and remodeling, relative abundance of histone PTMs, cytosine (DNA) methylation, and RNA interference, and we discuss examples of the particular mechanisms in the respective sections. Although molecular aspects of somatic memory are best well studied in the short-lived annual *Arabidopsis* (*Arabidopsis thaliana*), it may be of particular importance in long-lived perennial species (Lafon-Placette et al. 2018; Le Gac et al. 2018). Its existence is suggested by maintained changes of DNA methylation in the shoot apical meristem

(SAM) of poplar (*Populus* spp.) trees that have grown under different water availability (Lafon-Placette et al. 2018). In addition, winter-dormant SAMs of trees grown at different environmental conditions retain differentially methylated regions at genes involved in abiotic stress response, SAM organization, and phytohormone metabolism and signaling (Le Gac et al. 2018) suggesting that growth conditions during vegetative phase can be reflected in cells that will produce organs in the next vegetative season and may potentially influence performance and growth. It is of note, however, that global DNA methylation level changes occur during bud dormancy and break that are mediated by DNA demethylases (Conde et al. 2017), suggesting that active reprogramming occurs. Whether environmentally induced epialleles can escape the global DNA methylation reprogramming remains to be addressed.

1.2.2 Inter- and Transgenerational Memory

Transgenerational stress memory can be in principle mediated by transmission of structural variation in the genome, inheritance of chromatin states (or epialleles), and/or seed provisioning (or maternal effect) whereby different level of resources such as mRNA, hormones, proteins, starch, lipids, or other reserve molecules are stored in the seed based on the environmental conditions during growth of the maternal plant (reviewed in Herman and Sultan 2011; Pecinka and Mittelsten Scheid 2012; Pecinka et al. 2013; Heard and Martienssen 2014). Due to the difficulty in separating maternal effects from heritability of epialleles, transgenerational inheritance of acquired epialleles as means of environmental memory and its adaptive value has been debated (Boyko and Kovalchuk 2011; Mirouze and Paszkowski 2011; Paszkowski and Grossniklaus 2011; Pecinka and Mittelsten Scheid 2012; Ganguly et al. 2017). Taking into consideration also maternal effects or possible induced structural variation, intergenerational memory mechanisms can nevertheless contribute to adaptive transgenerational plasticity (Herman and Sultan 2011) and to rapid environmental adaptation in plants (Franks and Hoffmann 2012).

Transmission of acquired epialleles between generations is prevented by active resetting of chromatin states during sexual reproduction (reviewed in Paszkowski and Grossniklaus 2011; Heard and Martienssen 2014; Kawashima and Berger 2014; Iwasaki 2015). In mammals, extensive epigenetic reprogramming occurs during germline formation and early embryogenesis during which DNA methylation and histone PTMs are erased and thus examples of transgenerational inheritance of epialleles are rare (reviewed in Heard and Martienssen 2014). On the contrary, several features of plant development make plants more prone to transgenerational inheritance of acquired epialleles. First is the late developmental origin of the germline that forms from stem cells within the SAM, in which exposure of the somatic tissue to environmental conditions can be reflected. Nevertheless, it needs to be considered that mechanisms which restrict responses affecting genome and epigenome stability may operate with higher stringency in stem cells that give rise to the

germline than in vegetative tissue (Yadav 2009; Baubec et al. 2014). Second, stress-induced epigenetic changes have a chance to be copied and maintained during plant sexual reproduction. In plants, reprogramming (reduction) of DNA methylation mainly occurs in the companion cells, the vegetative cell nucleus in pollen and the central cell nucleus in the ovule, rather than in the nuclei (sperm cell and egg cell) that will fuse to form the zygote during fertilization (reviewed in Kawashima and Berger 2014). Still, considerable global epigenetic reprogramming does take place during gametogenesis, connected to histone replacement (Ingouff et al. 2007; Schoft et al. 2009; She et al. 2013; She and Baroux 2015) and DNA demethylation (Calarco et al. 2012). Despite the constraint imposed by epigenetic reprogramming during sexual reproduction for the transmission of acquired epialleles, examples of sexual transmission of epialleles are more abundant in plants than in mammals suggesting a higher potential for transgenerational epiallele inheritance (reviewed in Heard and Martienssen 2014). Finally, it is important to note that plants possess an immense capability of vegetative reproduction, which may increase the probability of epiallele retention and its later outgrowth into a sexually propagating individual.

1.2.2.1 Memory During Sexual Reproduction

Despite sexual reprogramming and other mechanisms that actively limit transgenerational inheritance of epialleles (Iwasaki 2015), natural epialleles that can be stable over sexual plant generations exist in plants (Cubas et al. 1999; Manning et al. 2006; Martin et al. 2009; Stam 2009). Much information on the inheritance of acquired and existing epialleles has been provided by genome-wide studies employing DNA methylation variation in natural accessions (ecotypes) (Dubin et al. 2015; Kawakatsu et al. 2016), in mutation accumulation lines (Becker et al. 2011; Schmitz et al. 2011), recombinant inbred lines (RILs) (Eichten et al. 2013; Schmitz et al. 2013), or the epigenetic RILs (epi-RILs) (Reinders et al. 2009; Teixeira et al. 2009; Johannes et al. 2009). These studies demonstrated that natural as well as some newly acquired DNA methylation epialleles can be inherited over several sexual generations and that DNA methylation at some loci can be re-established in the epiRILs to resemble the ancestral epiallelic states (Reinders et al. 2009; Teixeira et al. 2009). Hence, DNA methylation-based epialleles can be stably inherited mitotically and meiotically but are often reversible, especially if located close to TEs and small RNA-producing loci (Becker et al. 2011).

Alternative epialleles could serve as a source of variation for breeding purposes (Hofmeister et al. 2017). Indeed, epialleles can confer alternative transcription of their respective gene loci (Becker et al. 2011; Schmitz et al. 2011) and alter phenotypic traits of plants (Roux et al. 2011; Zhang et al. 2013b; Cortijo et al. 2014). Phenotypes associated with changes in chromatin states also affect traits that are of potential agronomic importance, including stress tolerance (Kooke et al. 2015; Verkest et al. 2015), disease resistance (Akimoto et al. 2007; Reinders et al. 2009), plant stature (Miura et al. 2009; Reinders et al. 2009; Johannes et al. 2009), root length (Soppe et al. 2000; Reinders et al. 2009; Johannes et al. 2009; Cortijo et al.

2014), transition to flowering (Soppe et al. 2000; Reinders et al. 2009; Johannes et al. 2009; Cortijo et al. 2014), senescence (He et al. 2018), flower sex determination (Martin et al. 2009), genetic incompatibility (Durand et al. 2012), fruit ripening (Manning et al. 2006), or yield (Hauben et al. 2009; Ong-Abdullah et al. 2015). However, the extent of purely epigenetic contribution to the observed phenotypes must be interpreted with care as the studied plant lines are not completely isogenic and genetic changes may accompany chromatin states connected to a particular epiallele (Pecinka et al. 2013). In some cases, structural changes to the genome can be induced by strong selective pressure imposed by stress (e.g., by chemical treatment, as is frequent during evolution of herbicide resistance—reviewed in Markus et al. 2018) or by activation of transposable elements (TEs) (discussed in Sect. 1.4.2). Even though combined effect of genetic and epigenetic change contributing to the desired phenotypic traits is not necessarily an obstacle and may be exploited for agricultural purposes (Yasuda et al. 2013), the nature of stress-induced epigenetic changes may be stochastic (Eichten and Springer 2015) and present an impediment to targeted crop improvement.

Transgenerational memory of abiotic stress observed in subsequent sexual generations of stress-exposed plants seems limited to one to two generations of unstressed sexual progeny of stressed plants. Activation of TEs induced by heat stress was only retained for maximum of several weeks in the treated plants but was not observed in the progeny (Pecinka et al. 2010). In a more extensive study the effect of several abiotic stress treatments was seen in the first or second generation after the treatment, but the appearance was stochastic and could represent experimental variation (Pecinka et al. 2009). Similarly, the resistance to several stresses (including heat, cold, flood, and UV-C) was elevated in the progeny of plants when both generations were subjected to stress but the effect was diminished in unstressed progeny (Boyko et al. 2010). Recently, Wibowo et al. (2016) observed enhanced resistance to hyperosmotic stress in the progeny of plants exposed to the stress for at least two consecutive generations. In the absence of the stress, however, the enhanced resistance was lost within two sexual generations (Wibowo et al. 2016), demonstrating transient retention of stress memory. Interestingly, repetitive stress over several generations does not always seem to correlate with improved phenotypic performance under stress. *Arabidopsis* plants subjected to drought conditions during five generations did not show any growth advantage to control plants (Ganguly et al. 2017). The only trait that showed significant memory through generations and perdured one generation after the stress was seed dormancy that was increased by drought (Ganguly et al. 2017).

1.2.2.2 Memory During Vegetative Reproduction

Epialleles can also be transmitted during vegetative propagation in vitro. Multiple economically important species are propagated vegetatively, producing large numbers of clonal progeny. Despite clonal origin, phenotypic variability occurs among individuals of the progeny, a phenomenon called somaclonal variation. Even though

somaclonal variation can in principle contribute to the emergence of advantageous traits and progeny improvement, it often leads to reduced plant vigor, and substantial quality and yield losses (reviewed in Miguel and Marum 2011). Somaclonal variation can be caused by different chromatin states, often associated with differences in DNA methylation (reviewed in Miguel and Marum 2011). Somaclonal variation may also be connected to genome structural rearrangements, as tissue culture in several crop species including rice (*Oryza sativa*) or maize may promote mobilization of TEs (reviewed in Negi et al. 2016), and other types of structural changes including polyploidization, aneuploidy, chromosomal mutations or DNA mutations (reviewed in Neelakandan and Wang 2012).

Three recent studies show that DNA methylation patterns can be maintained in plants regenerated from tissue culture in *Arabidopsis* (Wibowo et al. 2018), rice (Stroud et al. 2013), or maize (Stelpflug et al. 2014; Han et al. 2018). Importantly, the altered epiallelic states were retained in sexual progeny of plants obtained from these tissue cultures and DNA methylation changes were reflected in gene expression changes (Stroud et al. 2013; Han et al. 2018; Wibowo et al. 2018). In *Arabidopsis*, plants were regenerated from somatic embryos induced from either root or leaf and the original tissue-specific DNA methylation patterns persisted for two generations of sexual progeny of the regenerated plants. Especially the leaf of root-derived plants retained DNA methylation pattern of the original root tissue (Wibowo et al. 2018). These results suggest that DNA methylation epialleles established during tissue culture can be retained during regeneration and sexual propagation and that tissue of origin can be reflected in the regenerated plants and their sexual progeny. Although it remains unclear to what extent the changes in DNA methylation may be associated with genomic structural changes, these findings raise important considerations for massive clonal propagation of plants.

Information regarding inheritance of other than DNA-methylation dependent epialleles during vegetative propagation is scarce. Nevertheless, environmentally induced epialleles that are known to be stable somatically, such as the repressed form of the *FLOWERING LOCUS C* (*FLC*) (which will be discussed later in the chapter), can be maintained during vegetative propagation in vitro, changing the phenotypic traits of the regenerated plants by promoting early flowering (Nakamura and Hennig 2017). It is therefore possible that asexual propagation in tissue culture may allow for retention of histone PTMs but much more work is required in the future to gain more global insights.

In summary, epialleles (especially connected to alternative DNA methylation states) can be transmitted over generations both during sexual and vegetative plant propagation and can have an impact on plant phenotypes. The adaptive value of purely epigenetic, but not structural, variation and its contribution to evolution of populations under changing environmental conditions however remains to be determined (De Waele 2005; Franks and Hoffmann 2012).

1.3 Abiotic Stresses: Physiological Perspective

Although environmental stresses are usually combined in nature, most research so far has focused on application of a particular stress type. A compilation of major abiotic stresses and plant responses in relation to plant memory is summarized in the following section.

1.3.1 Drought and Desiccation

Drought is one of the factors limiting agricultural output that will be increasingly important due to the predicted climate change in next decades (reviewed in IPCC 2013). Therefore, drought-induced responses and stress memory in crops attract considerable attention in tackling negative effects of global warming.

In *Arabidopsis*, drought memory was studied by single or multiple desiccation stress treatments, followed by recovery (re-watering) periods of varying duration. In the seminal works from Avramova group (Ding et al. 2012, 2013; Liu et al. 2014a), *Arabidopsis* seedlings were treated with air-dry desiccation/rehydration cycles repeated up to four times. The samples were collected at pre-stress, stress, and recovery phases and subjected to gene expression and chromatin analyses. As a result, the group identified desiccation-responsive genes whose transcriptional and chromatin status is changed by the stress. Importantly, a subset of drought-responsive targets exhibited stress memory pattern, where response to subsequent stresses was altered in relation to the priming stress (Ding et al. 2012). Interestingly, categorized by the function, the biggest fraction of drought-memory genes is implicated also in response to salt, cold/heat, light, and abscisic acid (ABA) (Ding et al. 2013), highlighting a crosstalk between different stress signaling pathways. Another example of desiccation memory in *Arabidopsis* concerns drought tolerance induced at the seed stage. Imbibed seeds were treated with polyethylene glycol (PEG) at different developmental stages, followed by rehydration and growth/survival assessment during post-germination development. Strikingly, improved survival in PEG-treated plants was still present for at least 5–10 days after rehydration. A microarray experiment revealed significant subset of genes related to temperature- and hormone-response upregulated 3 days after PEG-treatment demonstrating continuous transcriptional response (Maia et al. 2011).

In crops, drought leads to morphological (e.g., reduced germination, plant height, plant biomass), physiological (e.g., reduced water content, photosynthetic activity, pigment content, membrane integrity), biochemical (e.g., accumulation of osmoprotectants like proline, sugars, antioxidants), and molecular (e.g., altered expression of stress-related genes) changes (reviewed in Farooq et al. 2012; Fahad et al. 2017). Rice, as submerged crop, is one of the most drought-sensitive species (Jaleel and Llorente 2009), in which drought-induced yield losses can amount even to 92% (Lafitte et al. 2007). Intermediate drought stress applied to rice seedlings causes

dehydration-induced oxidative cellular damage symptoms (Li et al. 2011). However, rice seedlings pre-treated with mild drought and re-watered before intermediate stress exhibited less pronounced oxidative damage as assessed by the levels of lipid peroxidation and selective antioxidants (Li et al. 2011). The beneficial effect of pre-treatment of rice seedlings suggests existence of drought memory mechanisms protecting against oxidative-stress caused by subsequently applied stronger drought. Wheat seedlings acclimated by dehydration, re-watered, and exposed to further water deficit showed limited membrane damage, retained water content, decreased accumulation of reactive oxygen species (ROS), compared to non-acclimated controls (Selote et al. 2004; Selote and Khanna-Chopra 2006, 2010). The authors correlated drought acclimation with levels of antioxidant enzymes that were induced by pre-treatment and maintained over re-watering period and triggering stress event (Selote and Khanna-Chopra 2006, 2010). In maize, drought memory was assessed by studying response to repetitive dehydration/rehydration cycles in seedlings. Plants exposed to multiple stress cycles exhibited improved water content in leaves as compared to single-stress controls. By comparing transcriptomic responses in maize and *Arabidopsis*, the authors identified not only conserved acclimation features, but also species-specific gene regulation patterns, indicating not only evolutionarily conservation but also divergence in drought stress response and memory (Ding et al. 2014). In potato (*Solanum tuberosum*), drought stress acclimation was shown to have positive effect on yield and overall plant growth. Plants exposed to two mild dehydration cycles before two complete soil dehydration showed reduced leaf wilting, cuticle accumulation, greater stem number and more open stomata under stress, compared to non-acclimated controls. In contrast, the authors did not observe acclimation effect on tuber weight and number under severe drought (Banik et al. 2016).

1.3.2 Osmotic Stress and Salinity

High salinity is one of the most detrimental factors for agricultural production on both, naturally saline soils and irrigated lands with high level of evaporation or insufficient water management. Salt-induced osmotic stress impairs plant growth by reduction of water uptake, stomatal closure, and decline in photosynthetic activity. In turn, ionic stress caused by specific salts taken up at above-optimum concentrations influences the homeostasis of essential ions, metabolic activity, and integrity of plasma membranes (reviewed in Sudhir and Murthy 2004; Rasool et al. 2012). Priming with mild salt treatment can increase the tolerance of model plants and different crop species to subsequent salt stress, improving the physiological and growth parameters connected to plant vigor and fitness.

Memory in salinity and osmotic stress responses in *Arabidopsis* were studied at both, somatic and *trans*-generational level. Regarding the somatic memory, Sani et al. (2013) reported that plants primed with low NaCl concentration accumulate less sodium in their shoots, have higher biomass and better survival after triggering

stress than control plants. The memory of initial stress was retained for at least 10 days and salinity-primed plants acquired tolerance also to drought, highlighting the crosstalk between the two stresses. Importantly for biotechnology applications, the plants did not exhibit obvious growth retardation effects after the priming stress, suggesting that memory did not come with a cost of overall plant vigor (Sani et al. 2013). Response to salinity stress in *Arabidopsis* was also related to proline content (Feng et al. 2016). Proline is an amino acid implicated in metal chelation, antioxidation and signaling, and its accumulation is positively correlated with tolerance to various stresses (Hayat et al. 2012). *Arabidopsis* plants primed by salt (NaCl) exhibited increased proline content upon subsequent stresses than non-primed controls. The effect was dependent on the transcription of the gene encoding the enzyme Δ1-pyrroline-5-carboxylate synthetase 1 (P5CS1) that mediates the rate-limiting step of proline biosynthesis pathway (Feng et al. 2016).

In wheat, priming of seedlings with low NaCl concentration led to increased tolerance to subsequent treatment with high NaCl concentrations. Specifically, primed plants exhibited efficiently reduced chlorotic symptoms, undisturbed photosynthetic activity, and improved osmotic potential upon high salt stress than non-primed controls (Janda et al. 2016). Higher tolerance to salinity stress was achieved in rice by pre-treatment of seedlings with sublethal NaCl dose. Primed plants showed better control of ion absorption, improved ion transport to leaves, less affected photosynthesis activity, and enhanced accumulation of osmolytes for osmotic adjustment than non-pre-treated controls (Djanaguiraman et al. 2006). In sorghum (*Sorghum bicolor*), priming of seedlings with NaCl led to improved growth upon severe salt treatment (Amzallag et al. 1990). In maize, priming treatment with low salt significantly reduced the detrimental effect of high salt stress manifested by less decreased chlorophyll concentration, water content, and stomatal conductance in comparison with non-primed plants (Pandolfi et al. 2016). Salt priming effect was also observed in other crops like pea (*Pisum sativum*—Pandolfi et al. 2012), potato (Etehadnia et al. 2010), or olive (*Olea europaea*—Pandolfi et al. 2017). In potato and olive, salt tolerance and priming effect were related also to cultivar type. In potato, the biggest effect of priming was seen for relatively salt-sensitive cultivars (Etehadnia et al. 2010), while in olive, priming had overall similar effect in improving salt tolerance, but affected plant organs to different extent, depending on the cultivar (Pandolfi et al. 2017).

1.3.3 Heat

Increase in temperatures is one of the major predictions from climate change models that will likely deeply impact on food security as it impairs plant growth, affects plant reproduction and, therefore, final yield (reviewed in Bäurle 2016).

In *Arabidopsis*, heat stress memory was studied mostly at the seedling stage. Current evidence suggests that heat stress memory in *Arabidopsis* seedlings can be kept up to 3 days after initial stress (Lämke et al. 2016; Brzezinka et al. 2016).

However, the memory strength may decay within hours of recovery (Charng et al. 2006b).

Works on heat stress memory in *Arabidopsis* served as an aid for similar studies in agronomic plant species. For example, an experimental setup established for *Arabidopsis* (Charng et al. 2006a, b) was applied to rice seedlings where the duration of the memory differed between cultivars (Lin et al. 2014).

The crosstalk between different stress types in crops was studied for heat, as priming stress, and cold or salinity as triggering stress. For example, barley plants subjected to high salt stress exhibit impaired growth, as measured by root elongation (Faralli et al. 2015). However, this response can be prevented by acute heat shock priming (Faralli et al. 2015). The beneficial effect of heat shock priming in protection against cold stress-mediated damage was observed in tomato (*Lycopersicon esculentum*). Harvested tomato fruits exposed to non-freezing cold conditions exhibit signatures of chilling injury, i.e. aroma loss, electrolyte leakage, failure to ripen, and oxidative stress (Malacrida et al. 2006; Biswas et al. 2016). However, post-harvest treatment of tomato fruits with higher temperature results in decreased chilling injury upon subsequent cold stress (Saltveit 1991; Zhang et al. 2013a).

Heat-stress memory has been frequently linked also to the tolerance to subsequent heavy metal exposure in crops. In wheat, priming heat shock was shown to mediate higher viability rate of seedlings upon subsequent injection of iron and cadmium salts to leaf segments (Orzech and Burke 1988). In wild tomato (*Lycopersicon peruvianum* L.) cell suspension cultures, acute heat shock prevented cell membrane leakage upon treatment with cadmium (Neumann et al. 1994). In rice, short-term heat pre-treatment led to reduced cadmium-induced chlorosis in seedlings (Hsu and Kao 2007; Chao et al. 2009; Chao and Kao 2010; Chou et al. 2012). Heat-shock-induced accumulation of antioxidative compounds is suggested to play a prominent role in protection against subsequent exposure to cadmium (Hsu and Kao 2007; Chao et al. 2009; Chao and Kao 2010; Chou et al. 2012). These studies indicate that heat pre-treatment can be efficient for priming against heat but also heavy metal, cold or salt stresses.

1.3.4 Cold

Low temperature is also one of the major factors determining locations of crop production and is periodically responsible for losses in crop yields (reviewed in Thomashow 1999). Exposure to low temperatures causes various phenotypic symptoms such as poor germination rate, chlorosis, reduced organ expansion, wilting, and inhibited reproductive development (reviewed in Yadav 2009). Cold memory in *Arabidopsis* can be triggered by persisting or oscillating low temperature stress (reviewed in Thomashow 1999; Markovskaya et al. 2008), both of which have an immense impact on plant fitness to seasonal and daily temperature changes in the environment.

Arabidopsis response to triggering cold stress was assessed after two different priming stress types, short-term cold stress (STC) or long-term cold stress (LTC), both followed by a 5 day-long recovery phase. Only LTC plants showed higher effective quantum yields of photosystem II and higher photochemical quenching after triggering stress, in contrast to STC plants (van Buer et al. 2016). The results indicate that long-term, but not short-term, priming allows better energy dissipation through photosystem II in response to cold.

Cold stress memory was studied in a number of chilling-sensitive agronomic species. Here, exposure to moderate temperatures before cold alleviates cold-induced negative effects on plant growth and development. For example, in rice, cold-priming prevents cold-induced impaired water uptake in roots, leaf wilting, and color bleaching (Ahamed et al. 2012). Priming of maize was shown to protect the photosynthetic apparatus from cold-induced damage. The authors used maize inbred lines of different cold-sensitivity to demonstrate a crucial role of cold priming in chilling-resistant high cold-tolerant varieties (Sobkowiak et al. 2016). Cold priming effect on different varieties was also studied in wheat (Charest and Ton Phan 1990). Cold treatment led to increased soluble protein content, decreased water content, and accumulation of proline even 30 days after cold. Most importantly, such cold memory effect was more pronounced in winter than in spring wheat cultivars. Cold priming has an effect in tolerance to subsequent exposure to freezing temperatures in winter wheat and also in winter and spring cultivars of canola (Trischuk et al. 2014).

Similar to heat priming, cold priming treatment was shown to increase resistance to further exposure to stress of other types. For example, cold priming results in better survival and growth of mustard seedlings exposed to salt and drought (Hossain et al. 2013), in alleviated photoinhibition and oxidative cellular damage caused by cadmium, copper or high light intensity in pea (Streb et al. 2008) and in increasing resistance to heat stress (Zhang et al. 2006a; Wan et al. 2009).

1.3.5 Ultraviolet (UV-B) Radiation

UV-B is one of the types of ultraviolet light and a natural component of solar radiation. Increased UV-B intensities are especially detrimental for plants due to their sessile lifestyle and obligatory requirement for sunlight. UV-B stress can be divided into low- and high-dose, and short-term (acute, seconds to hours) or long-term (chronic, hours to days) exposure (reviewed in Brown and Jenkins 2007; Lang-Mladek et al. 2012; Hideg et al. 2013). Whereas acute, high dose radiation causes severe detrimental effects and results ultimately in programmed cell death, chronic, low-dose UV-B causes effective activation of defense mechanisms and acclimation to UV stress (reviewed in Hideg et al. 2013).

Arabidopsis plants exposed to long-term low-dose UV-B exhibited stress memory even after 9 days of recovery period and showed morphological changes such as decreased rosette diameter, reduced inflorescence height, increased number of flow-

ering stems, and stimulated axillary branching (Hectors et al. 2007). However, such stress did not affect photosynthesis efficiency—increased pigment content compensated reduced leaf area, preventing substantial growth impairment (Hectors et al. 2007). *Arabidopsis* plants treated with a 2-h pulse for several days showed increased flavonoid content that eventually reaches a steady-state (Hectors et al. 2014). Such result suggests a role of flavonoids in long-term UV memory and acclimation.

The response to low-dose UV treatment and the UV stress memory was studied also in crops. Beneficial effect of low-dose UV-B was observed on morphological, physiological, and metabolic levels. Plant species showing long-term beneficial effect after UV-B stress range from crops (wheat, maize, rice) to commonly cultivated Brassicaceae (cabbage—*Brassica oleracea*, rapeseed—*B. napus*) and legumes (mungo bean—*Vigna radiata*, kidney bean—*Phaseolus vulgaris*, cowpea—*Vigna unguiculata*, soybean—*Glycine max*) (Thomas and Puthur 2017). Crop seeds treated with UV-B exhibit, i.e. increased germination, faster growth rate, elevated pigment content, and increased tolerance to other stresses (i.e., salinity, pathogens). For example, increased germination rate as a result of UV-B treatment was seen for maize (Wang et al. 2010); increased content of pigments for cabbage, beet (*Beta vulgaris*), kidney bean (Kacharava et al. 2009), soybean (Yanqun et al. 2003), mash bean (*Vigna mungo*—Shaukat et al. 2013), and rice (Olsson et al. 1998); and increased biomass for tartary buckwheat (*Fagopyrum tataricum Gaertn.*—Yao et al. 2007). Increased chlorophyll or carotenoid content was reported for UV-treated seedlings of rice (Xu and Qiu 2007), cowpea (Mishra et al. 2008), and bitter gourd (*Momordica charantia* L.—Mishra et al. 2009).

1.3.6 Chemical Agents

Instead of applying initial mild abiotic stress, stress memory in plants can be also induced by treatment with chemical compounds in a process called chemical priming. Such chemicals can be synthetic or of natural origin and include, i.e. amino acids, hormones, nutrients, pesticides, reactive oxygen-nitrogen-sulfur species (RONSS) (reviewed in Jisha et al. 2013; Savvides et al. 2016; Antoniou et al. 2016; Lutts et al. 2016). One of the advantages of using chemical agents to prime plants against environmental stresses is the robustness, enhancing plant resilience against many different stress types.

Chemical priming on *Arabidopsis* was assessed in a number of studies. Pretreatment of *Arabidopsis* seedlings with the non-protein amino acid β-aminobutyric acid (BABA) 1 day before, either high salt or drought treatment showed improved tolerance to subsequent stresses—lower wilting rate and water loss (Jakab et al. 2005). Interestingly, BABA is also a commonly used agent enhancing systemic acquired resistance (SAR) for pathogen protection, indicating that the compound triggers activation of a pathway common for biotic and abiotic stresses. *Arabidopsis* plants pre-treated with melatonin showed better growth following cold stress, manifested in fresh weight, root length and shoot length increase (Bajwa et al. 2014).

Melatonin increased expression of cold-inducible genes at different timepoints during stress (Bajwa et al. 2014), suggesting that the compound triggered a similar primed state at the transcriptomic level as a mild cold pre-treatment (van Buer et al. 2016).

Spermine is a natural polyamine synthesized in eukaryotic cells and it was reported to accumulate, along with the other polyamines, under abiotic stress conditions (reviewed in Rhee et al. 2007). Pre-treated *Arabidopsis* seedlings with exogenously applied spermine exhibited attenuated chlorosis in cotyledons compared to controls. The crucial impact of spermine on heat acclimation was also confirmed by genetic approaches—transgenic plants overexpressing spermine biosynthetic genes showed less inhibited growth upon heat shock, whereas knock-out mutants were hypersensitive to a high temperature (Sagor et al. 2013).

The exogenous application of chemical compounds on crops has frequently been used for seed priming, because seeds can be more easily treated and with a minor cost than the adult plants (reviewed in Jisha et al. 2013; Savvides et al. 2016; Lutts et al. 2016). Confirmed for a big range of various agronomic plants, chemical pretreatment of seeds can increase the rate and percentage of seed germination. In addition, it can have a beneficial effect in the longer term by improving seedling vigor, especially during growth under stress conditions (reviewed in Savvides et al. 2016; Lutts et al. 2016). However, there are also reports showing priming effect of chemicals, when applied at later developmental stages. The application of the chemical on a specific organ, for instance roots, leaves, or stems, or at specific developmental stage, such as seedlings, promoted a systemic response that will spread to protect other parts of the plant and not only the organs that were treated in different crops such as wheat (Hasanuzzaman et al. 2011; Shan et al. 2011; Turk et al. 2014), rice (Uchida et al. 2002; Saleethong et al. 2013; Mostofa et al. 2014), maize (Li et al. 2013), tomato (İşeri et al. 2013; Amooaghaie and Nikzad 2013), strawberry (*Fragaria* sp.—Christou et al. 2013, 2014a, b), oil rapeseed (Yıldız et al. 2013; Xiong et al. 2018), or tangerine (*Citrus* sp.—Shi et al. 2010).

1.4 Epigenetic Mechanisms of Abiotic Stress Response and Memory

Responses to stress result in genome-wide changes to chromatin structure and gene transcription or can be even associated with modifications to genomic sequence. Exposure to stress induces alterations at all levels of chromatin structure, including DNA methylation, nucleosome occupancy and composition, presence of histone variants as well as histone PTMs and global chromatin arrangement. Uncoupling the direct effects of stress on chromatin structure and nuclear architecture from its effects on gene transcription is very challenging, making a large part of evidence describing mechanisms of stress-induced changes correlative. In addition, even though the connections between chromatin rearrangement and response to various

stresses are well established, the inheritance of the stress-induced chromatin structure is less well understood.

1.4.1 Global Changes to Chromatin Structure

Various abiotic stresses lead to cytologically detected heterochromatin de-condensation and release of transcriptional gene silencing (TGS) from TEs and ribosomal RNA genes (rDNA) in different plant species. In *Arabidopsis*, prolonged heat stress causes decompaction of centromeric repeats and 5S rDNA and TGS release from specific TEs, hallmarked by the *COPIA78* family (Pecinka et al. 2010). Heat treatment of cold acclimated plants results in general de-repression of heterochromatic (centromeric and pericentromeric) repetitive elements (Tittel-Elmer et al. 2010) and heat or UV-B stress releases TGS of transgenes as well as endogenous loci (Lang-Mladek et al. 2010). Similarly, de-condensation of 45S rDNA clusters follows salinity and heat stress in rice (Santos et al. 2011) or heat stress in rye (Tomás et al. 2013), and loss of tandem repeat transcriptional silencing in heterochromatin knobs follows cold treatment in maize (Hu et al. 2012). De-condensation of heterochromatic regions and release of TGS may be a shared response to several types of abiotic stresses. One possibility is that this may represent increased need for ribosomal RNAs to support changes in protein synthesis or transcriptional reprogramming in specific genomic regions. The effects seem to be generally transient (Pecinka et al. 2009, 2010; Lang-Mladek et al. 2010; Hu et al. 2012; Iwasaki and Paszkowski 2014a) and may provide a time window that allows complex epigenetic and gene expression changes in response to stress (Pecinka et al. 2010). Interestingly, stress-induced TGS release is not necessarily connected to reduction of repressive chromatin marks at the affected loci (such as DNA methylation, H3K9me2—dimethylation of lysine 9 of histone 3—or H3K27me) (Lang-Mladek et al. 2010; Pecinka et al. 2010; Tittel-Elmer et al. 2010) and can be associated with transient reduction in nucleosome occupancy (Pecinka et al. 2010). Restoration of TGS to naïve state requires factors such as the nucleosome chaperone CHROMATIN ASSEMBLY FACTOR 1 (CAF-1) (Pecinka et al. 2010), the chromatin remodeler DECREASED DNA METHYLATION 1 (DDM1), the TGS regulator MORPHEUS' MOLECULE 1 (MOM1) (Iwasaki and Paszkowski 2014a), and components of the RNA-directed DNA methylation (RdDM) pathway (discussed in more detail in Sect. 1.4.2). Global alterations to chromatin structure in response to stress resemble chromatin changes during developmental transitions and may suggest functional connection between developmental and stress-induced reprogramming (reviewed in Probst and Mittelsten Scheid 2015). Even though the causal connection between the triggering stress, global chromatin architecture change and stress-induced gene transcription change is often unclear, strong correlative association is well documented. Exposure to abiotic stress factors such as salt, water availability, temperature, or UV-light results in global changes to histone modifications marks (for example, van Dijk et al. 2010; Hu et al. 2012; Sani et al. 2013; Forestan et al. 2018)

and/or DNA methylation (for example, Colaneri and Jones 2013; Secco et al. 2015; Wang et al. 2015b, 2016a; Eichten and Springer 2015; Wibowo et al. 2016; Ganguly et al. 2017) and is connected to global gene expression changes (for example, van Dijk et al. 2010; Sani et al. 2013; Eichten et al. 2013; Ding et al. 2013, 2014; Secco et al. 2015; Forestan et al. 2016; Wang et al. 2016a; Wibowo et al. 2016).

1.4.2 DNA Methylation Changes and the Role of TEs in Response to Abiotic Stress

Transcriptional activity of genes and especially TEs is affected by DNA methylation. In mammals, DNA methylation is found almost exclusively in the CG sequence context during somatic development. In plants, however, any cytosine can be methylated and three functional sequence contexts are distinguished. Cytosines in the symmetrical CG or CHG contexts as well as the non-symmetrical CHH (where H represents A, T, or C) context can undergo methylation (reviewed by Law and Jacobsen 2010; Du et al. 2015; Zhang et al. 2018a). Presence of DNA methylation in all contexts generally contributes to TGS. Genome-wide distribution of DNA methylation in plant genomes strongly correlates with the density of TEs where DNA methylation safeguards the genome from unwanted activity of repetitive elements (Kato et al. 2003; Zhang et al. 2006b; Lister et al. 2008; Cokus et al. 2008; Mirouze et al. 2009; Ito et al. 2011). In contrast, presence of only CG methylation, typically in the bodies of moderately transcribed genes, does not have repressive function in plants (reviewed in Bewick and Schmitz 2017). DNA methylation is catalyzed by three classes of DNA methyltransferases in plants. DNA METHYLTRANSFERASE 1 (MET1) is an evolutionarily conserved DNA-replication-coupled maintenance CG methyltransferase. CHROMOMETHYLASE (CMT) family harbors plant specific DNA methyltransferases, where CMT3 and CMT2 act as CHG and CHH maintenance and de novo DNA methyltransferases whose activities are coupled to the presence of H3K9me2 (Stroud et al. 2013; Zemach et al. 2013). Finally, DOMAINS REARRANGED METHYLTRANSFERASES (DRMs) are guided to the target sites by RdDM. In RdDM, 24 nt siRNAs are produced following transcription of target loci from a double-stranded RNA precursor by the joint action of the plant-specific DNA-dependent RNA polymerase IV (RNA pol IV, NRPD), RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) and DICER-LIKE3 (DCL3). The siRNAs are loaded into ARGONAUTE 4 (AGO4)-containing complex and paired with another plant specific DNA-dependent RNA polymerase V (RNA pol V, NRPE)-produced nascent transcripts, recruiting chromatin modifier complexes including DRMs (reviewed by Law and Jacobsen 2010; Du et al. 2015; Zhang et al. 2018a). RdDM is a pathway that provides a backup for other methylation pathways and can largely restore original methylation patterns if necessary (Teixeira et al. 2009; Baubec et al. 2014).

A large number of studies have addressed DNA methylation and gene expression changes induced by abiotic stresses in different model and crop plants. In general, exposure to abiotic stresses induces global changes in DNA methylation amount and distribution in different plant species. The changes affect not only the exposed plant generation but, in some cases, also their progeny (Bilichak et al. 2012; Boyko et al. 2010; Colaneri and Jones 2013; Eichten and Springer 2015; Jiang et al. 2014; Secco et al. 2015; Steward et al. 2002; Wang et al. 2015b; Wibowo et al. 2016; Yong-Villalobos et al. 2015). Whether a particular stress induces targeted changes to DNA methylation is not clear. In *Arabidopsis*, it was suggested that DNA methylation changes may be stress-type-specific (Wibowo et al. 2016). However, analysis of DNA methylation in individual maize plants exposed to various abiotic stresses showed that DNA methylation changes in response to abiotic stress are stochastic and unrelated to particular stress-type (Eichten and Springer 2015). Additionally, upon drought stress in five generations of *Arabidopsis*, only stochastic changes in DNA methylation without epiallele accumulation were observed (Ganguly et al. 2017), supporting the idea of DNA methylation changes observed during stress being mostly stochastic.

DNA methylation machinery may nevertheless represent an integral component of abiotic stress responses and memory. For example, expression of DNA methyltransferase genes is modulated by inorganic phosphate (Pi) availability in *Arabidopsis* and mutants in the methyltransferases *DRM1*, *DRM2* and *CMT3* or *RNAi MET1* plants display impaired response and hypersensitivity to Pi starvation (Yong-Villalobos et al. 2015). Similarly, *Arabidopsis nrpd2* mutants are hypersensitive to heat-stress (Popova et al. 2013). Transcription of RdDM and DNA demethylation genes responds to hyperosmotic stress (Wibowo et al. 2016) and intergenerational memory of stress-response genes is affected in *nrpd1a*, *cmt3*, and DNA demethylase mutants (Wibowo et al. 2016). Stress-induced changes in DNA methylation can affect the expression of stress-responsive genes (Colaneri and Jones 2013; Wibowo et al. 2016). In contrast, correlation between DNA methylation and gene expression of plants treated with drought stress was not observed (Ganguly et al. 2017). Additionally, stress-induced transcriptional changes at methylated loci are not always accompanied by DNA methylation changes (for example, Lang-Mladek et al. 2010; Pecinka et al. 2010; Tittel-Elmer et al. 2010; Song et al. 2012b). Thus, the connection between DNA methylation and stress-induced transcriptional changes is not uniform.

Direct effects of DNA methylation on gene expression (Fig. 1.2) can stem from the change of chromatin state at an affected locus or from altered properties of regulatory regions—over 75% transcription factors are affected by DNA methylation for their binding to DNA (O'Malley et al. 2016). Negative correlation between DNA methylation with gene expression in response to abiotic stress is mainly observed. For instance, cold treatment of maize seedlings induces hypomethylation at the genomic fragment *ZmMI1* in roots which correlates with its elevated transcription (Steward et al. 2002). Salt stress in soybean leads to the hypomethylation of promoter regions and transcriptional activation of several salt-responsive transcription factor genes (Song et al. 2012b). In contrast, increase in DNA methylation on

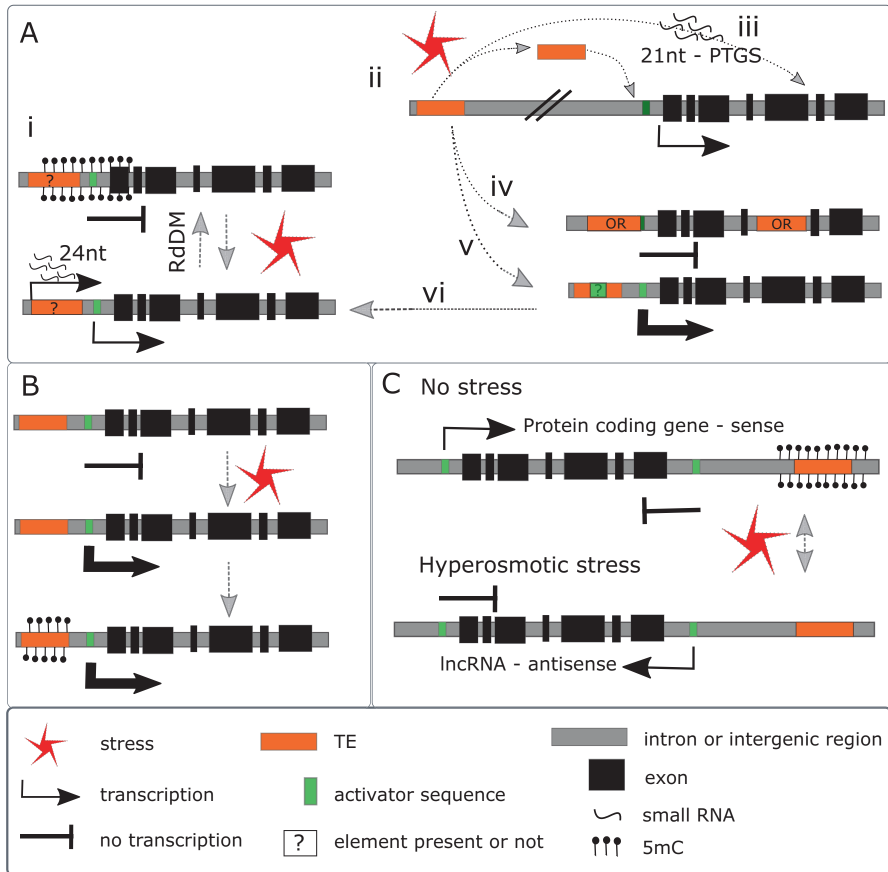

Fig. 1.2 Stress-induced changes to gene expression connected to DNA methylation and transposable elements (TEs). (**a**) The role of DNA methylation and TEs in affecting gene expression in response to stress. (i) Upon stress, global DNA methylation distribution and level can be changed, potentially affecting gene expression. Stress-induced changes in DNA methylation and associated gene expression are often connected to the presence of TEs or their residual sequences in the upstream regions of affected genes and to general release of transcriptional gene silencing (TGS) from TEs and other repetitive elements. This is associated with the production of 24 nt small RNAs. Silent (methylated) state can be re-established by RNA-directed DNA methylation (RdDM). (ii) TEs can affect genomic loci in-trans. (iii) TEs can be a source of 21 nt small RNAs that mediate post-transcriptional gene silencing (PTGS) in trans (McCue et al. 2012, 2013). (iv) Alternatively, upon very severe stress (and/or in combination with deficient RdDM), TEs can transpose and their de novo integration can disrupt regulatory regions or gene bodies, impairing the expression of a gene (for example, Ito et al. 2016). Alternatively, (v) TEs can integrate into gene regulatory regions, introducing novel stress-responsive elements that they carry (for example, Cavrak et al. 2014). By TE integration to the upstream region, the target gene can also become silenced by the in-trans activity of RdDM (vi). (**b**) Stress-induced gene expression can induce DNA methylation at adjacent TEs. This may serve as a mechanism that protects TE-rich genomes, such as in rice (Secco et al. 2015). (**c**) Gene expression can be downregulated upon stress by the loss of DNA methylation at downstream loci, which correlates with the transcriptional activation of antisense long non-coding RNAs (lncRNAs) (Wibowo et al. 2016)

stomata-specifying transcription factor genes *SPEECHLESS* (*SPCH*) and *FAMA* in response to low humidity was observed, corresponding with their downregulation and decrease in relative number of stomata (Tricker et al. 2012). DNA-methylation-coupled changes in gene expression can be connected to the presence of TEs or their partial sequences in the proximity or within protein-coding genes (Fig. 1.2a). TE transposition can be induced by some severe abiotic treatments including gamma-irradiation (Nakazaki et al. 2003) or hydrostatic pressure (Lin et al. 2006), potentiating the emergence of novel insertion sites. However, TE transposition does not seem to be a common response to abiotic stresses (reviewed in Negi et al. 2016). In *Arabidopsis*, heat stress-induced TE mobilization was observed only in mutants of the RdDM pathway (Mirouze et al. 2009; Ito et al. 2011, 2016), indicating stringent control of TE mobility. More frequently, abiotic stresses including salinity, flooding, heat, cold, or UV-light stress cause general release of TGS of endogenous repetitive sequences including TEs and can also increase the frequency of homologous recombination in *Arabidopsis* (Molinier et al. 2006; Pecinka et al. 2009, 2010; Boyko et al. 2010; Tittel-Elmer et al. 2010). Transcriptional activation of TEs in response to various abiotic stresses also occurs in different crop species, including maize (Makarevitch et al. 2015), oat (*Avena sativa*) (Kimura et al. 2001), durum wheat (*Triticum durum*) (Woodrow et al. 2010), or blood oranges (*Citrus sinensis*) (Butelli et al. 2012). In maize plants subjected to various abiotic stresses including heat, cold, UV-light, and high salt, activation of 20–30% of stress-responsive genes is associated with TGS release of proximal TEs that act as local enhancers (Makarevitch et al. 2015). In *Arabidopsis* subjected to hyperosmotic treatment, differentially methylated regions overlapping with genes that harbor proximal TEs were identified. Approximately 30% of these genes changed their expression in response to osmotic stress (Wibowo et al. 2016). In several rice varieties, the inverted-repeat TE *miniature Ping* (*mPing*) can locate into regions inducing the expression of genes responding to cold or salt stress (Naito et al. 2009; Yasuda et al. 2013).

In addition to general TGS release of TEs upon stress, specific stress-mediated activation of TEs can be conferred by stress-responsive elements present within the TE sequence itself. Heat, drought, and ABA-response elements are found in the proximity and within a TE-derived repetitive sequence upstream of *Arabidopsis* heat-responsive genes (Popova et al. 2013). The heat-responsive *Ty1/Copia*-type retrotransposon *ONSEN* (*COPIA78*) contains a target site for the plant heat-responsive transcription factor HFSA2, exploiting the plant's innate heat-stress response system for its activation (Cavrak et al. 2014). The transcription factor binding site is evolutionary conserved in *COPIA78* transposons and several other transposon families, making it apparently possible to trick the host genome in a similar way (Pietzenuk et al. 2016). Different TE families can be associated with different stress responses (Beguiristain et al. 2001; Makarevitch et al. 2015), supporting the notion that selective transcriptional activation of TEs in the upstream regulatory regions of stress-responsive genes may be an integral part of stress-type-specific responses in plants.

The interplay between stress-induced changes in gene expression and TE methylation can also act in an opposite direction (Fig. 1.2b). Stress-induced changes in DNA methylation at TE loci can follow altered transcription of neighboring stress-responsive genes, as is the case in Pi-starved rice plants (Secco et al. 2015). Here, the newly deposited DNA methylation in TEs does not restrict the expression of the neighboring genes but may serve as a protective measure against reactivation of TEs located close to highly transcribed genes. Interestingly, this response is species specific, not observed in *Arabidopsis*, perhaps reflecting the different genome organization in the two species, in particular the relatively low abundance of TEs in *Arabidopsis* compared to rice (Secco et al. 2015; Yong-Villalobos et al. 2015).

Due to mechanisms of DNA methylation maintenance and re-establishment following DNA replication (reviewed in Du et al. 2015), stress-induced DNA methylation changes have the potential to be maintained somatically and also inter- or transgenerationally. The maintenance of stress-induced DNA methylation changes over sexual generations, however, seems limited either to the generation of stress-exposed plants (somatic memory) or to the first generation of their progeny (intergenerational), being progressively reset to the pre-treatment state in the absence of stress (Pecinka et al. 2009; Boyko et al. 2010; Secco et al. 2015; Wibowo et al. 2016; Ganguly et al. 2017). This has been attributed to epigenetic reprogramming mainly in the male germline (Wibowo et al. 2016) and active resetting of DNA methylation by RdDM (Ito et al. 2011; Popova et al. 2013). In wild-type plants, the heat-induced transcription of *ONSEN* decreases during recovery after the stress but in plants carrying mutations in genes of the RdDM pathway, including *NRPD2*, *ONSEN* activity persists and retrotransposition occurs (Ito et al. 2011). NRPD2 is also required for the re-establishment of heat-released TGS and restoration of CHH methylation at RdDM target loci during resetting of heat stress-induced genes (Popova et al. 2013). RdDM therefore seems to be a key pathway involved in the resetting of the pre-stress DNA methylation patterns. DNA methylation may however be implicated also in stress memory, as intergenerational increase of resistance to hyperosmotic stress and transcriptional memory also depend on the NRPD1a, CMT3, or DNA demethylation (Wibowo et al. 2016).

In summary, stress-induced changes to DNA methylation and associated stress responses are mostly reversible in the absence of the initial stress. Somatic and intergenerational resetting of DNA methylation is in large governed by RdDM and sexual transmission of acquired chromatin states is mitigated by global epigenetic reprogramming in the germline. Nevertheless, TE mobilization potentiates transgenerational stability of altered DNA methylation connected with structural changes to the genome (Fig. 1.2a).

1.4.3 The Role of Small RNAs in Post-Transcriptional Regulation of Stress-Responsive Genes

Abiotic stresses including cold, drought, salt, nutrient deficiency, and oxidative stress induce the production of long non-coding RNAs and small RNAs of different classes (reviewed in Borges and Martienssen 2015) that affect the expression of stress-responsive genes (reviewed in Shukla et al. 2008; Sunkar et al. 2012; Kumar 2014; Zhao et al. 2016a) in *Arabidopsis* (Sunkar 2004; Zhou et al. 2008; Amor et al. 2009) and in crops including wheat or barley (reviewed in Alptekin et al. 2017), maize (Wang et al. 2014; Lunardon et al. 2016), rice (Liu et al. 2017), *Brachypodium distachyon* (Wang et al. 2015a), foxtail millet (*Setaria italica*—Wang et al. 2016b), or legumes (Trindade et al. 2010).

MicroRNAs (miRNAs) modulate stress-responsive gene expression through post-transcriptional gene silencing (PTGS). In PTGS 20–22 nt long miRNAs originating from RNA polymerase II-mediated transcription of *miRNA-coding* genes and processing of their transcripts by DCL1 incorporate into AGO1-containing RNA-induced silencing complex (RISC) that mediates cleavage of miRNA-targeted transcripts (reviewed in Borges and Martienssen 2015). The expression of stress-responsive miRNAs can be both upregulated or downregulated, affecting the expression of their target genes negatively or positively, respectively. For example, in *Arabidopsis*, drought reduces the expression of miR169 that targets a drought response-activating transcription factor NFYA5, increasing the abundance of the NFYA5 and contributing to drought resistance (Li et al. 2008). On the contrary, several conserved miRNAs are upregulated in response to drought stress in *Medicago truncatula* (Trindade et al. 2010). Among those are the miRNAs miR398 and miR408, whose upregulation by drought suppresses the abundance of their target transcripts, which code for proteins involved in copper homeostasis (Trindade et al. 2010). miR398 is upregulated in response to abiotic stresses targeting the copper metabolic genes in several species (Sunkar et al. 2012). Interestingly in *Arabidopsis*, where miR398 targets the genes *Zn/Cu SUPEROXIDE DISMUTASE 1* and *2* (*CSD1/2*) and the *Cu CHAPERONE FOR SUPEROXIDE DISMUTASE 1* (*CCS1*), it is downregulated under oxidative stress but upregulated in response to copper deprivation, regulating these genes in an opposing manner to fine-tune their dosage in response to abiotic conditions (Sunkar et al. 2006; Yamasaki et al. 2007; Beauclair et al. 2010).

In addition to miRNAs, other classes of small RNAs have been implicated in regulation of abiotic stress responses. For example, 21 and 24 nt natural antisense siRNAs (natsiRNAs) produced from overlapping transcripts of convergent gene pairs *SIMILAR TO RCD ONE 5* (*SRO5*) and a gene encoding Δ^1-pyrroline-5-carboxylate dehydrogenase (*P5CDH*) establish salt stress tolerance in *Arabidopsis* (Borsani et al. 2005). Interestingly, in addition to affecting gene expression *in-cis* TEs can influence stress-responsive gene expression also post-transcriptionally *in-trans* (Fig. 1.2c). This is demonstrated by the effect of TGS release of the *GYPSY*-type LTR retrotransposon *ATHILA6* following heat stress in *Arabidopsis*. Upon

transcriptional activation, 21–22 nt *trans*-acting siRNA (tasiRNA854) is produced from the *ATHILA6* locus that targets the transcript of *UBP1b*, encoding a stress granule-formation protein (McCue et al. 2012). Several other genes regulated in a similar fashion were identified, suggesting a more general mechanism of *in-trans* regulation of gene transcription following stress-induced release of TGS of TEs (McCue et al. 2013). In contrast to the traditional role in PTGS, 21 nt small RNAs in concert with AGO1 can also participate in the transcriptional activation of stress and hormone-responsive genes. AGO1 interacts with SWI3 and BSH, subunits of the SWI/SNF chromatin remodelers, and guided by the different classes of 21 nt RNAs (including miRNAs and tasiRNAs), the complex can be recruited to activate the target genes. The number of AGO1 targeted regions is enhanced by different biotic and abiotic triggers, including cold stress, and these are enriched for the stress-specific genes, indicating targeted effect (Liu et al. 2018a). In addition, in *B. distachyon*, stress-induced siRNAs target regulatory intronic regions, possibly affecting splicing (Wang et al. 2015a).

Interestingly, miRNAs can also be involved in the memory of abiotic stresses. Heat stress induces the expression of miR156, which correlates with the downregulation of miR156-targeted *SQUAMOSA PROMOTER BINDING PROTEIN–LIKE* (*SPL*) transcription factor genes. Maintained level of miR156 is required for the memory of recurring heat stress and miR156, an evolutionarily conserved miRNA in plants, may mediate crosstalk between responses to environmental conditions and plant development (Stief et al. 2014; Cui et al. 2014).

Antisense long non-coding RNAs (lncRNA) have been implicated in hyperosmotic stress response and memory (Wibowo et al. 2018) (Fig. 1.2c). Upon stress-induced hypomethylation, antisense lncRNA is transcribed from the locus encoding a metabolic sensing gene *CARBON/NITROGEN INSENSITIVE 1* (*CNI1*). This activation inversely correlates with the sense transcription of *CNI1* through a yet unknown mechanism. Interestingly, regions differentially methylated upon stress were enriched with proximal hyperosmotic stress-responsive antisense lncRNAs, indicating that this may be a more general mechanism of interplay between DNA methylation stress-response gene control (Wibowo et al. 2018).

Together, non-coding RNAs are emerging as integral components of stress response but also memory pathways. Overexpression of transgenic miRNAs has been used in *Arabidopsis* as well as crop species to successfully modulate sensitivity to various abiotic stresses (reviewed in Zhang 2015). The use of transgenic miRNAs or artificial miRNAs may present a promising approach to future crop improvement. At the same time however, more research is needed to decipher the mechanism of function of stress-response-associated miRNAs, to identify broader gene regulatory networks that are affected by the respective miRNAs and to distinguish general and tissue- or species-specific effects of miRNA expression (reviewed in Zhang 2015).

1.4.4 *Modifications of Histone–DNA Interactions by Chromatin Remodelers*

ATP-dependent chromatin remodelers use the energy of ATP hydrolysis to alter DNA–histone interactions and modify the accessibility to the genetic information. This action can have both positive and negative effects on the regulation of gene expression (reviewed in Clapier and Cairns 2009; Narlikar et al. 2013; Reyes 2014; Han et al. 2015). DNA-dependent ATPases of the SNF2 family act as the enzymatic subunit of large SWI/SNF (SWItch/Sucrose Non-Fermentable) multi-subunit complexes that have been involved in the alteration of nucleosome position and assembly (reviewed in Narlikar et al. 2013). SNF2 proteins were first discovered in yeast (Egel et al. 1984; Neigeborn and Carlson 1984), but they are conserved in animals (Flaus et al. 2006) and plants (Hu and Lai 2015; reviewed in Knizewski et al. 2008; Sarnowska et al. 2016). Plants have larger SNF2 families with more than 40 members (Flaus et al. 2006; reviewed in Knizewski et al. 2008; Hu and Lai 2015). Plant SWI/SNF complexes have been involved in the cellular reprogramming triggered by diverse abiotic stresses. Three of the four members of the SNF2 subfamily in Arabidopsis have been implicated in the regulation of stress responses and in the control of plant development upon abiotic stress. CHR12/MINUSCULE 1 (MINU1) is required to arrest plant development in response to moderate heat, salinity, or water deficiency stresses (Mlynárová et al. 2007) and the reduced germination phenotype of both *MINU1* and *CHR23/MINU2* overexpressing plants was enhanced under mild salt or mild temperature stress conditions (Leeggangers et al. 2015). BRAHMA (BRM) has an important role in the ABA-mediated post-germination growth arrest under water scarcity stress and is involved in repressing early stress responses during germination (Han et al. 2012; Peirats-Llobet et al. 2016). BRM directly represses the expression of the gene *ABA INSENSITIVE 5* (*ABI5*) and many of the phenotypes observed in *brm* mutants are due to an overactive stress response. These results also indicate the intricated link between environmental stress and development, as most of the *brm* developmental phenotypes were mitigated in the double *brm abi5* mutant. BRM direct repressive action upon *ABI5* was related with positioning of a nucleosome at the transcription start site (TSS) of this gene which may block initiation of transcription (Han et al. 2012). BRM and SWI3B, another subunit of the SWI/SNF complex in Arabidopsis (Sarnowski et al. 2002), are able to interact with HYPERSENSITIVE TO ABA1 (HAB1), a type 2C phosphatase that negatively mediates ABA signaling. However, *brm* and *swi3b* mutants showed opposite phenotypes to ABA, whereas *swi3c* mutant plants are ABA hypersensitive similarly to *brm* plants (Saez et al. 2008; Han et al. 2012). A possible scenario is that SWI3B contributes to the ABA response as part of a different complex (reviewed in Asensi-Fabado et al. 2017). BRM also interacts with ABA-activated Sucrose non-fermenting 1-Related protein Kinases (SnRKs) (Saez et al. 2008; Peirats-Llobet et al. 2016). Therefore, the current model proposes that ABA-induced phosphorylation of BRM by SnRKs and dephosphorylation by HAB1 may rapidly regulate the activity of the BRM-associated SWI/SNF complex without the requirement to evict

it from the chromatin of stress-related genes (Peirats-Llobet et al. 2016). However, BRM has been directly involved in activation and repression of many different stress-responsive genes (Archacki et al. 2016) and, therefore, more molecular data will be required to understand if phosphorylation/dephosphorylation of BRM is a rule in the transcriptional reprogramming mediated by its associated complex in response to abiotic stress conditions. In addition to ABA-mediated responses, BRM has also been involved in the regulation to heat stress through repression of a subset of heat-responsive genes (Buszewicz et al. 2016). The Rolled Fine Striped (*RFS*) gene of rice encodes a member of the Mi-2 subfamily (Cho et al. 2018). In *rfs-2* plants, essential genes involved in ROS scavenging are downregulated correlating with changes in particular histone modifications. However, it is still unknown if the changes in the covalent marks were directly or indirectly due to RFS. Although ROS accumulation occurs as a result of abiotic stresses, the possible role of RFS in the regulation of stress responses has not been elucidated yet (Cho et al. 2018). Alkaline Tolerance 1 (ALT1) is a rice protein that belongs to the Ris1 subfamily, which is a SWI2/SNF2 specific group of plants and fungi (Hu et al. 2013; reviewed in Knizewski et al. 2008). *alt1* mutants and *ALT1*-iRNA plants showed decreased sensitivity to alkaline stress probably due to an impairment of ROS homeostasis induced by this stress (Guo et al. 2014). Considering that alkaline salts have a more severe effect on crop growth and yield than neutral salts (Zhang et al. 2017), it will be very interesting to fully understand the molecular roles of plant-specific ALT1-like proteins as a way to improve plant resistance. The role of SWI2/SNF2 ATPases in the regulation of an environmental-induced molecular memory was indirectly revealed through its association to another DNA-dependent ATPase called FORGETTER 1 (FGT1) (Brzezinka et al. 2016). FGT1 is the single Arabidopsis member of a different family of chromatin remodelers called Strawberry Notch (Sno) (Majumdar et al. 1997; Brzezinka et al. 2016; Watanabe et al. 2017). Arabidopsis *fgt1* mutant plants were not able to maintain the expression of heat-shock memory genes after a heat shock stress and, subsequently, showed a decreased memory to passed heat shock events. FGT1 binds to heat-shock memory genes before application of the stress and its binding increases just after the stress correlating with an upregulation of target genes (Fig. 1.3). FGT1 interacts with BRM and CHR11 and CHR7 *in planta*. CHR11 and CHR7 are the two members of the Imitation Switch (ISWI) subfamily, which also belongs to the SNF2-group (reviewed in Knizewski et al. 2008; Li et al. 2017). In Arabidopsis, they regulate gametogenesis and have been involved in regulation of nucleosome spacing (Huanca-Mamani et al. 2005; Li et al. 2014b). Single *brm* and *chr11* mutants and the double *chr11−/−; chr7+/−* were also impaired in heat-shock acclimation and, as in *fgt1* plants, high heat-shock related gene expression was not sustained after heat shock (Brzezinka et al. 2016). Genome-wide analyses in nonstressed plants demonstrated that binding of BRM overlaps with FGT1 binding in heat-shock memory genes and transcriptional analyses had already pointing out a role of BRM in regulating the expression of heat-responsive genes (Brzezinka et al. 2016; Buszewicz et al. 2016). Considering the nature of FGT1 and its interaction with other main chromatin remodelers, an obvious possibility was that this protein acts in nucleosome remodeling at target

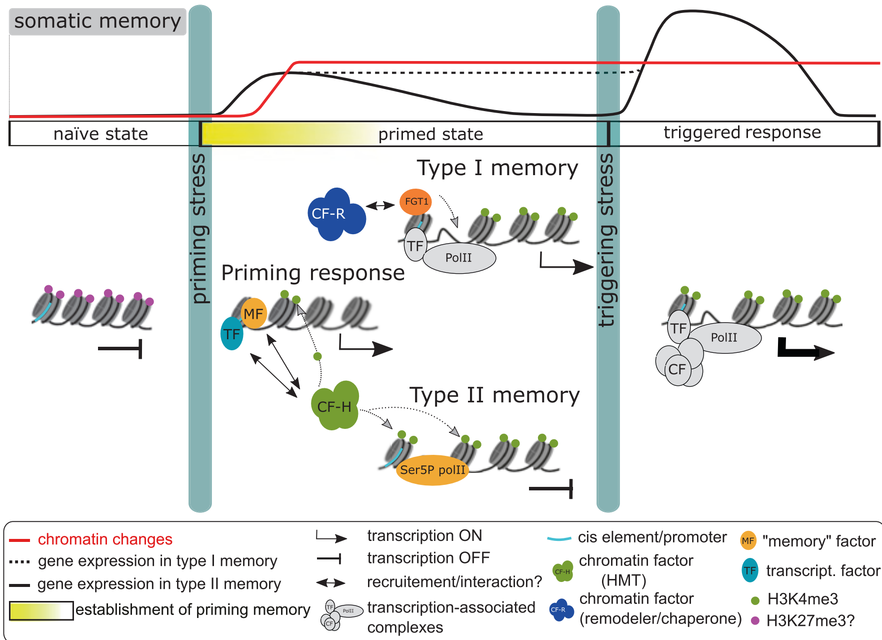

Fig. 1.3 Chromatin changes associated with transcriptional stress memory. The first encounter of stress (priming stress) induces initial transcriptional onset of stress response genes that is associated with modification of the naïve-state repressive chromatin structure towards accessible transcriptionally active structure (type I memory) or accessible transcriptionally inactive structure (poised—type II memory). Both these states predispose enhanced transcriptional response upon triggering stress. Type I memory is marked by continuous transcriptional activity of stress-response genes. This is, for example, connected to the recruitment of a putative helicase FORGETTER 1 (FGT1) to the transcription start sites (TSSs) of heat-response genes and recruitment of chromatin remodelers (CF-R, such as the SWI/SNF—Brzezinka et al. 2016). Type II memory is associated with chromatin changes that are uncoupled from elevated gene transcription. Type II memory can be mediated by different sets of factors. First, the stress-response transcription factors (TFs) that are responsible for the initial transcriptional activation during priming response. Second, the "memory factors" (MF) (such as the heat-shock factor HSFA2) Lämke et al. 2016) or the transcription factor HY5 (Feng et al. 2016) that are not required for the initial transcriptional activation but ensure the establishment and/or maintenance of heritable accessible chromatin structure, putatively by recruiting chromatin modifiers (CF-H, such as histone methyltransferase complexes—HMT). Type II memory chromatin is associated with RNA polymerase II phosphorylated on serine 5 (Ser5 pol II—Ding et al. 2012) and histone PTMs H3K4me3 but also H3K4me2 or H3ac

genes. To confirm this hypothesis, *fgt1*, *brm,* and *chr11−/−;chr7+/−* mutant plants showed an increase in nucleosomes at the TSS of heat-shock memory genes without stress conditions and in *fgt1* plants recovery of nucleosome DNA at the TSS during acclimation was faster than in WT plants. Therefore, FGT1 might be involved in poising target genes into a more easy-to-activate state and has been proposed that could act as a linker between SWI/SNF and ISWI remodeling complexes (Brzezinka et al. 2016). Why does heat-stress memory require the concerted action of different

chromatin remodelers? and are these complexes involved in the memory to other stresses? are still unaddressed questions that will require further analysis.

1.4.5 Histone Variants and Nucleosome Assembly

As one of the main components of the chromatin, histones play a very important role in transcriptional regulation. Different histone variants are described: canonical histones and other histone variants. While synthesis of canonical histones, and its incorporation to chromatin is mostly coupled to DNA replication, other histone variant genes are expressed independently of cell division and their exchange occurs in connection to transcription. The turnover of histones contributes to the regulation of gene expression enabling fast cellular reprogramming that occurs, for instance, during the response to environmental stresses (Deal and Henikoff 2011; Henikoff and Smith 2015; Jiang and Berger 2017; Talbert and Henikoff 2017). The structure and function of a nucleosome containing histone variants differs from that containing canonical histones. Further, canonical and histone variants can contribute to recruit specific proteins to chromatin (Koyama and Kurumizaka 2018). So far, most described histone variants belong to H3 and H2A families. Besides the canonical H2A, there are three other H2A variants: H2A.X and H2A.Z, also present in animals, and H2A.W, a plant-specific variant. The sequence diversity among these four H2A versions affects the DNA–histone and histone–histone interactions impacting chromatin stability (Deal and Henikoff 2011; Jiang and Berger 2017; Talbert and Henikoff 2017; Osakabe et al. 2018). H2A.Z fulfills an important role in transcriptional regulation; and, although its exact role is not fully clear yet, it seems to counteract DNA methylation (Zilberman et al. 2007). In *Arabidopsis*, H2A.X plays a role in DNA repair in euchromatic regions (Lorković et al. 2017; Osakabe et al. 2018). H2A.W is abundant at heterochromatic TE-enriched regions and acts in DNA repair at heterochromatic regions (Yelagandula et al. 2014; Lorković et al. 2017; Osakabe et al. 2018). H3.1 is the canonical H3, whereas the transcription-coupled H3.3 (Deal and Henikoff 2011; Jiang and Berger 2017; Talbert and Henikoff 2017), sperm cell-specific H3.10 (or MALE GAMETE-SPECIFIC HISTONE3, MGH3) (Okada et al. 2005; Ingouff et al. 2007, 2010), and the centromeric CenH3 (Lermontova et al. 2006; Ravi et al. 2011) are variants of H3. H3.3 is the closest related to canonical H3.1 that are differently targeted by chromatin modifying enzymes and deposited at distinct genomic loci (Stroud et al. 2012; Jacob et al. 2014). The bulk of information about histone variants comes from *Arabidopsis* and, for example, histone variants of cereals display some special features (Waterborg 1991; Shan et al. 2011; Cui et al. 2015; Hu and Lai 2015). Future studies in crops and other plant species are foreseen to reveal the conservation of histone variants and their functions in plants.

Current knowledge of the involvement of histone variants in responses to the environment comes from analyses of histone chaperone mutants. Histone chaperone complexes are usually well conserved among different organisms and are key in

depositing histones for nucleosome assembling dependently or separately of DNA replication (Deal and Henikoff 2011; Henikoff and Smith 2015; Jiang and Berger 2017; Talbert and Henikoff 2017). Among these complexes, the chromatin remodeling complex SWI2/SNF2-Related 1 (SWR1), highly conserved in eukaryotes, contributes to the replacement of H2A by H2A.Z at target genes. H2A.Z and components of the SWR1 complex modulate response to ambient temperature in monocot and eudicot plants coupling this response to developmental plasticity and yield (Kumar and Wigge 2010; Boden et al. 2013; Cortijo et al. 2017). Sensitivity of plants to increases in ambient temperature (i.e., still below stress threshold) relates to the presence of H2A.Z in specific genes. Thus, H2A.Z has been proposed as a key component of the thermosensory machinery in plants. At low temperature H2A.Z is enriched genome-wide, posing the target genes to dynamically respond to temperature increase. Upon temperature rise, the Heat Shock Factor A1 (HSFA1) family of TFs is recruited to chromatin and promotes removal of H2A.Z, increase in chromatin accessibility, and induction of transcriptional activation of temperature-responsive genes (Kumar and Wigge 2010; Cortijo et al. 2017). In addition to this role in perception of an environmental cue, H2A.Z also has a role in response to heat stress that is particularly harmful during seed development, strongly impacting plant yield. Using *B. distachyon*, Boden et al. (2013) showed a differential sensitivity of vegetative and reproductive organs in response to moderate heat stress that was associated with higher thermostability of H2A.Z nucleosomes in vegetative tissue and the transcriptome of *SWR1-RNAi* grains resembled that of wild-type grains under high temperatures (Boden et al. 2013). H2A.Z may also play a role in response to nutrient starvation. In SWR1 complex mutant plants, the decrease of H2A.Z in specific genes mimicked the phenotypes of plants grown under low Pi concentrations (Zahraeifard et al. 2018). In *Arabidopsis*, depletion of H2A.Z-containing nucleosomes from the chromatin of the *AtMYB44* gene has been correlated with the strong induction of this gene in plants grown under salt stress conditions (Nguyen and Cheong 2018). *h2a.z* mutants showed genome-wide misregulation of stress-responsive genes, indicating a broad contribution of H2A.Z to stress responses (Coleman-Derr and Zilberman 2012) and, indeed, in response to stress a genome-wide decrease of H2A.Z is observed (Kumar and Wigge 2010; Sura et al. 2017). In addition to the H2A.Z variant, a wheat histone variant called TaH2A.7, closely related to H2A.W of *Arabidopsis*, plays a specific role in drought tolerance. The expression of *TaH2A.7* is strongly induced under drought conditions and when overexpressed in *Arabidopsis* increases drought resistance (Xu et al. 2016). Therefore, further analyses in other plant species will be required to fully understand the roles of H2A.W-like variants in response to water deficiency. Another group of histone chaperone involved in stress responses is the Nucleosome Assembly Protein (NAP)-family. NAPs work as H2A and H2B chaperones, but are also involved in the assembly of H3/H4 dimers into the nucleosome and in assisting SWR1 in the deposition of H2A.Z (Dong et al. 2003; Jiang and Berger 2017). *Arabidopsis* mutants affected in the three constitutive *NAP1* genes were affected in their response to salt stress (Liu et al. 2009). *OsNAPL6*, proposed to encode a H3/H4 specific chaperone, has been related to resistance to different stresses in rice and

when overexpressed results in biomass and yield improvement under drought or salinity stress (Tripathi et al. 2016).

Three histone variants for the linker H1 (H1.1, H1.2, and H1.3) have been described and, among them, H1.3 seems to play a prominent role in the regulation of stress responses. H1.3 protein has shorter N- and C-termini which may affect its interaction with DNA (Over and Michaels 2014; Jiang and Berger 2017). Water deficiency induces the expression of the *His1.3* gene in *Arabidopsis*, levant cotton (*Gossypium herbaceum* L.), and tomato (Ascenzi and Gantt 1997; Scippa et al. 2000, 2003; Trivedi et al. 2012), but, interestingly, not in tobacco (*Nicotiana tabacum* L.—Przewloka et al. 2002). Low light conditions alone or in combination with water deficiency also increases expression of *His1.3* in *Arabidopsis* and lack of H1.3 impairs developmental adaptations to these combined stresses (Rutowicz et al. 2015). ABA-mediated induction of the *His1.3* gene during stress signaling has been proposed (Cohen et al. 1991; Bray et al. 1999). Although still not fully clear, H1.3 may play a role during the response to stress by competing with other H1 variants and destabilizing chromatin structure (Rutowicz et al. 2015).

Despite the demonstrated role of histone variants in immediate stress response, there is not much evidence connecting them to stress memory. Several examples nevertheless suggest involvement of histone variants and their chaperones in memory. ANTI-SILENCING FUNCTION 1 (ASF1) is a conserved histone H3-H4 chaperone that has been associated with both the sensitivity and memory of heat stress in *Arabidopsis*. Double mutant affected in the two orthologous *Arabidopsis ASF1* genes, *AtASF1a* and *AtASF1b*, was more sensitive to a first priming heat stress shock than wild-type plants and showed decreased priming response to a second more severe heat shock. Interestingly, the impairment of heat priming was more pronounced under long recovery lag periods, indicating a role of AtASF1 in memory maintenance. Expression and chromatin analyses demonstrated that AtASF1A/B may induce nucleosome disassembly at specific *Heat Shock Factor* (*HSF*) genes upon priming for heat stress, which creates an acclimated chromatin environment for higher and faster induction of the stress-responsive genes after a second stress treatment (Weng et al. 2014). In *Arabidopsis*, the H3.3 chaperone Histone Regulator A (HIRA) partially compensates the loss of CAF-1 (Duc et al. 2015; Muñoz-Viana et al. 2017) and it is plausible that other plant histone variants and chaperones aside ASF1 and CAF-1 play a role in the inheritance of transcriptional states. For instance, BRUSHY (BRU1)/TONSOKU/MGOUN3 is a nuclear protein of unknown molecular function that has been related not only to DNA damage repair, but also to stable transmission of chromatin and transcriptional states (Takeda et al. 2004; Brzezinka et al. 2018). *bru1* mutant developmental phenotypes resembled mutants affected in components of the DNA replication-coupled histone H3/H4 chaperone complex CAF-1 (Takeda et al. 2004). Furthermore, *bru1* mutants show defects in the maintenance of thermotolerance, a function that may be independent of CAF-1 and its activity on DNA repair (Brzezinka et al. 2018). Although it has been suggested that BRU1 could be involved in the inheritance of chromatin structure from mother to daughter cells through DNA replication, further evidence will be necessary to support this model (Takeda et al. 2004; Brzezinka et al. 2018).

1.4.6 *Histone Modifications*

Histone PTMs have a key role in regulating gene expression. Mainly N-terminal histone tail PTMs are currently implicated in transcription—some PTMs act as marks for transcriptional activation and others for repression (Zhao and Garcia 2015) but for some, the function in transcriptional modulation is still unclear. Histone PTMs affect chromatin structure (1) by altering nucleosome compaction and (2) by mediating or interfering with the recruitment of other proteins to the chromatin, among them chromatin remodelers (Bannister and Kouzarides 2011; Lawrence et al. 2016). PTMs can be highly dynamic or stably transmitted through cell division and multiple proteins involved in adding, removing, and reading them have been described (Hyun et al. 2017; Zhao et al. 2018). A single amino acid residue can be modified by different number of groups (e.g., lysine mono-, di- or trimethylation) or PTM types (e.g., lysine methylation and acetylation) and different PTMs can co-exist on the same histone or on different histones of the same nucleosome. In summary, all these possibilities add an extraordinary level of complexity to histone PTMs and their impact on gene expression (Bannister and Kouzarides 2011; Zhao and Garcia 2015; Lawrence et al. 2016).

1.4.6.1 Acetylation and Deacetylation of Histones During Responses to Abiotic Stress

The activity of histone acetyltransferases (HATs) and deacetylases (HDACs) is significantly involved in the responses to different abiotic stresses (Kim et al. 2015; Luo et al. 2017; Lämke and Bäurle 2017). Acetylation is a highly dynamic PTM and therefore chromatin of stress-related genes whose acetylation changed in response to abiotic stress may rapidly recover its naïve state (Kim et al. 2012; Friedrich et al. 2018). Histone acetylation is usually correlated with transcriptional activation and histone deacetylation with gene repression (Loidl 1994).

Transcriptomic analysis in different plant species indicates that HAT genes expression is regulated by ABA, salinity or drought stresses (i.e., rice (Fang et al. 2014); barley (Papaefthimiou et al. 2010); maize (Li et al. 2014a)). In *Arabidopsis* plants under drought stress a gradual increase in H3 acetylation in stress-responsive loci correlated with enhanced transcription (Kim et al. 2008). In addition, ADA2b, a putative component of the GENERAL CONTROL NON-REPRESSED PROTEIN 5 (GCN5) HAT complex, may also act in the response to high salt (Kaldis et al. 2011). Strikingly, it has been recently shown that under salinity stress GCN5 directly binds and increases H3 and H4 acetylation at genes involved in the biosynthesis of cell wall components and, hence, contributes to cell integrity under high salt conditions (Zheng et al. 2018). In peanut (*Arachis hypogaea*) activation of *AhDREB1*, an APETALA2/Ethylene Respond Factor (AP2/ERF2) involved in the activation of different stress pathways through acetylation under osmotic stress, has been proposed to enhance drought resistance (Zhang et al. 2018b).

GCN5 and ADA2b are also involved in the activation of *COLD-REGULATED* (*COR*) genes (Vlachonasios et al. 2003). A more recent paper from the same group showed that although cold-triggered induction of *COR* relies on GCN5 and ADA2b and correlates with elevated acetylation, the chromatin changes did not depend on ADA2b or GCN5 (Pavangadkar et al. 2010). This suggests that other HATs may be involved in this process. Increase in histone acetylation together with expression of specific stress-related genes has been shown to be induced by cold stress in rice and maize and specifically at repetitive sequences in maize (Hu et al. 2011, 2012; Roy et al. 2014). Considering that HATs are well conserved in different plant species, orthologs of these proteins could serve similar functions in crop species (Pandey et al. 2002; Aquea et al. 2010; Papaefthimiou et al. 2010; Liu et al. 2012; Aiese Cigliano et al. 2013; Fang et al. 2014).

In *Arabidopsis*, HATs mediate response to UV-B light (Fina et al. 2017), correlating with histone hyperacetylation at targets of the UV-B photoreceptor UVR8 (Velanis et al. 2016). *Arabidopsis* ASF1A/B has been implicated in H3K56ac coupled to nucleosome removal and stalled RNA PolII at specific *HEAT SHOCK PROTEIN* (*HSP*) loci under heat stress in *Arabidopsis* (Weng et al. 2014). Histone acetylation is also coupled to waterlogging stress response in rice (Tsuji et al. 2006).

HDACs are also very well conserved in different plant species (Luo et al. 2017) and can be induced (e.g., in barley—Demetriou et al. 2009) or repressed (e.g., in *Arabidopsis*—Sridha and Wu 2006; Luo et al. 2012a, b—or in rice—Fu et al. 2007) by ABA and salt. For instance, one of the HDACs in rice, the HDAC OsSRT701, modified H3K9ac levels on stress genes, whereas overexpression of *OsHDT701*, which encodes for another HDAC, increases resistance to high salinity and drought and overexpression of *OsHDA705*, results in lower resistance to salt and ABA (Zhong et al. 2013; Zhao et al. 2015, 2016b). *HD2C* overexpression in *Arabidopsis* has been related with drought and salinity stress resistance (Sridha and Wu 2006; Luo et al. 2012a). *HD2D* overexpression also conferred higher resistance to drought, high salt stresses and cold in *Arabidopsis* (Han et al. 2016). HDA9 may repress the response to high salt and drought, as in the *hda9* mutant many drought-related genes were upregulated and showed increased H3K9ac at the promoter of salt and drought-responsive genes (Zheng et al. 2016; Chen et al. 2016; Kim et al. 2016). *Arabidopsis* HDC1 interacts with HDA6 and HDA19 to form a putative complex able to deacetylate H3 in vitro (Perrella et al. 2013). Overexpression of *HDC1* reduces sensitivity to high salt and ABA reducing H3 acetylation levels and expression of salinity stress-related genes (Perrella et al. 2013). On the contrary, mutations in *HDA6*, *HDA19*, or *HDC1* result in plants more sensitive to high salt and ABA and decreased expression of salinity stress genes (Chen et al. 2010; Chen and Wu 2010; Perrella et al. 2013). HDA6 was also shown to play a role in drought tolerance (Kim et al. 2017), whereas HDA19 could help to increase plant resistance to different abiotic stresses (Ueda et al. 2018). In addition, HDA19 and HDC1 interact with MULTICOPY SUPPRESSOR OF IRA 1 (MSI1) (Derkacheva et al. 2013; Mehdi et al. 2015), which is a subunit of at least two different complexes, CAF-1 and POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) (Hennig et al. 2005). The HDA19-MSI1 complex reduces the level of H3 acetylation at ABA receptor genes

(Mehdi et al. 2015). MSI1 has also been shown to repress ABA responses in plants and its downregulation to enhance drought tolerance. The role of MSI1 in drought tolerance is probably independent of PRC2 and CAF1 (Alexandre et al. 2009). Thus, these results highlight the flexibility of chromatin-related proteins to form part of different complexes and contribute to different outputs.

HDA6 has also been related to the response to cold stress by freezing temperatures (To et al. 2011). HDA9 and its interactor POWERDRESS (PWR) have been involved in regulating developmental changes under high temperatures. PWR was required to promote H3K9ac in thermomorphogenic genes. In addition, transcriptional analyses demonstrated a link between PWR and H2A.Z (Tasset et al. 2018). Another example of interplay is the interaction between HD2C and SWI/SNF complex in the regulation of the response to heat stress (Buszewicz et al. 2016). HOS15 (for high expression of osmotically responsive genes) interacts with HD2C and together directly represses the expression of *COR* genes. Low temperatures induce HOS15-dependent HD2C degradation, probably through the recruitment of an E3 ligase complex, and decreases H3 acetylation of *COR* genes (Park et al. 2018). In maize cold treatment promotes the decrease of different acetylation marks correlating with upregulation of several HDACs (Hu et al. 2011). Although expression patterns of HDACs under abiotic stresses have also been analyzed in other crops (Luo et al. 2017), further data will be required to link these changes with transcriptional changes due to acetylation status of stress-related genes.

1.4.6.2 Methylation and Demethylation of Histones During Responses to Abiotic Stress

Although methylation can occur on any histone, the bulk of our knowledge for the role of this PTM in the modification of gene expression comes from the methylation of a few H3 residues (i.e., K4, K9, K27, and K36). The impact of histone methylation on transcription varies: H3K4me3, H3K9me3, and H3K36me3 are involved in activation; H3K27me3 switches off genes; H3K9me2 and H3K27me1 are enriched in stably repressed regions and usually related to DNA methylation (Roudier et al. 2011; Sequeira-Mendes et al. 2014). Histone methyltransferases (HMTs) are responsible for adding this PTM and histone demethylases (HDMs) for its active removal (Hyun et al. 2017). Therefore, methylation of histones is also a dynamic mark, despite showing a slower turnover than histone acetylation (Asensi-Fabado et al. 2017; Hyun et al. 2017), which may affect relative contribution to memory by these two PTMs.

Most HMTs are characterized by the presence of a conserved SET (Su(var), E(z), and Thritorax) domain that has allowed the identification of components of this family in different plant species, including important crops (Ng et al. 2007; Pontvianne et al. 2010; Aquea et al. 2011; Huang et al. 2011, 2016; Lei et al. 2012; Yadav et al. 2016). In maize, class V of *SET* genes, which is related to H3K4 methylation, was differentially expressed in response to osmotic stress (Qian et al. 2014). Analysis of the SET family in foxtail millet (*Setaria italica*), a millet crop cultivated

in arid regions and highly resistant to stress, demonstrated that most *SiSET* genes were upregulated under cold stress and several also responded to salt and dehydration stress (Yadav et al. 2016). In *Gossypium raimondii*, a putative contributor to allotetraploid cotton, three *SET* genes (*GrKMT1A;1α*, *GrKMT3;3,* and *GrKMT6B;1*) were induced by heat stress (Huang et al. 2016).

Arabidopsis plants subjected to drought stress showed an increase of H3K4me3 coupled to transcriptional activation on specific drought-stress related genes. Enrichment of this PTM followed RNA polymerase II (RNA Pol II) accumulation, indicating that in this case H3K4me3 was probably a consequence of high transcriptional rate (Kim et al. 2008). Although H3K4me3 decreased on stress genes during rehydration, low levels of this mark still above naïve levels were maintained. These results indicated a role of H3K4me3 as epigenetic mark for drought-responsive genes (Kim et al. 2012).

Genome-wide analyses of histone PTMs in *Arabidopsis* subjected to water deprivation showed changes in H3K4me3 enrichment that correlated with gene expression levels. However, stress-responsive genes showed broader H3K4me3 distribution not only after applying the stress, but also under unstressed conditions (van Dijk et al. 2010). The meaning of the differential H3K4me3 distribution along the bodies of stress-responsive genes is unknown, but it is tempting to speculate that it may mark specific stress genes for a prompt response under inductive conditions. Genome-wide analyses of rice plants grown under control and water scarcity conditions showed that only a small percentage of genes that showed different H3K4me3 enrichment under stress was differentially expressed. Therefore, changes in this mark were not necessarily coupled to changes in gene expression. As expected however, in stress-responsive genes that showed a change in both H3K4me3 and expression, the increase in H3K4me3 positively correlated with expression levels (Zong et al. 2013). Upon waterlogging in rice, changes in PTMs, including increase in H3K4me3, correlated with gene activation (Tsuji et al. 2006). Hence, these changes in PTMs during waterlogging may mirror transcriptional state of stress-responsive genes and are unlikely to be involved in priming.

ARABIDOPSIS HOMOLOG OF TRITHORAX1 (ATX1) is a HMT involved in the deposition of H3K4me3 and is key in the regulation of drought- and ABA-related genes (Ding et al. 2009, 2011). *atx1* mutants are hypersensitive to water deficiency (Ding et al. 2009, 2011) and show decreased ABA levels under both naïve and water stress (Ding et al. 2011). ATX1 binds to *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCDE3*), which plays an important role in ABA biosynthesis (Qin and Zeevaart 1999), and its binding to this gene increases under drought stress correlating with higher H3K4me3. Expression analyses showed that ATX1 is involved in both activation of ABA-dependent and -independent dehydration stress genes through H3K4me3 enrichment, but that may not be the only HMT involved in this regulation (Ding et al. 2011). ATX1 belongs to class III SET domain proteins, with ATX2 being its closest homolog (Pontvianne et al. 2010). It will be very interesting to see in future works whether ATX1 homologs also play a role in stress responses. *Arabidopsis* JMJ15, a Jumonji-family HDM, mediates response to salt stress. Overexpression of *JMJ15* increases salt resistance and leads

to downregulation of H3K4me2/me3 marked genes, many of which are stress-related genes (Shen et al. 2014). JMJ and other HDM proteins are well conserved in plants (Zhou and Ma 2008; Qian et al. 2015). However, our understanding of HDMs is limited and future works will be paramount to fully understand the activity of these proteins in different processes such as responses to stress.

In addition to H3 methylation, H4R3 symmetric dimethylation (H3K4sme2) has been shown to act as a repressive mark for gene expression during stress responses in *Arabidopsis*. Specifically, H4R3sme2 plays a role in calcium signaling in the response to drought. CALCIUM UNDERACCUMULATION 1 (CAU1)/PROTEIN ARGININE METHYLTRANSFERASE 5 (PRMT5)/Shk1 BINDING PROTEIN 1 (SKB1) is a H4R3 HMT able to directly repress the expression of *CALCIUM SENSOR* (*CAS*) that acts in detection of external cellular calcium. Increase of external calcium concentration induces a reduction in CAU1 and subsequently reduction of H4R3sme2 at CAS and activation of this gene. This cascade results in stomata closure to reduce water-loss through transpiration (Fu et al. 2013). A very recent paper from the same lab has shown a CAU1/PRMT5/SKB1 novel activity independent of CAS, which also promotes drought resistance indirectly inducing accumulation of proline (Fu et al. 2018). PRMTs are well conserved in rice and OsPRMT5 can also induce H3K4sme2 in vitro (Ahmad et al. 2011); therefore, a plausible hypothesis is that the molecular activity of this protein in dehydration tolerance is also conserved in crops.

1.4.6.3 Other PTMs Involved in Abiotic Stress Responses

Compared to acetylation and methylation, our knowledge about the role of other PTMs in gene expression and, in particular, in transcriptional regulation under stress conditions is limited. For instance, proteomic analyses of sumoylated proteins under different conditions indicated that heat stress induces H2B sumoylation. In addition, other important chromatin-related factors (e.g., SWI/SNF components, HMTs, GCN5, etc.) were also sumoylated after stress, suggesting that sumoylation may develop a more complex role in the regulation of chromatin-mediated stress responses (Miller et al. 2010).

In *Arabidopsis*, *HISTONE MONOUBIQUITINATION 1* and *2* (*HUB1/2*), which encode C3HC4 RING-type E3 ubiquitin ligases responsible for H2Bub1 (Cao et al. 2008), were induced under salt stress. Single *hub1* and *hub2* mutants and the respective double mutant were intolerant to high salt. H2Bub1 is required for microtubule de-polymerization in response to high salt stress through the activation of genes that mediate in this process. In addition, H2Bub1 was also required for activation of *MAP KINASE PHOSPHATE 3* and *6* (*MAP 3/6*) genes that play a central role in stress signaling (Zhou et al. 2017). Recently, the overexpression of *Arabidopsis HUB2* in transgenic cotton (*Gossypium hirsutum*) increased the performance of plants under drought conditions through H2Bub1 of drought-responsive genes, while decreased expression of *GsHUB2* increased water deficiency intolerance (Chen et al. 2018).

H3T3 phosphorylation (H3T3ph) levels increased in pericentromeric regions under drought stress and mutants affected in the kinases that phosphorylate this residue were sensitive to osmotic stress (Wang et al. 2015c). Using *Arabidopsis* and tobacco cell lines, Sokol et al. demonstrated that application of cold or salinity stress transiently induced H3S10ph in both cell types. This increase was followed up by H3 and H4 acetylation. These chromatin changes correlated with differential expression of stress-related genes (Sokol et al. 2007).

In summary, these data highlight not only the importance of other PTMs in defining stress responses, but also how far we still are to have the full picture of the contribution of PTMs to the capacity of plants to adapt to the environment.

1.4.6.4 Histone Modifications and Memory

At present, histone PTMs have mainly been implicated in somatic rather than intergenerational memory of abiotic stresses. Chromatin-related mechanisms of stress-induced gene transcription memory are subject of intense research (for recent reviews, see Bäurle 2017; Lämke and Bäurle 2017; Friedrich et al. 2018). Currently available results indicate that histone PTM-associated transcriptional memory is of relatively short duration in the range of 3–10 days following the initial abiotic stress treatment (Ding et al. 2012; Sani et al. 2013; Singh et al. 2014; Lämke et al. 2016). Most studies that focus on molecular mechanisms of transcriptional memory were performed using *Arabidopsis* seedlings, in which the range of several days may nevertheless represent a substantial number of cell divisions, indicating that histone PTMs can be mitotically transmitted and contribute to improved stress tolerance under conditions of recurring stress (Lämke and Bäurle 2017). Molecular mechanism of vernalization in *Arabidopsis* provides a well-studied example of how histone PTM-based chromatin state can be stably transmitted somatically and, if not reset, even inter-generationally. *FLC* encodes a MADS-box transcription factor serving as flowering repressor in *Arabidopsis*. During vernalization, upon sufficient time of cold treatment, repressive chromatin marked by the PRC2-deposited H3K27me3 forms at the *FLC* locus (Michaels 1999; Sheldon 2000; Bastow et al. 2004; De Lucia et al. 2008). The repressive chromatin is then transmitted during mitotic cell divisions for the rest of the plant life regardless of ambient temperature. Permissive chromatin state at *FLC* is only reset during embryogenesis by active removal of H3K27me3 mediated by the histone demethylase EARLY FLOWERING 6 (ELF6) which enables *FLC* activation and inhibition of precocious flowering in the next generation (Sheldon et al. 2008; Choi et al. 2009; Crevillen et al. 2014). The fact that in the absence of ELF6 the elevated amount of H3K27me3 is maintained inter-generationally (Crevillen et al. 2014) suggests that, in the lack of active resetting mechanisms, there is potential for sexual transmission of repressive histone PTMs.

Such a long-lasting somatic memory has not been observed in connection to abiotic stress and most examples come from transcriptional activation rather than repression of stress-responsive genes. Somatic stress memory can be mediated by

sustained expression of stress-responsive genes (type I memory—Figs. 1.1 and 1.3; reviewed in Bäurle 2017). An example is provided by the *HSP* gene *Hsa32* that is induced by heat in several plant species including *Arabidopsis*, rice (Charng et al. 2006a) and tomato (Liu et al. 2006). In contrast to other *HSP* genes (Scharf et al. 2012) whose expression declines quickly after the immediate stress response, the transcription of *Hsa32* declines at a slower rate. Hsa32 is not required for the immediate stress response but it is needed for the retention of acquired thermotolerance in plants after 2–3 days following a first heat exposure (Charng et al. 2006b). Similar to *Hsa32*, 40 other genes were identified in *Arabidopsis* whose transcription is induced following heat stress and remains elevated for another 2–3 days in the absence of the stress, defining a set of heat-stress memory genes (Stief et al. 2014).

Abiotic stress transcriptional memory can also be independent of continuously elevated transcriptional activity of genes, but initial transcriptional activation may induce a state that allows for altered gene activation upon repeated exposure to stress (type II memory—Figs. 1.1 and 1.3; reviewed in Bäurle 2017). Most often, memory of active transcription seems connected with the maintenance of elevated levels of H3K4me3 (Ding et al. 2012; Lämke et al. 2016), which correlates not only with the duration of heat-stress memory (Liu et al. 2018b) but also with H3K9 acetylation and H3K4me2 (Singh et al. 2014; Lämke et al. 2016; Liu et al. 2018b). High H3K4me3 occupancy and presence of RNA Pol II phosphorylated on serine 5 (Ser5P Pol II—PTM-associated with transcriptional initiation/early elongation and stalling of RNA Pol II) marks several dehydration-responsive genes in *Arabidopsis* that display higher transcriptional activity upon repeated exposure to dehydration but are not upregulated during periods of stress recovery (rehydration) (Ding et al. 2012). These were termed “trainable genes” and using the same experimental setup, two genome-wide studies identified extended sets of dehydration memory genes that displayed progressive up- or downregulation, loss of induction or loss of repression in response to repeated dehydration stress in *Arabidopsis* and in maize (Ding et al. 2013, 2014). While the memory of transcriptional activity correlated with high H3K4me3 and Ser5P Pol II (Ding et al. 2012; Liu et al. 2014b), the mechanisms and histone PTMs imposing memory of repression remain less clear. Similarly, heat-responsive memory genes in *Arabidopsis* are marked by increased levels of not only H3K4me3, but also H3K4me2 and H3K9ac (Lämke et al. 2016; Liu et al. 2018b). Chromatin at these genes is established by the action of the heat shock transcription factor HFSA2 (Lämke et al. 2016). HFSA2 is activated by heat but, similarly to Hsa32, it is not required for the immediate response to heat but for the retention of thermotolerance (Charng et al. 2006a). HSFA2 transiently binds to the promoter of the heat-stress responsive genes at early timepoints after exposure to heat stress but is dissociated from the promoters at later timepoints when elevated levels of H3K4me2/3 and transcription of the target genes persist. HFSA2 was therefore proposed to act as a “hit-and-run” transcription factor that is not required for the initial transcriptional activation, but recruits chromatin modifiers and promotes the establishment of active PTMs that are retained even after HFSA2 has dissociated from the locus (Lämke et al. 2016). High levels of H3Kac, H3K4me2, and H3K4me3 also mark cold, heat, or salt-primed genes (Singh et al. 2014) and retention of ele-

vated H3K4me3 is required for salt stress memory (Feng et al. 2016). During salt stress, the proline biosynthetic enzyme-encoding gene *P5CS1* is activated and the locus is marked by sustained high levels of H3K4me3 even during stress recovery. Interestingly, the maintenance of H3K4me3 during the recovery phase—but not the initial salt-induced transcriptional activation of *P5CS1*—requires light-dependent binding of the transcription factor ELONGATED HYPOCOTYL 5 (HY5) to a C/A-box element in the promoter of *P5CS1* that was found to be essential for the stress memory. These results support a model where the salt-responsive transcription factor mediates the initial stress-related gene activation but light-dependent HY5-mediated recruitment of H3K4me3 histone methyltransferase mediates the maintenance of the chromatin state as a part of the memory (Feng et al. 2016). Similar uncoupling of stress-response gene activation and memory was observed in the case of the drought-response gene *RD29B*, which depends on ABA-response binding factors (ABFs) for the priming (transcriptional memory) but also requires additional factors for its repeated induction (Ding et al. 2012; Virlouvet et al. 2014). This suggests that combination of environmental cues (light and salt stress) and distinct transcription factors may be implemented in initial gene activation, stress memory, and repeated induction of the genes.

Apart from gaining chromatin marks associated with accessible chromatin structure, genes that display enhanced activation upon stress exposure in primed plants may also lose repressive PTMs (Sani et al. 2013). Genome-wide profiling of several histone PTMs (H3K4me2, H3K4me3, H3K9me2, and H3K27me3) in roots of *Arabidopsis* seedlings primed by mild hyperosmotic treatment revealed shortening and fragmentation of H3K27me3 regions and limited changes to other PTMs, which were not globally reflected by changes in gene expression. Changes to H3K27me3 distribution persisted for another 10 days after the stress suggesting mitotic inheritance of the modified chromatin. The priming treatment enhanced the plant tolerance to subsequent stress exposure and the reduction of H3K27me3 in primed plants corresponded to elevated transcription of the root sodium transporter gene *HKT1* during subsequent stress exposure (Sani et al. 2013), suggesting direct functional connection between level of H3K27me3 and primed state.

In summary, abiotic stress memory mediated by histone PTMs seems to have a limited duration of several days. It is often associated with elevated levels of H3K4me3 that persist even during stress recovery and correlate with the duration of the memory. Histone methylation may be a PTM suitable to contribute to memory of past stress events considering its relatively slow turnover compared to other highly dynamic modifications such as acetylation that also commonly marks memory genes. Initial stress-response gene activation and establishment and/or maintenance of memory may require cooperation of distinct factors for full execution. It remains to be determined whether stress-induced transcriptional activation and memory establishment are two separated molecular phases governed by different subsets of factors in general, or whether different modes of memory establishment and maintenance exist.

1.5 Summary and Perspectives

Ongoing climate change and quickly growing world's population represent challenges for sustainability of agriculture and food production in many, often underdeveloped, regions (Adams et al. 1998; Mendelsohn 2008). The climatic and the demographic models predict even a greater challenge in the future (Tol 2018). Plants are one of the important factors that can help to mitigate negative effects of the climatic changes by reducing weather extremes and binding atmospheric CO_2. Furthermore, plants feed the world as the major source of carbon and energy for humans and domestic animals (Conway and Toenniessen 1999; Borlaug 1997). Therefore, understanding plant stress responses and developing new strategies allowing sustainable agricultural production under wide range of less predictable conditions is one of the big challenges in plant biology. In order to succeed, the strategies will need to involve a battery of measures, including modifications of the farming style and breeding new crop varieties with high yield under stress. Many breeding strategies are being discussed, ranging from the classical breeding to the possible use of genome editing techniques (Moose and Mumm 2008; Belhaj et al. 2015; Bortesi and Fischer 2015).

One way to prepare plants for the new challenges could be through the epigenetic modifications of DNA and histones in a transient or permanent manner and resulting in memory. Stress responses are accompanied by changes in gene expression, many of which were shown to be underlined by modifications at the chromatin level (reviewed in Chinnusamy and Zhu 2009; Iwasaki and Paszkowski 2014b; Avramova 2015). Most of the chromatin changes appear short lived. However, there are some which can last for days and in some cases even into the next generation(s). There is an ongoing discussion to what extent these changes could be influenced by other experimental factors, whether they are stochastic or specific response to stresses (Pecinka and Mittelsten Scheid 2012; Iwasaki and Paszkowski 2014b; Quadrana and Colot 2016; Ganguly et al. 2017). Evidence exists for both possibilities. Well-documented cases of beneficial plant memory include priming, which allows to prepare plants for the future stress conditions by application of a lower stress dose and/or activation of stress defense pathways by, e.g., chemical treatment (reviewed in Conrath et al. 2015; Bäurle 2016). Priming is already applied in agriculture and helps reducing economic losses. We foresee that the development of new priming methods and/or understanding molecular basis of priming will open new possibilities towards plant protection and can reduce economic losses due to stress. However, application of a recurrent stress during several generations does not always correlate with improved plant vigor and, therefore, memory may be only linked to specific traits (Ganguly et al. 2017).

It is very attractive to think about transgenerational reprogramming of plants to withstand many types of stress. However, success of such attempts is greatly limited by the endogenous machineries resetting any changes to the basic (pre-stress) situation through the checkpoint centers localized in the meristematic tissues and reproductive organs (Baubec et al. 2014; Iwasaki 2015). One possible way how a plant

could maintain epigenetic changes is through vegetative propagation. In addition, successful transmission of gained activation can be hampered for some loci upon genetic interaction with the repressed allele as demonstrated in multiple examples of paramutation (reviewed in Chandler and Stam 2004; Chandler and Alleman 2008). Evolutionary significance of (trans)generational stress memory is unknown. On the one hand, exposure to periodically occurring stress probably led to evolution of specific mechanisms, which became part of plant developmental program and possibly allowed colonizing new niches. The best described example is vernalization, which involves extensive and complex epigenetic regulation by multiple epigenetic pathways (reviewed in Song et al. 2012a). On the other hand, many stresses occur stochastically and it is impossible to predict them even using modern monitoring methods. Furthermore, multiple studies demonstrated that activation of stress response pathways requires energy and slows down growth, and, in case of severe and/or long-lasting stress, also yield (Fig. 1.1; reviewed in, e.g., Bechtold and Field 2018). Prophylactic long-term activation of the stress memory may lead to selective disadvantage compared to the less sensitive peers. Therefore, plants may constantly search for a balance between too little and too much stress responses. Epigenetic regulation is a perfect candidate, which could control both the duration and the amplitude of such response (reviewed in Lämke and Bäurle 2017). In addition, stress-induced epigenetic variation among individuals and their offspring may represent a bet-hedging strategy, where the variation increases the chances that at least some of the individuals will be programmed in the right way.

At present, most stresses are applied separately and in high doses in laboratory conditions. This is a perfect approach to pin down components of individual pathways and to understand their functions. However, such experimental setups may be very different from natural conditions where stresses occur in lower doses for longer time (chronic stress) and often in combinations, e.g. heat, drought, and high UV during a hot summer day. Therefore, understanding combinatorial effects of multiple stresses applied in natural-like conditions on plant performance, physiology, and epigenomes remains to be deciphered. It is also clear that the studies using different species may give different answers. While most of the early and also current information on plant memory comes from *Arabidopsis*, which allows accurate and fast testing of many hypotheses and still is essential in this aspect, not all trends could be confirmed in other species and various crops show also new epigenetic phenomena (Chandler and Stam 2004; Jablonka and Raz 2009; Quadrana and Colot 2016).

The toolbox for analysis of plant epigenetic changes and memory contains a continuously growing number of tools (Spillane and McKeown 2014). Recently, multiple ultrasensitive methods for analyzing transcriptome, DNA methylation, histone modifications and variants, chromatin packaging, etc. have been developed and can be directly applied to any species with existing genome assembly. Furthermore, the ongoing boom of the new technologies for genome editing offers great possibilities for modifications of the systems towards epigenome editing or directing specific modifications into the genomic regions of interest (Belhaj et al. 2015; Puchta 2015, 2017). The most promising approach is based on the CRISPR system, where Cas9 nuclease is guided by a specific RNA molecule to the target locus containing homol-

ogous sequence (Herrmann et al. 2015). Upon removing Cas9 nuclease activity and fusing Cas9 with epigenetic modifiers, potentially any genomic region could be targeted with chromatin modifications of interest (Gallego-Bartolomé et al. 2018). Current systems require stable transformation of the fusion construct. It is foreseeable that the development will be directed towards transient transformation systems, and even delivery of the ready-made modifier proteins, which will speed up the whole process for induction of epigenetic variation in plants. Along with these technical advancements, both the risks (if any) and the benefits in use of such systems need to be discussed with the public. General acceptance of the new technologies is an essential step, which needs to be achieved before applying such methods in agricultural production.

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Chapter 2
Plant Epigenetic Mechanisms in Response to Biotic Stress

Michael R. Roberts and Ana López Sánchez

Abstract The environment changes faster than the ability of genetic mutation and recombination to generate natural genetic diversity. In this context, epigenetic regulation of gene expression has the potential to provide organisms with an alternative mechanism for phenotypic variation by controlling the extent of plasticity that can be achieved in response to environmental changes. There is now substantial evidence suggesting roles for epigenetic regulation of several different aspects of the plant response to biotic stress. At the basic level of gene expression, posttranscriptional gene silencing mediated by small RNAs and chromatin remodelling controlling transcriptional gene silencing are essential for the induced resistance responses activated during pest and pathogen attack. Beyond this, there is also evidence that histone modifications and DNA methylation are associated with immune memory, or defence priming, such as systemic acquired resistance (SAR). In addition, recent evidence indicates that epigenetic modifications can also generate longer-term defence priming responses that can be inherited across generations. In this chapter, we will discuss the roles of epigenetics in these different modes of biotic stress resistance, and suggest ways in which we may in the future be able to exploit epigenetic systems for crop protection.

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2.1 Introduction

Like all living organisms, plants need to adapt to environmental changes in order to persist. Until the last decades, neo-Darwinian evolutionary theories assigned the origin of phenotypic variability to a set of characteristics determined by the genetic information. In the case of facing a change in the environment, different individuals within a population will have differential survival, depending of their characteristics, that will determine the genetic, and therefore phenotypic features of the new generations (Pigllucci 1996). Surprisingly, some recent observations lead different experts to claim for an implementation of this now classical new Darwinian perspective (Rando and Verstrepen 2007). For example, mutation rates are usually slower than environmental changes, and the phenotypic plasticity observed in natural populations wider than genetic variability. Therefore, an extra source of phenotypic plasticity is expected (Grativol et al. 2012). On the other hand, some environmentally induced adapted states seem to be relatively stable or even inherited for few generations without involving a change in the genetic information. This extra layer of relatively stable phenotypic plasticity has also been called epigenetic buffering (understood as 'something' beyond genetics). Epigenetic buffering could have a special importance in the case of plants, which because of their sessile nature, face threats to their survival and fitness from biotic stresses (O'Dea et al. 2016).

Against a pathogen attack, plants counter with a broad range of defence mechanisms. Plants possess some constitutive barriers to protect themselves against potential pathogens that are usually effective against a variety of microbes (Malinovsky et al. 2014). Along their evolutionary arms race for survival, pathogens have developed several strategies to overcome those defences and produce infections. In response to those, plants are able to actively induce defences when they identify a microbe or herbivore as a threat (Jones and Dangl 2006). This is associated with a reprogramming of gene expression. In some cases, once they have suffered a stress that induced their defences, plants are able to remember this first stress encounter. Then, in the case of recurrent stresses, the induced responses are faster and stronger (usually more effective). This is the concept of priming of defence responses, which involves a different control of gene expression and it is inevitably associated with a memory of the stress (Prime-A-Plant Group et al. 2006). Waddington in 1942 coined the term epigenetics to describe the study of phenomena in which the phenotypes observed in nature cannot be explained just by the understanding of their genotypes (Waddington 2012). He was studying development. At that time, the scientific community understood the genome as packages of information encoding specific features, but the control of that information (gene expression control) was an unknown. Of course, they could not imagine that part of the information stored in the genome works, in fact, by controlling gene expression (regulatory regions, transcriptional factors, etc.) and it is therefore genetic. Along the last decades, epigenetics has been redefined as phenotypic changes that can be transmitted through mitotic or even meiotic divisions in the absence of changes in the DNA sequence. Thus, epigenetics is associated with the control of gene

expression and certain memory. Importantly, some of the initially considered epigenetic mechanisms have been found to be encoded in the genome (miRNAs, chromatin modellers, histone variants, etc.). However, at all stages of induced immunity, the considered epigenetic mechanisms have been demonstrated to be of a key importance.

In this chapter, we will first introduce the different epigenetic mechanisms. We will focus on their role in controlling gene expression at the transcriptional and posttranscriptional level, contextualizing them by the use of some examples of their involvement in plant response to pest and diseases. Then, we will dedicate a section to assess the role of epigenetics in the memory of the stress and priming of defence responses. We will present and discuss publications supporting the role of epigenetic mechanisms in priming at different timescales, from short to long periods of time or even *trans*-generationally. Finally, we will summarize and discuss the potential application of epigenetics in the development of alternative programs for plant protection. We believe, this integrated view of epigenetics in plant defence and priming could be inspiring for a new generation of plant scientists aiming to understand plant defence mechanisms, as well as to develop alternative, hopefully more effective and sustainable, crop protection strategies.

2.2 Epigenetic Mechanisms Involved in Plant Defence

As originally defined, epigenetics allows plants to show different phenotypes with the same genotype. The underlying question for years was how? Nowadays, we know that the majority of what are considered epigenetic mechanisms are centred on the control of gene expression. From this perspective, the so-called epigenetic buffering, which is used to explain the extra source of phenotypic plasticity, does not involve a change in the information itself, but the different observed phenotypes are a consequence of modifying the speed and intensity at which genes are expressed (Grativol et al. 2012). Although epigenetics has traditionally been associated with repression of gene expression (reason why they are called 'silencing' mechanisms), we now also know of mechanisms considered as epigenetic that are able to promote or facilitate gene expression (Eamens et al. 2008; Matzke and Matzke 2000). This gene expression control can be imposed at either the transcriptional or posttranscriptional level. For this reason, epigenetic mechanisms are typically classified in two groups: those controlling gene expression at transcriptional level, known as 'transcriptional gene silencing mechanisms' (TGS), and those controlling gene expression at posttranscriptional level, 'posttranscriptional gene silencing mechanisms' (PTGS).

2.2.1 Posttranscriptional Control (PTGS): The Role of Small RNAs

In general terms, the main part of posttranscriptional epigenetic control is associated with the action of small RNAs (sRNA). Despite the fact that sRNAs pathways play important roles in developmental processes, they seem to have evolved originally from a defence mechanism against viruses and transposable elements to later start silencing endogenous genes (Borges and Martienssen 2015; Matzke and Matzke 2000). The first reports of epigenetic mechanisms in plant defence were examples of posttranscriptional gene silencing. Actually, reports of plant defence and priming processes as part of the cross-protection observed against viruses in the 1920s could be considered the very first recognized examples of epigenetic posttranscriptional gene silencing (Ross 1961; Waterhouse et al. 2001). They described how infections with relatively avirulent virus strains can induce protection against related virulent viruses. Unfortunately, at that time, the mechanisms were far from being discovered, and remained unknown for decades. It was not until the late 1980s that the first examples of posttranscriptional gene silencing mechanisms emerged, during experiments by plant biotechnologists trying to alter the colour of petunia flowers (Eamens et al. 2008). They observed how the expression of a transgene could trigger the silencing of the transgene and also homologous endogenous genes, by mechanisms involving small RNAs. Today we know both observations were related. It is generally accepted that all the epigenetic mechanisms involving RNA intermediates were originally part of the plant defence mechanisms against viruses. Virus transcription and replication is carried out inside the host cell, so the plants evolved the ability to detect exogenous nucleic acids as a potential threat (Waterhouse et al. 2001). Thus, plants first developed a system to defend themselves against viruses (exogenous RNAs). Then, the system was adapted to an endogenous control for gene expression and genome stability (controlling the movement of transposable elements—TEs—that are similar to viruses). That is the reason why advances in sRNA-mediated silencing mechanisms and plant defence have been feeding from each other during decades.

Derived from this original antiviral function, all small RNAs possess common features in their biogenesis. For example, most of the small RNAs required a longer double-stranded RNA (dsRNA) precursor molecule. This could be due to the fact that the 90% of plant viruses depend on a dsRNA molecule for their replication (Waterhouse et al. 2001). The dsRNAs are processed by DICER-LIKE proteins (DCLs) to generate fragments of 21–24 nt length. These molecules are then 2′-*O*-methylated by the protein HUA ENHANCER 1 (HEN1) at the 3′ end, which is a specific aspect of plant sRNAs (Yang et al. 2006). Finally, mature sRNAs are loaded into ARGONAUTE (AGO) proteins which can interact with other proteins to form the RNA-induced silencing complexes (RISCs; Fang and Qi 2016). In posttranscriptional gene silencing mechanisms, RISC complexes find the target mRNA by base pairing and affect its stability through mRNA cleavage (degrading the target mRNA), or repress its translation.

2.2.1.1 Silencing of Exogenous RNAs. The Special Case of Viruses

Due to its evolutionary origin, the majority of antiviral defences are triggered by exogenous RNAs (viral RNAs). Viral infection activates epigenetic mechanisms in order to destroy or silence the invading viral genome (Waterhouse et al. 2001). In Fig. 2.1 we represent a summary of the different silencing mechanisms against viruses as discovered in *Arabidopsis* (adapted from Ruiz-Ferrer and Voinnet 2009). These biological roles of the silencing components were mainly deciphered by analysing plant defective mutants (reviewed in Katiyar-Agarwal and Jin 2010; Seo et al. 2013). For RNA viruses (the majority of plant viruses), the viral genome is replicated by a viral replicase to generate a dsRNA molecule (Fig. 2.1a). This dsRNA would be processed by plant DCL proteins to trigger the production of sRNAs that would lead to viral silencing by degrading the dsRNA replication intermediates, the viral transcripts or impeding their translation. Viral transcripts (single-stranded RNAs—ssRNAs) can also trigger silencing by being copied to a dsRNA molecule by plant RNA-dependent RNA polymerases (RDR proteins), feeding the system. Those RDR proteins are also involved in the silencing of the viruses which have ssRNA genomes. In the case of the DNA viruses (Geminivirus—family Geminiviridae—for instance), the viral transcripts are copied to dsRNA by the plant RDR2 protein which in this case would mediate the production of sRNAs similar to the heterochromatic sRNAs, triggering the silencing of the virus at transcriptional level (Fig. 2.1b, and further discussed in Sect. 2.2; Raja et al. 2008). Moreover, viruses spread through the plant using the vasculature (Hipper et al. 2013). In this respect, a striking property of the sRNAs is their cell-to-cell mobility, increasing the efficiency of the silencing mechanism. Once the silencing is locally triggered in the

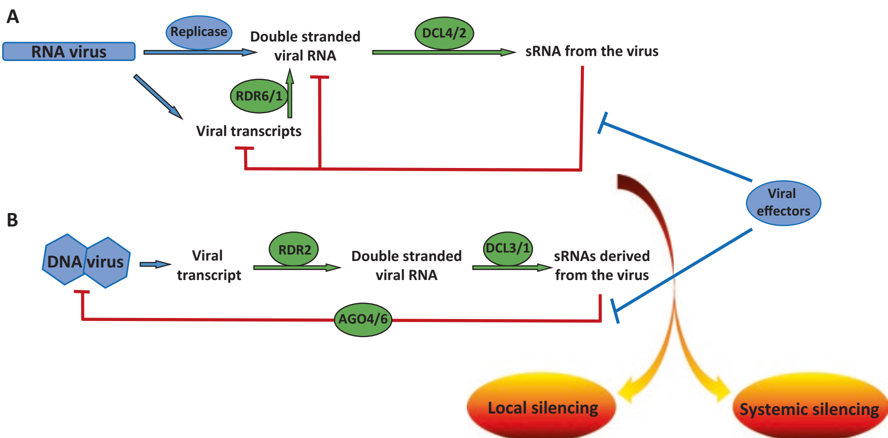

Fig. 2.1 Epigenetic mechanisms controlling viral infection. Adapted from Ruiz-Ferrer and Voinnet (2009). Epigenetic mechanisms against RNA (**a**) and DNA (**b**) virus. Pathogen components are highlighted in blue. Plant proteins are represented in green. Plant defence as a process is represented with red and orange colours

infected tissue, the sRNAs can travel through the plant xylem and phloem system and provide systemic resistance by silencing targets in distal parts of the plant (Chitwood and Timmermans 2010; Kalantidis et al. 2008). This systemic silencing has been broadly used by biotechnologists for decades, but more importantly, it has positioned the sRNAs as good candidates to be the mobile signal involved in some systemic resistance processes (Voinnet 2005).

2.2.1.2 Endogenous RNAs: From an Antiviral Defence to the Control of Endogenous Sequences

From these early mechanisms designed as antiviral defences, plants (and other organisms) evolved the capacity to use the silencing machinery in controlling other sequences (Waterhouse et al. 2001). This evolution leads to the sRNA-mediated regulation of endogenous genes and the suppression of transposable element (TE) movement. Nowadays there is an increasing number of different sRNAs recognized (Borges and Martienssen 2015). Several classifications have been proposed. On the basis of their biogenesis, we can consider two major classes: small interfering RNAs (siRNAs) and micro RNAs (miRNAs). Maybe the better-known of the two are the miRNAs, which are typically 20–22 nt length, transcribed by RNA polymerase II (Pol II) and processed by DCL1. Among the endogenous siRNAs there are many different subclasses. On the one hand, there are the hairpin-derived siRNAs (hp-siRNAs) and natural antisense siRNAs (natsiRNAs), both 21–24 nt length. On the other hand, the 'secondary siRNAs', including *trans*-acting siRNAs (tasiRNAs), phased siRNAs (phasiRNAs), epigenetically activated siRNAs (easiRNAs) and the long siRNAs (lsiRNAs). The lsiRNAs represent a class of endogenous siRNAs identified specifically in plant–pathogen interactions with a length of 30–40 nt. Finally, plants also produce heterochromatic siRNAs (hetsiRNAs), which from a classical view of the pathway are 24 nt length and generated by the plant-specific RNA-dependent DNA polymerase IV (Pol IV). hetsiRNAs are involved in transcriptional gene silencing, so they will be addressed later (see Sect. 2.2). Both miRNA and non-heterochromatic siRNAs bind the RISCs complexes and find the target mRNA (in this case a plant gene transcript), by base pairing. In the same way that the viral sRNAs are eliminated, the control of the target gene is achieved by mRNA cleavage (degrading the target mRNA) or repression of its translation.

The majority of the sRNA classes have been demonstrated to play a role in the control of plant defence at almost all stages. In general terms, once the pathogen is recognized by the plant, the induced defence process includes some conserved elements (Tsuda et al. 2008) such as the production of reactive oxygen species (ROS), which on specific occasions can lead to a hypersensitive response (HR) and apoptosis, an intracellular cascade mediated by MAP kinase proteins (MAPK) and hormonal signalling which generally involves a transcriptional reprogramming. Salicylic and jasmonic acid (SA and JA, respectively) are considered the two main hormonal pathways involved in plant defence. Plants adjust their immune system

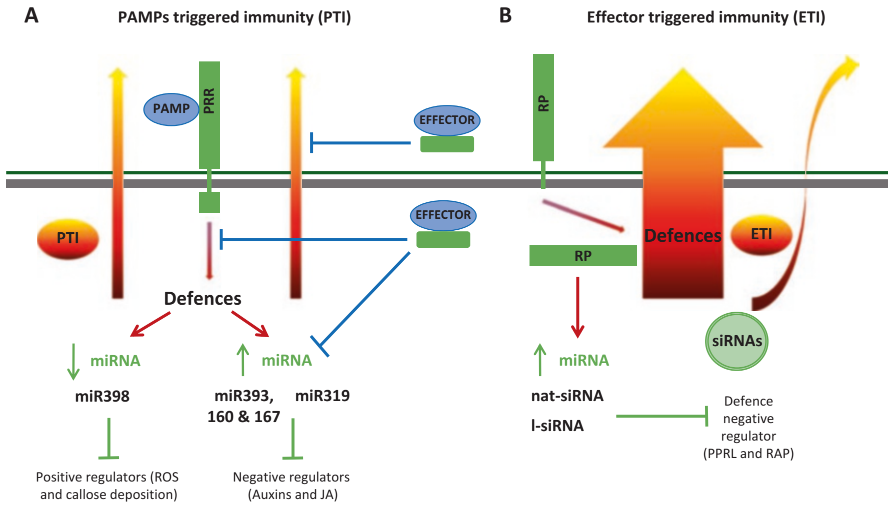

Fig. 2.2 Posttranscriptional epigenetic mechanisms involved in plant defence. The diagram represents some of the identified small RNAs controlling defence responses at the level of PAMP triggered immunity (**a**) and effectors triggered immunity (**b**). Pathogen components are highlighted in blue. Plant elements are represented in green. Plant defence as a process is represented with red and orange colours

depending on the lifestyle of the attacker they encounter. Generally, defence against attack by biotrophic pathogens, which feed from living cells, is mediated by the SA pathway. Conversely, necrotrophic pathogen infections, which kill the tissues to feed from them, or herbivores are resisted by the JA/ethylene (ET) pathway (Pieterse et al. 2012). An effective focus of the resources in defence is achieved by the prioritization of one of the pathways at the expense of the other, once the pathogen is recognized (Glazebrook 2005). As a consequence, it is common to find opposite phenotypes for different lifestyle pathogens when one of the pathways is active (Vos et al. 2015). In addition to SA, JA and ET, other hormones such as auxins, abscisic acid (ABA), cytokinins, gibberellins and brassinosteroids play a secondary, but nevertheless important, role in plant defence (Pieterse et al. 2012). These are general defence mechanisms triggered by the plant independently of the recognition of the pathogen.

It is generally accepted that there are two main routes by which the plant can activate these various defences, depending on the recognition of the pathogen. These are ‘PAMP triggered immunity (PTI)’ and ‘effector-triggered immunity (ETI)’. In Fig. 2.2, we contextualize some of the most important sRNA examples controlling PTGS described to date in both branches of plant defence.

sRNAs in PAMP Triggered Immunity (PTI)

The first branch in plant defence is triggered when the plant recognizes general microbial- or pathogen-associated molecular patterns (PAMPs), such as flagellin (common to different bacteria) or chitin (common to many fungi) by the use of transmembrane pattern recognition receptors (PRRs). This recognition leads into the PAMP triggered immunity (PTI) by the activation of a defence signalling cascade (Fig. 2.2a). miRNAs modulate different stages of the plant defences but they have a special contribution in PTI (Voinnet 2008). As a reflection, *dcl1* mutants (strongly impeded in the production of miRNAs) are effectively infected by the usually avirulent *hrcC* strain of *Pseudomonas syringae* DC3000 (Navarro et al. 2008). One of the first observations in this respect was a remarkable change in miRNAs populations during infection, and in response to exogenous PAMP applications. During PTI some miRNA species are repressed (represented as ↓ miRNAs in Fig. 2.2a). This is the case of miRNAs controlling positive elements in the defence response, like the miRNA398 (Fig. 2.2a; Jagadeeswaran et al. 2009). The repression of miR398 releases its targets (superoxide dismutases CSD1 and CSD2), and as a consequence, enhances callose deposition, reinforcing the cell wall and impeding the pathogen infection (Li et al. 2010). In contrast, other miRNAs are induced during PTI (↑RNA), which are usually the ones that control negative regulators. Maybe the one that could be considered the most relevant example until date is the case of the miR393. Transcription of miRNA393 is induced during PTI (by both flagellin treatments and *Pseudomonas syringae—Pst—*infections) and it is accompanied by a repression of its targets, which in turn causes repression of the auxin signalling pathway (Fig. 2.2a). Repression of the auxin pathway in this way seems to lead in resistance against *Pst* by hormonal crosstalk (Navarro et al. 2006). Apparently, by this hormonal crosstalk, the plant would prioritize the expense of the resources in defence over growth by repressing auxin pathway during a defence response. In fact, there are other miRNAs involved in the repression of the auxin pathway during PTI, like miR160 and miR167 (Fig. 2.2a; Zhang et al. 2011). The fine-tuning of the defence responses through a hormonal control is not exclusive of the auxin pathway. Actually, as introduced before, it is accepted in the field that plants tailor their defence responses in accordance with the attacker's lifestyle by the negative crosstalk of SA and JA pathways, the two main hormonal pathways involved in immune responses. This crosstalk is also under the control of miRNAs. This is the case of the miR319 which is induced in response to a/virulent hemibiotrophic bacteria (*Pst* DC3000, *Pst* DC3000 *hrcC,* and *Pst* DC3000 *avrRpt2*). miR319 represses JA pathway components, and its induction during defence responses against biotrophic pathogens could therefore have the objective of prioritizing SA-related defences at the expense of JA responses (Fig. 2.2a; Zhang et al. 2011). This kind of immunity has been demonstrated to stop the colonization of many different pathogens. However, some plant pathogens evolved to somehow interfere with these PTI mechanisms by the use of specific molecules called as effectors.

Host-adapted pathogens use effectors to suppress PTI and thus successfully colonize their host. There are few identified cases of pathogens manipulating the plant

silencing mechanisms as part of the immune system. One of the most relevant cases of pathogen effectors suppressing silencing comes once again from viruses. As effectors, viruses produce viral suppressors of RNA silencing (VSRs) to counteract their silencing (Fig. 2.1). Those are the best-studied examples of suppressors of silencing and are able to interfere with silencing at different stages (Fig. 2.1; Csorba et al. 2015). However, there are also a few identified cases of other pathogens manipulating the plant silencing mechanisms. This is the case of the miR393, and probably miR159, which are repressed by AvrPto effectors of *Pst* (Navarro et al. 2008; Zhang et al. 2011). One of the most interesting examples of this co-evolution comes from the discovering siRNAs produced by the pathogen *Botrytis cinerea.* The fungus produces siRNAs as effectors in order to hijack the plant silencing machinery, facilitating its infection (Weiberg et al. 2013). Nonetheless, along their shared evolutionary path, some plants have also acquired the ability to detect pathogen effectors, triggering the second branch of plant immunity.

sRNAs in Effector-Triggered Immunity (ETI)

Plant recognition of the presence of effectors triggers the second branch of plant defence, which is known as effector-triggered immunity (ETI). Effector detection is carried out by what are sometimes referred to as R proteins (from 'resistance proteins'). R proteins, also called guard proteins, can be intra- or extracellular and detect the presence of effectors either directly (e.g. by direct protein–protein interaction) or indirectly, via the outcome of the effector's interference with PTI. During ETI, induced defences are typically much stronger than PTI (Fig. 2.2b). One remarkable example of epigenetic control of ETI concerns the small interfering RNAs *nat-siRNAATGB2* and *AtlsiRNA-1* (Fig. 2.2). They were both found to take part in the ETI response against the strain *avrRpt2 of Pseudomonas syringae* pv *tomato*. In an avirulent interaction, the plant R protein RPS2 is able to detect the action of the effector avrRpt2 degrading the plant defence protein RIN4, and triggers an ETI response. As part of that system *nat-siRNAATGB2* and *AtlsiRNA-1* are induced and contribute to the releasing of the defences by the inhibition of *PPRL* (Katiyar-Agarwal et al. 2006) and *AtRAP*, both negative regulators of RPS2-related defences (Katiyar-Agarwal et al. 2007). Those are just some examples of the roles of silencing mechanisms along the co-evolutionary history between plants and their pathogens. Strikingly, some recent discoveries show how, as a last counteracting measure, some plant sRNAs can be transferred by extracellular vesicles to the pathogen in order to trigger silencing of virulence factors as part of ETI response (Fig. 2.2, Cai et al. 2018). This demonstrates once again the important role of the small RNAs in the plant–pathogen arm race.

2.2.1.3 The State of the Field, from *Arabidopsis* to Other Species

Unfortunately, even though the first studies started many years ago using tobacco as a model species, in the rise of epigenetics as a hot field during the last 20 past years, the majority of the research has been done in *Arabidopsis* plants. Infections of *Pst* in *Arabidopsis* have been consolidated as a model pathosystem. However, as translational science strategies are rapidly building on this fundamental knowledge, nowadays there is evidence coming from many different pathosystems, including crop species. For instance, the role of the silencing machinery against viruses has been investigated in rice against the rice stripe virus (Jiang et al. 2012). In *Brassica*, there has been identified a miRNA (bra-miR1885) which appears to be specifically targeted by viral effectors from TuMV virus. In addition, TuMV infections (but not TMV or CMV) induce bra-miR1885 levels which represses a defence-related protein, facilitating virus infection (He et al. 2008). It has also been reported that miR393 is conserved across different plant species such as rice and cucumber (Bian et al. 2012; Xu et al. 2017), and there is even evidence in soybean pointing to a conserved role of its function in PTI (soybean—*Phytophthora sojae* infections; Wong et al. 2014). Another notable example is the tomato miR482 family, which represses components of the plant basal defences and is down-regulated during bacterial and viral infections (Shivaprasad et al. 2012). Last decade there has also been an increment in the range of pathogen interactions analysed, including bacteria (Alizadeh et al. 2018; Zhao et al. 2013), fungi (Ellendorff et al. 2009; Li et al. 2014, 2016; Shen et al. 2014), oomycetes (Li et al. 2012), viruses (Li et al. 2012), cyst nematodes (Hewezi et al. 2008; Zhao et al. 2015) and other herbivores. Importantly, the majority of the crop studies started with genomic and transcriptomic analysis, with a strong in silico component (Guo et al. 2011; He et al. 2014; Jeyaraj et al. 2017; Kapoor et al. 2008; Lu et al. 2007; Pandey et al. 2008; Pérez-Quintero et al. 2012; Qiu et al. 2009; Radwan et al. 2011; Warren and Covert 2004; Xin et al. 2010; Yin et al. 2012). Much more work needs to be done in vivo to be able to include these epigenetic mechanisms as agronomical tools for the development of crop protection strategies.

2.3 Transcriptional Control (TGS): Chromatin Remodelling

Epigenetics can also modify gene expression at the transcriptional level. The DNA is compacted in the nucleus by association with proteins in what we know as chromatin (Kornberg 1974). The basic units of chromatin are called nucleosomes and are formed by an octamer of histone proteins (two of each H2A, H2B, H3, and H4; Van Holde et al. 1974) and approximately 146 bp of DNA, or 1.7 turns, wrapping the histone octamer. The DNA between nucleosomes is called linker DNA and for higher levels of compaction can be associated with histone H1 (Fig. 2.3a). Depending on the physicochemical interaction of the DNA and histone proteins in the nucleosome, the chromatin can have different levels of compaction (Fig. 2.3b). This

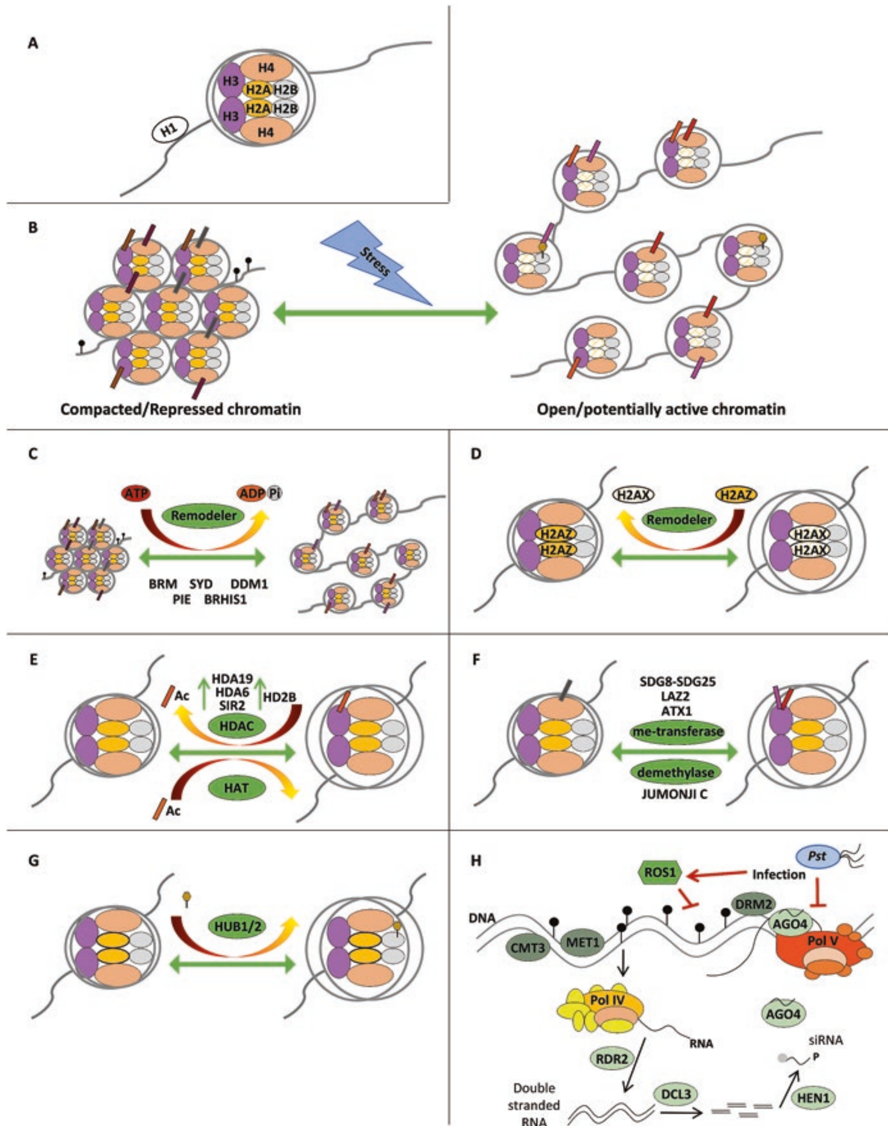

Fig. 2.3 Transcriptional epigenetic mechanisms involved in plant defence. (**a**) Representation of a nucleosome unit. The DNA wraps around an octamer of histone proteins (2× -H2A, H2B, H3 and H4-). H1 is located in the linker DNA (between nucleosomes). (**b**) The main part of the epigenetic mechanisms controlling defence at transcriptional level influences the chromatin compaction. In response to pathogen attack, plants activate epigenetic mechanisms to open the chromatin at the level of genes involved in defence responses, facilitating their expression and/or compacting chromatin regions containing defence repressors. DNA methylation, histone acetylation, histone methylation as a negative mark (for instance, H3K9me2), histone methylation as a positive mark (for instance, H3K4me3 or H3K36me), histone ubiquitination. (**c**) Some of the chromatin remodellers involved in plant defence. (**d**) Deposition of histone variants in plant defence processes. Fig. 2.3 (continued) Specifically, the case of the H2AZ is represented in the diagram. (**e**) Some histone acetylation examples in response to pathogen attack. (**f**) Histone methylation examples in response to pathogen attack. (**g**) Histone ubiquitination associated with plant defence. (**h**) DNA methylation changes mediated by the RdDM pathway and ROS1 in response to *Pst* infections

compaction is essential as, for the main part of the functions of DNA such as gene expression and replication, the DNA should be accessible to large protein complexes. However, the entire genome does not fit into the nucleus in the uncompacted state (Li et al. 2007). Thus, during interphase, the transcribed regions are uncompacted constituting what is known as open chromatin, while other regions are preserved (silenced) in very compacted chromatin regions. At the same time, the compaction of the DNA at some regions should be maintained, as this preserves the genetic information from damage and the jumping of TEs. In response to an environmental stimulus, the chromatin has been proposed to interpret the signal and facilitate the gene reprogramming (Fig. 2.3b; Badeaux and Shi 2013). As the chromatin compaction has been directly related to the transcriptional control of gene expression, all mechanisms modifying chromatin compaction are considered epigenetic mechanisms controlling transcriptional gene silencing (Fransz and de Jong 2011). Among such mechanisms, the most important are: chromatin remodellers, deposition of histone variants, histone posttranslational modifications and DNA methylation. Similar to the control of PTGS, chromatin compaction has been demonstrated to play a crucial role in the fine-tuning of defence responses. Importantly, the chromatin state has been proposed to be the mechanism underlying priming processes and memory of the stress, which will be addressed in Sect. 2.3.

2.3.1 ATP-Dependent Chromatin Remodellers

The ATP-dependent chromatin remodellers are multiprotein complexes that are able to disrupt the interaction between the DNA and histone proteins using energy provided by the hydrolysis of ATP molecules (Fig. 2.3c; Han et al. 2015). They are conserved cross-kingdom in eukaryotes. In plants, the ATPase function resides in proteins from the Snf2 superfamily. Members of the Snf2 superfamily involved in plant defence identified to date are: BRAHAMA (BRM), SPLAYED (SYD), DECREASE IN DNA METHYLATION 1 (DDM1), PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1), BIT-RESPONSIVE HISTONE-INTERACTING SNF2 ATPASE 1 (BRHIS1) and CHROMATIN-REMODELLING FACTOR 5 (CHR5). BRM is maybe the most canonical and well-studied chromatin remodeller in plants, and it has been widely studied in responses against abiotic stresses, where it controls abscisic acid (ABA)-related genes (Han et al. 2012; Peirats-Llobet et al. 2016). However, some defence-related genes have been observed as misregulated in the *brm101* mutant, pointing to a defence role of BRM, probably by crosstalk between ABA and SA hormonal pathways (Bezhani et al. 2007; Ramirez-Prado et al. 2018). SYD has been reported to play a role in the activation of JA/ET related defences. On the one hand, SYD seems to bind some promoter regions for JA/ET-related genes, probably promoting the opening of the chromatin. On the other hand, mutants defective in SYD are consistently more susceptible to the necrotrophic pathogen *B. cinerea* and unable to properly induce appropriate target genes (Walley et al. 2008). In addition, some SYD mutants are

more resistant to biotrophic pathogens such as *Hyaloperonospora arabidopsidis* (*Hpa*) and *Pseudomonas syringae* pv. *maculicola* (*P.s.m.*; Johnson et al. 2015). DDM1 plays a critical role in the maintenance of the DNA and histone H3 methylation pattern. In this case, DDM1-mediated chromatin opening does not recruit gene activators, but it seems to allow the recruitment of methyltransferases to its target regions, inducing gene silencing (Gendrel et al. 2002; Jeddeloh et al. 1998; Zemach et al. 2013). It therefore links the direct opening of the chromatin with changes in the histone protein modifications and DNA methylation. DDM1 has been related with the control of the SA-related defences and the silenced basal state of *RPP5*, a six-defence gene cluster (Yi and Richards 2007, 2009). Accordingly, the *ddm1* mutant plants are more resistant to *Hpa* (López Sánchez et al. 2016). Recently it has been reported how the *SNC1* gene from the *RPP5* cluster is also regulated by another ATP-dependent chromatin remodeller, CHR5. In this case, CHR5 acts as a positive regulator (classical chromatin remodeller function) opening the chromatin at the level of *SNC1* gene. The mutant *chr5* shows an inability to open the chromatin at *SNC1* level, exhibits increased nucleosome deposition along the whole genome, and hyper-susceptibility to virulent and avirulent strains of *Pst* (Zou et al. 2017). Finally, the chromatin remodellers PIE and BRHIS1, like DDM1, act as intermediaries for other chromatin modifications such as the deposition of histone variants and histone monoubiquitination, respectively (discussed later).

2.3.2 Deposition of Histone Variants: Histone Replacement

One of the mechanisms involved in chromatin remodelling is the replacement of the canonical histone by specific histone variants. Due to different physicochemical properties, the interaction of the histone variants with the DNA in the nucleosome can modify chromatin compaction and thus, gene expression at transcriptional level (Coleman-Derr and Zilberman 2012). That is the reason why the deposition of different histone variants has been proposed as a mechanism mediating responses to environmental changes (Talbert and Henikoff 2014). One of the most important examples for this mechanism is the case of the H2A.Z (Fig. 2.3d). First, March-Diaz et al. (2008) described how mutants defective in the H2A.Z coding genes, as well as the SWR1 chromatin remodelling complex, which facilitates its deposition (including the above-mentioned PIE protein), constitutively express SA-related genes and are more resistant against biotrophic pathogens. The authors suggested that the deposition of H2A.Z has a role in controlling the silencing of SA-related genes in a basal state (March-Díaz et al. 2008). Recent works have confirmed the role of this histone variant in repressing SA-related genes, but they report hyper-susceptibility for both biotrophic and necrotrophic pathogens (Berriri et al. 2016). However, the deposition of the histone variant H2A.Z seems to be key for the fine-tuning of the defence responses.

2.3.3 Modification of the Histone Proteins

Histone proteins can be posttranscriptionally modified at different residues, having an important impact in the chromatin state (Kouzarides 2007). More than 60 positions have been detected in the tail of the core histone proteins that can potentially carry posttranscriptional modifications (PTMs) of a variable nature. It is becoming clear that PTM of histone proteins imparts a dynamic and complicated regulation of gene expression, particularly in plant defence processes. This regulation is a consequence not just of the appearance of one PTM, as they can have an individually positive or negative contribution for chromatin compaction, but the consensus of several interacting PTMs, in what is called 'the histone code'. The PTMs that have been demonstrated to play a key role in plant defence are related with acetylation, methylation and ubiquitination of different residues (Fig. 2.3e–g). There have been very recent and comprehensive reviews in this field (Chen et al. 2017; Ding and Wang 2015; Ramirez-Prado et al. 2018). The majority of cases have been described once again in *Arabidopsis*, so here we will just outline some of the most notable examples to offer a general view.

2.3.3.1 Acetylation

Generally, the acetylation of residues in histone H3 and H4 proteins is associated with a relaxation of the chromatin (openness). As DNA is negatively charged, the addition of acetyl groups (also negative), loosen the nucleosome association, facilitating gene expression. Histone acetylation is carried out by histone acetyltransferases and the deacetylation by histone deacetylases (HATs and HDACs, respectively, Fig. 2.3e; Berger 2007). In *Arabidopsis*, two HDACs in particular, HDA19 and HDA6, have been linked with plant defence. Both of them are induced by necrotrophs and/or JA-related signals (for example, wounding; Zhou et al. 2005), suggesting some overlapping functions. *hdc19* mutants show increased susceptibility to necrotrophic pathogens and an inability to induce JA-related genes. This phenotype does not seem to be related with direct changes in the PTMs at the level of the JA-related genes, but by the hormonal crosstalk with the SA hormonal pathway (Choi et al. 2012; Koornneef et al. 2008). HDA19 seems to play a key role in the maintenance of the silent basal state of the SA-related genes. At the basal state, HDA19 seems to inhibit the acetylation of histone proteins at the *PATHOGENESIS-RELATED1* and *2* (*PR1* and *PR2*) defence gene loci (genes considered marker genes for the SA hormonal pathway). In fact, *hda19* mutants show enhanced expression of those genes and hyper-resistance against some biotrophic pathogens (even when originally there were contradictory results at this respect; Choi et al. 2012; Kim et al. 2008). Other histone deacetylases involved in plant defence belong to the SIR2 protein family. In *Arabidopsis*, *AtSRT2* controls the basal repression of the SA-related defences (Wang et al. 2010). Thus, histone deacetylations seems to cause a general repression at the level of different SA-associated defence genes.

However, some recent studies have described how during PTI responses, the phosphorylation cascade induced by MAPKs proteins can lead to the specific activation of the HDAC, HD2B. HD2B would deacetylate genic regions, inhibiting their expression and therefore contributing to the gene expression reprogramming during defence processes (Latrasse et al. 2017). Lastly, nitric oxide has been proposed as a repressor of the histone deacetylation during plant defence processes, mediating the hyperacetylation and contributing to the induction of defence-related genes (Mengel et al. 2017; Ramirez-Prado et al. 2018).

2.3.3.2 Methylation

As for acetylation, methylation of different residues of the histone proteins has been proven to play a crucial role in plant defence (De-La-Peña et al. 2012; Ramirez-Prado et al. 2018). It mainly occurs in lysine and arginine residues of histones H3 and H4. For each residue, from one to three methyl groups can be added (Bannister and Kouzarides 2005; Kouzarides 2007). These forms of histone methylation can differentially impact on the chromatin structure. Unlike the case of acetylation that usually involves chromatin relaxation, methylation as a PTM of histone proteins has been linked with both inhibition and priming of gene expression (Fig. 2.3f). The enzymes involved in histone de/methylation are very specific. The most notable examples related to plant defence come from the analysis of methyltransferases. This is the case, for instance, of *ARABIDOPSIS* HOMOLOG OF TRITHORAX (ATX1), which trimethylates the lysine 4 of histone 3 (H3K4me3) and positively regulates the expression of *WRKY70*, a transcriptional factor involved in SA hormonal pathway and postulated to be crucial to the SA-JA hormonal crosstalk (Alvarez-Venegas et al. 2007). LAZARUS2 (LAZ2) is another histone methyltransferase involved in trimethylation of lysine 36 of histone 3 (H3K36me3), which is required to activate an R gene involved in ETI responses against *Pst* (Palma et al. 2010). Recently, roles for demethylases in defence have also been reported. This is the case of Jumonji C demethylases. For example, the H3K9 demethylase JMJ27 is induced during bacterial infection, required for the resistance to the pathogen and the correct expression of defence-related genes (Dutta et al. 2017). A peculiar case is the demethylation of lysine 9 of histone 3 (H3K9me2). H3K9me2 has been traditionally considered as a repressive chromatin mark of TEs. Surprisingly, it has been reported that the levels of H3K9me2 at the defence gene *RPP7* can affect the selection of the polyadenylation site, and thus, the production of a different transcript (Tsuchiya and Eulgem 2013). As pointed out previously, the chromatin environment is determined not by just individual marks, but the appearance of different ones at the same time. Therefore, it is easy to imagine that there is co-regulation of the different PTM pathways. This was evident in the study of the methyltransferases SDG8 and SDG25. Mutants in those proteins are altered in plant defence against necrotrophs, biotrophs, and show differences in methylation of several residues of the histone proteins at the level of some defence-related genes (Berr et al. 2010; Lee

et al. 2016). They also display an altered pattern in H2B ubiquitination (Lee et al. 2016), which will be the subject of the next section.

2.3.3.3 Ubiquitination

Ubiquitination of proteins typically refers to the addition of the 76-residue peptide known as ubiquitin by the action of three consecutives enzymes, E1, E2 and E3 (Weake and Workman 2008). In general, those enzymes can add one or more residues of ubiquitin to the target proteins. Many different proteins can be ubiquitinated, but in the specific case of histone proteins, ubiquitination has only been found in the form of a single residue in H2A or H2B, and usually acts as a positive mark for gene expression marking open chromatin (Fig. 2.3g). As with the other PTMs, ubiquitination is reversible. *Arabidopsis* HUB1 and HUB2, the two RING E3 enzymes, have been reported to be required for plant defence against necrotrophs (Dhawan et al. 2009; Hu et al. 2014) and to play a certain role against biotrophic pathogens (Zou et al. 2014), probably through modifications of the cuticle (Ménard et al. 2014).

2.3.4 DNA Methylation

DNA methylation usually refers to the addition of a methyl group at the fifth carbon of the cytosine residues of the DNA. In addition to cytosine methylation, adenine methylation has also been reported in many different organisms, being a key factor for protecting prokaryotic DNA. However, adenine methylation has so far received little attention in plants (Liang et al. 2018). Cytosine DNA methylation in plants can be found in every sequence context and in different extents, depending on the species (Niederhuth et al. 2016; Takuno et al. 2016). Attending to the nature of the context, both symmetrical and asymmetrical contexts can be considered (Cokus et al. 2008). Symmetrical contexts are CG and CHG (H refers to A, T or C), where both strands of the DNA are methylated. Asymmetrical context is CHH. The maintenance of the DNA methylation pattern is carried out by the METHYLTRANSFERASE1 (MET1) in CG context and CHROMOMETHYLASE2 and 3 (CMT2 and CMT3) in CHG contexts. The maintenance of CHH methylation is mainly performed by an RNA-directed DNA methylation pathway (RdDM), and requires the constant production of siRNAs (Fig. 2.3h; Law and Jacobsen 2010). Probably as a consequence of its origin as defence systems against exogenous nucleic acids, the establishment of a de novo pattern in DNA methylation is primarily controlled by siRNAs and has some homologies with the PTGS mechanisms. The current view of the process in *Arabidopsis* includes an initiation phase (not included in the figure for simplicity reasons) in which it is likely that over-accumulated RNAs are copied to a double-stranded molecule by RDR6, and then

processed by DCL2 and DCL4 into 21–22 nt siRNAs. Probably these siRNAs trigger PTGS by binding to AGO1 or AGO2 proteins. However, in subsequent stages, they are loaded into AGO6, which directs the plant-specific DNA-DEPENDENT RNA POLYMERASE V (Pol V) and the DNA methyltransferase, DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), to the target regions in the genome to induce low levels of DNA methylation (Nuthikattu et al. 2013). After this initiation phase, the second branch of the RdDM pathway is activated (Fig. 2.3h). In this, the other plant-specific DNA-DEPENDENT RNA POLYMERASE IV (Pol IV) is involved in the production of RNA molecules from the targets that after being processed by RDR2, DCL3 and HEN1 will be loaded onto AGO4. The base pairing between the siRNA with Pol V-produced RNA transcripts enables the recruitment of DRM2 for establishment of DNA methylation (Matzke and Mosher 2014). DRM2-dependent CHH methylation requires the constant production of siRNAs, and ongoing activity by the Pol IV-RDR2-dependent RdDM pathway. Both the overall level of genome-wide DNA methylation and the pattern of methylation are controlled by a balance between DNA methylation and demethylation processes. Removal of DNA methylation can happen passively during replication, or actively by the action of DNA glycosylase/lyases, of which four have been identified to date in *Arabidopsis* (Zhu 2009). Among these, REPRESSOR OF SILENCING 1 (ROS1) is predominantly responsible for DNA demethylation in vegetative tissues. The primary functions of DNA methylation are controlling genome stability and gene expression. In general, the epigenetics community tend to associate DNA methylation at the level of promoter regions with repression of gene expression, while the consequences of methylation within gene bodies remain uncertain (Bewick and Schmitz 2017).

The first characterized roles for DNA methylation in plant defence came again from the defence against viruses, with several examples showing DNA methylation of the viral genome for the Geminivirus family (Blevins et al. 2006; Raja et al. 2008). The majority of plant viruses possess RNA genomes, but the Geminiviridae family genome is a single-stranded DNA. The silencing of the viral genome is carried out by the TGS mechanisms of the cell as a defence mechanism against exogenous nucleic acids. Therefore, Geminivirus genomes are silenced by part of the RdDM pathway (Fig. 2.1b). A beautiful example of plant–DNA virus interaction is seen between tomato and the TOMATO YELLOW LEAF CURL CHINA VIRUS. During their co-evolution, tomato plants first developed the ability of defend themselves from viral infection by methylating the viral DNA. Some virulent strains of the virus carry what is known as the betasatellite encoding βC1, which is a repressor of silencing used as an effector (Yang et al. 2011). However, resistant strains of tomato plants have developed the ability to polyubiquitinate βC1 to mediate its degradation via the proteasome. Nowadays, a range of different viral effectors acting as repressors of transcriptional silencing are known (Rodríguez-Negrete et al. 2013; Wang et al. 2014). Although the TGS assigned to the defence against Geminiviruses was thought to act at the level of DNA methylation of the viral genome, recent studies found that the role of the RdDM pathway in silencing the

viral genome is performed by triggering H3K9 methylation (Jackel et al. 2016). This reflects once again the crosstalk between different TGS mechanisms.

As in the case of the PTGS mechanisms, plants took advantage of the TGS defence system to control different endogenous sequences. In fact, the activation of antiviral defences has direct consequences in endogenous sequences (Castillo-González et al. 2015; Coursey et al. 2018), as the most important role of DNA methylation is controlling TE repression (considered invasive DNAs). The changes in chromatin caused by TE silencing can also modify the expression of some plant genes, and in our case of interest, defence-related genes. There has been increasing evidence for a role of the DNA methylation and demethylation machineries in controlling plant defence. On the one hand, plants trigger changes in DNA methylation during pathogen attack (Dowen et al. 2012; Pavet et al. 2006; Yu et al. 2013). In general terms, an active DNA demethylation process in response to infections of *Pst* and the application of PAMPs such as flagellin has been observed. This demethylation seems to be a consequence of the repression of some RdDM components and the active removal of methyl-cytosines by the protein ROS1 (Fig. 2.3h; Dowen et al. 2012; Yu et al. 2013). On the other hand, *Arabidopsis* mutants impeded in DNA methylation (such as *met1*, *drd1*, *cmt3* and mutants defective in Pol V) have been reported to show increased resistance to biotrophic pathogens like *Pst* and *Hpa* (Dowen et al. 2012; López et al. 2011; Yu et al. 2013). Correspondingly, with the SA-JA hormonal crosstalk, those same mutants show hyper-susceptibility against necrotrophic pathogens like *Plectosphaerella cucumerina* and *B. cinerea* (López et al. 2011; López Sánchez et al. 2016). Also in accordance, mutants in ROS1 protein, which cannot actively demethylate the DNA, show the opposite phenotype (hyper-susceptibility against biotrophs and enhanced resistance against necrotrophs). The exact mechanisms by which DNA methylation controls plant defences are not known and even when transcriptomic analysis of the mutants during infection point to changes in several genes, only in very limited cases, *cis*-regulation has been demonstrated (Le et al. 2014; Yu et al. 2013). In just a few specific cases, differentially methylated regions have been directly associated with the transcriptional control defence-related genes, for instance, being TEs located at the promoter regions of defence genes (Yu et al. 2013). Alternative *trans*-regulatory mechanisms have been proposed, which will need further research in the future. Importantly, even when mutants defective in DNA methylation are more resistant against biotrophic pathogens and they show a better induction of the SA-related defence genes, those mutants do not display constitutive expression of those genes (López et al. 2011; López Sánchez et al. 2016). These and other evidences involving chromatin states and the requirement of DNA methylation machinery in transgenerational phenomena have been crucial in determining their role in priming of induced defences and memory of stress, which will be assessed below.

2.3.5 *The State of the Field, from* Arabidopsis *to Other Species*

In line with the case of the PTGS mechanisms, there are not many studies carried out in non-model organisms. Here, we will introduce some of the works reported until now. The BRHIS1 chromatin remodeller from rice is a nice example. It has been demonstrated to repress defence-related genes and maintain them in the basal state. As part of the plant response to fungal pathogens/priming agents, plants repress *BRHIS1*, which would in turn favour the induction of the defence genes (Li et al. 2015). A few more examples have been reported of posttranslational modification of histone proteins. Also in rice, there has been nice work in the rice interaction with the pathogen *Magnaporthe oryzae*. The over-expression of the histone deacetylase HDT701 confers to the rice plants hyper-susceptibility to *M. oryzae* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Thus, HDT701 has been proposed as defence repressor, probably targeted by effectors, as its induction has been detected during infections (Ding et al. 2012a). There is also an elegant work in rice studying the Jumonji C histone demethylases. During *Xoo* infection, rice plants have been reported to induce the expression of 15 JmjC proteins (Hou et al. 2015). Among these, JMJ704 and JMJ705 have been demonstrated to play a key role for plant defence. Accordingly, a *jmj704* mutant is more susceptible, and *JMJ705* over-expression lines more resistant, to *Xoo* (Hou et al. 2015; Li et al. 2013). Both seem to play a role in repressing defence genes during basal conditions and releasing their expression during defence. On the one hand, JMJ704 seems to be important in maintaining low levels of the positive mark H3K4me2/3 in the defence genes during basal conditions (Hou et al. 2015). On the other hand, JMJ705 seems to induce the removal of negative marks such as H3K27me2/3 during infections (Li et al. 2013). Apart from rice, following a similar strategy to the genome-wide analysis of sRNAs, a whole-genome analysis during rust infection in common bean reported global changes in histone methylation and acetylation (Ayyappan et al. 2015). In addition, and built on the basis of the work in *Arabidopsis*, the histone ubiquitination pathway has also been studied in other species such as tomato (Zhang et al. 2015). In this study, the tomato homologs of the HUB1/2 proteins: SlHUB1 and SlHUB2 were identified. The authors demonstrated that SlHUB1/2 are required for defence against *B. cinerea* and seem to play a role in the crosstalk of the SA-JA hormonal pathways, probably by a combination of the cuticle properties and the priming of defence genes (Zhang et al. 2015). Lastly, consistent with the role of DNA methylation in response to biotrophic pathogens, treatments with 5-azadeoxycytidine in rice seedlings have been reported to show resistance to *Xoo*, a phenotype which correlated with the demethylation of specific regions, including a defence gene *Xa21G* (Akimoto et al. 2007). Again, even though the role of the different TGS mechanisms has been proved to be crucial for plant defence, more work is needed in order to apply the fundamental knowledge generated in models to crop protection programs.

2.4 Epigenetics Is Involved in the Memory of the Stress: Priming

In addition to the roles of small RNAs and chromatin remodelling in the regulation of immediate defence responses and defence-related gene expression, epigenetic processes also play key roles in longer-term defence priming. Priming refers to the immunological memory that can develop following stress exposure, such that responses to future stresses are more effective. The best-studied example of priming in relation to biotic stress is systemic acquired resistance (SAR). As well as transient up-regulation of defence genes in systemic leaves (which have not suffered the infection), SAR typically includes a priming element, such that defence responses are stronger and more rapidly induced in response to a secondary infection for up to several weeks following an initial pathogen infection (Klessig et al. 2018). More recently, evidence has accumulated that under some circumstances, longer-lasting priming memory can be established, which is then inherited by one or more future generations.

2.4.1 Short-Term Priming Memory

2.4.1.1 Changes in Defence Response Signalling Components

Several mechanisms have been proposed that could potentially generate the memory of stress that is required for priming. Short-term memory could be generated relatively easily by changes in the quantity or activity of signalling components required for the regulation of ETI and PTI (Conrath et al. 2015). For example, systemic leaves of *Arabidopsis* undergoing SAR display elevated levels of unphosphorylated (therefore inactive) mitogen-activated protein kinases MPK3 and MPK6. When inoculated with *P. syringae*, these primed leaves exhibit higher levels of MPK3/6 activity than non-primed leaves, due to their faster activation, as they are already synthetized (Beckers et al. 2009). The accumulation of transcription factors necessary for defence gene expression may be another similar mechanism for priming. Van der Ent et al. (2009) identified panels of *Arabidopsis* transcription factors that were up-regulated upon priming by either induced systemic resistance (ISR) triggered by *Pseudomonas fluorescens* or by root drenching with β-aminobutyric acid (BABA), a well-known chemical inducer of priming. They suggested that the different groups of transcription factors responsive to each of these priming treatments not only provide a mechanism for priming, but can also act as markers for different priming responses. The up-regulation of pattern recognition receptors (PRRs) involved in recognition of biotic attackers has also been suggested as a mechanism for priming (Tateda et al. 2014).

2.4.1.2 Chromatin Remodelling

Aside from the production of additional signalling molecules, the other main area that has received attention is epigenetic mechanisms for encoding stress memories.

As well as being essential for immediate, short-term transcriptional responses, chromatin remodelling via histone and DNA modifications also has the potential for conferring stable patterns of gene expression. Indeed, during development, epigenetic mechanisms are central to changes in gene expression associated with cell-type specialization (Heard and Martienssen 2014). As described above, regulation of defence gene expression involves various histone and DNA modifications which ultimately make genes more accessible to the transcriptional machinery. After a transient burst of stress-induced transcription, the chromatin of a defence-related gene may revert to the basal state, in which case it would be expected to show the same response characteristics to any subsequent experience of stress. Alternatively, if the reversion was only partial, then it might be possible that subsequent access of the transcriptional machinery would be less restricted. This would represent a primed state, in which chromatin modifications, brought about by previous transcriptional activation, leave a memory imprint. Primed genes might therefore be expected to reside on more open chromatin associated with altered levels of key histone and DNA modifications. To date, evidence from several different biotic and abiotic stress response systems has identified a range of histone marks and DNA methylation patterns, but in particular, reduced nucleosome occupancy and increased H3K4me3 are emerging as especially common features of primed stress-related genes.

Histone Modifications

Several authors have identified H3K4 hypermethylation associated with transcriptional memory/priming, most notably the trimethylation state. Key work linking histone modifications and defence priming came from Jaskiewicz et al. (2011), who demonstrated that local inoculation with *P. syringae* or treatment with benzothiadiazole (BTH) led to increases in H3K4me2 and H3K4me3, along with increased acetylation at a number of histone H3 and H4 lysine positions in the promoters of several WRKY genes. Histone H3K4me2/3 methylation is generally associated with a permissive transcriptional chromatin state (Berger 2007). Importantly, the increase in H3K4 methylation following BTH treatment was not associated with any immediate change in gene expression, but the affected WRKY genes exhibited augmented expression in response to a secondary stimulus. Furthermore, H3K4me3 hypermethylation was not observed in the SAR-deficient *npr1-1* mutant, whereas constitutively primed *cpr1* and *sni1* mutants showed constitutive high levels of H3K4me3 in the WRKY gene promoters. Similarly, mutants defective RNA polymerase V, which is required for initiation of DNA methylation in the RdDM pathway, also demonstrate constitutive priming of SA-dependent defence genes, and exhibit enhanced H3K4me3 at defence genes (López et al. 2011). Priming responses

following application of BABA in common bean were also linked with elevated H3K4me3 states at defence gene promoters (Martínez-Aguilar et al. 2016). Importantly, BABA treatment resulted in enhanced H3K4 trimethylation without any change in gene expression. The subsequent transcriptional activation of genes with this modification was primed, being higher in BABA-treated plants than in control plants, in response to bacterial infection (Martínez-Aguilar et al. 2016).

Other histone modifications may act alongside H3K4 methylation to establish priming. A role for histone acetylation in priming of PTI was identified by Singh et al. (2014a), who showed that priming for bacterial disease resistance was eliminated by mutation of *histone acetyltransferase1* (*HAC1*). Interestingly, the *Arabidopsis FLD* gene, which encodes a protein homologous to a human lysine-specific demethylase and also associated with histone deacetylase complexes, was identified in a genetic screen for mutants defective in the ability to express SAR (Singh et al. 2013). Mutants in *FLD* display wild-type levels of basal resistance to *Pst*, but do not exhibit priming of *PR1*, *WRKY6* and *WRKY29* gene expression following secondary inoculation of systemic leaves (Singh et al. 2013, 2014b). H3K4me2 appears to be the major substrate for FLD (Liu et al. 2007), but H3K4me2 methylation was decreased overall rather than increased in the promoters of *WRKY6* and *WRKY29* following challenge inoculation of an *fld* mutant (Singh et al. 2014b). Although the data do not identify a clear role for FLD in regulating histone modifications during priming of these genes, the authors suggested it may function as a negative regulator of an alternative histone demethylase that represses H3K4me2 methylation, and therefore priming, of defence genes.

As well as posttranslational modifications of histones, nucleosomes are also regulated by inclusion of variant histone proteins. In particular, H2A.Z has been linked with plant–pathogen resistance responses, although its precise function remains unclear. As noted in Sect. 2.2, March-Díaz et al. (2008) found that mutants defective in the SWR1 chromatin remodelling complex, which is responsible for substitution of canonical H2A with H2A.Z, are resistant to *P. syringae* and over-express a suite of SAR-regulated genes. More recently, different roles were identified for genes in the SWR1 complex and H2A.Z in SA and JA-mediated basal and effector-triggered immunity, indicating a complex interaction between H2A.Z substitution and immune regulation (Berriri et al. 2016). Overall, while loss of H2A.Z increases basal immunity, it reduces inducible responses. Whether it plays any role in priming of defence genes following induced resistance remains to be tested.

Nucleosome Occupancy

A second, related, common feature that has been identified in the promoters of primed genes is a more open chromatin configuration. Chromatin assembly factor CAF-1 is required for assembly of nucleosomes on newly replicated DNA, and mutants in CAF-1 subunits display pleiotropic developmental phenotypes (Ramirez-Parra and Gutierrez 2007). One of these phenotypes is constitutive priming of many

defence genes. Under normal growth conditions, the *fas2-4* mutant (a null allele for one of the CAF-1 subunits) exhibited constitutive expression of SAR-responsive genes. When grown under sterile conditions, these genes were no longer constitutively expressed, but displayed primed expression in response to SA treatment (Mozgová et al. 2015). CAF-1 therefore appears to be involved in repression of the primed state in wild-type plants. In the CAF-1 mutant, chromatin assays identified reduced nucleosome occupancy but increased abundance of H3K4me3 around transcription start sites (TSS) of several SAR genes, and similar chromatin states were observed in the same genes when priming was induced by either SA or BABA treatment (Mozgová et al. 2015). Interestingly, similar profiles were also detected in the promoters of genes exhibiting priming memory following drought stress. In this work, promoters of primed genes possessed stalled RNA polymerase II (PolII) and H3K4me3 hypermethylation (Ding et al. 2012b). Finally, genome-wide profiling of nucleosome positioning in response to SA treatment also identified reduced nucleosome occupancy at the TSS of SA-responsive genes (especially those regulated by *NPR1*), while SA-repressed genes showed nucleosomal enrichment (Singh et al. 2015).

DNA Methylation

Changes in DNA methylation are another obvious candidate mechanism for long-term stress memory. As discussed above, DNA hypomethylation has been detected following infection of *Arabidopsis* with *P. syringae* in several studies (Dowen et al. 2012; Pavet et al. 2006; Yu et al. 2013). This may play a functional role in immediate induced resistance responses, since hypomethylated loci centred around transposable elements are enriched in defence genes (Dowen et al. 2012). Yu et al. (2013) also identified demethylation of TEs near defence genes in response to treatment with the PTI-eliciting FLG22 peptide. This response was dependent on the DNA glycosylase *ROS1*. These stress responsive changes in methylation are consistent with the phenotypes of hypomethylated DNA methylation mutants, which are typically more resistant to infection (Dowen et al. 2012; Le et al. 2014; López et al. 2011; López Sánchez et al. 2016; Luna et al. 2012; Luna and Ton 2012). Whether mutants that suffer from extensive genome-wide hypomethylation genuinely reflect what happens following infection is open to question, but plants with more restricted regions of hypomethylation, such as in the so-called epiRIL (epigenetic recombinant inbred) lines generated by back-crossing hypomethylated mutants to wild-type *Arabidopsis*, can also exhibit altered responses to defence hormones and variations in resistance to pathogens (Latzel et al. 2012). Importantly, increased resistance in some DNA methylation mutants has been attributed to priming rather than constitutive SA-responsive gene expression (López et al. 2011; López Sánchez et al. 2016). Since DNA methylation is readily inherited during mitotic cell divisions, it provides a potential mechanism for long-term priming memory that can extend even into cells and tissues not present during the initiating stress.

2.4.2 Long-Term Priming Memory

Responses such as SAR are well documented to persist over several weeks, but in recent years, examples of defence priming that persist for much longer periods of plant development have also emerged. For example, seed treatments with elicitors including JA, chitosan and BABA resulted in defence priming that persists for many weeks (Haas et al. 2018; Strapasson et al. 2014; Worrall et al. 2012), while seedling root drenches with BABA prime long-lasting disease resistance in *Arabidopsis* and tomato (Luna et al. 2014; Wilkinson et al. 2018). Not only that, priming can extend from one generation to the next. One early report hinting at what is now referred to as transgenerational acquired resistance (TAR) in the case of SA-dependent disease resistance, or transgenerational immune priming (TGIP) more generally, suggested that tobacco mosaic virus (TMV) infection enhanced resistance in progeny of tobacco (*Nicotiana tabacum*; Roberts 1983). Other studies found that *Brassica spp*. suffering biotic stress produced seeds containing higher concentrations of glucosinolates compared with non-infested control plants (Lammerink et al. 1984; Shattuck 1993). Another series of papers demonstrated that insect herbivory on wild radish (*Raphanus raphanistrum*) enhanced resistance in seedlings of progeny plants (Agrawal 2001, 2002; Agrawal et al. 1999). The increase in resistance observed in these studies was transient and no mechanism was identified. Although intriguing in the context of the potential ecological benefits of TGIP, the examples described above could also be explained by simple maternal effects—the provisioning of seeds with altered resources from the mother plant in response to stress. While widely recognized as ecologically important, maternal effects are distinct from *bone fide* transgenerational inheritance, which enables the offspring generation to express phenotypes independently of any non-genetic contribution from the parental plants. Such transgenerational inheritance is most likely to be epigenetically encoded.

2.4.2.1 Transgenerational Immune Priming

Several examples have emerged over recent years which provide much stronger evidence for true transgenerational inheritance of biotic stress priming. As well as following pest and pathogen attack, priming can be induced by various chemical agents. One of the best studied among these is β-aminobutyric acid (BABA; Cohen et al. 2016). As well as within-generation priming, it was recently found that the progeny of plants primed either by BABA treatment or infection with avirulent *P. syringae* bacteria exhibited TAR. Offspring of treated plants were more resistant to infection by both *Pst* and *Hpa* because of primed SA-dependent gene expression (Slaughter et al. 2012). Intriguingly, offspring of BABA-primed parents were more responsive to BABA treatment than offspring of control plants, suggesting a ‘primed-to-be-primed’ phenotype. Similarly, treatment of barley (*Hordeum vulgare*) with the commercial resistance-inducing agent acibenzolar-*S*-methyl, or with saccharin, resulted in TAR against leaf blotch disease caused by the fungal pathogen, *Rhynchosporium commune* (Walters and Paterson 2012).

Luna et al. (2012) also reported TAR in *Arabidopsis* following repeated inoculations with virulent *P. syringae*. A key aspect of this work was that the authors were not only able to identify priming in the immediate offspring generation, but it could also be detected in the grandchildren of the infected plants. This demonstrates that priming memory can be inherited over at least one stress-free generation and eliminates the possibility that maternal effects alone are responsible for the increased resistance. Subsequent work from the same group now shows that priming can be detected, albeit weakly, even after two stress-free generations (Stassen et al. 2018), indicating that the phenomenon must be epigenetically regulated. In evolutionary terms, the gradual loss of priming after the initial stress episode would be expected in order to avoid excessive costs of priming and could readily be achieved through reversible epigenetic changes. In parallel with this work on transgenerational disease resistance, similar responses to herbivory were reported. terHorst and Lau (2012) found that both herbivore resistance and reproductive fitness were affected by parental exposure to insect herbivory in a field experiment with *Lotus wrangelianus*. Moreover, herbivory also resulted in JA-dependent transgenerational priming of defence against insects in both *Arabidopsis* and tomato (Rasmann et al. 2012).

2.4.2.2 Mechanisms for TGIP

The most likely mechanisms for true transgenerational priming effects are epigenetic. Similar to within-generation priming responses such as SAR, histone modifications could be detected in the promoters of the SA-regulated genes, *PR1*, *WRKY6* and *WRKY53* in plants derived from *P. syringae*-infected parents (Luna et al. 2012). Such histone modifications may well contribute to primed defence gene expression in TAR, but there is still wide debate over the roles that histone modifications might play in epigenetic inheritance between generations (Heard and Martienssen 2014). Much better understood is the ability of DNA methylation to be meiotically inherited, and DNA methylation has therefore been suggested as the more likely mechanism for encoding transgenerational epigenetic memory (Quadrana and Colot 2016). It has become increasingly clear over recent years that modifications to the DNA methylome can be maintained through the plant's lifespan and into subsequent generation(s), and can generate heritable phenotypic changes (Bossdorf et al. 2010; Johannes et al. 2009; Mathieu et al. 2007; Verhoeven et al. 2010). Accordingly, several studies have found that mutants affected in various regulatory mechanisms controlling DNA methylation show altered biotic stress resistance, and/or fail to establish transgenerational priming (López Sánchez et al. 2016; Luna et al. 2012; Luna and Ton 2012; Rasmann et al. 2012). These two modes of epigenetic regulation are not mutually exclusive, since histone modifications and DNA methylation are somewhat inter-dependent (Heard and Martienssen 2014).

The strongest current candidate for generating methylation-dependent stress memory is RNA-dependent DNA methylation (RdDM). As noted above, several studies have shown that mutations in genes involved in the RdDM pathway can have a direct impact on defence responses (López et al. 2011; Le et al. 2014; López

Sánchez et al. 2016; Luna and Ton 2012). RdDM-mediated methylation is initiated by the generation of 21–24 nt siRNAs by DICER-like proteins, and transgenerational priming responses to both biotic and abiotic stress have been found to require the production of siRNAs. Furthermore, offspring priming phenotypes triggered by different abiotic stresses in *Arabidopsis* required *DCL2* and/or *DCL3*, which are responsible for the production of 21/22 and 24 nt siRNAs, respectively (Boyko et al. 2010). Similarly, for biotic stress, the *dcl3–1* mutant failed to establish TAR against biotrophic pathogens (Luna and Ton 2012) and a *dcl2/dcl3/dcl4* triple mutant failed to establish transgenerational priming against herbivores (Rasmann et al. 2012). siRNAs are able to move systemically throughout the plant (Dunoyer et al. 2010; Lewsey et al. 2016; Molnar et al. 2010), and can therefore be viewed as candidates for long range priming signals both within and between generations, since reprogramming of methylation in germ line cells by siRNAs would allow inheritance by offspring tissues. Interestingly, sRNA populations have been reported to be significantly influenced by parental/grandparental environmental stress in both *Brassica rapa* and the dandelion, *Taraxacum officinale* (Bilichak et al. 2015; Morgado et al. 2017). Since pathogen infection triggers genome-wide methylation changes associated with reactivation of transposon sequences, generation of mobile siRNAs and subsequent RdDM presents a feasible mechanism for maintaining stress memories.

Active DNA demethylation also appears to be required for TAR. The DNA glycosylase ROS1 plays a major role in global demethylation, and was found to be essential for transgenerational memory in offspring of *Pst*-infected *Arabidopsis* (López Sánchez et al. 2016). Interestingly, the same authors found that ROS1 was not required for within-generation SAR. The nature of the changes imposed by ROS1-dependent demethylation and RdDM in response to biotic stress and the mechanisms by which they impact on defence phenotypes remain to be elucidated. Although methylome profiling of different generations of plants expressing TAR identified differentially methylated sites that correlate with ancestral stress experiences (Stassen et al. 2018), the analysis of these sites does not yet provide a clear insight into the mechanism of transgenerational priming.

2.4.2.3 Re-Setting Epigenetic Priming Memory

Epigenetically mediated transgenerational defence priming provides a novel system by which plants can display phenotypic plasticity in response to environmental stress, providing enhanced evolutionary fitness when parental environments are good predictors of offspring environments. To have evolved, the benefits of transgenerational phenotypic plasticity must outweigh any costs. Because plasticity is epigenetically mediated, it is readily reversible, such that when stress is not present, defence reverts to basal levels, thus minimizing costs. Costs would also be minimized when the signals initiating priming are good predictors of future stress. In other words, long-lasting priming would be expected only under strong, consistent stress, and should decay in the absence of stress. These predictions appear to hold

true in the limited instances where these ideas have been tested. Singh et al. (2014a) found that repeated, but not single, mild stress exposures provided short-lived priming of biotic stress resistance but was not sufficient for long-term priming. TAR induced by *P. syringae* infection of *Arabidopsis* was maintained at high levels when successive generations of plants were inoculated, but was gradually lost when only a single generation suffered disease (Stassen et al. 2018). The costs of transgenerational priming remain relatively poorly explored, but one clear cost is seen in the antagonism between the major SA- and JA-dependent defence pathways. TAR against biotrophic pathogens was associated with transgenerational increased susceptibility to necrotrophic pathogens (Luna et al. 2012), while transgenerational priming of herbivore resistance caused increased susceptibility to *P. syringae* infection (Singh et al. 2017). The storage of epigenetic marks reflecting stress memories of previous generations could therefore have deleterious impacts when offspring experience different stresses than their parents (Crisp et al. 2016; Iwasaki and Paszkowski 2014). Avoidance of such maladaptive memories requires a system for re-setting of epigenetic stress memories that can balance the forces imposing them. As an example to keep in mind, the *Arabidopsis FLC* locus, involved in vernalization, is epigenetically silenced in response to low temperature. The low temperature memory is re-set each generation, meaning that it is the experience of individual plants, not their ancestors, that determines flowering time. Mutation of the *ELF3* gene, a histone H3K27me3 demethylase, causes a failure of the ability to re-set the low temperature signal, meaning that offspring flower early regardless of environmental conditions (Crevillén et al. 2014). Other re-setting systems have also been identified. Two chromatin regulators, *DECREASE IN DNA METHYLATION1* (*DDM1*) and *MORPHEUS' MOLECULE1* (*MOM1*), prevent the transmission of memories of heat stress exposure from parents to their progeny. Genome-wide transcriptional signatures induced by stress were found in the subsequent generation in *ddm1/mom1* double mutants, but not wild-type plants (Iwasaki and Paszkowski 2014). More recently, MOM1 has also been identified as an epigenetic regulator of the expression of pattern recognition receptor (PRR) and nucleotide-binding leucine-rich repeat (NLR) genes in *Arabidopsis*. PRRs and NLRs act as receptors for activation of PTI and ETI, respectively, and therefore changes in their expression could potentially be a mechanism for defence priming. *MOM1* indirectly regulates PRR/NLR gene expression via an RdDM-dependent pathway for transposon silencing. Mutants deficient in *MOM1* displayed higher expression of PRRs/NLRs and were more resistant to bacterial infection (Cambiagno et al. 2018), consistent with the idea that *MOM1* might function antagonistically with defence priming. Such mechanisms of chromatin re-setting could prevent or constrain transgenerational priming of defence to optimize trade-offs between the costs and benefits of priming.

2.5 Potential Application of Epigenetics

World population is growing at a speed that places food security at risk at a global level (FAO 2009a). In this context, increasing crop yields by reducing losses caused by pests and diseases would appear to be essential. At the same time, the use of pesticides, which have had a major influence on protecting yields in the past, has been demonstrated to considerably contribute to soil degradation, ecosystem disturbance, climate change and even to have a negative impact on human health. Given this scenario, global entities are taking action to promote the development and implementation of alternative crop protection technologies and strategies (European Academies Science Advisory Council and Deutsche Akademie der Naturforscher Leopoldina 2014). Although when its impacts were first recognized, epigenetics represented an inconvenience in biotechnology, for example, because of unintended gene silencing effects (Napoli et al. 1990; van der Krol et al. 1990), today, the field of epigenetics presents a clear example of the importance of fundamental research in future applications (Connor 2002). On the one hand, advances in the understanding of epigenetic mechanisms have already brought novel tools for biotechnologists to analyse, control and tailor gene expression. On the other hand, the possibility to produce new stable (even heritable) phenotypes in the absence of genetic changes opens doors to new approaches avoiding transgenesis, which has important implications in view of the current regulatory framework around genetically modified organisms (GMOs) and their public perception. Moreover, boosting the plants' natural immune system has been suggested as the safest approach to improve crop yields while minimizing environmental impacts (Rapicavoli 2015; Dewen et al. 2017; Kothari and Patel 2004; Quintana-Rodriguez et al. 2018).

Bearing in mind that the origin of the epigenetic machinery seems to be linked to ancestral defence mechanisms, we believe the application of epigenetics to crop protection could provide significant breakthroughs in the development of future integrated pest management programs (Stenberg 2017). Here, we discuss some possible applications to translate the new knowledge of epigenetics to first, keep broadening our understanding of natural phenomena, and second, as a tool to a new generation of plant biotechnologists and breeders in the field of crop protection.

2.5.1 As a Tool in Research

Advances in the understanding of epigenetic mechanisms have significant potential to impact the future research of plant–pathogen interactions. Up to now, such understanding has enabled the development of various techniques to characterize and even manipulate the epigenome. Nowadays, we can detect small RNAs by northern blotting, hybridization with probes for detection and subtraction, PCR/qPCR, etc. (Boccara et al. 2017; Li and Zamore 2018; Ro and Yan 2010; Urbanek et al. 2015). Using the new advances in bioinformatics we can also sequence and map sRNAs in

different genomes, compare them in different species or even design specific systems to knock them down. Chromatin changes can be analysed by different techniques such as chromatin immunoprecipitation (ChIP; Furey 2012), DNase I treatments (Cockerill 2011), FAIRE (Simon et al. 2012) and chromatin conformation capture (3C; Dekker et al. 2002). There have also been enormous advances in the analysis of DNA methylation as a result of the development of bisulphite sequencing, which enables the detection of changes in DNA methylation at the level of individual cytosine residues across the whole genome. Other techniques, like ChIP analysis using antibodies against methyl-cytosines (MeDIP-ChIP), allow for the detection of regional differences in DNA methylation (Cortijo et al. 2014b). MeDIP-ChIP has lower resolution than bisulphite sequencing, but it can be used as an affordable method to detect regional differences in the whole genome, or, coupled to PCR, for the detection of differential methylation in discrete regions, which can be helpful in species for which the full genome cannot be sequenced. Another important set of tools is based on the differential activity of restriction enzymes in methylated and non-methylated DNA. This property of the endonucleases can be used for the detection of the whole-genome methylation level by southern blotting, in which no or minimum information about the DNA sequence is required. It can also be used in the design of chop PCR markers, which allow us to detect changes in DNA methylation at specific positions quickly and economically.

Currently, we can not only detect, but also modify the levels of DNA methylation. For example, following the identification of the proteins of the DNA methylation machinery, mutant plants are available with different and relatively stable patterns of DNA methylation that can be used for research and applied purposes. There are also several chemical reagents known that alter the epigenome. For example, 5-azacytidine (5-azaC), zebularine and sulfamethazine reduce the general levels of DNA methylation (Jones 1985; Zhang et al. 2012; Zhou et al. 2002). More targeted manipulation of the epigenome—epigenome editing—is now becoming possible as a result of new advances in molecular biology. By fusing proteins with DNA methylation/demethylation activities to sequence-specific DNA binding proteins such as zinc finger nucleases or the CRISPR/dCas 9 system, it is possible to target methylation/demethylation to specific target sites (Gallego-Bartolomé et al. 2018). Hence, our knowledge of epigenetics made it possible to develop these techniques, and we are now in a position to use them to speed up progress in understanding mechanisms of plant defence.

Now, we can also understand phenotypes that were baffling for decades, as they involved changes in gene expression in the absence of any change in DNA sequence. The use of the aforementioned techniques is accelerating the discovery of the epigenetic mechanisms controlling plant defence. It has opened the door to the identification of new natural epialleles, as well as the understanding of part of the observed natural variation found between species and ecotypes (Cubas et al. 1999; Niederhuth et al. 2016; Richards 2011; Turck and Coupland 2014; Vaughn et al. 2007; Zhai et al. 2008). It has also contributed to unravelling the evolution of the plant immune system. Since the plant immune system has been demonstrated to be controlled by epigenetic mechanisms at almost all levels, the study of the epigenotype will be

essential for the future understanding of plant defences and the translation of this new knowledge into crop protection strategies.

2.5.2 As a Tool in Biotechnology

Crop losses due to pests and diseases must be reduced to a minimum to ensure food security and sustainability in the coming decades. World population is expected to grow up to 9.7 billion by 2050. Thus, improving crop yields is a priority action for the world's public entities in order to produce more food on less land, avoiding the over-exploitation of natural ecosystems and the decline in biodiversity (FAO 2009b). At the same time, global climate change is introducing abiotic stress variables that promote outbreaks of different plant pathogens. To date, the use of pesticides has been the most successful agronomic strategy to protect crops from infections. Unfortunately, pests tend to easily acquire resistance to chemical pesticides. Moreover, the use of pesticides contributes to the disruption of natural ecosystems, degrading soils and contaminating water sources. It has also been demonstrated to be potentially toxic or carcinogenic, at the very least for workers who manipulate the products (Aktar et al. 2009). This provides additional impetus in the search for alternative strategies (European Commission 2009). Moreover, while biotechnologists focused their efforts on genetic engineering of plants to introduce new traits, there has been a strong public pressure against transgenesis and other forms of genetically modified organisms, especially in Europe (Collinge et al. 2010). With this in mind, some experts in the field agree that plant vaccination and priming (or plant immunization) is one of the most promising approaches in pursuit of designing safer and more sustainable crop protection programs (Kothari and Patel 2004; Rapicavoli 2015; Dewen et al. 2017; Quintana-Rodriguez et al. 2018).

As this chapter has described, the plant immune system and priming processes are epigenetically controlled at different levels. Given that it facilitates phenotypic changes while avoiding transgenesis, epigenetics appears to be an attractive tool/target for breeders and agronomical biotechnologists hoping for a new and more sustainable green revolution.

2.5.2.1 PDR: Pathogen-Derived Resistance

The first examples of the exploitation of epigenetics for plant protection were seen in the form of pathogen-derived resistance (PDR) or 'plant vaccination'. It had been known for some time that infections with some viruses triggered cross-protection against similar viruses (Hamilton 1980). The scientific basis for this observation was unknown for a long time, but resistance was assumed to be triggered by the production of viral components. Thus, PDR was proposed as a biotechnology strategy for plants in which pathogen-specific components could be altered and overexpressed in a non-functional form, or at the wrong developmental stage, to

somehow compete with the functional proteins produced by the virus (Sanford and Johnston 1985). Following the suggestion of this concept, there were a few successful cases of protein-mediated resistance (based on viral coat protein expression) that were probably unrelated to epigenetic mechanisms (Abel et al. 1986; Powell et al. 1990). In all cases, the mechanism involved the production of transgenic plants over-expressing genes (or modified versions of them) from the target pathogen. Surprisingly, in many subsequent cases, this approach was discovered to not be always associated with the levels of proteins of the transgene, but rather, RNA expression. In fact, instead of protein-mediated resistance, RNA-mediated resistance appeared to provide near complete immunity against, at least, the target virus. The basis of such RNA-mediated resistance remained obscure until it was linked with the co-suppression phenomenon observed in transgenic petunia plants (Lindbo et al. 1993). It then became clear that in most viral PDR cases, protection was due to the activation of gene silencing mechanisms against the pathogenic virus. With the discovery of the epigenetic mechanisms, PDR mediated by RNA expression has been improved (Prins 2003), optimizing the silencing of the pathogen. RNA silencing-mediated PDR is also now known as host-induced gene silencing (HIGS), which involves plant transformation with constructs expressing efficient siRNAs targeting different pathogen elements.

This strategy has been demonstrated to be effective not just against viruses in model organisms, but also other groups of pathogens and herbivores and in different crop species (Huang et al. 2016). Since its first uses in tobacco and *Arabidopsis*, PDR has been successfully applied to introduce virus resistance to crops such as papaya (against the papaya ringspot virus, PSRV; Gonsalves 1998; Jia et al. 2017), squash (against the Squash leaf curl virus; Taha et al. 2016), tomato (Schwind et al. 2009), cotton, rice, maize, corn, wheat and barley (Duan et al. 2012; Huang et al. 2016; Koch and Kogel 2014). Beyond its uses in plant-viral protection, it has been adapted to protect plants against many other different pest and diseases such as bacteria, fungi (Nowara et al. 2010; Nunes and Dean 2012), parasitic plants, nematodes or insects (Koch and Kogel 2014). Perhaps most remarkable is the work performed in plant–insect interactions, where HIGS-mediated resistance has been demonstrated following the ingestion of plant-produced siRNAs during insect feeding (Baum et al. 2007). Undoubtedly, the latest discoveries involving cross-kingdom sRNA trafficking will help us to better engineer PDR and/or HIGS, optimizing this kind of plant vaccination (Wang et al. 2016, 2017).

In some cases, PDR confers almost complete resistance. However, it also poses some problems. The first issue is that it involves transgenesis, which faces strong social opposition and is under the control of very restrictive legislation (Lucht 2015). On the other hand, alongside the benefits of resistance, there could be some deleterious consequences in the plant, due to the insertion and over-expression of the constructs. For example, the genomic insertions could disturb genic regions required for specific plant functions in response to environmental changes, which is difficult to predict or identify in field trials. A strong activation of silencing mechanisms could also have consequences for the natural roles of these pathways during plant development or responses. Finally, the expression of the transgene can be

modified by environmental changes, or even silenced due to its high expression (Martelli 2001; Zhao et al. 2014). Therefore, even though its high potential has been demonstrated for several decades, its success usually requires an important investment of time and optimization effort, which has restricted its successful commercial application to a relatively small number of specific cases.

2.5.2.2 Epigenetics Mediated Resistance

Since the role of DNA methylation in plant defence has only emerged relatively recently, commercial approaches that exploit epigenetics have not yet reached the market. However, the possibility to select or engineer plants at the epigenetic level to improve resistance without transgenesis makes this one of the most fashionable current strategies that is being actively pursued. In the same way that breeders have been searching for disease resistance genes in crop wild relatives and land races to develop introgression systems, we now know that a proportion of natural phenotypic variability is generated by the epigenome (Zhang et al. 2013). Given that some DNA methylation patterns appear to be stably inherited through meiosis, an attractive idea is the use of natural epigenetic alleles (epialleles) to provide traits for classical selective plant breeding (Gallusci et al. 2017; Hofmeister et al. 2017; Zhang and Hsieh 2013). In this respect, epialleles impacting on defence phenotypes from different backgrounds could be identified and introgressed (Ji et al. 2015).

There are a number of methods by which such epialleles can be identified. The first is through simple screening of populations of a single genotype for phenotypic variation. Repeated selection of an isogenic population of *Brassica napus* for individuals with elevated energy use efficiency (EUE) resulted in the isolation of 'epilines' with improved EUE and which gave increased yield (Hauben et al. 2009). The improvement in EUE appeared to come from altered DNA methylation profiles rather than genetic changes was inherited for eight generations and was stable over 3-year field trials. Later work used a similar strategy to isolate stable epilines with increased EUE and drought tolerance (Verkest et al. 2015). These lines showed increased expression of abiotic stress-related genes, and elevated H3K4me3 methylation at those genes. As well as simply screening for naturally occurring epigenetic variation, it is possible to create recombinant populations in which individual lines carry substantial portions of the genome with altered methylation patterns. Such lines are known as epigenetic recombinant inbred lines (epiRILs). EpiRILs can be generated from two varieties or ecotypes with similar genetic sequences but different epigenetic landscapes (Kawakatsu et al. 2016; Schmitz et al. 2013). The approach for isolating useful epialleles that provide novel traits assumes that novel methylation patterns carried by an epiRIL can be stably inherited and used for crop breeding. One method for generating novel epigenetic patterns is the generation of epiRILs by crossing mutants defective in DNA methylation machinery. Homozygous mutants develop a different epigenetic landscape (e.g. hyper- or hypomethylation), and following removal of the original genetic mutation by back-crossing, the resulting recombinants would carry a mosaic of different epigenetic marks at different

locations across the genome. This has already been applied successfully in *Arabidopsis* (Cortijo et al. 2014a; Johannes et al. 2009; Reinders et al. 2009), and several groups are currently working in epiRILs in crops like tomato, wheat and rice. Genome-wide epigenetic variability can also be induced by the application of DNA methylation inhibitors like sulfamethazine, 5-azacytidine (5-azaC) and zebularine (Jones 1985; Zhang et al. 2012; Zhou et al. 2002), or by introduction of novel DNA methylase/demethylase enzymes (Hollwey et al. 2017). An interesting alternative is to use natural instances of epigenome reprogramming. For example, plants regenerated from tissue culture often exhibit significant phenotypic variation, despite being clonal and therefore, in principle, identical at the DNA sequence level. This is known as somaclonal variation. Wibowo et al. (2018) recently found that Arabidopsis plants regenerated from root (but not leaf) cells were more susceptible to *Pst* and *Hpa* infection than non-regenerated controls. Genome-wide DNA methylation profiles also differed with the origin of regenerated plants and altered methylomes were inherited for at least three generations. Finally, where a priori information exists about desirable epigenetic traits, modifications at specific target loci can be achieved through the use of epigenome editing (Gallego-Bartolomé et al. 2018; Kungulovski and Jeltsch 2016) as described above. Despite the novelty of breeding with epialleles, it should not be forgotten that as for other forms of genetically encoded resistance, systems that result in constitutively elevated defences are likely to bring with them costs in yield in the absence of stress, due to the expense of allocating resources to defence. In nature, such costs are minimized by priming of defence, which could therefore be an attractive trait to target.

2.5.2.3 Epigenetic Priming

With the exception of the epigenome editing at specific sites, the approaches described above rely on natural or introduced random epigenetic variation as a source of natural variation from which to select desired traits, such as pest and disease resistance. However, the phenomenon of transgenerational immune priming described above, suggests that epigenetic resistance can be induced by exposure of plants to biotic stress. The primed state involves sensitization of defence responses following initial triggering of priming, but primed plants do not express higher levels of defence prior to attack by pests or pathogens, avoiding costs (van Hulten et al. 2006). Priming is therefore probably more similar conceptually to vaccination or immunization in vertebrates (Hilker et al. 2016; Mak et al. 2014) than PDR, as it involves a certain memory of the first stress-indicating signal. Priming falls within the concept of natural plant immunization, which has been suggested as one of the new targets for the next green revolution (Dewen et al. 2017; Quintana-Rodriguez et al. 2018).

The long-lasting nature of priming is one of its strengths for crop protection. Importantly, across shorter to longer periods, priming memory has been correlated with epigenetic changes (Jaskiewicz et al. 2011; López et al. 2011; Luna and Ton 2012). In fact, the majority of *Arabidopsis* mutants impeded in de novo DNA

methylation show resistance against biotrophic pathogens, but do not constitutively express defences. Rather, they show a constitutive priming phenotype. The lower cost of priming as a defence strategy is evident as such mutants do not usually show alterations in growth or development under control conditions. Thus, the already mentioned induced changes in the epigenetic landscape by the use of mutants, chemical compounds or epigenetic editing could be applied to trigger the epigenetic changes that induce the priming state. One successfully commercialized example is the use of seed treatments with elicitors of defence. Seed treatment with JA or BABA provides long-term priming of pest and disease resistance with either no or minimal costs in terms of growth and development (Paudel et al. 2014; Worrall et al. 2012). Obviously, the most natural priming induction would be to moderately stress plants in order to induce long-lasting priming, or even transgenerational priming. This approach does not involve the use of transgenesis, chemical treatments or any artificial systems, does not involve the constitutive induction of defences and is long-lasting. Priming should also be a sustainable approach, since it is not expected to artificially impact other species or the physicochemical properties of natural ecosystems. Moreover, it is expected to be more resilient to environmental changes as it is naturally responsive to them. Unfortunately, the phenotype induced by these methods is very variable and our poor understanding of the underlying mechanisms prevents the design of molecular markers to track resistance at the current time. Undoubtedly, more research is needed in this promising field to make it feasible and widely applicable. Advances in fundamental understanding, including the interactions between multiple stresses, durability of priming and the development of markers to trace the epigenetic changes are all needed to optimize its application in future crop protection programs.

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Chapter 3
The Role of Germinally Inherited Epialleles in Plant Breeding: An Update

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, mainly DNA methylation, are suitable for selection in plant breeding. This chapter is an update from a chapter published 4 years ago that highlighted that pure epigenetic variation (epigenetic differences across individuals not caused by DNA differences) is rare and often unstable and thus plays a small role in plant improvement. Our thesis has remained unchanged, but we supplement this text with additional examples, and we highlight those rare situations where pure alleles or facilitated epigenetic alleles (alleles that are caused by a DNA polymorphism but are maintained independently of that polymorphism) may be beneficial to plant improvement.

3.1 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 (Waddington 1942). In other words, epigenetics referred to mechanisms that enable the developmentally appropriate expression of genes. In Waddington’s conception, epigenetic information laid down in

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development is erased during gametogenesis, consistent with the Mendelian principle that genes passed across generations are unaltered by developmental or environmental stimuli. More recently, pure epigenetics has been described as meiotically heritable changes in gene function that are not due to differences in nucleotide sequence. Following this definition, an organism can impose chemical changes to DNA or chromatin within a germ cell, and these changes can be 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 epigenetic inheritance.

In this chapter, we first review how the inheritance of variable chromatin states (mainly variation in DNA methylation)—induced spontaneously, chemically, or genetically—can contribute to phenotypic variation. We then address the stability of epialleles across generations and the frequency at which stable epialleles occur in plant genomes. Finally, we highlight the role of epigenetic variation in plant breeding.

3.2 Changes in DNA Methylation Can Be Pure, Facilitated, or Genetically Dependent

Most epialleles characterized to date are marked by changes in the status of DNA methylation. These epigenetic modifications are easy to identify and the processes by which they are directed, maintained, and removed are well understood, making them attractive to investigate. Before examining specific instances of methylation-based epialleles and how they can be exploited in plant breeding, it is important to understand the relationship between cytosine methylation and the primary sequence of DNA. While pure epialleles do occur (i.e. those changes in DNA methylation that are purely epigenetic and have no dependence on DNA sequence), there are also epialleles that are either partially (facilitated epialleles) or fully (obligate epialleles) dependent on DNA polymorphisms and thus have a genetic dependence (Richards 2006). A solid example of a facilitated epiallele has been described for the *FOLT1* gene (Durand et al. 2012). *FOLT1* is a folate transporter whose expression can be silenced via increases in cytosine methylation. These changes in methylation are actually directed by truncated copies of a second, but related gene, *FOLT2*, that are located on a different chromosome. siRNAs originating from these truncated copies direct the hypermethylation at *FOLT1* and the subsequent silencing. If *FOLT1* silencing was completely dependent on the presence of the truncated copies of *FOLT2,* then this example would describe an obligate epiallele; however, silencing of *FOLT1* remains after the siRNA-derived loci are segregated away, indicating that

specific loci are necessary for initiation of the epiallele but are not required for its maintenance through generations. An example of an obligate epiallele has been elucidated by Woo et al. (2007). In this case, demethylation of centromeric repeats was identified in an Arabidopsis accession carrying a mutation in *VARIANT IN METHYLATION 1*. Occurring only in the presence of vim1, this epiallele is of the obligate category and is completely dependent on DNA polymorphism.

Facilitated and obligate epigenetic polymorphisms show greater stability than pure epialleles and are thus likely to be the most useful in plant breeding efforts geared towards generation of highly stable and heritable traits. Here, we describe a variety of pure and facilitated epialleles and bring attention to those types that are likely candidates for integration into crop improvement programs.

3.3 Meiotically Inherited Epigenetic Differences Can Cause Phenotypic Variation

Meiotically heritable epigenetic modifications are of interest to plant breeders because they can direct changes in phenotype that, in some cases, provide stable improvements to plant phenotype. In this chapter we focus on cytosine methylation (also commonly referred to as DNA methylation). The molecular processes underlying cytosine methylation are thoroughly described. The manipulation of DNA methylation via chemical treatment and through the use of genetic mutants is also well studied. While the examples of the positive relationships between changes in DNA methylation and changes in plant phenotype are numerous, it is important to understand how pure and facilitated epigenetic alleles are created and their transgenerational stability before determining their relevance to plant breeding. Here, we describe a variety of instances where changes in DNA methylation that have occurred spontaneously, following chemical treatment, at genes paired with certain alleles in heterozygotes, or in genomes with defective DNA methylation machinery, have resulted in changes to plant phenotype.

Some remarkable examples of pure epigenetic modifications have been found to occur spontaneously in nature (Table 3.1). One of the best known examples comes from a toadflax (*Linaria vulgaris*) mutant, originally described by Linnaeus, which 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), but instead 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 symmetric floral trait correlates perfectly with the cytosine methylation status of Sau3A restriction enzyme recognition sites (Cubas et al. 1999). In other words, plants with radially symmetrical flowers have high cytosine methylation upstream and within the coding sequences of *Lcyc*. In tomato, one

Table 3.1 Examples of epialleles described in this chapter

Species	Locus	Nature of change	Trait affected	Refs
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)
Rice	*QQS*	Spontaneous	Starch metabolism	Silveira et al. (2013)
Rice	*Epi-d1*	Spontaneous	Height	Miura et al. (2009)
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)
Strawberry	?	Induced (5azaC)	Flowering time and rosette diameter	Xu et al. (2016a, b)
Maize	*Spm*	Spontaneous	Anthocyanin production	McClintock (1958, 1965), Peterson (1966), Fowler and Peterson (1978), Banks et al. (1988), Fedoroff (1999)
Arabidopsis	*SUP*	Mutagen	Floral morphology	Jacobsen and Meyerowitz (1997), Ito et al. (2003)
Arabidopsis	*FWA*	Mutagen	Flowering time	Soppe et al. (2000)

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). More recently an epiallele of the *QSS* (*Qua-Quine Starch*) gene that is involved in starch metabolism in Arabidopsis has been identified (Silveira et al. 2013). This epiallele appears spontaneously in nature and is explained by methylation of repeat elements in the 5′ region of the gene (Table 3.1) (Silveira et al. 2013).

Treatment of plants with DNA methylation inhibitors, such as 5-azacytidine (5azaC) and 5-azadeoxycytidine (azadC), can induce pure epigenetic, heritable, phenotypic changes, though it is not always known if the resulting phenotypic changes are truly pure, facilitated, or because the inhibitors may be mutagenic (Fig. 3.1). For example, Fieldes (1994) induced heritable phenotypic changes in flax using a 5azaC treatment. Relative to untreated plants, the plants growing from treated seeds were often shorter, had fewer leaves on the main stem, and had reduced flowering times (Table 3.1) (Fieldes 1994). From first generation progeny of treated

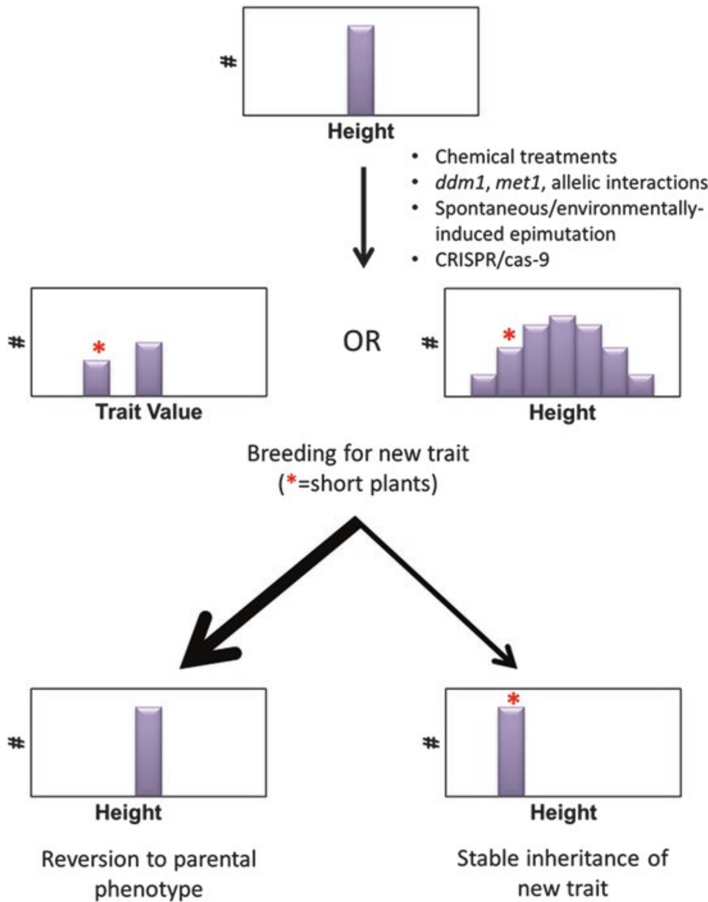

Fig. 3.1 Epimutations induced by several phenomena can generate phenotypic novelty that is in some cases stably inherited. Within the histograms above, the X axis represents a trait value for a plant, for example, plant height. The Y axis represents the number of individuals within a population with that trait value. New discrete or continuous trait values arise because of epimutation. The *asterisk* represents a new, favourable 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

plants, Fieldes 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 and many generations following that (Amyot 1997; House 2010). Additionally, 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 × rye hybrid plant, plants grown from seeds treated with 5azaC have a number of heritable, phenotypic differences relative to plants from untreated seeds (Table 3.1) (Heslop-Harrison 1990). Specifically, one 5azaC treatment resulted in plants that were taller than controls, had 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 the DNA demethylating agent 5-azadeoxycytidine (azadC) differed from plants grown from untreated seeds. Most remarkable was a line that was dwarf and flowered 10–14 days early (Table 3.1). Similarly, chemically induced heritable variation has been observed in 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 Melandrium (appearance of bisexual flowers on a normally dioecious plant) (Janoušek et al. 1996). Recently, 5-azaC was used to induce changes in cytosine methylation in strawberry (Xu et al. 2016a) and through repeated selection, stable early flowering lines were established (Table 3.1) (Xu et al. 2016b). These strawberry studies are of significance because they provide evidence that changes in cytosine methylation can be induced by chemical treatment, can alter important traits, and be selected via artificial selection.

Mutations within genes important for maintaining DNA methylation also act as epimutagens and generate facilitated, heritable epialleles. For example, the *Arabidopsis thaliana* gene *DDM1* (*Deficient in DNA Methylation 1*) encodes an ATPase chromatin remodeler 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; Singer et al. 2001; Miura 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 related to leaf structure, flowering time, flower structure, both increased and decreased apical dominance, and reduced internode length arise at high frequency (Kakutani et al. 1996). Some epialleles that appear within the *ddm1* mutant background, such as that determining the *bns* phenotype, are stably inherited (Kakutani et al. 1996; Soppe et al. 2000; Saze and Kakutani 2007). METHYLTRANSFERASE1 (MET1) is also required for propagating 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 alleles (Finnegan et al. 1996).

Some trait variation is also caused by allelic interactions between homologous alleles, which is also referred to as paramutation, a type of facilitated epiallele. While it is clear that there are genetic requirements for paramutation (Springer and McGinnis 2015), the complete mechanism underlying the process has yet to be determined. Studies of maize pigmentation inheritance have revealed a number of these scenarios. Brink (1956), who was studying anthocyanin biosynthesis in maize, noted the changing effects of alleles across generations. Specifically, he found that the effect of the *R-r* allele, which typically confers full pigmentation in seeds, varied in its effect on seed colour depending on an association with another allele, *R-st*, an allele which results in stippled pigmentation. If inherited with *R-st*, the resulting *R-st*/*R-r* progeny had lower-than-expected levels of pigmentation. When *R-st*/*R-r* was crossed with *r*/*r* individuals, the progeny carrying the *R-r* allele also had much reduced pigmentation compared to the expectation of fully pigmented seeds. In fact what Brink was observing was the paramutagenic effect of *R-st* on *R-r*. The paramutagenic effect was transferred to *R-r* and remained for several generations but did eventually revert after repeatedly being inherited in the absence of *R-st*. The *booster1* (*b1*) locus in maize (Coe 1966) also regulates the 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 (10–20-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). RNA-dependent RNA polymerase, *mediator of paramutation1* (*mop1*) is necessary for paramutation to occur (Alleman et al. 2006), indicating that double stranded RNA is very likely a key factor that changes paramutable alleles to paramutagenic alleles, though this research is ongoing (Springer and McGinnis 2015). Paramutation likely describes a very small percentage of epialleles (Eichten et al. 2013).

3.4 The Stability of Facilitated and Pure Epialleles Across Generations

Epialleles may, in certain circumstances, have two attributes that suggest utility in plant breeding. These useful epialleles have beneficial 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). Fieldes et al. estimated that 5–8% of cytosines were methylated in the early flowering lines, while 14% of cytosines were methylated within the control lines (14%) (Fieldes et al. 2005). Akimoto et al. (2007) reported that the dwarf trait generated by azadC treatment in rice 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). Although in these instances it has not been established if the cause of the phenotype of interest is purely epigenetic, these examples provide evidence for the stability of traits induced by treatment with DNA hypomethylating agents. The *Cnr* pure epiallele described above also has high stability. Between the years of 1993 and 2006, more than 3000 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′* epiallele in maize is also 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).

Despite the examples of persistent and pure epialleles, stable inheritance of traits caused by pure 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 and only in specific and rare instances remain stable over many generations (Fieldes 1994). In maize, *R′* (the paramutated, and temporarily paramutable version of *R-r*) can readily revert to *R-r* (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 1958, 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%) have 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). In rice, a metastable epiallele called *Epi-d1* has been identified (Miura et al. 2009) that confers silencing of the *DWARF1* gene and causes a short stature phenotype. Typical of pure alleles, *Epi-d1* plants are often chimeric, showing a combination of dwarf and wild-type tillers (Miura et al. 2009).

Two experiments in *Arabidopsis thaliana* 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 and these genotypes were inbred for six generations. Reinders et al. (2009) reported that induced changes in 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 1/3 to 1/10 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 facilitated or pure epigenetic differences. Nonetheless, some trait variation may be genetic, although almost certainly a small proportion. Parental lines, despite having a recent, shared common ancestor, likely have some polymorphic DNA sites. Mutations could also occur during inbreeding (Ossowski et al. 2010).

3.5 Obligate Epialleles Greatly Outnumber Facilitated and Pure Alleles Within Plant Genomes

While pure epialleles that have no relationship with genetic variation are remarkable and of interest, the reality is that many epialleles are associated with genetic variants. In Arabidopsis, the data ranges from a relatively small percentage (18%) of epialleles being associated with DNA sequence polymorphisms (Dubin et al. 2015), to a moderate level of association (35%) (Schmitz et al. 2013b), to a high level of association (~50%) (Hagmann et al. 2015). Interestingly, even the higher estimates for Arabidopsis are low in comparison to those in some field crops. Strong evidence for the imbalance in the frequency of pure epialleles to those with at least partial genetic dependence has been found in soybean. An analysis of 83 RIL and parent methylomes revealed that 91% of DMRs (differentially methylated regions) were associated with genetic differences between RILs, and the remaining 9% of DMRs possibly represent a combination of pure and facilitated epialleles (Schmitz et al. 2013a). Eichten et al. (2013) investigated DMRs in maize cultivars and found evidence for potentially pure epialleles, but also found evidence for regions that are differentially methylated and dependent on associated differences in genotype. More specifically, 51% of DMRs examined were associated with local SNPs (Eichten et al. 2013). The remaining DMRs may represent pure epialleles, but more likely are a combination of pure epialleles, alleles with trans-acting regulatory factors, and alleles for which there are not any SNPs in linkage disequilibrium (Eichten et al. 2013). In another study that examined the methylomes of maize genotypes B73, Mo17, and 9 RILs from a B73 × Mo17 cross, researchers determined that most of the CG methylation segregated with the parental genotype, indicating that those epivariants were associated with genetic variants (Regulski et al. 2013). Genomes with numerous repetitive elements, such as many crop genomes, may have a higher chance of generating new, beneficial epialleles than compact genomes with few such elements.

3.6 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 pure 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).

Nonetheless, plant breeding requires significant traits to vary and for selection on those traits to be effective. As noted above, facilitated and pure epigenetic differences can cause meaningful trait variation that is heritable. In addition, novel variation is generated far more quickly from single residue epimutation than from DNA mutations, and larger, stable differentially methylated regions arise at the same rate as genetic mutations (Becker et al. 2011). Thus, although pure and facilitated epimutations are rare and often unstable, these 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. The approach taken for generating novel epialleles within a plant population is dependent on the species, with genome size and propagation method being factors to consider. For example, while methods, such as epiRIL development, have been successful in plants with simple genomes, such as Arabidopsis, crop plants with larger and more complex genomes, such as maize (Li et al. 2014) and rice (Hu et al. 2014), have been recalcitrant to this method. Chemical treatments provide an efficient means for discovering effects of hypomethylation since there are no specific targets of the demethylation and the effects can be widespread. Such treatments have been used to effectively alter important traits in crop species (Sano et al. 1990; Heslop-Harrison 1990; Fieldes 1994; King 1995; Janoušek et al. 1996; Akimoto et al. 2007; Xu et al. 2016a, b), but it is possible that the underlying cause of the altered phenotype may still, in part or entirely, be due to genetic differences. More recent developments in technology are bringing forward methods for directing site-specific changes in methylation status that might provide a means for inducing stable changes in methylation within genes of interest. For example, the CRISPR-cas9 system has been used to increase methylation in a site-specific manner in mammalian cells that resulted in facilitated epigenetic silencing of target genes (Vojta et al. 2016). This technology will not only provide a means for directing specific increases and decreases in methylation that have already

proven to drive meaningful phenotypic changes, but will also provide a means for studying the direct effects of novel changes in methylation rather than relying solely on correlations between changes in methylation and changes in gene expression.

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Chapter 4
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 the *dominant, overdominant,* 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 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.

4.1 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

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botanist Joseph Koelreuter observed that F1 hybrid tobacco plants exceeded the height of their parents (Reed 1942). The first characterization of heterosis was performed 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 hybrid plants from crossed plants were typically taller and more vigorous than self-fertilized crosses (Darwin 1876). This phenomenon was later verified independently by George Shull (Shull 1908) and Edward East (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 among 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, the 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 agronomy 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.

4.2 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).

4.2.1 Dominance Model of Heterosis

The *dominance model* of heterosis proposes that following hybridization between genetically distant genomes, the F1 generation displays 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. 4.1a, 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).

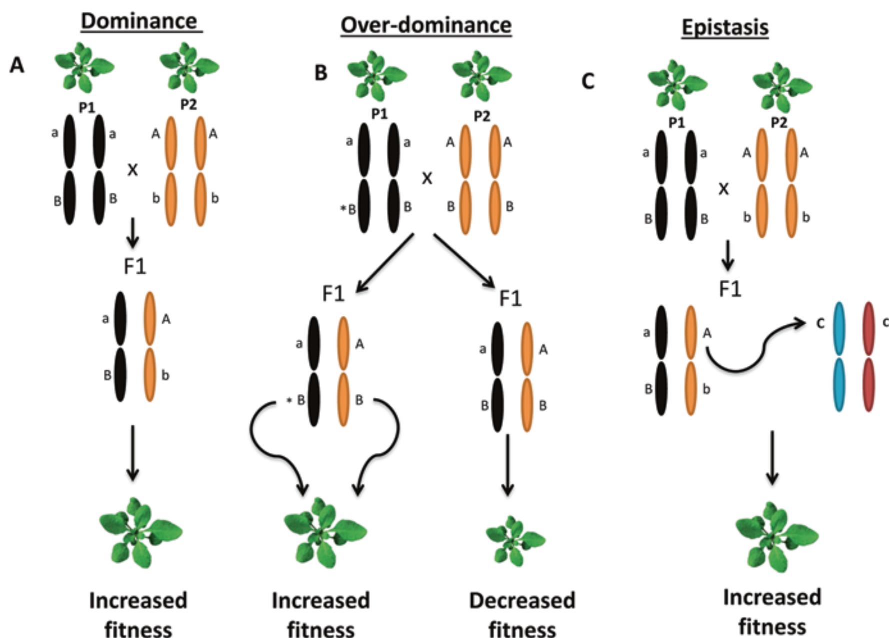

Fig. 4.1 Schematic of genetic models for explaining heterosis. (**a**) Dominance model; (**b**) Overdominance model; (**c**) Epistasis. For full descriptions, see text

4.2.2 Overdominance 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 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 overdominant) 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 *overdominance model* proposes that synergistic allelic interaction at particular heterozygous loci leads to superior performance in the F1 progeny. In Fig. 4.1b, *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 overdominant heterosis is the identification of cases of "single locus overdominance" (Mckeown et al. 2013a) such as that involving *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).

The identification of overdominant 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, where pseudo-overdominance 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 overdominance effects (Crow 1952; Schnable and Springer 2013). Heterosis due to epistatic interactions can also mimic overdominance (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 overdominant alleles to induce artificial overdominant heterosis as previously proposed (Mckeown et al. 2013a).

4.2.3 Heterosis, Epistasis, and Complexity

Whereas the overdominance model proposes that interactions at individual loci can induce heterosis (for example, by producing heterodimeric protein complexes with greater activity than a homodimeric complex), 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. 4.1c 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 overdominant heterotic QTL.

QTLs 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 quite 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 levels including additive, high- and low-parent dominance, overdominance, and underdominance (Swanson-Wagner et al. 2006). Some researchers have proposed that terms such as dominance, overdominance, 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 biological effects (Birchler et al. 2010).

4.3 Is There an Epigenetic Component to Heterosis?

Despite the successes of the *dominant, overdominant,* 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 the 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 arena 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.

4.3.1 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. 4.2a). 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 analyze 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

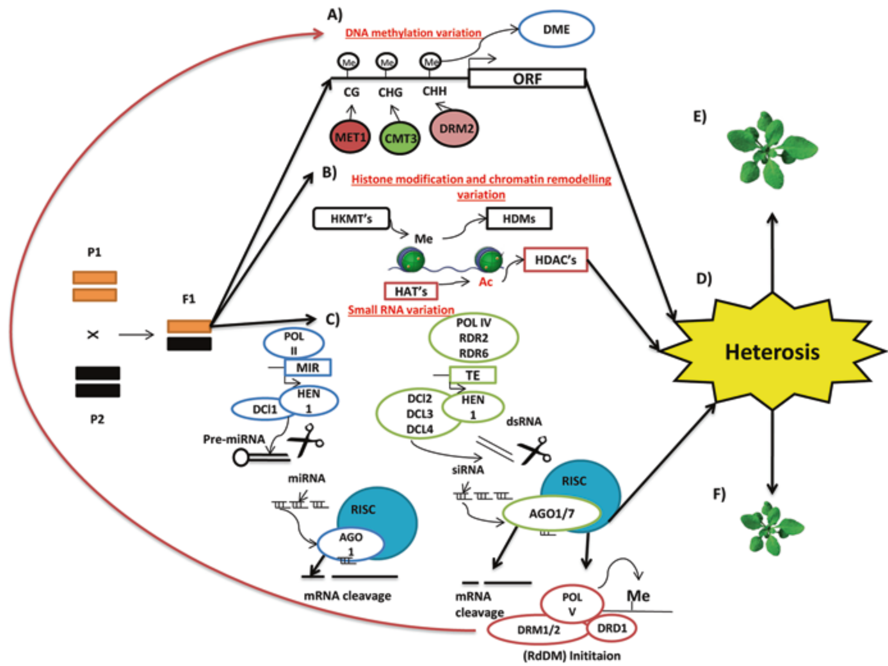

Fig. 4.2 A possible model linking epigenetics to the alteration of biological networks. Two distinct genomes hybridize to create a heterotic F1 hybrid: (**a**) Differential methylation patterns can occur in F1 hybrids where there is allelic variation at particular loci. Such methylation patterns are 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 (HKMTs), demethylases (HDMs), histone acetylases (HATs), and deacetylases (HDACs) 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

parents. In a similar approach Greaves et al. (2012) performed 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 were 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).

4.3.2 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 (HKMTs and HDMs), and acetylases and deacetylases (HATs and HDACs) (Fig. 4.2b) (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 overdominance 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.

4.3.3 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 RNAs (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 (miRNAs) and small interfering RNAs (siRNA) (Fig. 4.2c). 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 x* 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 sRNAs including miRNAs, while the role of MOP1 is restricted to the generation of 24-nt siRNA.

4.3.4 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. 4.3) 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

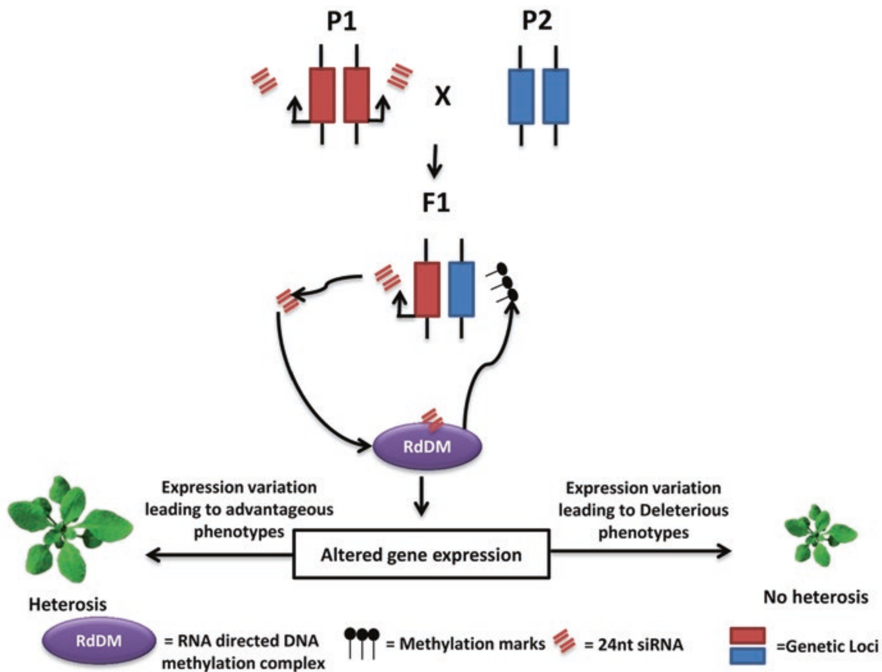

Fig. 4.3 Possible roles for methylation, siRNA, and RdDM in heterosis. (**a**) Two distinct genomes (P1, P2) with various levels of siRNA accumulation hybridize to create a heterotic F1 hybrid (F1). (**b**) Upon hybridization siRNAs can interact in cis or trans with genetic elements containing their 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

(C24, L*er*-0) and F1 hybrid lines (C24×L*er*-0, L*er*-0×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.

4.4 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 lato* 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.

4.5 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 5
Exploring the Role of Epigenetics in Cereal and Leguminous Crops Exposed to Abiotic Stress

Romesh Kumar Salgotra and Mehak Gupta

Abstract Epigenetics affects the gene expression due to chromatin structure changes without involving the DNA sequences. Epigenetic gene expression mechanisms play an important role in abiotic stress tolerance in plants. The mechanisms such as histone modifications, DNA methylation, and noncoding (nc) RNAs are the key elements of the epigenetic regulation machinery which leads to gene activation or gene silencing. Comprehensive literature showed the role of epigenetics controlling specific loci under environmental stresses in various plants. The epigenetic effects can be perceived on various developmental stages of plants in coping with the abiotic stresses. The whole genome-wide studies have led to unveil epigenetic effects of crop plants particularly cereal and legume in the era of high-throughput and next-generation sequencing (NGS) technologies. A number of epigenetics investigations are being carried out in cereals and legumes crops for abiotic stresses such as cold, drought, heat, salinity, etc. This chapter has compiled the latest improvements made in the field of epigenetics related to abiotic stresses focusing on cereal and legume crops. Moreover, development of crop varieties tolerant to abiotic stresses such as drought, cold, heat, high temperature, etc., is essential to sustain the crop productivity.

5.1 Introduction

Cereals and legumes are the important nutritional source of human being and feed for livestock. To feed the ever increasing population particularly in the developing and underdeveloping countries besides limited resources there is a need to increase the productivity of crops. Continuous threatening from global warming and various abiotic stresses such as drought, salinity, cold, heat, etc., have endangered food security (Maiti and Satya 2014). To cope with the ever increasing population and

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alarming environmental stresses, it is essential to develop crop varieties with high yield and quality tolerant to various stresses. These crops suffer from various biotic and abiotic stresses at different growth stages, resulting in decrease in crop yield and quality. Abiotic stresses cause modifications in the (de)methylation pattern at the coding region of some stress-responsive genes and regulate their expression, affect plant quality, and decrease plant productivity. By activating molecular networks such as signal transduction, stress perception, metabolite production, and expressions of specific stress-related genes, plants can overcome environmental stresses.

Epigenetics is defined as heritable modification in chromatin structure which intensely influences expression of genes but does not involve change in the DNA sequence (Fujimoto et al. 2012). The term epigenetics (Waddington 1942) was derived from e*pigenesis* to capture the reasonable presumption that a layer of mechanisms exist that reside above (*epi*) the level of the *genes* during organismal development that control their output in order to specify cell fate determination (Sweatt 2013). Mirouze and Paszkowski (2011) stated the term epigenetics as heritable variation in gene regulation resulting from covalent modifications of DNA and its associated chromatin proteins without changing the underlying nucleotide sequences. Hence, the term epigenetics can also be defined as the stable heritable phenotype resulting from changes in chromosome without alterations in the DNA sequence. The key epigenetic marks which regulate different plant traits in response to biotic and abiotic stimuli are cytosine methylation, histone modifications, and small RNA accumulation. These epigenetic changes are reported to be reversible in nature and provide speedy retort mechanisms to plants to withstand pathogen and environmental stress (Hewezi et al. 2017).

The mechanism of epigenetic regulation involves the modification of histones, DNA methylation, and the action of noncoding (nc) RNAs (Fig. 5.1). The DNA methylation, histone modifications, and nc RNAs such as either long nc RNAs or small RNAs lead to open or closed chromatin states associated with gene activation or gene silencing, respectively. The small RNAs include small interfering RNAs (siRNAs) and microRNAs (miRNAs). Epigenetic changes do not alter the genetic code sequence of DNA, but modify the activation of certain genes. The noncoding structure DNA itself or the associated chromatin proteins may be modified, causing activation or silencing. RNA interference (RNAi) mediates gene silencing at post-transcriptional level in a sequence-specific manner (Yang et al. 2017). This array of processes is clearly interconnected and almost certainly acts in a complex, interactive, and redundant fashion (Berger 2007).

With the advent of high-throughput techniques, a wealth of information on epigenetic regulation in crop plants is generated and in recent years, the rapid progress in next-generation sequencing (NGS) has led to the unveiling of epigenetic landscapes at genome-wide scale (epigenomes). There is growing aspiration to understand the stability and role of epigenetic regulatory systems in plants surviving under adverse environmental conditions (Geyer et al. 2011). The high-throughput techniques and NGS have helped to study the effect of epigenetics on various developmental stages of plants under biotic and abiotic stresses. The epigenetics

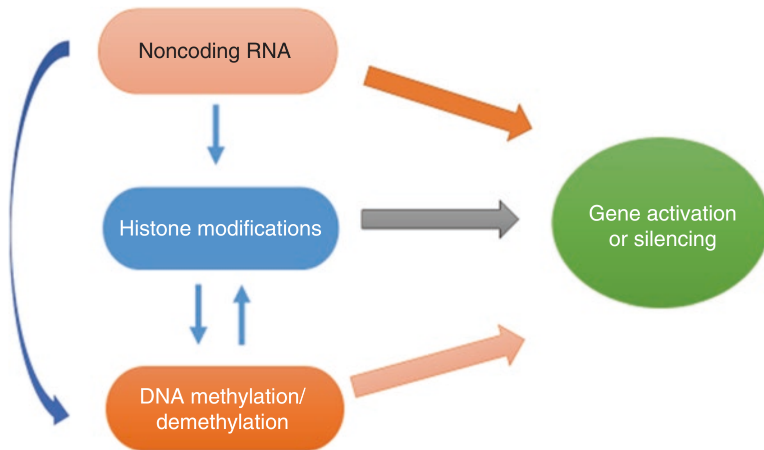

Fig. 5.1 Three epigenetic mechanisms, viz., DNA methylation, histone modifications, and noncoding (nc) long or small RNA molecules, lead to changes in chromatin structure without altering the underlying DNA sequence

also has importance in the transgenerational adaptive response to environmental stimuli of plants (Weinhold 2018). The burgeoning area of epigenetics and its role in abiotic stress is emphasized in this chapter in context of the role of chromatin regulators.

5.2 Abiotic Stresses

Various environmental stresses such as drought, cold, heat, and salinity affect growth and development of plants, which consequently hampers the plant productivity. Under environmental stresses low crop productivity results due to various changes in plants at genotypic and phenotypic levels (Asada 2006). However, plants have evolved an array of defense mechanisms to adapt to different stresses by quick and coordinated changes at transcriptional and post-transcriptional levels (Boyko and Kovalchuk 2008). The stress tolerance mechanisms have been reported to inherit over generations, though the inheritance mechanism may differ among plant species based upon intensity and duration of stress and the genetic composition of the plant species (Chen et al. 2010). Excessive abiotic stress due to drought, heat, salinity, etc., and/or limitation of an important mineral affect the genes that regulate the epigenetics mechanism (Fang et al. 2014). Bocchini et al. (2015) reported that chromatin modifications, methylation, chromatin remodeling, and RNAi mechanisms can rapidly regulate gene expression under stress. Bruce et al. (2007) reported that these modifications can be "memorized" by plant somatic cells after a stimuli to stress and further can be utilized as an epigenetic mark which can be inherited transgenerationally. When their progenies face stressful conditions,

the same epigenetic alteration will occur. Moreover, female gamete is responsible for causing epigenetic changes (Wibowo et al. 2016). The key factor in the plant's response to stress and its adaptation is the plasticity that transmitted from one generation to another generation, which can have a great impact on breeding programs (Fortes and Gallusci 2017). Peng and Zhang (2009) revealed that DNA methylation levels declined with some stresses like planting density, chilling, and successive subculturing, whereas an increase was found under salt stress.

5.3 DNA Methylation

The main epigenetically mark in eukaryotes is the addition of a methyl group on the 5 °C of the cytosine base to form 5-methylcytosine. Feng et al. (2010) reported that DNA methylation usually occurs in plants in three string contexts such as CG and CHG (symmetric) and the CHH (asymmetric). Niederhuth and Schmitz (2017) reported that genome stability, regulating global gene expression, and silencing deleterious transposon insertions are some of the important roles played by methylation. DNA methylation at promoter regions is related to gene silencing, while its demethylation leads to gene activation. Cokus et al. (2008) stated that the methylation at 5′ portion (promoter) and 3′ portion hampers gene expression, while expressed genes are methylated in the transcribed region (gene-body methylation). During plant development, specific enzymes facilitate the important role in demethylation and DNA methylation (Van Oosten et al. 2014). Attenuation of gene transcription occurs preferentially at C-G dinucleotide sequences in DNA sites and cytosine demethylation also occurs which can be again reconverted to unmethylated position.

In plants, two major types of methylation activities occurred such as *de novo* and maintenance. The *de novo* methylation is a process by which previously unmethylated cytosine residues are methylated, leading to the formation of new methylation patterns, whereas maintenance methylation is the process of maintenance of pre-existing methylation patterns after DNA replication (Chen and Li 2004). The mechanism of DNA methylation is governed by preferentially two types of enzymes, i.e., methyltransferases and demethylases. *De novo* methylation is established by DRM2 (domains rearranged methyltransferase 2) in the new DNA sequences generated after DNA replication, whereas MET1 (DNA methyltransferase 1) and CMT (chromomethylase) are responsible for maintenance of CG and CNG methylation, respectively (Chen et al. 2010). DNA glycosylases catalyze the removal of methyl group from cytosine residue (Cao et al. 2000). A central dogma of the epigenetics field has depicted that once DNA methylation patterns are established upon the genome in terminally differentiated cells, those modifications are permanent and essentially immutable.

Genome-wide profiling of DNA methylation is termed methylome, while the combination of both post-translational modifications of histone tails and methylation at cytosines along the genomes is termed as epigenome. Till date, most studies of

plant epigenomes have been focused on DNA methylation, which is more stable than post-translational modifications of histone tails. DNA methylation is distributed in the plant genomes, including heterochromatic and euchromatic regions (Gehring and Henikoff 2007). Higher levels of cytosine methylation are reported in the heterochromatic regions, containing transposons and other repetitive sequences, whereas comparatively lesser levels of cytosine methylation in euchromatic regions inhabiting genes and non-repetitive intergenic regions. The first report on global DNA methylation profiling of endosperm and embryo genomes demonstrated widespread reduction of DNA methylation in the endosperm, particularly at regions corresponding to transposable elements (TEs) and small RNAs (Zemach et al. 2010). DNA methylation studies at genome-wide levels are well studied in various cereal and legume crops (Zhong et al. 2013).

5.3.1 Methylation Under Abiotic Stress

Understanding the mechanisms of epigenetic regulation of plant growth and development could create new genetic variation for improving crop productivity as well as adaptation to stress environment. Stress can cause hypermethylation or hypomethylation of DNA. Drought-induced hypermethylation has been proposed to play a primary and direct role in reducing the metabolic activity in plant (Labra et al. 2002). They reported drought-induced hypermethylation and hypomethylation in drought-tolerant and drought-susceptible varieties of rice, respectively. Tan (2010) stated that the changed methylation level in maize exposed to osmotic and salt stress helps in stress acclimation. Stressful environment produces transgenerational epigenetic modifications leading to enhanced stress adaptability in future progenies (Ou et al. 2012). It has been reported that non-stressed progenies carrying modified methylation patterns acquired from the stressed parent rice plants exhibit enhanced stress tolerance (Kou et al. 2011). In addition to gene silencing, cytosine methylation is aimed at silencing of transposons as their activity can have disastrous effects on the plant, especially if they insert into a gene and disrupt its function. Steward et al. (2002) reported that the cold stress at seedlings stage in maize created the DNA demethylation at genome-wide level. The osmotic stress in rice was associated with a higher expression of specific genes due to proline biosynthesis, but also with a global DNA demethylation (Zhang et al. 2013), whereas in some cases, the imposition of cold stress did not necessarily related to DNA demethylation events, for example, in the case of *Medicago sativa* plant, the imposition of cold stress was associated with transcription activation of specific retrotransposons (Ivashuta et al. 2002). Several reports correlating DNA methylation dynamics with stress adaptation are available. A drought-sensitive genotype of horse gram (*Macrotyloma uniflorum*) shows higher methylation (Bhardwaj et al. 2013). Differential DNA methylation patterns were reported in Barley genotypes under drought particularly in drought related genes (Kapazoglou et al. 2013). These studies indicate that certain genomic regions may be more prone to differential methylation upon stress

imposition/relief eventually corresponding to a stress adaptation process. Distinct epigenetic variations were reported due to the presence of methylome variations in the population to tolerate abiotic stresses. Methylome variations are present in natural plant populations and may help individuals to better cope with different environments. Mayer et al. (2014) stated that high methylation levels were observed due to different cold acclimation capacities in *Cannabis sativa* varieties.

The epigenetic changes in crop genomes have been studied by many research groups. For genome-wide methylation studies, one of the basic methods is methylation sensitive amplification polymorphism (MSAP). It is a potent technique for studying the whole genome cytosine methylation changes in crops. The technique has been used for this purpose in various crops like maize, rice, and wheat. The study of methylation dynamics of the regions associated with transposable elements, in response to abiotic stress, can be helpful in understanding the trend of epigenomic changes specifically targeting gene flanking regions, which may not be reflected in the whole genome cytosine methylation analysis. Another technique for high-throughput methylation studies is methyl chip-on-chip. It involves the enrichment of methylated regions by immunoprecipitation of sonicated and denatured genomic DNA with an antibody specific for methylated cytosine, followed by hybridization on to chip. Using this technique, the methylome analysis for the stress-responsive genes gives us an idea about the genes being activated or silenced under stress. Also, histone PTMs are known to show dynamic covalent changes during stress (Bruce et al. 2007).

Earlier studies showed that the DNA methylation influences the various developmental processes. DNA methylation differences were observed in tissue and organ types in soybean and sorghum (Song et al. 2013). Although they supported the association of hypomethylation with higher gene expression nearby the gene, the difference between organ methylation was very little. It is yet unclear whether these were spontaneous in nature or were developmentally controlled DMRs (differentially methylated regions). MSAP revealed lower level of sorghum tissue methylation. Similar results with insignificant methylation changes across seven tissues except for the endosperm have been reported by Zhang et al. (2011). The studies on *A. thaliana*, rice, and maize endosperm showed a genome-wide hypomethylation (Waters et al. 2011).

5.4 Histone Modifications

Another epigenetic mechanism, histone modifications have significant role in various developmental stages of plant (Forderer et al. 2016). In plants, histone modifications are the second major category of epigenetic mechanisms. The histone proteins present in the nucleus exist largely as octameric complexes, which make up the core of the chromatin particle around which most DNA is wrapped, forming a three-dimensional histone/DNA complex. Individual isoforms of histone monomers can also be swapped in and out of the octamer, a regulatory mechanism referred to as

histone subunit exchange. Subunit exchange and post-translational modifications trigger either increases or decreases in transcription, depending upon the particular modification, the particular histone isoform involved, and even the context of other histone modifications in which the modification resides. A histone code concept aroused from these mechanisms, wherein modifications of histone are interpreted in situ as a combinatorial code regulating gene transcription rates at specific loci across the genome.

In order to modify transcriptional readout of the associated gene, the modifications of histones control these structures. According to Engelhorn et al. (2014) histone modifications refer to modifications on the N-tails of nucleosomal histones which consist of ubiquitination, phosphorylation, methylation, biotinylation, sumoylation, and acetylation on specific serine, arginine, threonine, and lysine residues. DNA associated developments are controlled only by histone post-translational modifications. Li et al. (2014b) stated that the histone tail modifications were the major control point for determination of chromatin structure and gene regulations. The chromatin change occurred due to modifications of these chemicals which lead to operation of chromatin, i.e., closing and opening and ultimately transcriptional regulations (Allis and Jenuwein 2015). Different developmental stages of plants such as response to stimuli, flower initiation, and development of seed are due to modifications of histones (Gallusci et al. 2016; Banerjee and Roychoudhury 2017). Chen et al. (2010) stated that ubiquitination, acetylation, and phosphorylation of histone tails are linked with gene activation and processes of biotinylation and de-acetylation are linked with gene inactivation. According to Law and Jacobsen (2010) methylation in histones is reported to control both activation and deactivation gene expression. The transcription and regulation of gene is affected by a number of methyl groups, sites, and degrees added to arginine and lysine residue (Ding et al. 2012). For example, in *Arabidopsis,* genes are inactive when H_3 methylation occurs at K9 and K27, and methylation of histone H3 at K4 and K36 is associated with actively transcribed genes (Nakayama et al. 2001; Li et al. 2012).

5.4.1 Histone Modifications Under Stress

The histone modifications lead to chromatin accessibility particularly in the promoter region of the gene, such as methylation or acetylation (Berger 2007; Kouzarides 2007). Expression changes in stress-responsive genes are due to modifications of histones which are transient in nature and response differentially under stress conditions (Zong et al. 2013). Zong et al. (2013) correlated the modifications of histones with induction of transcription genes under water stress conditions in rice plant. Another report by Tsuji et al. (2006) stated that in rice plant under submerged conditions acetylation increase of H_3 marks was associated with stress specific genes. Hu et al. (2012) specified that the increase of H_3K9ac, a mark of histone acetylation reported in the heterochromatic chromatin knobs, was correlated with the increase in transcription, whereas H_3K9me2 was associated with the

decrease in transcription. They further stated that these changes occurred with the reduction of different levels of DNA methylation.

5.5 Noncoding RNA

The opening and closing of chromatin occurred due to noncoding RNAs such as either long nc RNAs or small RNAs (siRNAs and microRNAs miRNAs) which is associated with gene silencing and activation, respectively. In general, ncRNAs function to regulate gene expression at the transcriptional and post-transcriptional level. Those ncRNAs that appear to be involved in epigenetic processes can be divided into two main groups: the short ncRNAs (<30 nts) and the long ncRNAs (>200 nts). Three major classes of short noncoding RNAs are microRNAs (miRNAs), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs). Both major groups are shown to play a role in heterochromatin formation, histone modification, DNA methylation, and gene silencing. Law and Jacobsen (2010) stated that the sequence-specific methylation is caused by double stranded RNA (ds-RNA) molecules and RNA-directed DNA methylation (RdDM). RNA interference (RNAi) is interrelated with the RdDM which is involved in the methylation of cytosine (Wassenegger et al. 1994; Meister and Tuschl 2004). In eukaryotic nuclear plants, miRNAs are small noncoding RNA structures which functions in RNA silencing and post-transcriptional regulation of gene expression (Lee et al. 1993; Maxwell et al. 2012). Xu et al. (2013) stated that eukaryotic plants have distinct classes of siRNAs, such as natural antisense siRNAs, heterochromatic-siRNAs, and trans-acting siRNAs and the siRNAs are facilitating gene silencing through methylation of histone and RdDM (Mosher et al. 2008). In crop plants and *Arabidopsis* a number of small RNAs have been identified by large-scale genome-wide and gene-specific studies. It has been observed that the siRNAs and small RNAs–miRNAs played a significant role in different developmental stages of plant under various environmental stress conditions. Bologna (2014) indicated that small RNAs control the regulation of genes which could be inherited under abiotic stress conditions.

5.5.1 Noncoding RNA Under Stress

The crop plant growth and productivity are reduced under biotic and abiotic stresses such as drought, heat, cold, and infections due to fungal, bacterial, and viral disease. To survive under these stresses, plant cell utilizes multi-gene regulation systems. Emerging evidence has revealed that ncRNAs play a critical role in the regulation of gene expression in response to stress conditions. Numerous novel antisense transcripts are accumulated due to abiotic stresses which are the major source of

siRNAs (Zeller et al. 2009). Hc-siRNAs (heterochromatic-siRNAs), siR441, and siR446 were found to be downregulated under abiotic stresses but show an increase in the creation of their precursors, entailing that the processing of siRNA precursors is inhibited that seems to be a mechanism of regulation due to stress responses (Yan et al. 2011).

The miRNAs play an important under abiotic stress resistance particularly under cold, heat, salinity, etc. Twenty-six new miRNAs showed upregulation or downregulation under abiotic stress in the small RNA analysis of *Arabidopsis* seedlings (Sunkar and Zhu 2004). In *Oryza sativa* under cold stress the miR319 was found to be downregulated, whereas upregulation of miRNAs was observed under cold stress in *Brachypodium* (Lv et al. 2010; Zhang et al. 2009). The salt and alkali stress tolerance enhanced due to overexpression of miR396 in rice and *Arabidopsis* (Gao et al. 2010); moreover, in these plants the miRNA concentration deviation was observed in response to stress (Gao et al. 2010).

5.6 Epigenetical Interventions in Cereal and Legume Crops

Cultivated crop plants are frequently exposed to stresses such as drought, cold stress, temperatures, heat, salinity, light intensity, cold stress, etc. These abiotic stresses distort growth of plant and ultimately reduced crop productivity. The transcriptional and post-transcriptional levels, including the epigenetic regulation of genes changes, are involved to cope with these stresses (Singh et al. 2015). Most of the abiotic stresses such as drought tolerance, salinity tolerance, and heat tolerance are controlled by the multiple gene action. It has been observed that transcriptional and post-transcriptional control of gene expression is controlled by siRNA, chromatin modifications, and DNA methylation (Angers et al. 2010). The epigenetic variations are also showing heritable variation for controlling these complex traits (Richards 2011). Epigenetic mechanisms have been associated with the regulation of stress-associated genes (Chinnusamy and Zhu 2009). As epigenetic regulation of gene expression can influence important crop traits and the creation of stably inherited epigenetic diversity could be a very powerful tool in crop improvement. Most known epigenetic variants are associated with loss of DNA methylation and correspond to gain of function variants. No direct link for phenotypic variation due to epigenetics has been detected so far, this does not mean that they do not play a role. In crops the number of examples of epigenetically controlled traits is increasing steadily (Table 5.1). Over the last two decades, significant variations in epigenetic phenotypes in plants have been recognized. A generator of epialleles, DNA methylation, could have important implications for the cereal and legume crops improvement against abiotic stresses. The following are some of the important cereal and legume crops in which epigenetical changes play an important role in against stresses.

Table 5.1 Examples of cereal and legume crops affected by epigenetics

Plant species	Type of stress	Epigenetics effect	Observations	References
Oryza sativa	Salt	T rDNA chromatin decondensation + genome-wide DNA methylation	Reduction in crop yield	Santos et al. (2011)
	Water-deficit	T H3K4me3 regarding the dehydrin genes	Reduction in crop yield	Zong et al. (2013)
	Submergence	T H3ac regarding the ADH1 and PDC1 genes	Reduction in crop yield	Tsuji et al. (2006)
	Cold tolerance	PCF5/PCF8	Reduction in crop yield	Yang et al. (2013)
		Hypermethylated in metastable Epi-d1	Reduction in plant height	Le et al. (2014)
	Osmotic	Genome-wide DNA methylation	Reduction in crop yield	Zhang et al. (2013)
	Salt stress	Demethylation at promoter region of *OsMYB91* gene and rapid histone modifications at *OsMYB9* locus	Reduction in crop yield	Zhu et al. (2015)
Triticum aestivum	Drought tolerance	miR170 miR171 miR172	Reduction in crop yield	Zhou et al. (2010)
	Heat	T rDNA chromatin decondensation	Reduction in crop yield	Santos et al. (2011)
	Heat stress	Increased histone demethylation of the various genes	Reduction in crop yield	Wang et al. (2016)
	Salt stress	Hypermethylation of cytosines at *HKT* genes	Reduction in crop yield	Kumar et al. (2017)
Zea mays	Cold	+ Genome-wide DNA methylation; nucleosome remodeling at tandem-repeat sequences with a: + DNA methylation; T H3K9ac; + H3K9me2	Reduction in crop yield	Steward et al. (2002)
	Cold	Differentially methylated	Reduction in crop yield	Shan et al. (2013)
	Cold	Methylated in rice/differentially methylated	Reduction in crop yield	Shan et al. (2013)
	Drought tolerance	PDH, POK, MAPK, PLD	Reduction in crop yield	Wei et al. (2015)
	Drought tolerance	11 different miRNA are upregulated under drought exposure	Reduction in crop yield	Kantar et al. (2011)
Hordeum vulgare	Terminal drought	Hc-siRNA-mediated hypermethylation at *CYTOKININ-OXIDASE 2.1* promoter	Reduction in crop yield	Surdonja et al. (2017)
	Drought tolerance	NFY-A	Reduction in crop yield	Ferdous et al. (2016)

(continued)

Table 5.1 (continued)

Plant species	Type of stress	Epigenetics effect	Observations	References
Glycine max	Drought tolerance	GmNFYA3	Reduction in crop yield	Ni et al. (2013)
Phaseolus vulgaris	Drought tolerance	NAF transcription factor ARF10	Reduction in crop yield	Sosa-valencia et al. (2016)
Vicia faba	Drought tolerance	Increased demethylation of *LOX, CDPK, ABC, GH,* and *PEPC* genes	Reduction in crop yield	Abid et al. (2017)
Vigna unguiculata	Drought tolerance	Transferase family protein leucine repeat rich transmembrane protein	Reduction in crop yield	Barrera-Figueroa et al. (2011)
Cicer arietinum	Drought tolerance	BHLH23 ERF/AP2	Reduction in crop yield	Hajyzadeh et al. (2015)
Macrotyloma uniflorum	Drought	DNA methylation dynamics	Reduction in crop yield	Bhardwaj et al. (2013)
Cannabis sativa	Cold	Methylome variation (locus-specific methylation and deacclimation)		Mayer et al. (2014)

5.6.1 Rice

Rice (Oryza sativa L.) is one of the most important staple food crops and is a primary source of food for world's population (Wang et al. 2018). Unfortunately, it is sensitive to climate changes, which leads to significant reduction in productivity. Genetic studies have been making great efforts to improve rice ability to handle environmental stresses (Jagadish et al. 2012). Epigenetic modification is controlled by epigenetic pathways, and mutations involved in disturbing the epigenetic pathways. This may lead to significant epigenetic and/or genetic changes. Due to epigenetic mechanisms and epigenomic variations accumulated during the long history of selection contribute to adaptation during the domestication of important crops like rice. Epigenetic regulations have been dissected in great detail in A. thaliana, but are still poorly characterized in rice (Deng et al. 2016). This is especially true for rice, whose genome is rich in epigenetic modifications and transposable elements (TEs) that are generally epigenetically silenced. This genetic variation awaits to be exploited for their potentials in generating a heritable source of variation for rapid environmental adaptation, which may hold tremendous importance for rice improvement under abiotic stresses.

The productivity in rice is influenced by extreme temperature, drought, cold, salinity, etc., and at various levels gene expression is involved in abiotic stress responses in different genotypes which could explain the resistant phenotype (Garg et al. 2015). In addition, Wang et al. (2016) showed that under the influence of DNA methylation a stable methylome has been observed in a drought resistant genotype

compared to a drought-sensitive genotypes. Zheng et al. (2017) stated that drought-induced epimutations are non-random and are inherited from one generation to another generation (Zheng et al. 2017). Recently, studies showed the DNA methylation patterns are affected in rice when the crop is exposed to heavy metals or pesticides in soil (Feng et al. 2016). Rice, whose genome is rich in epigenetic modifications and TEs (Chen and Zhou 2013; Song and Cao 2017), epigenetic pathways disturbance may dramatically change the epigenetic profile, and could therefore lead to phenotypic variation (Li et al. 2014a, b; Song and Cao 2017). The phenotypic impact of epigenetic changes in rice may be significantly amplified by TEs via creating both epigenetic and genetic variation (Song and Cao 2017). Now many genes involved in the pathways of establishing, maintaining, and removing DNA methylation, which is the most well-studied epigenetic marker so far, have been identified (Lanciano and Mirouze 2017).

Epigenetic pathways are relatively conserved between different plant species (Chen and Zhou 2013). In rice, *de novo* establishment of DNA methylation is carried out by the RNA-directed DNA methylation (RdDM) pathway (Lanciano and Mirouze 2017). During RdDM, small interfering RNAs (siRNA) are first produced, via several steps, from the target locus, and they then, with the help of a set of proteins, head to the target locus, where the target locus gets eventually methylated by an enzyme that is called "domain rearranged methyltransferase" (DRM) (Lanciano and Mirouze 2017). There are three DRM-encoding genes that have been identified in rice so far, *OsDRM2*, *OsDRM1a,* and *OsDRM1b*, with the last two not being expressed (Moritoh et al. 2012). The RdDM takes on two different forms in plant: canonical and non-canonical. The main difference between these two forms lies in the production of siRNA. Canonical RdDM is a proactive *de novo* methylation process, during which siRNAs production starts from the transcription of target locus by RNA polymerase IV (POL IV) (Lanciano and Mirouze 2017). Wendte and Pikaard (2017) reported that the non-canonical RdDM is a passive remedial strategy which takes action when regular transcription of the target locus has already occurred, and mRNAs produced via regular transcription processed into siRNA (Lanciano and Mirouze 2017).

Plant DNA methylation can be maintained by different mechanisms depending on the sequence context (Zhang et al. 2018). In rice, CG methylation is mainly maintained by the methyltransferase OsMET1-2 that is a possible ortholog of the mammalian DNMT1 enzyme (Lanciano and Mirouze 2017). DNMT1 recognizes hemi-methylated CG during DNA duplication and methylates the unmethylated cytosine in the newly synthesized daughter DNA strand (He et al. 2011). Apart from OsMET1-2, another methyltransferase, OsMET1-1, has also been identified in rice, and OsMET1-1 may have a minor and/or redundant role in maintaining the CG methylation (Lanciano and Mirouze 2017). The maintenance of rice CHG methylation is mostly the responsibility of the plant specific chromomethyltransferase OsCMT3a (Lanciano and Mirouze 2017). The rice chromomethyltransferase (OsCMT2) has also been identified; however, the function of OsCMT2 is still unknown (Lanciano and Mirouze 2017). Rice CHH methylation is mainly

maintained by OsDRM2 (Tan et al. 2016) that is also involved in the RdDM pathways (Pang et al. 2013). The *A. thaliana* ortholog of OsDRM2, DRM2, maintains CHH methylation through RdDM at RdDM target regions that include certain transposons and repeat sequences (He et al. 2011). Two rice chromatic remodeling proteins OsDDM1a and OsDDM1b lead DNA methylation. The chromatic remodeling proteins OsDDM1a/b act at CG and CHG methylation within both euchromatins and heterochromatins (Tan et al. 2016).

5.6.2 Maize

Maize (*Zea mays* L.) is another important staple food crop besides a model plant used for genetics, genomics, and other fundamental research (Bennetzen and Hake 2009). In maize, during seed development, gene expression is under epigenetic control (Berger and Chaudhury 2009). At early seed development stages, epigenetic mechanism plays a significant role in escaping the drought (Lu et al. 2013). In comparison to *Oryza spp.* and *Arabidopsis,* maize endosperm is hypomethylated and all three plant species, i.e., rice, maize, and *Arabidopsis,* have similar pattern for CG methylation (Zemach et al. 2010a; Cokus et al. 2008). The CHG and CHH methylation had significant differences between the plant species. In maize seeds sequence differentiation occurred among the plant species due to variation in the methylation-regulated transcription (Lu et al. 2015).

Significant variations in epigenetic phenotypes in maize plants have been recognized over the last two decades. Heritable epialleles are considered as a source of polymorphism and may have significant implications in crop improvement. Data show that F1 hybrids of maize are in general less methylated than their parental inbreds. Tani et al. (2005) explained the role of methylation in the expression of maize genes and performance of hybrids under different growth conditions with maize inbreds and hybrids. Repeated selfing for the isolation of inbreds, with emphasis on combining ability of inbreds, leads to gradual accumulation of methylated sites, which get released and/or when the inbreds are crossed to generate hybrid. Stressful growth conditions result in more methylated DNA, and such stress-induced methylation and suppression of genome activity could be at the core of higher yield of the hybrid (Kumar and Bhat 2014).

5.6.3 Wheat

Wheat is one of the most important cereal crops having global production of more than 700 million tonnes which provides 20% of the daily protein requirements, and calories for 4.5 billion people globally (Arzani and Ashraf 2017). Productivity of crop plants under abiotic stress such as salt is lagging behind because of limited

knowledge about epigenetic and molecular mechanisms in wheat plant. Salt stress affects metabolic processes in plants through impairment of water potential of cells, ion toxicity, membrane integrity and function, and uptake of essential mineral nutrients (Arzani and Ashraf 2016). The stress perception and signaling has been complemented with the stress-induced biochemical, physiological, and epigenetic changes (Kumar and Singh 2016; Kumar et al. 2017). The information about the biochemical, physiological, molecular, and epigenetic aspects of salt tolerance will not only be helpful in cloning of the genes involved in salt tolerance, development of transgenics, and better breeding programs, but also in screening germplasm toward breeding for saline conditions (Sairam et al. 2002).

The stress is sensed through cell membrane, transduced to various inducers to regulate structural and molecular alterations including H_2O_2 accumulation, induction of transcription factors, and molecular and epigenetic regulation of gene expression through transcriptional and/or translational reprogramming for protective defense mechanism (Kumar et al. 2017). Alleles/epialleles for the differentially expressed genes can be identified from the salt-tolerant genotype and validated in EpiRILs/mapping populations for their possible use in the stress wheat breeding program. Increasing evidences suggest the key role of genetic background and epigenetic changes in regulating expression of the stress-associated genes (Kumar et al. 2017). Expression level of HKTs regulated through genetic and epigenetic mechanisms rationalized the observed responses of wheat genotypes. Better understanding about the structural, functional, and regulatory control of HKTs may enable further improvement in salt tolerance of plants in future, and development of more salt-tolerant wheat crop varieties (Kumar et al. 2017).

5.6.4 Legumes

Legume crops play an important role in improving agricultural sustainability through increasing nitrogen use efficiency and enhancing soil fertility. Grain legumes being an important source of nutrition particularly protein for poor consumers and farmers suffers from various stresses. Hence, there is a need for novel approaches to develop improved versions of legume cultivars that are able to cope with a range of environmental stressors. Next-generation technologies are providing the tools that could enable the more rapid and cost-effective genomic and transcriptomic studies for most major legume crops, allowing the identification of key functional and regulatory genes involved in abiotic stress resistance. Therefore, it is essential to intensify legume improvement programs by using advanced breeding approaches and techniques, to develop new high-yielding legume cultivars that are able to cope with a range of environmental stresses.

5.6.4.1 Cowpea

Cowpea (*Vigna unguiculata*) is a good model crop for studying drought tolerance. Although microRNAs (miRNAs) play an important role in plant abiotic stresses in legumes like cowpea, Barrera-Figueroa et al. (2011) studied the role of miRNA and their associates in drought-tolerant genotypes. Earlier, it was not understood how miRNAs might contribute to different capacities of drought tolerance in different cowpea genotypes. The drought-associated microRNAs have been identified in cowpea genotypes (Barrera-Figueroa et al. 2011). A deep sequencing of small RNA reads was generated from two cowpea genotypes of a drought-sensitive and drought-tolerant that grew under well-watered and drought stress conditions. Barrera-Figueroa et al. (2011) reported that the miRNA expression was inconsistent in cowpea genotypes. They found that nine miRNAs were predominantly or exclusively expressed in one of the two genotypes, whereas some of the miRNAs were drought-regulated in only one genotype. They suggested that miRNAs may play important roles in drought tolerance in cowpea and may be a key factor in determining the level of drought tolerance in different cowpea genotypes.

5.6.4.2 Hemp

Hemp (*Cannabis sativa*) is one of the important crops which enhance the soil fertility. But this crop also suffers from various stresses like cold, temperature, drought, etc. The capacity to tolerate cold stress for adaptation in hemp plant is controlled under the molecular and epigenetic mechanisms. The molecular mechanisms underlying cold adaptation in hemp revealed higher levels of complexity of genetic, epigenetic, and environmental factors (Meyer et al. 2013). They reported that the hemp genotypes accumulated soluble sugars under cold stress which could be maintained at higher levels under this stress. These genotypes acclimated the most efficiently accumulated transcript levels of *COR* genes involved in *de novo* DNA methylation. Furthermore, these hardy hemp genotypes displayed significant increases in methylcytosine levels at *COR* gene loci when deacclimated, suggesting a role for locus-specific DNA methylation in deacclimation (Mayer et al. 2013).

5.6.4.3 Faba Bean

Faba bean (*Vicia faba* L.) is consumed as food for humans and animals because of its high content of protein. It plays a significant role in fixation of nitrogen through symbiotic relations. Numerous workers described the significant role of epigenetic in a plant under abiotic stress response. The histone modifications, sRNAs, DNA methylation, or longer noncoding RNAs are involved in epigenetic gene regulation, including chromatin regulation mediated (Meyer 2015). Under drought conditions it showed a high degree of correlation between changes in DNA methylation and gene expression DNA methylation modifications in faba bean cultivars, suggesting

a possible role of DNA methylation in faba bean in response to drought tolerance. Abid et al. (2017) stated that the expression pattern of drought stress response genes is influenced with DNA methylation. They identified potentially drought stress-related differentially methylated regions (DMRs) and provided a basis for further studies into the role of epigenetic regulation of faba bean responses to drought stress and other environmental stresses. High homology to various putative proteins has been observed which can be chosen for further characterization. Under drought stress conditions of faba bean the genome-wide epigenetic changes occurred in response to the stress (Abid et al. 2017).

5.6.4.4 Common Bean

Common bean (Phaseolus vulgaris L.) is known as grain of hope throughout the world as it is an important component of agriculture and feeds about 300 million people in tropics and 100 million people in Africa alone. It is rich in proteins, carbohydrates, micronutrients, and vitamin A and represents an important source of dietary protein for humans and animals. Micronutrient plays an important role in both animals and plants. Both iron and zinc deficiencies are the major problems faced by public health sector in the world. It is possible that common bean and other legumes may have particular strategies for gene regulation under stress conditions. It has become clear in recent years that many stress responses involve epigenetic components and we are far from understanding the mechanisms and molecular interactions. The recent description of the common bean genome (Schmutz et al. 2014; Vlasova et al. 2016) will provide invaluable knowledge for future PTGS studies. The knowledge of post-transcriptional regulation in common bean is mediated by the legume-specific miR1514a induced during drought stress (Sosa-valencia et al. 2016). miR1514a targets the transcript encoding NAC family of transcription factors, through cleavage and subsequent generation of secondary phasiRNAs. This process occurs during the exposure of adult plants to drought stress. Furthermore, based on an RNA-seq strategy downstream regulatory targets of the transcription factor NAC 700 were observed (Sosa-valencia et al. 2016). miR1514a is a legume microRNA that is induced in response to drought stress in common bean and shows differential accumulation levels in roots under drought stress conditions. The degradome analysis revealed that miR1514a targets the transcripts of two NAC transcription factors (TFs), Phvul.010g121000 and Phvul.010g120700. Furthermore, expression studies and small RNA-seq data indicate that only Phvul.010g120700 generates phasiRNAs, which also accumulate under drought conditions. They determined the functionality of NAC-derived phasiRNA associates with ARGONAUTE 1 (AGO1). In addition, a transcriptome analysis of transgenic hairy roots with reduced miR1514a levels revealed several differentially expressed transcripts involved stress responses which are regulated by the NAC TF and/or by phasiRNAs. They also demonstrated the participation of miR1514 in the regulation of a NAC transcription factor transcript through phasiRNA production during the plant response to drought stress (Sosa-valencia

et al. 2016). Moreover, the manipulation of the epigenetic processes involved in abiotic stress are key points in order to improve future plant breeding and crop productivity of legumes.

5.7 Conclusions

Epigenetic changes modify the activation of certain genes, but not the genetic code sequence of DNA which causes activation or silencing of gene expression. Under drought stress conditions, the defense mechanisms of a plant such as cellular pathways, morphological adaptations, inherent immunity, and specific signaling molecules are controlled by stress-responsive genes by transcribing and translating specific genes. The regulation of the expression of stress-responsive genes due to DNA modifications, chromatin alterations, and small RNA-based mechanisms provide another defense mechanism for plants under stress conditions. The epigenetic control in response to various abiotic stresses is now available due to rapid progress in high-throughput techniques and NGS in model and crop plants. The techniques such as methyl chip-on-chip and MSAP provide wealth of information for whole genome cytosine methylation changes in crops. These techniques have been used in various crops like maize, rice, wheat, and legumes to study methylation dynamics of the regions associated with transposable elements in response to abiotic stresses. Epigenetic can constitute another genetic engineering tool to be applied for defense mechanism in plant species under various environmental stresses. In future, epigenetics may be an important and alternate tool to develop transgenic plants to combat the abiotic stresses.

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Chapter 6
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.

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6.1 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 t/ha, this figure now stands at 9 t/ha today (source: Rothemstead Research). Improvements in agronomic technologies such as mechanised cultivation and the development of new and better fertilisers 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 t/ha for African farmland would tangibly impact on global food security (Professor Martin Parry, Rothamsted Research) and although the 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 bottleneck', 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.

6.2 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 8000 years ago (Huang et al. 2002). Archaeological evidence suggests that tetraploid (emmer) was the predominant dietary grain in pre-9500BC in this region, while the consumption of hexaploid grains began approximately 9500–7500 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 were then selected by early agriculturalists; presumably as this hybrid possessed superior traits compared to tetraploid emmer.

6.3 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 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 the 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 reorganisation 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).

6.4 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 5 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* suggests that as little as 0.17% of the D genome has been deleted during the past 8500 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 that 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 homoeologues 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 amongst 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).

6.5 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. (2003) 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.

6.6 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 that 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.

6.7 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 in 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.

6.8 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 two fold 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.

6.9 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 Lui and Adams (2007) 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 was first published in 2007, then subsequent works were published by Dong and Adams (2011), 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 bias in the expression of these homoeologues represents a generic stress response across a range of polyploidy species. Work by researchers such as Shoeva et al. (2014) is 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 amongst 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.

6.10 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 that variations in the expression amongst 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 amongst a panel of 16 different wheat varieties, and 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 homoeologues 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 that 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 amongst 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.

6.11 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 stabilisation (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 2000 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, a new diversity within the epigenome distinct from the originating progenitor plants.

6.12 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 7
Canonical Histones and Their Variants in Plants: Evolution and Functions

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Abstract The DNA found inside the nuclei of eukaryotic cells is complexed with histone proteins forming the polymer called chromatin. Chromatin is organized into repeating units, nucleosomes, which are comprised of DNA wrapped around an octamer of the core histones H2A, H2B, H3, and H4. Histones are encoded by multigene families organized as clusters in animals and algae, but as dispersed copies in the genome of higher plants. The bulk of histones are expressed during the S-phase of the cell cycle in order for them to be incorporated into the chromatin of the newly replicated DNA. In addition to these canonical histones, eukaryotic genomes also encode related histone variants. Histone variants are expressed independently of the cell cycle and replace canonical histones when chromatin is disrupted by processes such as transcription, DNA repair, recombination, etc. This chapter will review the core histone families H2A, H2B, H3, and H4 in higher plants. For each family, canonical histones and their variants will be described emphasizing their evolutionary origin and the roles they play in different chromatin-mediated processes. In the plant kingdom, the core histones families have diversified allowing some isoforms to maintain their original roles, but also the emergence of new variants with novel functions. Both conserved and plant-specific histone variants participate in all aspects of plant life including development, phase transitions, flowering, responses to abiotic stresses, and germline formation among others. Many of the processes regulated by histones involve agronomically important traits highlighting their potential as targets for crop breeding and biotechnology.

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7.1 Introduction

The DNA of all eukaryotes is organized inside the nucleus by histones and other proteins to form the dynamic polymer called chromatin. DNA packed into chromatin becomes less accessible and therefore a poor substrate for proteins involved in DNA-templated process. As a result, mechanisms modulating chromatin accessibility play a major role in the regulation of gene expression and other processes such as replication, DNA repair, and recombination among others. The basic unit of the chromatin is the nucleosome which is composed of approximately 146 bp of DNA wound 1.65 turns around an octamer of the core histones H2A, H2B, H3, and H4 (Luger 2003; Luger et al. 1997). Adjacent nucleosomes are connected by 20–80 bp of linker DNA resulting in the formation of linear arrays of nucleosomes that may be further organized into higher-order chromatin structures aided by the linker histone H1 (Luger 2003). Because the basic organization of the chromatin has remained constant throughout eukaryotic evolution, the structure of the core histones is the most conserved of all proteins. This is especially true for H3 and H4 whose positions in the nucleosome tolerate only a few amino acid changes (Malik and Henikoff 2003).

Each core histone contains two defined regions: the histone fold domain (HFD) and the histone tail domain. In addition, unique ordered-structure elements extending the HFD exist on each core histone (Arents et al. 1991). The HFD is a structural motif common to all core histones and is composed by three alpha-helices, α1, α2, and α3, each one connected by a loop, L1 and L2 (Arents and Moudrianakis 1995; Luger et al. 1997; Arents et al. 1991). The HFD and the ordered elements mediate the histone–histone and histone–DNA interactions that held the nucleosome together (Luger et al. 1997). The histone tails are relatively unstructured domains rich in basic amino acids that protrude out of the nucleosome (Luger et al. 1997). The histone tail domain corresponds to the N-terminal tail of all core histones, and the C-terminal tail of histone H2A. Amino acid residues in the tail domain are subject to numerous posttranslational modifications (PTMs) including acetylation, methylation, and phosphorylation among others (Berger 2007; Kouzarides 2007). Although its contribution to the nucleosome structure remains unclear, the histone tail domain modified by PTMs influences all the chromatin-mediated process including transcription, replication, recombination, and DNA repair (Kouzarides 2007).

The bulk of histones are loaded into chromatin during DNA replication. This occurs because histones are dissociated from the DNA to allow the progress of the replication fork while the newly replicated strands are reconstituted into chromatin immediately behind it (Margueron and Reinberg 2010). Both, old and new histones are deposited in the newly synthesized DNA although the mechanism of deposition is not fully understood (Margueron and Reinberg 2010). To supply enough histones to fold the newly replicated DNA into chromatin, the proper amount of histones need to be synthesized during replication. For this reason, histone genes are encoded by intronless multigene families that are tightly regulated to allow them to be highly expressed specifically during the S-phase of the cell cycle (Malik and Henikoff

2003). In addition to these "canonical histones," genes encoding related isoforms exist for each of the core histones. Incorporation of these "histone variants," which may differ by a few amino acids or completely diverge from the canonical histones, into nucleosomes will modify the properties of the chromatin (Jiang and Berger 2017). Furthermore, histone variants are expressed beyond DNA replication where they might replace canonical histones when chromatin is disrupted by nuclear process such as transcription or DNA repair (Jiang and Berger 2017). The incorporation of variants has the potential to confer distinctive properties to a chromatin domain, allowing histone variants to evolve to play roles in transcription, DNA repair, chromosome segregation, recombination, etc. In this chapter, we will explore the gene families encoding the core histones in the plant kingdom. An overview of the organization of histone genes in plants is first given. After that, the H2A, H2B, H3, and H4 gene families are examined looking first at their canonical isoforms and then the presence of variants, emphasizing the role(s) they may have acquired during plant evolution. The potential applications that histones and their variants may have in plant breeding and biotechnology are discussed.

7.2 Organization of Histones Genes in Plants

Histone genes are present in multiple copy numbers and their genome organization and distribution vary among different taxonomic groups. In animals, the four core histone genes (H2A, H2B, H3, and H4) are present in quartets. In some species, the linker histone H1 may also be present forming quintets. The organization of the core histone genes in the quartets and quintets is variable with all the genes located in the same strand or being present in pairs (H3–H4 and H2A–H2B) transcribed from divergent promoters. Quartets and quintets are organized in tandem and grouped into clusters, sometimes with both, quartets and quintets, present in the same genome. However, while clusters are present in all metazoans, tandem arrangements appear to become more heterogeneous during evolution to the point of almost disappearing in mammals (Eirín-López et al. 2009).

The copy number of histone genes also varies greatly in eukaryotes. *Saccharomyces cerevisiae* possesses only two copies of each core histone gene, whereas in the fission yeast *Schizosaccharomyces pombe* this number varies from one for H2B, two for H2A and three for H3 and H4 (Hereford et al. 1979; Smith and Andresson 1983; Matsumoto and Yanagida 1985). Conversely in the human genome, 60 genes coding for core histones are present (Marzluff et al. 2002). This number, however, is small compared with the genomes of other metazoans such as the sea urchin *Strongylocentrotus purpuratus* whose genome contains ~2000 genes encoding core histones and the H1 linker (Marzluff et al. 2006).

The histone gene complement in the plant kingdom is also variable in terms of copy number and organization. In the chlorophyte green algae, *Chlamydomonas reinhardtii* and *Volvox carteri*, histone genes are present in quartets organized in pairs (H3–H4 and H2A–H2B) that are transcribed from divergent promoters (Fabry

et al. 1995; Muller et al. 1990; Muller and Schmitt 1988). In Chlamydomonas, most of the quartets are grouped in clusters but a tandem arrangement is observed in very few of them. Histone genes belonging to quartets do not possess introns, and their transcripts are non-polyadenylated and end in a 3′ stem-loop structure resembling those of histone genes in animals (Fabry et al. 1995; Muller et al. 1990). These features suggest that these genes may encode replication-dependent canonical histones. Indeed, for H4 histone genes, replication-dependent gene expression has been demonstrated (Fabry et al. 1995). The Chlamydomonas genome also contains few histone genes that are not organized into quartets, some of which contain introns in their coding sequence. These features suggest that these genes may encode replication-independent histone variants.

Compared to animals and chlorophyte algae, histone genes in land plants display very different features. Histone genes of land plants are also present as multigene families. However, genes are not organized in divergently transcribed pairs, neither in quartets nor in quintets. Instead, every gene is transcribed from its own independent promoter and is distributed as interspersed copies throughout the genome (Chaboute et al. 1993). Histone genes encoding canonical histones do not contain introns (with the exception of H2A), and their transcripts are polyadenylated and lack the 3′ end palindrome characteristic of their animal and algae counterparts (Chaboute et al. 1988, 1993; Chaubet et al. 1988; Wu et al. 1989).

The contrasting structural and organizational differences between histone genes of animals and land plants have profound implications in the way these organisms regulate the expression of histone genes. In animals, transcriptional and posttranscriptional mechanisms, including pre-mRNA processing, translation, and mRNA stability control, of gene regulation ensure the correct expression of histone genes during the S-phase of the cell cycle (Rattray and Muller 2012). Given the similarities in organization of histone genes between animal and chlorophyte algae, related mechanisms of control have been suggested to be important for the later unicellular organisms (Fabry et al. 1995). In higher plants the lack of a 3′ end stem loop, and production of polyadenylated transcripts, led to the idea that regulatory mechanisms may act largely at the transcriptional level (Eirín-López et al. 2009; Chaboute et al. 1993). However, it has been demonstrated that posttranscriptional mechanisms are involved in the rapid decay of histone transcripts at the end of the S-phase even though they may be different from those acting on animals and algae (Kapros et al. 1995; Reichheld et al. 1998).

The number of core histone genes in plants also shows great variation. In the genome of the unicellular green alga *Ostreococcus tauri* 13 core histone genes were identified. From these, histones H2A and H3 were encoded by four genes each, whereas two and three genes were found for histones H2B and H4, respectively (Kawashima et al. 2015). The genome of higher plants, however, appears to contain a moderately larger number of histones genes than algae. For instance, the Arabidopsis and rice genomes contain 47 and 56 core histone genes, respectively (Table 7.1). Thus, it appears that the number of histones has increased over the course of plant evolution likely resulting in the emergence of new histones variants with novel functions. Genomes with exceptionally increased number of histone

Table 7.1 Core histone genes and proteins from Arabidopsis and rice

Histone/Subtype	Species										
	Arabidopsis thaliana[a]						*Oryza sativa*[b]				
H3	*Gene*	*Locus*	*Protein*[c]	*Length (aa)*	*No. of introns*[d]	*Expression peak*[e]	*Gene*	*Locus*	*Protein*[c]	*Length (Aa)*	*No. of introns*[d]
Canonical	HTR1	At5g65360	H3.1	136	0	G2	HTR702	Os01g64640	H3.1	136	0
	HTR2	At1g09200	H3.1	136	0	S	HTR703	Os06g06500	H3.1	136	0
	HTR3	At3g27360	H3.1	136	0	S	HTR704	Os11g05730	H3.1	136	0
	HTR9	At5g10400	H3.1	136	0	S	HTR705	Os04g34240	H3.1	136	0
	HTR13	At5g10390	H3.1	136	0	S	HTR706	Os05g36280	H3.1	136	0
							HTR708	Os06g06460	H3.1	136	0
							HTR713	Os06g06510	H3.1	136	0
H3.3	HTR4	At4g40030	H3.3	136	2	None	HTR711	Os03g27310	H3.3	136	3
	HTR5	At4g40040	H3.3	136	3	None	HTR716	Os04g37780	H3.3	136	4
	HTR8	At5g10980	H3.3	136	2	None	HTR712	Os06g04030	H3.3	136	1
CENH3	HTR12	At1g01370	CENH3	178	8	S	HTR707	Os05g41080	CENH3	164	6
H3-like	HTR6	At1g13370	H3.6	136	2	S	HTR701	Os06g06480	H3.701	143	1
	HTR7	At1g75610	H3.7	115	3	N.D.	HTR709	Os02g25910	H3.709	146	3
	HTR10	At1g19890	H3.10	137	3	None	HTR714	Os12g22650	H3.714	136	0
	HTR14	At1g75600	H3.14	136	3	S	HTR715	Os12g22680	H3.715	136	0
Pseudogenes	HTR7	At1g75610	–	115	3	–	HTR710	Os02g25940	H3.710	115	2
	HTR11	At5g65350	–	139	0	–					
H2A	*Gene*	*Locus*	*Protein*[c]	*Length (aa)*	*No. of introns*[d]	*Expression peak*[e]	*Gene*	*Locus*	*Protein*[c]	*Length (aa)*	*No. of introns*[d]
Canonical	HTA1	At5g54640	H2A.1	132	1	None	HTA702	Os08g33100	H2A.702	134	1
	HTA2	At4g27230	H2A.2	131	1	S	HTA703	Os12g25120	H2A.703	135	1
	HTA10	At1g51060	H2A.10	133	1	None	HTA708	Os07g36140	H2A.708	135	1
	HTA13	At3g20670	H2A.13	133	1	S	HTA709	Os07g36130	H2A.709	135	1

(continued)

Table 7.1 (continued)

Histone/Subtype	Species										
	Arabidopsis thaliana[a]						*Oryza sativa*[b]				
H2A.Z	HTA8	At2g38810	H2A.Z.8	137	2	S	HTA705	Os10g28230	H2A.Z.705	139	1
	HTA9	At1g52740	H2A.Z.9	135	1	None	HTA712	Os03g06670	H2A.Z.712	138	1
	HTA11	At3g54560	H2A.Z.11	137	2	S	HTA713	Os03g53190	H2A.Z.713	137	1
H2A.X	HTA3	At1g54690	H2A.X.3	142	1	None	HTA704	Os03g51200	H2A.X.704	137	1
	HTA5	At1g08880	H2A.X.5	143	1	S	HTA711	Os12g34510	H2A.X.711	138	1
H2A.W	HTA6	At5g59870	H2A.W.6	151	1	S	HTA701	Os01g31800	H2A.W.701	159	1
	HTA7	At5g27670	H2A.W.7	151	1	S	HTA706	Os05g38640	H2A.W.706	163	1
	HTA12	At5g02560	H2A.W.12	153	1	None	HTA707	Os05g02300	H2A.W.707	156	1
							HTA710	Os03g17100	H2A.W.701	159	1
Pseudogenes	HTA4	At4g13570	–	119	1	–	HTA714	Os04g13530	–	446	7
H2B	*Gene*	*Locus*	*Protein*[c]	*Length (aa)*	*No. of introns*[d]	*Expression peak*[e]	*Gene*	*Locus*	*Protein*[c]	*Length (aa)*	*No. of introns*[d]
	HTB1	At1g07790	H2B.1	148	0	None	HTB701	Os01g05950	H2B.701	153	1
	HTB2	At5g22880	H2B.2	145	0	S	HTB702	Os01g62230	H2B.702	139	0
	HTB3	At2g28720	H2B.3	151	0	None	HTB703	Os01g05900	H2B.703	153	0
	HTB4	At5g59910	H2B.4	150	0	S	HTB704	Os01g06010	H2B.704	155	0
	HTB5	At2g37470	H2B.5	138	0	S	HTB705	Os01g05970	H2B.705	153	0
	HTB6	At3g53650	H2B.6	138	0	S	HTB706	Os01g05630	H2B.706	153	0
	HTB7	At3g09480	H2B.7	126	0	S	HTB707	Os01g05610	H2B.706	153	0
	HTB8	At1g08170	H2B.8	243	0	None	HTB708	Os01g05990	H2B.708	153	0
	HTB9	At3g45980	H2B.9	150	0	S	HTB709	Os03g17084	H2B.709	152	0
	HTB10	At5g02570	H2B.10	132	0	None	HTB710	Os05g38560	H2B.710	118	1
	HTB11	At3g46030	H2B.11	145	0	S	HTB711	Os05g49860	H2B.711	152	0
							HTB712	Os08g38300	H2B.712	150	0
							HTB713	Os09g39730	H2B.713	194	0
							HTB714	Os01g31810	H2B.714	102	0
							HTB715	Os08g38289	H2B.715	56	0

Histone/Subtype	Species										
	Arabidopsis thaliana[a]						*Oryza sativa*[b]				
H4	*Gene*	*Locus*	*Protein*[c]	*Length (aa)*	*No. of introns*[d]	*Expression peak*[e]	*Gene*	*Locus*	*Protein*[c]	*Length (aa)*	*No. of introns*[d]
Canonical	HF01	At3g46320	H4.1	103	0	S	HFO701	Os10g39410	H4.1	103	0
	HF02	At5g59690	H4.1	103	0	G2	HFO704	Os07g36500	H4.1	103	0
	HF03	At2g28740	H4.1	103	0	G2	HFO705	Os04g49420	H4.1	103	0
	HF04	At1g07820	H4.1	103	0	S	HFO706	Os03g02780	H4.1	103	0
	HF05	At3g53730	H4.1	103	0	S	HFO707	Os09g26340	H4.1	103	0
	HF06	At5g59970	H4.1	103	0	S	HFO708	Os05g38740	H4.1	103	0
	HF07	At3g45930	H4.1	103	0	S	HFO709	Os01g61920	H4.1	103	0
	HF08	At1g07660	H4.1	103	0	S	HFO711	Os02g45940	H4.1	103	0
							HFO712	Os05g39050	H4.1	103	0
							HFO713	Os09g38020	H4.1	103	0
Variant							HFO714	Os05g38760	H4.2	103	1

[a]Data obtained from Talbert et al. (2012), Okada et al. (2005), Yi et al. (2006), and Bergmuller et al. (2007)

[b]Data obtained from Hu and Lai (2015)

[c]Proteins were named following the nomenclature proposed by Talbert et al. (2012)

[d]Only the number of introns of the most current gene model was considered

[e]Cell cycle expression data obtained from Menges et al. (2003). *S* peak of expression during S-phase, *G2* peak of expression during G2-phase, *none* no specific peak of expression during cell cycle, *N.D.* not determined

genes, like in the sea urchin in metazoans, have not been reported in plants. However, a caveat to this observation is that the model plants, Arabidopsis and rice, in which the complete histone gene complement has been studied are diploid species with small genomes. Polyploid species with larger genomes may have maintained duplicated histone genes leading to expanded histone gene families. This may have been the case of sugarcane (*Saccharum sp*.), which in its 10,000 Mbp, 50 times larger to that of Arabidopsis, polyploid (10–12x) genome contains at least 40 histone H3 genes and 24 H4 genes, a large number compared to the 15 H3 genes and 8 H4 genes encoded in the Arabidopsis genome (Table 7.1) (Moraes et al. 2015; Okada et al. 2005). Still, analysis of further plant genomes will be needed to shed more light into the expansion of histones genes in plants.

7.3 Core Histones in Plants

A multitude of histone genes and the proteins encoded by them were isolated and identified from early studies in plants. However, it was until the genome of several plants was sequenced that researchers could have a comprehensive view of the histone gene complement in plants. Several studies have identified canonical histones that share remarkable similarity to histones from organisms other than plants. In addition, histone variants deposited outside the S-phase of the cell cycle have also been found. As in other eukaryotes, the overwhelming majority of these variants correspond to the histones H3 and H2A, many of which have been functionally characterized, whereas little evidence exists for the presence of specialized forms of H4 and H2B.

7.3.1 Histone H3 Family

The *HISTONE 3 RELATED* (*HTR*) gene family is one of the best studied in plants in terms of their genes, encoded proteins, and posttranslational modifications. These studies have shown that even though the canonical H3 protein is very well conserved among eukaryotes, divergent H3 variants that play different roles in several DNA-templated process exist in plants (Ingouff and Berger 2010; Okada et al. 2005). Analysis of histone genes from Arabidopsis, rice, and other plant species suggests that four different forms of histone H3 proteins exist in land plants (Fig. 7.1). These include the canonical histone H3.1, the H3.3 variant, the centromeric H3 variant, and H3-like genes (Okada et al. 2005; Ingouff and Berger 2010; Hu and Lai 2015).

7.3.1.1 Canonical Histone H3

The canonical histone H3 or H3.1 from higher plants is remarkably similar to that of animals, fungi, and even lower eukaryotes (Waterborg 2012). A sequence comparison indicates that only four amino acid substitutions, F41Y, K53R, A90M, and A96C, differentiate the canonical histone H3 from plants and mammals. Interestingly, the five *HTR* genes from Arabidopsis encoding histone H3.1 do not contain introns and are expressed in tissues containing highly dividing cells and all but one are expressed during the S-phase of the cell cycle (Table 7.1) (Okada et al. 2005). In a similar way, the seven genes encoding H3.1 from rice also lack introns and several of them are expressed in highly dividing tissues (Table 7.1) (Hu and Lai 2015). All these characteristics indicate that indeed H3.1 genes encode replication-coupled H3 canonical histones deposited in chromatin during the S-phase of the cell cycle.

7.3.1.2 H3.3 Variants

H3.3 was the first variant of H3.1 identified by the characteristic amino acid changes A31T, F41Y, S87H, and A90L (Fig. 7.1a) (Waterborg 1990, 1991, 2012; Waterborg and Robertson 1996; Okada et al. 2005). In contrast to the intronless H3.1 genes, all the Arabidopsis and rice genes encoding H3.3 contain introns (Table 7.1) (Hu and Lai 2015; Okada et al. 2005; Chaubet et al. 1992). The same is true for the H3.3 genes identified in other plants (Waterborg 2012; Waterborg and Robertson 1996). In Arabidopsis, H3.3 genes do not show replication-dependent expression and are rather expressed throughout the cell cycle (Table 7.1). In addition, expression of Arabidopsis genes encoding histone H3.3 occurs not only in young, undifferentiated, but also in mature tissues suggesting that the expression of these genes continues after cell division ceases (Okada et al. 2005; Chaubet et al. 1992). Thus, H3.3 is a replication-independent variant that could be deposited into chromatin outside the S-phase of the cell cycle.

Histone H3.3 is an essential element of the plant chromatin likely involved in transcriptional regulation. Partial suppression of H3.3 expression causes pleotropic defects, whereas total loss of H3.3 is lethal (Wollmann et al. 2017). Genome-wide analysis of H3.1 and H3.3 distribution in Arabidopsis has shown that H3.3 is enriched in the body of actively transcribed genes with a bias towards the 3′ end, whereas H3.1 is associated with silent euchromatin and heterochromatic regions (Wollmann et al. 2012; Stroud et al. 2012). In addition to gene bodies, H3.3 is also enriched in promoter regions and downstream to the transcription termination site of a subset of genes (Shu et al. 2014). Deposition of H3.3 in gene bodies is positively correlated with transcriptional activity and may serve as a “memory” of transcriptional activity or may act as a mechanism facilitating the turnover or replacement of covalent modifications during developmental transitions (Wollmann et al. 2012; Stroud et al. 2012). The presence of H3.3 at promoter regions was, however, independent of transcription although RNA polymerase II was shown to associate with promoters enriched in H3.3 (Shu et al. 2014). Enrichment with H3.3 is believed to

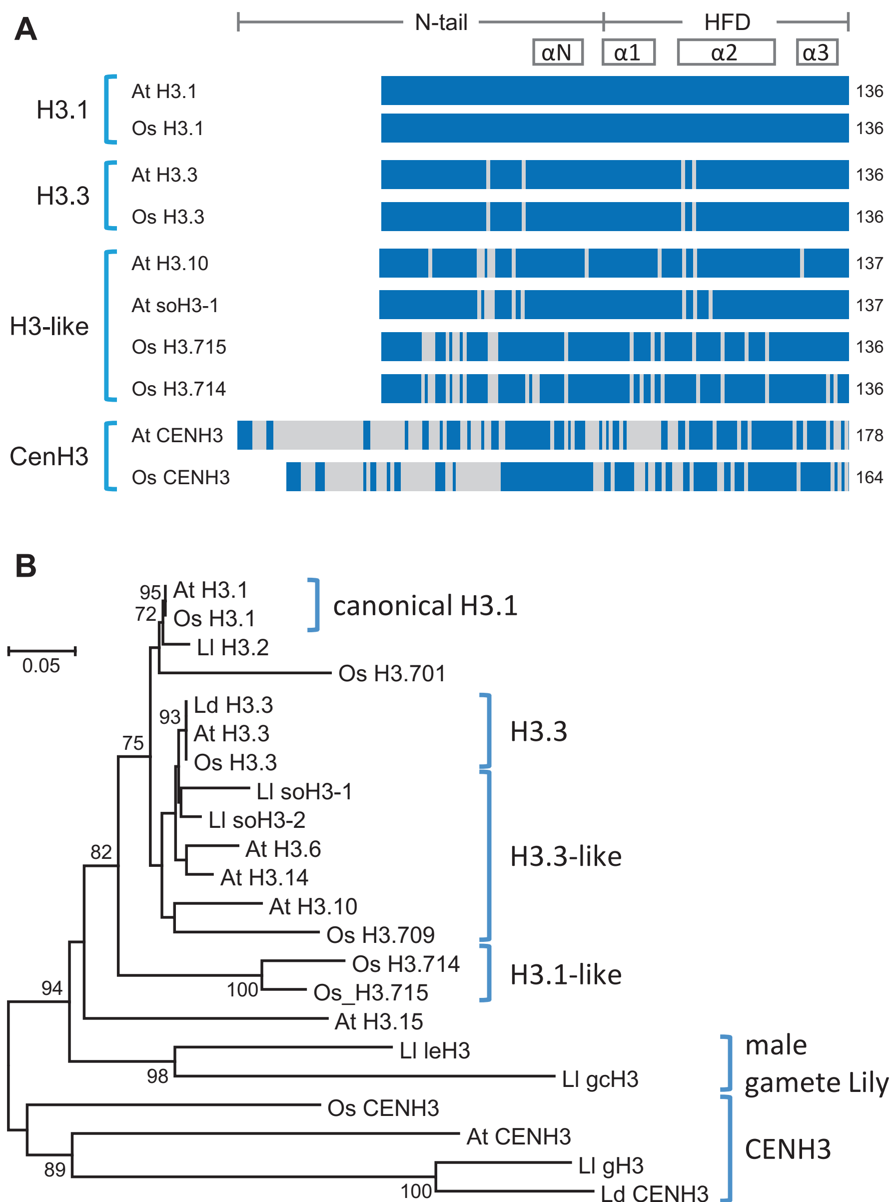

Fig. 7.1 The histone H3 family. (**a**) Amino acid conservation among representative histone H3 proteins. The position of the three α-helices (α1, α2, and α3) comprising the histone fold domain (HFD) and αN helix of the N-terminal tail domain (N-tail) is depicted on top. The different H3 variant types to which each protein belong is indicated to the left. Protein length in amino acids is shown to the right. Conserved residues are colored in blue. Proteins were drawn at scale. (**b**) Phylogenetic analysis of histone H3 proteins from Arabidopsis, rice, and lily. The different clades of H3 proteins are shown to the right of the tree. The tree was constructed using entire length of the

facilitate the access of transcription factors or the initiation complex to the promoter regions by relaxing chromatin folding (Shu et al. 2014). Similarly, deposition of H3.3 in the body of transcribed genes has been proposed to provide increased access of DNA methyltransferases, thereby allowing gene body methylation to occur in a transcription-dependent fashion (Wollmann et al. 2017). Taken together these observations suggest that one of the functions of H3.3 is to create a chromatin environment that is accessible to factors that modify and/or interact with the DNA. In fact, H3.3-containing nucleosomes show increased accessibility to DNase I indicating that H3.3 may actually interfere with higher-order chromatin folding (Shu et al. 2014). In addition, H3.3 deposition may promote an open chromatin by preventing the binding of the linker histone H1 to the bodies of transcribed genes (Wollmann et al. 2017).

Mass spectrometry analysis of specific peptides for H3.1 and H3.3 in Arabidopsis and sugarcane indicates that while H3.3 is enriched in modifications associated with transcriptional activity (K36 methylation), H3.1 contains higher levels of K27 methylation, a modification associated with gene silencing (Johnson et al. 2004; Moraes et al. 2015). The functional connotation of these modifications correlates with the genomic distribution of H3.1 and H3.3 and suggests a functional interplay between histone variants and posttranslational modifications. The four amino acid differences between H3.1 and H3.3 seem to be partially responsible for the differences in posttranslational processing and interaction with the chromatin, which in turn results in the different functional outcomes observed between H3.1 and H3.3. For instance, the presence of A (alanine) in H3.1 and T (threonine) in H3.3 at position 31 precludes the monomethylation of K27 (H3K27me1) in H3.3, but not H3.1, by the ARABIDOPSIS TRITHORAX-RELATED PROTEIN 5 and 6 (ATXR5, 6) (Jacob et al. 2014). This difference allows the inheritance of the heterochromatic mark H3K27me1 only at regions enriched in H3.1 while protecting regions associated with H3.3 (i.e., gene bodies and promoter regions) during replication (Jacob et al. 2014). The residue at position 31, together with 41, is also important for depletion of H3.3 at transcriptionally silent rDNA (ribosomal RNA genes) arrays, while amino acids 87 and 90 guide deposition of H3.3 at actively transcribed rDNA loci (Shi et al. 2011).

Fig. 7.1 (continued) histone H3 proteins, which were aligned with the ClustalX program (Larkin et al. 2007). This alignment was then used to infer a neighbor-joining tree using MEGA 7.0 (Kumar et al. 2016). Tree reliability was assessed by conducting a bootstrap test based on 1000 pseudoreplicates. Numbers on the nodes correspond to bootstrap values higher than 60%. The scale bar indicates the number of amino acid changes per site. Protein names are preceded by an abbreviation indicating the corresponding species as follows: At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Ld, *Lilium davidii*; and Ll, *Lilium longiflorum*. Accession numbers of the lily proteins used are: Ld CENH3, CUT18453; Ld H3.3, CUT18456; Ll gH3, BAA96098; Ll gcH3, BAE48427; Ll H3.2, BAE20250; Ll leH3, BAE48431; Ll soH3-1, BAE48433; and Ll soH3-2, BAE48435. Locus id of the H3 proteins corresponding to Arabidopsis and rice is given in Table 7.1. H3 proteins encoded by suspected pseudogenes, Arabidopsis *HTR7* and *HTR11*, and rice *HTR710*, were not considered

Even though the similarities in deposition patterns suggest conserved functions, this is likely the result of convergent evolution since the animal and plant H3.3 variants appear to have evolved independently (Waterborg 2012; Waterborg and Robertson 1996). Evidence for independent origins comes from differences in the exon/intron structure among plant and animal H3.3 genes (Waterborg and Robertson 1996). Furthermore, substitutions differentiating H3.1 from H3.3 in animals involve residues 31, 87, 89, and 90, whereas in plants changes in amino acids 31, 87, 90, in addition to 41, differentiate H3.1 from H3.3. This added to the fact that changes between H3.1 and H3.3 in plants and animals involve different types of amino acids are indications of distinctive evolutionary origins (Waterborg 2012; Waterborg and Robertson 1996; Malik and Henikoff 2003). Intriguingly, independent evolution of the H3.3 variant has occurred four times (in animals, plants, basidiomycetes, and alveolates) suggesting that with the emergence of multicellularity and a rise in organismal complexity, there is a need for histone proteins to become available for nucleosome assembly in specialized, non-dividing cells types (Waterborg 2012). Thus, emergence of an H3.3 variant may have been instrumental in the development of an increasing cell and tissue complexity associated with the advent of multicellularity (Waterborg 2012).

7.3.1.3 CENH3 Variants

The centromeric histone H3 (CENH3), or CENP-A (centromere protein A) in mammals, is a specialized histone variant that replaces canonical histone H3 at centromeric nucleosomes, thereby defining the chromosomal region that is known as the centromere (De Rop et al. 2012; Malik and Henikoff 2003; Comai et al. 2017). The centromere harbors the kinetochore, a complex to which the mitotic spindle attaches in order to separate the sister chromatids during mitosis (Malik and Henikoff 2003; De Rop et al. 2012). Deposition of CENH3 is necessary for the assembly of the kinetochore and thus allows for proper chromosome segregation (Howman et al. 2000; Blower and Karpen 2001). In plants, the first CENH3 variant, *HTR12*, was characterized in *Arabidopsis thaliana* (Talbert et al. 2002). Using immunolocalization with an anti-CENH3 antibody, Talbert et al. (2002) were able to demonstrate that CENH3 is localized specifically in the centromere of mitotic and meiotic chromosomes. CENH3 also co-localizes with the 180-bp satellite repeats and, in turn, these repeats can be specifically immunoprecipitated with the anti-CENH3 antibody (Nagaki et al. 2003; Talbert et al. 2002). CENH3 was also detected in interphase cells in the form of foci, found at centromeres and localized predominantly at the nuclear periphery, likely representing condensed centromeres (Talbert et al. 2002; Fang and Spector 2005; Lermontova et al. 2006). The localization pattern observed for CENH3 suggests that it is indeed a centromeric histone H3 variant. Since the discover of Arabidopsis CENH3, other plant H3 variants with a centromeric localization pattern similar to that of CENH3 have been identified in several plant species including sugarcane, tobacco, rice, maize, barley, and wheat (Nagaki and Murata 2005; Nagaki et al. 2004, 2009; Zhong et al. 2002; Sanei et al. 2011;

Yuan et al. 2015). Furthermore, in tobacco, rice, maize, the common bean (*Phaseolus vulgaris*), and the legume *Astragalus sinicus* co-localization of CENH3 variants with their corresponding centromeric DNA repeats has been demonstrated (Nagaki et al. 2004, 2009; Zhong et al. 2002; Iwata et al. 2013; Tek et al. 2011). Like many other histone variants, CENH3 is loaded into chromatin independently of replication (Lermontova et al. 2006, 2015). Arabidopsis CENH3 is instead deposited into centromeric chromatin during late G2 phase (Lermontova et al. 2006). The timing of deposition in Arabidopsis CENH3, and perhaps in most plants, is different from that of metazoans in which CENH3 is loaded during anaphase or early G1 phase (Lermontova et al. 2015).

Null mutations in the centromeric histone H3 variants from mammals (*CENP-A*) and Drosophila (*Cid*, centromere identifier) are embryo lethal (Howman et al. 2000; Blower et al. 2006). In Arabidopsis, a null mutation of CENH3, *cenh3-1*, also caused embryo lethality suggesting that it is an essential gene in animals and plants (Ravi et al. 2010). Downregulation of *HTR12* in Arabidopsis led to dwarf plants with a decreased number of cells as a result of reduced mitotic activity. These plants were also partially sterile, with smaller anthers and reduced pollen fertility (Lermontova et al. 2011). Defective pollen viability due to disturbed meiotic segregation is indicated by unequal separation of chromosomes, increased chromosome lagging, and the formation of micronuclei in pollen tetrads (Lermontova et al. 2011). Taken together these observations suggest that CENH3 participate in both, mitotic and meiotic cells divisions.

CENH3 is usually encoded by a single-copy gene in Arabidopsis, rice, and the majority of plant diploid species, even those that may have undergone past polyploidization events (Okada et al. 2005; Hu and Lai 2015; Lermontova et al. 2015). This suggest that after a duplication event a single *CENH3* copy is maintained, while the others become a pseudogene or may be completely lost. However, this is not always the case as two isoforms of CENH3, with different degrees of functionality, have been found in polyploids and few diploid species (Lermontova et al. 2015). For instance, two CENH3 variants have been found in the diploid species *Arabidopsis lyrata*, *A. halleri*, *Luzula nivea,* in few species of the genus *Mimulus*, *Pisum,* and *Lathyrus,* and in diploid barley (*Hordeum*) (Kawabe et al. 2006; Moraes et al. 2011; Finseth et al. 2015; Neumann et al. 2015; Sanei et al. 2011). Both CENH3 isoforms in *L. nivea*, *H. vulgare, M. guttatus*, *Pisum,* and *Lathyrus* show evidence of being transcribed. From these, the CENH3 paralogs from *M. guttatus* were proven to be expressed at the same levels in leaf tissues, while in *H. vulgare* the expression levels of both paralogs vary in a tissue-specific manner (Ishii et al. 2015; Finseth et al. 2015). Furthermore, CENH3 isoforms A and B from *L. nivea* displayed similar patterns of chromosomal localization, whereas in *P. fulvum*, *L. sativus*, and *L. latifolius*, CENH3-1 and CENH3-2 isoforms were shown to fully co-localize in the same domains in metaphase chromosomes (Neumann et al. 2015; Moraes et al. 2011). In a similar way, αCENH3 and βCENH3 variants from *H. vulgare* co-localize in mitotic and meiotic chromosomes in the form of discrete but intermingled subdomains (Ishii et al. 2015; Sanei et al. 2011). In allopolyploids, occurrence of two CENH3 isoforms is more widespread than in diploids. The prevalence of two

CENH3 variants has been reported in allopolyploids species of brassica (*Brassica*), cotton (*Gossypium*), sugarcane (*Saccharum*), wheat (*Triticum*), rye (*Secale*), and rice (*Oryza*) among others (Wang et al. 2011; Masonbrink et al. 2014; Nagaki and Murata 2005; Yuan et al. 2015; Evtushenko et al. 2017; Hirsch et al. 2009). In the allopolyploid species studied, the degree of expression of each copy varies widely from one copy being predominantly transcribed to both copies showing similar expression levels (Masonbrink et al. 2014). In *Gossypium* and *Triticum*, *CENH3* expression is biased to one of the copies, whereas in some *Oryza* species both copies are equally transcribed (Masonbrink et al. 2014; Yuan et al. 2015; Hirsch et al. 2009). Chromosomal localization for both CENH3 variants of *Triticum*, αCENH3 and βCENH3, has been determined using immunolocalization with antibodies specific for each variant (Yuan et al. 2015). These studies showed overlapping localization of αCENH3 and βCENH3 to most centromeric regions of the mitotic chromosomes, although in some cases centromeric regions enriched in only one type of variant were detected. Variability in the protein levels of each variant was also observed during mitosis and in interphase nuclei (Yuan et al. 2015). Thus, after an allopolyploidization event newly duplicated copies of *CENH3* are usually maintained in the genome, many of these pairs have even survived the large-scale genome rearrangements that lead to diploidization following polyploidy. Functional differentiation of these pairs seems to have been attained by changing the expression levels and/or tissue-specific expression of the copies. However, even in the presence of both isoforms, chromatin domains enriched in a particular type could be observed in some cases suggesting that functional differentiation at the protein level may also occur.

In contrast to the highly conserved canonical histone H3.1 and the H3.3 variant, CENH3 is highly divergent (Fig. 7.1). The protein consists of a N-terminal part that is extremely variable in terms of length and sequence, and a C-terminal portion that contains the histone fold domain (HFD) which shares a 50% homology to other histone H3s except at the loop 1 region which is longer than the canonical H3 (Malik and Henikoff 2003). During mitosis, the C-terminus of Arabidopsis CENH3 is sufficient for deposition into centromeres but an N-terminal tail is still necessary to assemble a functional kinetochore (Lermontova et al. 2006; Ravi et al. 2010). Meiosis, on the other hand, requires both, the C- and N- terminal moieties, for loading of CENH3 into chromosomes (Ravi et al. 2011; Lermontova et al. 2011). Overall, there appear to exist two different pathways for deposition of CENH3 into mitotic and meiotic chromosomes, the second requiring the N-terminal tail (Ravi et al. 2011).

Centromeric sequences are highly variable and rapidly evolving leading to divergence even in related taxa. In plants, and in animals, centromeres consist of complex arrays of tandemly repeated satellite sequences that extend for megabases (Henikoff et al. 2001; Lermontova et al. 2015). Thus, rapid variation in CENH3, and other centromeric proteins, is generated in response to changes in fast-evolving centromeric DNA (Talbert et al. 2004; Masonbrink et al. 2014). In Drosophila species, rapid variation in the Cid protein, the homolog of CENH3, is driven by adaptive evolution, measured as the prevalence of amino acid-changing nucleotide

substitutions over synonymous nucleotide changes (Malik and Henikoff 2001). Both the N-terminal tail and the longer loop 1 region of Drosophila Cid were found subjected to adaptive evolution (Malik and Henikoff 2001). Evidence of adaptive evolution or positive selection acting in CENH3 is also found in plants. In the Brassicaceae family, signs of positive selection were found in the N-terminal tail and the loop 1 region of CENH3, although in Arabidopsis positive selection was only detected at the N-terminal region (Talbert et al. 2002; Cooper and Henikoff 2004). In other plant clades, including the genus *Oryza* and *Mimulus*, adaptive evolution of CENH3 has also been demonstrated even after allopolyploidization events (Hirsch et al. 2009; Finseth et al. 2015). Thus, substitutions in CENH3 that complement changes in the centromeric repeats are quickly selected in order to maintain centromere functionality. Rapid evolution of the centromeric repeats is in turn driven by the competition between centromeric repeats during asymmetrical meiosis in females, a phenomenon known as centromere drive (Henikoff et al. 2001). If this hypothesis is correct, then suppression of centromeric drive should lead to loss of adaptive evolution in CENH3. Indeed, in clades with symmetrical meiosis that are not subject to centromere drive, positive selection of CENH3 is not as common as in clades with asymmetrical meiosis (Zedek and Bures 2016b). In addition, *Luzula* species having holocentric chromosomes, with centromeres running along the length of the chromosome, that suppress centromere drive, do not show any signs of adaptive selection in their CENH3 proteins (Zedek and Bures 2016a). This evidence suggests that centromere drive may be the force behind the recurrent positive selection observed in CENH3 (Zedek and Bures 2016a, b).

CENH3 is ubiquitous in eukaryotes suggesting that it may have originated in the last common ancestor of all eukaryotes. However, because of the rapid changes and the evolutionary constrains associated with CENH3 and canonical H3, respectively, the resulting phylogeny of the H3 lineage especially in the deep branches remains unresolved (Malik and Henikoff 2003; Postberg et al. 2010). Thus, whether all extant CENH3 originated from an ancestral form common to all eukaryotes or if CENH3 arose multiple times during evolution remains an open question (Postberg et al. 2010).

7.3.1.4 H3-Like Variants

H3-like genes include a set of H3 homologs that encode proteins with degenerate amino acid sequences but without a specific pattern of changes (Fig. 7.1) (Ingouff and Berger 2010). Some H3-like genes possess amino acid substitutions similar to those of the H3.3 variants and are called H3.3-like, while others resemble H3.1 at the same positions and are considered H3.1-like. Due to the sequence degeneration some H3-like genes may correspond to pseudogenes. For instance, in Arabidopsis *HTR7* and *HTR11* are considered pseudogenes because they are unlikely to produce functional histone H3 proteins. *HTR7* encodes a protein with a deletion of a section of the N-terminal tail domain and lacks any detectable gene expression (Okada et al. 2005). *HTR11* does not possess a promoter region, instead is transcribed from the

promoter of *HTR1* as a read-through transcript. However, the presence of a cryptic intron yields a transcript with a missing portion of the coding sequence of *HTR11* producing a protein with an N-terminal deletion that is likely to be non-functional (Okada et al. 2005). The other Arabidopsis H3-like genes *HTR6*, *HTR10*, *HTR14,* and *HTR15* are expressed in at least one plant organ and are expected to produce functional proteins. These H3-like proteins, with the exception of *HTR15*, are all intron-containing genes and only *HTR6* and *HTR14* are expressed in the S-phase of the cell cycle (Table 7.1) (Okada et al. 2005). In rice five genes, *HTR701*, *HTR709*, *HTR710*, *HTR714,* and *HTR715,* are considered H3-like proteins (Hu and Lai 2015). However, *HTR710* encoded a protein with a 20 amino acid N-terminal deletion and its expression could not be confirmed in rice suggesting that it may be a pseudogene (Hu and Lai 2015). The remaining genes, on the other hand, appear to encode functional H3 proteins but none of them share a clear homology to any of the H3-like proteins from Arabidopsis (Hu and Lai 2015). Even though there is no cell cycle expression data, the presence of introns in *HTR701* and *HTR709* indicates that they may encode histone variants. The features from H3-like genes from Arabidopsis and rice suggest that they may encode additional histone H3 variants regulating chromatin structure and DNA-templated processes. The lack of apparent consistency in their amino acid substitution patterns and of phylogenetic relationships among them may indicate that they may have emerged as specialized, divergent variants after lineage-specific duplications (Fig. 7.1).

The function of most of the H3-like genes is unknown, although at least two H3-like proteins from Arabidopsis, H3.14 and H3.10, appear to function in the gametophyte. H3.14 was detected in both male and female gametophytes. In pollen H3.14 was localized in the nucleus of the vegetative cell, while in the embryo sac H3.14 was found in the central cell but not in the egg cell nucleus (Ingouff et al. 2010). H3.14 remains uncharacterized, however, it was also expressed in young seedlings suggesting that whatever is function is, it is not restricted to the gametophyte (Ingouff et al. 2010). H3.10, on the other hand, is specifically expressed in the male-germline, where it is detected first in the generative cell and later in the sperm cells of bicellular and tricellular pollen, respectively (Okada et al. 2005; Ingouff et al. 2007). H3.10 protein, accumulated in the nuclei of sperm cells, is detectable until karyogamy, but soon after it is actively evicted from the zygote by a replication-independent mechanism. As a result, the chromatin modulating activity of H3.10 on the generative and sperm cells is reset after fertilization (Ingouff et al. 2007, 2010). H3.10 may have a role in specifying a sperm cell-specific transcriptome, a hypothesis supported by the presence of H3.3-like amino acid changes, Y31 H87 L90, in its primary sequence (Ingouff and Berger 2010; Okada et al. 2005). An alternative function for H3.10 would be in organizing sperm chromatin condensation (Ingouff and Berger 2010). In lily, five H3 isoforms, gcH3, gH3, leH3, soH3-1, and soH3-2, were found to localize in the generative cell of mature pollen (Okada et al. 2006; Xu et al. 1999; Ueda et al. 2000). Not all five isoforms are specific to the male gamete since the isoforms gcH3, leH3, and soH3-2 are also expressed in the uninucleate microspore (Okada et al. 2006). However, it is likely that all the five variants are incorporated into the chromatin of the generative cell. soH3-1 and soH3-2 are

H3.3-like variants, whereas gcH3, gH3, and leH3 are more divergent (Fig. 7.1) (Okada et al. 2006). soH3-1 and soH3-2 share only a couple of conserved amino acids changes with H3.10 suggesting that they may not have shared a common ancestor even though they all are male-germline-specific H3 variants (Fig. 7.1).

Proteins with gamete-specific functions and those involved in fertilization are frequently rapidly evolving and therefore diverge very quickly (Swanson and Vacquier 2002). gcH3 and leH3 share little homology with other male-germline-specific H3 variants suggesting that the specialization in the modulation of the sperm cell chromatin may have influenced the rate of evolution of these variants and led to their rapid divergence. In contrast, gH3 shares some sequence similarity with CENH3 from other plants (Okada et al. 2006). Indeed, phylogenetic analysis show that gH3 and a putative CENH3 from *Lilium davidii* (Ld CENH3) cluster together with CENH3 from Arabidopsis and rice suggesting that it may be a centromeric variant (Fig. 7.1b). Furthermore, CENH3 has been shown to localize in the generative and sperm cells of the male gametophyte in Arabidopsis (Ingouff et al. 2007). Nonetheless, gH3 is distributed throughout the generative cell nuclei which is in contrast to the centromere-associated dot-like distribution of CENH3 (Ingouff et al. 2007). Thus, the similarity between CENH3 variants and lily gH3 suggest that these proteins may be related. However, only further research will determine whether gH3 is the only CENH3 variant in lily or if it is one CENH3 isoform that was coopted for male-gamete-specific functions.

7.3.2 Histone H2A

Histone H2A (HTA) is the only core histone whose members possess N- and C-terminal tail domains (Malik and Henikoff 2003). Similar to H3, several variants have evolved in the plant H2A family, canonical H2A, H2A.Z, H2A.X, and H2A.W. Canonical H2A, H2A.Z, and H2A.X are variants present in the majority of eukaryotic groups, whereas the H2A.W variant is found exclusively in angiosperms (Malik and Henikoff 2003; Kawashima et al. 2015; Jiang and Berger 2017). In addition, H2A variants that are lineage-specific may have emerged during plant evolution. This is the case of the H2A.M variant found exclusively in the genome of non-flowering plants and the male gamete gH2A variant from lily (Kawashima et al. 2015; Xu et al. 1999; Ueda et al. 2000). The distinct H2A variants can be set apart by the amino acid composition and signature motifs present in their C-terminal tail domains (Kawashima et al. 2015). Furthermore, particular amino acid changes in the L1 loop and the docking domains are characteristic of the different H2A variants (Jiang and Berger 2017). Changes in the L1 loop and the docking domain have an impact in intra-nucleosomal interactions, thereby affecting nucleosome stability, whereas the C-terminal region may modulate chromatin accessibility (Osakabe et al. 2018). Thus, differences among histone H2A variants localized in these three regions may influence their function by leading to the formation of alternative chromatin states in the genomic regions in which these variants are deposited.

7.3.2.1 Canonical H2A

In contrast to the histone H3 family in which all the canonical H3.1 proteins are identical, four genes in Arabidopsis *HTA1*, *HTA2*, *HTA10,* and *HTA13* encode four different canonical histone H2As (Table 7.1) (Yi et al. 2006; Yelagandula et al. 2014). Similarly, the rice genome also encodes for four canonical H2As (Table 7.1) (Hu and Lai 2015). Canonical H2As from Arabidopsis and rice are very similar to each other with only a few amino acid differences between them. All of them also contain a cluster of acid amino acid residues in their C-terminal tail, a defining feature of canonical H2As (Kawashima et al. 2015). Phylogenetic analysis indicates that canonical H2As from Arabidopsis cluster together with the ones from rice, but they form separate groups suggesting that sequence variation among canonical H2As may have emerged in a lineage-specific fashion (Fig. 7.2).

The features of canonical H2A genes seem to differ from those of the canonical histones H3, H4, and H2B. For instance, only Arabidopsis *HTA2* and *HTA13* show preferential expression during S-phase of the cell cycle (Table 7.1). In agreement with their cell cycle expression, *HTA2* and *HTA13* were shown to be expressed in meristems and tissues with highly dividing cells, whereas *HTA1* and *HTA10* showed expression in several organs but not in mitotic tissues (Yi et al. 2006). Further analysis also shows that all *HTA* genes from Arabidopsis and rice contain at least one intron (Table 7.1). These observations suggest that canonical *HTA* genes share some characteristics observed in genes coding for replication-independent histone variants. Furthermore, presence of introns in the coding sequence of all histone *HTA* genes from Arabidopsis and rice indicates that this feature may be common to all flowering plants.

In Arabidopsis, canonical H2A is relatively depleted from heterochromatin and found mainly at euchromatic regions where it is distributed uniformly over gene bodies (Yelagandula et al. 2014). The pattern of H2A distribution at gene bodies resembles that of H3.1 suggesting that both may have been loaded into chromatin during replication (Yelagandula et al. 2014). However, in contrast to H3.1, enrichment of H2A over the body of actively transcribed genes also indicates that differences in the deposition dynamics of canonical histones may exist between H3 and H2A (Yelagandula et al. 2014).

7.3.2.2 H2A.Z Variants

H2A.Z is a conserved H2A variant present in the majority of eukaryotic lineages. Phylogenetic analysis clearly demonstrates that H2A.Z forms a well-supported monophyletic group that splits from canonical H2A very early during eukaryotic evolution (Malik and Henikoff 2003; Jarillo and Pineiro 2015; Kawashima et al. 2015). Early functional specialization and evolutionary conservation throughout major eukaryotic super-groups suggest that H2A.Z has essential functions that could not be provided by the canonical H2A or the other H2A variants (Malik and Henikoff 2003; Jarillo and Pineiro 2015).

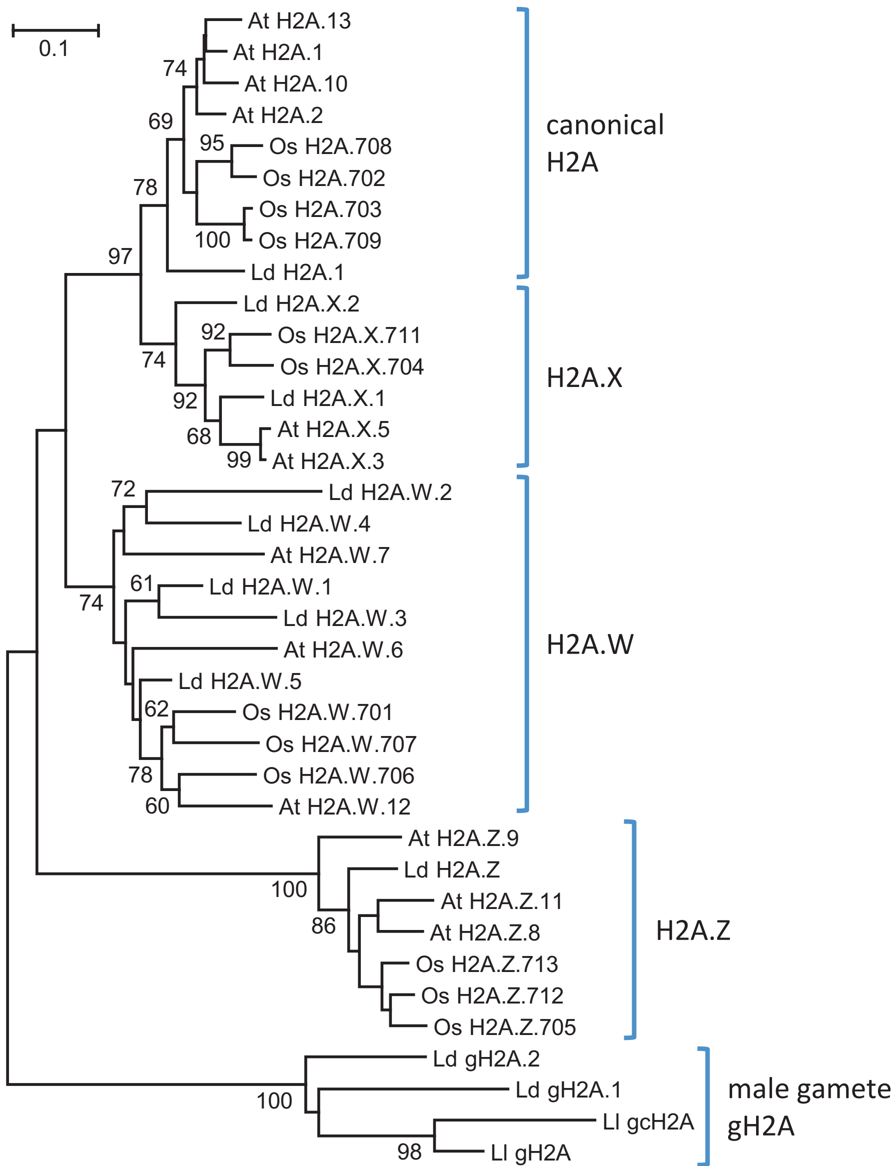

Fig. 7.2 Neighbor-joining tree showing the phylogenetic relationship among H2A proteins from Arabidopsis, rice, and lily. The different H2A subtypes are shown to the right of the tree. The phylogenetic tree was inferred from an H2A protein alignment using the neighbor-joining method with Poisson-corrected distances. Numbers on the nodes correspond to bootstrap values based on 1000 pseudoreplicates (only values higher than 60% are displayed). The scale bar indicates number of amino acid changes per site. The two initial letters in the protein names indicate the species as follows: At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Ld, *Lilium davidii*; and Ll, *Lilium longiflorum*. Accession numbers of the lily proteins used are: Ld gH2A.1, CUT18434; Ld gH2A.2, CUT18440; Ld H2A.1, CUT18443; Ld H2A.W.1, CUT18435; Ld H2A.W.2, CUT18437; Fig. 7.2 (continued) Ld H2A.W.3, CUT18444; Ld H2A.W.4, CUT18436; Ld H2A.W.5, CUT18441; Ld H2A.X.1, CUT18442; Ld H2A.X.2, CUT18439; Ld H2A.Z, CUT18438; Ll gcH2A, Q9XG56.1; and Ll gH2A, Q9LD75.1. Locus id of the H2A proteins corresponding to Arabidopsis and rice is given in Table 7.1. Proteins encoded by Arabidopsis *HTA4* and rice *HTA714*, suspected to be pseudogenes, were not included in the analysis

At the primary structure, plant H2A.Zs are characterized by having a short C-terminal tail domain containing a KD/E motif and a L1 loop that is less conserved with the other H2A variants (Kawashima et al. 2015; Malik and Henikoff 2003). Using these features and sequence conservation, H2A.Z from several plants could be unambiguously identified. For instance, the Arabidopsis and rice genomes were found to contain each three H2A.Z genes, *HTA8*, *HTA9,* and *HTA11*, and *HTA705*, *HTA712,* and *HTA713*, respectively (Table 7.1) (Yelagandula et al. 2014; Yi et al. 2006; Hu and Lai 2015). Phylogenetic analysis shows that even though Arabidopsis and rice H2A.Z proteins form a single clade, they do not group according to species suggesting that some of the duplications that gave origin to these proteins may have originated prior to their divergence (Fig. 7.2). In spite of this observation, plants harboring null mutations in either *HTA8*, *HTA9,* or *HT11* are phenotypically normal, while double, *hta9-1 hta11-1*, and triple mutants, *hta8-1 hta9-1 hta11-1*, show pleiotropic phenotypes indicating these genes are functionally redundant (Coleman-Derr and Zilberman 2012; March-Diaz et al. 2008).

H2A.Z function is linked to the modulation of euchromatin where it has been shown to regulate transcription, maintain a transcriptionally permissive chromatin environment, and protect euchromatic regions from the spreading of heterochromatin (Jiang and Berger 2017; Malik and Henikoff 2003). Genomic distribution of H2A.Z has been studied in Arabidopsis and rice. In these organisms, H2A.Z localizes in genes at euchromatic regions, while it is excluded from highly methylated heterochromatin and the body of genes with GMB (gene body methylation) (Zhang et al. 2017b; Yelagandula et al. 2014; Coleman-Derr and Zilberman 2012; Zilberman et al. 2008). H2A.Z deposition at individual genes depends on their expression. Usually actively transcribed genes are enriched with the canonical H2A but it becomes replaced by H2A.Z in less active genes (Yelagandula et al. 2014). Furthermore, two distribution profiles of H2A.Z along genes have been observed in plant genomes. In genes with moderate to high levels of constitutive expression, H2A.Z shows a prominent peak around the transcriptional start site (TSS), a smaller peak at the 3′ end and is depleted in between (Zhang et al. 2017b; Yelagandula et al. 2014; Coleman-Derr and Zilberman 2012; Zilberman et al. 2008). In contrast, H2A.Z throughout the gene body was found in genes with lower expression but with high responsiveness, a measure of differential expression among tissue types or environmental conditions (Zhang et al. 2017b; Yelagandula et al. 2014; Coleman-Derr and Zilberman 2012; Zilberman et al. 2008). For the latter case, there is a negative correlation between H2A.Z deposition at gene bodies and transcriptional activity (Zhang et al. 2017b; Yelagandula et al. 2014; Coleman-Derr and Zilberman 2012; Zilberman et al. 2008). Conversely, enrichment of H2A.Z close to the TSS appears to be associated with high levels of expression when present at moderate levels, but with lower levels of gene expression when the H2A.Z levels are higher or lower (Coleman-Derr and Zilberman 2012; Zilberman et al. 2008). Consequently, the deposition of H2A.Z at gene bodies will have a repressive effect in the expression of responsive genes, while H2A.Z loading at the TSS may have a broad function involving both housekeeping and responsive genes. In agreement with these observations, loss of H2A.Z, by depletion of H2A.Z proteins or mutations in the

subunits of the SWR1-like complex involved in H2A.Z loading, at responsive genes involved in phosphate deprivation (Arabidopsis and rice), systemic acquired resistance (Arabidopsis), osmotic stress (Arabidopsis), and response to warm temperatures (Arabidopsis and Brachypodium) led to a constitutive activation of these response pathways (Smith et al. 2010; Zahraeifard et al. 2018; Kumar and Wigge 2010; Boden et al. 2013; March-Diaz et al. 2008; Sura et al. 2017). In other instances, reduced transcriptional activity was found associated with disruption of H2A.Z at the TSS. For instance, in Arabidopsis depletion of H2A.Z at the *FLOWERING LOCUS C* (*FLC*) led to reduced expression of this floral repressor and to early flowering (Deal et al. 2007). In the same way, H2A.Z is required to maintain the expression levels of metabolic gene clusters (Arabidopsis), the microRNA genes *MIR156A* and *MIR156C* (Arabidopsis), and the genes *CPS1* (*Ent-copalyl diphosphate synthase 1*) and *GA3ox2* (*Gibberellin 3-oxidase 2*) involved in the gibberellin biosynthesis (rice) (Yu et al. 2016; Li et al. 2018a; Xu et al. 2018). H2A.Z has also been implicated in many biological other processes, including ethylene response (Arabidopsis) (Hu et al. 2011), meiotic crossover (Arabidopsis) (Choi et al. 2013), female meiosis (Arabidopsis) (Qin et al. 2014), response to day–night cycle regulation (rice) (Zhang et al. 2017a), anthocyanin biosynthesis (Arabidopsis) (Cai et al. 2018), and temperature control of pod shattering (Brassicaceae) (Li et al. 2018b). In all these cases, the increased or decreased gene activity observed as a result of H2A.Z deposition will depend on the gene targeted and whether H2A.Z is loaded in the gene body or the TSS.

The mechanisms of transcriptional modulation by H2A.Z are not completely understood. The most prevalent hypothesis is that H2A.Z deposition changes intra- and inter-nucleosomal interactions, thereby affecting chromatin accessibility. In vitro analysis in Arabidopsis H2A.Z suggests that nucleosomes containing H2A.Z are more unstable than those containing canonical H2A or the other variants (Osakabe et al. 2018). In vivo analysis shows that genes whose transcriptional activity depends on H2A.Z such as *MIR156*, *MIR164,* and *FLC* showed decreased nucleosome occupancy, tied to greater DNA accessibility, in the presence of H2A.Z (Choi et al. 2016). On the other hand, in responsive genes deposition of H2A.Z results in increased nucleosome occupancy at nucleosome +1 (relative to the TSS) and likely impaired access of the transcriptional machinery (Dai et al. 2017). Together with changes in nucleosome occupancy, presence of H2A.Z has also been demonstrated to impact posttranslational modifications in other histones. For instance, PHOTOPERIOD INDEPENDENT EARLY FLOWERING1 (PIE1), a subunit of the plant SWR1-like complex necessary for H2A.Z deposition together with PICKLE (PKL) and the histone methyltransferase CURLY LEAF (CLF) is part of a pathway necessary to promote H3K27me3 at a common set of repressed genes (Carter et al. 2018). Furthermore, H2A.Z stimulates the expression of *MIR156A* and *MIR156C* by facilitating the trimethylation of H3K4, but without necessarily changing nucleosome occupancy as in the case of *MIR156C* (Xu et al. 2018). These observations suggest that H2A.Z deposition leading to transcriptional activity or gene repression may be the result of different mechanisms acting to modulate chromatin accessibility. However, how H2A.Z deposition results in the

recruitment of repressive or promoting mechanisms of gene expression and whether these mechanisms cooperate to regulate similar functional outputs, or they act in a locus-specific fashion remains to be investigated.

7.3.2.3 H2A.X Variants

H2A.X is the variant most closely related to the canonical H2A (Fig. 7.2). Nonetheless, H2A.X can be differentiated from canonical H2A by the presence of a C-terminal motif, SQ(E/D)Φ, where Φ indicates a hydrophobic residue (Malik and Henikoff 2003). In all plant H2A.X, this motif is given by the sequence SQEF (Jiang and Berger 2017; Kawashima et al. 2015). H2A.X is a variant widely distributed among eukaryotic groups. However, phylogenetic analysis shows that in contrast to H2A.Z, H2A.X has arisen multiple times from canonical H2A during eukaryotic evolution (Malik and Henikoff 2003). H2A.X also evolved from canonical H2A in plants. H2A.X variants harboring the characteristic SQEF motif could be found throughout the group archaeplastidae. Nevertheless, a clear separation between canonical H2A and H2A.X could not be observed until early in the evolution of vascular plants (Kawashima et al. 2015). These observations suggest that even though a H2A.X variant emerged from canonical H2A as early as in unicellular green algae, additional diversification has occurred during land plant evolution (Kawashima et al. 2015).

In flowering plants such as Arabidopsis and rice H2A.X is encoded by two genes in each genome, *HTA3* and *HTA5*, and *HTA704* and *HTA711*, respectively (Table 7.1). In Arabidopsis, *HTA3* and *HTA5* are expressed in most organs tested suggesting that their expression is constitutive and that both genes are likely functionally redundant (Lang et al. 2012). H2A.X from Arabidopsis and rice group together according to species indicating these isoforms, which may be still under functional diversification, may have evolved after lineage-specific duplications (Fig. 7.2). The same seems to be true for the genome of other land plants in which the H2A.X paralogs from the same species tend to cluster together (Kawashima et al. 2015). However, this pattern may not be universal in the plant kingdom since H2A.X isoforms from *Lilium davidii* fall into separated clades in the phylogenetic tree (Fig. 7.2).

H2A.X has a conserved function in DNA repair in animals and plants (Kawashima et al. 2015; Malik and Henikoff 2003). In the H2A.X of these eukaryotes, the serine at the SQ(E/D)Φ motif is phosphorylated in the nucleosomes surrounding DNA double-strand breaks (DSB) (Friesner et al. 2005; Rogakou et al. 1998). The presence of nucleosomes containing phosphorylated H2A.X around DSBs is necessary for proper DNA damage repair and recruitment of repair proteins (Dona and Mittelsten Scheid 2015; Malik and Henikoff 2003). In agreement with H2A.X function in DNA repair, single mutants in Arabidopsis *HTA3* and *HTA5* are more sensitive to DNA damaging agents than the wild-type (Lorkovic et al. 2017). In addition, a double mutant in *HTA3* and *HTA5* displayed even higher sensitivity suggesting that both H2A.X isoforms are at least partially functionally redundant in DNA

damage repair (Lorkovic et al. 2017). Simultaneous downregulation of *HTA3* and *HTA5* also resulted in increased sensitivity to DNA damaging agents, although not as pronounced than the observed with the knock-out mutants (Lang et al. 2012). Thus, disruption of H2A.X led to the generation of plants with reduced ability to repair DNA damage suggesting a conserved function of H2A.X in DNA repair (Lorkovic et al. 2017; Lang et al. 2012). H2A.X isoforms were shown to interact with the E2Fa transcriptional activator indicating that both proteins may cooperate in the response to DNA damage in plants (Lang et al. 2012).

7.3.2.4 H2A.W and H2A.M Variants

H2A.W is a variant characterized by having a SPKK motif that resides in their C-terminal tail domain, which is the longest of all the variants (Kawashima et al. 2015; Yelagandula et al. 2014). H2A.W is a plant-lineage-specific variant that may have arisen early in the evolution of spermatophytes or seed plants (Kawashima et al. 2015). This observation is supported by the lack of homologs of H2A.W in unicellular green algae, liverworts, mosses, and lycophytes and the presence of distinctive H2A.W proteins in early spermatophytes from the genus *Ginkgo*, *Cycas,* and *Gnetum* (Kawashima et al. 2015). Instead of H2A.W homologs, Kawashima et al. (2015) identified a novel group of H2A variants, they named H2A.M, in the genomes of liverworts, mosses, and lycophytes. H2A.M variants are characterized by having a long C-terminal tail domain, rich in lysine, serine and acidic residues, not present in the other H2A variants (Kawashima et al. 2015). However, certain similarities between the C-terminal tail and the L1-loop of H2A.M and H2A.W variants and the clustering of H2A.M and H2A.W proteins in the phylogeny of H2A suggest that these two variants are related (Kawashima et al. 2015). Whether H2A.M and H2A.W emerged from the same ancestor or H2A.M in early basal land plants evolved gradually to become H2A.W in seed plants remains undetermined (Kawashima et al. 2015).

Arabidopsis possesses three H2A.W genes, *HTA6*, *HTA7,* and *HTA12,* while the rice genome has three isoforms encoded from four genes, *HTA701*, *HTA706*, *HTA707,* and *HTA710* (Table 7.1). All the Arabidopsis H2A.W isoforms possess a single SPKK motif in their C-terminal tail domains (Yi et al. 2006). In contrast, in rice all H2A.W isoforms, with the exception of H2A.W.707, have a duplicated SPKK motif (Hu and Lai 2015). Presence of a single copy of the SPKK motif seems to be true for all eudicots, whereas the monocots have been shown to possess 2 or even 3 copies of the same motif (Kawashima et al. 2015). Parallel phylogenetic analysis, one using Arabidopsis and rice, another using several plant species, and the one shown in Fig. 7.2, suggests that some of the H2A.W isoforms may have originated early in the evolution of this variant (Hu and Lai 2015; Kawashima et al. 2015). However, lineage-specific duplications that may have contributed to the expansion of H2A.W are also observed in some genomes of flowering plants (Kawashima et al. 2015).

The H2A.W variant functions primarily at heterochromatic regions. Genome-wide analysis of the distribution of Arabidopsis H2A.W.6 indicates that this H2A variant is excluded from gene bodies and is predominantly enriched at pericentromeric heterochromatin, transposable elements (TEs), and H3K9me2-rich regions (Yelagandula et al. 2014). In the same way, H2A.W.7 and H2A.W.12 were shown to localize to the heterochromatic chromocenters in Arabidopsis nuclei suggesting that all H2A.W variants are strongly associated with heterochromatin (Yelagandula et al. 2014). Localization of H2A.W in heterochromatic regions does not depend on H3K9me2 or DNA methylation, but it is reduced by a loss of a subunit of the CHROMATIN ASSEMBLY FACTOR 1 (CAF-1) complex, a H3–H4 chaperone responsible for chromatin assembly after DNA replication (Yelagandula et al. 2014; Benoit et al. 2018). Although CAF-1 may not be directly responsible for deposition of H2A.W, this observation still gives a strong indication that H2A.W is loaded into heterochromatin during replication (Benoit et al. 2018). Supporting this idea *HTA6* and *HTA7* were found to have S-phase specific expression during the cell cycle (Table 7.1). S-phase dependent deposition implies that H2A.W variants may be loaded at heterochromatic regions instead of canonical H2A during replication rather than the typical manner in which histone variants replace canonical histones after replication-coupled chromatin assembly is completed.

Single mutations in any of the H2A.W isoforms in Arabidopsis did not display any phenotype. Double mutants, *hta6 hta7* and *hta6 hta12*, and triple mutants, on the other hand, resulted in growth defects that were even more severe in the triple mutant (Yelagandula et al. 2014). These mutant analyses suggest that in spite of the early divergence of some isoforms, the three H2A.W paralogs in Arabidopsis are functionally redundant. Interestingly, analysis of nuclei in the double and triple mutants showed that loss of H2A.W led to dispersion of heterochromatic regions marked by H3K9me2 suggesting that H2A.W is necessary for heterochromatin condensation (Yelagandula et al. 2014). Even with such a marked effect on heterochromatin, loss of H2A.W did not result in a significant defect in the silencing of TEs but rather in an increase in CHG (where H is A, C, or T) methylation at these elements (Yelagandula et al. 2014). Increased CHG methylation may be a response to ensure the silencing of TEs in the face of a compromised heterochromatic structure caused by depletion of H2A.W (Yelagandula et al. 2014). Thus, the two pathways, H2A.W and CHG methylation, seem to act redundantly ensuring heterochromatic silencing in Arabidopsis.

H2A.W may facilitate heterochromatin formation by promoting long-range interactions between nucleosomes, an activity that requires the long C-terminal tail domain containing a SPKK motif (Yelagandula et al. 2014). Interestingly, motifs resembling SPKK are also present in other histones promoting chromatin condensation such as the linker histone H1, the sea urchin testis-specific H2B, and the macroH2A variant, raising the possibility that a common mechanism is used to regulate heterochromatin formation in all eukaryotes (Jiang and Berger 2017; Yelagandula et al. 2014).

Heterochromatic localization of H2A.W allows for novel specialized functions to arise among isoforms. This is the case for H2A.W.7 a variant involved in DNA

damage response at heterochromatic regions (Lorkovic et al. 2017). H2A.W.7 differs from the other two Arabidopsis H2A.W isoforms because it contains a SQ motif at its C-terminal tail domain (Lorkovic et al. 2017). The serine of this SQ motif is phosphorylated by the ATAXIA-TELANGIECTASIA MUTATED (ATM) kinase upon DNA damage induced by ionizing radiation and genotoxic agents such as bleomycin (Lorkovic et al. 2017; Roitinger et al. 2015). In addition, the loss-of-function mutant of H2A.W.7 is sensitive to genotoxic agents causing DSBs indicating that this isoform participates in the response to DNA damage (Lorkovic et al. 2017). Remarkably similar observations, including phosphorylation by ATM, were made for H2A.X variants, which also participate in DNA damage repair, suggesting that both histones have equivalent roles in this pathway. However, because of their localization, the role of H2A.W.7 in DNA damage repair is restricted to heterochromatin, while H2A.X function is confined to euchromatic regions (Lorkovic et al. 2017).

In spite of the similarity between H2A.W proteins, only H2A.W.7 contains the SQ motif and is involved in DNA damage response indicating functional diversification among Arabidopsis H2A.W variants. H2A.W.7-like variants involved in DNA repair may have emerged early in H2A.W evolution since isoforms with an SQ motif could be found in the genomes of some angiosperms and gymnosperms (Lorkovic et al. 2017). However, not all species of seed plants, especially those from grasses, encode H2A.W variants with an SQ motif (Lorkovic and Berger 2017; Lorkovic et al. 2017). Loss of H2A.W.7-like variants may be triggered, among other factors, by changes in heterochromatin organization that occur during plant evolution (Lorkovic et al. 2017). In these cases H2A.X or other core histone variants may substitute for H2A.W.7-like isoforms in DNA damage response at heterochromatin (Lorkovic and Berger 2017). How changes in heterochromatin organization may affect the selective pressure over the different H2A.W proteins so that novel functions may arise, or some isoforms may become dispensable, awaits further exploration.

7.3.2.5 gH2A Variants

The H2A isoform, gH2A, is a male-gamete-specific variant found in the genus *Lilium* (Ueda et al. 2000; Yang et al. 2016; Xu et al. 1999). gH2A and its paralog gcH2A are exclusively expressed in the generative cell of bicellular pollen in lily where they may assist during the chromatin reprograming of the male germline (Ueda et al. 2000; Xu et al. 1999; Ueda et al. 2005). gH2A and gcH2A are divergent variants that share ~50 and ~60% similarity with other H2A variants, respectively, but mostly restricted to the HFD (Ueda et al. 2000; Xu et al. 1999). Due to the small size of their C-terminal tail domain, gH2A isoforms are few amino acids smaller than any of the other H2A variants (Ueda et al. 2000; Xu et al. 1999). In addition, gH2A variants do not share any of the motifs that characterize the C-terminal tail domain of other H2As (Ueda et al. 2000; Xu et al. 1999) suggesting they are different from them. Indeed, phylogenetic analysis shows that gH2As cluster together

forming its own group (Fig. 7.2). Homology searches in monocots and other plant species did not yield any results suggesting that gH2A isoforms are restricted to the genus *Lilium*. As observed for many reproductive proteins, the function of gH2A in the male germline may have caused it to evolve fast and diverge quickly from other H2As. Still, this appears to have happened in the lily-specific lineage because no divergent H2A variants have been reported in other plants. Thus, gH2A may be considered a distinctive variant among the others in the H2A family that evolved specifically in *Lilium*.

7.3.3 Histone H2B Family

The histone H2B (HTB) family shows the least conservation of all core histones likely because it is less evolutionarily constrained than its counterparts (Chaboute et al. 1993; Malik and Henikoff 2003). In plants, the N-terminal tail domain is the most variable portion, whereas the C-terminal region containing the HFD is highly conserved (Bergmuller et al. 2007; Chaboute et al. 1993). In Arabidopsis, 11 *HTB* genes (*HTB1* through *HTB11*) encoding the same number of proteins were identified (Table 7.1). From the encoded proteins 10 showed an amino acid similarity higher than 80%, whereas one protein, H2B.8, was only 44% similar to the other H2Bs (Bergmuller et al. 2007). Similar to Arabidopsis, the rice genome was found to encode 12 histone *HTB* genes (*HTB701* and *HTB703–HTB713*), all of which are fairly well conserved with the exception of *HTA713* which is more divergent (Hu and Lai 2015). In a phylogenetic tree most of the plant H2B isoforms cluster together into a single group suggesting a monophyletic origin of the plant H2Bs (Malik and Henikoff 2003). Further analysis from Arabidopsis, rice, and *Lilium* indicates that most of the H2B isoforms tend to form clades according to species (Fig. 7.3). These observations suggest that the different H2B isoforms found were originated after lineage-specific duplications in these species. Supporting this idea, the N-terminal tails of H2B proteins were observed to be more conserved among isoforms within species that between Arabidopsis and rice (Hu and Lai 2015).

Arabidopsis *HTB* genes are transcribed from highly dividing tissues such as the shoot apex, while the expression of others was restricted to flowers and seeds (Bergmuller et al. 2007). From these *HTB9*, *HTB2*, *HTB11*, *HTB4*, and *HTB1* were the most highly expressed in cell suspension cultures and also their encoded proteins detected by mass spectrometry (Bergmuller et al. 2007). Interestingly, *HTB9*, *HTB2*, *HTB11,* and *HTB4* are all expressed specifically during the S-phase of the cell cycle (Menges et al. 2003) suggesting that they all encode canonical H2B proteins.

In contrast to H3 and H2A, H2B is less specialized with only a few variants described for this histone in all eukaryotes (Malik and Henikoff 2003). Three mammalian proteins, the testis-specific variants, TSH2B.1 and SubH2Bv, and the H2BE in olfactory neurons are the only H2B variants described so far (Santoro and Dulac 2012; Aul and Oko 2002; Montellier et al. 2013). Similarly, in plants only a single

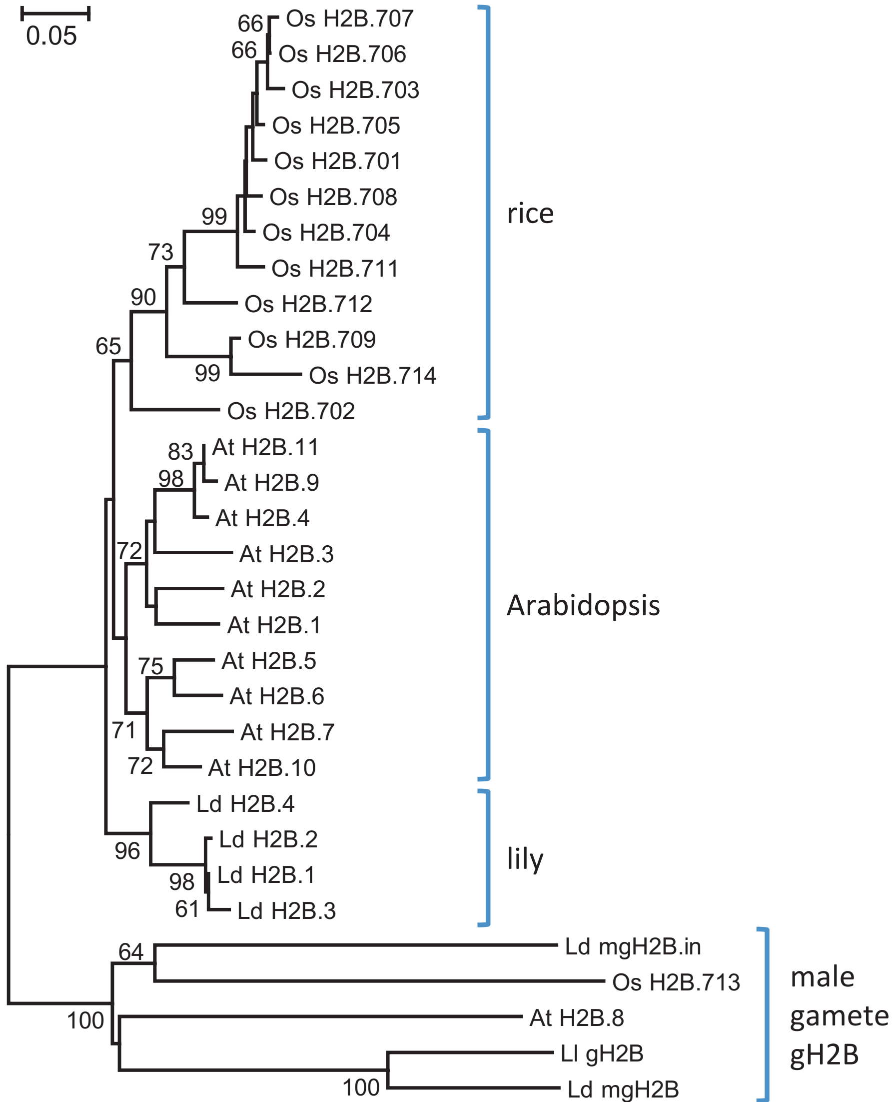

Fig. 7.3 Phylogenetic tree of histone H2B proteins from Arabidopsis, rice, and lily. The subgroups that could be distinguished in the tree are indicated to the right. For the phylogenetic analysis, H2B proteins were aligned with the ClustalX program (Larkin et al. 2007). This alignment was then used to construct a neighbor-joining tree with Poisson-corrected distances using MEGA 7.0 (Kumar et al. 2016). Numbers on the branches indicate bootstrap values, based on 1000 pseudoreplicates, higher than 60%. The scale bar refers to the number of amino acid substitution per site. The proteins used to construct the tree correspond to the following species: At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Ld, *Lilium davidii*; and Ll, *Lilium longiflorum*. Accession numbers of the lily proteins used are: Ld H2B.1, CUT18447; Ld H2B.2, CUT18451; Ld H2B.3, CUT18448; Ld H2B.4, CUT18446; Ld mgH2B, CUT18449; Ld mgH2B.in, CUT18450; and Ll gH2B, BAA96095. Locus id of Arabidopsis and rice H2B proteins used to draw the tree is given in Table 7.1. The proteins from the rice *HTB* genes, *HTB715* and *HTB710*, were not considered

male-gamete-specific H2B variant, gH2B, was described in the genus *Lilium* (Ueda et al. 2000; Yang et al. 2016). gH2B is highly divergent from the canonical H2B and is expressed in the generative cell of the bicellular pollen where it may be necessary for chromatin remodeling of the male germline (Ueda et al. 2000; Yang et al. 2016). As with other gamete-specific variants, divergence of gH2B may be caused by the fast-evolutionary rates characteristic of proteins involved in reproduction. Curiously, Arabidopsis and rice encode each a single H2B isoform (H2B.8 and H2B.713, respectively) that displayed a lower degree of similarity with all the other H2B proteins. Even though the divergence between these proteins makes alignment and phylogeny inference difficult, H2B.8 and H2B.713 still cluster together and with the lily gH2B variant (Fig. 7.3). Additionally, the highly divergent Arabidopsis H2B.8 variant is specifically expressed in pollen grains but not in sporophytic tissues (Hoffmann and Palmgren 2013). Thus, it is likely that these highly divergent H2B variants represent a group of male-gamete-specific H2B variants.

7.3.4 Histone H4 Family

The proteins of the histone H4 (HFO) family, together with H3, are among the most conserved proteins in eukaryotes, likely due to their intolerance to amino acids changes resulting from the structurally constrained position of H3 and H4 in the nucleosome (Malik and Henikoff 2003). In contrast to H3 in which several variants have arisen during evolution, virtually no H4 variants have been described (Malik and Henikoff 2003). Similar to other eukaryotes, the histone H4 from plants is also very well conserved, only two substitutions, I60V and R77K, differentiate the histone H4 from plants to that of calf thymus (Chaboute et al. 1993). H4 genes in plants are present in several copies encoding identical proteins. The genome of Arabidopsis, for instance, contains 8 genes (*HF01–HFO8*) that encode for a single histone H4 isoform (Table 7.1). The rice and sugarcane genomes were also shown to harbor 10 and 21 genes encoding a single H4 isoform identical to that of Arabidopsis (Moraes et al. 2015; Hu and Lai 2015). However, in spite of the extremely high conservation a few H4 isoforms have been observed in some plant genomes. H4 variants with a single amino acid replacement, G4D, I60V, and Y72C, were identified in wheat, soybean, and sugarcane, respectively (Moraes et al. 2015; Tabata and Iwabuchi 1984; Wu et al. 2009). Interestingly, the amino acid replacements I60V and Y72C are located in the α-helix 2 of the HFD, the only H4 region shown to accept some amino acid substitutions (Fig. 7.4) (Malik and Henikoff 2003). The H4 variants from wheat and sugarcane were shown to be expressed, while the soybean H4 variant was detected by mass spectrometry. These observations suggest that these are in fact functional variants and not pseudogenes. A more divergent H4 variant, especially at the N-terminal tail domain, was detected in the rice genome (Fig. 7.4) (Hu and Lai 2015). This variant is expressed at relative low levels but is upregulated upon light stimulus (Hu and Lai 2015). Yet, the most compelling evidence for a H4 variant was provided by Yang et al. (2016) who detected a somewhat divergent H4,

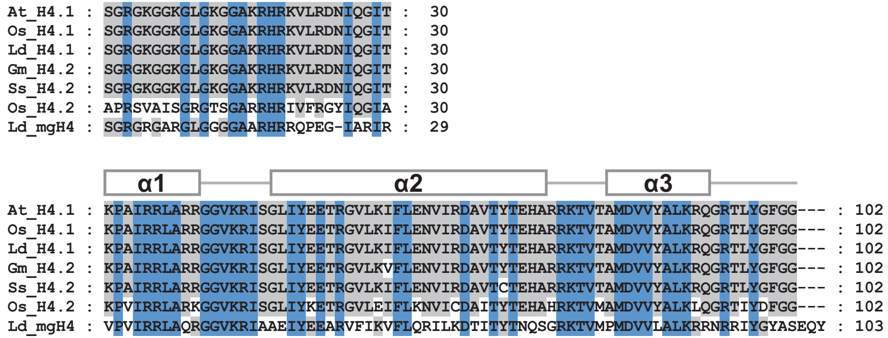

Fig 7.4 Amino acid sequence alignment of canonical and Histone H4 variants from several plants species. The upper part of the alignment corresponds to the C-terminal tail domain and the lower part to the histone fold domain (HFD). The three α-helices (α1, α2, and α3) comprising the HFD are indicated on the top of the HFD alignment. The degree of conservation is distinguished at three levels (100%, 70%, and not conserved). A two-letter abbreviation indicating the species precedes the name of each protein: At, *Arabidopsis thaliana*; Gm, *Glycine max*; Ld, *Lilium davidii*; Os, *Oryza sativa*; and Ss, *Saccharum sp*. Accession numbers of proteins used are as follows: Ld_H4.1, CUT18458; Ld_mgH4, CUT18459; and Ss_H4.2, CA145162. Locus id for At_H4.1, Os_H4.1, and Os_H4.2 is given in Table 7.1. Gm_H4.2, identified by Wu et al. (2009) by mass spectrometry, does not have an accession number

mgH4, in the proteome of generative and sperm cells of the lily pollen. mgH4, similar to gH2A, gH2B, and gH3, likely constitutes a male-germline-specific histone variant associated with establishment of a chromatin structure unique to the male germline. Thus, in spite of its evolutionary constrains some H4 variants appear to have emerged in plants. However, with the exception of one, all H4 variants were found in monocot genomes indicating that these variants may have appeared later in plant evolution or that there are some particularities in the chromatin organization of monocots that allow for H4 variants to arise. Furthermore, the lack of homology among H4 variants and their scattered presence in unrelated species suggest that they evolved mostly as species-specific variants.

7.4 Future Remarks

Histones are not only passive structural components of the chromatin but they are also important regulatory elements of nuclear processes that use DNA as a template. Chromatin can be modulated either by PTMs or by replacing histones with specialized variants, or both, resulting in a chromatin environment that will facilitate or negate access of enzymatic complexes to their target genomic regions. In plants, histones and their variants have been involved in controlling many aspects of the plant life cycle including development, flowering time, responses to abiotic stresses, specification of the germline, among others. Many of these processes are of

agronomical importance making histones attractive targets for plant breeding and crop biotechnology programs. Indeed, there have been two instances in which histones and their variants have shown potential applications in crop biotechnology.

The first of these technologies makes use of the histone variant CENH3 for the production of haploid plants. Chromosomes of plants harboring a CENH3 protein with compromised activity are not able to compete with wild-type chromosomes for the same spindle and are lost during mitosis leading to the generation of haploid cells (Ravi and Chan 2010). Based on this observation Ravi and Chan (2010) developed a haploid inducer (HI) strain in Arabidopsis that consisted of a *cenh3-1* null-mutant complemented with a partially functional *CENH3* transgene (GFP-tailswap). When the HI strain is crossed with a wild-type line the chromosomes of the parent containing the transgenic CENH3 protein are eliminated, while the wild-type is retained, resulting in aneuploid or haploid progeny. A similar strategy was used for haploid induction in maize demonstrating this technology may potentially be applied to crop plants (Kelliher et al. 2016). More recently it was discovered that Arabidopsis plants harboring single amino acid substitutions at CENH3 may also act as HI lines (Karimi-Ashtiyani et al. 2015; Kuppu et al. 2015). This last observation opens the possibility of using chemical mutagenesis or gene editing to create HI lines in different crop plants without the use of transgenes. Because haploid lines can be converted to diploids leading to 100% homozygosity, the application of CENH3-based technology will save a significant amount of time in the development of novel varieties in plant breeding programs.

Disruption of the *HTA1* gene in the Arabidopsis *rat5* mutant (resistant to transformation by *Agrobacterium tumefaciens*) resulted in resistance to Agrobacterium-mediated root transformation, whereas *HTA1* overexpression led to increased transformation efficiency of wild-type plants (Mysore et al. 2000). Overexpression of other *HTA*, some *HFO* and *HTR11* genes also caused increased efficiency in both Agrobacterium-mediated transformation and transient transgene expression (Tenea et al. 2009). Increased expression of histones has a protecting effect on the foreign DNA introduced into the cell which may allow higher expression of the DNA and improved recovery of transformed cells (Tenea et al. 2009). Although the exact mechanism by which overexpression of some histones, and others do not, has this enhancing effect on plant transformation remains poorly understood, this discovery has practical implications as the overexpression of histone genes could be used to increase transformation rates in recalcitrant plants. In support of this idea, overexpression of Arabidopsis *HTA1*, both stable and transient, was shown to improve Agrobacterium-mediated transformation frequency in rice (Zheng et al. 2009). Thus, transformation of many plant crops or recalcitrant phenotypes may be greatly improved by transforming genes of interest together with selected histones genes.

We are still far from completely understanding how the interplay between canonical histones and their variants led to the generation of specialized chromatin domains that contribute to the execution of the gene programs encoded in the genome. However, as we saw in some of the examples above, the understanding of the mechanisms underlying the function of histones in chromatin architecture and

regulation will be crucial in the development of novel varieties with sustained and even improved yields in the context of a changing environment.

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Chapter 8
Epigenetics of Light Signaling During Plant Development

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Abstract Light controls plant growth and development by directly impacting gene expression, physiology, and metabolism. For 20 years it has been known that light also targets epigenetic mechanisms to control different outputs. This research field is still at a relatively young stage, given its complexity and overall limitation to the plant model Arabidopsis. This chapter highlights major knowledge of the epigenetics of light signaling in Arabidopsis. Different developmental stages are discussed, including germination and early seedling development, control of the circadian clock and flowering, as well as hormone crosstalk and stress responses, and finally environmental memory. While most of the knowledge has been built up based on a laboratory plant model, studies on plants with commercial value are emerging. These studies show that some mechanisms using light signaling and epigenetic remodeling are conserved between different plant species, but other mechanisms show species specificity. The increasing availability of tools to study crops may allow the development of novel solutions for crop improvement by targeting epigenetic factors with light. This is of particular relevance in the future of agriculture, which will undoubtedly include indoor farming and the usage of artificial light.

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8.1 Introduction

Light is a broad regulator of plant growth and development. It impacts gene expression, metabolism, physiology, and morphology with tissue- and developmental stage-specific activity. Wavelengths from ultraviolet (UV) to far-red regions contribute to this regulatory control with independent, overlapping, synergistic, or antagonistic actions.

Light activity over plant growth is facilitated by photoreceptors, mediators between light perception and activation of internal light responses. It is known, although with still limited information, that different light receptors target epigenetic reprogramming, including chromatin condensation, histone modifications, and expression of noncoding RNAs (Fisher and Franklin 2011; van Zanten et al. 2012; Wang et al. 2014a; Perrella and Kaiserli 2016). Distinct light signaling pathways may also target similar epigenetic mechanisms but with light-specific effects (Guo et al. 2008). This feature may provide an additional level of plasticity to plants to allow for a more efficient response to light cues and changing light quality conditions. In addition, epigenetic remodeling provides a tool for the integration of light with additional environmental cues and internal signals, which is key for optimal plant growth and survival (Nicotra et al. 2010; Johansson et al. 2014; Legris et al. 2016; Xiao et al. 2017).

Known plant photoreceptors include the UV receptor UVR8, cryptochromes (cry), phototropins, LOV-domain sensors, and phytochromes (phy) (Folta and Carvalho 2015). Phytochromes are mostly responsive to red and far-red light, but also have roles in absorbing blue/near UV light. Cryptochromes, phototropins, and LOV-domain sensors are the main responders to blue light, while cryptochromes also interfere with green light absorption. Additional sensors, including a green light sensor, await identification.

Phytochromes play major roles during development, from the control of photosynthesis, to seed germination, flowering establishment, and shade avoidance responses. *Arabidopsis thaliana* (Arabidopsis) contains five phytochromes (phyA-E) with different sensitivities to light intensity, and distinct and overlapping activities during development. These molecules are synthesized in a biologically inactive, red-light absorbing, Pr, form. Upon red light perception Pr is converted into the biologically active, far-red light absorbing, Pfr (Pham et al. 2018). The photoreversibility of phy allows for a rapid response to fluctuating environmental conditions. An initial response upon phy activation involves the control of expression and activity of several transcription factors that then initiate specific signaling cascades controlled at the transcriptional and posttranscriptional levels. In addition, phy also mediates red and far-red light responses via epigenetic remodeling. PhyA activation, for example, is followed by an enrichment in acetylation of H3K9/14 (histone H3 lysine 9/14) and H3K27, as well as trimethylation of H3K4 at phyA-controlled transcription sites (Jang et al. 2011). Additional histone modifications have been reported, including increased H3K27me3 and decreased H3K27ac upon phyA repression. PhyB regulates nucleus size and heterochromatin condensation levels,

with obvious expected impacts on gene expression (van Zanten et al. 2010a, b; Snoek et al. 2017). This regulation may be achieved through controlled activity of the HISTONE DEACETYLASE6 (HDA6), which acts in cooperation with METHYLTRANSFERASE 1 (MET1) (Tessadori et al. 2009; To et al. 2011; Snoek et al. 2017).

Cryptochromes are additional regulators of photosynthesis and play roles during seed germination, flowering, and control of stomatal opening. Arabidopsis contains two cry, cry1 and cry2. Cry are activated upon blue/UV-A sensing through phosphorylation, which activates internal cascades involving transcriptional and post-transcriptional regulation. Cry also mediate light responses through epigenetic regulation. Similarly to phyB, cry2 regulates reversible chromatin compaction in response to low light, a function that is under control of phyB (van Zanten et al. 2010b). A target of cry2 may be a chromatin protein complex responsible for chromatin compaction, and that may include HDA6.

Knowledge on the effects of light on epigenetics during plant development is at a young stage. It has been poorly explored in other plants besides Arabidopsis. In this chapter we will focus on this model species, and on relevant examples that have connected light with plant epigenetics at different stages of plant development and during stress responses. We will go beyond the broad examples depicted in the introduction to describe known mechanisms in more detail. We will make an emphasis on the possibilities that further advancements in the field may allow extrapolation to other species, in particular to crop plants with commercial value, with possible benefits on plant quality and yield.

8.2 Plant Development

8.2.1 Seed Germination and Early Seedling Development

Seed germination and early seedling development are controlled by internal and external cues, including light and phytohormones. Light triggers seed germination, inhibits hypocotyl growth, and promotes chloroplast differentiation and initiation of photosynthesis. Two important hormones at this stage with antagonistic activities are abscisic acid (ABA) and gibberellic acid (GA) (Fig. 8.1a). ABA is a positive regulator of dormancy, whereas GA promotes plant growth and development (Koornneef et al. 2002). Light-activated phyB triggers a decrease in ABA and an increase in GA levels, resulting in the promotion of seed germination (Mazzella et al. 2005; Seo et al. 2008). PhyB-mediated changes in ABA and GA result from profound shifts at transcriptional levels in the two pathways. A transcription factor central in this process, which is degraded upon phyB light activation, is the basic helix-loop-helix PHYTOCHROME INTERACTING FACTOR 3-LIKE5 (PIL5). PIL5 is a phy-interacting protein that acts as a negative regulator of seed germination and inhibition of hypocotyl elongation (Oh et al. 2004). PIL5 activates ABA

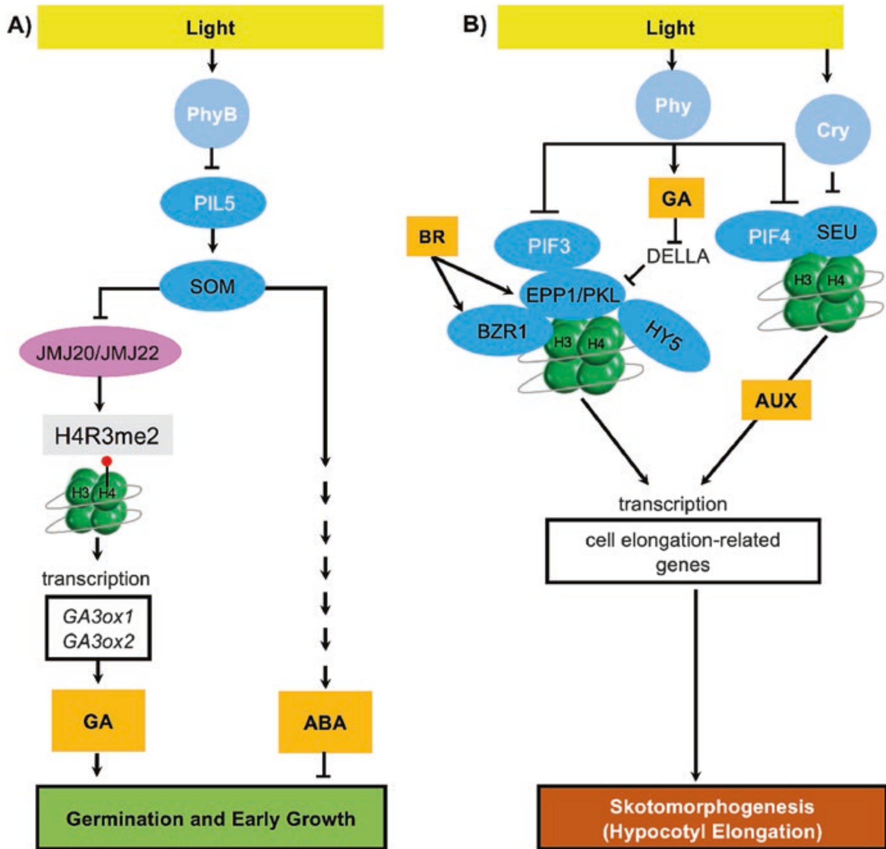

Fig. 8.1 Connecting light and hormone signaling with epigenetics during seed germination and hypocotyl growth. (**a**) PhyB is activated by light and triggers PIL5 degradation, releasing the repression of SOM on histone arginine demethylases, JMJ20 and JMJ22, which remove R3me2s on H4 in the chromatin region of GA genes (*GA3ox1* and *GA3ox2*), activating their transcription. The increase in GA promotes germination and early growth. In the absence of light, PIL5 activates ABA biosynthesis through SOM, and inhibits germination. (**b**) Light represses hypocotyl elongation by repressing PIF and other factors. In the dark, PIF3 acts together with EPP1/PKL and BZR1 to repress trimethylation of H3 of cell elongation-related genes. PIF4 interacts with SEU, blocking H3K4me3 methylation of auxin biosynthesis-related genes, interfering in this way with cell elongation. GA, gibberellic acid; ABA, abscisic acid; BR, brassinosteroids; AUX, auxins; H3-H4, Histones

biosynthesis and inhibits GA biosynthesis, partly through the positive modulation of SOMNUS (SOM), a nucleus-localized CCCH-type zinc finger protein (Fig. 8.1a) (Kim et al. 2008). SOM directly represses the histone arginine demethylases JUMONJI DOMAIN-CONTAINING PROTEIN 20 and 22 (JMJ20 and JMJ22). Upon light-induced derepression, JMJ20 and JMJ22 remove repressive symmetric histone H4 arginine 3 dimethylation (H4R3me2s) in the chromatin region of two GA metabolic genes, *GA3ox1* and *GA3ox2*, promoting an increase in bioactive GA,

and seed germination and early growth (Fig. 8.1a) (Cho et al. 2012). The regulatory effect of H4R3me2s on the expression of GA genes waits further clarification. The activity of histone arginine demethylase by Jumonji domain-containing proteins has been characterized in humans, highlighting the transferability of epigenetic studies and mechanisms between different organisms (Neff 2012).

Other hormones besides GA and ABA act on the crosstalk of light signaling and epigenetics during early plant growth. One known example is the signaling of brassinosteroids (BRs) via ENHANCED PHOTOMORPHOGENIC 1/PICKLE (EPP1/PKL), an ATP-dependent chromatin-remodeling factor of the chromodomain/helicase/DNA binding family (Fig. 8.1b). EPP1/PKL interacts with PHYTOCHROME INTERACTING FACTOR 3 (PIF3) and BRASSINAZOLE RESISTANT1 (BZR1) to promote hypocotyl growth by repressing trimethylation of H3K27 of cell elongation-related genes (Fig. 8.1b) (Zhang et al. 2008, 2012, 2014). DELLA proteins, negative regulators of the GA pathway, physically interact with PKL to repress its activity. PKL also regulates DNA methylation at *loci* targeted by RNA-directed DNA methylation (Yang et al. 2017). PIF4, in turn, interacts with the transcriptional regulator SEUSS (SEU) to regulate light, temperature, and auxin (AUX) signaling (Fig. 8.1b). Mutations in *SEU* affect H3K4me3 methylation at the AUX biosynthetic genes *INDOLE-3-ACETIC ACID INDUCIBLE 9* and *19* (*IAA9* and *IAA19*), interfering with proper cell elongation (Huai et al. 2018). Additionally, while PIF4 is part of the red/far-red light signaling pathway, SEU also responds to blue light, suggesting its extensive regulatory effect on epigenetics in response to changing light conditions.

Additional relevant examples of histone modifications include other specific trimethylation events and acetylation/deacetylation on a number of different genes and genomic regions, such as non-transposable element genes, intergenic regions, and transposable elements (Bertrand et al. 2005; Benhamed et al. 2006; Guo et al. 2008; Charron et al. 2009). For instance, the inhibition of light-induced seed germination mediated by PIF1 is partly achieved by increased levels of H3K36me3 at *PIF1* under the control of the H3K4 and H3K36 methyltransferase EARLY FLOWERING IN SHORT DAYS (EFS) (Lee et al. 2014). The HISTONE DEACETYLASE 15 (HDA15) is another interesting case study. HDA15 acts as a negative regulator of light-induced germination and photosynthesis establishment, but as a positive regulator of light repression of hypocotyl growth (Liu et al. 2013; Tang et al. 2017; Gu et al. 2017). In the dark, HDA15 interacts with PIF1 to lower histone H3 acetylation levels in genes involved in seed germination. Upon phyB activation, PIF1 is repressed and HDA15 activity on germination-related genes is dismissed. HDA15 also interacts with PIF3 to repress genes involved in chlorophyll biosynthesis and photosynthesis in dark-grown seedlings. Similarly to PIF1, PIF3 is repressed upon light exposure, ending HDA15 repressive activity, and allowing expression of target genes. In contrast, HDA15 acts as a positive regulator of light responses in the repression of hypocotyl elongation. In the hypocotyl, HDA15 interacts in a light-dependent manner with four NUCLEAR FACTOR-YC homologs (NF-YCs), NF-YC1, NF-YC3, NF-YC4, and NF-YC9, to decrease levels of histone H4 acetylation at the chromatin of positive regulators of hypocotyl elongation.

Under darkness, the NF-YC-HDAC15 complex is dismissed, increasing the levels of H4 acetylation at target genes and promoting hypocotyl growth. These three examples clearly suggest that the factors interacting with HDA15 define its biological role. Tissue- and developmental-specific interactions are therefore key to determine the output seen in terms of epigenetic reprogramming in response to light, even during this small developmental window of early plant growth.

A factor that may allow for hormone crosstalk during light responses is the Elongator complex. Elongator promotes RNA polymerase II-mediated transcript elongation through epigenetic modifications, including histone acetylation and DNA demethylation. Elongator complex was recently identified as a positive regulator of photomorphogenesis, and seems like an excellent candidate as integrator of light, hormones, and epigenetic reprogramming not only during early seedling establishment but also at various stages of plant development (Woloszynska et al. 2018a, b). Additional regulators integrate light responses and chromatin remodeling during early plant development, allowing for a massive rapid transcriptomic reprogramming, crucial for the transition to germination and photomorphogenesis. A critical challenge in research remains building detailed molecular networks that describe these regulatory mechanisms with direct targets of light signaling pathways and of chromatin-remodeling factors. While individual studies targeting specific molecules provide useful information, additional comprehensive studies are needed. Large-scale approaches may be a part of the solution, as seen with recent results obtained from RNA-sequencing, small RNA-sequencing, DNA methylation-sequencing (Methyl-seq or bisulfite sequencing), histone monoubiquitination profiling, and studies of nuclear architecture organization (Bourbousse et al. 2012, 2015; Narsai et al. 2017; Kawakatsu et al. 2017). Thousands of *loci* show dynamic changes at the epigenomic, transcriptomic, and alternative splicing pattern levels during the transition from a dormant seed stage to an active vegetative growth stage under the presence of light. Abundance of various small RNAs (sRNAs) populations, including microRNAs (miRNAs) and small interfering RNAs (siRNAs), correlates with this developmental transition. Modifications in specific siRNAs associate, for instance, with an extensive DNA demethylation towards the end of seed germination/early seedling development. Active changes in miRNAs are related to the control of gene expression by inhibition of translation or degradation of target transcripts. Changes in histone monoubiquitination and heterochromatin reorganization allow for rapid control over transcription. Challenging studies will be to identify individual molecular players in these pathways, and to add to the very few that have already been pinpointed, such as CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and DE-ETIOLATED 1 (DET1). COP1 and DET1 are required to maintain a decondensed state of heterochromatin in the dark, ensuring repression of genes needed for the transition to light growth. ELONGATED HYPOCOTYL 5 (HY5) is a master regulator of light signaling and a positive regulator of the transition to photomorphogenesis that also impacts light-regulated chromatin organization. For example, HY5 acts with EPP1/PKL to repress trimethyl H3K27 at target *loci* and regulate hypocotyl cell elongation (Fig. 8.1b) (Jing et al. 2013). HY5 also regulates expression of several miRNAs that in turn control the

transcript accumulation of different target genes (Zhang et al. 2011). Furthermore, *HY5* expression is in turn partly regulated via histone acetylation (Charron et al. 2009).

During germination and early seedling establishment, light acts together with other external cues, such as water uptake (imbibition), temperature, and nutrients, to regulate plant development. Light-specific epigenetic mechanisms may therefore be difficult to discern. Nevertheless, integrated analyses between different processes that regulate gene expression are essential to acquire more information on the role of light over epigenetics during early plant growth and the establishment of photosynthesis.

8.2.2 Circadian Clock

The circadian clock is an oscillator system synchronized approximately to a 24-h period that regulates rhythmicity of different developmental processes. From cyanobacteria to plants or humans, circadian clocks ensure more robust predictions and anticipated responses to environmental conditions, which contributes to higher fitness (Bell-Pedersen et al. 2005). Synchronization in plants is controlled externally by light and temperature, and these environmental inputs connect to internal interlocked regulatory feedback loops (Harmer 2009; Cui et al. 2014). Three chief loops exist in Arabidopsis: morning, central, and evening. The morning loop is initiated with the activity of the Myb transcription factors CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which induce expression of *PSEUDO-RESPONSE REGULATOR 7* and *9* (*PRR7* and *PRR9*), and the resultant products in turn repress *CCA1* and *LHY*. The central loop uses CCA1 and LHY to repress expression of the evening-phase gene *TIMING OF CAB EXPRESSION 1 (TOC1)*. The evening loop includes TOC1 to suppress accumulation of CCA1/LHY, and the activity of GIGANTEA (GI), EARLY FLOWERING 3 and 4 (ELF3 and ELF4), and LUX ARRHYTHMO (LUX). Each loop contains regulatory elements that act at the transcriptional, posttranscriptional, and posttranslational levels. These regulatory checkpoints have been reviewed extensively, therefore we will focus on the impact of light at the epigenetic level.

Chromatin remodeling is one of the mechanisms that allows the circadian clock to be connected to light signals, including photoperiod, and light intensity and quality (Stratmann and Más 2008; Barneche et al. 2014). Altered levels of histone acetylation, methylation, phosphorylation, and ubiquitination are associated with diurnal changes in expression of core clock and other genes, although the mechanisms governing these histone modifications require further clarification (Song and Noh 2012; Himanen et al. 2012; Malapeira et al. 2012; Barneche et al. 2014; Baerenfaller et al. 2016). The regulation of circadian clock through day-length is in turn important for additional developmental responses regulated by the circadian clock, allowing their indirect connection to photoperiod.

The 24 h-rhythmic histone acetylation at the *TOC1* promoter is under the control of various factors (activators and repressors) (Perales and Más 2007; Farinas and Mas 2011). At dawn, CCA1 represses *TOC1* by binding to its promoter and preventing acetylation, while during the day this binding decreases and is counteracted by factors that induce H3 acetylation, such as REVEILLE 8/LHY-CCA1-LIKE 5 (RVE8/LCL5). This allows *TOC1* to reach a peak of expression at dusk. During the night, histone deacetylases function to promote a *TOC1* promoter hypoacetylated state. It was recently shown that TOC1 interacts with PIF3 to repress its activity as a transcription factor (Soy et al. 2016). This finding establishes an important link between the circadian clock core components and the phytochrome photosensory pathway. One factor that may be recruited by CCA1 and LHY, to negatively regulate *TOC1* and other gene targets such as *GI*, is DET1, which binds to the non-acetylated tail of histone H2B (Benvenuto et al. 2002; Lau et al. 2011). Histone acetylation also seems to be necessary for differential expression of additional clock elements, such as *CCA1*, *LHY*, *PRR7*, *PRR9*, and *LUX* (Song and Noh 2012; Hemmes et al. 2012; Malapeira et al. 2012; Wang et al. 2013).

Proper H3K4 trimethylation at *LHY*, *TOC1*, and *CCA1* is essential for their accurate expression and functioning. H3K4me3 at the promoter of these genes might be mediated by the histone methyltransferase SET DOMAIN GROUP 2/ARABIDOPSIS TRITHORAX RELATED 3 (SDG2/ATXR3). Another epigenetic marker related to the expression of these clock genes is H3K36me2. In terms of the activity of histone demethylases, a reported example includes JUMONJI DOMAIN CONTAINING 5/30 (JMJD5/JMJ30) and its regulatory role over the pace of the circadian clock (Jones et al. 2010; Lu et al. 2011). *JMJD5/JMJ30* expression has a peak at dusk and is negatively regulated by direct binding of CCA1 and LHY to its promoter. In turn, JMJD5/JMJ30 acts in a feedback loop to control *CCA1* and *LHY* expression.

In the past 10 years, several reports have contributed to increase knowledge on the mechanisms that link epigenetics to light and the circadian clock. Further studies should focus not only on transcriptional regulation but also on regulation at posttranscriptional levels. For instance, the PROTEIN ARGININE METHYL TRANSFERASE 5 (PRMT5), which performs methylation on histones and Sm spliceosomal proteins, controls alternative splicing of *PRR7* and *PRR9* (Sanchez et al. 2010). Alternative splicing of *PRR7* and *PRR9* also requires the activity of the SKI-INTERACTING PROTEIN (SKIP) splicing factor (Wang et al. 2012; Cui et al. 2017), and disruption of *SKIP* affects the clock length. This splicing factor also regulates alternative splicing of salt stress-responsive genes, highlighting the possible crosstalk of different signaling pathways under the control of light (Feng et al. 2015). Such crosstalk is in fact addressed in further detail in Sects. 8.3, 8.4, and 8.5 of this chapter.

8.2.3 Flowering

Flowering ensures proper crop yield when fruits and/or seeds are harvested, and its correct timing maximizes yield and quality. It represents a fundamental transition during plant development from a vegetative to a reproductive stage, which ultimately relies on epigenetic regulation. Similarly to the previous sections, we will focus on the impact of light on these adjustments. Flowering is particularly sensitive to light quality and quantity, especially in plants that are photoperiod sensitive. Arabidopsis is a long-day plant, which means it flowers when exposed to long days and short nights, upon reaching adequate developmental maturity. Light, together with additional environmental factors, is perceived by plants, and along with endogenous cues, activates internal pathways that initiate flowering. Floral integrators, such as the florigen FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1), trigger the transition of the meristematic vegetative apex to a meristematic floral meristem. Immediately upstream of floral integrators, additional essential players communicate environmental cues to downstream pathways. These include the transcription factors FLOWERING LOCUS C (FLC) and CONSTANS (CO) (Blümel et al. 2015).

CO is the main responder to long day inputs. It is stabilized by cry2 and phyA during late afternoons and by the circadian clock to rhythmically induce *FT* expression under long days (Fig. 8.2) (Shrestha et al. 2014). CO directly binds to the *FT* promoter and also affects histone modifiers at the *FT locus* to regulate its expression (Fig. 8.2) (Wenkel et al. 2006; Gu et al. 2013; Wang et al. 2014b). For instance, CO physically interacts with MORF RELATED GENE 1 and 2 (MRG1 and MRG2), which bind to H3K4me3 and H3K36me3 at the *FT locus* to more robustly activate its expression (Fig. 8.2) (Xu et al. 2014; Bu et al. 2014). MRG1/2 also interact with the histone H4-specific acetyltransferases HAM and HAM2 to regulate histone acetylation at the *FT* promoter and 5′ region, which results in its high expression (Fig. 8.2). Moreover, CO associates at the *FT* distal promoter with NUCLEAR FACTOR-Y (NF-Y) transcription factors (Cao et al. 2014). NF-Y subunit C counteracts levels of H3K27me3 at the *FT* chromatin by interacting with and attenuating CURLY LEAF (CLF), a histone methyltransferase part of the Polycomb Repressive Complex 2, PRC2 (Liu et al. 2018). A CO-NF-Y complex additionally recruits the H3K27 demethylase RELATIVE OF EARLY FLOWERING 6 (REF6) to the chromatin of *SOC1* to reduce repressive H3K27me3 levels and induce flowering (Fig. 8.2) (Hou et al. 2014). The circadian clock also induces *FT* independently of CO: *GI* regulates processing of the microRNA miR172, which in turns upregulates *FT* (Jung et al. 2007).

At dusk, CO affects acetylation and methylation, repressing *FT* expression in order to prevent early flowering and ensure optimal flowering time (Gu et al. 2013; Wang et al. 2014b). For example, CO recruits the AFR-HDAC complex that includes two relatives of the yeast SAP30, SAP30 FUNCTION-RELATED 1 (AFR1) and AFR2. CO seems to enable recruitment of AFR-HDAC to *FT* by making *FT* chromatin accessible to the transcription factor AGAMOUS-LIKE 18 (AGL18), which

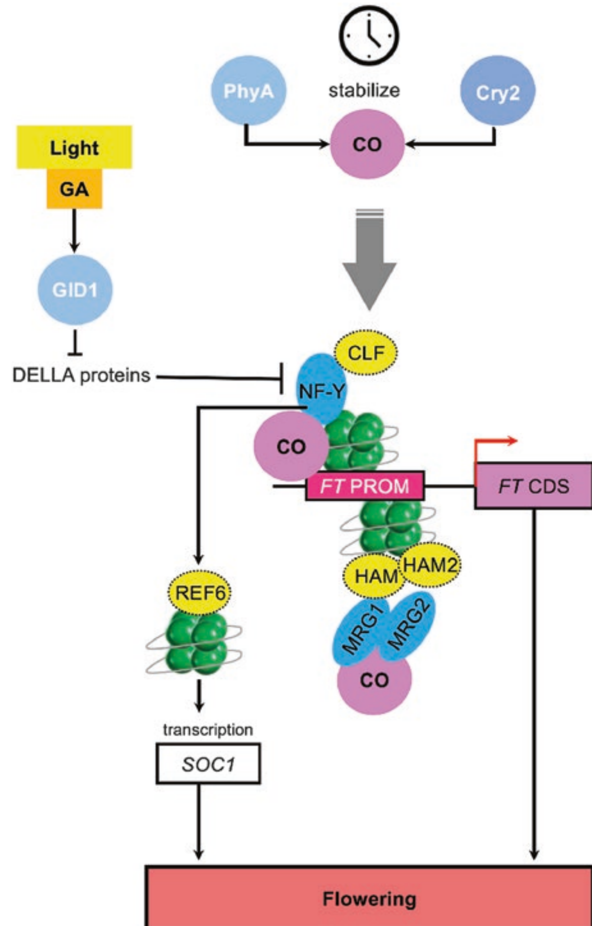

Fig. 8.2 Connecting flowering with epigenetics and the circadian clock, under long days. PhyA, cry2, and the circadian clock are involved in the stability of CO. During late afternoons, CO binds the *FT* gene promoter and affects histone modifiers at the *FT* locus to regulate its expression. In addition, the CO-NF-Y complex recruits REF6 to the chromatin of *SOC1* to induce flowering. GA-mediated degradation of DELLAs enhances NF-Y binding to the *SOC1* promoter and recruitment of REF6 to promote flowering. Histone modifiers: H4-specific acetyltransferases HAM and HAM2; histone methyltransferase CLF; and demethylase REF6. PROM, promoter; CDS, coding-sequence

then brings the complex to the *FT* chromatin (Gu et al. 2013). These examples are not exhaustive and show that distinct epigenetic mechanisms regulate *FT* in response to light. Current knowledge is not complete and further research is needed.

Both cry2 and phyB control transient chromatin compaction that regulates transition to flowering (Tessadori et al. 2007, 2009). Blue light-activated cry2 triggers a large-scale chromatin reorganization that includes decondensation of heterochromatic chromocenters and the chromatin of gene-rich regions (Tessadori et al. 2007). This process does not rely on CO, indicating that photoreceptors and CO may act together or independently on epigenetic modifications to regulate flowering in response to light. Comparing Arabidopsis accessions with different origins has allowed the identification of polymorphisms in *PHYB* and in the histone modifier *HISTONE DEACETYLASE 6* (*HDA6*) (Tessadori et al. 2009). These polymorphisms correlate with different levels of light-mediated chromatin remodeling. Chromatin

plasticity in response to light seems therefore to be associated with the plant capacity for environmental acclimation.

Under short days a CO-independent pathway ensures that plants do not transition to flowering. The histone deacetylase HDA9 maintains the transcription factor *AGAMOUS-LIKE 19* (*AGL19*) repressed by acting on H3K9 and H3K27 at this *locus* (Kim et al. 2013). AGL19 is a well-known inducer of the prolonged cold exposure pathway (vernalization) that also promotes flowering in Arabidopsis. The histone methyltransferase CLF is also known to act in the autonomous (developmental) pathway (Liu et al. 2018). These observations highlight the overlap of epigenetic regulatory mechanisms between different pathways that control flowering. The crosstalk of different pathways ensures proper flowering synchronization to different environmental cues.

8.3 Hormone Crosstalk

Plant hormones such as GAs, auxin (AUX), cytokinins (CKs), and brassinosteroids (BRs) control essential developmental processes and are essential for proper growth (Depuydt and Hardtke 2011). GAs promote cell elongation and are essential for seed germination, stem elongation, and floral development. CKs regulate cell proliferation, while AUX and BRs may be involved in both processes as well as in cell elongation. These hormones may share overlapping mechanisms and have synergistic or antagonistic activities. Hormonal action, from synthesis to sensing and signaling, often depends on epigenetic modifications to ensure rapid and effective biochemical responses. Described mechanisms include histone modification, chromatin remodeling, DNA methylation, and action of sRNAs (Yamamuro et al. 2016). Hormonal activity is well known to be linked to light cues (Lau and Deng 2010). We will focus here on examples of this crosstalk at the epigenetic level with an emphasis on hormonal action during plant development. Activity of stress-related hormones will be discussed in Sect. 8.4.

DELLA proteins are central components of the GA pathway. As negative regulators of GA, DELLAs repress GA-induced growth. DELLA proteins are degraded upon binding to the GA receptor GA INSENSITIVE DWARF 1 (GID1) in a GA-dependent manner, allowing the activation of GA-mediated responses (Fig. 8.2). DELLAs also interfere with light signaling and are stabilized, for example, during light repression of hypocotyl elongation (Achard et al. 2007). DELLAs block transcriptional activity of the negative regulators of photomorphogenesis PIF3 and PIF4 by binding to their DNA-recognition sites, and by promoting their degradation through the ubiquitin-proteasome system (Cao et al. 2005; Feng et al. 2008; de Lucas et al. 2008; Li et al. 2016). This mechanism allows the integration of GA and light cues in order to optimize plant growth under changing environmental conditions. During the floral transition NF-Y factors interact with CO in the photoperiod pathway and with DELLAs to regulate *SOC1* expression (Fig. 8.2) (Hou et al. 2014). GA-mediated degradation of DELLAs enhances NF-Y binding to the *SOC1*

promoter and recruitment of REF6, accelerating the transition to flowering initiated by long days. The flexibility in terms of the possible combinations of multiple NF-Y subunits with distinct properties and *trans*-acting partners may enhance plastic responses to shifts in external cues.

Under low ratios of red to far-red light, shade intolerant plants, including Arabidopsis, undergo a series of responses known as the shade avoidance syndrome (SAS). Green light sensing is also important to trigger SAS (Zhang and Folta 2012). Typical SAS signs include stem elongation, leaf hyponasty, and reduced branching (Yang and Li 2017). In natural environments, SAS helps plants escaping from neighbors in dense vegetation in order to maximize access to sunlight. In agricultural contexts, prolonged shading can severely affect crop yield. It is well known that SAS requires changes in gene expression, and is mediated by localized transport and fluxes of auxin that induce cell division at particular spots. Other hormones may also be involved, such as GAs, BRs, CKs, ethylene, ABA, strigolactone, salicylic acid (SA), and jasmonic acid (JA). The modulation of the auxin pathway is initiated by pools of phytochrome in equilibrium under low ratios of red to far-red light, as well as by signaling from the UV-receptor UVR8 and cryptochromes. Under shade conditions, photoreceptors stabilize PIF transcription factors, which in turn activate auxin biosynthesis, transport, and signaling (Hornitschek et al. 2012). PIF proteins are now emerging as recruiters of chromatin modulators under shade. PIF7 recruits MRG1/MRG2 that bind H3K3m3/H3K36me3 at the chromatin of shade responsive genes, and bring histone acetyltransferases (HATs) to nearby chromatin to induce histone acetylation and activate SAS-related gene expression (Peng et al. 2018).

A crosstalk between phytochrome, auxin, and JA signaling and chromatin remodeling has been established with the action of the cytoplasmic localized JA-conjugating enzyme FAR-RED INSENSITIVE 219/JASMONATE RESISTANCE 1 (FIN219/JAR1). FIN219/JAR1 acts synergistically with phyA to negatively regulate SAS, reducing *PIF5* expression and COP1 levels (Swain et al. 2017). In addition, FIN219/JAR1 accumulation is reduced under shade. The *fin219* mutant, an epiallele of *FIN29* with altered methylation patterns, is more sensitive to shade (Hsieh et al. 2000). While reduced *FIN219* levels as a result of the *fin219* mutation seem to be at the basis of altered SAS, it may be interesting to verify whether the altered methylation status itself interferes with hormonal signaling.

Light effects are not specific to aerial parts of plants, and studies on roots should be further explored. Far-red light detection in shoots, for example, reduces auxin signaling in cortex cells in roots and reduces lateral root outgrowth through activity of HY5 (van Gelderen et al. 2018). It is very likely that light-mediated epigenetic remodeling supports root development. Interestingly, the H3K27 methyltransferase CLF binds the chromatin of the auxin efflux carrier *PIN FORMED 1* (*PIN1*) and functions to reduce auxin maxima and regulate the establishment of lateral roots (Gu et al. 2014). CLF, as referred in Sect. 8.2.3, affects flowering in association with CO (Fig. 8.2). It may be of interest therefore to analyze effects of light on CLF activity in roots.

8.4 Stress Responses

As sessile organisms, plants have evolved complex mechanisms to cope with abiotic and biotic stress cues, which include the activity of hormones ABA, JA, SA, and ethylene, and the modulation of reactive oxygen species (ROS) (Verma et al. 2016). Reversible epigenetic modifications, at various plant developmental stages, integrate light and internal signals and allow for rapid responses to environmental stress, towards ensuring plant survival.

Light and ABA control guard cell dynamics and stomatal aperture, as well as water loss, which is particularly relevant under drought and high salinity. Exposure of plants to ABA and low light conditions induces expression of the linker H1.3 histone (Fig. 8.3) (Rutowicz et al. 2015). H1.3 controls DNA methylation under low light and drought conditions, together with other H1 variants, H1.1 and H1.2. Furthermore, *h1.3* mutant plants display reduced stomatal density and CO_2 assimilation rate, and are unable to trigger proper responses to drought. Finally, H1.3 activity in response to light does not depend on photoreceptors but on

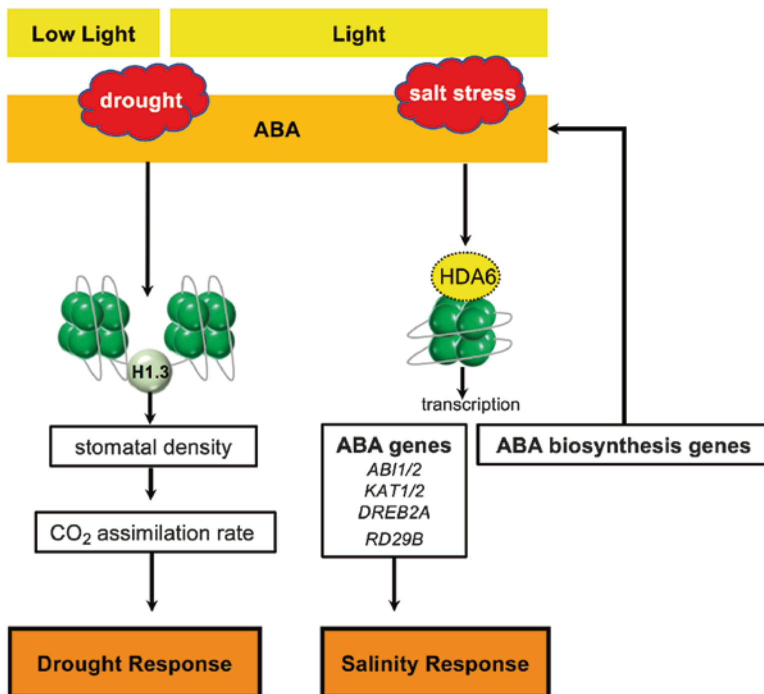

Fig. 8.3 Connecting hormones and stress signaling with epigenetics, an example with ABA. The response of plants to ABA and low light conditions induces the activity of the linker H1.3 histone. H1.3 controls DNA methylation during stomata development, which affects stomatal density and CO_2 assimilation rates and responses to drought stress. Salinity stress activates the ABA biosynthesis pathway through HDA6 and leads to increased ABA levels and ABA signaling

chloroplast-to-nucleus retrograde signaling. The epigenetic targets of H1.3 in the control of stomata opening under stress remain to be identified. Interestingly, histones of the H1.3-type subfamily are conserved in angiosperms but are absent in older plant lineages. This suggests that the mechanism using H1.3-ABA-light to control stomatal aperture, and possibly other biological functions using H1 histones, may have been important in the evolution of angiosperms, the largest group of plants. Additional epigenetic factors with roles in light responses and stomata regulation are yet to be found. An interesting candidate could be NF-YC9, given its activity in the ABA pathway and the regulation of stomatal aperture, and the roles of NF-Y members in chromatin remodeling (see Sect. 8.2.3) (Bi et al. 2017).

As referred in Sect. 8.2.3, the histone deacetylase HDA6 is a mediator of light-controlled chromatin compaction (Tessadori et al. 2009). Mutant plants in *HDA6* also display hypersensitivity to ABA and salt stress (Chen et al. 2010; Chen and Wu 2010). ABA and salt stress affect H3K4 trimethylation, H3K9 dimethylation, and H3 acetylation of several genes in the ABA pathway (Fig. 8.3). HDA6 is part of this regulatory process and may target ABA pathway genes, such as *ABI1/2*, *KAT1/2*, *DREB2A*, and *RD29B* (Fig. 8.3). Further studies are needed to connect the simultaneous response of HDA6, and of other histone deacetylases, to light and ABA. Light regulatory effects have in fact been identified in other histone deacetylases (HDAs), but more is known on the role of these factors in response to stress (Ueda et al. 2017). Arabidopsis contains 18 HDAs divided into three classes, and HDA6 is included in class I. HDA15, a HDA type II, mediates tolerance to salt stress, and its activity is regulated through subcellular compartmentalization by light-controlled protein shuttling between the cytoplasm and the nucleus (Alinsug et al. 2012). It seems that different families of HDAs show functional diversification and may act at an epistatic level to allow for nonselective histone deacetylases to acquire specificity upon particular environmental conditions.

Plant cells possess different mechanisms to cope with damaging UV light, which can include chromatin regulators. The UV RESISTANCE LOCUS 8 (UVR8) is a UV-B signaling component that helps in UV protection. UVR8 interacts with chromatin mainly via interaction with histone H2B in different genomic regions, including in the *HY5* promoter. UVR8 increases acetylation of K9 and K14 of histone H3 at UV-responsive *loci*, such as *EARLY LIGHT-INDUCIBLE PROTEIN 1* (*ELIP1*) (Cloix and Jenkins 2008; Velanis et al. 2016). UVR8-regulated chromatin modification also requires the activity of HY5. In addition, inhibiting activity of histone acetyltransferases prior to damage by UV-B reduces the capacity for DNA repair, highlighting the fundamental role of histone acetylation in plant survival under UV (Campi et al. 2012; Velanis et al. 2016). Known acetyltransferases with role in UV-B-induced damage repair and signaling include HAM1 and HAM2 of the MYST family, and HAG3 of the GNAT family (Fina and Casati 2015). On the other hand, the histone acetyltransferases HAC1 (HAC family) and HAF1 (HAF family) do not directly participate in damage repair but still have roles in signaling and plant responses to UV light (Fina et al. 2017). It may be possible that different families of histone modifiers have evolved specific functions to support plants in more efficient responses to UV stress conditions.

Synthesis of secondary metabolites, such as anthocyanins in the flavonoid pathway, provides a mechanism for protection under various stress conditions. UV and far-red light are well-known promoters of anthocyanin production, which is known to require epigenetic reprogramming. A study targeting the role of miRNAs in stress-induced anthocyanin biosynthesis identified miR858a as a positive regulator of this induction (Wang et al. 2016). miR858a inhibits translation of *ARABIDOPSIS MYB-LIKE 2* (*MYBL2*), a transcription factor functioning as a negative regulator of anthocyanin biosynthesis. The role of light is integrated via the activity of HY5, which activates *MIR858A* expression and represses *MYBL2* by binding to its promoter, as well as via histone demethylation and deacetylation.

8.5 Environmental Memory

A research field that is gaining relevance in the recent years is the study of epigenetic mechanisms that allow plants to keep track of past exposure to environmental conditions, and to transmit this memory to future generations (Baulcombe and Dean 2014; Buzas 2017; Lämke and Bäurle 2017; Bäurle 2018; He and Li 2018). Some authors divide plant memory into two parts: priming and memory. Priming refers to when a plant or a plant tissue is exposed to a particular environmental condition that triggers physiological conditions for its adaptation. Upon a later exposure (in a time window of hours or days) to the same environmental cue, this plant or tissue shows a more robust physiological response given its primed state, when compared to the first naïve organism. Plant memory describes the transmission of physiological priming from a primed plant or tissue to newly synthesized cells or tissues, not exposed to the priming condition, or to new generations.

Environmental memory has been described in response to various abiotic and biotic factors. There is growing evidence that epigenetics is a central regulator of plant memory. Various reports are emerging, and more studies are required, but it is becoming clear that this increasing knowledge may be useful for crop improvement. Climate change is associated not only with global warming but also with extreme and sudden shifts in environmental conditions. The latter may be in fact the major challenge to farmers. For proper yields, crops must be able to adjust growth to fluctuating weather. Targeting epigenetics mechanisms of plant memory in crop biotechnology may therefore be of high interest to plant producers in order to obtain plants that respond more rapidly to the environment and also are more resilient in their responses.

The most explored environmental conditions affecting plant memory include winter cold exposure and its effect over flowering (vernalization), and virus-induced silencing (Baulcombe and Dean 2014). In terms of light effects on plant memory, knowledge remains scarce, but a couple of studies have shown that light may impact epigenetic memory to a large extent. Excessive UV light is a stress signal that induces responses in plants such as the accumulation of secondary metabolites to protect cellular structures. Plants keep track of this primed state, as plants previously

exposed to UV show more robust responses to UV upon a second exposure (Müller-Xing et al. 2014). With the current depletion of stratospheric ozone, understanding the mechanisms of UV memory is of particular relevance. UV exposure is sensed and signaled by the UVR8 receptor, which activates downstream pathways that directly target, for example, *CHALCONE SYNTHASE* (*CHS*) in the flavonoid biosynthetic pathway (Müller-Xing et al. 2014). UV modulation of *CHS* requires epigenetic mechanisms, through at least increased histone acetylation (H3K9) at the *CHS locus* (Schenke et al. 2014). DNA damage repair pathways are also activated by UV, as a result of the UV damaging effect on DNA. This activation involves chromatin modifications and is linked to epigenetic memory (Molinier 2017). In another study analyzing plant responses to excessive white light, the authors failed to prove the hypothesis that altered DNA methylation patterns supported priming responses, including the synthesis and accumulation of photoprotective compounds (Ganguly et al. 2018). Nevertheless, it is still possible that other epigenetic mechanisms, such as histone modification, sustain priming to excessive white light. The histone acetyltransferase HAG1/GCN5 may be an interesting factor to analyze, given its described putative role in light-induced preparation of chromatin for priming inducible gene activation (Servet et al. 2010).

Drought and other stress conditions, such as exposure to high salt, low temperature, UV irradiation, heavy metals, phosphate starvation, and biotic cues, trigger proline accumulation (Szabados and Savouré 2010; Aleksza et al. 2017). The activation of proline synthesis is affected by light and phytohormones, such as ABA and BRs (Abrahám et al. 2003). Proline acts as an osmoprotectant that increases stress tolerance. Engineering of proline metabolism may bring interesting solutions to agriculture. Salinity-induced proline accumulation is memorable (Feng et al. 2016). This memory is dependent on light, is restricted to the shoot, and uses HY5 in the phyA pathway. Salt memory is based on the retention of increased H3K4me3 levels at *Δ^1-PYRROLINE-5-CARBOXYLATE SYNTHETASE 1* (*P5CS1*), which encodes the rate-limiting proline biosynthetic enzyme. HY5 binds to a C/A-box light responsive element at the *P5CS1* promoter and helps maintaining H3K4me3. There is still no evidence on how HY5 maintains H3K4me3 at *PCS1*. It may directly interact with H3K4 histone methyltransferases or demethylases or it may recruit the histone acetyltransferase HAT1/GCN5 to acetylate histones and activate light-responsive gene expression. It may also be of interest to assess whether modulating proline with light may target flowering quality, particularly in plants exposed to stress. This suggestion comes from the fact that proline is active during flower transition, male gametophyte, and seed development (Székely et al. 2008; Mattioli et al. 2009, 2012). In addition, FRIGIDA (FRI), which acts in the vernalization pathway upstream of FLC, increases *P5CS1* expression under drought (Chen et al. 2018).

Another guideline to be considered in future research for crop improvement may be to compare epigenetics of environmental memory, including of light memory, in wild and cultivated species. Specific mechanisms may be identified and provide novel solutions to obtain better crops.

8.6 Conclusions

Light regulates plant growth by directly impacting gene expression at various developmental stages and under different environmental conditions. Knowledge mostly obtained from Arabidopsis has shown that epigenetics is a fundamental tool in light signaling. Individual reports detailing specific molecular interactions have been helpful advancements and may also be complemented with parallel large-scale analysis.

Light and epigenetics in plants of agronomic importance have only just started being explored, and some similarities are often seen with the model Arabidopsis. The regulator of light responses HY5 has been identified in various plant species from green algae to flowering plants (Serrano-Bueno et al. 2017; Li et al. 2017). Given the master role of HY5 in light signaling and chromatin remodeling, it is an interesting target to further explore. Chromatin remodeling at the *FT locus* during the control of flowering is also conserved between Arabidopsis and other flowering species, including rice, soybean, and wheat (Blümel et al. 2015). Hormonal pathways display similarities as well between different plant species. Mechanisms that use histone modification also show similarities among organisms, from plants to yeast and humans. While we are at a point where some solid knowledge could be easily transferred to direct applications for crop growth, more studies are needed, particularly targeting specific crop species and possible exclusive gene expression regulatory mechanisms. Adaptation to distinct natural light environments, and different latitudes, may cause changes in light-mediated epigenetic responses that may then impact outputs measured as agronomic traits.

Indoor farming is likely a key solution for the future of agriculture. Usage of natural resources can be decreased, and large fields returned back to the wild. In the context of large urban areas, indoor farming may guarantee access to fresh and healthy produce by larger amounts of the population. Indoor crop growth facilitates a tighter control of environmental factors. Light environments, in particular, can be precisely controlled with the usage of light emitting diodes (LEDs). The large-scale commercialization of LEDs offers the possibility to effectively design light recipes to optimize crop growth and value. Near future breeding programs may consider including markers for light-responsive epigenetic remodeling.

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Chapter 9
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.

9.1 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.

Tomato belongs to the large and diverse *Solanaceae* family, also called Nightshades, which includes more than 3000 species from several habitats. Among them, major crops arose from the "Old World" (eggplant from Asia) and the "New

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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.

9.2 Epigenetic Studies on Tomato

9.2.1 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 nonripening* (*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. 9.1), 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 *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

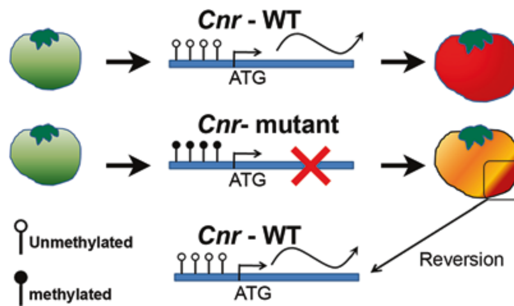

Fig. 9.1 Graphic representation showing how the natural epiallele *Cnr* prevents ripening, resulting in yellow fruits. Such epiallele is the result of changes in methylation status on CpG and CpHpG regions within the promoter and 5′-UTR of *SBP3*-like/*CNR* gene. Interestingly, some occasional revertant “ripening” sectors that have a wild-type ripening phenotype are observed in mutant fruits

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 epiallele 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 9.1). 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. 9.2). Future studies are needed to address whether tomato MET1 and CMT3 enzymes are indeed involved in the generation of the natural *Cnr* epiallele.

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* epiallele arose from 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

Table 9.1 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 Solyc01g006100	12 1
DRM-like[a]	De novo: CpG, CpHpG, CpHpH Maintenance: CpHpG, CpHpH	Solyc02g062740 Solyc10g078190	2 10

[a]Domains-rearranged methyltransferases-like proteins

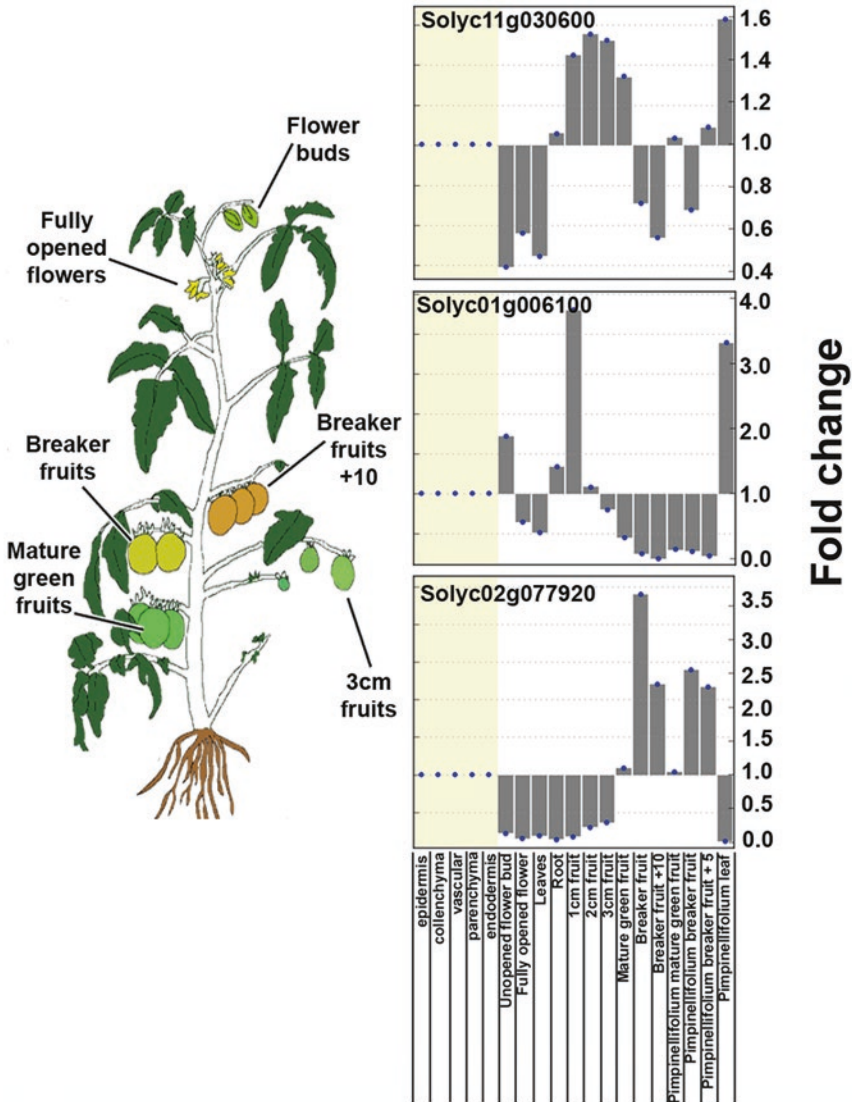

Fig. 9.2 Expression profiles of *SBP3*-like/*CNR* (Solyc02g077920), *MET1* (Solyc11g030600), and *CMT3-like* (Solyc01g006100) genes in different tissues and organs. The figure was generated using RNA-seq data from Tomato eFP Browser (http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi). Adult tomato plant showing tissues/organs analyzed is shown in the left panel

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 epialleles associated with natural changes in fruit development and ripening? A promising answer for this important biological and agronomical question may come from genome-wide analyses of the DNA methylation status during fruit development and ripening. Recently, Zhong and colleagues (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 includes 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 9.1) during fruit development (Fig. 9.2) 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 *Abscisic 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 and collaborators (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 analyses, 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 analyses 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.

9.2.2 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 four *DCL*, 10 *AGO,* and six *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 analyses 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, seven *SlDCL*, 15 *SlAGO*, and six *SlRDR* genes have been identified so far (Bai et al. 2012). One recent study conducted by Xian and coworkers (Xian et al. 2013) analyzed in detail 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 economic 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 seem 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 non-conserved 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 and coworkers (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 the 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 (Silva 2012). 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; Silva 2012). 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 et al. (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 analyses of the dominant mutant *Gob*, which contains a miR164-insensitive version of the *GOB* gene, the abovementioned 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.

9.3 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. 9.3).

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. 9.3). 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).

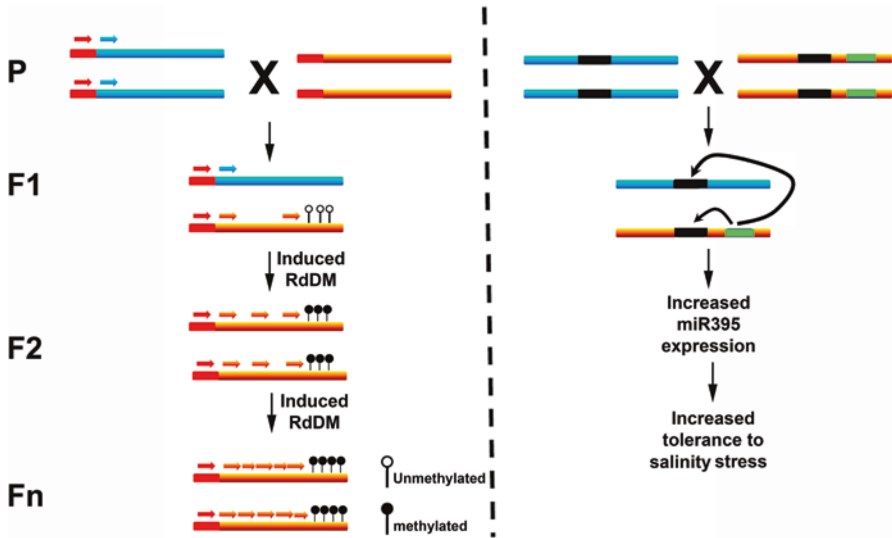

Fig. 9.3 Possible scenarios for epigenetic-based transgressive segregation. Left panel: interaction between allelic or non-allelic loci that share only limited sequence identity (red box) in F1 can lead to generation and spreading of siRNAs (small arrows), perhaps through a phenomenon called transitivity. 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 10
Targeted Epigenome Editing of Plant Defense Genes via CRISPR Activation (CRISPRa)

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Abstract Crop protection plays a central role in maintaining and increasing crop productivity. Many pathogens continue to affect crop production, however, and losses generated by pests must be halted. Thus, if farming is to support the human population, additional viable strategies for crop production and improved integrated pest management systems must be developed. Genome editing is an alternative to conventional breeding that can facilitate the molecular breeding of crops with desired properties. We propose here the implementation of targeted modification of epigenetic marks (epigenome editing via CRISPR activation or CRISPRa) to activate plant defense genes to confer resistance against pathogen attack. Work on CRISPRa in plants is lacking, although its potential application to crops is one of the greatest challenges in the field. Future exploitation of this approach in crop improvement programs will reduce important economic losses and benefit society.

10.1 Introduction

Since the transition from hunting and gathering to farming, humans have domesticated numerous plant species. Domestication of wild species of plants, through selective breeding, involves an array of evolutionary changes that may increase the fitness of the plant, but could decrease its survival in the wild. Mainly since the Green Revolution (GR), the selection of individuals with a desirable form of a gene (or allele), the breeding of high-yielding varieties or genotypes, the implementation

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of new mechanized agricultural technologies, and numerous technological advances have permitted crop production to increase and to supply the nutritional requirements of an ever-growing human population.

What made the GR possible, however, was a dependence on dangerous pesticides and fertilizers. Thus, some of the impacts and environmental criticisms of the GR include "soil deterioration caused by excessive fertilization" and the excessive use of pesticides (Briney 2018; Pellegrini and Fernández 2018). Despite this, many pathogenic bacteria, fungi, and viruses continue to affect crop production, and losses generated by pests must be halted. Thus, if farming is to support the human population, additional realistic strategies for crop production and improved integrated pest management systems must be developed as part of a new GR.

Crop protection consequently has a central role in maintaining and increasing crop productivity. Second-generation synthetic pesticides are useful for controlling pests, but they have many disadvantages (e.g., effects on human health, environmental damage, effects on beneficial organisms, evolution of resistance to the pesticide, elevated production costs, and risks to the environment, farmers, and consumers; Bai and Lindhout 2007; Bruce et al. 2016). Alternative answers to the use of pesticides include the development of new resistant crop cultivars, the use of biological control agents, the exploitation of plant activator agrochemicals to turn on natural plant defenses (Bruce 2010), and the use of genome engineering tools or gene editing, such as clustered, regularly interspaced, short palindromic repeat–CRISPR-associated protein (CRISPR-Cas).

The CRISPR system is a characteristic element of the genomes of most bacteria and archaea; it evolved as an adaptive defense mechanism and is involved in providing resistance to bacteriophages and immunity against foreign genetic elements (Barrangou et al. 2007; reviewed in Chandrasegaran and Carroll 2016). In bacteria, CRISPR loci are composed of several non-contiguous direct repeats separated by segments of variable sequences called spacers, which are generally adjacent to *cas* genes (CRISPR-associated). Specificity against phages or foreign genetic elements is controlled by the CRISPR spacer content, while the resistance is conferred by the Cas enzymatic machinery (Barrangou et al. 2007).

Consequently, the control and improvement of this system and its application in eukaryotic organisms has offered a ground-breaking tool for genome engineering. Most genome editing with the CRISPR system has used Cas9, an RNA-guided type II DNA endonuclease from *Streptococcus pyogenes* (Zaidi et al. 2017), and research is now being performed to optimize genome-wide specificities of CRISPR-RNA-guided nucleases (Kleinstiver et al. 2016a).

10.2 Genome Editing

Genome editing requires the creation of a DNA double-strand break (DSB) at the genomic locus that will be modified. In a living cell, these breaks can be repaired by one of at least two different pathways that are operative in nearly all cell types and organisms: non-homologous end-joining (NHEJ) and homology-directed repair

(HDR). NHEJ is error-prone and can lead to the insertion/deletion (indel) of some DNA base pairs. If indels occur within a coding exon, they can cause frameshift mutations or generate premature stop codons that could disrupt the open reading frame of coding sequences or disturb binding sites for regulatory proteins. DSBs also stimulate the HDR pathway when a DNA extra copy is present. Thus, NHEJ can be used to generate indels and HDR can be used to introduce specific mutations or insert desired sequences via recombination of DNA donor templates (Sander and Joung 2014). Alternatively, DSBs can be generated in a sequence-specific manner by a wide range of DNA-targeting nucleases, enabling the introduction of desired genome modifications, hence the term "genome editing" (Wu et al. 2018).

The first generation of genome-editing tools appeared in the early 1990s with the use of meganucleases, which contain recognition sites between 12 and 40 base pairs (bp) in length, meaning that their target sites are limited in a long DNA region. One of the first discovered meganucleases was I-*Sce* harboring an 18-bp recognition site. In 1993, Puchta and colleagues observed that I-*Sce* produced targeted DSBs in *Nicothiana plumbaginifolia*, and these were mainly repaired via double homologous recombination in the presence of a template sequence. These pioneer results opened the way for the future editing of plant genomes (Puchta and Dujon 1993). Soon, the first attempts to improve DSB-mediated mutagenesis arose. In 1994, Kim and Chandrasegaran reported the first successfully constructed chimeric-endonuclease, achieved by fusing the *Ultrabithorax* (Ubx) homeodomain of *Drosophila melanogaste*r (able to tightly bind to the 5′-TTAAT(G/T)(G/A)CC-3′ consensus DNA sites) to the cleavage domain of the *Fok*I restriction endonuclease. This hybrid endonuclease was shown to bind to the appropriate site on the DNA, the UBx homeodomain, and cut in a non-specific manner 9–13 nucleotides (nt) downstream of the DNA-binding domain. Two years later, they replaced the homeodomain with zinc-finger polypeptides (Kim et al. 1996). The particularity of these polypeptides is that each zinc-finger α-helix contains four variable amino acids at an invariable location, and these are capable of specifically binding to a 3-bp sequence in the DNA target. As the number of different known zinc fingers rapidly increased, a new race to obtain artificial endonucleases directed to specific targets started. Since then, artificial and functional zinc-finger nucleases (ZFNs) have been used to modify endogenous genes in a wide range of organisms, including *Arabidopsis*, maize, soybean, tobacco, fruit fly, zebrafish, rats, and human cells, among others (reviewed by Urnov et al. 2010). Several types of genetic modifications, such as point mutations, insertions, deletions, inversions, and substitutions, can be introduced with ZFNs, providing researchers with unprecedented tools for genetic manipulation. Nevertheless, the construction and optimization of poly-zinc-finger domains are very tedious and time-consuming (Quétier 2016).

In contrast, studies on the mechanisms of virulence used by the bacteria *Xanthomonas* to infect plants revealed to researchers a set of peculiar bacterial proteins able to directly bind to regulatory sequences on the host plant DNA and modify gene expression. This class of proteins (transcription activator-like effectors, TALEs) from the *Xanthomonas* genus represents the largest type III effector proteins and functions to transcriptionally activate host gene expression (Bi and Yang 2017).

TALEs share a similar structure: an N-terminal region that contains the secretion and translocation signal for the type III secretion system, a central repeat domain that confers DNA-binding specificity, two C-terminal nuclear localization signals, and a transcriptional activation domain (Boch and Bonas 2010). These TALE proteins encompass 13.5–25.5 central repeats of a 34- or 35-amino acid motif (the last 3′ unit is a 5′ half of a repeat). The amino acid composition of each repeat is strictly invariable, except at amino acid positions 12 and 13 (known as the repeat variable di-residue), which vary and specify the binding to A, C, G, or T nucleotides, according to a deciphered code: HD = C; NG = T; NH = G; NI = A; NS = A, C, G, or T; NN = G/A; and IG = T (Boch et al. 2009). This code is only slightly degenerated and allows the easy design of artificial TALEs able to bind to any DNA target determined by the user. TALE proteins can be easily engineered to fuse a nuclease domain, commonly *Fok*I, generating a new class of artificial nucleases called TALE-nucleases (TALENs). TALEN technology is faster and cheaper than ZFN, and has rapidly emerged as an alternative to ZFNs for genome editing. TALENs have gained much interest and relevancy because they can be easily and rapidly designed by researchers using the simple protein–DNA code, described above, relating a DNA-binding TALE repeat domain to individual bases in a specific target site. Since 2011, TALENs have been used to edit the genome of many organisms, including yeast, fruit flies, zebrafish, frogs, rats, silkworms, rice, barley, wheat, tomatoes, soybeans, maize, and human somatic and pluripotent stem cells (reviewed by Joung and Sander 2012; Quétier 2016). Many studies have shown that TALENs and ZFNs have comparable efficiencies when targeted to the same gene (Hockemeyer et al. 2011; Sander et al. 2011; Tesson et al. 2011). The use of TALENs has decreased due to several technical problems, however, such as laborious protein engineering, the reduced number of targets in a long DNA sequence, time consumption, and high cost.

10.2.1 A New Era in Genome Editing

In 1987, the presence of a peculiar DNA region composed of short direct repeats interspaced by short variable sequences and located close to a bacterial protein gene was reported (Ishino et al. 1987). Surprisingly, the variable sequences, called protospacers, corresponded to short sequences of foreign DNA that came from invading biological elements such as bacteriophages and plasmids. These arrays of DNA, then called clustered regularly interspaced short palindromic repeats or CRISPR, have since been found in many bacteria and archaea, and have been demonstrated to play an important role as a type of bacterial immune response (Doudna and Charpentier 2014). To date, there are six identified types (I–VI) of CRISPR systems across a wide range of bacterial and archaeal hosts, and these are divided into two classes that mainly differ in the architecture of their effector modules. Class 1 comprises type I and type III CRISPR systems, which are commonly found in Archaea and possess an effector module that is a complex of multiple proteins. Class 2 contains type II, IV, V, and VI CRISPR systems, and is

represented by a single multi-domain effector protein (Yan et al. 2018). This classification is yet to be completed, however, as researchers continue to discover new systems and redefine the classification system with subclasses, groups, and types based on comparative genomic analyses, structures, and biochemical activities of CRISPR components (Adli 2018). For simplicity, each CRISPR system consists of a CRISPR array, comprising short direct repeats interspaced by short variable DNA sequences (protospacers) and flanked by diverse CRISPR-associated *cas* genes (Makarova et al. 2015).

Type II is the best characterized CRISPR system. It consists of the Cas9 nuclease, the CRISPR RNA (crRNA) from the CRISPR array, and a required transactivating crRNA (tracrRNA) necessary for processing the crRNA arrays into small units and binding Cas9 nuclease to the DNA target. Each processed crRNA unit results in a 20-nt guide sequence and a partial direct repeat. The guide sequence directs the Cas9 to a complementary 20-bp DNA target, allowing the nuclease to make a DSB close to the binding site (Gasiunas et al. 2012). In *S. pyogenes*, the target DNA must be immediately upstream of a 5′-NGG triplet called the protospacer-adjacent-motif (PAM; Jinek et al. 2012), whereas other Cas9 orthologs may have different PAM requirements, such as 5′-NGGNG for *S. thermophilus* (Sapranauskas et al. 2011) and 5′-NNNNGATT for *Neisseria meningitidis* (Zhang et al. 2013) (Fig. 10.1a).

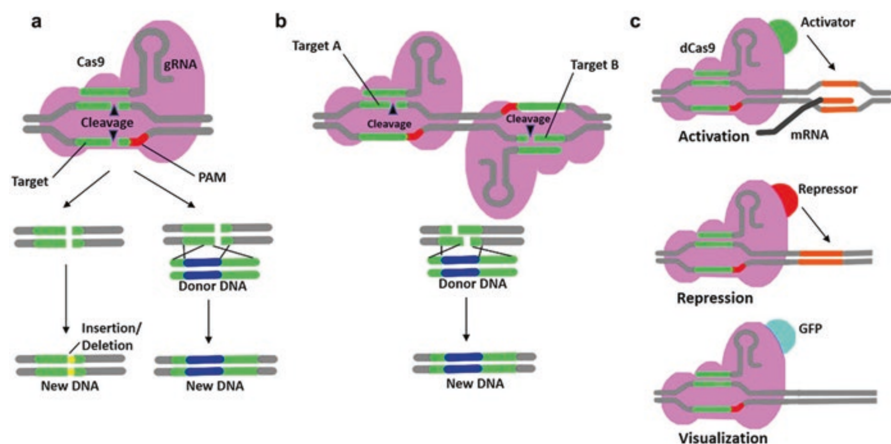

Fig. 10.1 Schematic representation of Cas9 activity and its modifications. (**a**) The CRISPR/Cas9 system induces artificial double-strand breaks (DSBs) via Cas9 protein. The DSBs can be repaired by non-homologous end-joining (NHEJ) and homology-directed repair (HDR), leading to gene modification and insertion. (**b**) Cas9 nucleases can be converted into DNA nickase (Cas9n) by substitution of its key amino acids, D10A or H840A, producing single-stranded DNA breaks. (**c**) Mutations in both catalytic residues modify Cas9 to an inactive dead Cas9 (dCas9). dCas9 is no longer able to cut DNA, but can still be directed to specific targets. For example, dCas9 can be directed to specific sites in promoter sequences, where it blocks transcription and knocks down gene expression; it can be fused to strong activating domains or effector domains to induce gene expression; or it can be fused to fluorescent proteins for live imaging

When a bacterium is infected by a virus, the CRISPR machinery cuts up and retains small pieces of the foreign DNA, storing the sequences as protospacers in the CRISPR loci, which are then transcribed and processed into short crRNAs. Each crRNA anneals to a tracRNA to form a ribonucleoprotein complex in conjunction with Cas9, allowing Cas9 to protect the bacterium against repeat invaders by seeking foreign DNA that is complementary to the harbored crRNA. If a match occurs, the Cas9 cuts the invading DNA via DSBs at the target locus (Urnov 2016). These site-specific DSBs are performed through Cas9's two conserved catalytic domains: HNH and RuvC, both are metal ion-dependent. Recognition and cleavage strictly require the presence of the PAM sequence in the non-target strand and depend on the base pair complementarity of the guide RNA template to the target DNA strand (Jinek et al. 2012). The HNH domain cleaves the target DNA strand (via a one-metal-ion mechanism), whereas RuvC cuts the non-target DNA (via a two-metal-ion mechanism; Zuo and Liu 2017). The type II CRISPR system has been reconstituted and applied in a wide range of organisms in order to achieve genome editing through the heterologous expression of *Sp*Cas9 (from *S. pyogenes*) and the required crRNA and tracrRNA components, both fused together to create a simplified chimeric single-guide RNA (sgRNA; Jinek et al. 2012). Moreover, *Sp*Cas9 can be directed to nearly any target of interest, as long as it is immediately upstream of the PAM sequence, by simply altering the 20-nt guide sequence within the sgRNA.

In the same way that ZFNs and TALENs have been used to promote DSBs, the artificial CRISPR/Cas9 system can be used to stimulate genome editing at a target genomic locus. Upon cleavage by Cas9 at a target locus, DSBs are re-ligated either by NHEJ repair, which frequently leaves indels scars, or, if a DNA template is also introduced, via HDR, consequently generating mutations (Ran et al. 2013). ZFNs and TALENs are based on specific polypeptide–DNA-binding, which is tedious to optimize, time-consuming, and costly. CRISPR/Cas9, however, is based on DNA–RNA hybridization and its results are simpler and cheaper than those of other methods of genome editing. Thus, CRISPR/Cas9 has emerged with novel advantages for genome engineering, including easy and simple design of sgRNAs for genomic targets, reduced off-target activity, low cost, rapidity, and the ability to achieve different targets in the same experiment, among others.

Today, CRISPR/Cas9 appears to be the most efficient system by which to achieve site-specific genome editing, by simply designing a short sgRNA. Since the initial publication of the potential use of CRISPR/Cas9 for gene-targeting and genome-editing purposes (Jinek et al. 2012), the system has enabled the genome edition of a wide range of organisms, including human cells (Mali et al. 2013), zebrafish (Hwang et al. 2013), yeast (DiCarlo et al. 2013), mice (Shen et al. 2013), fruit flies (Yu et al. 2013), fungi (Nødvig et al. 2015; Shi et al. 2017), and other species. The application of the CRISPR/Cas9-based genome-editing tool in plants has also been demonstrated since 2013 (Feng et al. 2013; Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013; Xie and Yang 2013), and numerous studies displaying applications of the CRISPR/Cas9 system in plants have been published (reviewed in Bortesi and Fischer 2015; Ma et al. 2016; Jaganathan et al. 2018; Soda et al. 2017).

Moreover, several modifications have been performed to improve the CRISPR/Cas9 system. For example, when CRISPR/Cas9 is used, certain possibilities for off-target activity are present because Cas9 is capable of cutting even when some mismatches in regions more distant from the PAM take place at the moment of DNA–RNA recognition (Doench et al. 2016). In order to improve targeting specificity, researchers have employed a system composed of two Cas9-nicking enzymes (a D10A mutant that cuts only one DNA strand) driven by a pair of sgRNAs complementary to opposite strands of the target site Fig. 10.1b). This strategy has been successfully implemented to reduce off-target activity by 50–1500 fold and increase on-target specificity in mice and *Arabidopsis* (Ran et al. 2013; Schiml et al. 2014). Cas9 nickase-based strategies have also been employed to reduce NHEJ-based mutations and robustly induce HDR across multiple target sites in human cells (Mali et al. 2013). Other improvements have involved the development of a multiplex CRISPR/Cas9 system to allow the co-expression of various sgRNA modules at the same time. For example, Xie et al. (2015) developed a method to produce numerous sgRNAs from a single polycistronic gene, separating each sgRNA module by a tRNA precursor so that the endogenous tRNA-processing system cleaves the RNA and efficiently releases the sgRNA. Other strategies for delivering sgRNAs from RNA transcripts have consisted of flanking the sgRNA modules with self-processing RNA molecules such as hammerhead type ribozyme or hepatitis delta virus ribozyme (Gao and Zhao 2014; Jacobs et al. 2014; Ryan and Cate 2014; Nødvig et al. 2015).

Furthermore, a point mutation in both catalytic domains (D10A and H840A) results in a catalytically inactive dead Cas9 (dCas9), which cannot cleave DNA but can still be directed to the target sequence (Jinek et al. 2012). Since its discovery, dCas9 has been exploited for a wide range of genome-targeting purposes beyond gene editing. For example, dCas9 has enabled several applications such as transcription regulation, epigenetic modifications, and genome imaging, among others (Fig. 10.1c). This has been possible due to the ability of dCas9 to strongly bind to the DNA target. Although dCas9 is no longer able to cut DNA, it can still be directed to specific targets for other purposes, for instance, the tight DNA–protein binding can interfere with the activity of other DNA-binding proteins, such as transcriptional factors or RNA polymerase II. This is the basis for the CRISPR interference (CRISPRi) approach, in which dCas9 is directed to specific sites in promoter sequences, where it blocks transcription and knocks down gene expression. Qi et al. (2013) demonstrated that dCas9 can repress the transcription of target genes either by sterically hindering RNA polymerase activity in the gene promoter or by reducing RNA polymerase processivity when targets are along the coding sequence. Later, Peters et al. (2016) reported a quantitative repression of essential target genes by CRISPRi in *Bacillus subtilis*, and Li et al. (2016) used CRISPRi to successfully repress gene expression in two Clostridia species. CRISPRi using dCas9 alone has been very effective in bacteria, but not in eukaryotic cells, likely because RNA polymerase II is more complex and more difficult to sterically hinder (Xu and Qi 2018).

In addition, dCas9 has been engineered with effector domains fused at the C-terminus, resulting in a stronger and more specific modulator than dCas9 alone. Gilbert et al. (2013) coupled dCas9 to a strong repressor complex such as Kruppel-associated box (KRAB), which is present in a large fraction of zinc-finger transcription factors (TFs) that mainly act as repressors in mammals, and showed that CRISPRi-mediated transcriptional repression is highly effective in human and yeast cells. In plants, Piatek et al. (2014) generated a synthetic transcriptional repressor by fusing the dCas9 C-terminus to the repressing SRDX domain and observed a markedly reduced expression of the targeted gene *pds* in *Nicothiana benthamiana* by using both dCas9 alone and the dCas9-SRDX synthetic transcriptional repressor. Thus, the repressor domain recruits TFs that could block transcriptional initiation or recruits TFs that could disrupt transcriptional elongation when the binding of chimeric dCas9-based transcriptional regulators takes place downstream of the transcriptional start site.

dCas9 has also been turned into a transcriptional activator by fusing strong activating domains to its C-terminus end. In this system, gene activation is favored by recruitment, via the activation domain, of TFs and co-factors at the promoter region of interest (Piatek et al. 2014). In this way, Gilbert et al. (2013) tested different effector domains fused to dCas9. They utilized four copies of the 16-amino-acid-long transactivation domain VP16 of the Herpes simplex virus, which constitute together the strong transactivator VP64, or a single copy of p65, an activation domain of the mammalian NF-κB TF. The authors showed that both dCas9-VP64 and dCas9-p65 can effectively activate gene expression (Gilbert et al. 2013). These results revealed a novel platform for efficiently regulating gene expression: the CRISPR activation (CRISPRa) system. Additionally, Perez-Pinera et al. (2013) utilized dCas9–VP64 fusion to activate the expression of endogenous genes by targeting human promoters with engineered sgRNAs.

In recent years, researchers have designed several strategies to exploit CRISPRa. The dCas9-VP64 system was further improved by adding several extra copies of the VP16 unit, resulting in stronger transactivators. Cheng et al. (2013) fused the VP160 (VP16 × 10) domain to dCas9 and demonstrated that this CRISPRa system can robustly induce gene expression in both human and mouse cells. Additionally, the authors observed a synergetic effect in gene induction when clusters of 3–5 sgRNAs were directed to the proximal promoters, nearby and strictly upstream of the transcription start site. Furthermore, when sgRNAs targeting multiple genes were simultaneously introduced into cells, the authors reported robust multiplexed endogenous gene activation (Cheng et al. 2013). In plants, Piatek et al. (2014) fused the strong activating EDLL or TAL domains to dCas9 to test transcriptional regulation in *N. benthamiana*. Their results showed that both activation domains can induce strong transcriptional activation in the target genes (Piatek et al. 2014). Also in plants, Lowder et al. (2015) described an efficient CRISPR-dCas9-based multiplexed transcriptional activation system by using dCas9–VP64 fusion in *A. thaliana, N. benthamiana*, and rice. They reported the effectiveness of this system in activating transcription of both protein-coding and non-coding genes by three independent sgRNAs for each target gene simultaneously. Targeting multiple distinct genomic loci for expression simultane-

ously resulted in an effective strategy for maximizing transcriptional control. In addition, targeting a single gene promoter with multiple sgRNAs showed a synergistic effect (Lowder et al. 2015). In another attempt to improve dCas9-mediated transcriptional activation, Chavez et al. (2015) coupled dCas9 to VPR, a tripartite transactivation complex composed of VP64, p65, and Rta activation domains (the latter from the Epstein–Barr virus), and demonstrated its effectiveness in activating several endogenous genes in human pluripotent stem cells, *Saccharomyces cerevisiae* cells, and cell lines of *D. melanogaster* and *Mus musculus.* VPR also allowed robust multi-locus activation at significantly higher expression levels than did VP64-based activators across the panel of tested genes (Chavez et al. 2015).

dCas9-based strategies have continuously undergone more sophisticated improvements. To this end, some strategies have also focused on engineering the sgRNA scaffold. For instance, Zalatan et al. (2015) introduced RNA-hairpin aptamers to the 3′ sgRNA structure. These aptamers can recruit specific RNA-binding proteins (RBP) and, consequently, transcriptional effectors can be fused to RBPs instead of to dCas9 protein. Zalatan's group introduced up to three different viral aptamers (MS2, PP7, and com) to the 3′ end of the sgRNAs in order to recruit their corresponding RBPs (MS2-coat protein [MCP], PP7 coat protein, and Com, respectively), fused to the VP64 activator domain. They observed significant gene expression when using each of the three RNA-binding recruitment modules, substantially greater than for the direct dCas9-VP64, and applied synthetic CRISPR-based transcriptional programs to successfully redirect the flux of a metabolic pathway in yeast (Zalatan et al. 2015). Other researchers have achieved successfully multiplexed activation of up to ten genes simultaneously in human cell lines by engineering sgRNAs (Konermann et al. 2015). Konermann and colleagues introduced aptamers at the two loops occurring in the sgRNA-dCas9 structure (stem loop and tetraloop) and tested the recruitment of MS2-VP64, which resulted in additive effects leading to increased gene expression (several folds) over dCas9-VP64. In the same work, they tested different combinations of dCas9 and MCP, fused either to the VP64 or p65 activator domains. Later, they expanded the possibilities of domain synergy by introducing the activation domain from human heat-shock factor 1 (HSF1) and found that an MS2-p65-HSF1 fusion protein combined with dcas9-VP64 strongly improved transcriptional activation (Konermann et al. 2015). They designated this system as synergistic activation mediator (SAM). Another innovative approach to improving CRISPRa technology was the SunTag strategy described by Tanenbaum et al. (2014), which consisted of fusing a peptide array of ten copies of the GCN4 epitope to dCas9 (dCas9-SunTag$_{10x_v4}$) and also fusing VP64 transcriptional activator to the C-terminus of its respective binding protein, specifically a single-chain variable fragment antibody. The researchers showed a robust transcriptional activation achieved by SunTag-dependent multimerization of transcriptional activation domains at endogenous promoter genes using a single sgRNA in human K562 cells (Tanenbaum et al. 2014).

Recently, a novel generation of engineered dCas9 has arisen focusing on the possibility to switch, input/output (I/O), the transcriptional regulatory system in order to enable a precise spatial and temporal control over the dynamics of gene expres-

sion. Two main strategies with this approach have been reported. The first couples dCas9 to chemical or optogenetic sensing domains, and the second couples dCas9 to ligand-sensing receptor domains. For instance, Nihongaki et al. (2015) fused the light-inducible photolyase homology region of cryptochrome 2 (CRY2PHR) from *A. thaliana* to the p65 activator domain and separately expressed the CRY2PHR-binding protein partner CIB1 fused to dCas9, so that upon blue light irradiation, CRY2 and CIB1 were heterodimerized and consequently the transcriptional activator p65 was recruited to the genomic target to activate gene expression. The result was a simple light-inducible system for spatially and temporally activating multiple user-defined endogenous genes (Nihongaki et al. 2015). In the same way, Gao et al. (2016) screened the dCas9–VPR fusion of some chemical- and light-inducible heterodimerization domains, and found that by using abscisic acid (ABA)-inducible ABI-PYL1 and gibberellic acid (GA)-inducible GID1-GAI systems, both derived from plant hormone signaling pathways, they were able to achieve strong inducible activation of endogenous genes in mammalian cells. Recently, an alternative strategy to achieve ligand-inducible control of dCas9 was reported by Nguyen et al. (2016), who utilized a split dCas9 fused to the ligand-binding domain estrogen receptor (ERT), which interacts with the cytosolic protein Hsp90. The dCas9-ERT-Hsp90 complex is retained in the cytoplasm until the addition of the ligand 4-hidroxytamoxifen, which disrupts the ERT–Hsp90 interaction, thereby allowing dCas9 translocation to the nucleus and enabling gene regulation (Nguyen et al. 2016). Many other sophisticated strategies, such as the MESA, Tango-GPCR, and ChaCha systems, make use of ligand-receptor pairs to switch on/off dCas9-mediated gene expression, enriching the repertory of CRISPR I/O systems and offering a promising toolkit with which to control gene expression by programmable signal inputs. Most systems, however, must still be optimized to improve efficiency and signal sensitivity, and reduce the size of the devices (reviewed by Xu and Qi 2018). Further attempts to improve CRISPR technology are rapidly and continuously emerging. Choosing an appropriate CRISPR strategy for genome editing or gene regulation mainly depends on the context of the research and the biological characteristics of the organism under study.

Cas9 technology from the type II CRISPR system has been applied to a broad range of studies with many purposes, rapidly eclipsing the previous genome-editing methods because of its advantages, which include versatility, ease of design, low cost, higher precision with reduced off-target effects, and increased speed. A new generation of nucleases has recently been discovered, however, and researchers are using them to upgrade CRISPR technology or to overcome minimal Cas9 constraints.

10.2.2 Cpf1: A New Generation of Nucleases

Cpf1 (CRISPR from *Prevotella* and *Francisella* 1 spp.), recently named Cas12a, is a monomeric nuclease belonging to the type V CRISPR group (Hirano et al. 2016). Three Cpf1 variants have been studied most intensively: *Fn*Cpf1 from *F. novicida,*

*As*CPF1 from *Acidaminococcus sp.* BV3L6, and *Lb*Cpf1 from *Lachnospiraceae bacterium*. The crystal structure of *Lb*Cpf1 and *As*Cpf1 has shown that Cpf1, similarly to Cas9, displays a bi-lobed structure with a central channel in which the RNA–DNA heteroduplex is bound. Unlike Cas9, however, Cpf1 contains only a single endonuclease domain (RuvC), which was recently proposed to cleave both DNA strands (Yamano et al. 2016). Cpf1 requires a T-rich PAM (5′-TTTV-3′ for *As*Cpf1 and *Lb*Cpf1, and 5′-TTV-3′ for *Fn*Cpf1; V=A/G/C) located at the 5′-end of the protospacer, in contrast to Cas9, which recognizes the G-rich PAM (5′-NGG-3′) located at the 3′-end of the protospacer (Fig. 10.2). In addition, Cpf1 makes a staggered cut 17–18 nucleotides distal from the PAM and generates a 5-nt 5′-overhang, whereas Cas9 generates a blunt-end cut 3 nt upstream from the PAM (Zetsche et al. 2015). Cpf1's staggered DSBs preferentially promote the HDR pathway, whereas the blunt-ended DSBs generated by Cas9, in the absence of a DNA donor, are mostly repaired by the NHEJ pathway (Chaudhary et al. 2018). Interestingly, Cpf1 harbors a distinct RNase activity for pre-crRNA processing into mature crRNAs, and therefore does not require tracrRNA or additional RNase III activity (Fonfara et al. 2016). Each processed crRNA is composed of a 19-nt-long repeat and a 23- to 25-nt-long spacer (protospacer), and forms a short stem loop structure in the direct repeat sequence. Thus, crRNA of Cpf1 is shorter than the sgRNA required for CRISPR/Cas9 (Zetsche et al. 2015). It has been reported that Cpf1 is more specific than Cas9 because it is highly intolerable to mismatches, at least in the first 18 nt adjacent to the PAM (Kim et al. 2016). Furthermore, Cpf1 is a perfect nuclease to target some AT-rich regions or peculiar AT-rich genomes, such as in *Plasmodium falciparum* chloroplasts or mitochondria (Chaudhary et al. 2018).

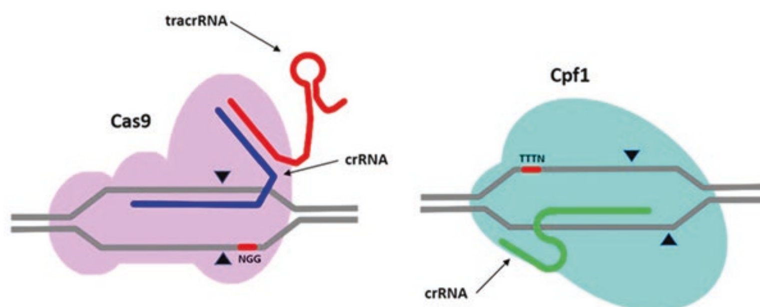

Fig. 10.2 Schematic representation of the Cas9 and Cpf1 nuclease technologies used for genome editing. (**a**) Cas9 recognizes the G-rich PAM sequence (5′-NGG-3′) located at the 3′-end of the protospacer and generates a blunt-end cut 3 nt upstream from the PAM. The blunt-ended DSBs generated by Cas9, in the absence of a DNA donor, are mostly repaired by the NHEJ pathway. (**b**) Cpf1 contains only a single endonuclease domain (RuvC), which cleaves both DNA strands. Cpf1 recognizes a T-rich PAM located at the 5′-end of the protospacer and creates a staggered cut 17–18 nucleotides distal from the PAM, generating a 5-nt 5′-overhang. Cpf1's staggered DSBs preferentially promote the HDR pathway. *Abbreviations: DSB* double-strand break, *PAM* protospacer adjacent motif, *NHEJ* non-homologous end-joining, *HDR* homology-directed repair

Early CRISPR/Cpf1 applications were performed in human cells and mice (Hur et al. 2016; Kim et al. 2016; Kleinstiver et al. 2016b; Zetsche et al. 2016), and rapidly extended to other organisms, including bacteria (Jiang et al. 2017; Yan et al. 2017; Li et al. 2018), yeast (Świat et al. 2017; Verwaal et al. 2017), plants (Endo et al. 2016; Hu et al. 2017; Kim et al. 2017; Tang et al. 2017; Wang et al. 2017; Yin et al. 2017), zebrafish, and *Xenopus* spp. (Moreno-Mateos et al. 2017). For instance, Hur's group used electroporation to introduce *As*Cpf1 and synthesized crRNA into mouse embryos, and obtained an efficiency of up to 64% (Hur et al. 2016). Kim et al. (2016) achieved knockout mice generated using *As*Cpf1 and *Lb*Cpf1 via microinjecting Cpf1 mRNAs with corresponding crRNA molecules into embryos. In bacteria, researchers achieved 86–100% efficiency in editing *Corynebacterium glutamicum* genes by using *Fn*CPpf1 combined with single-stranded DNA donors, and provided evidence that Cpf1 can be used for genetic engineering of bacteria that cannot utilize the CRISPR-Cas9 system (Jiang et al. 2017). In another example, CRISPR-FnCpf1-assisted recombineering was used to rapidly and efficiently generate point mutations, deletions, and insertions on chromosomes or native plasmids in *Escherichia coli*, *Yersinia pestis*, and *Mycobacterium smegmatis* (Yan et al. 2017). Cpf1 functionality was also demonstrated in yeast. Verwaal et al. (2017) tested the three Cpf1 orthologs *As*Cpf1, *Lb*Cpf1, and *Fn*Cpf1 for genome editing of *S. cerevisiae*. The authors reported efficiencies comparable with the CRISPR/Cas9 system for *Lb*Cpf1 and *Fn*Cpf1, whereas *As*Cpf1 editing efficiency was lower, indicating that Cpf1 variants can display different results, and therefore meticulous selection may be necessary (Verwaal et al. 2017).

CRISPR/Cpf1 has also been implemented for genome engineering in plants. For example, Endo and colleagues (2016) utilized codon-optimized *Fn*Cpf1 to perform site-specific mutagenesis in tobacco and rice. Their results showed that targeted mutagenesis occurred in transgenic plants and most observed mutations were deletions of the targeted region (Endo et al. 2016). Wang et al. (2017) successfully introduced site-directed mutagenesis by using *Fn*Cpf1 and *Lb*Cpf1 to target six sites of three distinct genes in rice. Their results showed that *Lb*Cpf1 had a higher efficiency than did *Fn*Cpf1 for all six targeted genes. These authors also tested four-unit crRNA arrays for multiplex gene targeting and observed mutation efficiencies of 44 and 67% for *Fn*Cpf1 and *Lb*Cpf1, respectively, at all four target sites simultaneously (Wang et al. 2017). Yin et al. (2017) knocked out a positive regulator of stomatal development in rice using *Lb*Cpf1 and found phenotypic changes similar to those observed in the CRISPR-Cas9 system. Tang et al. (2017) utilized *As*Cpf1 and *Lb*Cpf1 in rice, but detected little cleavage activity at six tested targets. The observed mutation frequencies ranged from 0.6 to 25% and mostly consisted of deletions resulting from error-prone NHEJ (Tang et al. 2017). Hu et al. (2017) demonstrated the potential of CRISPR-Cpf1 in plants by using codon-optimized *Lb*Cpf1 and *As*Cpf1 alongside the endogenous tRNA-processing system to simultaneously deliver various crRNA for respective targets in rice genes. They identified mutations in only 12% of transgenic plants with *Lb*Cpf1, whereas no mutations were detected with *As*Cpf1 (Hu et al. 2017). Later, Kim et al. (2017) described the delivery of *Lb*Cpf1 and *As*Cpf1 with *in vitro* synthetized target-

specific crRNAs into soybean and tobacco protoplasts, and determined that indels occurred at low frequencies of 0.0–11.7%. In view of some reports that showed a low CRISPR/Cpf1 efficiency, Moreno-Mateos et al. (2017) investigated the effect of temperature on Cpf1 activity. They demonstrated that CRISPR-Cpf1 allowed genome editing in *Danio rerio* and *X. tropicalis*, but observed that temperature highly influenced Cpf1 activity *in vivo*, which explained its lower activity in ectothermic organisms such as zebrafish, *Xenopus*, *Drosophila*, and plants (Moreno-Mateos et al. 2017).

Cpf1 nuclease possesses a domain that autocatalyzes specific cleavage of its precursor crRNA to yield mature crRNA guides. This feature has been applied to efficiently target multiple genes simultaneously using a single customized crRNA precursor. Zetsche's team was the first to use a customized pre-crRNA and *As*Cpf1 nuclease to simultaneously target up to four genes in HEK 293 human cells and three genes in brain mice (Zetsche et al. 2016). Further works have successfully employed the same strategy to multiplex gene edition via HDR or NHEJ repair, achieving high efficiencies at targeting multiple genes simultaneously in yeast, actinomycetes, plants, mice, and human cells (Świat et al. 2017; Verwaal et al. 2017; Wang et al. 2017; Zetsche et al. 2016; Li et al. 2018).

As with the Cas9-based CRISPRi system, Cpf1 has been engineered to catalytically inactivate its only nuclease domain, RuvC, by introducing two point mutations (D917A, E1006A) at the positions that are each implicated in DNA cleavage, leading to a mutated *Fn*Cpf1 without cleavage activity against both DNA strands, but still able to strongly bind the target DNA (Leenay et al. 2016). Considering that distinct domains have been characterized for the DNase and RNase activities of Cpf1, the inactivation of DNase activity has no influence on its RNase activity. Consequently, DNase-dead Cpf1 (ddCpf1) retains the capacity to process its precursor crRNA, as well as a customized CRISPR array (Zhang et al. 2017). ddCpf1 has therefore been employed to perform further CRISPRi strategies. In this context, Zhang's group employed a ddCpf1 paired with a crRNA array for multiplex gene repression of up to four genes in *E. coli,* showing that ddCpf1-mediated repression is of high specificity for single or multiple gene repression. Similarly, Tang et al. (2017) also deactivated the nuclease domains of *As*Cpf1 and *Lb*Cpf1 via D908A and D832A mutations, respectively. They then fused these dCpf1 to three copies of the strong SRDX transcriptional repressor and tested these in *Arabidopsis*, targeting a non-coding RNA promoter. They efficiently achieved transcriptional repression (approximately 90%) by using both dCpf1-SRDX species, and offered the view that, although *As*Cpf1is less potent as a nuclease, it effectively binds DNA (Tang et al. 2017). Recently, Li et al. (2018) efficiently utilized the DNase-deactivated *Fn*Cpf1 (ddCpf1) to achieve multiplexed gene repression. They used single customized crRNA arrays in *Streptomyces* strains and obtained effective gene repression, ranging from 48 to 95%, in four targeted genes. They also observed that crRNA targeting the site nearest to the start codon exhibited the highest repression activity (Li et al. 2018).

Dead Cpf1 (dCpf1)-based gene regulators have only been used to repress gene expression in a few bacteria and plants. To date, there are limited reports regarding dCpf1-based transcriptional activation. For instance, Tak et al. (2017) fused d*Lb*Cpf1

to the strong transcriptional activator VPR and targeted the promoters of three different endogenous genes in HEK293 human cells, using three customized crRNAs arrays for each one. The authors observed robust transcriptional activation with at least one crRNA for each of the three target genes. Thereafter, they engineered d*Lb*Cpf1 to convert it into a drug-inducible transcriptional modulator. For this, they created a split d*Lb*Cpf1 and fused one section to a four-DmrA tandem domain. The other section was fused to DmrC and p65 domains, in order to make activation dependent on the A/C heterodimerizer drug (Tak et al. 2017). These results open the way for new and improved approaches, as occurred with dCas9-based CRISPRa and CRISPR I/O.

Overall, information regarding the use of CRISPR/dCas9, or CRISPR/dCpf1, for gene activation and epigenome editing in plants remains lacking. Such studies are essential, however, for evaluating the relationship between chromatin modifications and transcriptional regulation, as well as the effectiveness of the targeted epigenetic regulators. Successful development of the above-mentioned fusions could enable new CRISPR tools to efficiently tune gene expression, as well as presenting other diverse biological applications.

10.3 Epigenome Editing

Epigenome editing refers to the targeted modification of epigenetic marks at particular genomic loci (Kungulovski and Jeltsch 2016) through the use of synthetic epigenome engineering tools (Thakore et al. 2016) or EpiEffectors (Kungulovski and Jeltsch 2016; Rienecker et al. 2016). These tools usually consist of a programmable DNA-binding domain fused to a catalytic domain from an epigenome-modifying enzyme (Hilton et al. 2015; Rienecker et al. 2016) or scaffolding effector domain (Thakore et al. 2016). As mentioned above, in the CRISPR/Cas9 system, the nuclease activity of the Cas9 protein has been eliminated by specific amino acid mutations at the RuvC-like and HNH domains (D10A and H840A, respectively; Jinek et al. 2012; Perez-Pinera et al. 2013; O'Geen et al. 2017), generating an RNA-guided DNA-binding protein with no enzymatic activity (dead or deactivated Cas9, or dCas9; O'Geen et al. 2017). The dCas9 protein is then fused to effector domains and can be directed to define genomic loci by specific base-pairing between an engineered sgRNA and the target DNA sequence to regulate gene transcription in a precise way (Hilton et al. 2015; La Russa and Qi 2015; O'Geen et al. 2017). For example, Guo et al. (2017) performed a CRISPR-ON (CRISPRa) system using a doxycycline-inducible dCas9-VP64-p65-Rta cassette to induce and control the expression of NANOG in human pluripotent stem cells. Similarly, fusion of dCas9 to the catalytic core of the human acetyltransferase p300 allows the targeted addition of the histone 3 lysine 27 acetylation activating mark (Hilton et al. 2015). Particularly, a dCas9$^{\text{p300 Core}}$ fusion protein has an increased transactivation capacity relative to dCas9VP64; it produces a higher transactivation

of downstream genes than does the full-length p300 protein, and activates transcription of endogenous genes from distal enhancer regions (Hilton et al. 2015). More recently, CRISPR-dCas9 was fused to histone deacetylases and tested in a murine neuroblastoma cell line, where deacetylation was observed, but the chromatin environment is an important element to consider (Kwon et al. 2017).

Furthermore, combining dCas9 to a transcriptional repressor domain can silence the expression of endogenous genes (CRISPRi; Gilbert et al. 2013; La Russa and Qi 2015). For instance, O'Geen et al. (2017) analyzed, in the human colon cancer cell line HCT116, dCas9 fusions with histone-modifying enzymes producing transcriptionally repressive marks and found that deposition of repressive histone marks via CRISPRi is feasible. In their experiments, however, transcriptional repression was independent of deposition of the expected repressive histone marks. Additionally, dCas9 can be fused to the DNA methyltransferase DNMT3A to add repressive DNA methylation marks. In this case, the activity of the dCas9-DNMT3A construct targets the CpG islands within the promoter regions of *IL6ST* and *BACH2* (important genes for *N*-glycosylation of IgG) and lowers their expression levels (Vojta et al. 2016). Another study provided the demonstration for targeted dosage control by epigenome editing (Rienecker et al. 2016). In plants, cytidine deaminase has been fused to CRISPR-Cas9, and tested in rice and tomato (Shimatani et al. 2017). Thus, up-regulation or down-regulation of target genes via artificial or synthetic transcriptional regulators is an attractive tool for targeted manipulation of the transcriptome.

Clearly, work on CRISPRa epigenome editing in plants is lacking, although its potential application in crops is one of the greatest challenges of the field (Liu and Moschou 2018). Epigenetic reprogramming in plants, or the ability to accomplish histone and DNA modifications, will have a novel effect on plant breeding. It may facilitate the control of quantitative phenotypic traits, help manipulate the metabolome to generate valuable nutrients, and lead to increased plant disease resistance and enhanced plant pathogen defense (Liu et al. 2017). In addition, inducible activation or repression of hub genes, homeotic genes, and defense genes could have enormous agronomical applications (even if the dCas9/sgRNA transgene must be present in the plant; Barakate and Stephens 2016).

It is extremely important, therefore, to engineer plant disease resistance and enhance plant pathogen defense without a negative impact on crop productivity by using CRISPRa. We believe that the recruitment of histone lysine methyltransferases (HKMT) to the promoter region of plant defense genes, via dCas9 or dCpf1 fusions, will modulate the epigenome and activate expression of individual target genes involved in plant defense against pathogens. Targeted epigenetic reprogramming and regulation of disease-responsive genes as a result of plant–pathogen interactions is significant because it is expected to help us understand (1) how the epigenetic component regulates plant gene expression and the plant phenotype; (2) how the epigenome operates as a powerful source of diversity for important agronomical traits; and (3) by what means its exploitation, in crop improvement programs, would benefit society.

10.4 Defense Priming and CRISPRa

Targeted deposition of chromatin modifications (epigenome editing via CRISPRa) holds great potential, and will be a powerful approach for functional studies of locus-specific chromatin modifications and their relationship to gene expression and plant defense against pathogens.

To selectively activate plant defense genes against pathogens, targeted epigenome editing can be achieved by fusing a set of epigenetic effectors to, for example, a plant dCas9 that is codon-optimized and catalytically inactive, in different conformations. As mentioned, Lowder et al. (2015) developed an easy-to-use, thorough molecular set of tools to consolidate and facilitate fast, wide-scale use of the CRISPR/Cas9 system for plant research. This toolbox is based on Golden Gate cloning and Gateway recombinant methods, and consists of a step-by-step protocol "to efficiently assemble functional CRISPR/Cas9 transfer DNA (T-DNA) constructs" for plant gene editing and transcriptional activation/repression of endogenous genes (Lowder et al. 2015). This system can be used to target transcriptional activation at specific plant defense genes by using dCas9 fused to the VP64 transcriptional activation domain or to the catalytic domain of epigenetic writers (e.g., HKMTs, histone acetyltransferases [HATs], etc.). In addition, it is feasible to use distinct inducible and/or constitutive promoters to test the dCas9 fusions during, for example, biotic stress (Fig. 10.3).

N-terminus and C-terminus dCas9 fusions to catalytic domains of, for example, HKMTs, HATs, or the VP64 transcriptional activator will be sufficient to produce and evaluate transcriptional active histone marks at different genomic loci (e.g., defense-related genes) and as a response to pathogen attack. Furthermore, the use of inducible versus constitutive promoters will allow the study of targeted and controlled defense responses.

Thus, to evaluate the effects of increased plant disease resistance and enhanced plant pathogen defense via CRISPRa, it is logical to study defense-related genes involved in systemic acquired resistance (Luna et al. 2012), which are also highly responsive to priming of defense (or induced resistance up-regulated by biological or applied chemical stimuli; Slaughter et al. 2012) and that have been evaluated in different plant species under various stressful conditions (Cohen et al. 2016). For example, genes such as pathogenesis-related gene 1 (*PR-1*; Zimmerli et al. 2000; Slaughter et al. 2012; Breen et al. 2017; Ramírez-Carrasco et al. 2017), WRKY transcription factor 29 (*WRKY29*; Jaskiewicz et al. 2011; Singh et al. 2014; Martínez-Aguilar et al. 2016), or *WRKY53* (Jaskiewicz et al. 2011; Hu et al. 2012; Luna et al. 2012; Martínez-Aguilar et al. 2016) are excellent candidates to be tested. These genes are all tightly associated with primed expression of defense mechanisms.

Epigenetic regulators associated with an active chromatin state and involved in the activation of plant defense genes are, for example, histone-modifying enzymes that "write" epigenetic marks associated with an active chromatin state in plants (e.g., HKMTs). These genes are mainly gene orthologs to: (1) the Arabidopsis trithorax 1 (*ATX1*) gene, coding for a histone H3 lysine 4 (H3K4me3) methyltransferase (Alvarez-

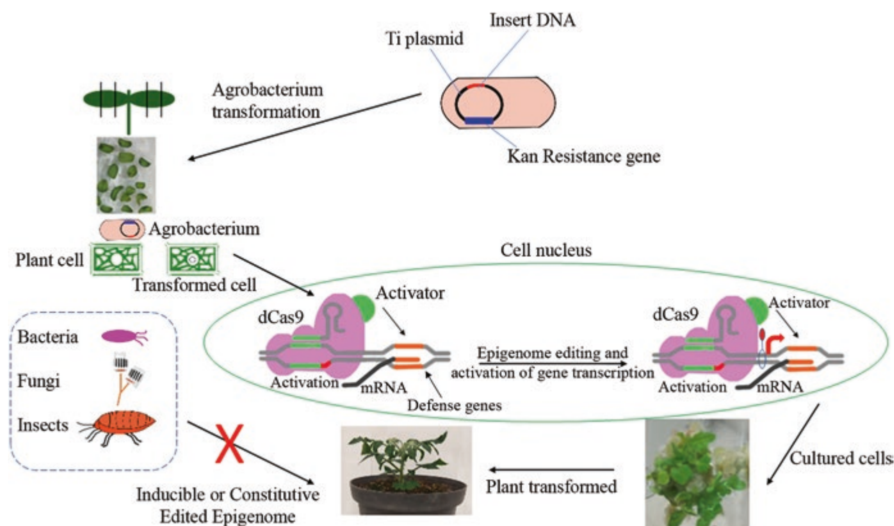

Fig. 10.3 Transcriptional activation of plant defense genes against pathogens in plants mediated by dCas9 fused to a transcriptional activation domain or to the catalytic domain of epigenetic writers. gRNAs and dCas9 fused to the transcriptional activation domain or to the catalytic domain of epigenetic writers are assembled into a T-DNA vector. Next, agrobacterium containing the assembled T-DNA is used to transform plant tissues. Selection is carried out for antibiotic resistance. The dCas9 synthetic transcriptional activator is guided to the target site by guide-RNAs. Activators and epigenetic writers stimulate transcription by recruiting and stabilizing diverse protein components of the eukaryotic transcriptional machinery. Plant tissues are collected, processed, and analyzed by RT-qPCR. Plant disease resistance and enhanced plant pathogen defense is analyzed by challenging the plants with insect pests and diseases

Venegas et al. 2003, 2007); (2) absent, small, or homeotic discs 1 homolog 2 (*ASH1 HOMOLOG 2* or *ASHH2*), a major H3K36 histone tri-methyltransferase that also methylates H3K4 (Xu et al. 2008); and (3) Arabidopsis trithorax-related 3 (*ATXR3*), the major enzyme responsible for H3K4me3 (Guo et al. 2010).

In addition, HATs can also be used for the activation of plant defense genes and any member of the following categories are excellent candidates: (1) HAG for HATs of the GNAT (GCN5-related N-terminal acetyltransferases) superfamily, (2) HAM for HATs of the MYST superfamily, (3) HAC for HATs of the CREB-binding protein family, and (4) HAF for HATs of the TATA-binding protein-associated factor family. Several HATs are related only to a certain development momentum or are tissue-specific; thus, selection of the correct HATs is a principal factor to take into account (reviewed in Wang et al. 2014). Nonetheless, practically any epigenetic writer can be fused to the dCas9 or dCpf1 proteins to regulate gene expression.

Currently, there are a few plant-specific vectors to be used for epigenome editing, which can be acquired from nonprofit global plasmid repository sites like ADDGENE. Specifically, it is possible to obtain plasmids (as empty backbone) to separately clone one, two, or three sgRNAs, or plasmids for the simultaneous assembly of two or three sgRNAs. In addition, a Gateway entry vector with pco-

dCas9-VP64 (plant codon-optimized, deactivated Cas9, VP64 activator fusion) is available, as well as a Multisite Gateway T-DNA entry vector (https://www.addgene.org/crispr/plant). Nevertheless, research on plant epigenome editing for gene activation (CRISPRa) remains missing, and information concerning the use of engineered dCas9 or dCpf1 under the control of inducible or constitutive promoters for the activation of plant defense genes is lacking.

10.5 Concluding Remarks and Future Perspectives

The simplicity and facility of CRISPR-Cas9 technology has many advantages over other genome-editing methods. This system is an excellent tool for genome and epigenome edition. An important issue working with plants is how to delivery and express the CRISPR system. Several transformation methods for plants have been developed (viruses, agrobacterium strains, or biolistics), but these methods must still be optimized. Genome and epigenome-editing tools provide new strategies for genetic and epigenetic manipulation in plants, by editing the epigenome and genes that confer the desired characteristics to the crop.

Targeted modification of epigenetic marks (targeted epigenome editing) to activate plant defense genes is a promising strategy for conferring resistance against pathogen attack. The recruitment of activating epigenetic writers and/or transcriptional activator domains via, for example, dCas9 or dCpf1 to the promoter region of plant-defense genes will allow positive epigenetic reprogramming and the manipulation of disease-responsive genes as a result of plant–pathogen interactions. This is significant because it will contribute to a broader understanding of the epigenetic mechanisms involved in plant–pathogen interactions, and help us understand how the epigenome operates as a source of diversity for essential agronomical traits.

To enhance sustainable agriculture, plants must be more vigorous and disease-resistant. Land and water are insufficient, however, and the excessive use of pesticides and fertilizers causes environmental damage. Thus, future exploitation of CRISPRa in crop improvement programs, in combination with biological control agents and the exploitation of elicitors of defense priming, will significantly reduce economic losses.

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Chapter 11
Chemical RNA Modifications: The Plant Epitranscriptome

Celso Gaspar Litholdo Jr. and Cécile Bousquet-Antonelli

Abstract RNA post-transcriptional modifications create an additional layer to control mRNA transcription, fate, and expression. Considering that they are non-genetically encoded, can be of reversible nature, and involved in fine-tuning gene expression, the landscape of RNA modifications has been coined the "RNA epigenome" or "epitranscriptome." Our knowledge of the plant epitranscriptome is so far limited to 3′-uridylation and internal m^{6}A and m^{5}C modifications in *Arabidopsis thaliana*. m^{6}A is the most abundant and well-studied modification on mRNAs, and involves the activities of evolutionarily conserved "writer" (methyltransferase), "reader" (RNA binding proteins), and "eraser" (demethylases) proteins. In Arabidopsis, m^{6}A is crucial for embryogenesis, post-embryonic growth, development, phase transition, and defense responses. Conversely to animals, our understanding of the roles of m^{6}A is limited to the finding that it is an mRNA stabilizing mark. Yet likely to exist, its roles in controlling plant mRNA maturation, trafficking, storage, and translation remain unexplored. The m^{5}C mark is much less abundant on the transcriptome and our knowledge in plants is more limited. Nonetheless, it is also an important epitranscriptomic mark involved in plant development and adaptive response. Here, we explore the current information on m^{6}A and m^{5}C marks and report knowledge on their distribution, features, and molecular, cellular, and physiological roles, therefore, uncovering the fundamental importance in plant development and acclimation of RNA epigenetics. Likely to be widespread in the green lineage and given their crucial roles in eukaryotes, the fostering of data and knowledge of epitranscriptome from cultivated plant species is of the utmost importance.

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11.1 Chemical RNA Modifications: A New Layer of Post-transcriptional Regulation

The pattern of gene expression of a cell is what determines its identity and activity. Maintaining its homeostasis is hence crucial for any organism. However, cells must also respond to developmental and environmental stimuli for organisms to develop and grow, or to acclimate to external conditions. In such cases, their pattern of gene expression needs to be adjusted, occasionally very fast. This reprogramming takes place simultaneously at the transcriptional (Kaufmann et al. 2010; de Nadal et al. 2011; Lelli et al. 2012) and post-transcriptional levels (Mata et al. 2005; Zhao et al. 2017; Schaefke et al. 2018). Post-transcriptional regulation is exerted at pre-messenger RNA (pre-mRNA) maturation (including transcription termination/polyadenylation and splicing), mRNA intracellular trafficking (including nucleocytoplasmic and sub-compartment localization), storage, stability, and translation. Regulation of the transcriptome is dependent on the primary genetic code, which provides local structures and short sequences, either for binding of proteins that form with the messenger RNA RiboNucleoProtein (mRNP) complexes or for complementary recognition by microRNAs (miRNAs).

In the last couple of years, the scientific community regained interest in RNA (in particular mRNA) chemical modifications, and recognized that they create an additional layer to the control of mRNA transcription and fate. RNA modifications are non-genetically encoded and can display a reversible nature. Their landscape on the transcriptome (in particular on mRNAs) of a cell has hence been coined the "RNA epigenome" (He 2010) or "epitranscriptome" (Meyer et al. 2012; Saletore et al. 2012).

In all three domains of life (Archaea, Bacteria, and Eukarya) as well as in viruses, RNAs carry chemical modifications. More than 110 distinct modifications (http://mods.rna.albany.edu/mods/) have been recognized across all domains of life and across all types of RNAs [mRNAs, ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), long noncoding RNAs (lnRNA), circular RNAs (circRNAs), and small noncoding RNAs (sRNAs)] but the roles of the vast majority of them remain unknown (Li and Mason 2014). Although highly debated until 2012, the existence of chemical modifications deposited on mRNAs is now well recognized and documented in several eukaryotes, such as yeast, mammals, and plants, as well as recently in bacteria (Deng et al. 2015; Hoernes et al. 2015). In addition to the 5′ cap and 3′-poly(A) tail, eukaryotic mRNA 3′-extremities can be modified by the non-templated addition of uridines (uridylation; de Almeida et al. 2018a; Li et al. 2016a) and/or carry internal modifications, which can be of over 15 different types (http://mods.rna.albany.edu/mods/; Song and Yi 2017).

The most common of the internal nucleotide modifications consists in the addition of a methyl group to the 2′-O position of the ribose moiety. In addition, up-to-date transcriptome-wide mapping on mRNAs and functional data are available on transcripts that can be edited by deamination of adenosine to inosine (A-to-I editing;

Yablonovitch et al. 2017; Sinigaglia et al. 2018) or carry N[1]-methyladenosine (m^1A; Dominissini et al. 2016; Li et al. 2016b), N[6]-methyladenosine (m^6A; Dominissini et al. 2013), 5-methylcytosine (m^5C; Squires and Preiss 2010), N[4]-acetylcytidine (ac^4C; Arango et al. 2018), pseudouridine (Ψ; Schwartz et al. 2014a; Carlile et al. 2014), or hydroxymethylcytosine (h^5mC) (Fig. 11.1a, b). Additional modifications include the N[6]-2′-O-dimethyladenosine (m^6Am) and 5-hydroxymethylcytosine

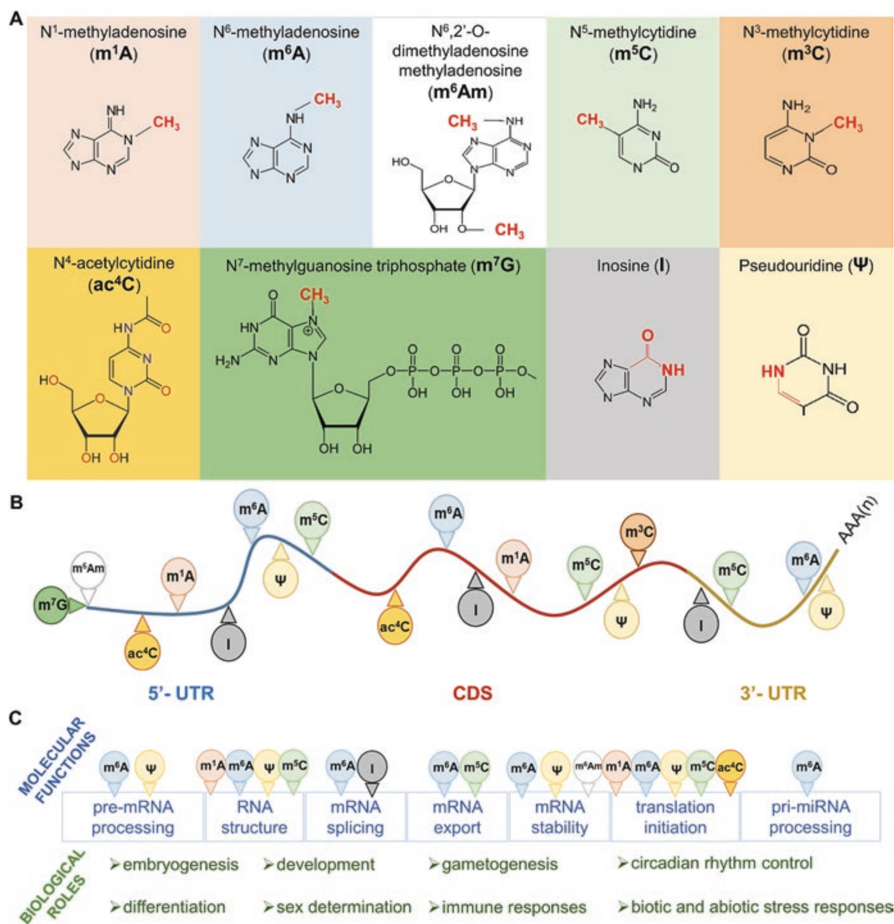

Fig. 11.1 The epitranscriptome landscape. (**a**) The major post-transcriptional modifications deposited on the transcriptome of mammalian cells are N[1]-methyladenosine (m^1A), N[6]-methyladenosine (m^6A), N[6],2′-O-dimethyladenosine methyladenosine (m^6Am), N[5]-methylcytosine (m^5C), N[3]-methylcytosine (m^3C), N[4]-acetylcytidine (ac^4C), N[7]-methylguanosine triphosphate (m^7G), inosine (I), and pseudouridine (Ψ). (**b**) An RNA polymerase II transcribed RNA is represented, including the 5′-cap structure, which is a modified 7-methylguanosine (m^7G) linked via an unusual 5′ to 5′ triphosphate linkage to mRNA, and the 3′-end poly(A) tail (AAAn). For each particular RNA chemical modification, a representation is shown in relation to mRNA position (5′ UTR, blue; CDS, red or 3′ UTR, yellow). (**c**) The molecular consequences of each RNA marks and the biological roles of these modifications are also represented. It is important to mention that only m^6A and m^5C modifications have been identified so far in the plant epitranscriptome

(h^{5}mC) (Song and Yi 2017; Frye et al. 2018). These modifications can regulate all steps of an mRNA life (Fig. 11.1c) and can even recode open reading frames (Powers and Brar 2018). Several were proposed to be of a dynamic nature (i.e., they can be erased) and their profiles found to be distinct across development or in response to stress exposure. At the organism level, RNA modifications are required for differentiation, development, gametogenesis, sex determination, embryogenesis, circadian rhythm control, immune response, biotic and abiotic stress responses (Fig. 11.1c, Sinigaglia et al. 2018; Song et al. 2017).

Except for the 5′-cap and poly(A)-tail, our knowledge of the plant epitranscriptome is so far limited to uridylation (de Almeida et al. 2018a, b), m^{6}A (Luo et al. 2014; Li et al. 2014b, 2018), and m^{5}C (Cui et al. 2017; David et al. 2017). Plant mRNAs are likely to carry other types of modifications but their existence and roles remain to be explored. A-to-I editing though is absent from the plant nuclear transcriptome but organelle transcripts (chloroplast and mitochondria) carry C-to-U edited bases, and in ferns and mosses also U-to-C changes (Takenaka et al. 2013). Excellent reviews have recently been published on the synthesis, molecular, cellular, and physiological roles of uridylation (de Almeida et al. 2018a, b), and organelle editing (Takenaka et al. 2013). We will hence focus the present chapter on the features and functions of the internal m^{6}A and m^{5}C modification of messenger RNAs in plants.

11.2 Roles and Features of the m^{6}A Mark in Plants

11.2.1 General Features of the m^{6}A Mark

The m^{6}A mark is the most abundant and widespread of mRNA modifications. It has been profiled on the polyadenylated transcriptome of the yeast *Saccharomyces cerevisiae* (Schwartz et al. 2013) and of various human and mouse cell lines and tissues (Dominissini et al. 2012; Meyer et al. 2012; Fustin et al. 2013; Schwartz et al. 2014b; Wang et al. 2014; Chen et al. 2015). In higher plants, it has been mapped on rice callus and leaves (Li et al. 2014b), in two distinct ecotypes of *Arabidopsis thaliana* (Luo et al. 2014), and in mature leaves (Anderson et al. 2018), 5- and 14-day old seedlings (Shen et al. 2016; Duan et al. 2017) and across several organs (leaves, flowers, and roots; Wan et al. 2015) of Arabidopsis Columbia-0 ecotype. Consistent with the evolutionarily conserved nature of the m^{6}A mark, several of its features were found to be conserved across organisms and tissues. Transcriptome-wide, m^{6}A represents 1–1.5% of the total number of adenosines on polyadenylated transcripts. It mostly localizes in the 3′-UTRs, following the stop codon and in the last exons of transcripts (Ke et al. 2015). A nucleotide sequence context around m^{6}A is shared across eukaryotes. Indeed, m^{6}A is mainly confined at the consensus RRACH (where R = A/G and H = U > A > C) and found in 70% of the cases at GAC. In mammals at least, the m^{6}A mark was detected on most, if not all, polymerase II transcribed RNAs, including primary transcripts of miRNAs (Alarcon et al. 2015), lnRNAs, circRNAs, and mRNAs (Meyer et al. 2012; Dominissini et al. 2013; Schwartz et al. 2013).

In plants, a thin layer chromatography analysis of the m^{6}A/A ratio on the polyadenylated transcriptome of Arabidopsis shows that it ranges from 0.9% in roots and leaves to 1.4% in flowers (Zhong et al. 2008) and that it is not randomly distributed, but mostly enriched at the 3′-end of transcripts (Bodi et al. 2012). Subsequently, next generation sequencing (NGS) profiling of the polyadenylated transcriptome found, both in rice and Arabidopsis, that the vast majority of the m^{6}As peaks occur in the 3′-UTRs or overlap the stop codon (Li et al. 2014b; Luo et al. 2014; Anderson et al. 2018; Shen et al. 2016; Wan et al. 2015). These studies in rice and Arabidopsis also found that 10–15% of the detected m^{6}A peaks are located around the start codon (Li et al. 2014b; Luo et al. 2014; Shen et al. 2016). The presence of some m^{6}A marks around the start codon and in 5′-UTR is not restricted to plants, for instance, this has been observed in certain mammalian cells types and growth conditions (Dominissini et al. 2012; Zhou et al. 2015). Most of the m^{6}A peaks were found to carry the RRACH consensus suggesting that this sequence motif is necessary also in plants for the deposition of the mark. However, recent findings support the idea that m^{6}A sites could occur in sequence contexts other than RRACH [such as "GGAU" or URUAY (R = G > A, Y = U > A)] in Arabidopsis (Luo et al. 2014; Anderson et al. 2018; Shen et al. 2016; Wei et al. 2018) and rice (Li et al. 2014b). Whether other types of plant RNA polymerase II transcripts (such as pre-miRNAs, lnRNAs, and sRNAs) are modified with m^{6}A remains to be explored.

In mammals and flies at least, the m^{6}A mark is deposited co-transcriptionally by a conserved heteromultimeric complex called the "writer" complex and can be reverted to unmodified adenines by demethylases tagged as "erasers" (see Sect. 11.2.2; Fig. 11.2). At the molecular level, the most prevalent role of m^{6}As is to influence the binding of proteins to their RNA targets. They can either act to repel or attract RNA binding proteins (RBPs), the latter of which are known as "m^{6}A readers" (Arguello et al. 2017; Edupuganti et al. 2017). Readers convey the m^{6}A signal by directly controlling the fate of their RNA target and/or by recruiting effector proteins. The m^{6}A mark recruits readers by two main processes. First, the reader may carry a YTH domain, an evolutionarily conserved RNA binding motifs whose folding forms a pocket that tightly accommodates the m^{6}A residue (see Sect. 11.2.4; Fig. 11.2). Alternatively, the presence of m^{6}A may positively influence the recruitment of RBPs by: (1) increasing their affinity for their RNA binding region, or (2) acting through alteration of RNA structures in a mechanism called "m^{6}A-switch" (Zhou et al. 2016; Roost et al. 2015; Liu et al. 2015).

11.2.2 The Plant Writer and Eraser Systems

In 1994, Bokar and colleagues characterized and partially purified an mRNA N^6-methyltransferase from HeLa cell nuclei. They found that it comprises a multisubunit complex composed of two fractionable subcomplexes: MT-A (200 kDa) and MT-B (875 kDa) containing the S-adenosyl-methionine-binding site and the RNA binding site, respectively (Bokar et al. 1994). The MT-A subcomplex carries on a 70 kDa component, the methyltransferase player that was identified and named

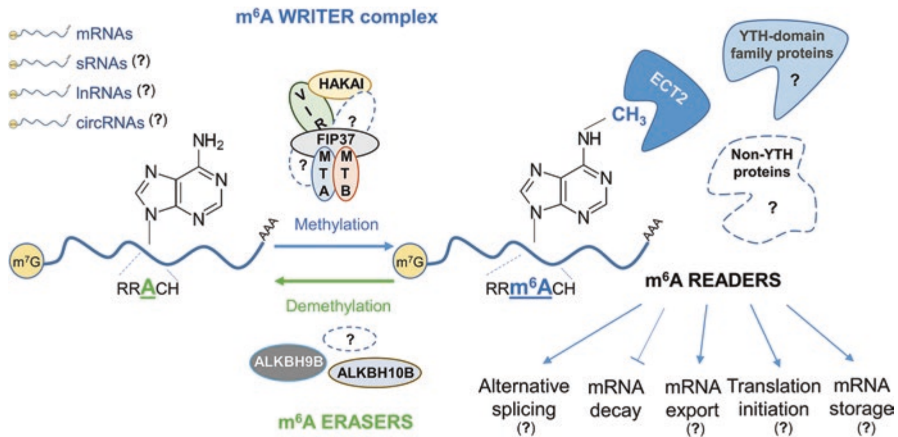

Fig. 11.2 The m⁶A modification regulatory system. The m⁶A mark is found in most, if not all, RNA polymerase II transcribed RNAs, including messenger RNAs (mRNAs), small RNAs (sRNAs), long noncoding RNAs (lnRNAs), and circular RNAs (circRNAs); except the latter, all contain the modified m⁷G nucleotide at the 5′-end and poly(A) tail at the 3′-end. A nucleotide consensus sequence RRACH (R = A/G, H = U > A > C) is mainly the site for the m⁶A writer complex, which includes the subunit methyltransferase proteins MTA, MTB, FIP37, VIR, and HAKAI. m⁶A-RNA pol II RNA demethylation is carried by two eraser enzymes, ALKBH9B and ALHBH10B. So far only m⁶A readers carrying a YTH-RNA binding domain have been identified in plants, which include the recently characterized ECT2 protein. The molecular role of m⁶A mark depends on the reader protein that binds to the modified nucleotide, generally in animals, directing the RNA to alternative splicing, mRNA decay, mRNA export, translation initiation, or mRNA storage. Question mark (?) indicates the unknown features of the plant m⁶A regulatory system

MT-A70 (Bokar et al. 1997). MT-A70 is conserved across eukaryotes and is known as METTL3 in mammals (Liu et al. 2014), IME-4 (Inducer of Meiosis-4) in *S. cerevisiae* (Yadav and Rajasekharan 2017) and *Drosophila melanogaster* (Lence et al. 2016), and MTA70 in *A. thaliana* (Zhong et al. 2008).

Purification of the writer complex from animals (human and fly) confirmed that it is an heteromultimeric complex, whose catalytic core is composed of two RNA methyltransferases (METTL3 and METLL14) and the cofactor WTAP (fly Fl(2)d). METTL3 and 14 physically interact with each other and their association has a synergetic effect on the complex catalytic activity (Liu et al. 2014). METTL3 is the catalytically active component while METTL14, which has a degenerate methyltransferase site, plays a scaffolding role that is critical for substrate recognition (Wang et al. 2016; Śledź and Jinek 2016). The animal writer complex contains other subunits: VIRMA (fly Virilizer), RBM15/RBM15B (fly spenito), ZC3H13 (fly Xio/Flacc), and HAKAI (Ping et al. 2014; Yue et al. 2018; Haussmann et al. 2016; Lence et al. 2016; Guo et al. 2018; Knuckles et al. 2018; Patil et al. 2016). ZC3H13 bridges the mRNA binding factor RBM15 to WTAP (Knuckles et al. 2018) and VIRMA mediates preferential methylation by recruiting the METTL3/METTL4/WTAP core complex to 3′-UTRs and near the stop codons (Yue et al. 2018).

Up to now, data on the plant writer complex comes from *A. thaliana* (Table 11.1; Zhong et al. 2008; Bodi et al. 2012; Ruzicka et al. 2017). Following the discovery

Table 11.1 The Arabidopsis m^{6}A modification regulatory system

Function	Name	Arabidopsis locus	Mammalian homolog	Biological role	References
mRNA m^{6}A writer	MTA	AT4G10760	METTL3	Embryo development	Zhong et al. (2008) Bodi et al. (2012)
	MTB	AT4G09980	METTL14	Embryo development	
	FIP37	AT3G54170	WTAP	Meristem maintenance	Shen et al. (2016)
	VIRILIZER	AT3G05680	KIAA1429	Embryo development	Ruzicka et al. (2017)
	HAKAI	AT5G01160	HAKAI	Embryo development	
mRNA m^{6}A eraser	[a]ALKBH9A	AT1G48980	ALKBH5	–	Duan et al. (2017)
	ALKBH9B	AT2G17970	ALKBH5	Viral infection response	Martínez-Pérez et al. (2017)
	[a]ALKBH9C	AT4G36090	ALKBH5	–	Duan et al. (2017)
	[a]ALKBH10A	AT2G48080	ALKBH5	–	
	ALKBH10B	AT4G02940	ALKBH5	Flowering	
	Name	*Arabidopsis locus*	*YTH domain evolutionary subtype*	*Biological role*	*References*
YTH m^{6}A readers	[a]ECT1	AT3G03950	DF-A	Calcium-mediated signaling	Ok et al. (2005)
	ECT2	AT3G13460	DF-A	Leaf and trichome morphogenesis	Scutenaire et al. (2018), Wei et al. (2018), Arribas-Hernández et al. (2018)
	ECT3	AT5G61020	DF-A	Leaf and trichome morphogenesis	
	ECT4	AT1G55500	DF-A	Leaf morphogenesis	Arribas-Hernández et al. (2018)
	[a]ECT5	AT3G13060	DF-B	–	Ok et al. (2005), Scutenaire et al. (2018)
	[a]ECT6	AT3G17330	DF-C	–	
	[a]ECT7	AT1G48110	DF-C	–	
	[a]ECT8	AT1G79270	DF-B	–	
	[a]ECT9	AT1G27960	DF-B	–	
	[a]ECT10	AT5G58190	DF-B	–	
	[a]ECT11	AT1G09810	DF-C	–	
	[a]ECT12	AT4G11970	DC-B	–	Scutenaire et al. (2018)
	[a]CPSF30-L	AT1G30460	DC-A	[b]Nutrient uptake/ oxidative stress response	Scutenaire et al. (2018), Li et al. (2017a, b)

[a]These uncharacterized genes are potential players of m^{6}A regulation

[b]CPSF30-L isoform contains most of the short form polypeptide fused at its C-terminus with a canonical YTH domain of the DC-type; however, it is unknown if the biological role involves the m^{6}A and the CPSF30-L reader function

by Bokar et al. (1997) that the methyltransferase activity of the writer complex was carried by METTL3, further characterization of the complex remained incomplete. It is in 2008 that the team of Rupert Fray ran the first functional study of an MTA70 protein and also identified FIP37 (the Arabidopsis homolog of WTAP) as a component of the writer complex (Zhong et al. 2008). Further biochemical characterization of the Arabidopsis writer complex showed that it also contains MTB (the plant homolog of METTL14), VIRILIZER, and HAKAI (Ruzicka et al. 2017). The Arabidopsis writer complex hence closely resembles the animal complex, but, whether it contains additional factors in particular homologs of RBM15 and ZC3H13 remains to be explored. Every component of the Arabidopsis complex is found in the nucleoplasm. However, their nucleoplasmic distribution changes between root meristematic cells and cells in the root elongation zone. While showing a nucleoplasmic diffuse pattern in non-differentiated cells, they localize to nuclear speckles in dividing cells (Ruzicka et al. 2017). These observations support the idea that m^6A deposition is likely co-transcriptional in plants, as in animals, and that the activity of the writer complex might be regulated. Total or partial loss of any of the five components, except for HAKAI, of the Arabidopsis writer complex drastically decreases the total levels of m^6A in polyadenylated transcripts (Zhong et al. 2008; Ruzicka et al. 2017). HAKAI is not required for plant viability (see Sect. 11.2.3) and shows only a 35% reduction of m^6A levels in loss-of-function mutants. Except for MTA70, which based on evolutionary analyses (Bujnicki et al. 2002) is a *bona fide* methyltransferase and homolog to METTL13, the molecular roles that other components carry out inside the writer complex remain to be uncovered in plants.

The m^6A epitranscriptomic mark was proposed to be dynamic following two reports that identified mammalian FTO (fat mass and obesity) and ALKBH5 (the alkylation repair homolog protein) as specific RNA m^6A demethylases, both *in vitro* and *in vivo* (Jia et al. 2011; Zheng et al. 2013). They both belong to the AlkB subfamily of Fe(II)/ α-Ketoglutarate-dependent dioxygenases superfamily that has 9 members (ALKBH1-8 and FTO) in humans (Xu et al. 2014a). Enzymes of the ALKB family excise the methyl group through a two-step oxidative alkylation process and can act on DNA or RNA. Both FTO and ALKBH5 are found in nuclear speckles, suggesting that erasing of mRNA m^6A is mostly nuclear (Jia et al. 2011; Zheng et al. 2013). In mice, loss of FTO leads to increased m^6A levels and is associated with several metabolic disorders and cell differentiation (Zhoa et al. 2014), while loss of ALKBH5 also affects m^6A levels and is characterized by impaired fertility resulting from spermatocyte apoptosis (Zheng et al. 2013). These findings indicate that these two demethylases function in different physiological processes and strongly suggest that they are crucial for the development and reproduction.

The Arabidopsis genome codes for thirteen proteins of the ALKB family, among which, based on sequence alignment, five (ALKBH9A, 9B, 9C, 10A, and 10B) are potential homologs of the mammalian ALKBH5 m^6A-RNA demethylase (Table 11.1; Mielecki et al. 2012; Duan et al. 2017). The Arabidopsis genome codes for a sixth putative homolog of human ALKBH5 (AtALKBH10C), but it is most likely not an active demethylase as it has a degenerate catalytic site (our unpublished data).

Besides Arabidopsis, these enzymes can be found in agronomically important plants, for instance, the presence of *ALKB* demethylase orthologues was detected in *Nicotiana sylvestris* (Li et al. 2018), *Zea mays*, *Oryza sativa*, *Marchantia polymorpha*, and *Solanum lycopersicum*. No homolog of the FTO demethylase was found to exist in plant genomes (our unpublished data). Based on transcript level measurements, *ALKBH9B, 9C,* and *10B* are the most expressed of all five Arabidopsis *ALKBH5* genes. Across development, it is always one (or few) of these three genes, whose transcript levels show the highest expression. In seedlings and leaves (juvenile, adult, and cauline), *ALKBH9B*, *9C,* and *10B* mRNAs show similar levels and are by far the most highly expressed genes. In buds and young siliques, *9B* and *10B* are almost the sole demethylases to be expressed and they show similar levels. Finally, *9B* is nearly the only demethylase expressed in the apical meristem and *10B* is by far the major eraser gene to be expressed in flowers and matured siliques. Recently, *in vitro* assays showed that ALKBH9B and 10B have m^{6}A-demethylase activities on RNA (Duan et al. 2017; Martínez-Pérez et al. 2017) and 10B was shown to have a demethylase activity *in planta* on polyadenylated transcripts (Duan et al. 2017). ALKBH10B-mediated mRNA demethylation is required for the proper transition from vegetative to reproductive stage. This is at least in part linked to the role of ALKBH10B in demethylating, in a timely manner, transcripts required for the floral transition and as a result stabilizing them (Duan et al. 2017). Arabidopsis ALKBH9B, was so far not found to affect m^{6}A levels *in vivo*, but one cannot exclude the possibility that it works redundantly with other ALKBH5 orthologues, such as ALKBH9C. ALKBH9B was found to influence m^{6}A abundance on the viral genome of Alfalfa mosaic virus (AMV) and regulate its infectivity (Martínez-Pérez et al. 2017).

It is important to note that the dynamic nature of m^{6}A on mRNAs (e.g., the erasing of the m^{6}A marks on mature cytoplasmic transcripts) is still highly debated in the scientific community (Rosa-Mercado et al. 2017). Nonetheless, mRNA demethylases were found to exist and to be evolutionarily conserved, their downregulation and overexpression shown to significantly alter the pattern of m^{6}As on the polyadenylated transcriptome, and their loss to have drastic physiological impacts. Hence, they have roles to play in m^{6}A-based post-transcriptional regulation, however, where and how do they intervene remains to be understood.

11.2.3 m^{6}A Physiological, Cellular, and Molecular Roles

The biological consequences of m^{6}A methylation are multiple, but a common feature of most organisms is that it has pleiotropic physiological functions and is necessary for reproduction, differentiation, growth, development, biotic and abiotic stress responses. Arabidopsis is no exception to this. Except for HAKAI, loss-of-function and hypomorphic mutants of any of the constituents of the plant writer complex show total to drastic decrease of the levels of m^{6}A on the polyadenylated transcriptome and display identical phenotypes (Ruzicka et al. 2017). Complete loss of the

m^{6}A mark results in embryogenesis defects leading to lethality of the embryos, whose development is arrested at the globular stage (Vespa et al. 2004; Zhong et al. 2008; Bodi et al. 2012; Shen et al. 2016; Ruzicka et al. 2017). Downregulation of N^6-methyladenosines at post-embryonic stages has drastic pleiotropic consequences. Plants show delayed growth and development with reduced apical dominance (Bodi et al. 2012; Shen et al. 2016; Ruzicka et al. 2017). Seedlings with reduced levels of m^{6}A show an over proliferation of the vegetative shoot apical meristem (SAM), accompanied by a dramatic delay in leaf emergence and aberrant leaf morphology (Shen et al. 2016; Arribas-Hernández et al. 2018). Plantlets, with very low levels of m^{6}A, fail to develop a reproductive SAM and eventually die (Shen et al. 2016). Hypomethylated plants also show trichome morphogenesis defects, with leaves accumulating overbranched trichomes, due to abnormally high ploidy levels (Vespa et al. 2004; Bodi et al. 2012; Scutenaire et al. 2018). Root growth and development also require normal m^{6}A levels. Indeed, hypomethylated mutants show reduced root growth, aberrant gravitropic responses, abnormal root cap formation, and deficient vascular development (linked to defective protoxylem development).

The m^{6}A mark and its control is also most likely necessary not only for the response of the plant to viral infection (Martínez-Pérez et al. 2017; Li et al. 2018) but also for environmental growth conditions and stress exposure (Luo et al. 2014; Anderson et al. 2018). In Arabidopsis, the viral RNA of AMV was found to have m^{6}A residues upon infection and to be demethylated *in vivo* by ALKBH9B (Martínez-Pérez et al. 2017). Loss of ALKBH9B provokes a hypermethylation of the viral RNA and downregulates AMV replication and infectivity. The current model suggests that m^{6}A could control AMV viral infection by signaling the viral transcript to the nonsense mediated decay (NMD) pathway. This m^{6}A-based response to viral infection is likely not restricted to AMV (Martínez-Pérez et al. 2017), nor to Arabidopsis. Recently, a report by Li et al. (2018) correlated endogenous m^{6}A-levels to tobacco mosaic virus (TMV) infection in *N. tabacum*. Upon infection, global m^{6}A content decreased and the levels of transcripts coding for putative homologs of ALBKH5 and MTA70 were, respectively, up- and downregulated. These observations support a putative m^{6}A-mediated control of viral infection in tobacco as well. Methylome profiling of the transcriptomes of two Arabidopsis accessions [Can-0 (from Canary Islands) and Hen-16 (from Northern Sweden)] shows that most methylation peaks are shared by both ecotypes, supporting the crucial role of m^{6}A-mediated regulation in development. Nonetheless, a portion of the detected methylated sites are specific to each ecotypes, and the presence of m^{6}A correlates with highest expression levels of the marked genes. Considering the Can-0 and Hen-16 are originally from very distinct climates, one can postulate that m^{6}A could play a role in plant acclimation to the environment (Luo et al. 2014). Along the same idea, a recent work by the Gregory lab (Anderson et al. 2018), profiled m^{6}A on the transcriptome of salt treated Arabidopsis leaves and found that upon stress, transcripts coding for salt and osmotic stress response proteins gain m^{6}A and are stabilized. This supports a role of m^{6}A in promoting the plant response to stress, at least salinity.

Our understanding of the molecular and cellular bases of m^{6}A physiological functions in plants is so far quite modest and limited to their role in the control of cytoplasmic mRNA stability (Luo et al. 2014; Shen et al. 2016; Duan et al. 2017; Wei et al. 2018; Anderson et al. 2018). At the global transcriptome scale, m^{6}A acts to stabilize transcripts by preventing their endonucleolytic cleavage (4–5 nt upstream to the mark) and subsequent 5′-3′ digestion by XRN4, the plant homolog of XRN1 (Anderson et al. 2018). This is coherent with previous observations showing that the m^{6}A mark correlates with elevated transcript levels (Luo et al. 2014). However, this is opposite to the situation in animals where the m^{6}A mark is an mRNA-decay triggering signal at the global level (Ke et al. 2015, 2017). This transcriptome-wide observation does not stand for all Arabidopsis mRNAs, as there are cases where the presence of m^{6}A directs a signal to turnover. Shen et al. (2016) found that the lack of m^{6}A on two key SAM regulators (*WUSCHEL* and *SHOOTMERISTEMLESS*) prevents the timely degradation of their transcripts and proper regulation of SAM proliferation. Furthermore, ALKBH10B-mediated demethylation was found to stabilize transcripts of *FLOWERING LOCUS T* (*FT*), *SPL3,* and *SPL9*, which are key regulators of the floral transition (Duan et al. 2017).

In animals, m^{6}A also acts as a translation stimulatory signal, at transcriptome-wide level, and is known to control a handful of alternative splicing events (Lence et al. 2016; Haussmann et al. 2016), directs primary miRNA transcripts to processing (Alarcon et al. 2015), and acts directly on chromatin, where it contributes to DNA repair (Xiang et al. 2017) and to the XIST-dependent gene silencing (Patil et al. 2016). Whether m^{6}A also acts on these processes in plants remains to be explored.

11.2.4 The Plant m^{6}A Readers: YTH-domain-Containing Proteins

So far, only one type of m^{6}A readers has been recognized in plants: those containing YTH domains. The YT521-B Homology domain (YTH) is a highly structured conserved RNA binding domain among eukaryotes. After being first identified as a human splicing factor, YT521-B proteins carrying a YTH domain (now called YTHDC1) were further identified and classified as DC type (YTH-domain-containing protein) and DF type (YTH-domain family proteins), depending on the subcellular localization (Imai et al. 1998; Hartmann et al. 1999; Stoilov et al. 2002; Zhang et al. 2010). A recent analysis of YTH domains from yeast, metazoan and Viridiplantae, found that they are of two evolutionary types: the DC-type group comprising YTH domains of human YTHDC1 and 2 and the DF-type group containing human YTHDF1-3 (Scutenaire et al. 2018).

The structural resolution of YTH domains from yeast and animal proteins showed that both DC- and DF-type motifs adopt a conserved canonical fold of three α-helices and six β-strands that creates an aromatic pocket (formed with three highly conserved tryptophan residues) that tightly accommodates m^{6}A (Li et al. 2014a; Luo and Tong 2014; Theler et al. 2014; Xu et al. 2014b; Zhu et al. 2014; Xu et al. 2015).

Sequence comparisons support that the m^{6}A-binding mode of the YTH domains is largely conserved across eukaryotes (Scutenaire et al. 2018).

In plant genomes, genes coding for YTH-domain proteins experienced a large expansion with thirteen genes in Arabidopsis (Table 11.1). Viridiplantae YTH-proteins also carry DC- and DF-type domains that are further subdivided into two (DCA and DCB) and three (DFA, DFB, and DFC) subgroups, respectively. This observation suggests that plant YTH domain likely underwent neo-functionalization and that they are not fully redundant (Scutenaire et al. 2018).

In plants, all the functional work done on YTH-domain m^{6}A readers is from Arabidopsis. Arabidopsis YTH domain was initially identified in two proteins found to directly bind the CIPK1 (Calcineurin B-like-interacting protein kinase 1) calcium-dependent kinase. Eleven proteins were found to share the YTH domain at their C-terminus and called ECT1 to 11 (for evolutionarily conserved C-terminal region) (Ok et al. 2005). Subsequent searches identified two additional proteins, which are of the DC-type (while ECT1-11 is of DF-type): ECT12 of unknown function and CPSF30-L, which is encoded by the long isoform of the gene encoding CPSF30, the cleavage and polyadenylation subunit factor 30 (Addepalli and Hunt 2007).

The physiological and molecular roles of ECT proteins have been just recently explored with the first functional analysis of a plant m^{6}A reader, the Arabidopsis ECT2 protein. *In vitro* and *in planta* assays showed that ECT2 binds to m^{6}A-containing RNAs and requires an intact aromatic pocket (Scutenaire et al. 2018; Wei et al. 2018). *ECT2* transcript is the most abundant and ubiquitously expressed of all ECTs, nonetheless, the pattern of expression of its protein is distinct (Scutenaire et al. 2018; Wei et al. 2018; Arribas-Hernández et al. 2018). Consistently with its expected role as m^{6}A reader, *ect2* loss-of-function mutants, although not displaying dramatic phenotypes, recapitulate some of the defects observed in hypomethylated plants. First, ECT2 and its m^{6}A-reading activity were found to be required for proper trichome morphogenesis (Scutenaire et al. 2018; Wei et al. 2018; Arribas-Hernández et al. 2018). In the absence of ECT2, or the sole presence of a mutant allele coding for a protein with a mutated aromatic pocket, trichomes are over-branched—a phenotype that arises from increased ploidy levels. ECT3 was also found to be required for normal trichome morphogenesis, acting together (but not redundantly) with ECT2. ECT2 and ECT3 were also found to act redundantly to ensure the timely emergence and proper leaf formation. This role also requires their m^{6}A reading activities (Arribas-Hernández et al. 2018). Leaf morphogenesis also requires ECT4 but solely in backgrounds where both ECT2 and ECT3 are absent.

The loss of ECT2 induces the rapid downregulation, through degradation, of three trichome-morphogenesis transcripts (*TTG1*, *ITB1,* and *DIS2*) that carry m^{6}A. This observation is consistent with the role of ECT2 as m^{6}A reader, as in its absence, the m^{6}A-signal is likely improperly decoded and transcripts targeted for degradation. Furthermore, it also suggests that aberrant trichome morphogenesis could be, at least in part, the consequence of the improper expression of these three transcripts (Wei et al. 2018).

In planta, ECT2 accumulates mostly in the cytoplasm, but is also found in the nucleus. Upon stress-induced downregulation of translation initiation (heat and

osmotic stresses), ECT2 relocalizes to stress granules, which are messenger ribonucleoprotein particles (mRNPs) triage and storage centers, also containing factors of the translation machinery. The formation of cytoplasmic foci upon stress is also a feature of ECT4, but not ECT3, which is coherent with the presence in ECT2 and ECT4 (but not ECT3) of YPQ-rich regions, reminiscent of that found in human YTHDF proteins and aggregation-prone factors. The dynamic and complex subcellular distribution of these readers suggests that they might decode the m^6A signal in several post-transcriptional processes, such as splicing/maturation and/or nucleocytoplasmic export step.

11.3 The m^5C Epitranscriptomic Mark in Plants

Compared to m^6A modification, m^5C is less abundant and much less research has been conducted so far. Transcriptome-wide m^5C represents 0.4% of the total number of cytosines on human polyadenylated transcripts (Squires et al. 2012), whereas m^6A represents 1–1.5% of the adenosines on mRNA (Ke et al. 2015). This cytosine methylation mark is widespread and mainly detected in tRNAs and rRNAs, affecting RNA conformational structure and translational process (Chow et al. 2007; Motorin and Helm 2010; Squires and Preiss 2010), but it was also identified in mRNAs and noncoding RNAs (Squires et al. 2012). Consensus sequence for m^5C sites has been distinguished in Archaea, and until recently, none were found in animal and plant species (Edelheit et al. 2013). However, two enriched sequence motifs around m^5C sites were recently detected in Arabidopsis, with the most significantly enriched motif at the consensus HACCR (where $H = U > A > C$ and $R = A/G$) (Cui et al. 2017). Additionally to the consensus motif, David et al. (2017) suggested that RNA secondary structure may also be important to confer methylation at m^5C sites, by demonstrating that a 50-nucleotide sequence flanking at m^5C site is essential for methylation in a transient expression system in *N. benthamiana* (David et al. 2017).

The Arabidopsis transcriptome-wide profiling of m^5C-containing RNAs has been recently mapped by two distinct approaches. First, David et al. (2017) identified more than a thousand m^5C sites in mRNAs, lnRNAs, and sRNAs by RNA bisulfite sequencing, using several tissues and RNA methyltransferase mutants. Quantitative differences in methylated sites between roots, shoots, and siliques revealed a dynamic pattern to suggest a tissue-specific function of m^5C modification (David et al. 2017). The second approach, using RNA immunoprecipitation followed by deep-sequencing, also revealed a tissue-specific regulation of m^5C in various tissues and at different developmental stages (Cui et al. 2017). Thousands of m^5C sites were found to be enriched around start and stop codons of thousands of expressed genes in young seedlings (Cui et al. 2017).

Two classes m^5C writer proteins were identified in eukaryotes, the transfer RNA aspartic acid methyltransferase 1 (TRDMT1) [also known as DNA methyltransferase 2 (DNMT2)] found in yeast, plants, and animals (Goll et al. 2006; Burgess et al. 2015), and the yeast tRNA specific methyltransferase 4 (TRM4) [also known as the

human NOP2/Sun domain protein 2 (NSUN2)] (Motorin and Grosjean 1999; Auxilien et al. 2012). The Arabidopsis genome encodes eight potential m^{5}C methyltransferases, two are the TRM4-like proteins, TRM4A and TRM4B (Chen et al. 2010; Cui et al. 2017), from which the latter has been already characterized in plants (David et al. 2017; Cui et al. 2017). Further analysis was undertaken, using loss-of-function mutants for the tRNA-specific m^{5}C methyltransferase (TRM4B), revealing that m^{5}C modification is required for proper root development and oxidative stress responses. David et al. (2017) observed defects in primary root elongation due to impaired cell division at the meristematic tissue, and showed that loss of TRM4B increases sensitivity to oxidative stress and decreases tRNA stability. Accordingly, Cui et al. (2017) showed that TRM4B loss-of-function mutants exhibit downregulation of key genes of root development, namely *SHORT HYPOCOTYL 2* (*SHY2*) and *INDOLE ACETIC ACID-INDUCED PROTEIN 16* (*IAA16*), which is positively correlated with the stability and m^{5}C modification in their transcripts (Cui et al. 2017).

Together, these studies identified the m^{5}C modification as another important methylation mark on RNA that has an impact on plant development and adaptive responses. Further research is needed to elucidate the mechanisms and functional roles of m^{5}C-mediated regulation of protein-coding genes, and to perhaps identify potential m^{5}C readers and erasers. A recent study showing that an Arabidopsis RRM motif-containing ALY protein preferentially binds to an m^{5}C-modified RNA (Pfaff et al. 2018) has encouraged future research efforts on this potential m^{5}C reader. Arabidopsis ALY protein family functions on mRNA export, and *aly* mutant plants exhibited various defects in vegetative and reproductive development, including shorter primary roots, altered flower morphology and reduced seed production (Pfaff et al. 2018). Altogether, it seems that the m^{5}C modification may influence protein-coding genes with widespread consequences for the development and stress responses.

11.4 Concluding Remarks

The advances of new technologies, such as sequencing-based transcriptome-wide mapping, revolutionized the field of RNA chemical modifications and permitted to unveil a novel layer in the control of gene expression that is now known as epitranscriptomics or RNA epigenetics. Advances on animal epitranscriptomic regulation have been dazzling in the past years and several epitranscriptomic marks (including m^{1}A, m^{5}C, m^{6}A, m^{6}Am, ac^{4}C, or h^{5}mC) have been mapped transcriptome-wide in different cell types and environmental conditions. We learned from animal studies the crucial importance of these regulatory marks that control constitutive cellular processes and allow their reprogramming to permit organism development and acclimation. In plants, our current understanding of epitranscriptomics is limited to the m^{6}A and m^{5}C-based regulations in a single model plant. Nonetheless, Arabidopsis studies revealed that in plants also these modifications are crucial to growth and

acclimation. It is hence now a necessity to foster more knowledge on this novel field of biology in model, but also in cultivated plants.

A first step is to get a global vision of the nature and patterning of chemical modifications on the polyadenylated transcriptome of plants. With the advent of global approaches such as LC-MS/MS or next-generation sequencing, one is now capable of not only knowing the nature and relative abundance of mRNA modifications but also to decipher their distribution on each expressed genes. Such repertoires might easily be obtained from diverse species, organs, environmental conditions, and even populations. We anticipate these data to give insights on the role and agronomical importance of RNA epigenetics, as did, for example, the 1001 Arabidopsis epigenomes. Analyses and comparisons of these repertoires will give us clues regarding the interplay that exists between the various marks or their respective importance in acclimation and growth.

Of course, several fundamental questions remain to be addressed in model plants that will contribute to our understanding of the importance of RNA epigenetics in crop development and resistance to stressful conditions, encountered in cultivated fields. What are the actors (writers, readers, and erasers) of the different epitranscriptomic mark-based regulations? Understanding the molecular, cellular, and physiological roles of these actors will help comprehend the role of the mark and the interplay between marks. As an example, data already obtained from Arabidopsis studies on the features and role of the m^6A mark can be exploited to understand the importance of this mark in cultivated species. With the advent of genome editing technologies, reverse genetic approaches on proteins of the writer complex, m^6A-readers, and erasers can easily be conducted.

RNA epigenetics in animals is no longer an emerging field but a fast growing new topic of biology that appeals to more and more scientists. Of course, several deficiencies in the epitranscriptomic control of gene expression were linked to cancers and diseases. In plants, the m^6A mark controls development at the embryonic and post-embryonic stages, and very likely required for defense against viral infections and stress responses. The community of plant scientists interested in RNA epigenetics is so far quite small and must grow to foster sufficient knowledge to understand this novel extremely complex field of biology.

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Chapter 12
The Role of Small RNAs in Plant Somatic Embryogenesis

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Abstract In plants, differentiated somatic cells can revert their identity to pluripotent, reprogrammed cells in order to optimize growth and development depending on external conditions and in aid of overcoming their limitations as sessile organisms. Different modes of regeneration include tissue repair, de novo organogenesis and somatic embryogenesis (SE). The latter usually comprise the formation of proliferating pluripotent cell masses called callus. Identification and characterization of genes involved in the SE process allows the exploitation of distinctive features that make a tissue susceptible to change its normal cell fate and produce new plants massively.

Small RNAs (sRNAs) are non-coding RNA (ncRNA), 20–24 nucleotides long molecules involved in plant development, reproduction and genome reprogramming. Likely, the enormous variety of operating sRNA pathways contributes to the plant phenotypic plasticity. Two main sRNAs classes are defined by their modes of biogenesis: a class in which the precursor is a single-stranded, hairpin loop forming RNA (hpRNA), mainly represented by microRNAs (miRNAs) and a class in which the precursor is a dsRNA molecule (dsRNA) comprising several small interfering RNAs (siRNAs).

sRNAs, especially miRNAs, are common regulators of transcription factors (TFs) essential for plant meristem maintenance, growth and proliferation control, and with recently uncovered role in somatic to embryonic cell reprogramming. Although the siRNA function in plant development and SE has been much less explored, recent findings shape out their relevance in organ patterning and stress responses, both involved in cell plasticity. This review focuses on compiling and integrating the described function of miRNAs and siRNAs as a molecular basis in establishing cell dedifferentiation and further plant regeneration in economically relevant crops.

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12.1 The SE Process

12.1.1 General Description

Somatic embryogenesis (SE) is an alternative plant reproduction process where embryos are produced from somatic tissues through an initial cell dedifferentiation promoted by exogenous signals (Elhiti et al. 2013). Dedifferentiated cells are able to proliferate maintaining their totipotential state (embryogenic masses or callus) and can further develop into a whole plant if the exogenous signal is removed.

SE has great impact on plant biotechnology and is widely used for clonal propagation, transformation or somaclonal variation. In addition it constitutes a valuable model to study early developmental features of embryogenesis, molecular aspects of cell transition during differentiation and hormone responses (De-la-Peña et al. 2015).

While zygotic embryogenesis initiates upon fertilization and comprises a series of molecular events underlying morphogenesis and embryo patterning, SE results from differentiated somatic tissues, which gain on embryogenic competence as a response to imposed external stimuli. For example, in maize, immature embryos have proven to display greater competence to achieve totipotency. In this plant species embryogenesis commitment requires dedifferentiation and further establishes cell proliferation prior to plant regeneration (Garrocho-Villegas et al. 2012).

12.1.2 Known SE Markers

The first step in SE induction is cell dedifferentiation where the cell fate of particular differentiated cells returns to a totipotent ground state as a response to external stimuli. The process is related to stressful conditions, such as temperature change, high phytoregulator concentration, light deprivation and others (Elhiti et al. 2010; Kumar and Van Staden 2017). The imposed stress and exogenous hormones induce gene expression reprogramming, particularly through AUXIN RESPONSE FACTORS (ARFs), AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA), Helix-Loop-Helix (bHLH), LATERAL BOUNDARY DOMAIN (LBD) and other transcription factors (TFs) to direct cells towards dedifferentiation (Yang et al. 2012; Elhiti et al. 2013; Ge et al. 2016).

Upon promoting dedifferentiation, the achievement of embryogenic potential is crucial for further plant regeneration through SE. Several markers correlate with enhanced embryogenic potential. These include LEAFY COTYLEDON (LEC1 and LEC2), WUSCHEL (WUS) and BABY BOOM (BBM) genes (Su et al. 2009; Elhiti et al. 2010; Lowe et al. 2016). LEC1 and LEC2 are required to activate endogenous auxin biosynthesis, which consequently up-regulates the expression of WUS and SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) (Elhiti et al. 2013).

Induced totipotent embryogenic tissues further activate signalling towards cell division and meristematic fate. Genes involved in successful proliferation program include cell cycle regulators and signal transduction components. Exogenous phytohormones, present in the callus proliferation medium, contribute to enhanced SHOOT APICAL MERISTEM (STM), CYCLIN DEPENDENT KINASEs (CDKs) and WUS gene expression (Elhiti et al. 2010). On the other hand, negative regulators of WUS, such as CLAVATA (CLV1, CLV2 and CLV3) repress meristematic cell proliferation and promote differentiation (Elhiti et al. 2013).

More recently, small RNAs (sRNAs) have emerged as master regulators for most of the above-mentioned transcription factors. Their role in the SE process has been intensively studied in model and agronomical plant species over the last decade (Luo et al. 2006; Shen et al. 2013; Wu et al. 2015; Szyrajew et al. 2017).

12.2 sRNAs Classification, Biogenesis Pathways and Functions

12.2.1 sRNA Classification

Major sRNA groups include small interfering RNAs (siRNAs) and microRNAs (miRNAs). Further classification separates siRNAs in: hairpin-derived siRNAs (hp-siRNAs), natural antisense siRNAs (nat-siRNAs), secondary siRNAs and heterochromatic siRNAs (hc-siRNAs). miRNAs and hp-siRNAs derive from single-stranded RNA precursors that form a stable hairpin loop, while other siRNAs originate from double-stranded RNA (Axtell 2013). Plant genomes usually present several individual genes encoding miRNAs from the same family and in few cases the miRNA originates from transcripts of protein-coding genes (reviewed in Budak and Akpinar 2015). Secondary siRNAs include phased- and trans-acting siRNAs. Phasing is a consequence of successive DCL processing that initiates at particular site within the dsRNA precursor determined by specific miRNA targeting (Fig. 12.1). Secondary siRNAs that act *in trans* to direct silencing of distinct mRNA targets are termed tasiRNAs. Most known tasiRNAs are also phased (Axtell 2013). Heterochromatic siRNAs commonly derive from plant transposable elements (TEs) and trigger important epigenetic mechanisms (Borges and Martienssen 2015).

12.2.2 sRNA Biogenesis Pathways

All sRNA production requires DICER-LIKE (DCL) enzymes to produce 21–24 nt long RNA duplexes with 2 nt overhangs at the 3′ ends from precursors. Duplexes are protected by 2′-methylation at their 3′ ends by HUA ENHANCER 1 (HEN1). These duplexes are recognized by ARGONAUTE (AGO) proteins in complex with

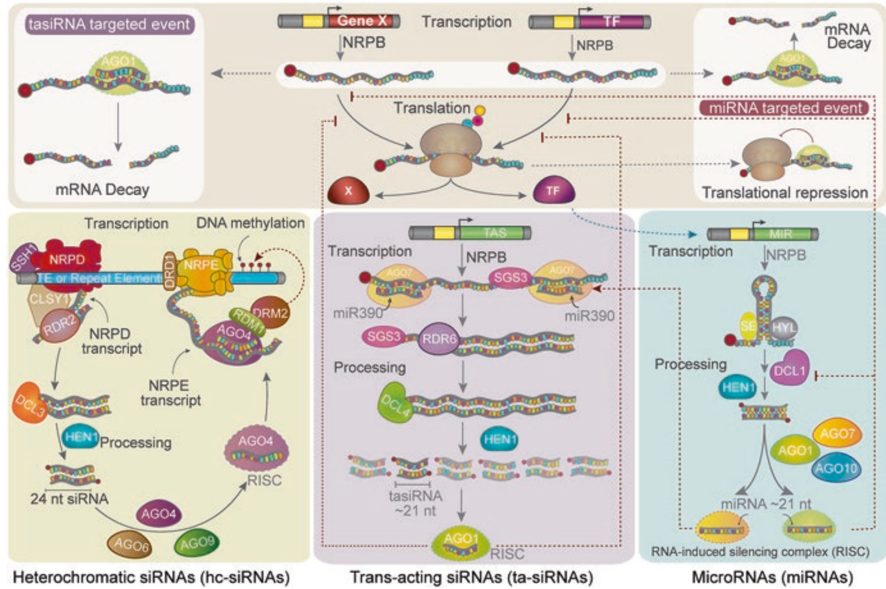

Fig. 12.1 Small RNA biogenesis pathways, interconnection and mechanisms of action. Lower panels represent the distinct origin (genetic loci) and biogenesis of hc-siRNAs, tasiRNAs and miRNAs (from left to right). The upper panel represents distinct modes of action and targets for tasiRNAs and miRNAs. Interconnection between miRNAs, tasiRNAs and targeted transcription factors (TFs) is shown by dotted lines. NRPB, RNA Pol II; NRPD, RNA Pol IV; NRPE, RNA Pol V; DCL, Dicer-like; AGO, Argonaute; RDR, RNA-dependent RNA polymerase; HEN1, sRNA methylase HUA enhancer 1; DRM2, domain rearranged methylase 2

other partners to select the mature sRNA strand and target protein-coding or non-coding RNAs by sequence complementarity. There are several comprehensive reviews on plant sRNA production and action (Bologna and Voinnet 2014; Borges and Martienssen 2015). Commonly, sRNA-charged AGO constitutes an RNA-induced silencing complex (RISC) that usually exerts post-transcriptional gene silencing (PTGS) by either transcript degradation or translational inhibition (Rogers and Chen 2013). However, the particular subclass hc-siRNAs promotes transcriptional gene silencing (TGS) and RNA-directed DNA methylation (RdDM), which is very important for TE control in plants. sRNAs originating from dsRNA precursors require the action of RNA-dependent RNA polymerase (RDR) and other stabilizing proteins for their biogenesis. The pathways depicted in Fig. 12.1 show major steps and particular DCL, AGO and RDR family members, as well as other enzymes required for miRNA, tasiRNA and hc-siRNA production, genetically dissected in the model plant *Arabidopsis thaliana*. Several mutants for these proteins have also been identified in agronomical crops such as rice and maize, unravelling specialized functions for some of them (Nagasaki et al. 2007; Nobuta et al. 2008; Chitwood et al. 2009; Thompson et al. 2014).

12.2.3 sRNA Mechanisms of Function

Plant microRNAs regulate target RNAs by nearly perfect complementarity with sequences within any region of the transcript (Axtell 2013). miRNA-guided RISC preferentially induces target cleavage generating fragments at the targeted sequence that could follow up experimentally by degradome analyses (Ding et al. 2012; Yang et al. 2013). However, there are several examples of targets reduced at protein level, but not affected at mRNA level, due to miRNA action (Chen 2004; Brodersen et al. 2008; Beauclair et al. 2010). These studies suggested translational repression as a second way of action for plant miRNAs (Fig. 12.1). The extent of miRNA-target complementarity has been considered as premise to turn the balance towards either slicing (perfect) or translational repression (imperfect) in animals. However, the two modes of miRNA action have been shown to simultaneously operate in plants independently of the grade of complementarity (Aukerman and Sakai 2003; Beauclair et al. 2010).

Trans-acting small interfering RNAs (tasiRNAs) derive from precursor TAS genes transcribed by RNA pol II (Fig. 12.1). A miRNA drives the initial processing; the cleaved fragment is converted to dsRNA by RDR6 and sliced by DCL4 to 21-nucleotide siRNAs in a phased arrangement from the miRNA cleavage site (Xia et al. 2017). Four different TAS genes have been identified in *Arabidopsis thaliana* (Fei et al. 2013), but TAS3 is the most conserved and well-studied in different plants. Initial cleavage of TAS3 transcript is promoted by miR390 charged on AGO7, and some of the derived tasiRNAs target several members of the ARF3/4 family. This pathway is known as miR390-TAS3-ARF and related tasiRNAs are termed tasiR-ARFs (Dotto et al. 2014).

Heterochromatic siRNAs (hc-siRNAs) originate from repeat-rich loci and TEs. Initial transcription by plant-specific RNA Pol IV (NRPD) and conversion of the transcript to dsRNA by RDR2 are required (Matzke and Mosher 2014). Then DCL3 processes the precursor to 23–24 nt duplexes, which are exported to the cytoplasm where they are loaded onto members of the AGO4 clade and returned to the nucleus (Borges and Martienssen 2015). AGO4-hc-siRNAs are recruited to homologous loci transcribed by plant RNA Pol V (NRPE) to deposit repressive chromatin marks, such as 5-methyl cytosine at asymmetric CHH context and histone H3K9 methylation. Usually 23–24 nt long hc-siRNAs represent the most abundant sRNA class in many plant species. In maize, mutation of RDR2 causes important reduction of 23–24 nt hc-siRNAs accompanied by increase of 21–22 nt siRNAs, including some miRNAs and tasiRNAs (Nobuta et al. 2008). This and other loss-of-function mutants in the RdDM pathway are not associated with major developmental defects suggesting that transcriptional silencing involves several layers of regulation.

12.3 miRNA Role in Plant Somatic Embryogenesis Induction

microRNAs are important regulators of plant developmental switches. Their role in SE induction relies on targeting central TFs that determine tissue differentiation. They also act as sensors of imposed stress conditions during dedifferentiation, phytohormone signalling and responses, as well as in embryogenic potential acquisition. In *Arabidopsis thaliana*, miR165 and miR166 target the CLASS III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIP III) TFs PHABULOSA (PHB) and PHAVOLUTA (PHV), which are positive regulators for LEC2 expression. On the other hand, miR160 regulates ARF10, ARF16 and ARF17 involved in auxin signalling during SE induction (Wójcik et al. 2017). In addition, miR393 controls the levels of auxin receptors TIR1 and AFB2 (Wójcik and Gaj 2016). The relevance of these miRNAs in the context of auxin signalling pathways is described with more details below.

A pioneer study developed in rice revealed that some miRNAs are particularly enriched in dedifferentiated tissues (Luo et al. 2006). Such finding was followed by reports based on next-generation sequencing (NGS) techniques approaching miRNA abundances during SE embryogenesis induction, plant regeneration or between tissues with distinct embryogenic potential (Shen et al. 2013; Wu et al. 2015; Szyrajew et al. 2017). A common finding for these studies was that development-related miRNAs (miR156, miR159, miR164, miR166 and miR172) tend to decrease their levels upon dedifferentiation, while stress-related (miR319, miR396, miR397, miR398 and miR408) increase (Fig. 12.2). Other miRNAs related to auxin responses (miR160, miR167, miR169 and miR390) may show transient increases depending on the stage of dedifferentiation induction. On the other hand, during plant regeneration through SE, stage-specific miRNA patterns and their target regulation oppose the dedifferentiation status revealing important roles for miR156, miR159, miR164 and miR168 in *Citrus sinensis* (Wu et al. 2011), *Larix leptolepis* (Zhang et al. 2012), *Dimocarpus longan* (Lin and Lai 2013) and *Zea mays* (Chávez-Hernández et al. 2015). However, it is important to highlight that each plant species requires particular in vitro culture conditions and some of the conserved miRNAs might display species-specific patterns.

12.4 Relevance of sRNAs in Auxin Responses and Homeostasis

12.4.1 The Auxin Signal Transduction Pathway

Auxins are the most widely studied phytoregulators in plants (Sanan-Mishra et al. 2013). They are involved in plant growth, cell division, elongation and differentiation, apical-basal axis formation, embryogenesis, meristem formation and tropism (Hrtyan et al. 2015; Kasahara 2016; Mutte et al. 2018). Indole-3-acetic acid (IAA)

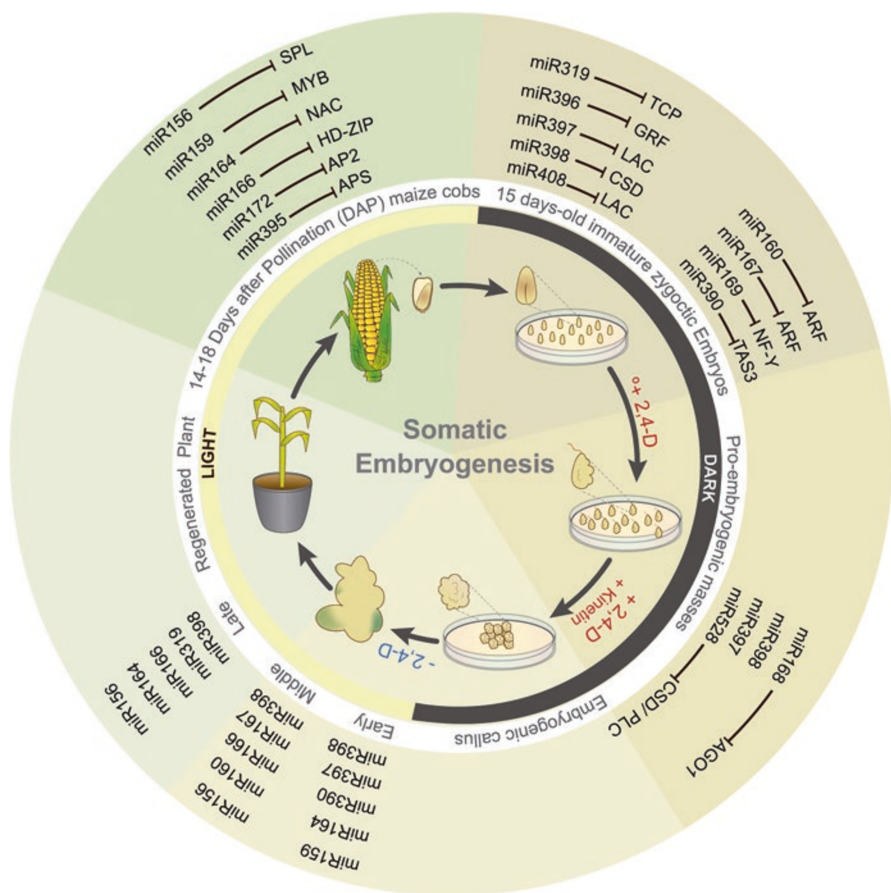

Fig. 12.2 sRNAs role in somatic embryogenesis (SE) and plant regeneration (exemplified with maize). SE induction and dedifferentiation is represented at the top of the circle using as explant immature embryos and 2,4-D/darkness as stimulus. Further embryogenic callus proliferation establishment includes kinetin in addition to 2,4-D (Garrocho-Villegas et al. 2012). The bottom part represents differentiation induction of proliferating callus by phytoregulators removal in the presence of photoperiod. The circle is completed by plant regeneration and reproduction. At each stage, the most abundant miRNAs detected for maize and other plant species (details in Table 12.2) are shown on the external circle together with their proposed roles in regulating TFs or proteins crucial for the SE process

is the most common natural auxin and the final product of general auxin biosynthesis mechanisms. There are also synthetic auxins: 2,4-dichlorophenoxy acetic acid (2,4-D) and 1-naphtaleneacetic acid (NAA). 2,4-D mimics IAA in the perception and signalling, except for cell-to-cell auxin transport mechanisms (Fukui and Hayashi 2018; Mutte et al. 2018).

Auxin regulatory networks operate by three dynamic processes: (1) auxin biosynthesis and inactivation; (2) cell-to-cell auxin transport (auxin polar transport)

and (3) final signal transduction (Fukui and Hayashi 2018). The first two are related with the balance of auxin concentrations in specific tissues at certain developmental stages, whereas the third one represents the final response to auxin perception, which consists in the transcriptional activation or repression of a wide range of genes (Sanan-Mishra et al. 2013).

Exogenous auxin influx takes place by passive diffusion or by AUXIN RESISTANT 1/LIKE AUXIN (AUX1/LAX) transporters (Fig. 12.3). Auxin efflux is carried out through PIN-FORMED (PIN) efflux carriers and ATP-BINDING CASSETTE subfamily B/MULTIDRUG RESISTANCE/P-GLYCOPROTEIN (ABCB/MDR/PGP). The influx/efflux conforms the auxin polar transport system responsible to maintain auxin levels and gradients between cells. The transporters (AUX/LAX, PIN and ABCB) have particular spatiotemporal expression and subcellular localization to determine the specific auxin gradients during plant growth and development (Barbosa et al. 2018; Fukui and Hayashi 2018; Zhao 2018).

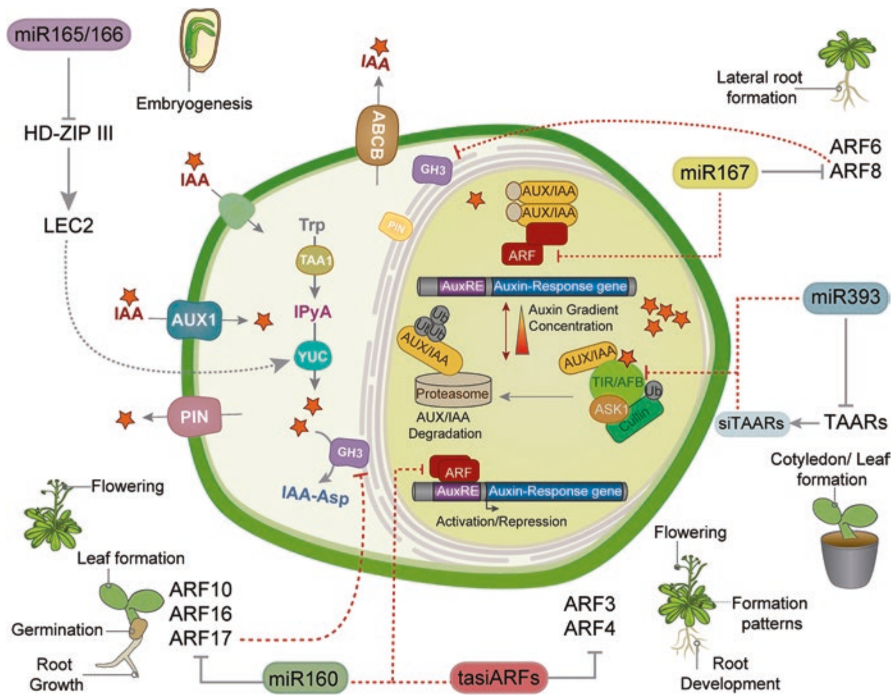

Fig. 12.3 sRNA-mediated regulation on plant auxin signalling pathway**.** The image represents the main Arabidopsis auxin signal transduction pathway explained in Sect. 12.4. miR160 controls IAA degradation by inhibiting ARF10/16/17 during seed germination and plantlet establishment. miR165/166 indirectly affects IAA biosynthesis by repressing HD-ZIP III TFs which are required to promote LEC2 and YUC expression. miR167 inhibits ARF6/8 involved in auxin-responsive gene expression, particularly during lateral root formation. miR393 represses TAAR expression and promotes generation of TAAR-derived siRNAs during cotyledon leaf formation. Opposite gradients of tasiR-ARFs and their ARF3/4 targets help to establish leaf pattern formation, appropriate root development and flowering

Auxin biosynthesis and inactivation has been characterized mostly in *Arabidopsis thaliana*. However, homologous pathways are highly conserved in plants. A Trp-dependent pathway produces endogenous IAA by Trp conversion in two sequential steps: (1) Trp is converted to indole 3-pyruvic acid (IPyA) by the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1/TRYPTOPHAN AMINO TRANSFERASE RELATED (TAA1/TAR gene family); (2) Enzymes from the flavin monooxygenase family YUCCA (YUC) catalyse the conversion of IPyA to IAA (Fig. 12.3). The second conversion is the rate-limiting step because of strict YUC availability and spatiotemporal regulation. Auxin catabolism and inactivation are as well important to maintain optimal endogenous levels for certain processes. The Gretchen Hagen 3 (GH3) family of IAA-amide synthetases conjugate IAA to amino acids and in such form the auxin can be degraded. Conjugation is reversible depending on the amino acid identity (Kasahara 2016; Fukui and Hayashi 2018; Zhao 2018).

Auxin signal transduction triggers transcriptional regulation of many gene families. It involves intracellular receptors, transcriptional activators and repressors (ARFs) and auxin-responsive genes. The TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALLING F-BOX (TIR1/AFB) receptor family are responsible for the perception of intracellular auxin levels. TIR1 is an F-box protein that conforms the SCF-type complex (Skp1, Cullin, and F-box protein-type), which act as ubiquitin-ligases responsible for protein ubiquitination and further degradation via the proteasome (Sanan-Mishra et al. 2013; Wang et al. 2018). At high auxin levels, TIR1/AFB receptors promote interaction between the SCF-type complex and the ARF inhibitors AUX/IAA. Such interaction promotes AUX/IAA degradation and ARF release to regulate auxin-responsive genes bearing DNA cis-elements (AuxRE) in their promoters (Leyser 2018; Kim et al. 2018; Wang et al. 2018). Auxin-responsive genes include GH3s, SAURs (SMALL AUXIN UP-REGULATED RANKs) and AUX/IAA. Gene repression or activation depends on ARF identity (Sanan-Mishra et al. 2013; Roosjen et al. 2018).

12.4.2 miRNAs Related to Auxin Responses

From the 23 ARF family members identified in *Arabidopsis thaliana*, at least five are regulated by miRNAs (Mallory et al. 2005). Arabidopsis miR160 targets ARF10, ARF16 and ARF17. The loss of miR160 target site in any of these ARFs results in developmental defects, such as reduced ABA sensitivity during germination (ARF10), defective root cap development and alteration in lateral root formation (ARF10 and ARF16); embryo symmetry anomalies, leaf shape defects, premature and abnormal inflorescence development and root growth impairment (ARF17) (Mallory et al. 2005; Wang 2005; Liu et al. 2007). Interestingly, ARF17 represses GH3 transcription, thereby affecting the intracellular auxin inactivation (Fig. 12.3). In addition, miR167 represses ARF6/ARF8, which also regulate GH3 transcription. Surprisingly, it has been reported that miR167 promoter displays AuxRE elements probably regulated by other ARFs. Therefore, miR160/167 nodes seem to have

complementary roles at least in root development (Rubio-Somoza and Weigel 2011; Sanan-Mishra et al. 2013; Hrtyan et al. 2015).

Another miRNA involved in auxin regulation is miR393. The targets of this miRNA belong to the family of F-box proteins, including four members of the TIR1/AFB2 clade of auxin receptors (TAARs). Cleavage of TAAR transcript promotes the production of secondary siRNAs (siTAARs) which regulate the final expression of each TAARs from where they were originated and other unrelated genes, generating auxin accumulation and developmental abnormalities of leaves and cotyledons (Si-Ammour et al. 2011; Singh et al. 2018). On the other hand, miR165/166 indirectly affect IAA biosynthesis through inhibiting the activation of YUC transcription by LEC2 (Fig. 12.3; Wójcikowska et al. 2013; Wójcik et al. 2017).

12.4.3 tasiRNAs Involved in Auxin Responses

As described in Sect. 12.2, tasiRNAs targeting ARF3/4 are commonly known as tasiR-ARFs (Dotto et al. 2014; Xia et al. 2017). The tasiR-ARF highly conserved regulatory mechanism is required for proper leaf development, as well as juvenile to adult phase changes (Guilfoyle and Hagen 2007). Other functions are related to flower development and lateral root formation under normal and salt stress conditions (Marin et al. 2010; Hrtyan et al. 2015; He et al. 2018). Before lateral root initiation, miR390 expression is activated in xylem cells and promotes tasiR-ARFs production to repress ARF3/4 transcripts in the new primordium. This provokes endogenous auxin level alteration at particular sites, required for lateral root formation and appropriate plant growth (Marin et al. 2010). A recent report in Poplar (Populus spp.) showed that osmotic stress inhibits auxin signalling to enhance lateral root formation through miR390 expression stimulation and tasiR-ARFs accumulation (He et al. 2018).

12.5 Other siRNAs in Cell Dedifferentiation and Proliferation Establishment

12.5.1 Epigenetic Regulation in SE

Epigenetic mechanisms coordinate gene reprogramming for the acquisition of totipotency during dedifferentiation of somatic cells (Miguel and Marum 2011; Elhiti et al. 2013). Such reprogramming is partly achieved by DNA methylation and histone modifications in response to environmental and stress conditions to achieve the developmental switching in somatic cells as adaptation to the external cues (Henderson and Jacobsen 2007; Huettel et al. 2007; Neelakandan and Wang 2012).

DNA methylation is an essential epigenetic mechanism that regulates and maintains gene expression programs (Milutinovic et al. 2003). In plants, cytosine methylation occurs in the context of CG, CHG and CHH (H = A, T or C) and is catalysed by METHYLTRANSFERASE (MET), CHROMOMETHYLASE (CMT) and DOMAIN REARRANGED METHYLTRANSFERASE (DRM) (Cao and Jacobsen 2002; Fehér 2015). Several factors influence DNA methylation during plant SE (Elmeer and Hennerty 2008; Joshi et al. 2008) and both, hyper- and hypomethylation, play crucial roles in somatic embryo development (Chakrabarty et al. 2003; Nic-Can et al. 2013). Early research in plant SE determined that high auxin concentrations and nitrogenous compounds like L-proline, commonly present in in vitro culture media, affect the DNA methylation status, and hence gene expression (LoSchiavo et al. 1989). DNA methylation inhibition correlates with SE competence impairment and loss of regeneration capacity in *Medicago truncatula* (Santos and Fevereiro 2002) and *Daucus carota* (Yamamoto et al. 2005). Therefore, certain levels of DNA methylation have to be maintained for a proper course of SE (De-la-Peña et al. 2015).

Chromatin remodeling has been widely reported for plant somatic cell dedifferentiation, organogenesis, embryogenesis and regeneration (Grafi et al. 2007; Valledor et al. 2010). This process allows TFs and chromatin modifiers to access DNA and exert gene expression control. The regulation is mediated by particular histone N-terminal methylations, acetylations, ubiquitinations and phosphorylations (Kouzarides 2007). During SE induction in *Coffea canephora* the H3K9me2 repressive mark was absent, while H3K4me2 and H3k4me3 activation marks increased. Additionally, after the first week of induction, the levels of H3K27me2 and H3k27me3 repressive marks were also substantially reduced (Nic-Can et al. 2013). Reduction in repressive histone modifications has been associated with genes encoding TFs involved in cell differentiation, such as BBM1, LEC1 and WUSCHEL-RELATED HOMEOBOX4 (WOX4) to promote successful SE induction (Lafos et al. 2011). In addition, a mutant for the chromatin modifier PRC2, which directly binds H3K27me3 and promotes repressive chromatin remodeling, tends to develop embryo-like structures from differentiated tissues (Ikeuchi et al. 2015). This supports the role of transcriptional repression in preventing dedifferentiation of mature somatic cells and suggests de-repression is needed to achieve cellular dedifferentiation and SE progression. Although all these studies have pointed out the relevance of dynamical plant chromatin regulation during SE, the mechanisms underlying the epigenetic plasticity required for cell totipotent status have been still poorly explored.

12.5.2 sRNA Impact on Epigenetic Landscapes

Plant hc-siRNAs (Fig. 12.1) are involved in heterochromatin formation and transcriptional gene silencing by guiding sequence-specific DNA and histone methylation through RdDM (Matzke and Mosher 2014; Borges and Martienssen 2015). In

Arabidopsis thaliana, RdDM targets genomic loci for de novo DNA methylation through DRM2 (Zhang and Zhu 2011; Saze et al. 2012). Reports from several plant species have illustrated hc-siRNA-mediated epigenetic regulation, their role in chromatin organization and transcription silencing during different developmental stages and stimuli. It has been shown that hc-siRNAs participate as mobile elements for inter-tissue epigenetic regulation. In grafting experiments using wild-type and mutant plants, unable to produce hc-siRNAs, the movement of these sRNAs was detected from wild-type-to-mutant tissues to induce *novo* DNA methylation (Molnar et al. 2010; Tamiru et al. 2018). Also, hc-siRNAs mediate transgenerational epigenetic regulation. Prior fertilization, cells surrounding germline undergo DNA demethylation leading to the transcriptional activation of endogenous TEs (Zemach and Zilberman 2010). TE reactivation triggers the formation of hc-siRNAs that move into the germ cells and ensure epigenetic silencing of TEs in the embryo (Olmedo-Monfil et al. 2010; Kumar and Van Staden 2017). Also, mutants for components of hc-siRNA biogenesis were related to decondensation of pericentromeric repeats and depletion of H3K9me2 at chromocenters leading to genome instability (Pontes et al. 2009). The relationship between RdDM and chromatin remodelers has been demonstrated in maize (Fu et al. 2018). Mutants for CMT or the nucleosome remodeler DDM1 exhibited decrease in RdDM activity and nearly complete loss of both, 24 nt hc-siRNAs and CHH-methylation. Curiously, the loss of 24 nt hc-siRNAs was accompanied by a dramatic increase of 21 and 22 nt siRNAs mapping to heterochromatic loci in the genome. However, these siRNAs apparently are unrelated to DNA methylation and RdDM.

12.5.3 hc-siRNAs and Other siRNAs during SE

Despite all reports that have linked epigenetic regulation by sRNAs with plant development, to date very few studies have approached the implication of hc-siRNAs and other siRNAs in SE regulation. While investigating sRNA roles in synchronic SE of *Larix leptolepis*, an overrepresentation of 24 nt siRNAs was observed for synchronous embryos suggesting their participation in SE synchronism, a crucial hallmark in plant tissue culture (Zhang et al. 2014). Likewise, genome-wide analysis of sRNAs in non-embryogenic and embryogenic tissues of 'Valencia' sweet orange (*Citrus sinensis*) SE indicated that 24 nt siRNAs exhibited lower abundance in the non-embryogenic callus (Wu et al. 2015). In addition, plant regeneration through rice SE revealed DNA hypomethylation associated with 24 nt hc-siRNAs loss (Stroud et al. 2013). Also, in immortalized Arabidopsis cell suspension cultures, particular heterochromatic regions were hypomethylated and TEs became activated (Tanurdzic et al. 2008). However, the 24 nt hc-siRNAs were significantly reduced only for particular TEs.

In maize, the 24 nt sRNA population importantly decreased during the establishment and maintenance of embryogenic callus for the Tuxpeño VS-535 cultivar (Alejandri-Ramírez et al. 2018). However, 21–22 nt populations were not affected. Interestingly, the 24 nt-long hc-siRNAs derived from retrotransposons decreased

only transiently during callus proliferation establishment, concomitant with 22 nt increases. Unexpectedly, such changes were accompanied by reduction in the expression of some transposons, suggesting that TE regulation might be needed for proper establishment of embryogenic callus and the acquirement of proliferative status. Moreover, the role of other maize siRNAs was revealed while studying factors determining the frequency of embryonic callus formation in the Chinese maize inbred line 18-599R (Ge et al. 2017). Surprisingly, some 24 nt siRNAs mapping to promoter gene regions were significantly up-regulated and correlated with hypermethylation of the corresponding target genes during different stages of embryogenic callus induction and formation. This further resulted in decreased expression of the target genes. All these data only expose the tip of an iceberg that represents the largely unknown role of hc-siRNAs and other siRNAs for gene expression regulation during plant SE. Whether these siRNAs act through the RdDM pathway remains to be demonstrated.

12.6 sRNAs in Plant Regeneration Through Somatic Embryogenesis

12.6.1 Comparison Between Somatic and Zygotic Embryogenesis

Numerous studies have shown the resemblance between somatic and zygotic embryos in terms of morphological, histological, physiological, biochemical and genetic features. However, somatic embryos are more exposed to stress than their zygotic counterparts, accumulate less storage compounds and do not experience a growth arrest but germinate precociously (Winkelmann 2016).

Several proteins act as multifunctional regulators in both, zygotic and somatic embryogenesis. These include WUS, LEC1/LEC2, BBM1 and the AGAMOUS-LIKE 15 (AGL15) TFs (Fehér 2015). Not only the key regulators are common, but also the overall gene expression patterns of somatic and zygotic embryos are similar. When the cotton somatic and zygotic embryo transcriptomes were compared, the expression patterns of genes associated with metabolism, cellular processes and embryo development were found to be greatly similar (Jin et al. 2014). However, the main gene expression difference for in vitro cultured embryos resided within the stress-related gene class.

The study of regulatory molecules and connected gene networks during SE is of great significance for the long-term understanding of embryogenic competence and plant regeneration capacity, which is indispensable for crop improvement. While key role of miRNAs in zygotic embryogenesis was early demonstrated for *Arabidopsis thaliana* (Nodine and Bartel 2010; Armenta-Medina et al. 2017), their central function in somatic embryogenesis is starting to shape for different plant species (Chen et al. 2011; Wu et al. 2011; Zhang et al. 2012; Li et al. 2012; Lin et al. 2015; Yang et al. 2013; Chávez-Hernández et al. 2015; Zhang et al. 2017b).

12.6.2 Plant Regeneration Pathways

Based on the capability to regenerate whole plants from a variety of tissues or cells, such as leaf, pollen, root and endosperm cells, it is often claimed that all plant cells are totipotent. However, experimental data are scarce to sustain this statement. In vitro regeneration may progress through pre-existing stem cells in the plant body, and totipotency has been demonstrated only for certain, mostly young or partly differentiated tissues (Fehér 2015).

Also it has been suggested that dedifferentiation process includes the developmental switch of the explant cells to a pericycle cell-like functioning (Sugimoto et al. 2010). This implies that the early step in organogenesis involves cell redifferentiation to a distinct cell type, rather than to an 'undifferentiated/dedifferentiated' state (Horstman et al. 2017).

Somatic embryogenesis mainly follows two paths of regeneration depending on the developmental stage of the explant and culture conditions. That means somatic embryos can develop directly from the explant or indirectly from callus. The development of embryos is regularly indirect going through a pro-embryogenic cell mass (PEM) or embryogenic callus phase and only limited cells of the callus can form embryos (Fehér 2015). For example, cells that have undergone only a few divisions, such as asymmetrically dividing stem cells, can rapidly re-establish a removed stem cell niche of the root tip, the callus induced on Arabidopsis immature zygotic embryos can produce somatic embryos and the callus initiated from pericycle stem cells retains its ability to regenerate shoots.

For successful shoot regeneration from in vitro induced callus, it has been shown that lateral root primordial features are required and precede de novo shoot formation (Radhakrishnan et al. 2018). Morphology, cellular organization and molecular markers, such as WUSCHEL-related homeobox 5 (WOX5), SHORT-ROOT (SHR), SCARECROW (SCR), PLETHORA (PLT1/2), PIN1 and others, support the root identity of callus tissues (Sugimoto et al. 2010; Kareem et al. 2015). This is in accordance with the crucial role of auxin concentration in callus formation and further plant regeneration.

12.6.3 Pattern Formation During SE

During plant regeneration through SE, stem cells need cues to establish the conventional plant developmental patterning. Coordinated cell division and differentiation are required throughout plant regeneration to obtain a whole plant. Due to the existence of rigid walls limiting cell migration and rotation, pattern formation depends on positional information. Hence, the 'on-site' differentiation of newly formed cells comprises fundamental cell-to-cell communication. Molecules facilitating such events include peptides, phytohormones, transcription factors and small non-coding RNAs (Hisanaga et al. 2014).

The beauty of sRNA-mediated cell-to-cell signalling resides in avoiding the use of specific receptors and energy consuming sequential steps of signal transduction pathways preceding gene expression responses. Instead, it utilizes highly specific nucleotide base paring for direct suppression of target mRNA expression (Fig. 12.1). The mechanism underlying sRNA transference across the cell wall possibly involves plasmodesmata (PD).

12.6.3.1 sRNAs Involved in Shoot Apical Meristem Formation

Direct in vitro shoot regeneration is de novo committed by cytokinin (Radhakrishnan et al. 2018). This process is characterized by a clearance of epigenetic marks at the WUS locus. Upon transfer to cytokinin-rich medium, repressive histone mark H3K27me3 is gradually removed from the locus coincident with WUS expression at shoot regeneration sites. In *Arabidopsis thaliana*, WUS is expressed at the organizing centre (OC) located at the SAM inner stem cell layer (L3) and the corresponding protein moves to more external L2 and L1 layers to activate the production of CLV3, which eventually attenuates WUS expression (Schoof et al. 2000; Lee and Clark 2013). This feedback loop maintains the size of SAM stem cell pool constant, but does not explain how exactly the cellular organization operates in the context of stem cell division.

Recent reports have nicely demonstrated that both, CLV3 and B-type ARABIDOPSIS RESPONSE REGULATORs (ARRs) partnered by HD-ZIP III TFs, are required for WUS enhanced expression during shoot regeneration (Zhang et al. 2017a). Furthermore, regulation of HD-ZIP III TFs by miR165/166 restricts the regionalization of ARRs and miR394 acts as a positional cue by repressing the F-box protein LEAF CURLING RESPONSIVENESS (LCR) at the internal layer, where it interferes with CLV3 expression (Knauer et al. 2013). This repression allows stem cell maintenance and supports the precise interplay between cytokinin and auxin at the SAM.

Additional regulation by miR156 contributes to shoot regeneration potential. As Arabidopsis plants age, they lose their ability to regenerate shoots mostly due to reduced miR156 levels and up-regulation of its target SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) TFs (Zhang et al. 2015). Particularly, SPL9 directly interferes with the function of B-type ARRs, impairing the cytokinin response and consequently shoot regeneration. Plants overexpressing miR156 showed increased shoot regenerative ability and for longer periods. Interestingly, miR156 also declines during long-term callus subculture in maize (Dinkova and Alejandri-Ramirez 2014) and citrus (Long et al. 2018). In the last study, it was demonstrated that miR156 overexpression or the SPL9 orthologous gene knockdown rescued the embryogenic capacity of aged citrus callus supporting the central role of this miRNA-target module in SE regulation.

12.6.3.2 sRNAs Implicated in Root Apical Meristem Development

Similar to shoot regeneration, plant root regeneration is also guided by spatial complementary hormone domains (Efroni et al. 2016). However, low cytokinin:auxin ratios can promote root organogenesis, but not shoot regeneration. The root apical meristem (RAM) formation occurs in an auxin-dependent fashion during the early globular embryonic stage. The meristematic region is formed by stem cells organized around mitotically inactive cells called quiescent centre (QC). The root meristem size varies between species. While Arabidopsis has a small meristem with only four QC cells, maize RAM has 500–1000 cells (Jiang et al. 2010). Most studies on RAM establishment regulation have been done in Arabidopsis. Auxin polar transport from shoot to the root is carried out by PIN efflux transporters and generates auxin maximum at the root tip for expression of TFs at the stem cell niche, PLTs, SCR, SHR and WOX5 (Honkanen et al. 2017).

Impairing the sRNA biogenesis machinery (null *dcl1* mutant) displays early embryo patterning defects, including both SAM and RAM establishment (Nodine and Bartel 2010). Such defects mostly occur due to a precocious up-regulation of TFs that promote differentiation before the pluri-potential cell state can give rise to different cell types. Most of the miRNAs involved in auxin signalling (Fig. 12.3) are crucial for root tissue pattering. Particularly, miR165/166 are produced in the endodermis layer of root meristem, and move into other cell layers where HD-ZIP III TFs dose-dependent suppression is required for protoxylem and metaxylem specification (Carlsbecker et al. 2010).

12.6.3.3 sRNAs Involved in Tissue Polarity

The tasiRNA class was the first described mobile sRNAs acting in a cell non-autonomous manner. Particularly, tasiR-ARFs participate in leaf polarity by establishing opposite gradients for ARF3/4 and HD-ZIP III TFs (Chitwood et al. 2009). miR390 promotes tasiR-ARFs production at the adaxial layers of leaf primordia and they are spread in a gradient decreasing towards the abaxial side. ARF3/4 promote abaxial identity through positive regulation of miR165/miR166, which in turn represses HD-ZIP III TFs. This pathway is conserved in land plants and it is involved in the normal development of leaves, lateral roots and flowers. Moreover, HD-ZIP III mRNA accumulation, defined by miR165/miR166-dependent suppression, is restricted to the central-apical domain of globular stage embryos to promote SAM specification and to ensure cells at the basal pole to be correctly destined to root meristem (Smith and Long 2010). miR165/miR166 act cell non-autonomously from the basal part of the heart stage embryos and subsequently promote the apical fate at the subsequent stages (Miyashima et al. 2013). As additional control mechanism, AGO10 sequesters miR165/miR166 to protect HD-ZIP III mRNA (Zhu et al. 2011). Such regulation provides a novel mechanism by which the graded distribution of sRNAs is translated into an array of cell fates through a miRNA-dependent gene expression control.

12.6.4 sRNAs Abundance Switches in Somatic Embryo Development

sRNA role in the development of somatic embryos and plant regeneration is often overlooked, while significantly more attention has been paid to these molecules during the induction phase of SE. Most of the available research has focused in comparing gene expression programs at dedifferentiated status (i.e. embryogenic callus and or non-embryogenic callus) versus globular, heart and torpedo somatic embryos differentiating upon appropriate stimulus. During most recent years, global analyses using microarrays or next-generation sequencing (NGS) technology compared the presence of conserved and species-specific miRNAs at particular developmental stages of somatic embryos. However, most of the studies use pooled sRNA from the somatic embryos and embryogenic cultures to generate their library, so considerable information might be lost (Table 12.1).

Somatic embryo developmental stages have been established according to each species-specific plant regeneration method. While some species display distinguishable globular, heart, torpedo and cotyledonary embryos, others have assigned early, mid, late embryo or first, second, third stages in a temporary line starting from the differentiation induction. In spite of such heterogeneity, available sRNA data suggest that plant conserved miRNAs exert common functions during somatic embryo development (Fig. 12.2). With greater detail, we have summarized characteristic miRNA abundances reported at different developmental stages of plant regeneration for several species in Table 12.2. All data correspond to the analyses of

Table 12.1 Developmental stages of SE in different crops and type of analysed sRNAs

Specie	Developmental stage	Methods	sRNA	Reference
Oryza sativa (rice)	Differentiated callus	Northern blot NGS	miRNAs, tRNA-derived	Luo et al. (2006) Chen et al. (2011)
Citrus sinensis L. Osb. (orange)	Globular and cotyledon-shaped somatic embryo	qRT-PCR	miRNAs	Wu et al. (2011)
Larix leptolepis (larch)	Early, middle, late single embryo and cotyledonary embryo	NGS qRT-PCR	miRNAs	Zhang et al. (2012)
Liriodendron tulipifera (hybrid yellow poplar)	Stages of embryos: E5–E9	NGS Microarrays	miRNAs	Li et al. (2012)
Dimocarpus longan (longan)	Globular, torpedo-shaped, cotyledonary embryos	NGS, qRT-PCR	miRNAs tasiRNAs	Lin and Lai (2013) Lin et al. (2015)
Gossypium hirsutum (cotton)	Globular, torpedo, cotyledon-stage embryo	qRT-PCR	miRNAs	Yang et al. (2013)
Zea mays (maize)	First stage, second stage, plantlet	Northern blot qRT-PCR	miRNAs	Dinkova and Alejandri-Ramirez (2014) Chávez-Hernández et al. (2015)
Lilium pumilum DC. *Fisch.*	Globular, torpedo and cotyledon-stage embryos	NGS qRT-PCR	miRNAs	Zhang et al. (2017b)

Table 12.2 miRNA expression during somatic embryo differentiation in diverse plants

Different plant groups			Developmental stages of somatic embryo					
			Early stage		Middle stage		Late stage	
			Globular		*Heart*	*Torpedo*	*Cotyledonary*	
Angiosperms	Dicots	Orange[a]	miR156 miR159 miR164	miR390 miR397	miR394		miR166 miR167 miR398	
		Longan[b]	miR397a miR398b		miR159a,b,c,f miR160a miR398a,b	miR159a,b,c miR160a miR167a miR390a miR398b	miR156a miR159a,c miR167a	miR397a miR398b
		Cotton[c]	miR164 miR390				miR156 miR167 miR390	
		Lilium[d]	miR396 miR397 miR168 miR319			miR156 miR164 miR166 miR171	miR160 miR166 miR319	miR390 miR397 miR398 miR482
	Monocots	Maize[e]	*First stage*		*Second stage*		*Plantlet*	
			miR156 miR164 miR168 miR397	miR398 miR408 miR528	miR159 miR167 miR168	miR319 miR408 miR528	miR156 miR164 miR397 miR398	
		Rice[f]	miR150 miR156 miR157 miR158 miR159	miR164 miR166 miR167 miR169 miR394				

Different plant groups		Developmental stages of somatic embryo				
		Early stage	Middle stage		Late stage	
		Globular	*Heart*	*Torpedo*	*Cotyledonary*	
Gymnosperms	Larch[g]	*Early embryo*	*Middle embryo*		*Cotyledonary embryo*	
		miR162 miR168a, b miR171a,b,c	miR171a,b,c		miR159a,b,c miR160 miR162 miR166 miR167 miR168a,b	miR171a,b miR390 miR397 miR398

[a]Wu et al. (2011), Wu et al. (2015), and Long et al. (2018)
[b]Lin and Lai (2013), and Lin et al. (2015)
[c]Yang et al. (2013)
[d]Zhang et al. (2017b)
[e]Dinkova and Alejandri-Ramirez (2014), and Chávez-Hernández et al. (2015)
[f]Chen et al. (2011)
[g]Zhang et al. (2012)

bulk tissues composed mostly of heterogeneous cell types at each stage and might not reflect the precise miRNA expression switches. Recently, a new protocol was developed in *Arabidopsis thaliana* SE to visualize miRNA expression in a whole mount tissue using in situ hybridization (Wójcik et al. 2018). The application of such technique would be of utmost significance since, as discussed above, sRNA and target cell-specific distribution determines particular cell fates in tissues committed to the SE program.

For most plant species shown in Tables 12.1 and 12.2, early stages of differentiation are featured by the expression of miR159, miR164 and miR397. miR164 targets CUC2, a member of the plant-specific NAC domain (NAM, ATAF1/2 and CUC2) TF family, with important roles in plant development and stress responses (Aida et al. 1997). miR159 controls the transcript levels of MYB factors during seed germination and abiotic stress (Reyes and Chua 2007) and miR397, miR398 and miR408 regulate copper-dependent enzymes, such as superoxide dismutases (SOD) laccases and plantacyanin in response to copper deficiency (Abdel-Ghany and Pilon 2008; Sunkar et al. 2012).

It is well known that abiotic stress plays crucial role in modulating differentiation during SE. miR397 and miR398 are particularly abundant at early or late SE developmental stages for most of the analysed plant species. For example, miR398 increased during cotyledon-shaped embryo morphogenesis in orange and during formation of early staged embryo in larch. In *Dimocarpus longan* SE, miR398b, but not miR398a is highly expressed at heart-shaped and torpedo-shaped embryos. However, miR398b levels decreased during cotyledonary embryo development, leading to CSD accumulation and promoting embryo maturation (Lin and Lai 2013; Lin et al. 2015). For maize and rice, miR397, miR398, miR408 and the monocot-specific miR528 were present in both, dedifferentiated and differentiated tissues (Luo et al. 2006; Chen et al. 2011; Chávez-Hernández et al. 2015; Alejandri-Ramírez et al. 2018). Most of them were abundant at initial differentiation stages, but further decreased in the regenerated plantlet. Interestingly, laccases targeted by miR397 or miR528 have been associated with cell wall lignification and thickening during secondary cell growth (Constabel et al. 2000; Sun et al. 2018). Hence, miRNA-mediated down-regulation of laccases might associate with cell wall loosening in dedifferentiated tissues and early differentiation stages (Fig. 12.2).

The miR390-tasiR-ARF-ARF3/4 regulation also seems to operate in SE differentiation. miR390 was abundant at early globular-shaped embryo formation in *Citrus sinensis* (Wu et al. 2011) and *Gossypium hirsutum* SE (Yang et al. 2013), at heart and torpedo embryonic stages in *Dimocarpus longan* and in cotyledonary embryos for *Larix leptolepis* (Lin et al. 2015; Zhang et al. 2012). Correspondingly, *Dimocarpus longan* TAS3 and ARF4 exhibited their lowest expressions at the cotyledonary stage and reached their peaks in globular embryos. Interestingly, the miR390 primary transcript and TAS3 precursors were up-regulated by the synthetic auxin 2,4-D in a concentration-dependent manner (Lin et al. 2015).

Another miRNA participating downstream of auxin signalling, miR166, increased at later stages in *Citrus sinensis* cotyledon-shaped embryo morphogenesis (Wu et al. 2011, 2015), *Lilium pumilum* torpedo-shaped and cotyledonary embryos

(Zhang et al. 2017b) and *Larix leptolepis* cotyledonary embryos (Zhang et al. 2012). For *Dimocarpus longan* SE it was suggested that changes in miR166c* levels might be caused by alterations of endogenous gibberellin GA3 concentrations leading to the inhibition of early embryonic cell differentiation and globular embryo formation (Lin and Lai 2013). However, in *Oryza sativa*, miR166 increment was observed at early SE stages of differentiation (Chen et al. 2011).

The other two miRNAs that regulate ARFs, miR160 and miR167, would be expected to display auxin-dependent, tissue-specific expression patterns. Interestingly, miR160 was barely detectable at early, but highly expressed during heart- and torpedo-shaped embryonic stages of *Dimocarpus longan* SE (Table 12.2; Lin and Lai 2013). On the other hand, *Larix leptolepis* miR160 showed greater abundance at the cotyledonary embryo stage (Zhang et al. 2012). miR167 levels also increased during cotyledonary and mature embryonic stages for *Citrus sinensis*, *Dimocarpus longan*, *Gossypium hirsutum* and *Larix leptolepis* (Wu et al. 2011; Zhang et al. 2012; Yang et al. 2013; Lin et al. 2015). However, this miRNA showed contrasting behaviour in SE depending on the plant species and in vitro culture conditions. For example, rice miR167 decreased when cells, cultured in the presence of auxins, were transferred to an auxin-free medium (Yang et al. 2006), whereas Longan miR167 was undetectable in a medium containing 2,4-D (Lin and Lai 2013). In cotton and maize, miR167 also exhibited up-regulation in the dedifferentiated tissues (Yang et al. 2013; Alejandri-Ramírez et al. 2018). Whether miR167 participates in the SE process through regulating its targets ARF6/8 in response to external auxin levels remains to be elucidated.

As previously mentioned, miR156 mostly regulates tissue embryogenic potential through its SPL targets. It is required at early zygotic embryogenesis (Nodine and Bartel 2010) and during early SE (Long et al. 2018). However, its levels also progressively increased at later differentiation stages for cotton, Longan and maize SE (Table 12.2). A perfect inverse expression pattern was found for the SPL transcript and miR156 during cotton embryo development (Yang et al. 2013). On the other hand, in maize plant regeneration, miR156 also showed initial increase coincident with its target reduction during the differentiation process (Chávez-Hernández et al. 2015). Such behaviour supports its central role in SE for different plant species.

12.7 Conclusions and Perspectives

SE is a noteworthy model to study early developmental features of embryogenesis, molecular aspects of cell differentiation, and is a powerful tool for plant biotechnology. Exploring the role of different sRNA classes in this process constitutes a promising tool to understand the basis of totipotency as well as to achieve successful plant regeneration through the process. Recent progress of sRNA research in agricultural plants has been emphasized on trait regulation, stress responses and reproduction. Taking into account that SE covers a response of the plant to stressful conditions aiming to preserve its potential to further grow and reproduce in the

future, it represents a unique system to challenge our knowledge on developmental molecular cues. Although *Arabidopsis thaliana* has been a great model for sRNA pathways dissection, it urges to extend this research to diverse economically relevant plants. In this sense, SE represents an excellent model to understand sRNA cell-specific fate, target regulation, responses to phytoregulators, stress and differentiation stages. Further exploration of particular to SE sRNA regulatory nodes would provide insights into the development of appropriate tools for crop improvement.

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Chapter 13
Somatic Embryogenesis: Polycomb Complexes Control Cell-to-Embryo Transition

Fátima Duarte-Aké, Geovanny Nic-Can, and Clelia De-la-Peña

Abstract Reproduction by fertilization gives rise to evolutionary adaptation. All of the mechanisms that underlie this process, such as the regulation of embryo formation and seed development, are interesting for scientists and biotechnologists in their effort to breed agronomically important species. On the other hand, we know that the environment has different effects at different stages in plant development, changing their epigenomes. Among epigenetic regulators of development transitions in plants, there are two protein complexes involved in cell fate regulation, Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). Although the interactions of these epigenetic modulators are extensively studied in flowering and vernalization process, their influence on embryo formation and somatic embryogenesis (SE) has only recently been investigated. Therefore, in this chapter we discuss the epigenetic regulation by PRC1 and PRC2 during embryo formation and development. Also, we discuss the most important findings in both zygotic embryogenesis and SE regulation by PRC complexes.

13.1 Introduction

Regardless of the plant species, developmental history is preserved in the embryo, which culminates when the embryo is formed in a process called zygotic embryogenesis (ZE). ZE requires a double fertilization process, one to generate the zygote

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and another to develop the endosperm (Lafon-Placette and Köhler 2014). In dicotyledonous plants, the embryonic stages are defined morphologically as globular, heart, torpedo, and cotyledon. Nonetheless, in several plants, the embryos can form directly from a somatic cell in the unfertilized gametes (Rodriguez-Leal and Vielle-Calzada 2012). In this process, plant cells can change their somatic fate, acquire totipotency, and re-differentiate into a whole plant through a complex phenomenon known as somatic embryogenesis (SE). SE shows that embryo development is not restricted to fertilization in plants and allows us to think that some regulation mechanisms can be switched on in a particular way.

In fact, during both ZE and SE, the developing embryos pass through the same embryogenic stages. However, they differ considerably during the onset of the early molecular and biochemical programs. For instance, zygotic embryos are surrounded by a nourishing tissue called endosperm and possess a stalk-like suspensor structure that provides nutrients to the embryo. Additionally, the endosperm is required to support the synthesis of small messengers such as cysteine-rich peptides and small interference RNAs to regulate the formation of pre-embryo patterning and to reinforce the silencing of transposable elements to sustain the early events of ZE (Costa et al. 2014; Hsieh et al. 2018). In contrast to ZE that requires gamete fusion and all of the above conditions, during SE the plant cells can be induced by in vitro protocols to become totipotent through chemical and mechanical stimuli to allow the development of ectopic embryos. Although several studies in this field hypothesize the existence of a signal or several signals that trigger the development program in SE, there remain questions that cannot be answered. For instance, which are the signals that allow a reset and redefinition of the plant cell identity in order to begin a new developmental program? Perhaps the key to SE is found in the preparation of the cells to enter embryogenic competition, since there is much evidence in the literature that makes it clear that the success of SE depends on the induction process. In this process, the cells experience a considerable stress caused mainly by the ratio between auxins and cytokinins, which triggers the accumulation of key development transcription factors (TF) that could contribute to stabilizing the transition from somatic cells to embryo development.

The expression of underlying genes in the embryogenic pathway requires a complex regulation that acts at the chromatin level. Sophisticated epigenetic mechanisms including DNA methylation and histones' posttranslational modifications can change the state of chromatin, which has been considered as a crucial step that might involve the acquisition of pluripotency or totipotency through hormonal stimuli and developmental pathways (Florentin et al. 2013; Grafi 2003; Verdeil et al. 2007). These changes in chromatin remodeling can be carried out by a select group of proteins promoting the regulation of important development-related genes. For instance, polycomb group (PcG) complexes play a critical role in genome-wide regulation of key developmental genes that promote the phase transitions and cell fate determination in plants and animals (Köhler and Hennig 2010; Bemer and Grossniklaus 2012; He et al. 2011). In plants, populations of stem cells are maintained in the meristems, in which cells are constantly dividing in order to produce new cells that renew continually produced organs such as leaves or flowers. To achieve the production of new cells, the correct gene expression patterns involved in

cellular proliferation or differentiation are tightly regulated by PcG machinery (Bemer and Grossniklaus 2012). In addition, PcG proteins also control several developmental pathways including seed development, flowering time, and vernalization (Chanvivattana et al. 2004; Holec and Berger 2012; Köhler and Villar 2008). In the model plant *Arabidopsis thaliana*, PcG proteins have been classified into two multiprotein complexes: Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2, respectively). Both are involved in the repression of genes through histone modifications (Makarevich et al. 2006; Molitor et al. 2014). It was found that PCR2 is implicated in the regulation of pluripotency-related genes in order to maintain the repressed transcriptional state of target genes through cell division. However, PRC2 does not act alone, but works in cooperation with PRC1. The functionality of these complexes is complementary since PRC2 acts as a histone methyltransferase that methylates lysine 27 on histone H3 (H3K27me3) (Cao et al. 2002), and this epigenetic mark is in turn recognized by PRC1, which displays a monoubiquitination activity on histone H2A at lysine 119 (H2AK119Ub1) (Schatlowski et al. 2008). Therefore, both complexes contribute to the subsequent chromatin compaction that prevents the binding of transcription factors to DNA.

The evidence found from different mutants of PRC1 and PRC2 complexes suggests that PcG complexes have important roles in the maintenance of the repression genes for the correct cell differentiation in embryo formation. Therefore, they might provide important clues about how plant somatic cells can dedifferentiate into a totipotent embryonic cell. For this reason, in this chapter we discuss the regulation of embryogenesis and seed development by the polycomb group, comparing the advances in this field with the works that expose different clues to unravel the process of the beginning of SE.

13.2 Polycomb Multiprotein Repressive Complexes and Their Key Role in the Change of Cell Identity

PcG proteins were first discovered more than 50 years ago in *Drosophila melanogaster*, in which these complexes played an essential role in the regulation of thorax development through the correct expression of homeotic (*HOX*) genes [reviewed in (Morey and Helin 2010)]. *HOX* genes are a regulatory family coding for specific proteins that act as TF. These TF are characterized by sharing a homeodomain (HD), which is responsible for the recognition of and binding to a specific motif of DNA. In insects, the transcriptional activation of *HOX* genes leads to segmental determination. However, when the function of PcG is lost in PcG mutants, the HOX has a differential accumulation, while the expression of *HOX* gene was detected, even in the absence of PcG activity. Spatially, gene expression was distributed in a different region of the fly body, causing the incorrect formation of the *Drosophila* thorax. This finding indicated that PcG acts by repressing target genes (in this case the *HOX* gene family) as a cellular memory mechanism through successive cell divisions (Zheng and Chen 2011) to assure the correct body development.

The PcG proteins exist in distinct multiprotein complexes, of which PRC1 and PCR2 have been two of the most characterized, biochemically and genetically. These complexes work as transcriptional repressors of several 1000 genes throughout histone modifications (Köhler and Villar 2008; Sanchez-Pulido et al. 2008).

13.3 The Polycomb Repressive Complex 1, PRC1

In *Drosophila,* the repressive complex of PRC1 is formed by four core subunits: polycomb (PC), polyhomeotic (PH), posterior sex combs (PSC), and sex combs extra (SCE), also known as dRING. Additionally, this complex contains proteins such as sex combs on midleg (SCM), ZESTE, and general transcription factors (GTFs) (Breiling et al. 2001; Francis et al. 2001; Franke et al. 1992; Mohd-Sarip et al. 2002; Shao et al. 1999). It is important to highlight that there is evidence that the genes that code the proteins that compose the PRC1 complex originated in early animal evolution, as they are present in several insects as well as vertebrate species. However, some PRC genes are absent in species from other phyla. For example, except *SCM*, all of the genes that form part of the PRC1 complex are absent from the genome of the nematodes *Caenorhabditis elegans* and *C. briggsae*. On the other hand, PSC, dRING, and SCM are not found in the urochordata *Oikopleura dioica* (Schuettengruber et al. 2007). In the case of mammals, the PRC1 complex contains the Chromobox protein family (CBX 2, 4, 6, 7, 8) that carries out the PC function (Morey et al. 2012). Also, there are 3 PH homologs: PH1, PH2, and PH3 (Tonkin et al. 2002) and six PSC homologs. These proteins together are known as PcG RING fingers and include the BMI1A-C proteins. The last homologs of SCE proteins are RING1A and RING1B (Schoolemmer et al. 1997). In general, the function of PRC1 in *Drosophila* and mammals is to bind to H3K27me3 marks (Pc subunit) (Fischle et al. 2003) and catalyze the ubiquitination of H2A Lys-119 (RING subunit) (Wang et al. 2004; de Napoles et al. 2004), shaping a stable heterochromatin state and repressing all of the machinery of transcription (Holec and Berger 2012).

However, in contrast to *Drosophila* and mammals, plant genomes do not contain homologs of the animal PRC1 complex. For instance, in the *Arabidopsis* genome, the PRC1 does not contain PH and PC homologs of mammal components. Several proteins are thought to perform the function of the PRC1 complex. In fact, the plant chromodomain protein LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) (Fig. 13.1), also known as TERMINAL FLOWER 2, is considered to be a PRC1 component in plants because this protein binds in vitro (Gaudin et al. 2001; Turck et al. 2007) and co-localizes with the H3K27m3 mark in vivo (Zhang et al. 2007). In general, the principal function of LHP1 is to bind to euchromatic sites and silencing genes, including the PcG target genes (Fig. 13.1a, d). Additionally, the *lhp1* mutant phenotype is quite distinct from the phenotypes of PRC2 component mutants (Gaudin et al. 2001; Takada and Goto 2003), with the exception of an impact on flowering time (Mylne et al. 2006; Sung et al. 2006). Two other proteins, VERNALIZATION 1 (VRN1) and EMBRYONIC FLOWER 1 (EMF1), which bind and act together with

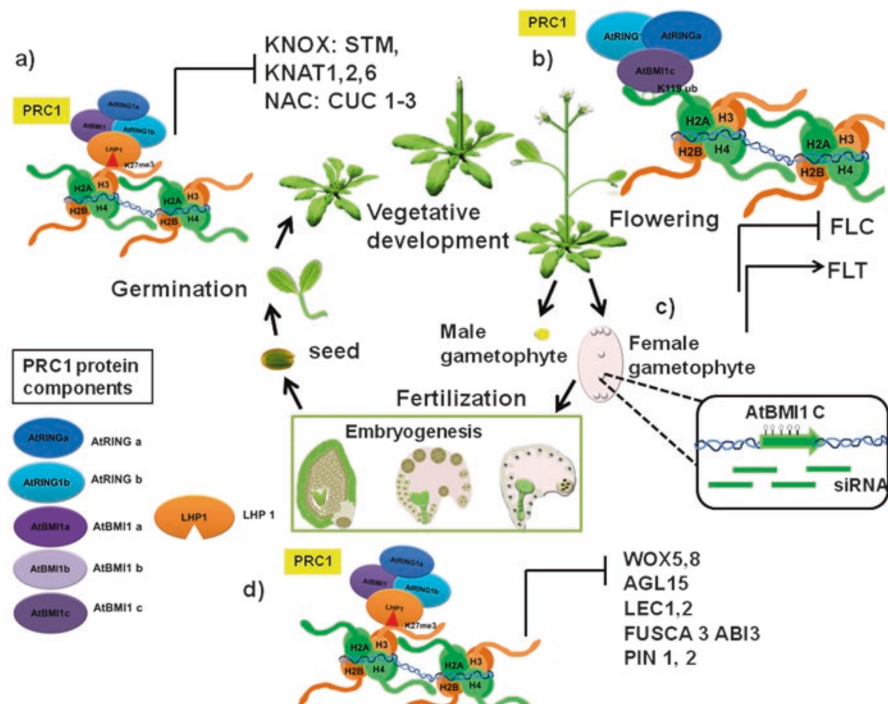

Fig. 13.1 PRC1 components and regulation of genes during different developmental stages of *Arabidopsis thaliana*. The PRC1 complex formed with AtRING1a, AtRING1b, AtBMI1a, and LHP1 promotes the H3K27me3 mark's silencing of the *KNOX* genes such as *STM*, *KNAT1*, *2*, *6*, and *CUC1–3*, a process that controls organ formation in the vegetative stage (**a**). During flowering in *A. thaliana*, the flowering repressor FLC is silenced by the PRC1 complex, thus recruiting the K119Ub to control flowering initiation and the expression of FLT (**b**). The *AtBMI1c* is maternally imprinted in endosperm (**c**). During embryogenesis, *WOX5*, *8*, *AGL15*, *LEC1*, *2*, *FUS3*, *ABI3*, and *PIN1* and *2* are silenced by the H3K27me3 mark recruited by the PRC1 complex (**d**)

LHP1, AtBMI1a, and AtBMI1b (Bratzel et al. 2010), have been proposed to be involved in PRC1-like functions (Calonje et al. 2008; Mylne et al. 2006). In this sense, Calonje et al. (2008) reported that EMF1 participates in the PcG-mediated gene silencing of the flower homeotic gene AGAMOUS (AG) during vegetative development in *A. thaliana.* Several proteins with a similar activity to PRC1 components are specific to plants (Holec and Berger 2012). Therefore, not all PRC1 homologs are conserved among species, so it may not be surprising that PRC1 function in plants is mediated by mechanisms distinct from that described in mammals.

For a long time, it was though that PRC1 is not necessary for stable repression in plants, since they are sessile organisms, and it was expected that they must be able to respond quickly to environmental signals. However, this appears not to be the case because recently some components of this complex have been identified (Sanchez-Pulido et al. 2008) implying that other factors have better control of gene expression. For instance, the belief that the lack of PCR1 and the modification that

it catalyzes (H2AK119ub) may be at least partly responsible for the greater dedifferentiation potential of plants compared to animals is widely accepted. In contrast to animals, which form a germline early in development, plant gametes are produced from vegetative cells that will acquire the competence to undergo gametogenesis. Therefore, plant cells need to remain competent in order to dedifferentiate. Stable maintenance of gene repression is probably less important or even unfavorable during plant development. This could explain why plants lack of all of the components of a PRC1-like complex (Köhler and Villar 2008). However, the paradigm is changing, and there is evidence showing that plant PRC1 RING finger homologs are present, suggesting a conservation of function for these proteins between animals and plants.

In *Arabidopsis,* PRC1 finger RING proteins were identified by analyzing the domain structure of mammalian BMI1 (B cell Moloney murine leukemia virus insertion region 1 protein) and RING1 proteins (Sanchez-Pulido et al. 2008). The same authors found that these proteins maintain a significant sequence similarity in RING finger domain at their N-terminal region and ubiquitin-like (Ubq-like) domain at the C-terminal region, which was named the RAWUL domain (Ring finger and WD40 associated Ubq-like). In *Arabidopsis*, ive PCR1 RING finger homologs have also been identified, two of them homologs to RING1, AtRING1A and AtRING1B, and three homologs to BMI1, AtBMI1A (also named DRIP1, DREB2A-INTERACTING PROTEIN1), AtBMI1B (also named DRIP2), and AtBMI1C.

The principal characteristics of PRC1 and PRC2 complexes and their role in zygotic embryogenesis (ZE) and somatic embryogenesis (SE) process in plants are described below.

13.3.1 AtRING1A, B and AtBMI1A, B Regulate Plant Embryonic and Stem Cell Development

The RING finger proteins are active E3 ubiquitin ligases in *Arabidopsis* and the recombinant AtRING1 and AtBMI1 have the ubiquitin ligase activity in vitro. However, in vivo H2A monoubiquitination has not been demonstrated so far. On the other hand, the identical phenotype of *atbmi1* and *atring1* mutants suggests the need to establish H2A ubiquitination (Bratzel et al. 2010). Bratzel et al. (2010) and Chen et al. (2010) demonstrated that double mutants of *atring1a/b* and *atbmi1a/b* present a de-repression of embryonic traits during vegetative development (Fig. 13.1a). The *atring1a atring1b* double mutants present a phenotype with shoot apical meristem (SAM) defects. These mutants display embryonic callus formation and/or pickle-root phenotypes, which can occur simultaneously within the same plant.

Among key regulatory genes involved in embryogenesis that are regulated for these RING finger proteins are *WUSCHEL-related homeobox 5* (*WOX5*) and *WOX8*, which are crucial for root apical meristem (RAM) function and basal embryo axis cell fate termination (Fig. 13.1d). According to expression analysis into *atring1a*

atring1b double mutants, Breuninger et al. (2008) found other proteins that are regulated by PRC1 components, such as *AGAMOUS-like MADS-box protein 15* (*AGL15*; an embryonic competence enhanced factor gene) and several regulatory genes of embryogenesis, such as the AP2-like ethylene responsive factor *BABY BOOM* (BBM), *LEAFY COTYLEDON 1* (*LEC1*), *LEC 2*, *FUSCA 3* (*FUS3*) *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), and auxin transporters such as *PIN-FORMED 1* (*PIN1*) and *PIN2*. This finding reinforces the active participation of PcG complexes in the regulation of embryogenesis (Fig. 13.1d).

Furthermore, Chen et al. (2010) determined that AtBMI1A-C can bind with AtRINGA or AtRINGB (Fig. 13.1a, b, d) and, in addition, AtBMI1C (Fig. 13.1c) can bind with LHP1 (Fig. 13.1a, d). This suggests that AtRING and AtBMI1 proteins have non-redundant functions within a PRC1 complex, which is crucial for the maintenance of differentiated somatic cell fate during postembryonic plant development. Additionally, working on mutants, Chen et al. (2010) found high expression levels of *AtBMI1A*, *AtBMI1B,* and *AtBMI1C* in the *atring1a atring1b* mutants, and the expression of *AtBMI1C*, *AtRING1A,* and *AtRING1B* is elevated in the *atbmi1a atbmi1b*, suggesting a self-regulatory feedback mechanism.

On the other hand, the maintenance of meristem function is a primordial event during embryo development. The maintenance of the pluripotent cell population to generate all organs of the vascular plant is the role of class 1 KNOTTED-LIKE homeobox (KNOX) TF (Jackson et al. 1994). The appropriate regulation of this gene is crucial for correct organ development in plants. In multiple studies it was found that KNOX TF are epigenetically regulated in some differentiation processes (De la Peña et al. 2012). However, new insights about epigenetic regulation by PRC1 components have been revealed. For instance, AtRING1A and AtRING1B have the capacity to bind to LHP1 and they together are involved in the repression of class 1 *KNOX* genes for the regulation of shoot stem cell activity (Xu and Shen 2008) (Fig. 13.1a). Among class 1 KNOX TF, *SHOOT MERISTEMLESS* (*STM*), *BP/KNAT1* (*KNOTTED-LIKE 1* from *Arabidopsis thaliana*), *KNAT2* (*KNOTTED-LIKE 2* from *A. thaliana*), *KNAT6,* and the NAC-domain transcription factor genes *CUP SHAPED COTYLEDON 1* (*CUC1*), *CUC2* and *CUC3* are up-regulated in *atring1a atring1b* double mutants (Fig. 13.1a). This suggests an important role of the PRC1 complex in maintaining a closed chromatin state and reprising *KNOTTED* and *CUC* genes for the correct renewal of meristem cells and organ formation (Fig. 13.1a).

13.3.2 MtLHP1, MtRING1, and MtVRN1 as Probable PRC1 Gene Markers of SE

The role of PRC1 proteins in SE has not been elucidated yet. However, it is known that PRC1 participates actively during SE in *Medicago truncatula*. Orlowska and Kępczyńska (2018) found interesting results in *M. truncatula* from differential expression analysis of *LHP1*, *RING1*, *BMI1*, *EMF1,* and *VRN1* by comparing

embryogenic (E) and non-embryogenic (NE) lines. The authors revealed that the lower expression level of all of these *PRC1* genes in the E line probably triggers processes leading to the formation of embryogenic cells. Conversely, the higher expression of all *PRC1* genes tested in the NE line may be responsible for the inhibition of SE induction because most of the genes that code for the proteins that make up the PRC1 complex are silenced during SE. *MtBMI1* expression in both E and NE lines during the induction phase was almost identical, suggesting that BMI1 is not necessary to repress the embryogenic program in *M. truncatula*. On the other hand, the expression of *MtVRN1* gradually increased in the E line, which was related to embryo formation during the differentiation phase, whereas the expression of *MtVRN1* in the NE line was unchanged, and the embryo was not formed. The authors concluded that these findings indicate that *MtVRN1* participates by modulating embryo development events (Orlowska and Kępczyńska 2018).

13.4 Polycomb Repressive Complex 2, PRC2

Among the PcG complexes, PRC2 is highly conserved and well characterized in both animals and plants. Each subunit in the *Drosophila* PRC2 complex has several paralogs in *Arabidopsis*. For instance, *CURLY LEAF* (*CLF*), *SWINGER* (*SWN*), and MEDEA (*MEA*) are orthologous of the *Drosophila* Enhancer of zeste E(z) protein, which is a SET domain protein that possesses histone methyltransferases activity for histone H3 lysine 27 trimethylation (H3K27me3) (Chanvivattana et al. 2004; Makarevich et al. 2006). Whereas *EMBRYOGENIC FLOWER* (*EMF2*), *FERTILIZATION-INDEPENDENT SEED2* (*FIS2*) and *VERNALIZATION2* (*VRN2*) are homologs of the Suppressor of zeste12 Su(z)12 protein; *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) is homolog of Extra Sex combs (Esc) and MULTYCOPY SUPRESSOR OF IRA HOMOLOG1-5 (MSI1-5) corresponding to p55 [reviewed in (Köhler and Villar 2008)]. In accordance with molecular and genetic evidence, it has been proposed that at least three forms of PRC2 exist in plants such as FIS2, VRN2, and EMF2, and each complex acts at specific developmental phases in *Arabidopsis*. The FIS2 complex, which contains MEA/SNF, FIS2, FIE, and MSI1, regulates early seed development while the VRN2 complex, integrated by SWN/CLF, VRN2, FIE, and MSI1, acts in response to prolonged cold treatment and induces flowering (vernalization). In the case of the EMF2 complex, integrated by SWN/CLF, EMF2, and FIE, it acts in the embryo and during subsequent sporophyte development [reviewed in (Köhler and Villar 2008; Deng et al. 2018)]. However, it is unknown how the readout and specificity of PRC2 complexes are established.

PRC2 proteins are a chromatin-modifying complex that catalyze the activity and dynamic of the H3K27me3 mark, which is a major silencing mechanism in plants and has a key role in cellular identity and cellular memory (Zhang et al. 2007; Butenko and Ohad 2011; Ikeuchi et al. 2015). This epigenetic mark is enriched in genes with tissue-specific expression patterns or genes that are induced by biotic or

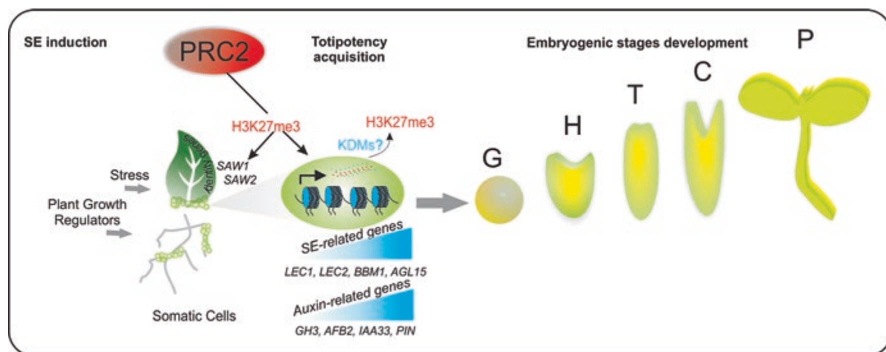

Fig. 13.2 Possible reprogramming of H3K27me3 during somatic embryogenesis (SE). PRC2 provides a mechanism to maintain cell identity. However, during SE induction, a genome-wide reprogramming of H3K27me3 is critical in changing the cell fate and acquiring cellular totipotency. Integration of multiple stimuli, such as stress and plant growth regulators, could be interpreted by PRC2 and KDM (histone demethylase) proteins to begin chromatin remodeling. This remodeling in the chromatin switches on the expression of SE-related transcription factors *(LEC1*, *LEC2*, *BMM1*, and *AGL15)* as well auxin-related genes (*GH3*, *AFB2*, *IAA33*, and *PIN)* in order to ensure the embryogenic program consistently transitions through the embryogenic stages [globular (G), heart (H), torpedo (T), and cotyledonary (C)] to whole plant (P)

abiotic stresses, which suggests that H3K27me3 has a dynamic function in plant development in *Arabidopsis*. This dynamic regulation has been observed through different developmental processes, from differentiation of SAM to leaf formation (Lafos et al. 2011), endosperm development (Weinhofer et al. 2010), the embryo-to-seedling phase transition (Bouyer et al. 2011), transition from vegetative to reproductive growth, flowering time regulation (Farrona et al. 2011), callus formation (Ikeuchi et al. 2013), reacquisition of embryonic potential (Nic-Can et al. 2013), and others. All of these findings show that reprogramming of H3K27me3 is required for tight regulation of the life cycle of plants including the embryogenesis process (Fig. 13.2).

13.4.1 Reprogramming of PRC2 Complex During Acquisition of Embryonic Traits

It has been known for some time that the application of plant growth regulators, mainly auxin and cytokinin, can induce the reprogramming of cells to establish a new developmental pathway and regenerate a whole plant. However, the role of PRC2 in the acquisition of totipotency remains mostly unknown. SE requires dramatic changes in cell identity in order to modify its cell fate to an embryogenic cell, which is accompanied by changes in expression of several genes. For instance, early phases of SE are characterized by the expression of ZE regulators such as *LEC1*, *LEC2*, *AGL15*, *BBM1*, and *WUSCHEL*, which, when overexpressed, lead to the

generation of somatic embryos from vegetative tissues (Lotan et al. 1998; Boutilier et al. 2002; Zuo et al. 2002; Braybrook et al. 2006; Thakare et al. 2008). Notably, the early developmental stages of somatic embryos are accompanied by histone modifications that lead to the modulation of the expression of TF involved in the embryogenic program (De la Peña et al. 2015) (Fig. 13.2).

There is evidence showing that PcG complexes are essential for maintaining mitotically stable gene repression. In plants, more than 25% of the protein coding genes are dynamically regulated by PcG (Lafos et al. 2011). Although H3K27me3 was first described as targeting only transcribed regions of single genes (Zhang et al. 2007), a more recent study highlights the importance of H3K27me3 as an additional silencing mechanism to regulate transposable element genes and a large fraction of miRNAs during the differentiation of *Arabidopsis* (Lafos et al. 2011; He et al. 2012). Consistently, the loss of function of two plant E(z) homologous PRC2 components, *CLF* and *SWI*, causes strong defects in organ identity, leading to the generation of callus-like tissue and ectopic somatic embryo formation (Chanvivattana et al. 2004). Similar results have also been observed in the early seedlings of *emf2vrn2 and fie*, in which somatic embryos can be clearly distinguished, and this capacity is based on the failure of repression of embryonic regulators such as *LEC1*, *LEC2*, *AGL15*, *ABI3,* and *FUS3* (Schubert et al. 2006; Bouyer et al. 2011; Ikeuchi et al. 2015). Furthermore, the lack of activity of MEA, another homolog of E(z), impairs the histone methyltransferase activity of the E(z) protein, allowing the up-regulation of *FUS3,* indicating that MEA, CLF, and SWN share at least a common target gene (Makarevich et al. 2006). These findings support the role of PRC2 as one of the principal factors involved in cell identity to maintain stable repression of key genes related to the embryonic program. It appears that a specific embryogenic status can be attained by decreasing the H3K27me3 patterns. The loss or reduction of H3K27me3 levels on the locus of both *LEC1* and *BBM1* during early events of SE induction in *Coffea canephora* supports this idea (Nic-Can et al. 2013).

13.4.2 PRC2 Does Not Act Alone: Plant Growth Regulators and Other Molecules Allow Embryo Formation

In most plant cultures, the application of auxin to the culture medium changes the balance of endogenous auxin modifying indole-3-acetic acid (IAA) metabolism in the cells, and is a crucial step for the induction of SE (Nic-Can and Loyola-Vargas 2016; Nic-Can et al. 2016). Thus, the increase of endogenous auxin has to be accompanied by the activation of genes involved in its biosynthesis, transport, signaling, and perception at a specific level to reach the embryogenic potential. This implies that auxin homeostasis is at the center of somatic embryogenesis induction. Therefore, a possible explanation for the generation of somatic embryos and the callus-like tissue in *emf2vrn2* or *clfswn* double mutants could be misregulation of auxin homeostasis-related genes, which would increase the endogenous auxin to a level where the pro-embryogenic cells can acquire the embryogenic potential (Schubert et al. 2006; Bouyer et al. 2011).

There is evidence that supports the idea that the reentry to the cell cycle and acquisition of totipotency activates the auxin biosynthesis pathway including TRYPTOPHAN synthases, nitrilases, and specialized cytochrome P450, as well as genes encoding conjugating enzymes, *GH3-2*, *GH3-3*, auxin receptors such as *AUXIN F-BOX 2 (AFB2)*, *AFB5*, and signaling including *IAA20, IAA29,* and *IAA33* (Chupeau et al. 2013). Interestingly, it has also shown that the entire auxin signaling pathway, including biosynthesis and transport, is regulated by H3K27me3 (Lafos et al. 2011) (Fig. 13.2). Further investigation is required to determine how the PRC2 complex regulates auxin-related genes during the beginning of SE.

There are some reports that show a correlation between hormone stimulus and PRC2 activity. For instance, in *A. thaliana* SE, Mozgová et al. (2017) found that the absence of PRC2 by itself is not enough to achieve full cell dedifferentiation required by SE, but that PRC2-depleted somatic cells respond to external hormone and stress treatments by becoming competent for SE. Using inducible activation of PRC2 in PRC2-depleted cells with hormone treatments, Mozgová et al. (2017) demonstrate that transient reduction of PRC2 activity allows SE formation. The authors propose that lowering the PRC2-imposed epigenetic barrier combined with hormonal stimuli allows ectopic co-activation of key developmental regulators and establishment of embryogenic potential in plant vegetative tissues, opening possibilities for novel approaches to cell reprogramming.

13.4.3 H3K27 Methylation and Demethylation: A Flexible Strategy for Organ Identity

In *Arabidopsis*, it has been shown that reprogramming of H3K27me3 is critical for pluripotency acquisition. For instance, during the leaf-to-callus transition, H3K27me3 levels first decrease certain auxin-pathway genes. This finding suggests that demethylation mechanisms might be involved in the transcriptional reactivation of auxin-related genes (Fig. 13.2). Although the exact molecular mechanism remains to be elucidated, it has been determined that overexpression of *RELATIVE EARLY FLOWERING 6* (REF6), a demethylase of H3K27me3, displays a phenotype reminiscent to *emf2*, in its ability to generate a callus-like structure (Lu et al. 2011). These findings suggest a dynamic of H3K27me3 through methylation and demethylation mechanisms to repress or activate genes at the same time during callus formation.

He et al. (2012) showed that reprogramming of H3K27me3 to direct cell fate transition may behave in a tissue-dependent manner. In contrast to the root explants, leaf explants and cotyledons of the *clf swn* and *emf2* mutants are defective in forming callus. This suggests that PCR2-mediated H3K27me3 is critical to suppressing the expression of leaf identity genes including *SAWTOW 1* (*SAW1*), *SAW2*, and some members of the *TCP* family TF, whereas the roots of the mutants formed calli normally (He et al. 2012) (Fig. 13.2). In addition, fully differentiated plant cells can still dedifferentiate and generate somatic embryos once PRC2-mediated epigenetic

repression is removed. Under this circumstance, expression of *WOUND INDUCED DEDIFERENTIATION3* and *LEC2* leads to callus formation and embryogenesis, suggesting that PRC2 activity is required to prevent unscheduled dedifferentiation and maintain the cell identity (Ikeuchi et al. 2015). Taken together, these results highlight the role of PRC2 in the reprogramming of gene activity as a crucial step in changing the somatic cell traits and establishing the embryogenic program.

13.5 Conclusions

In this work, we provide an explanation for how PRC1 and PRC2 could be impacting chromatin remodeling and influencing the on/off switch for TF related to the acquisition of cellular totipotency. It is known that PcG are key regulators in all stages of plant development, and the role of PcG in the cellular reprogramming that guides the cells through SE is starting to be understood. The studies carried out in *M. truncatula* make us speculate that the role of PRC1 has been overestimated; instead, it seems to be a major player in the regulation of SE. Still, it is unknown if there is a synergistic relationship between PRC1 and PRC2, both masters of cellular memory in plants.

It is well accepted that totipotent potential requires the exposure of somatic plant cells to strong stress and non-physiological concentrations of plant growth regulators. Related to this idea, we now know that PRC2 may respond to auxin stimulus in *Arabidopsis* SE. In addition, the loss of function of some components of both PRC2 and PRC1 leads to ectopic expression of SE-related transcriptional factors, which promote somatic embryo generation. For instance, the loss-of-function mutants of some components of both PRC2 and PRC1 lead to ectopic expression of *LEC1* and *LEC2*, *AGL15*, *BBM*, *WUS,* and *WOX5*, which promote cell dedifferentiation and callus development, as well as somatic embryo generation.

These findings provide strong evidence for the important role of PcG complexes as one of the major ways to maintain the stable repression of embryo-related genes in somatic cells. However, the role of PRC1 and PRC2 complexes in the gene regulation process that triggers somatic cells to change their cell fate is not yet well understood. For this reason, it is important to review the principal findings and create new hypotheses about epigenetic regulation in this in vitro process.

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Chapter 14
An Overview of the Epigenetic Landscape of the Male Germline

Cristiane S. Alves

Abstract The challenges faced in agriculture for improving crop yield and overcoming natural barriers are becoming more complex due to environmental changes and population growth. Improving agriculture will, in many ways, require a better understanding of the genetics and epigenetics behind plant adaptation and inheritance of desirable traits. In order to do so, it is essential to understand the mechanisms of germline regulation. The epigenetic mechanisms that orchestrate chromatin remodeling include DNA methylation, histone modifications, and small RNAs that act in synergy to modulate gene expression and regulatory elements. In pollen, these mechanisms are still poorly understood, but nevertheless, are coming to light.

14.1 Introduction

For centuries, crop improvement has been one of the crucial goals for humanity survival. Due to climate change and the growing rate of human population, better and faster strategies for increasing crop yield are necessary to feed human populations for the years to come. Crossing plants for acquiring desirable traits has been the main strategy to accomplish this task. As breeders select desirable phenotypes and not the type of underlying molecular variation, these traits can be either genetic or epigenetic (Springer 2013).

Another approach used by breeders is to introduce new alleles through mutagenesis or transgenic modification. Additionally, epigenetic mechanisms can shape transgenic performance, either by silencing inserted transgenes or by modulating the epigenetic status of a particular gene; consequently, all these strategies could be used to acquire desirable traits (Springer 2013).

In flowering plants, gametes develop within the floral primordia that arise from postembryonic stem cells of the shoot and floral meristems, keeping some undifferentiated cells from early embryogenesis until floral determination

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(Feng et al. 2013). Additionally, the floral primordia have the ability to generate somatic tissues, such as leaves and somatic branches. Consequently, plant germ cells might be exposed to somatic modification and transmit these somatic marks to the next generation (Feng et al. 2013; Schmidt et al. 2015).

The paternal germline derives from a pollen mother cell (PMC) that undergoes two divisions, meiosis I and meiosis II, resulting in four haploid microspores (Fig. 14.1). An additional asymmetric mitotic division subsequently results in the

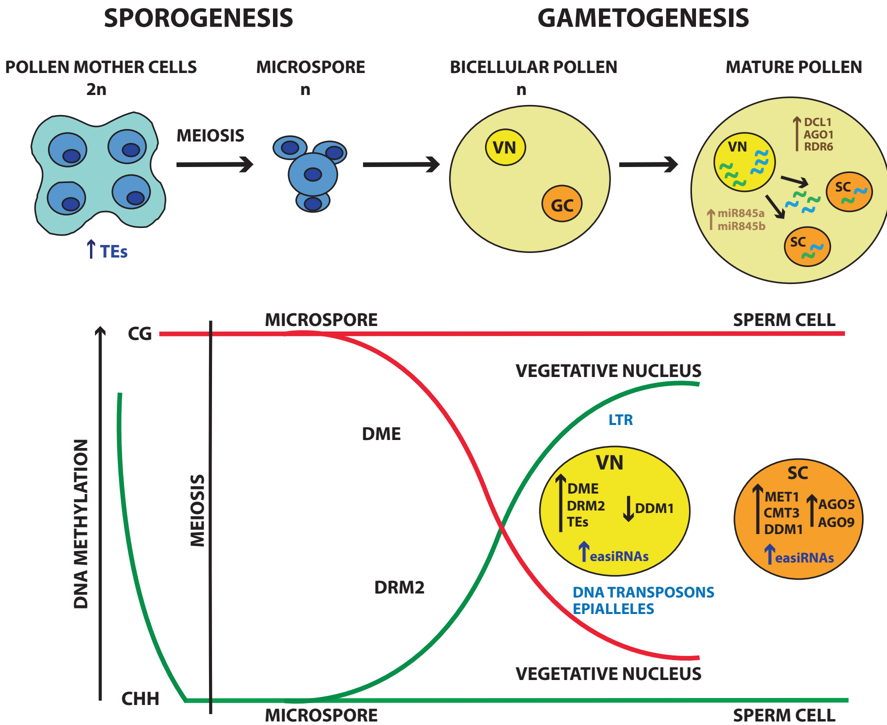

Fig. 14.1 *Arabidopsis thaliana* male gametogenesis epigenetic reprogramming. In the flower, the PMC is produced in a position-dependent manner from somatic cells in the male reproductive tissues. Meiosis takes place and generates microspores; the microspore undergoes asymmetrical division to give rise to the vegetative nucleus (VN) and the generative cell (GC). The GC divides again to create two sperm cells (SC), which leads to the mature pollen. The mCHH levels decline from the microspore to the SC; nevertheless, the level of mCG remains stable after meiosis, which is consistent with the expression of genes such as *MET1*, *CMT3,* and *DDM1* involved in DNA methylation. Transcription of TEs started to accumulate in the VN but not in SC. The VN loses mCG and restores mCHH especially at LTR retrotransposons. In the mature pollen, miR845a and miR845b play role in the biogenesis of 21 and 22nt easiRNAs by targeting retrotransposons. *DCL1*, *AGO1*, and *RDR6* are up-regulated, as well as *AGO5* and *AGO9* are also up-regulated in mature pollen. easiRNAs accumulate in SC at the same time that VN losts heterochromatin and TEs start to reactivate, due to the activity of DDM1 and DME. easiRNAs are generated in the VN and travel to the SC, where they target TEs, also 24-siRNAs from transposable elements flanking imprinted genes accumulate in SC

formation of the generative cell (germ cell—GC) and vegetative cell (VC) that exit the cell cycle in G_0. The generative cell divides again, producing isomorphic sister sperm cells (SC) enveloped in the cytosol of the larger vegetative cell (Fig. 14.1). The vegetative cell, a terminally differentiated cell type, eventually undergoes directional growth to form the pollen tube. The pollen tube is a morphological feature that guides the delivery of both sperm cells to the ovule, where double fertilization of the egg and central cell gives origin to the developing embryo, and endosperm, respectively. The central cell is diploid, hence the endosperm is a triploid extra-embryonic tissue where gene imprinting and dosage occurs, processes required for proper seed development (McCormick 1993; Berger and Twell 2011).

Chromatin remodeling is the dynamic process by which chromatin structure is modified restricting or allowing access to genomic DNA and regulatory elements, and thereby controlling gene expression. Epigenetic modifications affecting chromatin properties include DNA methylation, histone modifications, and chromatin modifiers, as well as microRNAs (miRNAs) and small interfering RNAs (siRNA). Epigenetic variation in plants can be inherited by the next generation through germline transmission, leading to phenotypic effects (Jablonka and Raz 2009). During male gametogenesis there is a decrease in global gene expression, at the same time pollen-specific transcripts raise, somatic transcripts are selectively silenced, possibly due to miRNA activity (Honys and Twell 2004). Moreover, functionally different transcripts arise from the vegetative nucleus (VN) and sperm cells, while VN is enriched with pollen tube growth and pollen germination transcripts (Pina et al. 2005), the SC undergo a long DNA replication phase that last until fertilization, with the predominance of transcripts dedicated to DNA repair, cell cycle transition, and ubiquitin-mediated protein degradation (Borges et al. 2008).

Another layer of regulation modulating locus accessibility is the covalent attachment of a methyl group to a cytosine. DNA methylation (mC) is associated with genetic regulation, cell memory, silencing of transposable elements, genomic imprinting, and repression of pseudo-elements coming from duplicate sequences (Bird 1995; Yoder et al. 1997; Colot and Rossignol 1999). Methylation patterns are established and maintained via an appropriate functional DNA methylation machinery. DNA methylation can be inherited across cell division without changes in DNA sequence, therefore it is defined as an epigenetic modification. An important aspect of DNA methylation in plants is that it can arise in three sequence contexts: CG, CHG, and CHH, in which H can be A, T, or C (Kawashima and Berger 2014).

To guarantee the integrity of the genome for the next generation, the germline should be free of errors. In addition, germline reprogramming is a key to allow totipotency in the zygote. Reprogramming erases epigenetic signatures acquired in response to the environment and during organismal development. Without reprogramming, epigenetic marks will be inherited across generations and allow epialleles (alternative chromatin states) to be inherited and accumulate across generations. This can have adverse effects, such as the release of silenced TEs (transposable elements) that may be harmful to the integrity and homeostasis of the genome (Martienssen and Colot 2001; Lippman et al. 2003; Slotkin et al. 2009;

Borges et al. 2012; Calarco et al. 2012). On the other hand, epialleles can also be beneficial, and their epigenetic inheritance can lead to evolutionary adaptations (Johannes et al. 2008, 2009; Weigel and Colot 2012).

The idea that the environment influences heredity exists in the evolutionary view for centuries. In the early nineteenth century, the evolutionist Jean-Baptiste Lamarck proposed the "Theory of inheritance of acquired characteristics," wherein the use or disuse of an organ led to its amplification or atrophy and the next generation inherits the phenotype (Springer 2013; Blake and Watson 2016). The theory proposed by Lamarck makes special sense in the Plant Kingdom, since plants generate germ cells from somatic tissue, potentially accumulating long-term environmental influences while in animals, the organism saves a dedicated germ cell line for this purpose (Springer 2013; She and Baroux 2015; Blake and Watson 2016).

Epigenetic inheritance is widespread, this phenomenon could be partially explained because sperm cells reprogramming occurs in asymmetric cytosine -CHH-methylation, while after fertilization CHH methylation is reestablished by small RNAs that come from the maternal side and disseminated through the embryo (Calarco et al. 2012; Ibarra et al. 2012). Moreover, throughout this process, small RNAs play an important role in modulating transcriptional and translational dynamics from individual developmental stages (Borges et al. 2011).

Inheritance of epigenetic changes through the germline (i.e., transgenerational) also occurs in unicellular and other multicellular organisms. DNA methylation is often associated with the inheritable changes in genomic expression leading to diversity and adaptation. In plants, DNA methylation is established and maintained by DNA methyltransferases. METHYLTRANSFERASE1 (MET1) is responsible for symmetric CG methylation after DNA replication by recognizing hemimethylated CG sites (Law and Jacobsen 2010). CHROMOMETHYLTRANSFERASE3 (CMT3) and CROMOMETHYLTRANSFERASE2 (CMT2) maintain CHG and CHH methylation via the chromo and BAH domains that recognize methylated histone H3 tails. CHG methylation is mostly correlated with H3K9 (histone H3 lysine 9) methylation (Du et al. 2012). Conversely, the H3K9 methyltransferases KRYPTONITE (KYP), SU(VAR)3-9 HOMOLOG 5 (SUVH5), and SUVH6 bind to CHG and CHH methylation to catalyze H3K9me2 (Du et al. 2015). De novo DNA methylation in all contexts is catalyzed by DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2) (Cao and Jacobsen 2002).

Plants also display a complex and still not completely understood pathway in which de novo DNA methylation is triggered by small RNAs (sRNAs), the RNA dependent DNA methylation (RdDM) pathway. Briefly, RNA polymerase IV-dependent transcripts, mostly from TEs (Castel and Martienssen 2013), are converted to double-stranded RNA by RDR2 (RNA dependent RNA polymerase II) and cleaved into 24nt siRNAs by DCL3 (DICER-LIKE 3). Following processing, the 24nt-siRNAs are loaded into AGO (ARGONAUTE) effector complexes, including AGO4, AGO6, and AGO9. Next, RNA polymerase V produces longer noncoding transcripts used as scaffolds for recruiting additional RdDM factors, including 21, 22, and 24nt siRNA-loaded ARGONAUTE proteins and several accessory proteins that are still not well understood, involving canonical and non-canonical pathways. Finally, these interactions direct the recruitment of DRM1

(DOMAINS REARRANGED METHYLTRANSFERASE1) and DRM2 which methylate DNA in the three contexts (Hamilton and Baulcombe 1999; Cao et al. 2003; Henderson and Jacobsen 2007; Zhong et al. 2014; Borges and Martienssen 2015).

For standard DNA methylation, the SNF2 nucleosome remodeler DDM1 (DECREASE DNA METHYLATION1) is required (Jeddeloh et al. 1999; Lippman et al. 2004). DDM1 works by moving along the DNA and altering nucleosome composition and placement, allowing other proteins to gain access to heterochromatic DNA (Ryan and Owen-Hughes 2011). DDM1 mediates DNA methylation in all contexts independently of the RdDM pathway by refuting the linker histone H1 (Zemach et al. 2013; Lyons and Zilberman 2017).

Among the chromatin regulating factors, the epigenetic state is also mediated by histones and histone post-translational modifications (PTMs) that dynamically change alongside DNA methylation to mark and reprogram the genome. Histones are the architectural proteins that pack the DNA into nucleosomal units (Henikoff et al. 2004). There are five histone families—H1, H2A, H2B, H3, and H4—which are subject to PTMs. Histones and their modifications became the focus of research interest as a result of the discovery of the histone code and its significance for chromatin modulation. The histone code is the result of covalent PTMs: methylation, acetylation, phosphorylation, ubiquitination, and poly-ADP-ribosylation that takes place at the N-terminal tails (and also the C-terminal tail of H2A) of histones. The outcome of PTMs can influence gene expression by altering chromatin structure or recruiting other histone modifiers (Jenuwein and Allis 2001).

Histone variants are also subjected to PTMs. They are substitutes for the core canonical histones that can confer specific structure and function to the nucleosome (Mariño-Ramírez et al. 2005). Canonical histones are expressed during the S-phase of the cell cycle and incorporated to chromatin in a DNA replication-dependent manner, while histone variants are expressed through the cell cycle in a replication-independent mode (Bernatavichute et al. 2008; Law and Jacobsen 2010). Histone variants are expressed at different developmental stages and are connected to specific processes. For example, in Arabidopsis pollen, MGH3 is a male gamete-specific H3 variant (Okada et al. 2005) that integrates the regulatory pathway of germ cell cycle progression (Brownfield et al. 2009). Furthermore, histone H3 variants replace canonical H3 in both vegetative and sperm cells (Ingouff et al. 2007; Schoft et al. 2009).

The reprogramming of the vegetative nucleus leads to the accumulation of small RNAs and activation of transposons in the gametes, reinforcing the germline imprinting events and transposon silencing (Slotkin et al. 2009; Hsieh et al. 2009). Conventionally, heterochromatin is considered transcriptionally inactive, while euchromatin is transcriptionally active. In the last decades, this concept has changed due to the abundance of heterochromatic transcripts found in the germline cells, involved in TEs control and germ-cell fate (Creasey and Martienssen 2010). Euchromatin correlates with low levels of mCG (Lister et al. 2008), while heterochromatin is highly methylated in all contexts (Henderson and Jacobsen 2007). Heterochromatin in plants consists mostly of transposable elements and related tandem repeats (Lippman et al. 2004). In the germline, the PMC has reduced heterochromatin and shows TE (transposable elements) transcriptional reactivation

(Yang et al. 2011; She and Baroux 2015). On the other hand, the vegetative nucleus heterochromatin is decondensed while the sperm cells have tight condensed heterochromatin (Calarco et al. 2012).

Evolution can be driven by TEs, ubiquitous elements within the eukaryotic genome that have the ability to control gene expression and generate mutagenesis through transposition (Chuong et al. 2017). In 1961, Barbara McClintock showed in maize that the transposable elements Activator and Suppressor Mutator could cycle between active and silent states and be inherited through generations. These elements are frequently controlling color genes, allowing the genetic identification of both *cis* (transposons) and *trans*-acting (transposase) regulatory factors (McClintock 1961).

It was in plants that TE-related silencing across generations was described for the first time (McClintock 1957). TEs are subjected to epigenetic silencing, presumably due to the harmful outcome correlated with its activity (Lippman et al. 2004). TEs have the ability to interrupt gene function, damage the chromosome, increase in copy number, and overpass host gene number (Creasey and Martienssen 2010). In plant chromosomes, meiotic recombination frequency alters dramatically in gene-dense euchromatin and suppressed within centromeres, enriched by TEs. In Arabidopsis, centromeric and pericentromeric regions are enriched for CG DNA methylation (Stroud et al. 2014), which contributes to TEs silencing.

Transgenerational epigenetic inheritance is more common in plants than in animals, which undergoes robust germline reprogramming (Soppe et al. 2000; Manning et al. 2006; Martin et al. 2009; Durand et al. 2012). Yet despite the fact that genes, transgenes, and TEs remain methylated over generations in plants, some epigenetic reprogramming does occur during sexual reproduction (Heard and Martienssen 2014; Kawashima and Berger 2014).

14.2 Epigenetic Mechanisms in Pollen

Germ cells developed mechanisms to guarantee the proper resetting of epigenetic marks and chromatin remodeling prior to the transmission to the next generation. Silencing of transposable elements and heterochromatin formation are important pathways in this process. Epigenetics marks are also involved in mechanisms beyond reprogramming: defending the genome against TEs on one hand, and having functional centromeres on the other.

14.2.1 Small RNAs in Pollen

In pollen, small RNAs are important components of the plant epigenetic reprogramming machinery, altering transcriptional and translational dynamics (Borges et al. 2011). The miRNA pathway is important to regulate multiple biological functions

such as development, the response to biotic and abiotic stress, as well as hormone response (Bartel 2004; Chen 2005; Martin et al. 2010; Khraiwesh et al. 2012; Sunkar et al. 2012). miRNAs act by cleaving their specific complementary mRNA targets and are also able to repress translation (Chen 2005; Brodersen et al. 2008). MicroRNAs are present and active in mature pollen; additionally, there is a consistent expression of genes connected to the miRNA pathway such as *DCL1* (*DICER-LIKE1*), *AGO1* (*ARGONAUTE1*), and *RDR6* (*RNA DEPENDENT RNA POLYMERASE6*) (Kidner and Martienssen 2005; Grant-Downton et al. 2009). In sperm cells, genes related to the small RNA pathway and DNA methylation—*MET1*, *DDM1*, *AGO9*, and *AGO5*—are enriched in mature pollen compared to sporophytic tissues (Borges et al. 2008; Slotkin et al. 2009).

According to the parental conflict theory that could be described as the struggle between maternal and paternal genome dosage (Moore and Haig 1991), paternally inherited microRNAs might provide a direct mechanism to regulate maternally expressed inhibitors of embryo growth (Spielman et al. 2001). Indeed, it is possible that in Arabidopsis SC small RNAs are delivered during fertilization. For example, transcripts from *SSP* (*SHORT SUSPENSOR*) accumulate in *Arabidopsis* SC and are translationally suppressed before fertilization, yet translated only in early zygotic development (Bayer et al. 2009). Paternal miRNA may be delivered at fertilization, playing important roles such as signaling molecules or triggering early zygotic patterning and endosperm development, providing an efficient reprogramming mechanism in the early development (Borges et al. 2011).

The reactivation of TEs in the vegetative nucleus leads to the accumulation of small interfering RNAs (siRNAs), while the accumulation of TE-derived siRNA can lead to TE silencing in sperm cell, targeting gene silencing in gametes (Slotkin et al. 2009). In the VN, *DDM1* is down-regulated, allowing expression of transposons, whose transcripts are subsequently processed by the RNA interference (RNAi) pathway into 21nt siRNAs, which are then also found in the SC. There is probably an unknown mechanism of communication between the VN and the SC, considering that the 21nt-siRNAs produced in the VN target TEs in the SC, where they are highly methylated and transcriptionally silenced, leading to the possibility that these 21-siRNA are mobile and transmitted from VN to SC (Slotkin et al. 2009; Martienssen 2010).

During epigenetic reprogramming in *Arabidopsis* pollen, the biogenesis of 21 and 22nt easiRNA (epigenetically activated siRNA) takes place. easiRNA is another class of secondary siRNA derived from transcriptionally reactivated transposable elements, and still poorly understood. In wild-type VN and *ddm1* mutant inflorescence, easiRNAs accumulate from the retrotransposon *ATHILA6* 3’UTR (untranslated region) (Slotkin et al. 2009). These small RNAs accumulate in sperm cells at the same time that the heterochromatin from VN is lost and TEs start to reactivate (Slotkin et al. 2009). In *ddm1* mutants, *DDM1* levels are down-regulated and methylation of H3K9 is replaced by methylation of H3K4, DNA methylation is lost and TEs start to become active, triggering the biogenesis of easiRNA (Nuthikattu et al. 2013; Creasey et al. 2014). Among other TEs, *Gypsy* and *Copia* retrotransposons are targeted by miRNAs, particularly by miR845a (21nt) and miR845b (22nt) and

generate easiRNAs in *Arabidopsis* pollen (Borges et al. 2018). Potentially, these molecules are generated in the vegetative nucleus, where TEs are reactivated and easiRNAs travel to the sperm cells, targeting TEs to promote genome stability of the next generation (Martinez et al. 2016). Intriguingly, the well-known miR156, miR159, miR172, and miR859 were also recognized to generate secondary siRNA from TEs mRNA targets, likely able to target TEs (Ronemus et al. 2006; Creasey et al. 2014).

Another class of small RNAs that plays a role in the male gamete is phased siRNA (phasiRNA). They are produced in the germinal cells and persist throughout pollen differentiation and maturation (Zhai et al. 2015). Secondary phased siRNAs are triggered by 22nt miRNA generating 21nt and 24nt phasiRNA. In monocotyledons, these sRNAs are generated from *PHAS* precursors, transcribed by RNA polymerase II, subsequently cleaved by miR2118 to generate 21nt-phasiRNAs and by miR2275 to generate 24nt-phasiRNA. The *PHAS* 3'mRNAs are then converted into a double-stranded RNA by RDR6 and processed by DCL4 and 5 (Song et al. 2012a, b). In grasses, this RNA class is prevalent in anthers, during early development and meiosis (Zhai et al. 2011; Arikit et al. 2013; Komiya et al. 2014). In rice and maize, 21nt-phasiRNAs accumulate in anthers before meiosis, during cell fate specification, while 24nt-phasiRNAs accumulate during meiosis (Nonomura et al. 2007; Zhai et al. 2015). Additionally, phasiRNAs are essential for male fertility (Zhai et al. 2015; Kakrana et al. 2018); however, no targets have been found so far for this class of sRNA, leaving the biological role of these intriguing molecules an open question.

Another interesting possibility for sRNA function in the germline is the parental epigenetic contribution to the next generation, where sRNAs from one parent could be required to silencing incoming TEs from the other (Klattenhoff and Theurkauf 2008). Heterochromatin reprogramming, like genome imprinting, could produce a parent-specific defensive barrier against interspecific and interploidy hybridization. Also, it is possible that sRNAs from the male germline are delivered into the next generation, and once more, bringing the Lamarckian inheritance to the spot, since the activation of many TEs may respond to environmental cues (Creasey and Martienssen 2010).

Small RNAs play important roles in pollen development and maintenance. However, the complex network of interrelation among the different pathways remains unknown. With the aid of the new sequencing techniques, novel classes of regulatory molecules and layers of regulation are beginning to unravel.

14.2.2 DNA Methylation

Methylation of cytosine residues plays important roles in the maintenance of genomic stability, control of gene expression, and imprinting (Law and Jacobsen 2010). Epigenetic consequences of DNA methylation include modification of alternative splicing and transcription. These effects can respond to environmental

cues in a reversible way (Richards 2011) without changes in DNA sequence (Jablonka and Raz 2009; Law and Jacobsen 2010).

Throughout male gametogenesis, DNA methylation patterns undergo reprogramming. There is a decrease in the mCHH levels from the microspore stage to the sperm cell stage while mCG levels remain stable (Fig. 14.1). Moreover, in contrast to the sperm cells, the vegetative nucleus loses mCG and restores mCHH at specific TE loci. These changes correlated with the expression of DNA methylation enzymes: the chromatin remodeler DDM1, which is involved in DNA methylation in heterochromatic regions, is found in SC, but not in VN; MET1 and CMT3 are expressed only in SC; DRM2 and DME (DEMETER), a DNA glycosylase enzyme involved in DNA demethylation, are expressed in the VN (Kawashima and Berger 2014). These observations reinforce the idea of the presence of specialized reprogramming machinery in the male germline.

RdDM is one of the pathways that guide DNA methylation on the male germline. RdDM is highly complex and the major small RNA-mediated epigenetic pathway in plants (Hamilton and Baulcombe 1999; Qi et al. 2006). RdDM has many biological functions, including transcriptionally repressing genes and transposons, related to intercellular communication as well as in stress response and reproduction (Borges et al. 2012; Calarco et al. 2012). The complex maintenance machinery ensures the perseverance of established mC through cell division and across generations (Law and Jacobsen 2010; Matzke and Mosher 2014; Lewsey et al. 2016). In Arabidopsis, for example, DNA methylation patterns after 30 generations of single seed descent were found to exhibit a rate of CG methylation per site change per generation considerable higher than nucleotide mutation (Schmitz et al. 2011).

The reprogramming of CG methylation in the vegetative nucleus is not clear; however, the mechanism overlays with chromatin remodelers. CG methylation is reduced in the vegetative nucleus, likely because of the reduced expression of *MET1* (Jullien et al. 2012). Furthermore, *DDM1* the main regulator of constitutive heterochromatin and TEs is not expressed in the VN (Slotkin et al. 2009). Moreover, H3K9me2 plays an important role aiding in the silencing of TEs in sperm cells, yet is not found in VN. Methylation in both somatic and pollen cells is maintained through similar mechanisms; however, the maintenance of mCG is more efficient in pollen, even though CG methylation level is similar among vegetative, sperm, and leaf cells (Hsieh et al. 2016). The lack of the H3K9me2 mark, required by CMTs enzymes to play its role, implies that mCHH and mCHG in the VC may mostly rely on the RdDM pathway (Hsieh et al. 2016).

Variation of methylation between pollen and soma could be an inevitable outcome of unique selective pressures. On one hand, gametes have the potential to undergo unlimited cell divisions, which will keep a strong selection to retain efficient methylation maintenance. On the other hand, somatic cells will divide limited times which demands just enough methylation activity to maintain TEs in control and other methylation functions from collapsing. These differences may occur because of the maintenance fluctuations rather than the developmental reprogramming (Hsieh et al. 2016).

In the vegetative nucleus, DME, ROS1 (REPRESSOR OF SILENCING1), DML2 (DEMETER-LIKE2), and DML3 (DEMETER-LIKE3) are expressed (Schoft et al. 2011). DME is required for demethylation of TEs and tandem repeats that surround the imprinted maternally expressed genes *MEA* (*MEDEA*) and *FWA (FLOWERING WAGENINGEN)* that are usually expressed from the maternal allele in the endosperm but are also expressed in the VN (Schoft et al. 2011). Moreover, several hypomethylated regions are targeted by ROS1/DML2/DML3 and distinctive hypomethylated regions by DME in the VN and microspore, suggesting that these DNA glycosylases are responsible for the loss of mCG in the VN (Calarco et al. 2012).

In rice sperm cells, nearly all classes of chromatin-modifying genes are up-regulated (Russell et al. 2012). Furthermore, there is evidence that somatic alterations in rice DNA mC patterns are inherited and maintained in the germline possibly through the DOMAINS REARRANGED METHYLTRANSFERASE (DRM) pathway, increasing the evidence that transcriptional expression is fine-tuned by mC in a plastic manner, and suggesting that the Lamarckian inheritance concept could be right in this instance (Akimoto et al. 2007).

The differential methylation patterns leading to the upsurge of epialleles occur naturally or as a response to environmental cues. In either way, non-Mendelian segregation of epialleles can be observed when these alleles undergo paramutation, an allelic interaction in which one allele leads to a heritable change in the expression of the homologous allele (Della Vedova and Cone 2004). These phenomena illustrate the importance of epigenetic variation and paramutation in phenotypic variation (Greaves et al. 2012; Hövel et al. 2015).

In the SC, when some epialleles are in a pre-methylated state at the CG context, these same alleles are hypomethylated in the leaf of the parental line. One possible explanation is that CG hypermethylation at some loci (Becker et al. 2011; Calarco et al. 2012) is the default state at undifferentiated cells that will give rise to gametophytes, depending exclusively on MET1 for its maintenance, which will pass on to the germline, but its stability requires RdDM and 24nt siRNA accumulation (Borges and Martienssen 2013).

In an interesting experiment, EpiRILs (epigenetic recombinant inbred lines) were constructed by crossing Arabidopsis with distinct DNA methylation profiles, *ddm1* mutant and wild-type plants, then backcrossing the progeny by single seed descendants. The reactivated hypomethylated chromosomal segments generated by these mutants were tracked across at least eight generations, resulting in a high heritability for complex traits such as flowering time and plant height, without selection (Johannes et al. 2009).

The possibility to track epialleles led the way for identification of epiQTLs (epigenetic quantitative trait loci) where a QTL influences the chromatin state in either cis or trans, while classical genetics analysis of QTLs takes into account phenotypic variations due to changes in DNA sequences. Therefore, integrating these two approaches—genetics and chromatin-level information—now provides a more comprehensive view to generate, and track the maintenance of, phenotypes over time (Johannes et al. 2008).

14.2.3 Histone Variants and Modifications

The Arabidopsis pollen mother cell is characterized by a global dynamic change in the nucleosomal organization and chromatin modifications, the differential fate in mature pollen cells rely on the chromatin organization—VC has a large and diffuse nucleus, compared to the SC smaller and condensed nucleus (She and Baroux 2015). The correct assembly and accessibility of chromatin also depends on histone variants and on the covalent PTMs of histones.

Both in animals and plants, the histone variant H3.3 replaces H3.1 at transcribed *loci* where it replaces H3.1 during transcriptional elongation (Tagami et al. 2004; Okada et al. 2005; Ausió 2006; Wollmann and Berger 2012; Stroud et al. 2012; Biterge and Schneider 2014; Jiang and Berger 2017). Furthermore, H3.3 organizes chromatin both in transcribed *loci* and in promoter regions (Shu et al. 2014). These dynamic alterations make it easier for global changes in chromatin structure and histone modification to occur (Wollmann et al. 2012). During Arabidopsis pollen development, the H3.1 five copies and the H3.3 three copies show differential expression (Ingouff et al. 2007; Borg and Berger 2015), both H3.1 and H3.3 are present in the microspore chromatin, after division H3.1 is not found in mature pollen. The chromatin from SC is almost entirely consisted of the H3.3 and H3.10 variants (Borg and Berger 2015). However, it is not expected that in SCs H3.1 is absent, since a new phase of DNA replication takes place before fertilization (Durbarry et al. 2005), suggesting that H3.1 synthesis is separated from proliferation during SC development. Therefore, through male gametogenesis, other regulatory pathways appear to control the dynamic expression of H3 isoforms, shaping the unique chromatin landscape from the male germ cells (Borg and Berger 2015).

Pioneering studies in the monocot lily described a broad range of specific male gamete histone variants that replace H2A, H2B, H3, and H4 somatic histones, such as gH2A, gH2B, gH3, gH4 (Ueda and Tanaka 1995; Ueda et al. 2012; Yang et al. 2016), gcH2A, gcH3 (Xu et al. 1999), leH3, soH3-1, and soH3-2 (Okada et al. 2006). Nevertheless, the biological role of these variants remains to be fully understood. However, the acquisition of histone variants specific to the germline reinforces the idea of distinctive chromatin functions between the SC and the VC (Yang et al. 2016). In the lily chromatin, H3K4 (histone 3 lysine 4) is hypermethylated in the GC and hypomethylated in the VN, while H3K9me2 is weekly distributed in the GC, probably H3 variants play role in distinctive chromatin assembly among the cell types during pollen development, as well as in male-specific transcriptional activation (Okada et al. 2006).

The SC-specific histones appear to be unique among species, for example, Arabidopsis genome contains 15 histone H3 genes, among them CENH3 and H3.10, also known as MALE GAMETE-SPECIFIC HISTONE3 (MGH3), are found in centromere and sperm cell chromatin, respectively, whereas the rice genome displays 16, including the MGH3 homolog H3.709 (Borg and Berger 2015). Moreover, SCs from rice express a distinctive and diverse set of histones H2B (Russell et al. 2012). Despite the apparent conservation of histone H3 male gamete-

specificity, there are minor, but important differences found in the basic amino acids of the N-terminal domain, the target region for most H3 PTMs (Russell et al. 2012; Borg and Berger 2015). For example, the R26-K27-S28 motif, location of important modifications, is not conserved in the rice histone variant H3.709. In this histone variant, this motif is present but contains a nine amino acid long insertion that is nonexistent in other histones such as H3.1, H3.3, and MGH3 (Borg and Berger 2015). Gamete-specific proteins diverge fast and their adaptive evolution could drive speciation via generation of fertilization barriers (Swanson and Vacquier 2002).

In Arabidopsis SC chromatin, MGH3 is under the control of a male germline-specific MYB transcription factor DUO1 (Rotman et al. 2005) that is expressed at the beginning of the pollen development. DUO1 is required for the regulatory network that controls SC differentiation within the mitotic entry of the germ cell, MGH3 activity follows DUO1 expression after microspore division (Brownfield et al. 2009). The expression of both DUO1 and MGH3 before meiosis II implies that the regulatory network that controls the germ cells specification begin soon after asymmetric division (Rotman et al. 2005; Okada et al. 2005; Borg et al. 2009), besides MGH3 specific and abundant expression in the SC suggests that this histone variant may play important role in chromatin structure in the germline (Borg et al. 2009). MGH3 promoter contains four DUO1 binding motifs (wAACCGy), and two of them are required for MGH3 activation by DUO1 in the germline (Borg et al. 2011). At the same time, DUO1 also controls the expression of a duet of zinc-finger proteins DAZ1/DAZ2 (DUO1-ACTIVATED ZINC FINGER1/DUO1-ACTIVATED ZINC FINGER2), key to intermediate germ cell mitotic entry and gamete differentiation (Borg et al. 2014).

The histone variant CENH3 is a main component of centromeres in eukaryotes and it is important for kinetochore assembly and chromosome segregation (Henikoff and Furuyama 2012). The Arabidopsis centromeric heterochromatin of the vegetative nucleus undergoes decondensation and loses the histone variant CENH3, the H3K9me2 mark, and centromeric identity (Schoft et al. 2009). In addition, the VN exits the cell cycle after microspore division (Borg et al. 2009). CENH3 does not undergo post-translational modification, which may contribute to the loss of centromeric heterochromatin in the vegetative nucleus (Schoft et al. 2009). Furthermore, H3K27me1 is still present in centromeric regions in the VN, but still retains non-CG methylation leading to transcriptional silencing probably through control of the RdDM/DRM2 pathway and 24nt siRNAs generated from centromeric regions (Schoft et al. 2009). DRM2 is expressed specifically in the vegetative nucleus, but not in the sperm cell (Calarco et al. 2012).

In Arabidopsis vegetative nucleus, SDG4 (SET DOMAIN GROUP4) is one of the enzymes responsible for the maintenance of methylation in H3K4 and H3K36—marks related with active euchromatin—and regulates the expression of genes that play role in pollen germination and pollen tube elongation (Cartagena et al. 2008). Likewise, SET DOMAIN GROUP2 (SDG2) mediates H3K4 trimethylation in the VN to control pollen germination and pollen tube elongation as well. Moreover, SDG2 is required for the expression of the transposable element *ATLANTYS1* in the VN (Pinon et al. 2017).

The linker histone H1 globally reduces heterochromatic DNA methylation in all contexts (Zemach et al. 2013). H1 is present in SC and absent in the VC, yet does not increase heterochromatic methylation in pollen (Hsieh et al. 2016). In heterochromatic TEs, the increased efficiency of mCG might be because of the reduced levels of H1, probably with a specific mechanism that differs from genes and euchromatic regions, where loss of H1 does not facilitate mCG (Hsieh et al. 2016).

During chromatin reorganization in PMC from Arabidopsis, there is an eviction of the linker histone H1 (She and Baroux 2015), consistent with chromatin decondensation, followed by an increase in nuclear size and reduction of the heterochromatin content. This is a *ddm1*-like phenotype, where TEs are activated after the loss of heterochromatin (Slotkin et al. 2009) and may assist the rapid CENH3 turnover in the PMC (Schubert et al. 2014). Furthermore, in the PMC, there is a reduction in the heterochromatin domains, likewise, a decrease of the H3K27me1 mark. Reduction of H3K27me3 (a repressive mark) and increase of H3K4me2 (a permissive mark) suggest a distinctive epigenetic landscape. SDG2 may also play role in the PMC epigenetic landscape (She and Baroux 2015).

Acetylation of lysine residues on the N-terminal tail of histones neutralizes their positive charge, decreasing the affinity for the negatively charged DNA strand, changing the conformation of chromatin and therefore altering gene accessibility. Hyperacetylated histones are usually correlated with gene activation, while hypoacetylation with gene silencing. HDAs (histone deacetylases) act together with corepressors in multiprotein chromatin modifiers complexes (Mehdi et al. 2016; Perrella et al. 2016). In *Arabidopsis*, some members of the HDA family are associated with the silencing of transposable elements, transgenes, and ribosomal RNA (Lippman et al. 2003; Probst et al. 2004). This family also plays a role in both euchromatin and heterochromatin, and may inhibit de novo DNA methylation in CG context (Hristova et al. 2015; Zhang et al. 2015). Moreover, these enzymes are involved in male fertility in maize (Forestan et al. 2018). Histone acetylation may participate in the germline epigenetic reprogramming, although its role still remains to be investigated.

14.3 Transposable Elements

TEs comprises Class I—retrotransposons which replicate through RNA and cDNA—that can be divided into LTR (long terminal repeats) and non-LTR, and Class II—DNA transposons which replicate via a DNA intermediate—that does not necessarily require transcription of the DNA elements (Underwood et al. 2017). In Arabidopsis, the LTR retrotransposon family Athila occupies 2.7% of the genome and is one of the building blocks of the centromere and the center of Arabidopsis epigenetic regulation, potentially playing an important role in speciation (Slotkin 2010). Athila elements, along with other TEs, are epigenetically reactivated in the VN, in part due to the lack of DDM1 (Slotkin et al. 2009). Additionally, Athila is not

controlled by sRNAs in the plant body, nevertheless in the female gametophyte is (Olmedo-Monfil et al. 2010). Taken together, the reactivation of Athila in the pollen and its regulation in other tissues clearly suggest a distinct regulation mechanism and a specific biological function in the pollen, possibly to make the necessary substrate—mRNA—to generate easiRNA to the effective silencing of TEs in the next generation (Slotkin et al. 2009).

In maize and *Arabidopsis*, TEs become active in the PMC, accompanied by a reduction in heterochromatin and changes in histone modifications (Wang and Köhler 2017). Additionally, TEs accumulate only in the VN and not in the SC (Borges et al. 2008), accompanied by novel transposition events in pollen DNA, but not in the subsequent progeny, thus reinforcing the notion that they are not active in the SC (Creasey and Martienssen 2010). Dynamic changes in mC during male gametogenesis include increases in non-CG methylation in the VN, and siRNAs homologous to the retrotransposons LTRs (long terminal repeats) are found in the vegetative nucleus, while 21 and 24nt siRNAs are found in sperm cells. In the SC, non-canonical RdDM pathways modify these elements (Borges et al. 2012). In rice, the same mechanisms may be present, as genes from distinct RNA silencing pathways are upregulated (Russell et al. 2012). The sources of TEs control during plant reproduction comprise changes in DNA methylation along with small RNA in specific tissues or cell types (Slotkin et al. 2009; Calarco et al. 2012).

A cooperation between H3K9me2, non-mCG dependent on CMT2, CMT3, and RdDM is established to maintain TE expression under control (Stroud et al. 2014); therefore, an upregulation of TEs in the male meiocyte indicates that DNA and H3K9 methylation are reduced before meiosis. Moreover, TE activation in pollen does not lead to genome instability and TE transposase activity, suggesting the presence of another layer of regulation to keep these elements from harming the genome (Slotkin et al. 2009; Calarco et al. 2012; Creasey et al. 2014).

Another potential mechanism to control TEs in pollen could be through the still poorly understood tRNA derived fragment (tRF) pathway. tRFs have been identified in different species and cell types, ranging from 13 to 30 nucleotides long; these molecules are processed from mature tRNAs in 5' tRFs, 3'CCA tRFs, and tRNA halves (Lee et al. 2009; Alves et al. 2017; Martinez et al. 2017; Schorn et al. 2017), although the biogenesis pathway for most tRFs is still unknown. These sRNAs are able to target TEs both in mouse stem cells and *Arabidopsis* pollen. In mouse, 3'CCA tRFs are able to target and inhibit retrotransposons by binding retrotransposons primer site, which is where a tRNA can bind and prime their reverse-transcription. Therefore, tRFs competing for the primer site can inhibit the transcription of these elements (Schorn et al. 2017). Pollen-specific 19 nucleotides 5'tRFs target TE mRNAs in *Arabidopsis*. Furthermore, the accumulation of 19nt-5'tRF in reproductive tissue/pollen is conserved among plants and there is evidence that suggests that 5'tRFs in pollen are processed by DCL1 (Martinez et al. 2017).

Arabidopsis sperm cells retain CG and CHG methylation while CHH methylation is lost, accompanied by extensive epigenetic remodeling of the VN cell (Slotkin et al. 2009). TE reactivation occurs in Arabidopsis, maize, and rice pollen, and could indicate a conserved mechanism among land plants (Nobuta et al. 2007; Slotkin

et al. 2009). The VN undergoes extensive histone variant substitution, losing canonical histones and CENH3, likely contributing to TE activation (Ingouff et al. 2007; Schoft et al. 2009). In rice sperm cells, nearly all classes of chromatin-modifying genes are up-regulated (Russell et al. 2012), and somatic changes in mC are inherited and maintained in the germline (Akimoto et al. 2007).

14.4 Imprinting

Imprinting is a phenomenon where one of the parental alleles is preferentially expressed over the other and has the potential to generate advantageous traits but still is poorly understood. This epigenetic singularity leads to parent-of-origin differentiated expressed alleles inheritance in several plant species, including maize, rice, and *Arabidopsis* (Luo et al. 2011; Waters et al. 2011; Pignatta et al. 2014). In plants, it occurs mostly in the endosperm, and hundreds of imprinted genes have been identified so far (Gehring et al. 2011; Luo et al. 2011; Wolff et al. 2011; Zhang et al. 2016; Yuan et al. 2017). After fertilization, the endosperm is originated from a triploid cell, containing the diploid maternal cell and one haploid sperm cell. The expected ratio of maternal and paternal expression is 2:1, therefore imprinted genes could differ from the probability where maternally expressed genes (MEGs) or paternally expressed genes (PEGs) diverged the expected ratio. Imprinting can be determined by suppression or activation of MEGs or PEGs. Studies have shown that MEGs are preferentially expressed in the endosperm while PEGs could be detected in the endosperm as well as in other tissues during development, suggesting that PEGs and MEGs could be regulated by different mechanisms (Waters et al. 2013; Pignatta et al. 2014; Zhang et al. 2016).

PEGs may be involved on the postzygotic hybridization barrier in the endosperm, indicating a major role in plant speciation (Wolff et al. 2015). In rice, a set of PEGs regulates endosperm development and nutrient metabolism, improving seed development and offspring fitness (Yuan et al. 2017; Pignatta et al. 2018).

Imprinted genes are usually bordered by TEs—which are frequently highly methylated—and could be affected by TEs methylation machinery that possibly overlaps the genes edges (Martienssen et al. 2004; Radford et al. 2011). It is not clear how regulation of imprinted parental genes occurs, but studies suggested that TEs could be the trigger for this phenomenon (Martienssen et al. 2004; Gehring et al. 2009; Wolff et al. 2011).

In Arabidopsis VN, TEs are target by DME (DEMETER), ROS1 (REPRESSOR OF SILENCING1), DML2 (DEMETER-LIKE2), and DML3 (DEMETER-LIKE3)—DNA demethylation enzymes—causing them to lose CG methylation (Lister et al. 2008; Calarco et al. 2012). In the SCs, 24nt easiRNAs corresponding to some of these elements accumulate, especially in TEs regions that flank MEGs (Calarco et al. 2012), probably playing role in the RdDM pathway from those cells. To illustrate this complex mechanism, there are examples such as *SDC* (*SUPPRESSOR OF DRM2/CMT3*) that is active only when the flanking sequences

are not methylated (Henderson and Jacobsen 2007), and the PEG *PHE1* (*PHERES1*) that is expressed only when a tandem repeat downstream of the coding region is methylated (Makarevich et al. 2008). In the VN, tandem repeats flanking both genes lose methylation. In the SC, these regions also lose mCG, although retain mCHH and accumulate 24nt easiRNAs, while imprinted genes are protected from the global loss of methylation.

The multidomain protein complex FACT (facilitates chromatin transaction) interacting with nucleosome components to initiate and elongate transcripts also is involved with DME at imprinted genes in Arabidopsis (Ikeda et al. 2011). Mediated by the linker histone H1, DME requires FACT for DNA demethylation especially in TEs regions with high CG content and nucleosome activity, enriched for heterochromatin marks, such as H3K27me1 and H3K9me2. So far, this mechanism is known to occur in the female central cell, but not for the male VN. This observation is particularly interesting because both cell types are separated from its somatic precursor by one cell division and have decondensed chromatin (Frost et al. 2018), demonstrating the specific epigenetic regulation mechanisms developed by maternal and paternal germlines.

14.5 Environmental Response and Inheritance

Plants are able to modulate gene expression to fine-tune biotic and abiotic stress responses. The rise of temperature triggered by climate change is deeply affecting plant farming worldwide: for example, the estimation is that for each 1 °C of increase in temperature, there will be a 10% decrease in rice yield (Peng et al. 2004).

Pollen grains are exceptionally delicate, particularly sensitive to elevated temperatures, and the mechanisms that underlie this stress response are still poorly understood. Heat stress response in tomatoes triggers the accumulation of small non-coding RNA (sncRNAs), transfer RNAs (tRNAs), and small nucleolar RNAs (snoRNAs) during post-meiotic and mature stages of pollen development (Bokszczanin et al. 2015). In *Arabidopsis*, the increase in temperature reduces the expression of the gene *SGS3* (*SUPPRESSOR OF GENE SILENCING3*), involved in the RNA interference (RNAi) pathway, therefore decreasing the accumulation of siRNAs. Moreover, heat stress induces a transgenerational epigenetic inheritance (Zhong et al. 2013). During pollen development, heat stress response can also trigger shifts in global DNA methylation together with methyltransferase expression (Solís et al. 2012). In *Brassica napus* microspores, DNA methylation levels and TEs activity change during heat stress (Li et al. 2016). Arabidopsis epigenetic silencing of transposable elements can also endure the consequences of heat stress through the RdDM pathway (McCue et al. 2015; Matsunaga et al. 2015). However, there is no evidence that the mechanism that regulates these alterations is of adaptive value (Lamke and Baurle 2017).

Twenty-four nucleotide hc-siRNAs (heterochromatic siRNA) derived from TE could be involved in pollen development and epigenetic regulation of the stress

response (Bokszczanin et al. 2015). The 24nt hc-siRNAs also participate in the RdDM machinery (Calarco et al. 2012; Zhou et al. 2018), associated with transcriptional gene silencing, they act by modulating DNA and histones modifications, while the 21nt siRNAs and microRNAs play role in transcriptional and post-transcriptional regulation (Brodersen et al. 2008). During heat stress in tomato, there is a loss of abundance of 22nt-sncRNAs in post-meiotic and mature pollen, which may be due to reduction in the production or degradation of these sRNAs. These 22nt-sncRNAs likely play a similar role as the 21nt-siRNA generated from TEs in Arabidopsis, also the difference in the length of sncRNAs in the different stages of pollen development is due to their different functions (Bokszczanin et al. 2015).

Environmental cues can lead to changes in gene expression by alterations in chromatin structure at specific responsive genes and/or the biogenesis of small RNAs (Hirsch et al. 2013). The majority of epigenetic stress-related alterations are only detected in somatic cells and rapidly disappear, although methyl cytosine (mC) and H3K27me3 (trimethyl histone H3 lysine 27) induced by stress can last one stress-free generation (Lamke and Baurle 2017).

During pollen development, microgametogenesis is the stage where mitosis occurs. Mitotic inheritance of epigenetic traits can be explained through the interplay among small RNA, maintenance DNA methyltransferases, and other chromatin modifiers, working together to retain the epigenetic information into the next cell division, preserving tissue integrity and correct function.

Variation in epigenetic marks, such as gain or loss of DNA methylation on a specific gene, can lead to silencing or activation of the affected gene altering its phenotype (Bond and Baulcombe 2015). There are a few examples that illustrate heritable epimutation in plants: the famous *Linaria vulgaris* example, in which the floral symmetry changes due to hypermethylation and transcriptional silencing of *Lcyc* (*Linaria cycloidea-like*) (Cubas et al. 1999), as does fruit color in the tomato locus *Colorless non-ripening* (*Cnr*) (Manning et al. 2006). An additional alteration that may affect *L. vulgaris* phenotype is a depletion of a TE approximately 10 kb from the *Lcyc* gene; however, it is not clear how this depletion could affect the phenotype. Besides, in many cases TEs mediate this epigenetic silencing, for example, at the *hcf106* (*high chlorophyll fluorescence106*) locus in maize (Martienssen et al. 1990), at the melon transcription factor gene *CmWIP* (Martin et al. 2009) and *Arabidopsis FWA* (Soppe et al. 2000), resulting in gene silencing in *cis*. *Cis*-regulatory elements are frequently within or near the target loci, while *trans*-regulatory elements play a regulatory role in a distant position from where they are transcribed, such as small RNAs. Small RNAs can cause epimutation by silencing the Arabidopsis gene *FOLT1* (FOLATE TRANSPORTER 1) (Durand et al. 2012) and homologous genes are methylated by RdDM pathway. However, most epialleles cause no phenotype and can only be detected by molecular means. From an evolutionary biology perspective, an extra layer of generation of heritable variation within complex traits may explain the rapid adaptation to environmental changes seen in natural populations (Pál and Miklós 1999). As yet, there is no

evidence that these epigenetic variations are subject to natural selection or have adaptive value (Manning et al. 2006; Hirsch et al. 2013).

Epialleles can also be induced by environmental challenges, such as biotic or abiotic stress. The heritability of these epigenetic alterations might be an interesting adaptive mechanism. External changes can lead to modifications in gene expression by alterations in chromatin structure at specific responsive genes and/or the biogenesis of small RNAs (Hirsch et al. 2013). The majority of epigenetic stress-related changes are only detected in somatic cells and, after a few days, these effects disappear. Although there are a few observations demonstrating the heritability of the epigenetic marks mC and H3K27me3 after stresses such as hyperosmotic, iron deficiency, bacterial infection, chemical stressors, and caterpillar herbivory, these transgenerational epigenetic alterations are reset after one stress-free generation (Lamke and Baurle 2017).

Some hypomethylated epialleles can be stably inherited, but after a few generations the methylation levels can be restored by an RNAi dependent pathway, because sperm cells can retain mCG and mCHG during differentiation, while a lower level of mCHH is retained during mitosis (Teixeira et al. 2009; Calarco et al. 2012). Methylation levels are restored by DRM2 guided by pollen 24nt siRNA in the VN prior to fertilization (Calarco et al. 2012; Ingouff et al. 2017).

On one hand, epialleles often arise throughout stress conditions, on the other, they arise naturally on a given population. There are numerous features in germline reprogramming to make sure that the next generation is going to be viable and fertile; however, it is not known how and why this natural variation occurs, also when they are fixed in the population and what their advantages in terms of adaptability are.

14.6 Perspectives

Rapid introgression of desired traits is the ultimate goal for increasing the quality of crops. Enhancing productivity by improving yield with larger seeds, more branches, and more fruits is imperative to feed the population worldwide. So far, breeders rely mostly on genetic techniques and test-crossing on the field to achieve this goal. With expanding molecular biology and big data techniques, a new world of epigenetic features is now beginning to unravel. The possibility to understand how epialleles, methylation levels, and other epigenetic mechanisms underlying desirable crop traits are inherited across generations is imperative to teach us how to manipulate them and to achieve the best crop production. Part of this modulation happens in the male germline that acquired complex and intricate chromatin regulation mechanisms. The differences between the vegetative nucleus and sperm cells are remarkable and we just have started to shed light on the germline regulation and male inheritance. More studies on these mechanisms are needed to understand the complex world of the male germline.

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Chapter 15
Epigenetics in Forest Trees: Keep Calm and Carry On

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Abstract Trees are sessile and long-lived organism so they have to rapidly adapt to dynamic and unfavorable environments (drought, soil salinity, heat, …) for ensure their survival. Acclimation is mostly related to epigenetic regulation mechanisms that act responding to environmental stimuli and thus regulating gene expression during leaf development, floral transition, bud dormancy, and climate change induced abiotic stress response. Also, environmental stresses have been related to the transgenerational inheritance of epigenetic marks, called epigenetic memory. Epigenetic variation complements natural genetic variation as a source of phenotypic and functional diversity in plants, resulting in a phenotypic plasticity including also traits of transgenerational inheritance. This chapter provide an overview about how epigenetic mechanisms act, the memory role and new epi-variates definition that combined will help us to create new biotechnological tools for forest trees productivity improvement.

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15.1 Introduction

Trees' long lifespan, growth in dynamic environments, and sessility make their development influenced by both biotic and abiotic factors. Contrary to annual plants, in most latitudes trees must survive to winter colds and to summer droughts in a loop that can last for thousands of years (think in some pines, firs, or junipers). During this time trees encounter unfavorable environmental situations such as temperature increasing, altered precipitation regime, soil salinity, and high light intensity due to climate change with the impossibility to move to more favorable environments. In consequence, they have to be able to adapt rapidly to new conditions to ensure their survival. The integration of external stimuli, which should be reflected in a reprogramming of gene expression that leads to adaption, can follow different pathways but in last term they are coordinated by a differential epigenetic modulation (Köhler and Springer 2017).

This modulation is also coordinated with the change of stress-responsive genes expression, becoming a crucial process for responding to environmental stimuli and regulating cell growth and differentiation (Chinnusamy et al. 2014). 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, the role of small RNAs is getting more importance for modulating epigenetic responses. This regulation is composed of a highly interactive network of sRNA-directed DNA methylation, histone, and chromatin modifications, all of which control transcription (Simon and Meyers 2011). These mechanisms are dynamic and can be reverted or adapted to particular environmental situations for maintaining gene and genome activities (Kim et al. 2015a), constituting a link between genotype and phenotype (Schmitz and Ecker 2012). The study of global changes in DNA methylation or specific histone PTMs has allowed the characterization 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). Furthermore it was recently discussed how epigenetic variation complements natural genetic variation as a source of phenotypic and functional variation in plants (Kawakatsu et al. 2016; Slotkin 2016).

In the current context of climate change, the study and understanding of the stress response and acclimation mechanisms in plants became mandatory for their stress response improvement. Principal physiological mechanisms in response to stress and later adaptation have been described in many forestry species. Good examples of well-described physiological stress response and acclimation are heat and UV stress response in *Pinus radiata* (Escandón et al. 2017, 2018; Pascual et al. 2017) and drought and heat stress response in *Eucalyptus globulus* (Jesus et al. 2015; Correia et al. 2018). In contrast, 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.

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 has raised a significant interest in stress-responsive epigenetic mechanisms. In addition, environmental stresses can occur repeatedly, so it has been suggested that plants have a stress memory supporting the stress adaptation. Epigenetic memory is based on the defense priming by playing a more rapid and stronger response to abiotic stress (Kinoshita and Seki 2014; Lämke and Bäurle 2017). Stress memory is still supported by few researches in forestry species (Le Gac et al. 2018), and it has been proposed as a way to improve environmental stress adaptation of cultivars (Springer and Schmitz 2017). Knowing these epigenetic marks will provide important information about how natural populations will survive in the current climate change context. Nevertheless, forests are composed by genetic and environmentally heterogeneous populations that make the epigenetic underlying mechanisms poorly understood (Bräutigam et al. 2013).

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. This variation is closely related to the epigenetic memory, the transgenerational inheritance of epigenetic marks, discussed long-time by scientific community but a widely accepted fact nowadays. Although the underlying mechanisms in natural populations are still poorly understood even in model species because of the difficulty of characterizing epigenetics in genetically and environmentally heterogeneous populations, some advances have been recently achieved in forest species. This chapter provides some of the last advances in forest epigenetic mechanisms and memory, and its implications as potential new tools for plant breeding and conservation as a way to select or induce new epi-varieties adapted to changing ecosystems.

15.2 Epigenetic Regulation in Plant Development and Environmental Responses

15.2.1 Epigenetics Implications in Tree Leaf Development

Plants are subjected to a series of transitions in their development cycle. Three clearly defined stages are embryonic, postembryonic, and growth. The latter is characterized by different patterns of growth in terms of differentiation of cells, organs (heteroblasty), and the gain of physiological competence. The heteroblasty has been described for woody species of the genus *Pinus* (Climent et al. 2006), *Acacia* (Forster and Bonser 2009), and *Eucalyptus* (James and Bell 2001). The most described type of heteroblastic variation is the change of the vegetative phase (Poethig 1990), which implies changes in the characteristics of leaves, stems, and buds (Rasmussen 1986).

In recent years, the mechanisms implied in the regulation of the leaf juvenile-to-adult transition have been discovered in model herbaceous species Arabidopsis (Wilson-Sánchez et al. 2014) and some of their players were uncovered also in forest trees, a first step for understanding the control of this essential developmental event in the life cycle of plants.

The role of DNA methylation has been reported in different tree systems and developmental stages. As a general rule DNA methylation levels increase with age in meristematic regions, while juvenile meristems are less methylated than adults, as it was described in *Prunus persica* (Bitonti et al. 2002) or *Pinus radiata* (Fraga et al. 2002). Vegetative to reproductive phase change seemed to be the triggering point for increasing DNA methylation in these species. Developing *Pinus radiata* needles also showed a lower DNA methylation level compared to mature needles (Valledor et al. 2010). Epigenetic marks of specific developmental stages have been reported for promoters of key genes implied in needle development (Valledor et al. 2015). In Arabidopsis, the changes in its epigenome during leaf development were clearly identified, and also the effects of knocking out methyltransferases (met1 and drm1 drm2 cmt3) in 5 weeks (Zhang and Jacobsen 2006) and wild plants of 25 days

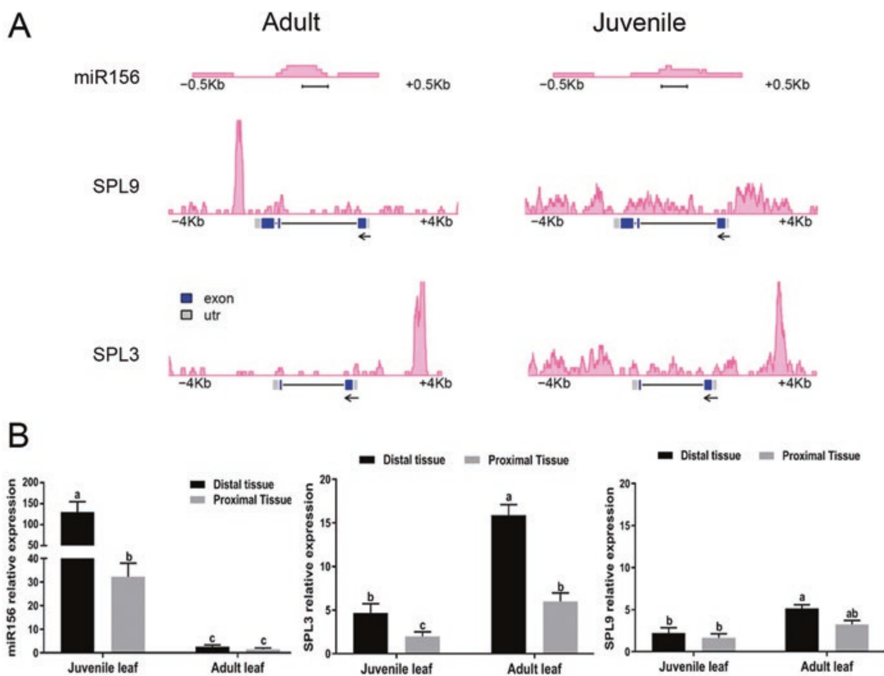

Fig. 15.1 (**a**) Methylation pattern at vegetative phase control genes. Normalized signal plots (pink, MEDIp) for adult (left) and juvenile (right) leaves. Arrows indicate direction of transcription. (**b**) Expression levels of miR156, SPL3, and SPL9 in juvenile and adult leaves of *E. globulus*, distal and proximal zones. Expression was normalized with miR171a (miR156) or EF1a and actin (SPL3 and SPL9). Averages of three replicates and three trees are shown for each tissue, two-way ANOVA

(Zilberman et al. 2007) identifying the differential methylation of SQUAMOSA PROMOTER PROTEIN-LIKE SPL10. These results suggest that the genes of the pathway miR156-SPL could be regulated by DNA methylation (Xu et al. 2018).

In *Eucalyptus globulus,* the levels of transcribed miR156 (associated with juvenility) were reduced during phase transition, with an increase of the transcriptional levels of SPL3 and SPL9, involved in adult traits. The comparison of the transcription expression profiles with DNA methylation profiles (Hasbún et al. 2016), both in adult and juvenile leaves, revealed a correlation between methylation of putative promoter regions and transcript abundance. Methylation levels on juvenile tissues correlated with lower transcription levels of the SPL3 and SPL9 genes (Iturra 2018) (Fig. 15.1).

Conversely the epigenetic mechanism that has been addressed in more reports is the modifications of histones. In Arabidopsis, an increase in the trimethylation of the histone H3 lysine 27 (H3K27me3), mediated by the chromatin remodeler CHD3 PICKLE (PKL) (Zhang et al. 2008), could contribute to the addition of H3K27me3 to genes miR156. This associated with a temporary decrease in the acetylation of H3K27 would contribute to the decrease of miR156 (Xu et al. 2015). Considering the developing *Pinus radiata* needle, primordia show a greater abundance of marks related to the expression of euchromatin genes, such as AcH3 or H4K4me3. These marks are progressively lost during needle development and replaced for repressive marks such as H4K9me3 (Valledor et al. 2010). Furthermore, these marks can be associated with gene specific methylation status and gene expression (Fig. 15.2).

In Arabidopsis, a decrease in the transcription of miR156 is related to an increase in the histone mark H3K27me3 and a decrease in the mark H3K27ac in the region after the beginning of the transcription (TSS) (Xu et al. 2016b, c). It is also reported that a decrease in the monoubiquitination of histone 2a (H2Aub) and H3K27me3 in the TSS region of miR156 prolongs the juvenile phase (Picó et al. 2015). Arabidopsis H3K4 methyltransferase TRITHORAX7 (ATXR7) joins a region adjacent to the TSS of miR156 and deposits the H3K4me3, which activates the transcription miR156 (Xu et al. 2017). The ambient factors (light and photoperiod) play an important role in the regulation of the genes SPLs and in the induction of miR156, by means of the acetylation of acetyltransferases of histones (HAT) of the type Spt-Ada-Gcn5-acetyltransferase (SAGA), they could be an important aspect in the transition of vegetative phase. This mechanism of control is prior to the post-transcriptional regulation mediated by miR156 in the phase change (Kim et al. 2015b).

The mechanism associated with chromatin remodeling has been addressed in recent years. It has been described that the transcription of miR156 is promoted by the remodeler nucleosomal BRAHMA (Xu et al. 2016c) and the complex SWR1, which exchanges the variant of histone H2A.Z by H2A (Choi et al. 2016). This indicates that H2A.Z promotes juvenile vegetative identity, and would also promote the expression of MIR156 by facilitating the deposition of H3K4me3 (Xu et al. 2017). The remodeling protein of nucleosomes PICKLE (PKL) is associated with the remodeling and deacetylation complexes of nucleosome (Ho et al. 2013), which binds to the adjacent TSS region of MIR156 by repressing its transcription (Xu et al. 2016b).

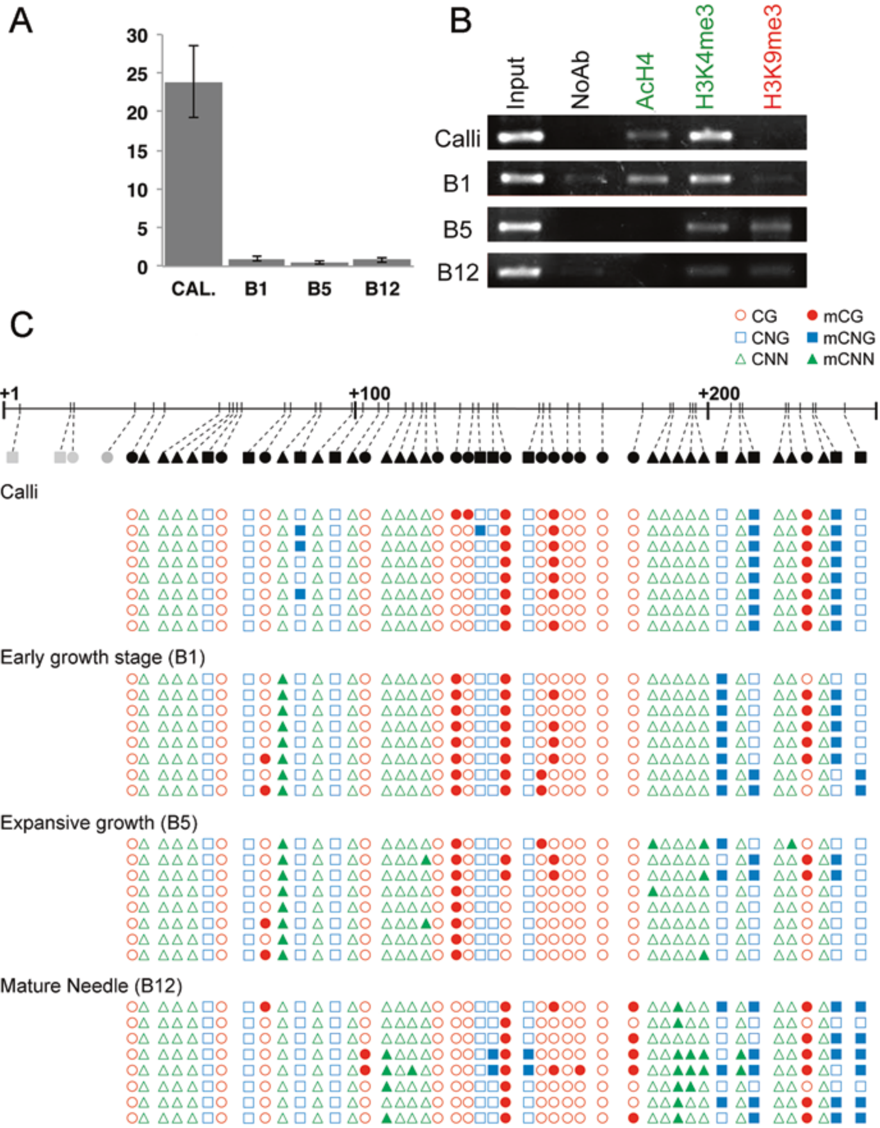

Fig. 15.2 (**a**) Expression level of *Pinus radiata CSDP2* gene in four developmental stages (cal., calli; B1 leaf primordia; B5 needles in expansive growth; B12 mature needles). (**b**) Chromatin immunoprecipitation of promoter region of CSDP2 in the same tissues. Two marks associated with gene expression (AcH4 and H3K4me3) and one to repression (H3K9me3) have been tested. Amplification band represents the presence of this mark in the analyzed region. (**c**) Site specific DNA methylation of promoter region of analyzed gene. All cytosines were analyzed, CG were represented as circles, CNG as squares, and CNN as triangles. Filled symbols represent a mC. This figure was adapted from Valledor et al. (2015)

15.2.2 Epigenetic Regulation of Floral Transition

Transition from vegetative to floral buds is a critical physiological change during plant development that determines the survival of the flowering species. Floral transition is achieved through a complex genetic network and regulated by multiple environmental and endogenous cues. A striking example of how epigenome reacts to environment involves the induction of flowering by exposure to low winter temperatures in Arabidopsis and many other flowering plants. These epigenetic modifications include DNA methylation, histone modifications, and the production of small RNA (sRNA) that mediate epigenetic modifications (Hepworth and Dean 2015).

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 shows 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 (Farrona et al. 2008; Hepworth and Dean 2015). Histone methylation participates in repression of expression of inhibitor of flowering FLC during cold. This epigenetic change is mediated by a conserved repressive complex, POLYCOMB REPRESSIVE COMPLEX 2 (PRC2). An intronic non-coding RNA, called COLD ASSISTED INTRONIC NON-CODING RNA (COLDAIR), is required for the vernalization-mediated epigenetic repression of *FLC*. COLDAIR physically associates with PRC2 and targets PRC2 to FLC (Heo and Sung 2011). In annual species, such as Arabidopsis, this histone methylation is stably inherited through mitosis after returning from cold to warm temperatures allowing the plant to flower continuously during spring and summer until it senesces. However, in perennial species, histone modifications rapidly disappear 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). In perennial and woody species, such as Azalea (*Rhododendron sp*), Meijón et al. 2010 showed that DNA methylation and histone H4 acetylation (Fig. 15.3a) have opposite and particular dynamics in the apical buds during the transition from vegetative to reproductive phase. The description of the global DNA methylation and histone H4 acetylation levels and immunodetection of 5-mdC and AcH4 in addition to a morphological study have delimited four basic phases in the development of the azalea bud identifying a stage of epigenetic reprogramming which showed a sharp decrease of whole DNA methylation (Fig. 15.3b). DNA methylation and histone modifications have been revealed as hallmarks that establish the functional status of chromatin domains and confer the flexibility of tran-

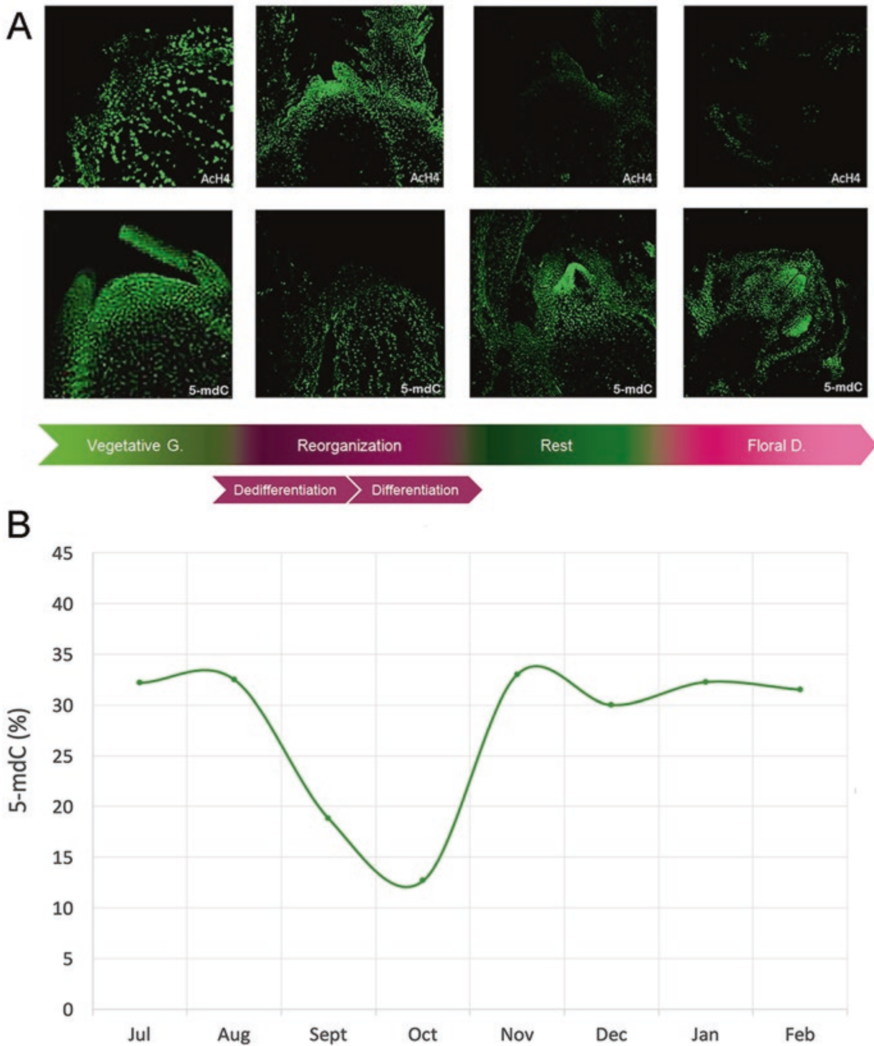

Fig. 15.3 (**a**) Immunodetection of 5-mdC and AcH4 along annual azalea bud development and (**b**) their global levels of DNA methylation (5-mdC %) from July to February. Adapted from Meijón et al. (2010)

scriptional regulation necessary for plant development and adaptive responses to the environment (Hepworth and Dean 2015).

In the last years, a crucial link between the epigenetic modifications associated with floral transition and those associated with stress tolerance was found (Yaish et al. 2011). Plants interact with their environment and accordingly modify their flowering programs. This research showed that plants use common and parallel epigenetic modification pathways in order to modify the expression of genes that are

involved in stress tolerance and flowering processes. Together these changes underlie intricate mechanisms that ensure plant survival and optimize reproductive success under a variety of stress conditions (Yaish et al. 2011).

On the other hand it has been recently demonstrated that these epigenetic modifications may also provide a mechanistic basis for a stress memory, enabling plants to respond more efficiently to recurring stress or even to prepare their offspring for potential future assaults (Lämke and Bäurle 2017).

15.2.3 Bud Dormancy

Bud dormancy is an adaptive mechanism allowing plants to endure periodic/seasonal chilling or drought in a quiescent status (de la Fuente et al. 2015). This response adjusts to seasonal variation through the perception of change into different environmental variables (Shim et al. 2014). Irradiance is the inducing factor for tropical species (Borchert et al. 2015), while for temperate ones it is the photoperiod and/or temperature (Lloret et al. 2018). This process is best characterized into temperate species where short days and/or temperature change induces bud set, growth arrest, and final endodormancy (Cooke et al. 2012). Moreover, low temperature exposition time and degree mediate these species endodormancy break and their variation affects phenological traits and productivity (Kumar et al. 2017).

Based on the same triggering stimuli, signaling system after bud dormancy holds onto different elements. Between these, phytohormones (Chao et al. 2017) and developmental factors such as *FLOWERING LOCUS T* (*FT*) in angiosperms (Porto et al. 2015; Maurya and Bhalerao 2017), and *FLOWERING LOCUS T/TERMINAL FLOWER1* like (*FTL1*) in gymnosperms (Gyllenstrand et al. 2007; Klintenas et al. 2012; Carneros et al. 2017) stand out. These genes are the most representative elements into temperature and day length dependent vegetative growth control, bud set and for angiosperms also floral induction control (Gyllenstrand et al. 2007; Hsu et al. 2011; Maurya and Bhalerao 2017; Chen et al. 2018). Bud endodormancy is *FT*/*FTL1* related (Hsu et al. 2011; Avia et al. 2014) but also with MADS-box *FLOWERING LOCUS C* (*FLC*) like genes known as *DORMANCY ASSOCIATED MADS-BOX* (*DAM*) genes. *FLC* controls flower repression (Gu et al. 2013) and vernalization (Bastow et al. 2004) in Arabidopsis. Likewise, some related *DAM* genes expression correlates with dormancy imposition and its vernalization like chilling dependent break in forest species (Lloret et al. 2018).

Environment induced fluctuations on the aforementioned genes along the bud developmental process imply the differential regulation of large gene sets (Porto et al. 2015; Chao et al. 2017) and a concomitant proteome (Xu et al. 2016a) and metabolome (Michailidis et al. 2018) reshape. In the previous section the epigenetics mechanisms that regulate bud phase change have been described; however, histone fold status might be also involved into the dormancy break required chilling sensing (Friedrich et al. 2018). Dormancy induction is associated with a global increase in repressive DNA methylation (Santamaría et al. 2009) and generally

repressive histone modifications with some histone deacetylases, methylases, and ubiquitinases upregulating on this stage (Maurya and Bhalerao 2017). Conversely, dormancy break is related to DNA methylation decrease and histone acetylation increase, correlating with DNA methyltransferases downregulation and histone acetylases upregulation (Santamaría et al. 2009; Kumar et al. 2016). Breaking this general scheme, histone acetylases as chestnut *GCN5L* upregulate under bud dormancy (Santamaría et al. 2011) and histone deacetylase genes upregulate during dormancy break pointing to the existence of specific epigenetic regulation mechanisms over regulatory genes. As an example of both this system specificity and epigenetic control over bud dormancy, some FLC-like DAM genes develop a promoter focused enrichment in H3K27me3 marks towards dormancy break allowing its silencing during the growth phase (de la Fuente et al. 2015). This resembles FLC repression upon chilling exposition during vernalization in Arabidopsis (Bastow et al. 2004). Interestingly, dormancy related DNA methylation has shown their chilling intensity sensitiveness. In apple, dormancy break is linked to a more intense demethylation after high chilling than after low chilling, and related to productivity and fruit quality (Kumar et al. 2016, 2017).

Although this developmental process shares multiple elements between species, there are also multiple evidences of its variability. Most of these changes can be addressed to genotypical variation, from profound changes as those separating gymnosperms and angiosperms bud development regulation to small ones like the latitudinal differences in FT-like *PaFT4* mediated growth arrest and bud set regulation in Norway spruce populations (Gyllenstrand et al. 2007). Besides this, epigenetic mechanisms are behind observed differences in some cases. *Picea abies* have shown the capacity to change its bud phenology upon environmental conditions during embryonic development through differential and stable epigenetic regulation of FT-like *PaFT2* between other targets (Carneros et al. 2017). miRNA mediated epigenetic regulation would be after this temperature dependent epigenetic tuning of *Picea* bud phenology (Yakovlev and Fossdal 2017). Moreover, methylation pattern and methyltransferases coding genes expression have been found differential between different *Pinus sylvestris* ecotypes and related to embryogenic environmental conditions (Alakärppä et al. 2018).

15.2.4 Epigenetic Regulation in a Changing Environment

Through this chapter we have shown the link between the different environmental inputs plants can sense and how they regulate gene expression by inducing epigenetic changes. These environmental inputs have slowly changed for thousands of years, and plants have evolved upon their changes. However, the negative effects of climate change in the form of altered temperatures and rains can be considered especially worrying for forest outcome, since these climatic variations are happening in a very short timeframe, impeding species to fully adapt. Thus, description of the mechanisms underlying bud set, dormancy, and dormancy release is mandatory

to identify key elements into this process allowing plants to tune their phenotype to a changing environment. The easiness, specificity, stability, or inheritance, and relation to bud dormancy make epigenetics mechanisms a promising place to start. Understanding and tuning bud phenology would enable us to prepare for and avoid climate associated problems selecting/engineering tolerant genotypes. The potential generation of epitypes or the directed selection of natural variants would also increase plant biomass production or release some crops from their phenological boundaries.

Besides representing a key mechanism during growth and developmental processes, adjustment and control of gene expression is especially important when plants are subjected to unavoidable environmental stressors (Yaish 2017). The phenotypic plasticity offered by reversible epigenetic marks constitutes an essential factor especially considering the adaptive capacity of long-lived organisms such as woody plants and the framework of rapid climate change (Plomion et al. 2016). Since 2014, when the main studies dealing with epigenetic alterations occurring in important tree species were reviewed by our group (Pascual et al. 2014), many others have been conducted. The advances in next-generation sequencing technologies have considerably boosted the research focusing on the involvement of epigenetic alterations to stress conditions. Most of the available studies are, therefore, conducted on species/genus with a sequenced genome. For example, *Populus trichocarpa* genome was the first sequenced tree genome, and most of the studies considering the subject of epigenetic alterations due to environmental stress were conducted with *Populus spp*.

The first single-base resolution methylome of *Populus trichocarpa* under control and drought stress conditions (Liang et al. 2014) revealed that the methylation levels of cytosines, upstream 2kp, downstream 2kb, and in repetitive sequences significantly increased after drought treatment. They suggested that DNA methylation may not be associated with cis-splicing but rather with trans-splicing and found a relation in transcription factors with transposable elements showing reduced methylation and expression levels or increased methylation and expression levels after drought treatment.

A different group examined the involvement of epigenetic mechanisms in phenotypic plasticity towards soil water availability in *Populus* × *euramericana* employing microarray chips (Lafon-Placette et al. 2018). Several regions were identified for each water regime and associated with differential gene expression. Interestingly, highest variations of both gene expression and DNA methylation were associated with rewatering. These authors observed that changes in methylation were particularly in the body of expressed genes and to a lesser extent in transposable elements, revealing that phenotypic plasticity was accompanied by coordinated variations in DNA methylation, gene expression, and specific genes involved in hormone pathways (Lafon-Placette et al. 2018).

An acute drought stress (7 and 11 days after water withholding) and relief (2 h and 3 days after rewatering) were monitored in leaves of *Eucalyptus globulus* by quantifying several biochemical markers of oxidative stress and DNA methylation patterns (Correia et al. 2016). Water withholding imposed a mild oxidative stress, an

increased global 5-methylcytosine distribution, and a high number of specific demethylation events, while rehydration showed a decreased global DNA methylation and lipid peroxidation shortly after 2 h (Correia et al. 2016). These results showed a parallel induction of redox and complex DNA methylation changes occurring during drought stress imposition and relief in eucalypts (Correia et al. 2016).

In order to decipher the molecular mechanisms that *Pinus halepensis* (Aleppo pine), one of the most drought-tolerant pine species, uses to withstand drought, Fox et al. (2018) performed large-scale physiological and transcriptome analyses at six physiological stages: pre-stomatal response, partial stomatal closure, minimum transpiration, post-irrigation, partial recovery, and full recovery. These authors found a strong transcription of retrotransposons during recovery from drought and argue that the activation of transposable elements might be partially related to the differential expression of several methylation-related transcripts, which implies an epigenetic regulation of gene expression during drought stress in *P. halepensis* (Fox et al. 2018).

The effect of temperature stress in *Populus simonii* was analyzed by using methylation-sensitive amplification polymorphisms and quantitative reverse transcriptase PCR (RT-qPCR) to uncover changes of methylation and expression of miRNA genes (Ci et al. 2015). The authors found that 25.38% of methylation sites changed in response to abiotic stress and identified 1066 sites that were differentially methylated in response to heat and cold stress, from which seven were miRNA genes (Ci et al. 2015). Their results suggest that DNA methylation may regulate the expression of miRNA genes, likely through the gene-silencing function, as a strategy to maintain cell survival under abiotic stress conditions (Ci et al. 2015).

Also using *Populus* (specifically, roots of *Populus* × *canadensis* I-214), Ariani et al. (2016) compared changes in epigenetic modifications under excess Zn using chromatin immunoprecipitation sequencing (Chip-Seq) for two histone modifications associated with highly expressed genes (H3K4me3) and repressed genes (H3K27me3) together with RNA-Seq transcript abundance to examine how epigenetic modifications would affect gene expression. On one hand, their analyses revealed that genes with an H3K4me3 modification are generally highly expressed, and that H3K4me3 modifications were enriched in genes involved in carbon (C) catabolism, nitrogen (N) metabolism, and in regulation of sub-cellular vesicular trafficking in roots under excess Zn condition. On the other hand, genes with an H3K27me3 modification on the 50-UTR are mainly low expressed and H3K27me3 modifications were enriched primarily in genes involved in photosynthetic processes (Ariani et al. 2016). As the authors stressed, the understanding of epigenetic modifications in response to excess Zn in *Populus* roots constituted a starting point for improving phytoremediation potential of this species (Ariani et al. 2016).

In 2018, Volkova et al. analyzed genetic and epigenetic changes in Scots pine (*Pinus sylvestris* L.) populations from areas that were chronically irradiated for more than 30 years. By using amplified fragment length polymorphisms and ultra-performance liquid chromatography coupled with mass spectrometry, their results

showed that the genetic diversity was significantly higher at the radioactively contaminated areas in comparison to the reference site, and that the genome of pine trees at 4 of the 7 affected sites was also significantly hypermethylated (Volkova et al. 2018).

The importance of dynamic epigenetic mechanisms, such as DNA methylation and histone modifications, in plant adaptation to different biotic stresses has also been explored (Espinas et al. 2016). Although most studies addressing this matter focus on model species like Arabidopsis or tomato, a few studies on woody species are already available. Gene expression patterns of miRNAs in *Populus trichocarpa* plantlets inoculated with the poplar stem canker pathogen, *Botryosphaeria dothidea*, were analyzed by miRNA Array, real-time quantitative PCR for miRNAs and their targets, and miRNA promoter analysis (Zhao et al. 2012). The authors found 12 upregulated miRNAs and any downregulated in the stem bark of *P. trichocarpa* and provided a potential co-regulatory network and a putative miRNAs-transcription factors feedback regulatory network, which were developed to describe post-transcriptional regulation in the pathological development of poplar canker disease (Zhao et al. 2012).

Sollars and Buggs (2018) performed a genome-wide DNA methylation analysis in several ash trees (*Fraxinus excelsior*) and Manchurian ash (*F. mandshurica*) genotypes, with different susceptibility to ash dieback (caused by the fungus *Hymenoscyphus fraxineus*). The authors found that the overall level of cytosine methylation in the leaf methylome of *Fraxinus excelsior* is similar to leaves of *Populus trichocarpa*. They also found higher methylation in transposable elements as opposed to non-mobile elements and identified 1683 significant differentially methylated regions between the high and low susceptibility genotypes of *F. excelsior* trees.

15.3 Epigenetics and Natural Variation: New Insight to Unveil Adaptive Mechanisms

The capability of different provenances of the same species to adapt to very different niches demonstrates the enormous plasticity of genotypes. To date, hundreds of polymorphisms or genes related to natural variation have been identified, mostly in model species (Arabidopsis and some crops). In Arabidopsis, recent studies involving the re-sequencing of thousands of ecotypes and the availability of genome-wide association tools allowed to elucidate the molecular bases of phenotypic differences related to plant adaptation to distinct natural environments and to determine the ecological and evolutionary processes that maintain this variation (Alonso-Blanco et al. 2016). Natural variation can thus be defined as the intra-specific phenotypic variation caused by spontaneously arising mutations that have been maintained in nature by an evolutionary process such as artificial and natural selection

(Alonso-Blanco et al. 2009). In the same way as gene variants, epigenetic regulation can also explain the observed differences between populations (Dubin et al. 2015; Kawakatsu et al. 2016; He et al. 2018). With the discovery that epigenetic regulation of 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 (Skinner 2015).

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).

15.3.1 Epigenetic Control of Natural Variation

The determination of the sources and the role of natural variation has always been recognized as a priority for plant evolutionary biology studies (Richards et al. 2017; Henderson and Salt 2017), embracing the enormous diversity present within wild plants (Alonso-Blanco et al. 2009). Analyzing and understanding the natural variation in wild species was the starting point to elucidate the molecular bases of phenotypic differences related to plant adaptation to distinct natural environments. This genetic variation also exists in more domesticated species, which allow the exploitation of desirable traits in agriculture (Henderson and Salt 2017). Several functional polymorphisms and genes involved in natural variation have been identified in crops development and physiology and associated with important plant traits including genes related to plant morphology, architecture, fruit and seed structure, yield, and quality traits improved by successive breeding (Alonso-Blanco et al. 2009). Phenotype diversity has also been reported in forest trees and the observation of high levels of within-stand phenotypic and molecular diversity has been a concern in forest population genetic studies (Scotti et al. 2016). As static but long-lived organisms growing under temporal and spatial contrasting environment conditions, trees are particularly exposed to many challenging situations during their life span (Avramidou et al. 2015). The maintenance of genetic and phenotypic variability may be a question of adaptation and survival for forest trees (Scotti et al. 2016).

The question in debate is to what extent genetic information contributes or influences a specific phenotypic trait. What do we currently know on the question of natural variation?

It has recently become clear that heritable phenotypic variation results from the mutual yet differential contribution of genetic and epigenetic variation. The notion that variation does not need to be based just on DNA sequence polymorphism is

already commonly accepted (Balao et al. 2018). Epigenetics increasingly occupies a pivotal position in our knowledge of inheritance, natural selection, and consequently, evolution with implications across many fields of biology (Burggren 2016; Richards et al. 2017). The theory claims that epigenetic marks can control adaptive phenotypes but it is not presently understood the relative potential of epigenetic variation in comparison to genetic variation for the contribution to certain traits, especially across generations (Burggren 2016). Epigenetic regulatory mechanisms can facilitate changes in gene activity and fine-tune gene expression patterns, thus enabling plants to survive and reproduce successfully in unpredictable environments. Current knowledge is based upon studies in the model species Arabidopsis (Alonso-Blanco et al. 2016; Richards et al. 2017; Aller et al. 2018) taking advantage of its short life cycle and benefiting from genome sequence, powerful genomic resources, and access to high-throughput phenotyping platforms (Richards et al. 2017). These works push up a new research field in which the theoretical ability of epigenetic variation to influence the heritable variation of complex traits is gaining power in the study of plant adaptation (Rodríguez-Leal et al. 2015; Aller et al. 2018). The use of model species allows the discover of the underlying mechanisms of epigenetic dynamics but these studies still have a limited ecological realism (Richards et al. 2017) and are carried out under controlled conditions. Available works with non-model species (that lack extensive genomic resources) in natural environments are still insufficiently explored and thus welcome. Extensive variation of DNA methylation patterns within a species has been uncovered from studies of natural variation (Zoldoš et al. 2018) and suggests that epigenetic variation might be important for ecological studies. Unfortunately, most species used for epigenomic studies are annual herbaceous plants, and epigenome dynamics has been poorly investigated in perennial woody plants (Fortes and Gallusci 2017). The dynamics between epigenetic variation in addition to genetic variation and environment as a mechanism of adaptive plasticity in natural plant populations still needs further research (Richards et al. 2017; Aller et al. 2018; Lele et al. 2018; Zoldoš et al. 2018). Environmentally shaped phenotypic plasticity is thought to play an important role in the adaption of plants to contrasting habitats particularly after postembryonic development (Pikaard and Scheid 2014); however, the transient or heritable nature of the variation (thus potential adaptive) should be clarified. It is also premature to establish whether or not the reported epigenetic profiles are under genetic control and the degree of such control (Richards et al. 2017). One thing is clear, plants are unable to escape their surroundings and are forced to cope with changeable and often unfavorable growth conditions; therefore, epigenetic changes and transgenerational epigenetic inheritance might play an important role in plant response to stress and ecological adaptation (Meyer 2015) that deserves attention. This may be especially important for long-lived organisms with complex life cycles such as forest trees (Bräutigam et al. 2013).

15.3.2 *Linking Epigenetic and Phenotypic Variation in Forest Species*

Trees are long-lived organisms that have to deal with heterogeneous habitat conditions. This imposes limits on natural selection under rapidly changing climate conditions. Phenotypic plasticity is the ability of a genotype to generate and display different phenotypes in response to variation in the environment (Forsman 2015). For the adaptation, phenotypic plasticity plays an important role in the environmental adaptation of trees, so epigenetic variation as a mechanism of adaptive plasticity in natural plant populations needs further research as epigenetic marks and their relation to phenotypic traits are still unexplored to date (Verhoeven et al. 2016; Lele et al. 2018). There are several studies in Arabidopsis about the relationship between epigenetic and phenotypic variation in response to environmental stimuli including also traits of transgenerational inheritance (Cortijo et al. 2014; Kooke et al. 2015; Liu et al. 2015; He et al. 2018), but just a few in trees (Bräutigam et al. 2013).

In tree forest species, phenotypic plasticity due to epigenetic variation plays a key role in long-term abiotic stress adaptation. Analysis of the involvement of epigenetic mechanisms in the winter-dormant shoot apical meristem of poplar (Populus × euramericana) clones in memory of the growing conditions faced during the vegetative period showed that variations in global DNA methylation between conditions were genotype dependent and correlated with biomass production capacity (Le Gac et al. 2018). Also in poplar (*Populus simonii*), it has been described that variation in genomic methylation in natural populations is associated with leaf shape and photosynthetic traits (Ci et al. 2016). This study provides an association analysis to study the effects of DNA methylation on plant development indicating that epigenetics, environmental, and genetic factors are linked and affect both poplar growth and development.

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. 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). Alakärppä et al. (2018) have reported variations in global DNA methylation and gene expression between three Scots pine suggesting a contribution to the local adaptation and the enhancement of fitness of trees under rapidly changing climatic conditions. Studies at transcriptional level in Norway spruce embryos from the same genotype exposed to different epitype-inducing temperatures showed several epigenetic regulators with principal role in epigenetic memory, supporting that both DNA and histones methylation and sRNAs are crucial for the epigenetic memory establishment (Yakovlev et al. 2016).

The long generation time of trees is a drawback and reduces the number of available studies considering epigenetic inheritance. So far, we have learnt that breeders must take care about the different lots of seeds generated for progeny selection from the same parent genotype, since the temperature and day length conditions should be similar for each lot in order to obtain similar progenies. This phenomenon is not only of important for breeding but has evolutionary significance for conservation of forest genetic resources. Contrary to crop and herb model species, there are only a few published examples linking tree epigenetics and environment being necessary more experiments. Nevertheless, some of them are ongoing for understanding both stress adaptive mechanisms underlying epigenetics and phenotypic variation in forest species however much more work is needed to gain enough knowledge to understand these processes.

15.4 Concluding Remarks and Further Perspectives

Since our first review (Pascual et al. 2014), the evidences proving the pivotal role of epigenetic regulation linking environment, gene expression, and phenotype in forest species have increased. Epigenetics mechanisms can respond to internal (i.e., leaf development through hexoses accumulation) and external (long-term cold exposure) sensing mechanisms. Furthermore, recent advances in epigenetics demonstrate how some traits can be modulated during embryogenesis, seedling stages, or even before fecundation. This last effect is particularly striking since, although priming effects are well known, the possibility of a transgenerational inheritance of these marks and the definition of new epi-varieties could quickly help to improve tree stress tolerance. Despite the difficulty of working with tree species, many fundamental questions have been answered in the recent years as it was described above. However, deepening in the epigenetic regulation of complex adaptive traits such as long-term metabolic adaption to stressful environments or in transgenerational epigenetics effects will possibly provide an unprecedented breakthrough and will be supposedly reached in the near future. All this knowledge will be applicable to develop new biotechnological tools for breeders and forest managers towards the improvement of the efficiency and productivity of our forests.

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