Signaling and Communication in Plants

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Epigenetic Memory and Control in Plants

Signaling and Communication in Plants

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Editors

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Preface

Epigenetics has emerged as a fundamental theme underlying alterations in expression of the genetic information without any obvious changes in DNA sequence. As such, epigenetics affect all aspects of the organism's life, including growth, development, and response to biotic and abiotic factors. The essence of epigenetics results from multiple reversible chemical modifications occurring on the DNA and on its packaging histone proteins that bring about modulation of chromatin structure and consequently to modulation of its function (e.g., gene expression, DNA replication, and recombination). This book highlights recent advances in our understanding of epigenetic mechanisms as a major determinant through which internal and external signals such as those occurring during hybridization (cross breeding), flowering time, reproduction, and response to stress communicate with plant cells to bring about activation of multiple nuclear processes and consequently to plant growth and development. The outcome of these processes may persist for generations long after the initial cues have expired and may contribute to plant evolution.

Each chapter addresses diverse aspects of plant development from the viewpoint of epigenetics. It begins with a general historical perspective by Grafi and Ohad on the field of epigenetics, from the discovery of "epicytosine" (5′ methylcytosine)—a minor constituent in acid hydrolysates of eukaryotic DNA and ϵ-N-methyl lysine in acid hydrolysates of histones to the discovery of the enzymes involved in modifying DNA and histone proteins. Emphasis is given to the experimental tools used by researchers in plants to assess the importance of epigenetic markers such as DNA methylation to plant development and the tools used to uncover the chromatin modifier genes involved in determining chromatin states (restrictive or permissive).

Fransz and colleagues address the flexibility evolved in plants to adapt to changes in their environment highlighting chromatin reorganization as a major means in plant adaptability to environmental cues that bring about transcriptional reprogramming. The authors discuss the available literature on how environmental and endogenous signals instigate large-scale chromatin remodeling in plants and how this results in acclimation to a changing environment, with a focus on the model plant *Arabidopsis thaliana*.

In her nobel article "The significance of responses of the genome to challenge," Barbra McClintock (1984) highlighted the potential for genetic instability that may be induced following exposure of cells to stress. Boyko and Kovalchuk discuss recent advances in understanding dynamic changes that occur in plant chromatin and smRNA populations during exposure to stress and their contributions to stress acclimation and plant survival.

Saijo and Reimer-Michalski discuss plant immunity, highlighting the potential epigenetic basis underlying transcriptional reprogramming during and after immune response, with a particular focus on the role of dynamic changes in chromatin configuration. The authors highlight recent studies that point to the role of chromatin-level control in the establishment and maintenance of transcription-repressive or -permissive states for defense-related genes.

The timing to flower and commit to the reproductive phase represents an important aspect in the life cycle of plants enabling reproduction under favorable conditions. Zografou and Turck provide a comprehensive review on the epigenetic regulation of flowering time summarizing the regulation of the floral repressor *FLOWERING LOCUS C* (*FLC*) of *A. thaliana*, for which the impact of chromatin modification on the molecular memory has been well studied. The authors also discuss differences in the regulation of the *FLC* and its ortholog *PERPETUAL FLOWERING 1* from *Arabis alpina*, a perennial relative of *A. thaliana*, as well as the impact of chromatin structure on the regulation of *FLOWERING LOCUS T* (*FT*).

Jerzmanowski and Archacki highlight hormonal signaling in plants and animals from the epigenetics viewpoint. The authors examine the similarities and differences between plant and animal nuclear receptor systems with the aim of revealing analogies that could help identify possible intersections between plant hormone signaling and epigenetic mechanisms.

Seeds are the end products of reproduction commonly derived from fertilized ovules in gymnosperm and angiosperm plants. Many seeds enter a period of dormancy to ensure germination under optimal conditions and consequently seedling survival. Soppe and colleagues provide an overview on the role played by epigenetic mechanisms in seed dormancy and germination in *A. thaliana*.

Houben and colleagues discuss the importance of histone modifications by phosphorylation for cell cycle progression highlighting the kinases involved in histone phosphorylation.

RNA interference (RNAi) was first discovered in *Caenorhabditis elegans* injected with double-stranded RNA (dsRNA) leading to silencing of genes sharing high sequence homology with the injected dsRNA. Since this ground-breaking discovery, small RNAs 20–30 nucleotides in length were found to play an important role in genome organization and function in a variety of organisms ranging from yeast to plants and animals mediating transcriptional and posttranscriptional silencing processes. Vaucheret and colleagues give a detailed review of the studies that uncovered the mode of action of the different classes of small RNAs during the development of plants.

Huh and Rim review the current knowledge on DNA demethylation and gene imprinting in flowering plants. The authors focus on endosperm gene imprinting and highlight epigenetic regulatory mechanisms involved in gene imprinting including DNA methylation and demethylation and histone modifications.

The book is concluded with a chapter addressing transgenerational epigenetic inheritance in plants, a phenomenon in plant evolution often refers to "Lamarckian inheritance," that is, the "inheritance of acquired characters." Sano and Kim discuss the idea that epigenetically acquired traits induced upon environmental stresses, are sometimes transmitted to their offspring. The authors argue that transgenerational epigenetic inheritance is confirmed if three requirements are fulfilled: (1) acquired characters are beneficial for the organism; (2) inheritance of acquired characters extends over three generations; and (3) responsible genes are identified.

Epigenetics has become central in regulating growth and development of higher organisms. The dynamic nature of epigenetic marks demonstrates the extent of flexibility that might be retained in somatic cells, which enable them to change fate. Plants are well suited for "Lamarckian evolution," that is inheritance of acquired traits induced epigenetically. First, epigenetic changes acquired during vegetative growth are not erased during the reproductive phase as they are in animals and, second, plants have remarkable ability to reproduce vegetatively from somatic cells. Such cells are often subjected to various stress conditions that might induce heritable epigenetic modifications that could lead to phenotypic variation. Thus acquired traits induced by epigenetic changes may be transmitted to the next generations and might play a role in plant evolution.

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Plant Epigenetics: A Historical Perspective

Gideon Grafi and Nir Ohad

Abstract The chemical marks that provide the major means by which epigenetics manifests its effect on chromatin structure and function have been discovered long ago almost along with the invention of the term epigenome by Conrad H. Waddington. However, it had to wait several decades before the connection between epigenetics and chemical modifications of DNA and histone proteins has been established. Many of the modifying enzymes responsible for the dynamic modifications of DNA and histones such as histone methyltransferases and histone demethylases have only recently been identified and molecularly characterized. This introductory chapter provides a historical view on epigenetics: when and how it has begun and where it is going.

1 Introduction

The term epigenotype was first introduced by Conrad H. Waddington to demonstrate the sum of interrelated developmental pathways that enable one genome to give rise to multiple epigenomes and consequently to multiple cell types that make up the whole organism. Nowadays, the term epigenetics is commonly referred to all kinds of heritable, chemical modifications on the DNA (cytosine methylation) or on histone proteins (e.g., acetylation, methylation) bringing about modulation of

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chromatin structure and function. Also, in recent years, small RNAs have been emerged as key players in controlling epigenetic landscapes throughout the plant genome. In this introductory chapter, we provide a historical perspective on several aspects of epigenetics in general as well as emphasizing the experimental tools used by researchers in plants to assess the importance of epigenetic markers such as DNA methylation to plant development and to uncover the chromatin modifier genes involved in determining chromatin states (restrictive or permissive).

2 DNA Methylation

The pyrimidine 5-methylcytosine has first been identified by Johnson and Coghill (1925) in the hydrolytic products of nucleic acids of tubercle bacillus (*Mycobacterium tuberculosis*). Based on the optical properties of the crystalline picrate, they found that the base fraction of this hydrolysis contains in addition to cytosine also 5-methylcytosine. It had to wait more than 20 years for 5-methylcytosine to be (re) discovered in nucleic acids of higher eukaryotes. Hotchkiss (1948) repeatedly observed a minor constituent in the chromatographic patterns from acid hydrolysates of a preparation of calf thymus DNA, which he designated "epicytosine," which was assumed to be 5-methylcytosine. Later, by using paper chromatography, Wyatt (1951) has reported on the occurrence of 5-methylcytosine in nucleic acids derived from plants and animals; 5-methylcytosine cannot be found in DNA from microbial sources. Chemical analysis of rye germ DNA showed that the distribution of cytosine and 5-methylcytosine is uneven and both do not randomly substitute for each other in polynucleotide chains; often 5-methylcytosine was found to have a preferential association with guanylic acid (Shapiro and Chargaff 1960). This unique nucleotide arrangement could not be attributed to the activity of the DNA polymerase inasmuch as the enzyme was found to freely catalyze the incorporation of pyrimidine and purine analogues (such as 5-methylcytosine and hypoxanthine, an intermediate of purine nucleotide biosynthesis) into DNA without distinguishing between the "natural" base and its analogue (Bessman et al. 1958). Thus, the nonrandom distribution of 5-methylcyosine along the DNA chain raised the proposition that cytosines are methylated by specific DNA methyltransferases after being incorporated into the DNA. The first evidence for a methylase activity in plants that is directed to cytosine was reported by Kalousek and Morris (1969) who found this activity in crude extract of nuclei from pea seedlings. In these experiments, the authors showed that *S*-adenosyl-L-methionine is the methyl donor and the product of the reaction was identified as 5-methylcytosine. Generally, the extent of cytosine methylation in plants is higher than in animals ranging from a tenth to a third part of all cytosines depending on the plant species (Wagner and Capesius 1981).

Besides 5-methylcytosine, there is evidence for the occurrence of N6-methyladenine in higher plants, which appears to be found mostly in mitochondrial DNA (reviewed in Vanyushin and Ashapkin 2011). Yet, the biological significance of this "trace base" for chromatin structure and function is largely unknown.

3 The Biological Significance of Cytosine Methylation

The finding that 5-methylcytosine does not exist as a precursor in the biosynthetic pathway and that cytosines are methylated nonrandomly after their incorporation into the DNA chain suggests that cytosine methylation might possess a regulatory role in chromatin structure and function. While most evidence related to its role in modulating gene expression was essentially correlative, a direct evidence was obtained from in vitro gene transfer experiments. Accordingly, gene sequences that were methylated in vitro remained methylated and transcriptionally silent when introduced into cultured cells whereas unmethylated sequences were transcribed (Vardimon et al. 1982; Stein et al. 1982). Also the use of methylation inhibitors and mutants in animals established the role of DNA methylation in the regulation of gene expression and genomic imprinting (Li et al. 1993). Hence, treatment of cells with 5-azacytidine resulted in alteration of gene expression and cell differentiation, while mutant mice deficient in DNA methyltransferase activity displayed abnormal expression of imprinted genes (reviewed in Robertson and Jones 2000).

Earlier works using 5-azacytidine or 5-azadeoxycytidine demonstrated the importance of proper DNA methylation for chromatin organization and gene expression. Treatment of *Vicia faba* root tips with the abovementioned inhibitors resulted in uncoiling of specific chromosomal segments and chromosome aberrations (Fucík et al. 1970). It has been shown that in certain T-DNA-containing tobacco cells, T-DNA suppression is associated with heavy methylation, whereas treatment with 5-azacytidine significantly reduced the level of T-DNA methylation leading to T-DNA expression and phytohormone-independent growth (Amasino et al. 1984; John and Amasino 1989). The role played by DNA methylation in gene silencing was further supported by treatment with 5-azacytidine of protoplasts and callus cultures derived from tobacco lines containing a silent GUS (beta-glucuronidase) gene. Among 14 lines with silent GUS that were examined, 11 lines showed GUS reactivation following exposure to 5-azacytidine. Notably, two lines showed GUS reactivation under culture conditions in the absence of 5-azacytidine (Weber et al. 1990) due perhaps to stress-induced epigenetic reprogramming brought about by tissue culturing (Madlung and Comai 2004; Miguel and Marum 2011).

It should be noted, however, that the so-called methylation inhibitors such as 5-azacyctidine and 5-azadeoxycytidine may exert a broader effect on cellular processes besides DNA methylation, which makes it difficult to relate a given developmental effect to the lack of DNA methylation. For example, 5-azacytidine can be processed to nucleoside triphosphate and can be incorporated into both DNA and RNA, and thus besides DNA methylation, it affects multiple cellular processes including protein and nucleotide syntheses (reviewed in Christman 2002).

It was thus necessary to take a more direct, genetic approach for assessing the biological significance of DNA methylation, namely, the use of DNA methylation mutants. Pioneering work pursuing a genetic approach to the problem of DNA

methylation were first reported by Vongs et al. (1993) and later by Finnegan et al. (1996), each employing different methodology. Vongs et al. (1993) screened mutagenized populations of *Arabidopsis thaliana* for plants whose centromeric repetitive DNA arrays (180 bp repeats) are susceptible to digestion by endonucleases sensitive to cytosine methylation. In this screen, three hypomethylation, recessive mutants were isolated and two mutants appeared to be alleles of a single locus, which was designated DDM1, for decrease in DNA methylation1. The *ddm1* mutant showed 70 % reduction in cytosine methylation, both at CpG and non-CpG contexts. These mutant plants grew essentially normally with no notable growth perturbation (Vongs et al. 1993); morphological abnormalities were developed in *ddm1* mutant only after several generations of self-pollination (Kakutani et al. 1996). In *ddm1* mutant, the DNA methyltransferase activity and the level of the methyl donor *S*-adenosylmethionine (SAM) were comparable to those found in wild-type plants (Kakutani et al. 1995). DDM1 was later found to be required for maintaining gene silencing in *Arabidopsis* (Jeddeloh et al. 1998) and to encode a SWI2/SNF2 chromatin remodeling factor (Jeddeloh et al. 1999), thus providing evidence implicating chromatin remodeling in maintaining DNA methylation. Finnegan et al. (1996) took a different approach to address the importance of DNA methylation for gene silencing and plant growth and development. The authors generated transgenic *Arabidopsis* plants expressing an antisense construct for DNA methyltransferase gene MET1, the major Dnmt1 class of maintenance cytosine methyltransferase in *Arabidopsis*. These transgenic plants displayed reduced cytosine methylation in CpG context as well as a number of phenotypic and developmental abnormalities, including reduced apical dominance, smaller plant size, altered leaf size and shape, decreased fertility, and altered flowering time (Finnegan et al. 1996). It should be noted that MET1 was later isolated by the Richards lab using the Southern blot screen for mutants with centromeric repeats susceptible to digestion by the methylation-sensitive endonuclease, *Hpa*II (Kankel et al. 2003). In this screen four additional DNA hypomethylation mutants were identified, two of which were recessive and allelic and were originally designated *ddm2-1* and *ddm2-2*. These mutations were found to disrupt the *MET1* cytosine methyltransferase gene and renamed *met1-1* and *met1-2* that displayed 70 % and 50 % reduction in cytosine methylation in TCGA sites, respectively. Notably, despite of the significant reduction in cytosine methylation in *ddm1* and *met1* mutants, flower-specific genes such as *SUPERMAN* and *AGAMOUS* became hypermethylated in these mutants (Jacobsen and Meyerowitz 1997; Jacobsen et al. 2000).

To further explore the molecular machinery involved in DNA-methylation-induced gene silencing, plant biologists have taken a genetic approach, in which *Arabidopsis* mutants with a notable phenotype resulted from methylation and silencing of a given gene were screened for suppression of the mutant phenotype in EMS-mutagenized populations. Screening of EMS-mutagenized population of transgenic *Arabidopsis*, in which hygromycin phosphotransferase (*hpt*) is stably silenced, revealed several suppressor mutants (designated *som4*, *5*, *6*, *7, and 8*) capable of derepressing the activity of transcriptionally silenced *hpt* gene and thus conferring hygromycin resistance (Scheid et al. 1998); these mutations were found to be alleles of *ddm1* (Jeddeloh et al. 1999).

Using this approach, additional chromatin modifiers playing a central role in epigenetic control of gene expression were discovered. Accordingly, Steve Jacobsen and colleagues have used the *clark kent* epimutants caused by hypermethylation and consequently silencing of the flower developmental gene *SUPERMAN* (*SUP*). These mutants displayed a notable flower phenotype of increasing number of stamens and carpels (Jacobsen and Meyerowitz 1997). Stable *clk* (*clk-st*) mutant plants were mutagenized by EMS and screened for suppressor mutants having wild-type flower phenotype. This screen identified 12 *clk-st* suppressor mutants, in which nine are loss-of-function alleles of the *CHROMOMETHYLASE3* (*CMT3*) gene, a plant-specific DNA methyltransferase responsible for maintaining cytosine methylation in the CHG context (Lindroth et al. 2001). At the same time, Judith Bender and colleagues used a similar approach in an attempt to identify genes responsible for methylation and silencing of an endogenous reporter gene. Here, they followed the PAI2 tryptophan biosynthetic gene whose methylation and silencing resulted in accumulation of tryptophan pathway intermediates and in a blue fluorescent plant phenotype that can be visualized under ultraviolet (UV) light. By screening an EMS-mutagenized population, Bartee et al. (2001) have isolated 11 loss-of-function alleles in the *CMT3* gene that showed reduced methylation (particularly at non-CG cytosines) and enhanced expression of the reporter PAI2 gene and consequently strong reduction in blue fluorescence. Interestingly, despite of global reduction in CHG methylation, *cmt3* mutants grew normally and displayed wild-type morphology even after multiple generations of inbreeding (Lindroth et al. 2001; Bartee et al. 2001), suggesting that CHG and CG methylation may be partially redundant in gene silencing (Lindroth et al. 2001).

De novo DNA methyltransferases in plants were first identified in a search of *Arabidopsis* and maize databases for genes sharing similarity with the catalytic domain of the mammalian de novo methyltransferase Dnmt3. Because these genes display a novel arrangement of the conserved catalytic domains, they have designated in *Arabidopsis DOMAIN REARRANGED METHYLATION1* (*DRM1*) and *DRM2* genes (Cao et al. 2000). In *Arabidopsis* plants carrying T-DNA insertional mutations in both genes *DRM1* and *DRM2* (*drm1 drm2* double mutant), maintenance methylation was persisted while de novo symmetrical (CpG, CpHpG) as well as asymmetrical methylation was blocked in flower genes *FWA* and *SUP*, pointing to DRM1 and DRM2 gene products as the major de novo methylation enzymes in *Arabidopsis* (Cao and Jacobsen 2002). Later, *DRM3* gene was identified as DRM2 paralog having a mutated, inactive catalytic domain, which is required for normal maintenance of non-CG DNA methylation, for establishment of RNA-directed DNA methylation triggered by repeat sequences, and for accumulation of repeat-associated small RNAs (Henderson et al. 2010). DRM3 appears to be functional equivalent to the mammalian Dnmt3L whose catalytic domain is inactive and functions in modulation of the de novo DNA methyltransferase activities of Dnmt3a and Dnmt3b (Wienholz et al. 2010); particularly, Dnmt3L was found to be involved in the establishment of maternal genomic imprints in mice (Bourc'his et al. 2001; Hata et al. 2002).

4 Interpretation of the DNA Methylation Signal

The way by which the DNA methylation signal is interpreted into a specific chromatin state has been illuminated with the identification of DNA methylation binding proteins (MBPs). The first protein capable of binding specifically methylated CpG sites independently of DNA sequence was MeCP1—a 120 kDa protein widely distributed in mammals; this protein, however, requires multiple symmetrically methylated CpG sites for strong binding to DNA (Meehan et al. 1989). MeCP1 was later found to play an important role in the methylation-mediated repression of gene transcription both in vitro and in vivo (Boyes and Bird 1991). A second protein named MeCP2 was later isolated for its capacity to bind methylated CpG sites. However, unlike MeCP1, MeCP2 was capable of binding a single symmetrically methylated CpG site and displayed transcriptional repression activity on both methylated and unmethylated templates (Lewis et al. 1992). The minimal methyl-CpG-binding domain (MBD) of MeCP2 was found to contain 85 amino acids capable of binding exclusively DNA that contains one or more CpG methylated sites (Nan et al. 1993). Later it was found that mutations in the gene encoding for X-linked MeCP2 are the cause of some cases of Rett syndrome (Amir et al. 1999) — a neurodevelopmental disorder causing mental retardation particularly in females. The dissection of the domain (MBD) responsible for binding methylated CpGs together with the availability of various plant genome sequences allows the identification of multiple putative genes encoding for MBD-containing proteins (The Chromatin database, http://www.chromdb.org) and their initial characterization (Zemach and Grafi 2003; Scebba et al. 2003; Berg et al. 2003; Ito et al. 2003). Thus far, among the 13 putative MBD encoding genes in *Arabidopsis thaliana*, the products of three genes, namely, AtMBD5, AtMBD6, and AtMBD7, were shown to have functional MBD capable of binding one or more symmetrically methylated cytosine exclusively in the CpG context; their possible mode of action in regulating chromatin structure and function is summarized in several review articles (Springer and Kaeppler 2005; Grafi et al. 2007; Zemach and Grafi 2007).

Besides the MBD group of proteins, several other proteins were found to bind methylated cytosine in various sequence contexts. Among them is a group of proteins containing the SRA (SET- and Ring-associated) domain, which was originally found in the human ICBP90 (inverted CCAAT box-binding protein of 90 kDa) to mediate binding to methyl-CpG (Unoki et al. 2004). Interestingly, several plant histone methyltransferases of the Su(var)3-9 homolog (SUVH) group, such as KYP/SUVH4 and SUVH5 bind directly to methylated DNA in all sequence contexts (Johnson et al. 2007; Rajakumara et al. 2011), thus further substantiating the link between DNA methylation and histone methylation in *Arabidopsis* plants. VARIANT IN METHYLATION 1 (VIM1) is a member of a small gene family, encoding proteins that contain PHD, RING, and SRA domains, initially found in mammalian proteins implicated in regulation of chromatin structure and function. The gene was isolated in a screen for hypomethylated

centromeric repeats in 89 different strains of *A. thaliana* that display natural variation in DNA methylation; Borky-4 (Bor-4) strain was found to be hypomethylated in both CpG and CHG (where H = A, T or C) contexts displaying decondensation of centromeric chromatin (Woo et al. 2007). VIM1 was found to bind, via its SRA domain, methylated cytosine in both CpG and CHG contexts; its capacity for interaction with recombinant histones (H2B, H3, H4, and HTR12) in plant extracts was also demonstrated (Woo et al. 2007).

The third group of methylated DNA-binding proteins is the kaiso and kaiso-like proteins, thus far found only in mammals. Kaiso requires at least two symmetrically methylated CpG sites for binding through its three Krüppel-like C2H2 zinc fingers and appears to act as a methylation-dependent transcriptional repressor in transient assays (Prokhortchouk et al. 2001). Blast search of the human genome for proteins containing kaiso-like zinc fingers identified two kaiso-like proteins, ZBTB4 and ZBTB38, which were found to bind methylated DNA in vitro and in vivo; both proteins are capable of binding a single methylated CpG site and to repress the transcription of methylated templates (Filion et al. 2006).

5 Histone Modifications

In the nucleus, the DNA interacts with core histone proteins (two of each of H2A, H2B, H3, and H4) to form the basic structural unit of chromatin, the nucleosome. The possible role of histones as regulators of the genetic activity has been speculated by Stedman and Stedman (1951). Later, biochemical evidence has demonstrated the inhibitory role imposed by histones on chromatin function. Accordingly, histones were found to inhibit DNA-dependent RNA synthesis in chromatin isolated from pea embryos; the removal of histones from chromatin resulted in an increased rate of RNA synthesis (Huang and Bonner 1962). Likewise, experiments performed in calf thymus nuclei showed that histones do play a role in the regulation of nuclear RNA synthesis via a complex mechanism (Allfrey et al. 1963). It was found that the degree of inhibition was dependent on the type of histone and its concentration. Hence, the arginine-rich histone fractions, which contain histones H3 and H4, strongly inhibited nuclear RNA synthesis while lysine-rich fractions (contains H1, H2A, and H2B) were essentially ineffectual (Allfrey et al. 1963). This activity might be related to the capability of H3 and H4, in the absence of lysine-rich histones, to form an octamer made of four H3–H4 dimers that can complex with DNA and retain many of the properties of the chromatin (Simon et al. 1978). However, it has been noted that histone–DNA complexes can occur without inhibition of RNA synthesis raising the possibility that specific and presumably reversible chemical modifications of histone proteins, taking place at the nucleosomal level, provide the means for switching on or off RNA transcription at various loci along chromosome arms (Allfrey and Mirsky 1964).

Initial studies of amino acid composition of acid hydrolysate of histones from various animal sources revealed the presence of a small amount of an unidentified substance that was eluted from an ion-exchange column as a small peak adjacent to

lysine (Crampton et al. 1957; Rasmussen et al. 1962). Comparing the behavior on ion-exchange chromatography of histone hydrolysates with that of the *Salmonella typhimurium* flagellin, known to contain ϵ-*N*-methyl lysine (Ambler and Rees 1959), it was suggested that the unidentified substance is ϵ-*N*-methyl lysine. Later, ϵ-*N*-methyl lysine was also found in histone preparation from wheat germ (Murray 1964). Also, the complete sequencing of histone H4 from calf and pea revealed two "unusual" amino acid residues, ϵ-*N*-acetyllysine (K16) and ϵ-*N*-methyl lysine (K20) (DeLange et al. 1968). A comprehensive view on plant histone acetylation from a historical perspective can be found in a recent review article by Waterborg (2011) — among the pioneers in plant epigenetics. Besides acetylation and methylation of specific lysine residues of histone proteins, also phosphorylation of the hydroxyl group of seryl or threonyl has been reported (Kleinsmith et al. 1966; Marushige et al. 1969). Later it has been shown that histone proteins can undergo multiple posttranslational modifications including acetylation, methylation, phosphorylation, ubiquitination, and ribosylation that alter the structure of chromatin and its function (van Holde 1989; Wolffe 1992).

Earlier studies pointed out that in a variety of tissues, the state of chromatin condensation as well as chromosome morphology correlates with the degree of histone posttranslational modification. Accordingly, highly condensed chromatin such as that occurring in micronuclei of *Tetrahymena* (Gorovsky et al. 1973) or in mature avian erythrocytes (Ruiz-Carrillo et al. 1974) was found to contain less acetylated histones than nuclei with diffused chromatin. These observations support the hypothesis that chromatin structure and function is regulated at least partly by the strength of interaction between basic histone side chains and the acidic DNA backbone. Enzymatic activities capable of transferring in vitro methyl and acetyl groups from *S*-adenosyl-L-methionine and acetyl CoA, respectively, to histone proteins were found in soluble extracts of rat organs (Kaye and Sheratzky 1969).

The genetic approach has been used quite intensively to pinpoint chromatin modifier genes whose products involved in epigenetic control of gene expression. Perhaps the most known example is the position effect variegation (PEV) in *Drosophila*, which resulted from chromosomal rearrangement leading to translocation of euchromatic genes into close proximity with heterochromatin causing these genes to be silenced in a metastable manner. As a result, individuals carrying this chromosomal rearrangement display a mosaic phenotype. The best example of PEV in *Drosophila*, first described by Muller (1930) more than 80 years ago, involves chromosomal rearrangement juxtaposing the *white* locus to heterochromatic region of the X chromosome (w^{m4}) resulting in variegated eye phenotype. The use of EMS and X-ray mutagenesis allowed mass isolation of several hundred PEV modifier mutations, namely, suppressors (Su) and enhancers (E) of variegation [Su(var) and E(var), respectively)] corresponding to about 150 genes (reviewed in Schotta et al. 2003). Some of the genes involved in PEV have been isolated and molecularly characterized including Suv(var)2-5 and Suv(var)3-9 that encode for heterochromatin protein1 (HP1) and histone H3 lysine 9 methyltransferase, respectively (Eissenberg et al. 1990; Rea et al. 2000).

As mention above, plant biologists used a genetic approach to pinpoint chromatin modifier genes whose product involved in gene silencing. Using the epimutant *clk-st,* Jacobsen and colleagues isolated 12 *clk-st* suppressor mutants, in which nine were loss-of-function alleles of the *CMT3* gene (Lindroth et al. 2001) and three alleles appeared to be loss-of-function mutations in the *KRYPTONITE* (*KYP*)/*SUVH4* gene encoding for histone H3 lysine 9 (H3K9) methyltransferase (Jackson et al. 2002). Similarly, Bender and colleagues used a genetic screen for mutations that disrupt silencing of the endogenous gene PAI2. This screen yielded seven loss-of-function alleles in the *SUVH4* gene, which encodes for a SET-domain protein with H3K9 methyltransferase activity (Malagnac et al. 2002). Interestingly, both *kyp* and *suvh4* mutants conferred reduced cytosine methylation, particularly at non-CG contexts, on *SUP* and *PAI2* genes, respectively, suggesting that H3K9 methylation and DNA methylation are coupled. Indeed, in *Neurospora crassa*, dim-5 gene that encodes for H3K9 methyltransferase was found to be required for DNA methylation as well as for normal growth and full fertility (Tamaru and Selker 2001); trimethylation of H3K9 by DIM-5 HMTase was found to mark chromatin regions for cytosine methylation (Tamaru et al. 2003). Genetic analysis in mammalian cells also demonstrated a link between DNA methylation and histone methylation. Accordingly, progeny of Dnmt3l−/− female mice completely lacks maternal DNA methylation at imprinting control regions (ICRs) and dies early during embryonic development (Bourc'his et al. 2001). Lack of DNA methylation was associated with a significant decrease in repressive histone modifications, thus providing a mechanistic link between DNA and histone methylation at ICRs (Henckel et al. 2009).

6 Polycomb Group Proteins and Histone Modifications

Polycomb group (PcG) proteins were initially identified in *Drosophila melanogaster*, found to take part in long-term repression of homeotic (*Hox*) genes via chromatin remodeling (Struhl 1981; Sathe and Harte 1995).

In animals, at least three distinct multisubunit polycomb repressive complexes (PRCs) were identified: polycomb repressive complex 2 (PRC2), polycomb-like PRC2 (Pcl-PRC2), and polycomb repressive complex 1 (PRC1) (Papp and Muller 2006; Muller and Verrijzer 2009). Initiation of gene silencing is catalyzed by methylation of histone H3 at lysine 27 (H3K27me) mediated by PRC2 and related Pcl–PRC2 complexes (Cao and Zhang 2004). PRC1 binds to the methylated histone (Fischle et al. 2003) establishing a stable repression of PcG target genes, by catalyzing monoubiquitination of histone H2A at lysine 119 (H2AubK119) (Shao et al. 1999). Histone modifications, such as H3K27me3 and H2Aub, play a key role in repressing gene expression, probably by preventing RNA-transcript elongation (Stock et al. 2007). PRC1 and to a lesser extent PRC2 also mediate compaction of the chromatin (Muller and Verrijzer 2009), which limits accessibility of transcription factors, including SWI/SNF-class ATP-dependent chromatin remodelers (Shao et al. 1999; Francis et al. 2004). These activities lead subsequently to repression of target genes through consecutive cell divisions.

The Drosophila PRC2 complex contains four core protein subunits: enhancer of zeste E(z), serving as the catalytic subunit, methylating H3K27 via the SET [Su (var), E(z), Thritorax] domain; extra sex comb (ESC) containing seven WD-40 domains; suppressor of zeste 12 [Su(z)12] containing the C2H2 zinc finger domain; and the nucleosome remodeling factor 55-kDa subunit (Nurf55, known also as p55) (reviewed by Schuettengruber et al. 2007).

In recent years it became evident that the transcriptional regulation mediated by PcG proteins is a general mechanism, which has been conserved along evolution and is involved in establishing and maintaining gene expression patterns both in animals (reviewed by Schwartz and Pirrotta 2008) and plants (Mosquna et al. 2009; Kohler and Villar 2008; Butenko and Ohad 2011).

The first characterized plant PcG gene *CURLY LEAF* (*CLF*), homologs of *E*(*z*), was identified among *Arabidopsis* mutant plants (Goodrich et al. 1997). The *clf-2* mutant display altered flower morphology and early flowering due to ectopic expression of the MADS-box homeotic gene *AGAMOUS* (*AG*), thus indicating that wild-type CLF takes part in regulation of *AG* expression.

Novel genetic screens aimed at identifying regulatory genes controlling *Arabidopsis* seed and fruit development yielded mutants that cause parent-of-origin effects on seed development and allow autonomous endosperm development in the absence of fertilization. These mutants revealed lesions in three loci. Based on their phenotype, these mutants were designated fertilization-independent endosperm (FIE) (Ohad et al. 1996) and fertilization-independent seed (FIS) (Chaudhury et al. 1997).

Subsequent cloning of *MEA*, *FIE*, and *FIS2* genes revealed that they encode homologs of animal PcG proteins. MEA is a SET-domain protein homologous to the *Drosophila* E(z) (Grossniklaus et al. 1998; Kiyosue et al. 1999; Luo et al. 1999), FIE encodes a WD-40 protein homologous to the *Drosophila* ESC (Ohad et al. 1999), and FIS2 is a C2H2-type zinc finger protein homologous to the *Drosophila* Su(z)12 (Luo et al. 1999). Further genetic screens lead to the identification of additional *Arabidopsis* PRC2 members. Thus, the *Arabidopsis* genome encodes for three *E*(*z*) paralogs containing the SET domain, namely, *CURLY LEAF* (*CLF*), *SWINGER* (*SWN*) (Chanvivattana et al. 2004), and *MEDEA* (*MEA*). Members of the *Su*(*z*)*12* family encoding for zinc-finger protein, including *EMBRYONIC FLOWER 2* (*EMF2*), *VERNALIZATION 2* (*VRN2*) (Gendall et al. 2001), and *FERTILIZATION-INDEPENDENT SEED 2* (*FIS2*). Members of the WD-40 motif proteins include *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) and *MULTICOPY SUPRESSOR OF IRA 1* (*MSI1*) (Kohler et al. 2003a).

Genetic, molecular, and biochemical evidences lead to the current understanding that at least three PRC2 complexes harboring different paralogs of the E(z) and Su (z)12 proteins families are likely to coexist in *Arabidopsis*. Each of these proposed complexes controls a particular developmental program (Hsieh et al. 2003; Katz et al. 2004; Chanvivattana et al. 2004; Sung and Amasino 2004; Guitton and Berger 2005; Makarevich et al. 2006; Pien and Grossniklaus 2007; Schatlowski et al. 2008; Kohler and Villar 2008; Kim et al. 2009).

The role of each of the proposed PRC2 complexes during the plant life cycle and their effect on gene expression and developmental programs will be discussed in this book.

As in animals, the PcG function in *Arabidopsis* is required for the methylation of H3K27 at different loci (Kohler et al. 2003b; Bastow et al. 2004; Jullien et al. 2006; Gehring et al. 2006; Turck et al. 2007; Zhang et al. 2007). In support of this hypothesis is the finding that intact SET domain is necessary for the functions of AtCLF and AtMEA proteins (Makarevich et al. 2006; Schubert et al. 2006). In addition it was shown that *Arabidopsis* PRC2 complexes repress homeotic transcription factors, such as members of the homeobox *KNOX* family (Katz et al. 2004; Xu et al. 2008). These results suggest for conserved function of the PcG complexes during ontogenesis in both plant and animal kingdoms. The above reports also reveal the critical role PcGs play in establishing and maintaining cell identity during the plant life cycle.

7 Interpretation of the Histone Modification Signaling

The histone code hypothesis suggests that chemical modifications of histone proteins that bring about changes in chromatin structure are not simply modulating the histone–DNA interaction but acting as recognition sites for the recruitment of proteins or protein complexes that in turn alter chromatin structure and function (Strahl and Allis 2000; Jenuwein and Allis 2001). Accordingly, the bromo domain often found in histone acetyltransferases binds acetylated lysine, while the chromo domain was shown to have preference to methylated lysines. However, proteins have high specificity for binding to a particular modified residue within the histone tail; for example, in animals, the chromo-containing HP1 protein binds to di-/trimethylated H3K9, while the chromo-containing polycomb proteins bind exclusively to trimethylated H3K27. In *Arabidopsis*, however, LHP1 binding was not specific to a particular modified residue as it could bind to H3K9me2 (Zemach et al. 2006) as well as to H3K27me3 (Exner et al. 2009). In *Arabidopsis*, H3K27 methylation mediated by the PcG complex has a profound impact on silencing gene expression (Turck et al. 2007; Zhang et al. 2007; Oh et al. 2008). However, this mark is only one out of a diverse range of histone modifications giving rise to an elaborated code established by posttranslational modifications. It has been shown in animals that methylated lysines such as H3K4me1, H3K4me2, H3K4me3, H3K36me, or acetylated H3 and H4 (H3Ac and H4Ac) are associated with active chromatin. In contrast, silent chromatin is associated with H3K9me1, H3K9me2, H3K9me3, H3K27me1, H3K27me2, or H3K27me3 (Roudier et al. 2009).

A recent comprehensive study by Roudier et al. (2011) describes mapping of eight histone modifications (H3K4me2 and 3, H3K27me1 and 2, H3K36me3, H3K56ac, H4K20me1, and H2Bub) using a tiling microarray covering the whole *Arabidopsis* genome sequence at 165 bp resolution. This dataset was combined with maps for H3K9me2, H3K9me3, H3K27me3, and DNA methylation described

previously (Turck et al. 2007; Vaughn et al. 2007). Collectively these 12 marks have revealed 4 main chromatin states covering ~90 % of the genome under nonstress conditions.

A first chromatin state (CS1) corresponds to transcriptionally active genes that are typically enriched in the trimethylated forms of H3K4 and H3K36. Two additional states correspond to two distinct types of repressive chromatin. H3K27me3-marked repressive chromatin (CS2) is mainly associated with genes under PRC2-mediated repression, while H3K9me2- and H4K20me1-marked repressive chromatin (CS3) corresponds to classical heterochromatin, which is almost exclusively located over silent TEs. The fourth chromatin state (CS4) is characterized by the absence of any prevalent mark and is associated with weakly expressed genes and intergenic regions. It should be noted, however, that global analysis of histone modifications along the genomes of *Arabidopsis* and rice (Deal and Henikoff 2010; He et al. 2010; Roudier et al. 2011) revealed a fifth chromatin state characterized by both repressive and permissive marks (e.g., H3K27me3 and H3K4me3), known as bivalent state (reviewed in Grafi et al. 2011). This chromatin state first identified in animal stem cells (Azuara et al. 2006; Bernstein et al. 2006) suggests a model in which transcription of tissue-specific regulatory genes is "primed" but held in check until specific differentiation signal dictates either activation (e.g., recruitment of H3K27 demethylases) or silencing (e.g., recruitment of H3K4 demethylases) of the gene locus (Lan et al. 2008).

8 Concluding Remarks

Although the term epigenome and epigenetic markers have already known for more than 80 years, most work on epigenetics has been done during the last 12 years with the discovery of chromatin-modifying enzymes involved in modification of histone proteins. Intriguingly, the discovery of chromatin modifier genes was essentially relied on genetic analysis of mutants in *Drosophila*, which were described more than 80 years ago by Muller (1930) that involve chromosomal rearrangement that placed the *white* locus to heterochromatic region of the X chromosome (w^{m4}) resulting in variegated eye phenotype. Similarly, the genetic approach has been the major tool taken by plant biologists to uncover major players involved in modifying chromatin and in regulating gene expression. Since then our understanding of plant epigenetics has increased remarkably. Chromatin Immunoprecipitation (ChIP) and large-scale sequencing allowed to map modified histones along the entire genome in *Arabidopsis* and rice as well a PcG binding sites and putative DNA targets sequences. To better understand these epigenetic processes, one would need to reveal the mechanism(s) by which the different epigenetic marks are targeted and placed specifically on a particular histone residue at a specific chromosomal site and how they are removed allowing for high dynamic range of chromatin states. Also, the interaction between different epigenetic mechanisms

and the sequence of events leading to the establishment of a particular chromatin state at a given loci within a given cell at a given time still needs to be unraveled.

The combined knowledge of mapping the histone code and DNA methylome and the relation between them will help to address the above problems. With this respect the extensive effort to map the methylome (Zemach et al. 2010) and its relation to RNA polymerase function (Wierzbicki et al. 2012) integrated with full mapping of histone marks will allow to advance our understanding as to how these mechanisms are coordinated to facilitate epigenetic regulation of gene expression in plants.

Finally, our knowledge on epigenetics is limited to a few plants such as *Arabidopsis* and rice. Conceivably, plants have evolved a plethora of epigenetic mechanisms to enable gene functionality in different genomic environments as well as to allow plants to withstand their natural habitats (Granot et al. 2009). Gene discovery in wild plants has become feasible, particularly with the development of next-generation sequencing (NGS) technologies. By employing NGS, it is possible to rapidly obtain low-cost de novo genomic and transcriptomic data for any non-model plant species and to study its unique epigenetic makeup.

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Environment-Induced Chromatin Reorganisation and Plant Acclimation

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Abstract Plants have developed a striking flexibility to adapt to changes in their environment, as they cannot flee from detrimental conditions. At the same time, they are also able to exploit favourable conditions to their benefit. This great adaptability is underlain by versatile regulation of gene transcription. It has become apparent that numerous signals, ranging from biotic (e.g. pathogen infections) to abiotic (e.g. shade, heat) environmental stimuli but also endogenous developmental signals, affect the compactness of chromatin, a process that is associated with transcriptional reprogramming. The mechanisms by which these signals induce the changes in chromatin condensation and, in return, whether chromatin compaction contributes to physiological acclimation to a changing environment are currently not well understood. In this chapter we discuss the available literature on how environmental and endogenous signals instigate large-scale chromatin remodelling

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in plants and how this results in acclimation to a changing environment, with a focus on the model plant *Arabidopsis thaliana*.

1 Introduction

Plants continuously face challenges and opportunities from their environment. As a consequence, they have established various strategies in the course of evolution to adjust their growth and development under changing environmental conditions. Such acclimation responses include, e.g. the timing of important developmental phase transitions like seed germination and floral transition, but also more subtle responses such as elongation growth of stems under dense canopies to optimise light capture. These responses are typically associated with transcriptional regulation, as many genes need to be activated (transcribed) to control the "new" state. Conversely, genes for which transcription is only required in the "old" state need to be repressed. In recent years, it was found that developmental phase transitions and perception of a changing environment are often associated with reversible, large-scale changes in chromatin compaction in planta (Exner and Hennig 2008; Jarillo et al. 2009; Fransz and De Jong 2011). A causal connection between extensive changes in chromatin compaction and transcriptional reprogramming, however, is still elusive.

In this chapter, we review and summarise the available literature on the effects of environmental and developmental signals on the structural organisation of plant chromatin. Special attention is given to global chromatin compaction in *Arabidopsis thaliana* and its emerging role in acclimation to changing endogenous and exogenous conditions.

2 Chromatin Organisation in *Arabidopsis thaliana*

In plants, as well as in other eukaryotes, nucleosomes form the organisational unit of chromatin. Each nucleosome consists of a histone octamer, containing two H2A–H2B dimers and one H3–H4 tetramer, around which two turns (146 bp) of DNA are wrapped. Neighbouring nucleosomes are connected via linker DNA, on which histone H1 resides to condense the chromatin further to the so-called 30 nm fibre and subsequent higher order compaction levels (Dorigo et al. 2004; Wu et al. 2007). Repositioning, replacements, and changes in the composition of nucleosomes (i.e. different histone variants) can influence transcriptional activity of a locus. In addition, covalent epigenetic modifications, including cytosine methylation (5-mC) and histone acetylation (Ac), methylation (Me), phosphorylation, or ubiquitination, determine the transcriptional activity of a locus by controlling differential accessibility of the DNA sequence for the transcription machinery (Tessadori et al. 2004; Naumann et al. 2005; Fransz et al. 2006; Liu et al. 2007).

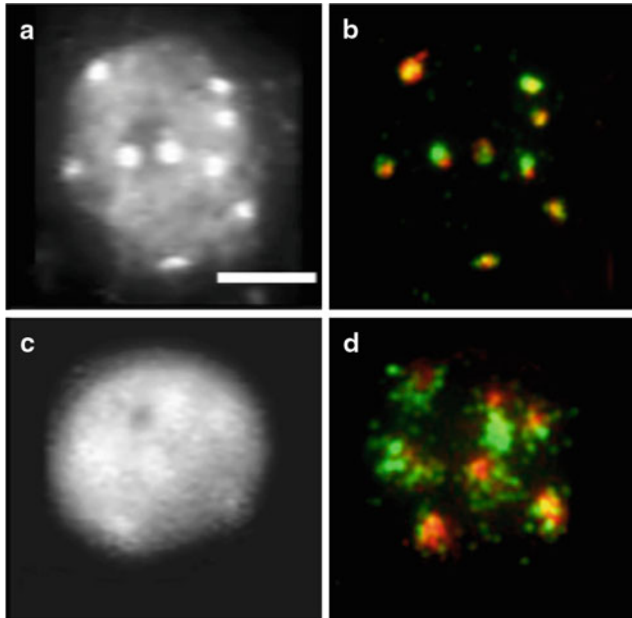

Fig. 1 Nuclear phenotypes of rosette leaf in *Arabidopsis*. Demonstration of typical DAPI staining (**a**, **c**) and FISH signals (**b**, **d**) in mesophyll cell nuclei under normal growth conditions (**a**, **b**) or following exposure to biotic and abiotic stress conditions (**c**, **d**). Chromocenters are compact and stained brightly with DAPI (**a**). Heterochromatin repeat sequences of the centromere (180 bp, *red*) and pericentromere (transposons, *green*) colocalise to chromocenters. Note that in stressed leaves nuclei lose the strict chromatin organisation in chromocenters due to decondensation of the centromere (*red*) and pericentromere (*green*) repeats. Scale bar is 5 μm

Based on the specific combination of the epigenetic marks, a classification of chromatin types into four groups (*C*hromatin *S*tate; CS1-4) has been reported for the model plant *A. thaliana* which roughly represent active genes (CS1), repressed genes (CS2), silent (transposon) repeat elements (CS3), and intergenic regions (CS4) (Roudier et al. 2011). *Arabidopsis* has a characteristic nuclear organisation in which different patterns of chromatin compaction are distinguished after staining with fluorescent dyes such as 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst (Fig. 1a). The interphase nucleus displays highly compact "heterochromatic" domains, known as chromocenters (Tessadori et al. 2004; Fransz et al. 2006; Fransz and De Jong 2011).

Fluorescence in situ hybridization (FISH) revealed that chromocenters are typically enriched in major repeat sequences including (peri)centromeric repeats, transposable elements, and ribosomal genes (Fig. 1b, d) (Table 1). Immunostaining showed that chromocenters typically contain silencing epigenetic marks including 5-mC and H3K9Me2 (Soppe et al. 2002; Naumann et al. 2005; Fransz et al. 2002, 2006). In contrast, euchromatic regions are enriched in gene activating epigenetic marks such as H3K4me3 and histone acetylation. The relative compaction level of

Table 1 Chromatin conformation of various genomic sequences in *Arabidopsis* nuclei under different genetic, environmental, and developmental parameters

		Gene[a]	Multicopy transgene (HPT)	Pericentromeric, non-Tes	Pericentromeric, Tes	Pericentromeric, 5S rDNA	180 bp repeat (centromere)	45S rDNA (subtelomere)	References
Mutants	Wild type	–	+	+	+	+	+	+	Fransz et al. (2002), Probst et al. (2003), Tessadori et al. (2007a)
	ddm1; met1-1	–	–	–	+	+	+	+	Soppe et al. (2002)
	hda6	–	nd	–	nd	–	+	+/–	Probst et al. (2004)
	mom1	–	+	+	nd	nd	+	+	Probst et al. (2003)
Environment	Low light	–	nd	–	–	–	–	+/–	Van Zanten et al. (2010a, b)
	Heat	–	–	nd	nd	–	–	nd	Pecinka et al. (2010)
	Bacterial infection	–	nd	–	+/–	–	–	+	Tessadori unpublished results
Development	Protoplast	–	–	–	–	–	–	+/–	Tessadori et al. (2007a)
	Cultured cell	–	nd	+/–	+/–	+	+	+	Tessadori et al. (2007a)
	Seeds (mature)	–	nd	+	nd	nd	+	+	Van Zanten et al. (2011)
	Seedling	–	nd	–	nd	–	+	+	Mathieu et al. (2003)
	Floral transition	–	nd	–	nd	–	+	+	Tessadori et al. (2007b)

+ fluorescent signal is condensed and colocalises with chromocenter; – fluorescent signal is dispersed and outside chromocenter; +/– both condensed and dispersed signals have been observed; *nd* not determined

[a]The majority of genes are situated outside chromocenters (Soppe et al. 2002; Fransz et al. 2006)

chromatin can be easily quantified by calculating the relative heterochromatin fraction (RHF), which is the fluorescence intensity of all chromocenters over the total intensity of the nucleus (Soppe et al. 2002; Tessadori et al. 2004), or by calculating the Heterochromatin Index (HX), which is the ratio of chromocenter-rich nuclei (Fig. 1a) over the total number of nuclei (Tessadori et al. 2009).

3 Environmental Control of Chromatin Compaction

3.1 Light Control of Chromatin Organisation

Because of their photoautotrophic nature, plants primarily rely on light for their growth and survival. Signals that indicate changes in the light environment are crucial for the timing of developmental phase transitions such as germination, floral induction, and senescence (reviewed in Sullivan and Deng 2003; Kami et al. 2010). The corresponding light properties include wavelength composition, light intensity, daily photon-flux, light direction, and photoperiod, which are detected via photoreceptor proteins. In *Arabidopsis*, blue light and UV/A are sensed via PHOTOTROPIN1 (PHOT1) and -2, CRYPTOCHROME1 (CRY1), CRY2, and ZEITLUPES (ZTL, FKF1, and LKP2). Red and far-red light signals are sensed by a family of PHYTOCHROME proteins (PHYA-E) (reviewed in Chen et al. 2004; Franklin and Quail 2010). UV/B is sensed via UV RESISTANCE LOCUS 8 (UVR8) (Rizzini et al. 2011). The signalling networks downstream of the photoreceptors are relatively well understood. Key roles have been identified for the photomorphogenesis repressor complex; COP/DET/FUS, the positive regulator ELONGATED HYPCOTYL (HY5)/HY5 HOMOLOG (HYH); and the PHYTOCHROME INTERACTING FACTORs (PIF1-5) (reviewed in Jiao et al. 2007). How light signals influence gene activity via modulation of chromatin structure, however, remains elusive. This issue is the topic of two recent reviews (Fisher and Franklin 2011; Van Zanten et al. 2012a).

Natural variation in chromatin compaction phenotypes has been identified using natural *A. thaliana* accessions (Tessadori et al. 2009). The compaction level under standard light conditions correlated with the latitude of geographic origin of the individual accessions. Subsequent analysis of climate data, followed by empirical confirmation, revealed that (local) variation in light intensity explains the observed variation in chromatin compaction. The sub-tropical accession Cape Verde Islands-0 (Cvi-0), for example, showed low chromatin compaction under control light conditions, while increased light levels re-established chromatin compaction. Quantitative Trait Locus (QTL) analysis using a L*er* x Cvi-0 Recombinant Inbred (RIL) population yielded chromatin compaction QTLs containing *PHYTOCHROME B* and *HISTONE DEACETYLASE-6* (*HDA6*) as underlying genes (Tessadori et al. 2009). Indeed, *phyB* knock-out lines display low chromatin compaction levels, while Cvi-0 carries a less functional *PHYB* allele (Filiault et al. 2008). Moreover, the reduced

chromatin compaction phenotype of Cvi-0 under standard light conditions was not complemented with the Cvi-0-*PHYB* allele but with the *PHYB* allele from L*er*. This demonstrates that PHYB is a positive regulator of chromatin compaction and most likely explains the QTL. Similarly, the *hda6* (*sil1*/*not*) mutant has a low chromatin compaction level and, like Cvi-0, shows decreased 5-mC and H3K9Me2 at the ribosomal genes (Probst et al. 2004) (Table 1). Sequencing revealed sequence polymorphisms in the *HDA6* promoter of Cvi-0 compared to L*er*, which is predicted to result in a truncated-HDA6 protein in Cvi-0 (Tessadori et al. 2009).

In agreement with the notion that light intensity can become limiting for chromatin compaction, reduction of light intensity by 90 % (from 200 to 15 μmol m^2 s^{-1}) resulted in a dramatic, but reversible, decrease of chromocenters (Van Zanten et al. 2010a) (Table 1). Intriguingly, the same was observed under low levels of blue light and in light with a reduced red-to-far-red ratio. These signals are associated with the induction of the "shade avoidance response", a suite of functional traits imposed by neighbouring vegetation, resulting in outgrowth of diminished light conditions (Franklin 2008; Kami et al. 2010). These observations therefore point to ecological and adaptive significance of chromatin compaction. In this context it should be mentioned that the blue light receptor CRY2 is also a positive regulator of low light-induced chromatin decompaction, since mutant cry2 plants fail to display chromatin decondensation upon low light treatment. Moreover, CRY2 decorates anaphase chromosomes (Cutler et al. 2000; Yu et al. 2009), indicating that the signal pathway of low light perception to chromatin changes is a short and perhaps direct process. PHYB has genetic background-specific effects. Interestingly, low light stabilises CRY2 protein levels (Van Zanten et al. 2010a).

The best-described example connecting light directly to chromatin is the observation that DET1 controls inhibition of non-acetylated core histone H2B (Benvenuto et al. 2002), via light-mediated recruiting of HISTONE ACETYLTRANSFERASES (HATs) and direct interaction with DAMAGED DNA BINDING PROTEIN 1 (DDB1) (Schroeder et al. 2002). Also UVR8 binds to nuclear chromatin by interacting with H2B presumably to control UV/B-mediated gene expression (Cloix and Jenkins 2008). Several other links have been shown between histone (de)acetylation and light sensing and signalling networks. For example, the histone acetyl transferase GENERAL CONTROL NON-REPRESSIBLE 5 (GCN5) and the histone deactylase HD1 mediate light-regulated gene expression (Benhamed et al. 2006). Seedling de-etiolation (transfer of seedlings from dark to light) is accompanied by changes in the histone marks H3K9ac, H3K4me3, H3K9me2, and H3K27me3, which result in changes in expression of light-regulated loci (Guo et al. 2008; Charron et al. 2009). Light-regulated transcription factors, including ELONGATED HYPOCOTYL 5 (HY5) and HY5-HOMOLOGOUS (HYH) and their downstream networks, appear associated with H3K9ac. Increased levels of this activating epigenetic mark were identified at various light-regulated loci in *det1-1* and *cop1-4* mutants (Charron et al. 2009). It has been proposed that E3 ubiquitin ligase complexes, including COP1, play a central role in controlling gene activity via large-scale chromatin remodelling (Van Zanten et al. 2012a). Indeed, the *axr1-12* mutant, lacking the active subunit

of the RUB1 activating enzyme that controls protein degradation via COP1-COP10-CSN-mediated repression of photomorphogenesis (Schwechheimer et al. 2002), exhibits constitutive decondensation of chromatin under standard light conditions (McLoughlin and Fransz unpublished; Van Zanten et al. 2012a). Together, these data indicate that light-mediated changes in chromatin compaction affect acclimation at the whole plant level to changing environmental light conditions.

3.2 Temperature Effects on Chromatin Organisation

Temperature is a key determinant of geographic distribution of species and has a major impact on plant life. Yet, little is known about the mechanisms by which plants perceive and signal temperature (Penfield 2008; Mittler et al. 2012). In an elegant paper by Kumar and Wigge (2010), however, it was shown that the histone H2A variant H2A.Z acts as thermosensor. Mutants in *ACTIN-RELATED PROTEIN 6* (*ARP6*), which are disturbed in H2A.Z positioning, showed exaggerated acclimation responses to high-temperature stress (i.e. enhanced hypocotyl and petiole elongation). Higher temperatures appear to reduce H2A.Z occupancy at the promoter region of heat-responsive genes, thereby directly linking temperature to transcriptional activation. Thus, instant changes in temperature are directly perceived and translated into relevant acclimation responses via changes in local chromatin compaction. Moreover, temperature also affects global chromatin compaction, since long-term heat stress results in a decrease of the heterochromatin index, similar to the low light situation mentioned above (Pecinka et al. 2010). The chromatin response is accompanied by a reduction of nucleosome occupancy and reactivation of silenced repetitive elements without loss of 5-mC, while only minor changes in histone modifications were noted (Lang-Mladek et al. 2010; Pecinka et al. 2010; Tittel-Elmer et al. 2010). Apparently, heat stress conditions can control chromatin organisation, nucleosome positioning, and transposon activity without strong interference of epigenetic modifications. Upon recovery from heat stress, nucleosomes were reloaded through CHROMATIN ASSEMBLY FACTOR1 (CAF1) action and *de novo* H3K9 dimethylation, which resulted in restoration of TGS. Interestingly, the original chromatin compaction level was not recovered, in contrast to low light-induced chromatin decompaction (Van Zanten et al. 2010a). The ability of plants to induce chromatin decompaction upon heat stress seems to be a general phenomenon as short-term heat stress also resulted in chromatin decondensation at the 45S rDNA loci in Monocotyledonous rice (*Oryza sativa* spp. *Japonica* Nipponbare) (3 h; 42°C) and wheat (2 h; 42°C) (Santos et al. 2011). However, temperature stress does not always lead to chromatin reorganisation, since meristematic tissues and newly formed leaves lack the reduced chromatin compaction response to heat stress (Pecinka et al. 2010). In addition, no chromatin decondensation was observed under freezing stress (−4°C for 24 h), although a

release of TGS has been reported in *Arabidopsis* plants exposed to cold (Lang-Mladek et al. 2010). Also the chromatin compaction of the 45S rDNA region in rice plants remained unaltered under cold stress (Santos et al. 2011).

3.3 Chromatin Decondensation Upon Biotic Infestation

Interactions of plants with other organisms can be detrimental to plant fitness, as is the case with plant pathogens, pests, and herbivores. On the other hand several beneficial symbiotic relations evolved between plants and bacteria or fungi (Denison and Kiers 2011). For example, association of roots with arbuscular symbiotic mycorrhizae enhances nutrient uptake and disease resistance, while rhizobium bacterial symbionts living inside root nodule cells of legumes fix nitrogen for plant uptake.

Symbiotic relations can result in large-scale chromatin decondensation of the host cells. This has been demonstrated in roots of leek (*Allium porrum*) (Berta et al. 1990; Lingua et al. 1999) and tomato (*Lycopersicon esculentum*) (Berta et al. 2000) upon infection by arbuscular mycorrhizae hyphae. Similarly, DAPI staining and DNase I digestion revealed chromatin dispersion and activation in mycorrhizae-infected pea (*Pisum sativum*) root cells (Sgorbati et al. 1993). Comparable effects were reported for rhizobial infections, at least in root nodule nuclei of pea (Bers et al. 1992).

There is ample evidence that plant defence against pathogens and acquired immunity require epigenetic regulation and chromatin remodelling factors to control resistance-associated genes (reviewed in Van den Burg and Takken 2009; Alvarez et al. 2010). Recently, it was demonstrated that induced resistance to pathogens is trans-generational heritable and stable through meiosis in *Arabidopsis* (Luna et al. 2012; Rasmann et al. 2012; Slaughter et al. 2012) and tomato (Rasmann et al. 2012). Infection with *Pseudomonas syringae* pv. tomato DC3000 triggers massive DNA hypomethylation in *Arabidopsis* nuclei (Luna et al. 2012). It has been proposed that transgenerational inheritance of induced resistance depends on such global hypomethylation events as this may affect genes that control primary defence regulators such as *NON EXPRESSOR OF PR1* (NPR1) and *PATHOGENESIS-RELATED GENE1* (*PR1*) (Luna et al. 2012; Slaughter et al. 2012).

Reports that pathogens have a direct impact on dynamic global chromatin organisation in the absence of cell death are surprisingly scarce. Perhaps the only well-documented case is from Pavet et al. (2006), who demonstrated that *P. syringae* pv. tomato DC3000 triggers chromatin decondensation in *Arabidopsis* (Table 1). Interestingly, this response was not observed in plants infected by the *hrpL* mutant of *P. syringae*. HrpL encodes an alternative RNA polymerase sigma factor that regulates the expression of many virulence genes. The inability of the hrpL mutant to generate a chromatin response indicates that pathogen-derived HrpL plays an active role in the initiation of plant chromatin decondensation.

4 Developmental Control of Chromatin Compaction

4.1 Chromatin Compaction and Decompaction During Developmental (de)Differentiation

As development progresses, cells become more differentiated and specialised. Plant cells have the ability to change cell fate after differentiation. In contrast to animals, each cell in a plant can in principle regain stem cell properties and regenerate into a new individual. Perhaps not surprisingly, this flexibility is reflected in the dynamic compaction of chromatin. Changes in chromatin compaction have been observed throughout plant development. For example, mesophyll cells of 2-cm-long rosette leaves in *Arabidopsis* show a 27 % higher heterochromatic fraction than younger leaves of 1 cm (Tessadori et al. 2004). Similarly, tobacco leaves that are induced to senesce in a controlled manner by exposure to prolonged darkness displayed characteristic features of cellular dedifferentiation including chromatin decondensation (Damri et al. 2009). Intriguingly, the severity of the decondensation correlated with the length of the darkness period, similar to the light-induced reorganisation of chromatin in *Arabidopsis* (see above). However, the response in tobacco is different from the response in *Arabidopsis* to low light levels, since reduction of nucleosome occupancy was observed in tobacco leaves after 12 days of darkness, whereas in *Arabidopsis* no changes at the nucleosomal level were observed (Van Zanten et al. 2010b). Chromatin changes have also been correlated with cell differentiation in root tissue. Nuclei in the root elongation and differentiation zone of Rhoeo (*Rhoeo discolor* Hance) contain a higher fraction of condensed chromatin than nuclei in the meristem region (Ruch and Rosselet 1970). Together, these data suggest that compaction level of chromatin is related to differentiation state.

Perhaps the most extreme situation of dedifferentiation in plants is the formation of protoplasts from differentiated tissue. Nuclei of *Arabidopsis* and cucumber (*Cucumis sativus* L.) protoplasts display severe decondensation of chromatin and loss of chromocenters (Grafi et al. 2007; Tessadori et al. 2007a; Ondrej et al. 2009, 2010). Similar observations have also been reported for tobacco protoplasts (Zhao et al. 2001). An intriguing correlation was reported between chromatin decondensation levels during protoplast formation and reactive oxygen species (ROS) levels in cucumber (Ondrej et al. 2010). Treatment of protoplasts with ascorbic acid, which prevents oxidative stress, resulted in stronger recondensation of the chromatin during recovery. Therefore, oxidative stress may be involved in decondensation of chromatin during the isolation of protoplasts and perhaps during other stresses (e.g. low light, high temperature) as well.

During protoplast isolation the pericentric heterochromatin becomes fully decondensed (Table 1). This is demonstrated in *Arabidopsis* by FISH with repeat sequences, including 5S rDNA, transposable elements, and the 180 bp centromere repeat (Tessadori et al. 2007a). The only chromocenter left, in a partly compacted

state, consisted of 45S rDNA repeats (Table 1). Reformation of all chromocenters occurred during prolonged cell culture and subsequent microcallus formation, indicating that the decondensed state of chromatin is transient. Remarkably, restoration of (hetero)chromatin compaction follows a stepwise process that correlates with the length of the repeat array, which suggests that number and size of the tandemly arranged repeat elements play an important role in the decondensation and recondensation process (Table 1). The molecular mechanism of this process remains to be elucidated.

In general, chromatin compaction is associated with decreased transcriptional activity (Tiang et al. 2012). Support for this hypothesis comes from FISH analysis of the *glabra2* locus in *Arabidopsis* roots (Costa and Shaw 2006). This locus appears accessible to FISH probes when the gene is transcribed, whereas the inactive locus gave no fluorescent signal. The result suggests that compact chromatin impedes gene expression. However, chromatin decondensation does not always lead to activation of silent genes, and *vice versa,* genes in compact chromatin can be transcribed. The silent *HPT* multicopy array of a transgenic plant (line A) displays a compact heterochromatic minichromocenter in rosette leaves which becomes decondensed in protoplast nuclei (Fig. 2a, b). However, *HPT* expression could not be detected in protoplasts (Tessadori et al. 2007a). Hence, the *HPT* locus is transcriptionally inactive in both condensed and decondensed chromatin. The opposite situation has also been reported. The same *HPT* locus is active in two different mutants, ddm1 (*DECREASE IN DNA METHYLATION 1) and mom1* (*MORPHEUS MOLECULE 1*), of which *ddm1* exhibits constitutively decondensed chromatin, whereas *mom1* has condensed chromatin (Fig. 1c, d, Probst et al. 2003) (Table 1). These four different combinations of chromatin compaction and gene activity underline that the correlation between gene activity and the chromatin compaction state is more complex than generally assumed.

4.2 Chromatin Dynamics During Floral Transition

The life history of annual plants, such as *Arabidopsis*, is determined by the timing of major developmental phase transitions. These include the transition from vegetative to reproductive meristem, or from embryo to dry seed, but also the transition from seed to seedling. All have to occur in the proper season and need to be synchronised within a natural population to secure reproductive success. Perhaps because massive transcriptional reprogramming is required, phase transitions are often accompanied by major changes in epigenetic marks. Vernalisation-mediated (i.e. exposure to a prolonged period of cold) epigenetic silencing of the floral repressor *FLOWERING LOCUS C* (*FLC*) is well understood and has been discussed in many reviews (Dennis and Peacock 2007; Exner and Hennig 2008; Jarillo et al. 2009; Yaish et al. 2011). Chromatin reorganisation is also associated with developmental transitions. Chromatin was less condensed in induced meristematic nuclei of *Sinapis alba* L. compared to vegetative meristems during floral transition

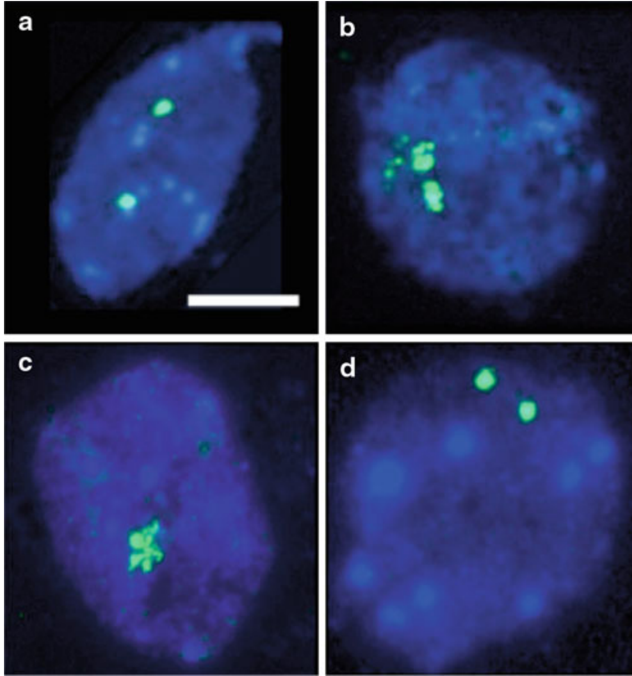

Fig. 2 Condensed and decondensed chromatin with active and inactive transgenes. FISH assay with a transgenic multicopy *HPT* array (*green*) and counterstained with DAPI (*blue*). (**a**) The silent *HPT* locus is condensed in leaf nuclei under standard growth conditions. *HPT* localises at minichromocenters close to bright chromocenters. (**b**) The silent *HPT* locus is decondensed in mesophyll protoplasts. (**c**) The active *HPT* locus is decondensed in leaf nuclei of the *ddm1* mutant. (**d**) The active *HPT* locus is condensed in leaf nuclei of the *mom1* mutant. (**c**) and (**d**) were modified from Probst et al. (2003), with permission. Scale bar is 5 μm

(Havelange and Jeanny 1984). Similarly, decondensation of chromocenters was noted 4 days before bolting, under both short day and floral-inductive long day photoperiods, in several *Arabidopsis* accessions (Tessadori et al. 2007b). After bolting, the chromocenters reformed, endorsing the reversibility of the decondensation process. During floral transition the centromeric repeats and 45S rDNA sequences remained condensed in chromocenters, whereas 5S rDNA and other pericentromeric, but also euchromatic sequences, were dispersed (Table 1). In addition, 5-mC abundance remained unaltered, but signals became dispersed over the nucleus. The transcription factors *CONSTANS (CO)* and *FLOWERING LOCUS T* (*FT*) control flowering by mediating expression of the floral meristem identity genes. Mutants in these factors, however, showed a wild-type-like chromocenter decompaction upon bolting. In contrast, *cry2* mutants did not show chromatin decondensation upon bolting, indicating that this blue light photoreceptor is a major controller of chromatin decondensation during the floral transition, analogous to its role in low light-induced chromatin compaction (see above).

Because *cry2* mutants do eventually flower (although delayed), chromatin decompaction is apparently not an absolute prerequisite for the floral transition (Tessadori et al. 2007b).

4.3 Chromatin Compaction in Reproductive Cells

Major changes in the differentiation state of cells occur during gametogenesis. In flowering plants, haploid reproductive cells are derived through meiosis in the male and female floral organs, the anthers (pollen) and ovules (embryo sac), respectively. At maturity, the male gametophyte consists of three cells: two gametic sperm cells within a vegetative cell. The female embryo sac contains the egg, the central cell, and five accessory cells; two synergid cells facilitating fertilisation and three antipodal cells. A body of evidence shows that gamete development in both anther and ovule is associated with dynamic changes in chromatin compaction and epigenetic reprogramming. The female egg cell and central cell display decondensed chromocenters compared to antipodal and synergid cells. In addition, developing endosperm is characterised by low chromatin condensation (Baroux et al. 2007). If and how interactions with the environment influence chromatin compaction is not known. The male sperm cell has compact chromatin with distinct chromocenters, whereas the vegetative nucleus has decondensed chromocenters (Schoft et al. 2009; Baroux et al. 2011). Interestingly, chromatin decondensation in the vegetative nucleus is accompanied by extensive derepression of transposable elements and accumulation of small RNAs of Athila retrotransposons (Slotkin et al. 2009). The authors suggest that the reactivation of the transposon elements is required to silence transposons in the neighbouring sperm cell. Small RNA from transposable elements may play a role in this process (McCue et al. 2012)

4.4 Chromatin Compaction During Seed Maturation and Germination

Seeds have very low metabolic activities and represent a distinct stage in the life cycle of flowering plants. Seed development starts after fertilisation and is characterised by three major phase transitions: (1) from embryogenesis to dry seed (seed maturation), (2) from dry seed to seedling (germination), and (3) the switch from heterotrophic to photoautotrophic growth (seedling establishment). These transitions are accompanied by major changes in chromatin compaction and involve complex epigenetic signalling mechanisms. This topic is elaborately discussed in this book by van Zanten et al. in chapter "Epigenetic signalling during the life cycle of seeds".

Germination is marked by an increase in transcriptional activity. For example, during the first 18 h after induction of germination the gene transcription activity in wheat (*Triticum aestivum*) is low (Yoshida and Sasaki 1977; Sugita and Sasaki 1982), but thereafter a sharp increase in template activity and endogenous RNA polymerase activity is observed (Sugita and Sasaki 1982). Germination is characterised by changes in chromatin structure. Chromatin in germinating pea was more susceptible to DNase II endonuclease compared to dry seeds (Grellet et al. 1977). Also, the amount of histones per unit DNA decreased. Together, these observations suggest that chromatin in plant seeds is more compact than in young seedlings. Indeed, quantification of chromatin compaction, using the above-mentioned heterochromatin index (HX), confirmed that the chromatin is highly condensed in cotyledons of dry *Arabidopsis* seeds (Van Zanten et al. 2011), whereas 2-day-old *Arabidopsis* seedlings lack visible chromocenters (Mathieu et al. 2003). Strikingly, chromocenters have already re-established 4 days after germination. The process of chromatin decompaction is coupled to seed germination rather than to imbibition (rehydration) of seeds, since imbibition alone is not sufficient to establish chromatin decompaction (Van Zanten et al. 2011). Seed maturation in *Arabidopsis* takes about 10 days and is physiologically characterised by accumulation of storage reserves, by induction of seed dormancy, and, during the last 3 days, by almost complete dehydration. Chromatin compaction gradually increases during the seed maturation period. A mutant lacking *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), which regulates seed development, did not show changes in chromatin compaction during seed maturation, indicating that the chromatin compaction is an actively controlled process (Van Zanten et al. 2012b).

The changes in chromatin organisation are accompanied by changes in the spatial distribution of methylated DNA sequences. At the beginning of seed maturation 5-mC signals are dispersed (Van Zanten et al. 2011), while in dry seeds, 5-mC is concentrated on chromocenters (Table 1). Similarly, during seed imbibition/germination, when chromatin decondenses, 5-mC sequences become dispersed over the nucleus (Mathieu et al. 2003; Van Zanten et al. 2011), while 4 days after germination, they colocalise with conspicuous chromocenters (Mathieu et al. 2003). The same observations were reported for repetitive DNA sequences, such as the 180 bp centromere repeats and transposon elements. They show increased compaction during seed maturation and decondensation upon germination. Taken together, several phase transitions can be characterised from embryo to seedling based on sequential decondensation and recondensation events. The embryo development stage characterised by decondensed chromatin is followed by seed maturation that is associated with strong chromatin condensation. Seed germination is accompanied by chromatin decondensation and is followed by chromatin recondensation at later seedling stages.

The reorganisation of chromatin during seed formation co-occurs with dramatic decrease in nuclear size during early seed maturation, well before major dehydration of maturing seeds happens. The latter indicates that nuclear shrinkage is not an indirect result of dehydration, but is an active, developmentally controlled process. Accordingly, nuclei of *abi3* mutant seeds did not show a decrease in size

(Van Zanten et al. 2011). Nevertheless, since nuclear size reduction was also observed during dehydration of the resurrection plant *Craterostigma plantagineum* (Van Zanten et al. 2011) and during the transition from the wet to the dry season in *Zygophyllum dumosum* Boiss (Granot et al. 2009), it was concluded that nuclear shrinkage and chromatin compaction are an adaptation to withstand desiccation in dry seeds (Van Zanten et al. 2011). Accordingly, chromatin condensation is also one of the features associated with desiccated leaf lamina cells in the moss *Polytrichum formosum* (Proctor et al. 2007).

Interestingly, changes in chromatin compaction during seed maturation and upon imbibition and germination take place independently of changes in nuclear size. This is demonstrated by the observation that chromatin compaction increases after the reduction in nuclear size and decreases quickly upon imbibition, whereas the restoration of nuclear size takes more time. Moreover, the *little nuclei 1* (*linc1*) *linc2* mutant, which has constitutive small nuclei throughout its life history, still displays changes in chromatin compaction during all tested stages of seed development and germination similar to wild type (Van Zanten et al. 2011).

The establishment of desiccation tolerance occurs simultaneously with the onset of dormancy during seed maturation in *Arabidopsis*. This explains the inability of viable seed to germinate despite optimal environmental conditions. Genetic analyses using mutants with different dormancy levels demonstrated that changes in nuclear size and chromatin compaction are not associated with dormancy in *Arabidopsis* (Van Zanten et al. 2011). In seeming contrast, observations on germinating and dormant *Phaseolus vulgaris* L. seeds suggested that nuclei shrink towards the dormant phase, which is reversed by imbibition (Kater 1927). In addition, a study on seeds of *Phaseolus lunatus* var. lunonanus and corn (*Zea Mays* var. Indentata) showed that nuclei in dormant seed were shrunken compared to germinated seeds, suggesting that chromatin is more compact in the dormant seed. After germination, nuclei became more turgid, regularly shaped, and more spherical (Middendorf 1939). Cotyledons and root tissue of dormant Peanut (*Arachis hypogaea* L.) seeds had higher heterochromatin contents compared to germinated green cotyledons and roots (Hillon and Miksche 1982). Finally, dormant meristems of onion bulbs (*Allium cepa*) had smaller nuclei and a higher fraction of dense chromatin than proliferating ones (Sans and De La Torre 1979). However, these studies provide no functional evidence that the observed changes in nuclear size and chromatin compaction are associated with dormancy.

5 Concluding Remarks and Perspectives

An increasing number of reports over the past decade have demonstrated that plants display a striking flexibility in chromatin organisation under different environmental conditions and upon major developmental switches. The similarities in chromatin dynamics in response to the different conditions suggest that a common molecular mechanism operates downstream of the signalling molecules. A number

of proteins have been identified. These include photoreceptors, histone modifiers, DNA methyltransferases, and chromatin remodellers. Remarkably, light stress and floral transition have the same key player, CRY2, that controls the switch to chromatin decondensation. Since CRY2 is a major interactor of the E3 ligase, COP1, E3 complexes have been proposed to be downstream components (van Zanten et al. 2012a). E3 ligase complexes target chromatin proteins such as transcription factors for degradation (Lyzenga and Stone 2012) and are in the signalling pathway of many stress and developmental events. How these complexes affect chromatin reorganisation remains to be elucidated. We are only beginning to understand the molecular networks that control chromatin compaction in response to endogenous and exogenous signals.

The question arises what is the biological relevance of the chromatin decompaction and recompaction events? Scientific data are lacking, although recent studies point at the control of transposon activity. Stress responses are often accompanied by the release of transposon activity (Arnholdt-Schmitt 2004; Madlung and Comai 2004). Derepression of transposons has also been reported under stress conditions that induce changes in chromatin compaction and during developmental switches (see above). In this context, it is remarkable that stress and developmental signals specifically affect the compaction level of (pericentric) heterochromatin, where the majority of transposable elements (TEs) reside. Reactivation of these TEs may trigger a silencing mechanism that modulates the stress response of the plant via siRNAs (McCue et al. 2012). The repetitiveness of the heterochromatic sequences is a prominent feature. It has been proposed that the repetition level is at the basis of molecular processes involving non-coding RNAs, whose role in gene and transposon regulation and heterochromatin establishment appears important (Martienssen 2003; Irvine et al. 2006; Wierzbicki et al. 2008; McCue et al. 2012). Such a mechanism may also explain the repeat array-dependent sequence of the chromatin recondensation events (Table 1, Tessadori et al. 2007a). Whether non-coding RNAs would also hold a role in the decondensation and re-establishment processes remains to be determined. These are important challenges for the future that will have to be addressed before we can fully appreciate the biological function of chromatin (de)condensation.

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Epigenetic Regulation of Genome Stability in Plants in Response to Stress

Alex Boyko and Igor Kovalchuk

Abstract Stress and acclimation responses in plants are mediated by genome-wide changes in gene expression, cellular proteome, and metabolome. In the past years, a significant progress has been made in understanding how epigenetic and smRNA pathways control and orchestrate these changes. Epigenetic marks modify the properties of chromatin and change gene transcriptional states on a scale of the entire genome to a single specific gene. These marks allow for the greater plasticity and adaptability of plant genomes to changing environmental conditions. As DNA methylation is well recognized as an epigenetic mechanism that largely controls stress-induced changes in the plant transcriptome, the crucial role of changes in chromatin structure and levels of small RNAs (smRNAs) becomes more apparent. In this chapter, we summarize and discuss recent advances in understanding dynamic changes that occur in plant chromatin and smRNA populations during exposure to stress. Where possible, we provide experimental evidence supporting direct contributions of these changes to stress acclimation and plant survival.

1 Introduction

Plants are sedentary organisms. The prolonged nature of environmental conditions that continuously influence plant growth (sometimes over many generations of plants) poses significant challenges to the plant's defense systems. Unlike the majority of higher eukaryotes that can leave their environment, many plants cannot use escape-avoidance tactics to minimize the damaging influence of stress. Thus, it is natural that plants possess both short-term response systems and long-term

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defense strategies allowing them to cope with acute and chronic stresses. Undoubtedly, such physiological plasticity can be only achieved by well-orchestrated genome-wide changes in gene expression and metabolome composition that should occur within a short period of time (Cook et al. 2004; Kaplan et al. 2004; Lee et al. 2005; Oono et al. 2006; Shinozaki et al. 2003; Sung and Amasino 2004). In other words, it is not the changing of genetic information but the manipulating of the expression of the existing gene pool via epigenetic regulatory pathways that allows plants to survive stress and adapt to new growth conditions.

The main strength of plant response to stress is the ability to rapidly alter homeostasis. This may require the following: massive changes in the number and the amount of metabolites produced as well as changes in their de novo synthesis and re-compartmentalization; dynamic alterations in mRNA and protein synthesis and turnover; and balancing salt concentrations, pH levels, levels of hormones, etc. The majority of these events are controlled by epigenetic mechanisms operating in somatic cells, including changes in DNA methylation and histone modifications as well as repositioning of histone and nonhistone chromatin-binding proteins in the nuclear matrix. A great role in response to stress is also played by small (sm)RNAs, including small-interfering RNAs (siRNAs) that are able to alter DNA methylation in sequence-specific manner and micro-RNAs (miRNAs) that promote sequence-specific mRNA degradation. All these mechanisms are critical for immediate plant survival.

The stress response is not limited to the exposed plants, and stress can influence the growth and development of the immediate progeny. It is possible that the effects of various environmental cues on somatic and meristem cells of plants can be reflected in the form of various smRNAs and epigenetic marks that form a molecular basis for epigenetic memory. This could mediate the transmission of environmental memories from ancestral plants to their progeny, thereby preparing them for new growth conditions (transgenerational responses in plants and mechanisms mediating these responses are covered by Sano and Kim in this book chapter “Transgenerational Epigenetic Inheritance in Plants”; see also Boyko and Kovalchuk 2010, 2011a, b). Epigenetic mechanisms responsible for transgenerational responses to stress may include the following: heritable but reversible changes in DNA methylation, various histone modifications, and chromatin remodeling. Indeed, changes in the distribution of any (or all) of these epigenetic marks can be regulated by a number of physiological and developmental stimuli including stress (Boyko and Kovalchuk 2008).

Maintaining genome integrity is an important component of plant response to stress. The wide spectrum of various internal and external stresses (Arnholdt-Schmitt 2004; Madlung and Comai 2004) continuously exerts a negative influence on plant genome stability. Genotoxic effects of these stresses are usually associated with the directly or indirectly induced changes in free radical metabolism (Vranova et al. 2002), thus resulting in oxidative DNA damage that can be mutagenic (Blokhina et al. 2003). For instance, the hydroxyl radical triggers the formation of DNA–protein cross-links and the release of free bases from DNA, thus generating DNA single- and double-strand breaks and apurinic/apyrimidinic sites. Generally, reactive oxygen species (ROS) are produced in the electron transport chains in chloroplasts and mitochondria in the process of photosynthesis and

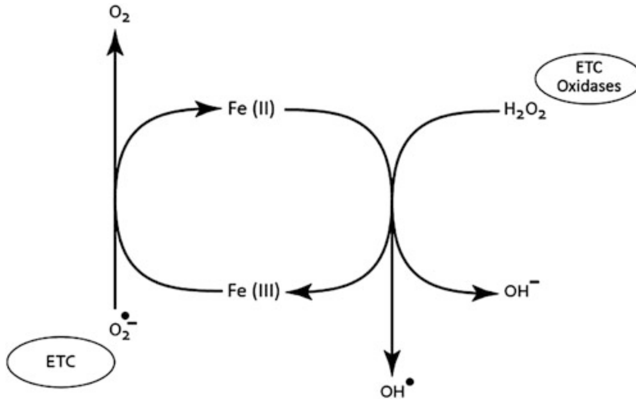

Fig. 1 Biogenesis of hydroxyl radical (•OH) in plant cells: the Haber–Weiss and Fenton reactions. Superoxide anion (•O_2^-) and hydrogen peroxide (H_2O_2) are constantly produced in electron transport chain (ETC) of mitochondria and chloroplasts. Additionally, cellular oxidase enzymes (e.g., NADPH) generate hydrogen peroxide. Both ROS molecules, superoxide anion and hydrogen peroxide, can be converted into a highly reactive hydroxyl radical (•OH) via the Haber–Weiss and Fenton reactions that occur in the presence of a ferrous ion

cellular respiration as well as in organelles with a high metabolic activity such as lysosomes and peroxisomes (Fig. 1). Under favorable conditions, a number of enzymatic and nonenzymatic antioxidants scavenge ROS, thereby maintaining a delicate balance of ROS in plant cells. However, various abiotic stresses including heat and cold, drought and desiccation, salinity, exposure to heavy metals, UV, intensive light, air pollutants, nutrient deprivation, and pathogen attacks can lead to a rapid accumulation of ROS. It appears that such a generalized response to stress is associated with the role of ROS as secondary messengers in stress-activated signal transduction cascades.

DNA repair is an important functional mechanism that ensures the maintenance of genome integrity at multiple levels. These include scanning and the identification of actual damage followed by global and/or local chromatin relaxation, the recruitment of the repairosome, and actual repair steps. DNA repair leads to the reestablishment of a similar (or sometimes different) transcriptional status of chromatin that may include changes in DNA methylation and histone modifications. Since there is a possibility that chromatin compaction can function as a buffer in the ability of various factors to access and damage DNA, it is plausible to think that genome stability of a given chromosomal region can be relaxed by choosing different DNA repair pathways and by introducing or removing various epigenetic modifications.

In the recent years, ample progress has been made in understanding the role of epigenetic mechanisms in regulating plant responses to stress. Since the response to stress involves transcriptional activation and repression at various genomic loci, changes in the chromatin structure play the most active role in this process. Chromatin decondensation involves the action of ATP-dependent remodeling

complexes, covalent modifications of histones, the deposition of histone variants, and/or changes in cytosine methylation. The genome-wide deposition of many epigenetic histone marks is region-specific and has important regulatory functions. In fact, over 18 % of *Arabidopsis* genes contain H3K27me3 in their promoters (Zhang et al. 2007), where it is believed to mediate tissue-specific gene expression patterns. Furthermore, H3K27me3 serves as a binding site for the LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) protein that reinforces transcriptional repression in euchromatin (Libault et al. 2005; Zhang et al. 2007; Turck et al. 2007). This interaction may also be important for the response to environmental changes since LHP1 is involved in the regulation of flowering time triggered by temperature changes.

DNA methylation is probably the best-studied epigenetic mechanism involved in the regulation of gene expression and stress response. DNA methylation is maintained by a highly complex network of molecular mechanisms, which display a great sensitivity to various developmental and environmental cues. DNA methylation can guide the deposition of other epigenetic marks and direct the activity of chromatin-remodeling complexes (Zilberman et al. 2007). Upon stress exposure, transcriptional induction of stress-specific genes frequently correlates with a decrease in DNA methylation at their loci, thus suggesting that the induction of stress-related genes under natural stress conditions may require sequence-specific DNA demethylation (Boyko and Kovalchuk 2008, 2011a). Since hypomethylated loci are more prone to genomic rearrangements (Bassing et al. 2002), it is plausible to suggest that a stress-induced increase in DNA methylation represents a defensive response that limits the occurrence of genome rearrangements under stress conditions. The highly dynamic nature of DNA methylation landscapes is maintained by the presence of several specific DNA glycosylases that remove methylated cytosines from DNA through the process of base excision repair (reviewed in Zhu 2009). Importantly, the ROS1 DNA demethylation pathway could be directed to the specific genome loci using smRNAs bound to the ROS3 protein (Zheng et al. 2008). This suggests an important functional link between smRNA biogenesis, DNA demethylation pathways, and genome stability during response to stress.

DNA methylation plays a key role in restricting transposon movements and preventing genome instability. Transposon activation in response to stress is often associated with a decrease in DNA methylation at transposon loci. The presence of transposons at their neo-insertion sites may exert some transcriptional control over neighboring genes resulting in their transcriptional responsiveness to stress (Ito et al. 2011) and even may cause the appearance of new phenotypes (Hashida et al. 2003, 2006). Noteworthy, changing DNA methylation is not an absolute prerequisite for the transcriptional response to stress. Recently, several experimental studies showed that the activation of a genomic sequence could occur without loss of DNA methylation (Pecinka et al. 2010). Similarly, heat, freezing, and UVB treatments could release transgene silencing only by altering histone occupancy and inducing histone H3 acetylation (Lang-Mladek et al. 2010). These studies suggest an important independent role that plant chromatin may play in the control of gene

expression during stress response. Below, we discuss recent advances in the understanding of chromatin dynamics in plants and changes in smRNA populations upon exposure to stress; their role in plant protection against stress is also discussed.

2 The Role of Histone Modifications in Stress Response

Histone modifications form a layer of epigenetic information that is highly interactive and responsive to developmental and environmental cues. Changing the histone code can mediate local changes in chromatin relaxation or compaction. There are several distinct molecular levels at which epigenetic information can be recorded using histones. As histones form nucleosomes, the exchange of canonical histones with specialized variants can alter transcriptional properties of chromatin (Talbert and Henikoff 2010). Similarly, the ATP-dependent chromatin remodelers can change nucleosome positions by moving the histone core with respect to the DNA sequence, thus allowing an easier access of the general transcriptional machinery to the targeted gene (Rando and Ahmad 2007). Next, numerous post-translational modifications in the N-terminal tails of histones alter their physical properties and change histone–DNA and protein–protein interactions in chromatin (Berger 2007). The most common histone tail modifications include acetylation, methylation, phosphorylation, ubiquitination, biotinylation, and sumoylation. Histone acetylation acts directly by loosening histone association with DNA but also indirectly by recruiting bromodomain-containing proteins leading to transcriptional activation. Histone methylation helps recruit other effector proteins and their complexes and can be associated with either transcriptional repression or activation. For instance, the trimethylated histones H3K4me3 and H3K27me3 are associated with transcriptional activation and silencing, respectively. Whereas trimethylation of H3K4me3 is mediated by the *Arabidopsis* trithorax group (trxG) protein ATX1 complex at the 5′ end of actively transcribed genes, trimethylation of H3K27me3 is mediated by the polycomb repressive complex 2 (PRC2) and leads to transcriptional repression of developmentally important genes and transcription factors (Pien and Grossniklaus 2007). The presence of H3K4me3 can facilitate the recruitment of histone acetyltransferases and chromatin-remodeling complexes leading to a decrease in the nucleosome density at the target loci and an increase in gene expression. Similarly, H3K27me3 serves as a binding site for LHP1 protein that further reinforces transcriptional repression in euchromatin (Fig. 2).

The transcriptional regulation of many stress-responsive genes depends on the activity of histone-modifying enzymes and chromatin-remodeling complexes (Chinnusamy and Zhu 2009). A number of histone deacetylases (HDACs) have been implicated in defense responses against various pathogens. Plant HDACs that belong to reduced potassium dependency protein 3/histone deacetylase 1 (RPD3/HDA1) and HD2 classes can be selectively inhibited by HC toxin of *Cochiobolus* (*Helminthosporium*) *carbonum* leading to histone hyperacetylation in susceptible corn cultivars. In *Arabidopsis*, the AtHDAC19 gene is induced in a similar manner

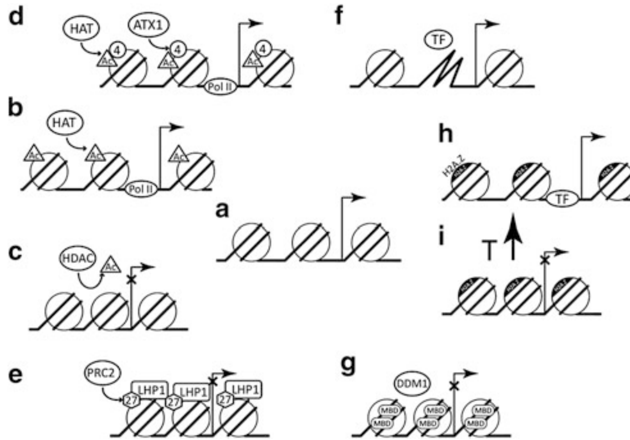

Fig. 2 The common types of structural changes in plant chromatin and their effects on gene expression. In plant cells, DNA is wrapped around a histone protein core forming nucleosomes (**a**) that allow tighter packing of DNA in the nucleus. In addition, chemical modifications of core histone proteins and repositioning of nucleosomes play an important functional role in gene expression control by mediating local changes in chromatin relaxation or compaction. While histone acetylation by HATs loosens histone association with DNA leading to transcriptional activation (**b**), histone deacetylation mediated by HDAC results in transcriptional repression of targeted genes (**c**). In contrast, histone methylation can have both activating and repressive effects as it helps recruit other effector proteins and their complexes. For example, histone H3K4 trimethylation by the ATX1 complex results in transcriptional activation facilitated by the recruitment of HATs and chromatin-remodeling complexes leading to a decrease in nucleosome density at the target loci (**d**). On the contrary, histone H3K27 trimethylation by the PRC2 complex results in gene repression and facilitates LHP1recruitment that further reinforces transcriptional repression in euchromatin (**e**). While the DDM1-mediated binding of MBD proteins to methylated DNA helps reinforce chromatin silencing (**g**), the unloading of histone proteins from nucleosome may have a dual impact on gene expression as it may allow the access of either activating or repressive transcription factors (TF) (**f**). Finally, transcriptional properties of chromatin can be also modified by incorporating various histone variants. The H2A.Z variant of histone H2A is often enriched within genes which expression is altered by high temperature stress (**i**). Due to the thermal instability of H2A.Z-containing nucleosomes, the high temperature loosens the tight wrapping of DNA in a nucleosome. This results in exposure of the gene promoter, thereby facilitating the access of transcriptional activators or repressors (**h**)

by the fungus *Alternaria brassicicola* and exogenous application of jasmonic acid (JA). Overexpression of the AtHDAC19 gene enhances fungal resistance through the apparent activation of the ethylene-responsive factor 1 (ERF1), whereas silencing of the gene increases fungal susceptibility. The HDA19 enzyme might also be involved in *Arabidopsis* resistance to the bacterial pathogen *P. syringae*. The proposed mechanism may involve a decrease of histone acetylation through interactions of HDA19 with WRKY38 and WRKY62 transcription factors. The locus-specific transcriptional suppression of these two WRKY genes results in the activation of the SA-dependent pathway and enhances resistance to bacterial pathogens. Similar to HDA19, the activity of *Arabidopsis* HDA6 that plays an important role in the transcriptional gene silencing pathway can be induced by JA and ethylene (Zhou et al. 2005).

Transcriptional activation of stress-responsive genes can be also mediated by the activity of histone acetyltransferases (HATs) that interact directly with activating transcription factors. Indeed, the histone acetyltransferase HAC1 is required for transcriptional upregulation of the gene encoding heat-shock protein HSP17 (Bharti et al. 2004), and the histone acetyltransferase GCN5 interacts with the CBF1 transcriptional factor that activates cold-responsive gene transcription (Stockinger et al. 2001). Similarly, the light-dependent transcriptional induction of the pea plastocyanin gene correlates with an increase in histone acetylation in the promoter and 5′ gene coding region (Chua et al. 2003). The natural ability to develop fast acclimation responses to UV-B light displayed by maize landraces originating from high-altitude environments requires rapid changes in the expression of a number of UV-B-responsive genes. Here, the transcriptional induction is mediated by significant enrichment in acetylated histones H3 and H4 at gene promoters and transcribed regions, thereby facilitating quick recruitment of transcriptional activators (Casati and Walbot 2008).

In developing seeds, the level of abscisic acid (ABA), another plant hormone and pathogen response factor, is regulated by histone H2B monoubiquitination (Chinnusamy et al. 2008). ABA can induce the expression of genes necessary for seed maturation either directly or through HDAC repression. Overexpression of the AtHD2C gene, a member of the HDAC family that is normally downregulated by ABA, results in the enhanced expression of ABA-responsive genes belonging to the LEA-class and increased tolerance to salinity and drought conditions (Sridha and Wu 2006). In addition, ABA regulates stress-responsive gene expression and stomatal responses through HDACs and HOS15-dependent histone deacetylation, as well as through the ATP-dependent SWItch/Sucrose NonFermenting (SWI/SNF) chromatin-remodeling complexes. The HOS15 protein interacts with histone H4 and is important for H4 deacetylation (Zhu et al. 2008). Moreover, *hos15* mutants are hypersensitive to freezing stress. ABA is also very likely to regulate the abiotic stress response through DNA methylation and siRNA pathways, although this remains to be shown.

Histone methylation, especially methylation of histone H3 at lysines 4 and 9, is an essential component of a gene expression regulatory network. The upregulation of the WRKY70 transcription factor was shown to be associated with H3K4 methylation (Alvarez-Venegas et al. 2007). In *Arabidopsis*, WRKY70 stimulates SA-mediated responses and represses JA-mediated responses. Infection with *P. syringae* results in the accumulation of H3K4me2 and H3K4me3 as well as in the reduction of H3K27me2 levels on WRKY70 nucleosomes, thus leading to the transcriptional induction of the WRKY70 gene. In two other examples, the transcriptional activation of submergence-inducible ADH1 and PDC1genes in rice and drought-responsive genes in *Arabidopsis* is reversibly mediated through histone H3K4 methylation and H3 acetylation (Kim et al. 2008; Tsuji et al. 2006).

The deployment of different histone variants to nucleosomes may serve as another strategy for regulating gene transcription in response to stress. The histone H2A variant H2A.Z was shown to be involved in the temperature stress response in *Arabidopsis* (Kumar and Wigge 2010); it was also associated with the downregulation of the expression of phosphate starvation response genes (Smith et al. 2010) and

genes that mediate systemic acquired resistance in *Arabidopsis* (March-Diaz et al. 2008). At moderately high temperatures, tight wrapping of H2A.Z and the amount of H2A.Z are reduced at the promoter of heat-responsive genes, such as HSP70, allowing an access of transcriptional activators or repressors (Fig. 2). A similar effect was observed in Drosophila where exposure to temperature stress resulted in nucleosome depletion at HSP70 loci. Additionally, in tomato, negative effects caused by drought stress could be alleviated by the deposition of the HIS1-S linker histone variant (Scippa et al. 2004). The overexpression of an active form of the AREB1 transcription factor that positively regulates the *Arabidopsis* homolog of HIS1-3 linker histone resulted in ABA hypersensitivity and increased tolerance to drought in *Arabidopsis* (Fujita et al. 2005).

3 The Role of Chromatin-Remodeling Factors in Stress Response

Another complex level of dynamic epigenetic regulations that mediates an efficient response to developmental cues and environmental factors consists of the redistribution of heterochromatin and euchromatin in the nucleus, nucleosome positioning, differential binding of chromatin-interacting proteins (excluding histones) and methyl-CG-binding domain proteins (MBDs) to DNA. The localization of MBDs at specific nuclear domains is mediated by the decrease in DNA methylation 1 (DDM1) protein (Zemach et al. 2005) and promotes heterochromatin formation and gene silencing (Ben-Porath and Cedar 2001; Zemach and Grafi 2007). DDM1 is a member of the SWI2/SNF2 DNA helicase family that is involved in the control of DNA repair, recombination, gene expression, and replication (Havas et al. 2001). Consistent with the role of DDM1 in mediating cross talks between DNA methylation and histone and chromatin modifications, *ddm1* mutant plants exhibit a genome-wide loss of DNA CG methylation (Jeddeloh et al. 1999) and a decrease in H3K9 methylation (Gendrel et al. 2002). Chromatin condensation plays a critical role in the maintenance of transcriptional gene silencing at repetitive elements. At the same time, a number of constitutively expressed genes contain nucleosome-depleted regions in their promoters (Zhang et al. 2007). The removal of nucleosomes from specific genomic locations in response to stress can be both an active and a passive process. The fact that the original nucleosome loading and epigenetic regulation of repetitive elements are restored fairly quickly upon the recovery from stress supports the active nature of this mechanism. Alternatively, a passive loss of nucleosomes at specific genomic locations could still occur due to DNA replication and transcription.

A recent study by Pecinka et al. (2010) showed that long-term exposure of *Arabidopsis* to heat resulted in the activation of some repetitive elements (Pecinka et al. 2010). Surprisingly, the activation occurred without loss of DNA methylation and with only minor changes to histone modifications. Repetitive elements were

primarily activated by the loss of nucleosomes and heterochromatin decondensation. The recovery from stress was characterized by nucleosome loading and transcriptional silencing of these repeats. Interestingly, plants deficient in chromatin assembly factor 1 (CAF-1) with impaired chromatin assembly functions displayed a considerably delayed recovery stage and nucleosome loading. Also, a substantial dissociation of heterochromatin was observed beyond the recovery phase when silencing and nucleosomes had been reinstalled. The loss of heterochromatin was detected in differentiated tissues of plants exposed to heat, and it lasted in exposed leaves until they showed signs of senescence. Heat-induced decondensation of chromocenters and the general loss of nucleosomes could allow for a better accessibility of DNA to transcription complexes. Indeed, several chromatin-remodeling proteins were shown to mediate UV-B acclimation in natural maize populations grown at high altitudes (Casati et al. 2006).

A similar heterochromatin decondensation was observed in 2-day-old *Arabidopsis* seedlings in response to dedifferentiation in cell culture, although regular chromocenters were still formed in a stepwise process after a longer culture period (Tessadori et al. 2009). Though being insufficient for repeat activation, the loss of heterochromatin also occurred in older plants upon floral transition during their development. Therefore, local changes in heterochromatin condensation not only represent a normal physiological and developmental process but also function as an (un)specific response to stress. Indeed, if plants were exposed to low-light stress, heterochromatin decondensation was more permanent and directed toward genome regions containing repetitive elements (Tessadori et al. 2009). The reversibility of these changes was confirmed by prolonged culturing of plants exposed to low-intensity light; at higher light intensity, the previously observed heterochromatin decondensation was eliminated. One way to interpret these data is that heterochromatin decondensation at genomic repeats can be a common response to stress in plants. However, Pecinka et al. (2010) argue that it does not seem to be the case as the authors did not observe the heterochromatin decondensation phenotype after freezing (−4°C for 24 h) and UV-C irradiation (3,000 J/m^2) stresses (Pecinka et al. 2010). In fact, exposure to abiotic stress may interfere with the capacity of plants to withstand biotic stress. Even moderately increased temperatures can reduce resistance to pathogen stress. In this case, exposure to long-term heat stress results in the activation of some repetitive elements followed by transcriptional repression and silencing of repetitive loci carrying clusters of resistance genes (Pecinka et al. 2010).

Heterochromatin decondensation in response to heat stress seems not to occur equally in all plant tissues as nuclei of meristematic cells do not undergo heat-induced decondensation. These findings may indeed reflect a naturally occurring event since heat stress responses are usually transient in nature and largely localized in somatic tissues only. This indicates the existence of some sort of a safeguarding mechanism for minimizing epigenetic and possibly genetic changes in the meristem cells and restricting the stress-induced transcriptional activation of heterochromatin-embedded genes to somatic cells only. It lends further support to the hypothesis that heterochromatin decondensation is a controlled process that occurs either during specific stages of plant development or in response to specific stresses such as heat and high light intensity stress.

The transcriptional activation of repeats that occurs without changes in DNA methylation resembles the situation previously observed in plants deficient in the activity of various chromatin modifiers, such as DDM1, MOM1, FAS1, FAS2, BRU1, and RPA2, which all exhibit various degrees of activation of repetitive elements. *ddm1* mutants display higher sensitivity to UV-C and gamma radiation as compared to wild-type or *met1* mutant plants (Shaked et al. 2006). Consistent with a role of DDM1 in the control of the distribution of MBD proteins, RNAi knockout of MBD101 results in a hypersensitive response to UV-B exposure in *Zea mays* (Casati and Walbot 2008). Similarly, *bru1* mutants are hypersensitive to genotoxic stresses and display an increased frequency of intrachromosomal homologous recombination (HR) (Takeda et al. 2004). The expression of another gene, MIM1, involved in the structural maintenance of chromosomes and required for efficient HR is significantly increased by DNA-damaging treatments (Hanin et al. 2000). Interestingly, changes in the expression level of a single chromatin-remodeling factor may have a significant genome-wide impact on the expression of other chromatin proteins. RNAi mutant *Zea mays* plants deficient in a chromatin-remodeling complex protein CHC101 display extensive changes in transcription of other chromatin proteins (Casati and Walbot 2008). Chromatin-remodeling factors also play a role in de novo DNA methylation mediated by the RNA-dependent DNA methylation (RdDM) pathway. A plant-specific SNF2-like chromatin-remodeling factor defective in RNA-directed DNA methylation 1 (DRD1) is an important component of the DRD complex which, together with Pol V, mediates the amplification and reinforcement of siRNA production and de novo DNA methylation at the siRNA targeted site (Simon and Meyers 2011). Both *polV* and *drd1* mutants display decondensation of pericentromeric repeats and depletion of the repressive H3K9me2 mark at centromeres, which suggests their contribution to the recruitment of histone-modifying complexes to chromatin (Pontes et al. 2009).

4 Small Noncoding RNAs and Regulation of Genome Stability

In the recent years, ample progress has been made in understanding the role of smRNAs in the establishment and maintenance of epigenetic landscapes throughout the genome and in controlling the processes of transcription and translation. Small RNAs are *trans*-acting molecules that can reversibly modify gene expression in a sequence-specific manner at transcriptional (siRNAs and the RdDM pathway) and posttranscriptional (miRNAs) levels (Carthew and Sontheimer 2009; Malone and Hannon 2009; Voinnet 2009). Small RNAs are highly sensitive to developmental and environmental cues and can influence DNA methylation and distribution of histone modifications as well as facilitate the recruitment of chromatin modifiers to their genome targets (Bourc'his and Voinnet 2010; Hammoud et al. 2009; Khraiwesh et al. 2010; Saze 2008; Wu et al. 2010).

Four main smRNA groups can be distinguished based on their biogenesis pathways, structure, and biological functions (Vazquez 2006). These include the following: miRNAs, *trans*-acting short-interfering RNAs (ta-siRNAs), natural-antisense siRNAs (nat-siRNAs), and repeat-associated siRNAs (ra-siRNAs). Details of the biogenesis of these noncoding RNAs and their mode of action are covered by Martínez de Alba et al. in the chapter "Small RNA-Mediated Control of Development in Plants."

Short-interfering RNAs guard genome integrity by the dynamic control of numerous transposons dispersed throughout the plant genome. It is not surprising that there is a strong correlation between siRNAs and DNA methylation. The balanced activity of siRNA-directed DNA methylation and ROS1 DNA demethylation pathways may be required to reversibly modulate gene expression in nondividing cells (Lister et al. 2008; Penterman et al. 2007). DNA demethylation pathways are necessary to maintain a proper composition of smRNA populations. Indeed, triple *ros1dml2dml3* mutants display an altered composition of smRNA populations due to de novo methylation of previously active DNA regions located in the proximity of ta-siRNA-generating loci (Lister et al. 2008).

The hypersensitivity of siRNA biogenesis mutants to genotoxic stress (Yao et al. 2010) supports their contribution toward the maintenance of genome stability. Despite these findings, the overall data about the involvement of these molecules in stress response are still rather limited. Moreover, no information about direct regulation of the genome stability by noncoding RNAs exists. It is also unknown whether specific miRNAs can target genes involved in DNA repair.

The significance of siRNAs in genome control is hard to underestimate. The RdDM pathway is an important component of the gene regulatory network that uses siRNA-derived signals to modify transcription of target genes and control the mobility of plant transposons. In fact, at least one third of all methylated loci in the *Arabidopsis* genome is controlled by the siRNA pathway (Lister et al. 2008). A recent study by Zheng et al. (2008) suggested that siRNAs could also direct sequence-specific DNA demethylation through the ROS1 pathway (Zheng et al. 2008). A strong correlation between siRNAs and DNA methylation is not surprising. The balanced activity of siRNA-directed DNA methylation and ROS1 DNA demethylation pathways may be required to reversibly modulate gene expression in nondividing cells in response to stress (Lister et al. 2008; Penterman et al. 2007). A broad spectrum of siRNAs originating from transposons can target various stress-tolerance genes (Hilbricht et al. 2008). Furthermore, due to siRNA mobility (Chitwood et al. 2009; Dunoyer et al. 2010; Molnar et al. 2010), transposon-derived siRNAs may regulate gene expression in distant non-effected plant organs, thus mediating a systemic response to stress and possibly acclimation.

Recent studies showed that siRNAs can act as mobile signals and effect transposon and DNA methylation in distant tissues (Chitwood et al. 2009; Dunoyer et al. 2010; Molnar et al. 2010). These observations add an additional degree of complexity to the system of epigenetically mediated transcriptional control and may be a key component in understanding the molecular mechanisms behind stress-induced systemic responses, including systemic acquired resistance and

virus-induced gene silencing. Moreover, siRNAs may provide a plausible mechanism for recording the memories of environmental conditions and mediate the transgenerational epigenetic inheritance of gene expression patterns associated with these memories. By using grafting experiments with *dcl2*, *dcl3*, and *dcl4* mutants, Molnar et al. (2010) demonstrated that mobile siRNAs could indeed direct DNA methylation in recipient cells (Molnar et al. 2010). These important findings were supported by Dunoyer et al. (2010) who, by using labeled siRNA duplexes, demonstrated cell-to-cell movement of siRNA duplexes (Dunoyer et al. 2010). Together, these two studies confirmed the role of siRNAs as mobile silencing signals between plant cells. The function of siRNAs as mobile silencing signals is not restricted to somatic cells. The activation of transposons and production of 24-nt siRNAs in the pollen vegetative nucleus and in the central cell during female gametogenesis may serve to reinforce transposon silencing in the sperm, egg cells, and developing embryo (Hsieh et al. 2009; Mosher et al. 2009; Slotkin et al. 2009).

5 Conclusion

Being sedentary in nature, plants constantly interact with the environment. Since the environment continuously undergoes dynamic changes, plants possess the molecular mechanisms that permit their quick adaptability. The latter is achieved by using a wide array of epigenetic regulations that allow rapid and reversible changes to the existing transcriptional and metabolic cellular profiles, thus creating a molecular basis for fast acclimation responses to stress. Dynamic changes in plant chromatin allow rapid modifications of gene expression by regulating the accessibility of the gene promoters to various transcription factors. The transcriptional activation of many stress-related genes requires changes in histones and nucleosome occupancy and/or a decrease in DNA methylation levels. Furthermore, the gene expression output is fine-tuned by a large variety of stress-inducible smRNAs that may affect gene expression at both transcriptional and posttranscriptional levels. Importantly, transcription-permissive epigenetic changes to DNA, histone proteins, and chromatin may also attract DNA damaging agents, thus delivering an additional challenge to the DNA repair machinery. In contrast, the deposition of particular epigenetic marks that make genomic loci inaccessible to transcription factors and the DNA repair machinery could play a critical role in preserving genome integrity if exposed to stress. Indeed, multiple correlations exist between the deposition of epigenetic marks, the accessibility of DNA to transcription factors, and the DNA repair machinery. Thus, it is plausible to believe that epigenetic pathways not only regulate gene expression but also maintain genome integrity and stability during the response to stress. It is a challenge for future studies to unravel these links and provide a better understanding of how stress-induced changes in plant chromatin and smRNA populations could protect the plant genome and mediate adaptations to nature.

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Epigenetic Control of Plant Immunity

Yusuke Saijo and Eva-Maria Reimer-Michalski

Abstract Plant immunity relies on two cell autonomous immune pathways present in each cell and on systemic signals emanating from local challenged sites, which enhance immunity in distal unchallenged cells. Activation of these different immune branches entails extensive transcriptional reprogramming of a largely common set of defense-related genes, leading to the termination or restriction of pathogen propagation at the cost of plant growth. Emerging evidence points to a role of chromatin remodeling and dynamics as a key mechanistic basis for timely and appropriate activation of immune response in plants. One such phenomenon that appears to be under epigenetic control involves defense priming that is conditioned upon immune activation or interactions with beneficial microbes. In defense priming, target defense-related genes are not actively transcribed but poised for a greater and/or faster activation upon second stimulation. Moreover, a growing list of nuclear-localized pathogen effectors also implies their possible role in the alteration of host chromatin configuration for virulence promotion. Epigenetic control of defense-related genes seems to represent an as-yet-underexplored interface during plant–pathogen interactions.

1 Introduction

Plants as sessile organisms cope with a wide range of microbes in an environment, including infectious pathogens that can cause disease. In addition to constitutive physical and biochemical defense barriers, plants have evolved an elaborate multi-layered innate immune system to resist the majority of pathogenic microbes. Based on the feeding lifestyles, plant pathogens are largely classified into three classes:

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biotrophic pathogens feed on living plant cells, necrotrophic pathogens actively destroy and kill host cells to obtain nutrients, and hemi-biotrophic pathogens switch their feeding styles between the two and require living host cells during part of their life cycle (Glazebrook 2005). Plants selectively activate appropriate immune response according to the infection styles of the pathogens encountered, which is achieved at the cost of growth-related physiological processes. In addition, tradeoffs exist between different immune branches, in which the activation of one branch negatively influences another branch. This also comes at fitness costs beyond the direct energy costs required for defense execution. Critical components of plant immunity, in particular in the interactions with biotrophic and hemi-biotrophic pathogens, include two classes of immune receptors that detect nonself molecules or altered host cellular states upon pathogen challenges. Immune receptors, upon the recognition of their specific ligands, trigger a set of cellular outputs including extensive transcriptional reprogramming during immune activation. This signaling process is influenced and fine-tuned by a network of phytohormones that are also engaged in the adaptation to different abiotic stresses in the environment, thereby allowing plants to coordinate between different stress responses and growth. At present, it is thought that all living plant cells possess these immune components and, thus, the ability to detect and react to pathogens (Jones and Dangl 2006).

One receptor class consists of the so-called pattern recognition receptors (PRRs) that detect molecular structures typically conserved in many microbial species, designated microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs). MAMPs include bacterial flagellin, the elongation factor EF-Tu, lipopolysaccharides (LPS), peptidoglycans, and components of fungal cell walls such as chitin fragments (*N*-acetyl-chitooligosaccharide oligomers) (Boller and Felix 2009; Segonzac and Zipfel 2011). MAMP perception by cognate PRRs triggers immune response that restricts the invasion and/or multiplication of potential infectious microbes, termed MAMP-triggered immunity (MTI), which provides a first line of inducible basal defenses against pathogens (Boller and Felix 2009; Segonzac and Zipfel 2011). MTI activation is accompanied by a stereotypic set of defense-associated cellular outputs, such as changes of ion fluxes across the membranes, production of reactive oxygen species (ROS) into extracellular apoplastic spaces, MAPK activation, ethylene production, callose deposition, but also extensive transcriptional reprogramming and metabolic changes. Loss of single PRRs renders plants more susceptible to adapted and non-adapted pathogens, providing evidence for the significance of MTI in plant immunity (Segonzac and Zipfel 2011).

For successful infection, adapted pathogens avoid and/or suppress MTI activation with a series of effectors injected into the host, which promote virulence in the absence of their immune recognition. As a strategy to counteract them, plants evolved a second class of intracellular immune receptors, termed the disease resistance (R) proteins, that detect the structure or actions of cognate pathogen effectors encoded by avirulence (*AVR*) genes that are typically isolate specific. Nucleotide-binding leucine-rich repeat (NB-LRR) proteins represent the major class of R proteins. NB-LRR receptors are classified into two subclasses defined

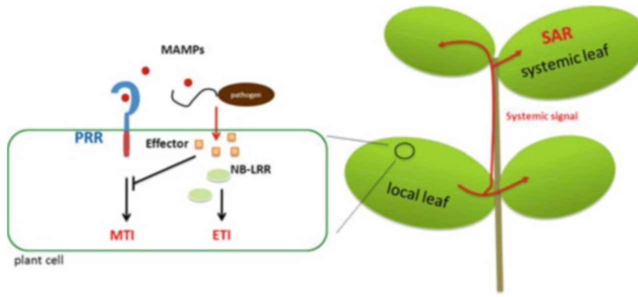

Fig. 1 Different immune branches in plant immunity. A first layer of inducible defenses is mounted by pattern recognition-receptors (PRR) at the membranes, upon the recognition of microbe-associated molecular patterns (MAMPs), termed MAMP-triggered immunity (MTI). Successful pathogens overcome MTI by evading PRR recognition and/or by secreting effectors into the cell that suppress MTI. Plants have evolved R proteins, of which the dominant class is represented by nucleotide-binding domain Leu-rich repeat (NB-LRR) proteins. Direct or indirect recognition of effectors leads to effector-triggered immunity (ETI). MTI and ETI activation both trigger the release of a systemic signal, which in turn leads to systemic acquired resistance (SAR)

by their N-terminal domains, namely, Toll/Interleukin-1 Receptor (TIR) and coiled-coil (CC) domains. Effector recognition by R proteins triggers more dramatic immune response than MTI, designated effector-triggered immunity (ETI) (Fig. 1). ETI typically culminates in hypersensitive response (HR), a host cell death at attempted challenge sites (Chisholm et al. 2006; Jones and Dangl 2006). Like MTI, ETI also entails extensive transcriptional reprogramming of a largely overlapping set of defense-related genes. However, of note, these target genes in general undergo faster, greater, and/or more prolonged expression during ETI than during MTI (Tao et al. 2003; Caldo et al. 2004). The differences of transcriptional outputs between MTI and ETI in the amplitude and kinetics rather than in target genes per se lead to the notion that signaling events and outputs (e.g., transcriptional reprogramming) of MTI are accelerated by R protein-triggered signaling during ETI (Tao et al. 2003). However, the mechanistic basis for the differences remains almost unknown. In this respect, it should be noted that immune function of a subset of NB-LRR receptors requires their direct actions in the nucleus (Shen et al. 2007; Garcia and Parker 2009), although not all NB-LRR receptors function in the nucleus, which suggests a close functional link between their triggered ETI signaling and the nuclear machineries engaged in the control of gene expression (Shen et al. 2007; Garcia and Parker 2009). It is therefore conceivable that differential nuclear events underlie the aforementioned differences in the mode of transcriptional reprogramming between MTI and ETI.

Both MTI and ETI at local challenged sites trigger the release of a systemic signal (s), of which the identity remains elusive or controversial, to induce an enhanced state of cellular immunity at distal non-challenged sites, designated systemic acquired resistance (SAR) (Dempsey and Klessig 2012). SAR is long lasting, occasionally even for the lifetime of the plant, and effective against secondary

infection by a broad range of pathogens (Durrant and Dong 2004). SAR is characterized by, e.g., accumulation of the defense-related phytohormone salicylic acid (SA) and the increased expression of a number of *pathogen-related* (*PR*) genes, encoding defense-related proteins such as antimicrobial peptides (*Arabidopsis thaliana PR-1*). As a master regulator for SA-based immunity and SAR, NON-EXPRESSOR OF PR GENES1 (NPR1) has been identified (Durrant and Dong 2004). Upon defense elicitation, NPR1 undergoes cellular redox state-dependent oligomer disassembly that is followed by its translocation to the nucleus, thereby interacting with members of the TGA family of basic Leu-zipper-type transcription factors (TFs) in the control of defense gene expression (Dong 2004; Durrant and Dong 2004). This represents a key mechanism that couples SA/SAR signaling with extensive transcriptional reprogramming. Moreover, as a possible basis for the long-lasting nature of SAR, subsets of defense-related genes are primed, rather than activated, in systemic unchallenged sites. The so-called defense priming holds target genes in an inactive or transiently active state but poised for faster and/or greater activation upon a subsequent pathogen attack (Conrath 2011). However, it remains elusive whether, and if so, how the aforementioned differences between MTI and ETI in transcriptional reprogramming at directly challenged sites influence the extent of SAR and/or of priming response in distal non-challenged sites.

In this chapter, we consider the potential epigenetic basis underlying transcriptional reprogramming during and after immune response, with a particular focus on the role of dynamic changes in chromatin configuration. We highlight recent studies that point to the role of chromatin-level control in the establishment and maintenance of transcription-repressive or -permissive states for defense-related genes. For the role of non-coding RNA or RNA quality control in transcriptional reprogramming, please refer to recent reviews on the topic in plants (Kanno and Habu 2011; Yaish et al. 2011; Naqvi et al. 2012).

2 Integration of Immune Receptor-Triggered Signaling with Gene Expression in the Nucleus

Protein phosphorylation cascades seem to couple signal inputs, whether upon extracellular recognition of MAMPs (MTI) or intracellular recognition of specific effectors (ETI), to gene transcription machineries in the nucleus (Tena et al. 2011). In mammals, direct outputs of MAPK signaling activated upon diverse stimuli involve histone H3 phosphorylation to condition subsequent transcriptional reprogramming (Clayton and Mahadevan 2003). In yeast, the MAPK Hog1 interacts with the Swi/Snf chromatin-remodeling complex REMODELS STRUCTURE of CHROMATIN (RSC), which then induces its recruitment to stress-responsive promoters (Mas et al. 2009). In *Arabidopsis*, phosphorylation activity of histone H3 and histone variant H2A.Z has been described for MPK3 and MPK6, two of major MAPKs activated in response to diverse biotic and abiotic stresses

(Feilner et al. 2005). Therefore, it is plausible that MAPKs provide a direct link between immune receptor-triggered signaling and chromatin configuration changes during immune response in plants as well.

Another key basis for signal integration in the nucleus seems to be provided by nucleocytoplasmic trafficking of defense signaling components and TFs including also a subclass of NB-LRR immune receptors per se (Meier and Somers 2011). Several NB-LRR receptors that require nuclear localization for their immune function include the tobacco TIR-NB-LRR receptor N, the *Arabidopsis* TIR-NB-LRR receptor RPS4, and the barley CC-NB-LRR receptor MLA that confer resistance to tobacco mosaic virus, the phytopathogenic bacterium *Pseudomonas syringae* expressing the type III secretion (T3S) effector AvrRps4, and the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* expressing cognate AvrMLA effectors, respectively (Burch-Smith and Dinesh-Kumar 2007; Shen et al. 2007; Garcia et al. 2010). Only a small portion of these NB-LRR receptor pools is localized in the nucleus, yet it plays an essential role for mounting ETI, since their enforced nuclear exclusion disables their immune function (Burch-Smith and Dinesh-Kumar 2007; Shen et al. 2007; Garcia et al. 2010). A critical nuclear action of these NB-LRR receptors involves physical interaction with DNA-binding TFs that regulate immune response, although the precise biochemical outcome of their interactions remains unclear to date (Burch-Smith and Dinesh-Kumar 2007; Shen et al. 2007).

The *Arabidopsis* EDS1 defines an essential non-receptor component for TIR-NB-LRR receptor-conditioned ETI. EDS1 acts as part of protein complexes with the basal defense regulators PAD4 and SAG101 (Wiermer et al. 2005), but again the precise biochemical function of the EDS1 complex(es) remains unclear. In addition, EDS1 interacts with the NB-LRR receptors RPS4, RPS6, and SNC1, but also with the phytopathogenic bacterium *Pseudomonas syringae* effector AvrRPS4 (Bhattacharjee et al. 2011; Heidrich et al. 2011). EDS1 shuttles between the cytoplasm and nucleus, with a small pool localized in the nucleus. Again, this small nuclear pool of EDS1, together with nuclear localization of RPS4 and AvrRPS4, is required for transcriptional reprogramming and ETI to bacterial infection that are conferred by RPS4 (Bhattacharjee et al. 2011; Heidrich et al. 2011). This reinforces the notion that critical events of ETI signaling for defense execution take place within the nucleus, and further implies that perturbations of host nuclear processes by pathogen effectors are monitored by NB-LRR receptors.

In line with this, genetic studies in *Arabidopsis* have revealed the genetic requirements for the components of the nuclear pore complexes in pathogen resistance, including *MODIFIER OF SNC1 6* (*MOS6*) encoding importin α3, and *MOS3* and *MOS7*, respectively, encoding homologs of the nucleoporin Nup96 and Nup88. MOS7 is required for proper nuclear accumulation of SNC1, EDS1, and NPR1 (Cheng et al. 2009). This further argues for the functional significance of the access of immune regulators to the nucleus and gene transcription machineries (Garcia and Parker 2009).

Of note, the aforementioned signaling from the membrane/cytoplasm to nucleus and nuclear processes is under the influence of a complex network of defense-related phytohormones. In general, salicylic acid (SA)-dependent defenses are

effective against biotrophic and hemi-biotrophic pathogens, while jasmonic acid (JA) signaling together with ethylene (ET) confers effective defenses against necrotrophic pathogens and insect herbivores. These phytohormones also contribute to plant adaptation to different abiotic stress cues in a fluctuating environment. The outcome of these phytohormone interactions differs in a context-dependent manner, providing a basis for fine-tuning of immune response according to the type of pathogens encountered and the prevailing environmental conditions (Glazebrook 2005; Spoel and Dong 2008; Robert-Seilaniantz et al. 2011; Pieterse et al. 2012). Together, all these aspects of immune response predict the need for the mechanisms that can rapidly and flexibly reprogram the expression of large sets of genes at once.

3 Chromatin Remodeling and Histone Replacement in Plant Immunity

One effective way to meet such requirements in transcriptional reprogramming can be achieved through changes of chromatin configuration in eukaryotic cells. The smallest packaging unit of chromatin is termed nucleosome that consists of two copies of histone H2A, H2B, H3, and H4 wrapped by approximately 147 bp of DNA (Zhang and Reinberg 2001). The structure and function of chromatin is regulated by multiple mechanisms, including DNA methylation, ATP-dependent chromatin remodeling, replacement of histone variants, and posttranslational histone modifications such as methylation, acetylation and ubiquitination. Several of the above mechanisms have been implicated in the modulation of immune response in plants (Alvarez et al. 2010; Ma et al. 2011; Berr et al. 2012).

Replacement of histone H2A.Z with canonical histone H2A occurs through the action of a multi-subunit complex termed SWR1 in yeast and SRCAP in humans (Krogan et al. 2003; Mizuguchi et al. 2004; Cai et al. 2005). H2A.Z is typically found in the nucleosomes flanking the transcription start sites (Zilberman et al. 2008). In *Arabidopsis*, disruptions of a SWR1-like complex (containing PIE1) and of two of the three histone variant H2A.Z-coding genes (*HTA9* and *HTA11*) cause in non-elicited plants transcriptional upregulation of SA-responsive SAR marker genes, spontaneous cell death, and enhanced immunity to bacterial infection (March-Diaz et al. 2008). These findings point to a role of H2A.Z deposition in the establishment and/or maintenance of transcription-repressive chromatin configuration on the target SA regulons. This might provide means by which plants avoid detrimental precocious activation of immune response in the absence of pathogens.

Genetic evidence also points to a role of several components of ATP-dependent chromatin-remodeling complexes in the repression or attenuation of these SA regulons and SA-based immunity to pathogens. These complexes contain the catalytic SUCROSE NONFERMENTING2 (SNF2) ATPase subunit. Out of the 42 SNF2 ATPase family members annotated in the *Arabidopsis* genome, loss of the following members results in enhanced expression of SA-responsive genes and/or enhanced

basal immunity response to biotrophic or hemi-biotrophic pathogens: SPLAYED (SYD) and BRAHMA (BRM) of the SNF2 subfamily, PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1 (PIE1) of the SWI/SNF-RELATED1 (SWR1) subfamily, and DECREASED DNA METHYLATION1 (DDM1) of LSH subfamily (see below).

Upon bacterial challenge of *syd* mutant plants as well as in non-elicited *brm* plants, hyper-activation of SA-responsive genes including *PR1* has been observed (Bezhani et al. 2007; Walley et al. 2008). The phenotypic differences between the two mutants might reflect that SYD and BRM have a partially overlapping function, but yet a distinct set of target genes (Bezhani et al. 2007; Walley et al. 2008). It should be also noted that the upregulation of SA regulons is accompanied by reduced expression of JA/ET-inducible genes in *syd* plants, suggesting that the SA–JA antagonism also contributes to the observed alterations of transcriptional reprogramming in the mutant. In addition, direct SYD recruitment was selectively detected in the promoters of some of the affected JA/ET-responsive genes, i.e., *VSP2* and *MYC2* but not *PDF1.2a*. These results suggest that most of the observed mutational effects might be indirect (Walley et al. 2008). To date, the precise mechanisms by which SYD and BRM regulates defense-related genes still remain largely unknown. The presence of a bromodomain in BRM1 and ability to bind histones in vitro implies its potential interactions with acetylated histones (Farrona et al. 2007). Future studies will be needed to clarify the above suggested models.

DDM1 is required to maintain DNA methylation along the genome, although there is no proof for its direct DNA methyltransferase activity (Jeddeloh et al. 1999). Various genetic and epigenetic alterations accumulate in the progeny of hypomethylated *ddm1* plants, causing the so-called *bal* effects that are characterized by dwarfism, curled leaves, and enhanced disease resistance that are dependent on EDS1. This is accompanied by derepression of several NB-LRR receptor-encoding genes from the *RPP5* locus, of which that of *SNC1* is responsible for the *bal* effects (Yi and Richards 2007, 2009). The *RPP5* locus includes *SNC1*, which has been originally identified through mutagenic suppressor screens for *npr1* mutant that is defective in SA-based immunity. The gain-of-function *snc1* allele rescues the SA signaling defects of the *npr1* mutant (Li et al. 2001; Zhang et al. 2003). In the absence of DDM1, duplication of a 55-kb region occurred between several clustered NB-LRR-encoding genes within the *RPP5* locus, which increases the copy number and thus expression levels of *SNC1*. (Yi and Richards 2009). Comparative genome and phylogenetic studies suggest that many of *NB-LRR* genes were generated as a consequence of tandem gene duplication events (Baumgarten et al. 2003; Meyers et al. 2003). DDM1 might serve to prevent recombination between repeat sequences from the *RPP5* cluster and thus to maintain genomic stability. This might allow plants to accommodate highly related but slightly variant repeat sequences in a cluster of homologous genes, while avoiding their mis-expression that can be detrimental to the plant. This might also serve sources for the evolution of sequence-related immune receptor-coding genes.

A separate study has revealed that MOS1, a large protein of an evolutionarily conserved BAT2 domain, can antagonize DDM1 function thus promoting *Snc1* expression (Li et al. 2010). In *mos1* loss-of-function mutant plants, *Snc1* expression

is lost and also its associated effects, i.e., constitutive autoimmunity activation, are lost. However, the expression of *Snc1* is de-repressed in *mos1* plants upon the disruption of DDM1. Of note, rather reduced DNA methylation levels were observed along the *Snc1* promoter in *mos1* plants despite the fact that *Snc1* expression was repressed. This result together with the insufficiency of *ddm1* mutation alone to enhance *Snc1* transcript levels (Li et al. 2010), points to the complex nature of controlling the expression of the *NB-LRR* gene.

Recent genome-wide DNA methylation profiling of *Arabidopsis* plants exposed to bacterial pathogens has revealed that differentially methylated cytosines (DmCs) were enriched in gene-rich but depleted in gene-poor regions along the genome, suggesting a role of these methylation changes in transcriptional control (Dowen et al. 2012). Interestingly, although CG and CHG (where H is A, C, or T) methylations were similarly altered in response to SA and avirulent (ETI triggering), or virulent *Pseudomonas syringae* strains, the changes of CHH methylation levels were unique to the infection of the virulent bacterial strain among the tested stimuli, implying that differential DNA methylation patterns are associated with effective or noneffective immune response. Consistent with this, subsets of defense-related genes are mis-expressed and antibacterial immunity is enhanced in *met1-3* and *drm1 drm2 cmt3* mutant plants that are globally defective in maintenance of CG methylation or non-CG methylation, respectively (Dowen et al. 2012).

4 Histone Modifications During Plant Immune Response

Recent studies have uncovered an edge of dynamic changes of histone modifications during immune response and presented genetic evidence for a role taken by several histone modifiers and remodelers important for plant immunity. In general, histone modifications associated with active (transcription-permissive) chromatin include histone H3 that is mono-, di-, or tri-methylated on Lys-4 (H3K4me1, H3K4me2, or H3K4me3, respectively), H3K36me3, or acetylated H3 and H4 (H3Ac and H4Ac, respectively), and those typical of silent (transcription-repressive) chromatin include H3K9me1, H3K9me2, H3K9me3, H3K27me1, H3K27me2, or H3K27me3 (Fuchs et al. 2006; Kouzarides 2007; Pfluger and Wagner 2007; Roudier et al. 2009). In *Arabidopsis*, epigenome mapping studies with a focus on 11 histone modifications (H3K4me2, H3K4me3, H3K9me2, H3K9me3, H3K27me1, H3K27me2, H3K27me3, H3K36me3, H3K56ac, H4K20me1 and H2B ubiquitination) and DNA methylation have revealed that four different combinations cover ~90 % of the genome under non-stress conditions (Roudier et al. 2011). It seems likely that different combinations/patterns of histone modifications differentially influence chromatin structure and transcriptional competence of the target loci. The functional outcomes (whether permissive or repressive for gene transcription) of histone modification patterns can also vary according to the positions of these modifications with respect to the gene structure and the genomic context (Fuchs et al. 2006; Kouzarides 2007; Pfluger and Wagner 2007; Roudier et al. 2009).

All three H3K4me marks occur almost exclusively on gene coding sequences and are associated with active chromatin (Zhang et al. 2009). Increased H3K4 methylation, together with H3K9- and H3K14-acetylation, was detected at the *PR1* locus in non-elicited *sni1* mutant plants (Mosher et al. 2006). This seems to in part account for the recovery of *PR1* expression by the *sni1* mutation despite the absence of NPR1 (Li et al. 1999). An elevation of these histone H3 modifications also occurs in wild-type plants 48 h upon the application of the SA analogue benzo(1,2,3) thiadiazole-7-carbonic acid S-methyl ester (BTH). These findings indicate that the nuclear protein SNI1 antagonizes NPR1 function as a repressor of these histone modifications and thus of *PR* gene expression in SA-based immunity (Li et al. 1999). However, of note, another independent work fails to detect such an increase of H4K4me3 in the *PR1* locus within 24 h after SA application (Alvarez-Venegas et al. 2007). This leads to a notion that active *PR1* transcription is followed by the elevation of H3K4me3 and H3Ac levels, which in turn contributes to keep the *PR1* chromatin in an active state. Thus, H3K4me3 and H3Ac might be associated with the establishment of a memory for the expression of defense-related genes (see below).

The major subclass of Lys-specific histone methyltransferase (HMTase) is SET (Su[var]3-9, Enhancer of Zeste, Trithorax) domain-containing enzymes, which catalyze mono- (me1), di- (me2), and/or trimethylation (me3) of different Lys residues on histone H3 and/or H4 (Hennig and Derkacheva 2009). For not all but some of *Arabidopsis* SET domain HMTase members tested, loss of their function results in alterations of immune response, pointing to their selective assignments to the modulation of plant immunity.

The Polycomb group (PcG) protein complex Polycomb Repressive Complex2 (PRC2) mediates H3K27me3 and thus sustains a transcription-repressive state of chromatin (Margueron and Reinberg 2011). The four core PcG subunits of PRC2 are defined by E(z), Su(z)12, Esc, and p55 in *Drosophila*. In *Arabidopsis*, homologs for these PRC2 components exist: the SET domain-containing E(z) homologs MEDEA, CURLY LEAF (CLF), and SWINGER (SWN); Su(z)12 homologs EMBRYONIC FLOWER (EMF), FERTILIZATION INDEPENDENT SEED2 (FIS2), and VERNALIZATION2 (VRN2); Esc homolog FERTILIZATION INDEPENDENT ENDOSPERM (FIE); p55 homologs MULTICOPY SUPPRESSOR OF IRA 1–5 (MSI1–MSI5). Although their catalytic activity has not been demonstrated, genetic evidence points to their role as the determinants for H3K27me3 levels in *Arabidopsis* (Liu et al. 2010; Jeong et al. 2011). Genetic evidence also points to pleiotropic roles of PcG proteins throughout the plant life cycle, including gametogenesis, fertilization, seed development, vegetative development, floral transition, and flower organogenesis (Kohler and Aichinger 2010; Butenko and Ohad 2011; Holec and Berger 2012). Genome-wide chromatin co-immunoprecipitation (ChIP) analysis revealed that approximately 4,400 genes (~18 %) are positive with H3K27me3 in non-stressed seedlings, suggesting the global impact of this histone mark in the control of gene expression in *Arabidopsis* (Zhang et al. 2007; Pontvianne et al. 2010). However, to date, the functional significance of PcG proteins has not been vigorously tested in plant immunity.

In *Drosophila*, as opposed to PRC2 function, Trithorax group (TrxG) proteins confer positive effects on transcription by mediating H3K4 tri-methylation. The

aforementioned genome-wide ChIP analysis revealed that 12.1 % of the *Arabidopsis* genome carries H3K4me3 under the normal laboratory growth conditions (Zhang et al. 2009). The *Arabidopsis* genome encodes five TRITHORAX (trx)-like proteins (ATX1 to ATX5), which are characterized by a SET domain and a PHD domain, and seven Trx-related proteins (ATXR1 to ATXR7) (Tamada et al. 2009). ATX1 and ATX2 have been demonstrated in vitro to possess H3K4 tri- and di-methylation activity, respectively (Saleh et al. 2008; Sang et al. 2009). An *Arabidopsis* ortholog of *Drosophila* Trithorax group (trxG) H3K4 trimethylase, ATX1, acts as a positive regulator for basal defense to bacterial infection and for the expression of a high proportion of defense-related genes, including *PR* genes (Alvarez-Venegas et al. 2006). Transcriptional activation of *WRKY70*, encoding a TF that acts for balancing SA–JA signaling crosstalk, is correlated with ATX1 binding and ATX1-dependent H3K4me3 signatures at the *WRKY70* promoter, suggesting that this gene defines one of ATX1 target genes in immune response (Alvarez-Venegas et al. 2007). By contrast, ATX1 binding was not detected on the *PR1* locus, implying that ATX1 confers the broad effects as the sum of indirect consequences, e.g., through the upregulation of defense-related TF-coding genes. Besides H3K4 methylation activity, ATX1 also serves to recruit the TATA-binding protein and RNA polymerase II (Pol II) to the target promoters including that of *WRKY70* (Ding et al. 2011). Upon the initiation of transcription, phosphorylated Pol II engaged in transcriptional elongation seems to recruit ATX1 to the transcribed gene region, where ATX1 tri-methylates histone H3. In addition to these trxG homologs, non-conserved proteins also seem to be engaged in antagonizing PRC2 function in plants (Aichinger et al. 2011). These findings suggest that less conserved, diverged mechanisms collectively mediate the equivalent function of *Drosophila* trxG in plants.

Suppressor screens for an *Arabidopsis* lesion mimic mutant, *accelerated cell death11* (*acd11*), have revealed SDG8 (also named ASHH2), a homolog of the yeast H3K36 di-/tri-methylase SET2, that is required for basal expression of *NB-LRR* genes including *RPM1*, RPM1-conditioned ETI, and basal immunity to bacterial infection (Palma et al. 2010). In both non-elicited and benzothiadiazol (BTH)-treated *sdg8* plants, H3K36me3 levels remain low on the locus encoding the NB-LRR protein LAZ5, in association with its lowered expression. Therefore, these findings suggest that SDG8-mediated H3K36me3 serves to establish and/or maintain a transcription-permissive chromatin state on subsets of *NB-LRR* gene loci. SDG8 also plays a crucial role in plant immunity against necrotrophic fungal pathogens through H3K36me3-mediated activation of subsets of JA/ET-inducible genes (Berr et al. 2010). However, consistent with multi-catalytic activity of SDG8 not only for H3K36me2/3 (Grini et al. 2009) but also for H3K4me3 (Cazzonelli et al. 2009), loss of SDG8 (ASHH2) also seems to influence H3K4me2 and H3K4me3 levels on the *PR1* promoter upon bacterial challenge (De-La-Pena et al. 2012). The requirement of H3K9me3 for SDG8 activity has been also described in shoot branching of *Arabidopsis* (Dong et al. 2008). Future studies will be needed to clarify whether SDG8 directly catalyzes H3 methylation on all these Lys residues.

5 Defense Priming

In defense priming, immune response is held in an inactive or less active state but competent for more rapid and/or strong activation upon subsequent stimulation [reviewed in (Conrath 2011; Pastor et al. 2012)]. This is often accompanied by the sensitization of immune response to lower doses of defense triggers or even to stimuli of otherwise non-eliciting activity. An advantage of defense priming, compared to direct defense activation, involves sustained enhancement of host immunity at low fitness costs (van Hulten et al. 2006). Defense priming occurs upon MTI or ETI activation, colonization of nonpathogenic microbes, or wounding. Chemical compounds have also been identified to act as a trigger for defense priming upon their application on plants, such as β-aminobutyric acid (BABA). The molecular basis for defense priming remains poorly understood, but recent studies suggest a role of histone modifications, in addition to metabolic changes (accumulation of inactive precursors/derivatives for defense-promoting metabolites), modulation of defense-related hormone crosstalk, and enhanced expression of MAPKs and TFs (Conrath 2011; Pastor et al. 2012). In this chapter, we put a particular focus on defense priming that is based on changes in chromatin configuration for defense-related genes.

Histone modifications and H2A.Z replacement have been considered as a molecular basis for priming of SAR-related genes (van den Burg and Takken 2009). As mentioned above, the induction of JA/ET-inducible defense-related genes upon JA application or challenges with necrotrophic fungal pathogens is accompanied by an increase of H3K36me3 levels at the promoters of these genes in an SDG8-dependent manner (Berr et al. 2010). It is of great interest to understand whether this leads to the establishment of primed states, i.e., the acquisition of immune memories, of these genes. Moreover, using BTH as a mimic of SAR trigger, a recent study demonstrated a correlation between systemic priming of SA-inducible *WRKY TF*-coding genes and changes in several histone modifications. In *Arabidopsis*, low-dose BTH application did not activate *WRKY29* and only slightly activated *WRKY6* and *WRKY53*, in a manner reflecting their transcriptional reprogramming in systemic non-challenged leaves during pathogen-triggered SAR (Jaskiewicz et al. 2011). However, these transcript levels were greatly elevated upon water infiltration 72 h after BTH pretreatment or in systemic (distal, non-challenged) leaves 72 h after local bacterial challenges, whereas they remain low in mock controls. Primed plants exhibit an increase of H3K4me3 levels in the promoters of these WRKY genes, which occurs in an NPR1-dependent manner, suggesting that a histone-based memory underlies defense priming.

Regarding defense priming, another important question involves the heritability of the established primed states on target defense-related genes. Trans-generation inheritance of stress adaptation has been well documented for abiotic stress (Chinnusamy and Zhu 2009). However, to date, only a few recent studies support this possibility for biotic stress. Recent studies show that primed states for defense-related target genes and immune response can be transmitted to the following generations when the parent plants were exposed to pathogen challenges or exposed

to priming triggering molecules. For instance, trans-generational SAR mounted upon bacterial challenges was sustained over one stress-free generation in *Arabidopsis* (Luna et al. 2012). This is accompanied by a shift in the balance of SA–JA signaling, i.e., enhanced SA responsiveness and reduced JA responsiveness, without significant changes in the corresponding phytohormone levels. The increase of H3K9Ac on the promoters of SA-inducible priming target genes and of H3K27me3 on a JA-inducible promoter points to a role of these histone modifications as a molecular basis for such differential primed states between SA and JA pathway genes. Moreover, trans-generational SAR occurs in non-primed *drm1 drm2 cmt3* mutant plants that show reduced levels in non-CG DNA methylation, although the genomic regions and genes undergoing this DNA hypomethylation remain to be determined (Luna et al. 2012). Nevertheless, this raises the possibility that DNA hypomethylation also facilitates the trans-generational heritability. It would be of great interest to determine the sequential order and functional relationship between histone modification changes and DNA methylation changes. ETI activation and BABA application also confer defense priming that is heritable to the following generation (Slaughter et al. 2012). Not only *Arabidopsis* but also tomato plants exposed to JA or insect herbivory exhibit priming of JA-inducible genes and trans-generational insect resistance, in a manner requiring the JA receptor COI1 (Rasmann et al. 2012). This phenomenon also requires intact RNA-dependent DNA methylation pathway (Rasmann et al. 2012), again pointing to a role of DNA methylation changes as an underlying basis. However, carefully designed experimentation will be needed to unambiguously clarify whether the trans-generation heritability of defense priming is exclusively based on changes taking place on the chromatin-level rather than stress-induced genetic changes which may interfere with chromatin organization (Pecinka and Mittelsten Scheid 2012).

6 Target Genes of Defense Priming

Our molecular genetic work on *Arabidopsis* suggests that a separation of initial and sustained activation phases of MTI occurs in the presence of mal-folded PRR (Lu et al. 2009). In an ER glucosidase II β-subunit allele, designated *rsw3*, sustained transcriptional reprogramming, and host immunity to bacterial infection are impaired despite almost intact co-activation of other early MTI-associated outputs such as a ROS burst, MAPK activation, ET production, and initial transcriptional reprogramming. This points to the importance of sustained transcriptional reprogramming as a critical step in mounting effective immunity. Thus, it is conceivable that the target genes of this sustained transcriptional reprogramming would be closely associated with defense execution.

Genome-wide transcriptome analysis has revealed an inventory of defense-related genes, including *PR1*, that are mis-regulated in the mutant and thus define targets of sustained PRR signaling during MTI (Ross and Saijo et al., unpublished).

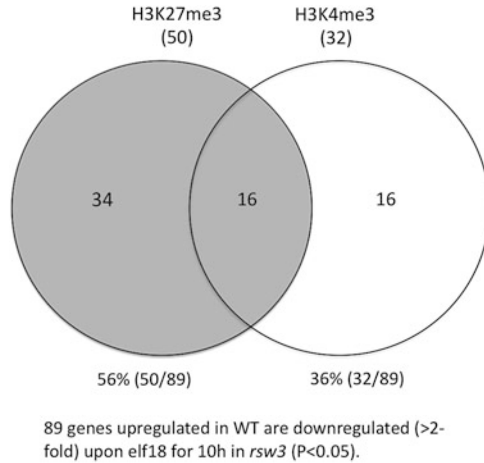

Fig. 2 In silico analysis for H3 methylation on defense-related genes in non-elicited *Arabidopsis* seedlings. The Venn diagram shows the number of *Arabidopsis* genes carrying H3K27me3 and/or H3K4me3 out of 89 genes that are upregulated in a late MTI phase in WT plants but not in *rsw3* plants (Lu et al. 2009). Further in silico comparative analysis suggests their close association with defense execution in diverse plant–pathogen interactions. The database is publicly available at the Jacobsen Lab Web site, USA (https://www.mcdb.ucla.edu/Research/Jacobsen/LabWebSite/P_EpigenomicsData.shtml)

In silico database analysis of these genes suggests that they are activated upon direct defense execution in diverse *Arabidopsis*–pathogen interactions, but remain at low expression levels in systemic tissues during SAR (Ross and Saijo et al., unpublished). Thus, these genes are also expected to include the target genes of systemic defense priming. Interestingly, these genes carry the transcription-repressive H3K27me3 and -permissive H3K4me3 histone modifications more often (56 % and 36 %, respectively) than expected (Fig. 2). The two mutually antagonistic chromatin marks are set by PcG and trxG protein complexes, respectively, and are typically associated with a gene-autonomous memory of transcription. This implies a role of these transcription memory-associated histone methylations in defense priming. This model is also consistent with the early studies on several *WRKY* genes (Jaskiewicz et al. 2011). Future studies will be required to reveal potential dynamics of these and other related histone modifications in the priming target loci during and after immune activation and to gain insight into the significance of the described chromatin-level changes in defense priming.

7 Conclusions and Prospects

Prompt and robust activation of pathogen-specific immune response is crucial to effectively repel the pathogens encountered. On the other hand, stringent control of the strength and spatiotemporal spreading of defense activation are also crucial to

minimize its negative influence on plant fitness. Recent progress, in particular in the reference plant *Arabidopsis*, has illuminated the potential importance of chromatin modification and remodeling as a means by which plants can meet these demands. However, the underlying mechanisms still remain largely unknown to date.

The engagement of histone modifications in establishing and reinforcing reversible and/or heritable patterns of gene expression has been well documented in plant development (Berr et al. 2011; Holec and Berger 2012). By contrast, the role of these regulations had not gained much interest of researchers in plant immunity until recently. However, the wealth of genetic resources and genetic tractability available in the model plant–pathogen interactions, e.g., between *Arabidopsis* and *Pseudomonas syringae*, would provide a great advantage for this emerging field as a model system for future epigenetic studies.

We propose the following stepwise regulation of histone modifications associated with transcriptional activation and attenuation of defense-related genes during immune response in plants. (1) In the absence of pathogens (or their derived elicitors), these genes are kept in a transcriptionally inactive or a basal state that is ensured by transcription-repressive or partially permissive chromatin configuration, respectively. (2) MAMP recognition, as an initial alert for the presence of potentially infectious pathogens, triggers a shift in chromatin configuration from the repressive to permissive state which either prevents the spreading of repressive histone marks and/or allows a rapid access and action of transcriptional activators. (3) Elevation of the strength of immune signaling beyond the activation threshold leads to massive activation of gene transcription, which in turn recruits defense-inducible TFs and histone modifications that would facilitate and/or reinforce the transcription of defense-related genes. (4) Following initial transcriptional changes, the persistence of active MAMP-triggered signaling or a distinct mode of signaling upon pathogen recognition (e.g., ETI signaling) leads to robust activation of gene transcription. This might be established by further spreading or acquisition of transcription-associated histone modifications and/or possibly by long-range interactions of distal genomic regions. By contrast, the absence of restimulation turns off gene transcription, which is eventually followed by the restoration of transcription-repressive (or basal, less permissive) patterns of histone modifications. (5) Upon sustained activation of gene expression (including certain posttranscriptional steps), transcription-coupled active histone modifications are firmly established and/or widely spread, which allows their persistence even after the removal of defense triggers. (6) Such long-lasting histone modification states keep the altered activation threshold, thereby providing a basis for a chromatin-level memory of immune response.

There are still many gaps in our knowledge to be filled for testing this model. To identify an inventory of target genes for systemic priming and to decipher histone modification patterns corresponding to particular chromatin states, genome-wide comparative analysis for transcriptomes (by RNA sequencing to cover possible changes in mRNA quality and non-coded RNA expression) and epigenomes (by ChIP-sequencing for different histone marks) during immune response and systemic priming will be a prerequisite. This would allow us to have a better picture of the underlying molecular events and to further generate new testable hypotheses.

This genome-wide analysis should be extended to obtain the transcriptome and epigenome profiles during MTI and ETI activation in an otherwise identical experimental platform, which is available, e.g., in *Arabidopsis–P. syringae* interactions. This is expected to gain insight into the mechanisms that are causative for the earlier described quantitative differences in transcriptional reprogramming between the two modes of immunity. It is possible that ETI skips or strengthens some of the stepwise regulatory processes proposed above. Of note, pathogens also seem to manipulate these host processes during infection. The transcription activator-like (TAL) effectors of the bacterial phytopathogen *Xanthomonas* species directly bind to specific promoter sequences in the host nucleus and activate target genes, which are otherwise repressed during immune response, for bacterial virulence promotion (Boch and Bonas 2010). This suggests the existence of host chromatin modulation activity that allows TAL effectors to access and transcribe the target genes. In addition, a growing number of effectors have been described for different pathogens that are localized in the host nuclei. It is conceivable that some of these effectors influence host gene transcription by altering chromatin configuration. Functional studies and host target identification of these effectors are expected to clarify these possibilities.

Furthermore, immune activation, whether in MTI or ETI, at directly challenged sites is linked to the activation of SAR and systemic priming in distal non-challenged sites. It will be interesting to determine whether MTI and ETI lead to significant differences in the target genes, strength, associated histone modifications, or combinations thereof of systemic priming. The overrepresentation of H3K27me3 and H3K4me3 marked by PcG and trxG proteins, respectively, at the defense-related gene loci implies a role of these modifications as a switch between non-primed and primed chromatin states of these genes in immune response. Of note, the transcripts for priming target genes accumulate barely above the background levels in systemic tissues upon defense priming, although both histone modifications typically act as a gene-autonomous memory of the preceding transcription states (Margueron and Reinberg 2011). It is of great importance to determine whether the stable acquisition of these histone modifications requires initial transcriptional reprogramming of target genes in systemic tissues as well or not.

The molecular links remain enigmatic between immune receptor-triggered signaling and chromatin modifiers/remodelers that participate in transcriptional reprogramming and priming of defense-related genes. The aforementioned genome-wide profiling of transcriptome and epigenome is expected to provide a new inventory of marker genes and histone modifications that would be valuable in further in-depth studies. In parallel, the chromatin modifiers and remodelers need to be identified that play a rate-limiting role in immune response. In this respect, the implementation of conditional gene knockout systems will be required to unambiguously assess the role of these chromatin regulators which cause dramatic pleiotropic effects during plant development and growth when they are permanently compromised.

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Epigenetic Control of Flowering Time

Theo Zografou and Franziska Turck

Abstract The decision to flower and commit to the reproductive phase requires that plants remember seasonal cues and keep a clock on their developmental age. Molecular memories have been explained by the bistable expression of genes. Bistable genes are switched from an expressed to a repressed state or vice versa in response to a primary stimulus, which is not required to maintain the switched state. Alterations in chromatin structure, orchestrated by covalent modifications of histones, are part of the molecular mechanism leading to bistable gene expression. For histone modifications that play a crucial role in molecular memories, the term "epigenetic chromatin marks" applies since bistable states can be maintained throughout mitosis. In the following chapter, we will first outline the regulation of the floral repressor *FLOWERING LOCUS C* (*FLC*) of *Arabidopsis thaliana*, for which the impact of chromatin modification on the molecular memory has been well studied. Differences in the regulation of the *FLC* and its ortholog *PERPETUAL FLOWERING 1* from *Arabis alpina*, a perennial relative of *A. thaliana*, are discussed. Last, the impact of chromatin structure on the regulation of *FLOWERING LOCUS T* (*FT*) is presented to illustrate that chromatin regulation can be important in regulatory networks that do not require bistability.

1 Introduction

Appropriate timing of flowering is crucial for successful plant reproduction. Plant species have adapted different strategies for flowering depending on their ecological niche. Rapidly cycling annual plants use the seed stage to survive adverse climatic conditions, whereas perennial plants are firmly established in their habitat

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and flower repeatedly (Albani and Coupland 2010). In both cases, correct seasonal timing is of essence and achieved by integrating external signals such as day length, light quality, and temperature into the decision to flower (Sung and Amasino 2004a; Amasino 2010; Srikanth and Schmid 2011; Turnbull 2011). Many plants are dependent on vernalization, a prolonged period of cool temperature, before they fully commit to the reproductive phase. However, the integration of temperature over time has also been implicated in bud dormancy and bud break, not regulatory steps in the decision to form flowers but important for the correct timing of reproduction (Horvath et al. 2003). In addition to external signals, internal cues participate in the decision to flower (Srikanth and Schmid 2011). Many plants experience a juvenile stage during which they are incompetent to respond to environmental flower-promoting signals (Bergonzi and Albani 2011). On the other hand, prolonged growth at a mature stage may lead to flowering in the absence of external floral-promoting cues (Hornyik et al. 2010).

Taken together, the decision to flower and the commitment to the reproductive phase comprise many aspects of memory, where plants remember previous periods of cool or warm temperatures and keep a timer on their developmental age. Furthermore, once committed to flowering, plant meristems do not usually reverse this decision even if the inductive stimulus is no longer perceived (Adrian et al. 2009). This raises the question by which molecular mechanisms plants acquire and recall a memory.

Molecular memories have been explained by bistable changes in gene expression, where a primary stimulus regulates a switch in the expression state of a target gene but is not required for the maintenance of this state (Kundu and Peterson 2009). Often, stable expression changes are correlated with local alterations in the chromatin structure and covalent modifications of histones, which may participate in establishing the memory. In particular Polycomb group (PcG) protein complexes have been shown to confer stable gene repression that is maintained in absence of repressive stimuli even throughout cell division (Fig. 1, right branch) (Morey and Helin 2010; Beisel and Paro 2011). Trithorax group (trxG) protein complexes act antagonistically because their activities are required for gene expression (Fig. 1, left branch) (Schuettengruber et al. 2011). The core PcG and trxG complexes methylate different lysine residues of histone H3, and the resulting modifications have been tagged as epigenetic chromatin marks because of their role in maintaining expression states in animals throughout cell divisions (Turner 2002). The pathways are evolutionary conserved and also mediate long-term memory of gene expression in plants (Farrona et al. 2008). Other chromatin-modifying activities are associated with PcG and trxG complexes and impart additional chromatin modifications that contribute to repression or induction of target genes. In particular the functions associated with trxG complexes are an integral part of the transcriptional process per se so that it is difficult to separate trxG components and transcriptional machinery (Kornberg 2007). The exact contribution of each chromatin modification to the actual "epigenetic" memory of transcription is not always firmly established although a picture emerges that a combination of features is required (Young et al. 2010; Scharf and Imhof 2011).

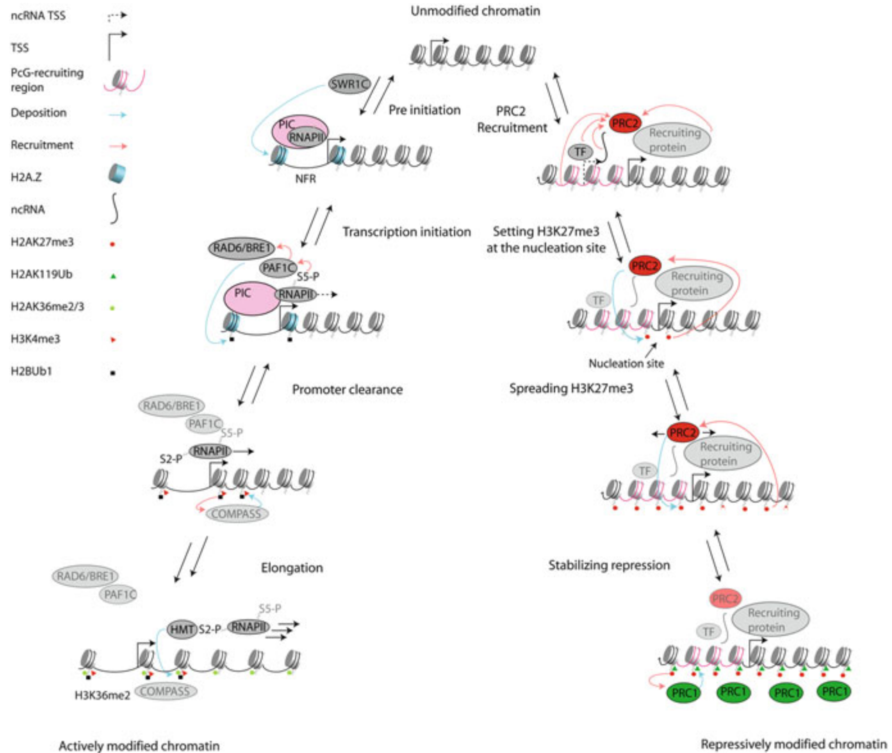

Fig. 1 Evolutionary conserved chromatin modifications and pathways implicated in gene expression regulation. *Left branch*: Transcription activation. At transcriptional start sites, the SWR1 chromatin-remodeling complex replaces nucleosomes containing canonical H2A with those containing H2A.Z. Because of its large size, assembly of the pre-initiation complex (PIC) requires a nucleosome-free region (NFR), formation of which appears to be promoted by H2A.Z nucleosomes. Transcription initiation is accompanied by phosphorylation of the Serine 5 (S5-P) residues within the repeated motif of the carboxy-terminal tail (CTD) of RNA polymerase II (RNAPII). S5-P among other signals serves as a docking site for the PAF1 complex (PAF1C), which in turn contributes to the recruitment of the RAD6/BRE1 complex, which mono-ubiquitinates H2B (H2Aub1) at nucleosomes located within the proximal promoter. H2Bub1 is a recruitment signal for the COMPASS complex. COMPASS encompasses an HMTase activity that trimethylates lysine 4 of histone H3 (H3K4me3). Elongation of RNA is sometimes paused, and RNAPII may require the action of HMTase that tri- or dimethylates lysine 36 of H3 (H3K36me2, me3) to overcome stopping signals. Complexes containing H3K36 HMTases are recruited via S2-phosphorylation (S2-P) of the CTD repeats. *Right branch*: Transcription repression: Polycomb Repressive Complex 2 (PRC2) is recruited either by the action of DNA-binding transcription factors, ncRNAs, or other mechanisms. PRC2 contains an HMTase that trimethylates lysine 27 of H3 (H3K27me3). Starting from an H3K27me3 nucleation site, which is linked to a PcG-recruiting region, the H3K27me3 spreads to flanking regions because the PRC2 recognizes its own target modification and is further activated by binding to the modified H3. Polycomb Repressive Complex 1 (PRC1) is recruited by H3K27me3 and further modifies chromatin by ubiquitination of lysine 119 (or related positions) of H2A (H2AK119ub) through two components that contain RING domains. H2AK119ub is important for chromatin compaction and subsequent gene repression

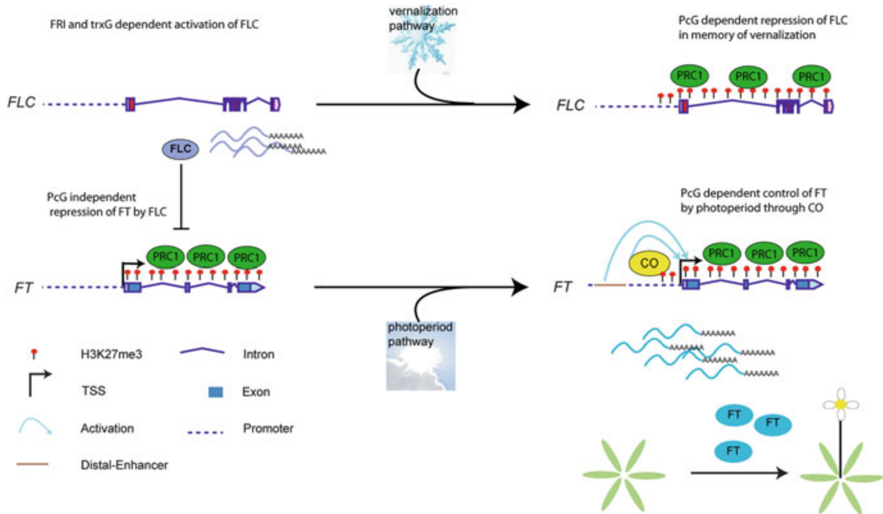

Fig. 2 Flowering regulation through epigenetic pathways in *Arabidopsis thaliana*. The MADS factor FLC is a key repressor of flowering that binds to the *FT* promoter and first intron to prevent activation of this gene through the photoperiod pathway. High FLC levels can repress *FT* independently of PRC2 and PRC1. *FLC* transcription is stably downregulated during vernalization by a process dependent on PRC2 and PRC1; it is also stably downregulated in very mature plants in the absence of vernalization by the autonomous pathway, which does not require PRC2 and PRC1. Once *FLC* is repressed, *FT* is activated in long days through the action of the transcription factor CONSTANS (CO), despite the continued presence of H3K27me3 and PRC1 across the locus and throughout the promoter. A distal enhancer, located 5.7 kb upstream of the transcriptional start site and outside of the H3K27me3 target region is required for *FT* activation by CO. FT protein is the mobile florigen signal that migrates from its site of production in the phloem companion cells of leaves to the shoot apical meristem to induce flowering

2 The Flowering Network in *Arabidopsis*

The gene network regulating flowering has been best characterized in the model plant *A. thaliana* (*Arabidopsis*). The following gives a succinct description of the regulatory pathways to introduce the players that have been mechanistically studied at the chromatin level and will be the focus of the following subchapter.

Analyses of induced mutants in *Arabidopsis* lead to the identification of five distinct pathways that promote flowering and which are interconnected by floral pathway integrator genes. These integrators receive inputs from several flowering pathways and are decision-makers as their induction commits the plant to flowering (Fornara et al. 2010; Srikanth and Schmid 2011). The vernalization and photoperiod pathway perceive environmental signals, whereas the autonomous and miR172/miR156 pathways are predominantly driven by internal programs. The gibberellic acid (GA) pathway acts both in parallel and together with the photoperiod pathway to induce flowering (Porri et al. 2012). The vernalization and autonomous pathways are interconnected through FLOWERING LOCUS C (FLC), a MADS-box transcription factor that serves as dominant floral repressor (Fig. 2). Flower-promoting signals that

are perceived by the photoperiod and GA pathways are ignored before *FLC* has been stably downregulated by the action of either the vernalization or autonomous pathway (Fig. 2). Both the vernalization and the autonomous pathway result in stable "epigenetic" downregulation of *FLC*, but the molecular mechanisms to achieve this overlap only marginally (Farrona et al. 2008).

Arabidopsis accessions can be separated into vernalization-dependent biannuals and vernalization-independent annuals, which respond to photoperiod and GA signals without requiring a prolonged cold period. Prior to vernalization, biannually growing *Arabidopsis* accessions express much higher levels of *FLC* than annuals, but both express similar low levels after vernalization (Michaels and Amasino 1999; Sheldon et al. 1999). A functional copy of *FRIGIDA* (*FRI*) is required for high-level *FLC* expression (Johanson et al. 2000). Natural variation at the *FLC* and *FRI* loci is the major cause of annual life habit in the genus (el-Assal et al. 2004; Le Corre 2005; Salome et al. 2011; Strange et al. 2011).

FLC is a potent repressor of flowering because it directly represses various floral pathway integrator genes and acts both in leaves and the shoot apex (Michaels and Amasino 1999; Searle et al. 2006). A direct target gene of FLC in leaves is *FLOWERING LOCUS T* (*FT*), which encodes the mobile florigen signal that moves from the leaves to the shoot apex to induce flowering. The arrival of FT protein from the phloem changes the nature of the apical meristem irreversibly from vegetative to reproductive (Fig. 2). *FT* perceives signals from the photoperiod pathway through the transcription factor CONSTANS (CO), which promotes *FT* expression in long days (LDs) but not in short days (SDs). CO protein is unstable in the dark and CO transcription shows a circadian oscillation. Only in LDs light perception and high transcription coincide at the end of the day and sufficient CO protein can accumulate to activate *FT* (Turck et al. 2008). *SUPPRESSOR OF CONSTANS 1* (*SOC1*), a floral integrator gene that is activated not only through FT by the photoperiod pathway but also directly by the GA pathway, is also directly repressed by FLC, thus adding a second layer of interconnectivity to the floral network.

3 Vernalization and the Memory of Winter

3.1 FLC Encodes the Memory of Winter in Arabidopsis

For a better description of the molecular events that take place during vernalization at the whole seedling level, the vernalization response of *FLC* can be broken down to three consecutive phases occurring during the cold period (VE I–III) and a post-vernalization phase corresponding to the return to warm ambient temperatures (see Fig. 3). It is important to point out that this sequential and coordinated view of events is slightly misleading because the vernalization response seems to be cell autonomous, and as detailed below, individual cells could respond to cold stochastically and with different kinetics (Angel et al. 2011; Satake and Iwasa 2012). During VE I, *FLC* transcription is not perceivably altered, but cold is somehow sensed qualitatively and integrated over time (Wollenberg and Amasino 2012). Temperature ranges most

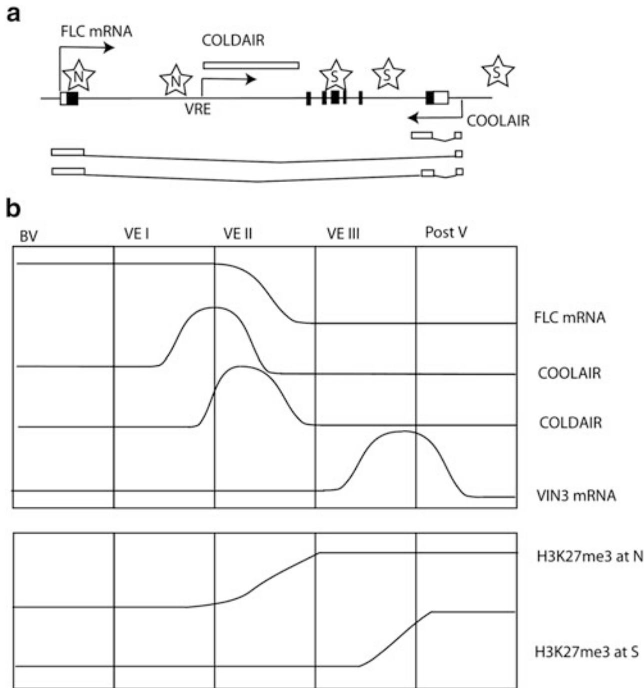

Fig. 3 *FLC* regulation by ncRNAs and PRC2 during vernalization. (**a**) Transcripts encoded by the FLC locus. Coding sense transcript of *FLC* with *boxed exons*, UTRs in *white*, and coding regions in *black*, and introns as *lines*. Transcription start sites are indicated by *directed arrows*. Location of COLDAIR transcript indicated above, COOLAIR transcripts below the locus as *white boxes* (exons) and *lines* (introns). Stars indicate locations of H3K27me3 nucleation (N) and spreading regions (S). *VRE* (*Vernalization Responsive Element*) at COLDAIR promoter. (**b**) Kinetic of molecular events at *FLC* during vernalization. Five depicted phases are before vernalization (BV), vernalization (VE) phases I, II, and III, and post-vernalization (post-V). The kinetics of *FLC*, *COOLAIR*, *COLDAIR*, and *VIN3* transcript accumulation are depicted as indicated in the top part, the bottom part of the graph models the level of H3K27me3 at nucleation (N) and spreading sites (S)

effective for vernalization are between 2 and 7°C, with some natural variation in the perception of vernalization-promoting temperatures (Wollenberg and Amasino 2012). During VE II, *FLC* is transcriptionally downregulated, but the process is still reversible as an interruption of vernalization at this stage will lead to substantial reactivation of transcription. VE III defines the phase when *FLC* repression becomes irreversible. In the post-vernalization phase, *FLC* repression appears to further reinforce in most tissues but is leaky in mature leaves, which coincidentally have reduced rates of cell division (Finnegan and Dennis 2007).

3.1.1 Sense and Nonsense Noncoding RNAs Transcribed from the *FLC* Locus

Two noncoding transcripts are transcribed from the *FLC* locus during VE I–II, and roles for their participation in the molecular memory were suggested (Swiezewski

et al. 2009; Heo and Sung 2011b). The transcripts appear to be produced in consecutive transient waves (see Fig. 3a, b) (Heo and Sung 2011b). First, a group of antisense transcripts collectively denominated *COOLAIR* are produced from a promoter located in the 3′ downstream region of *FLC* (Swiezewski et al. 2009). *COOLAIR* exists in various forms and splice variants that encompass either the entire *FLC* locus or terminate in its 3′ region (see Fig. 3a) (Swiezewski et al. 2009). *COOLAIR* peaks at the end of VE I and is downregulated in parallel to *FLC* during VE II. The decrease of *COOLAIR* and *FLC* steady state levels correlates with an induction of *COLDAIR*, which is an unspliced noncoding RNA (ncRNA) transcribed from within the first *FLC* intron (see Fig. 3a) (Heo and Sung 2011b). The peak of *COLDAIR* expression correlates with the highest rate of *FLC* and *COOLAIR* downregulation. The simultaneous full downregulation of *COOLAIR*, *COLDAIR*, and *FLC* marks the end of VE II (see Fig. 3b).

Both the *COOLAIR* and *COLDAIR* promoters have been shown to confer cold-inducible transcription if used to drive the expression of reporter genes in transgenic *Arabidopsis* (Swiezewski et al. 2009; Heo and Sung 2011b). In addition, cold-induced repression of sense transcript was observed when a *FLC* downstream region that included the *COOLAIR* promoter was fused to the 3′ end of a reporter gene driven by the strong *CaMV 35S* promoter (Swiezewski et al. 2009). The data indicate that antisense transcription from the *COOLAIR* promoter is sufficient to downregulate *FLC* during the cold induction. In contrast to *FLC*, repression of the heterologous reporter gene was never irreversible even after a prolonged period of cold indicating neither the antisense transcript alone nor a persistent downregulation confers a memory to the heterologous locus (Swiezewski et al. 2009).

Despite the observation that *COOLAIR* is sufficient to downregulate *FLC* transcription, it is not clear whether this mechanism is actually always required. Reporter gene constructs expressed under the control of the *FLC* promoter but lacking the 3′flanking region were shown to be stably downregulated during cold provided that they also contained a large part of the first *FLC* intron (Sheldon et al. 2002). In addition, the analysis of transgenic plants with T-DNA insertions at various positions within the *FLC* locus indicated that the expression of *COOLAIR* is not required for the molecular vernalization response since constructs that disrupted the antisense transcripts did not preclude a sustained *FLC* downregulation (Helliwell et al. 2011). Possibly, *COOLAIR* acts redundantly with other mechanisms in downregulating *FLC*, and deciphering its particular role in the vernalization process requires additional experimental conditions or the analysis of more genetic backgrounds.

3.1.2 ncRNAs Recruit PcG Proteins to FLC

The observation that its first intron is required for stable *FLC* downregulation argues for an involvement of the intronic *COLDAIR* transcript in the process (Sheldon et al. 2002; Sung et al. 2006a). *COLDAIR* has some unusual features that distinguish it from typical RNA polymerase (RNAP) II transcripts. Transcribed

form the second half of the first *FLC* intron, *COLDAIR* is extremely low in abundance so that ESTs have not been isolated and the transcript was never detected in microarray based approaches. *COLDAIR* does not possess a 3′ polyadenylated tail, but has a 5′ Cap structure (Heo and Sung 2011b). This is somewhat similar to noncoding transcripts that are transcribed by RNAP IV and RNAP V and serve as a scaffold for the recruitment of chromatin-modifying complexes (Haag and Pikaard 2011). However, chromatin immunoprecipitation (ChIP) data indicate that *COLDAIR* is transcribed by RNAP II (Heo and Sung 2011b). It was suggested that the function of *COLDAIR* in *FLC* downregulation is linked to the recruitment of POLYCOMB REPRESSIVE COMPLEX 2 (PRC2). The PRC2 core complex is conserved between animals and plants and contains four proteins, which are in all cases but one encoded by small gene families in *Arabidopsis* (Table 1). PRC2 complexes locally modify chromatin by trimethylating lysine 27 of histone H3 (H3K27me3) (see Fig. 1, right branch) (Margueron and Reinberg 2011). Animal PRC2s were shown to bind to ncRNAs through their Enhancer of Zeste (E(Z)) component. In addition to the SET domain, which is the catalytic histone methyl transferase (HMTase), E(Z) proteins feature a SANT and a cysteine-rich (CXC) domain, the latter important for RNA binding (Zhao et al. 2008). In *Arabidopsis*, CURLY LEAF (CLF) and SWINGER (SWN) are two partially redundant E(Z) homologs (Schatlowski et al. 2008). CLF protein alone was shown to directly bind RNA through its CXC domain but without sequence specificity (Heo and Sung 2011b). A pull-down experiment performed with biotinylated *COLDAIR* sense or antisense transcript and extract from vernalized plants showed that the presence of other proteins, presumably PRC2 components, increases the specificity of RNA binding, because only the sense construct could pull down a CLF:GFP fusion protein expressed in the extract (Heo and Sung 2011b). Based on their data, Heo and Sung proposed a model in which induction of *COLDAIR* would recruit a PRC2 complex containing CLF to a region in proximity or upstream of the *COLDAIR* transcript. This recruitment would lead to a local induction of H3K27me3 at the sites of PRC2 recruitment during the cold (Heo and Sung 2011a). Using an antisense approach to silence the expression of *COLDAIR*, cold-induced recruitment of CLF to the *FLC* locus and the local increase of H3K27me3 in the cold could be suppressed (Heo and Sung 2011b). However, the *COLDAIR* antisense plants still partially downregulated *FLC* in the cold and flowered earlier when vernalized for long periods of cold. Most likely, *COLDAIR* acts redundantly with other molecular mechanisms to coordinate the vernalization response at *FLC* (Buzas et al. 2012).

3.1.3 PHD-Finger Proteins and PcG Protein Complexes

The impact of PRC2 in the *FLC*-mediated vernalization response was first demonstrated through the cloning of *VERNALIZATION 2* (*VRN2*), which encodes for one of four PRC2 core components (Gendall et al. 2001). Although *vrn2* mutant plants display downregulation of *FLC* in response to cold, they are unable to

Table 1 PcG proteins in drosophila, mammals, and *Arabidopsis*

Complex	Drosophila	Mammals	*Arabidopsis*	Domains	Function
PRC2	Enhancer of Zeste [E(Z)]	EZH2	CLF (AT2G23380), SWN/EZA1 (AT4G02020), MEA (AT1G02580)	SET domain, CXC domain, homology domains I and II	H3K27me3 HMTase
	Suppressor of Zeste 12 [Su (Z)12]	SUZ12	VRN2 (AT4G16845), EMF2 (AT5G51230),FIS (AT2G35670)	C2H2 zinc finger, VEFS domain	Stimulates H3K27me3 HMTase activity
	ESC ESCL	EED1, EED2, EED3, EED4	FIE (AT3G20740)	WD40	Stimulates H3K27me3 HMTase activity
	Nucleosome-remodeling factor 55	RBAP48/RBBP4, RBAP46/RBBP7	MSI1 (AT5G58230)	WD40	Nucleosome binding, also part of (CAF1) complex
PRC1	Sex combs extra (RING) [SCE]	Ring1a, Ring1b/RNF2	RING1A (AT5G44280), RING1B (AT1G03770)	RING finger (C3HC4 zinc finger)	E3 ubiquitin ligase for H2A or cofactor
	Posterior sex combs [PSC]	BMI, MEL16	BMI1A (AT2G30580), BMI1B (AT1G06770), BMI1C (AT3G23060)	RING finger (C3HC4 zinc finger)	E3 ubiquitin ligase for H2A or cofactor
	Polycomb [Pc]	Cbx2, Cbx4, Cbx6, Cbx7, Cbx8	LHP1/TFL2 (AT5G17690)	Chromo, Chromoshadow	Binds trimethylated H3K27me3
	Polyhomeotic [PH]	Phc1, Phc2, Phc3	not present	SAM domain, C2C2 zinc finger	unknown
			EMF1 (AT5G11530)	Not identified	unknown
PHD-PRC2	Polycomb-like [PCL]	PHF1/PCL1, MTF2/ PCL2, PHF19/PCL3		Two PHD fingers and a tudor domain	Associates with PRC2 to increase H3K27me3 HMTase activity, PRC2 recruitment
			VRN5/VIL1 (AT3G24440), VIN3 (AT5G57380), VEL1/VIL2 (AT4G30200), VEL2/VIL3 (AT2G18880), VEL3/VIL4 (AT2G18870)	PHD finger, Fibronectin	Associates with PRC2 to increase H3K27me3 HMTase activity

Alternative names are separated by a slash and gene ID for *Arabidopsis* is given in brackets

Abbreviations: *BMI1* B lymphoma Mo-MLV insertion region 1, *CAF* chromatin assembly factor 1, *CBX* Chromobox protein homologue, *EED* embryonic ectoderm development, *ESC* Extra sex combs, *ESCL* Extra sex comb like, *EZH* enhancer of Zeste homologue, *MEL18* melanoma nuclear protein 18, *NSPC1* nervous system Polycomb 1, *PCL* Polycomb-like, *RBAP* retinoblastoma-binding protein, *SUZ* suppressor of Zeste, *RNF* RING finger protein, *PHF* PHD Finger protein, *CLF* CURLY LEAF, *SWN* SWINGEREZA: ENHANCER Of ZESTE ARABIDOPSIS, *MEA* MEDEA, *VRN* VERNALIZATION, *EMF* EMBRYONIC FLOWER, *FIS* FERTILIZATION INDEPENDENT SEED, *FIE* FERTILIZATION INDEPENDENT EMBRYO; *LHP1* LIKE HETEROCHROMAIN PROTEIN 1, *VIN* VERNALIZATION INSENSITVE, *VEL* VRN5-LIKE, *VIL* VIN3-LIKE

maintain the repression after a return to warm temperatures (Gendall et al. 2001). VRN2–PRC2 complex binds to the *FLC* locus during vernalization (Gendall et al. 2001). In addition to the core PRC2 components, proteins belonging to a PHD-finger family associate with VRN2–PRC2 during vernalization. These proteins were shown to stimulate the HMTase activity of PRC2 at *FLC* (Sung and Amasino 2004b; Wood et al. 2006; Greb et al. 2007; De Lucia et al. 2008). VERNALIZATION 5 (VRN5) is constitutively expressed, and loss of function of this PHD-finger protein causes a delay in *FLC* downregulation during the cold that is followed by a partial to complete reactivation during post-vernalization (Sung et al. 2006b; Greb et al. 2007). VRN5 physically interacts with VERNALIZATION INSENSITIVE 3 (VIN3) (Sung and Amasino 2004b). *VIN3* expression is gradually increased in response to prolonged cold during VE III (see Fig. 3b). Plants with mutations in *VIN3* show reduction in *FLC* expression almost as in WT plants in the cold but fully reverse the repression after a return to warmer temperatures (Sung and Amasino 2004b; Greb et al. 2007).

3.1.4 The Pattern of H3K27me3 During the Vernalization Response

Despite the induction of *VIN3* during vernalization as well as the increased activity of VIN3–PRC2 and the recruitment of the complex to *FLC* by *COLDAIR*, the increase in H3K27me3 at *FLC* is relatively mild during the actual cold phase (see Fig. 3b) (Finnegan and Dennis 2007). Notably, during cold, H3K27me3 increases only locally at a region corresponding to the transcription start site (TSS) and the proximal part of the first exon (see Fig. 3b) (Finnegan and Dennis 2007; Angel et al. 2011). This H3K27me3-enriched region is located at the *FLC* TSS and therefore upstream of *COLDAIR* transcript and promoter. Such locally enriched H3K27me3 regions have also been observed in animals and have been described as nucleation sites or nucleation regions (Talbert and Henikoff 2006). Nucleation sites are functionally linked, and sometimes identical to, regions of primary PRC2 recruitment. In animals it has been shown that H3K27me3-nucleation sites can be relatively distant to PcG-recruiting regions, which correspond to loci encoding for PRC2-associated ncRNAs. It is believed that three-dimensional interaction of chromatin, also called chromatin looping, can tether nucleation sites to the ncRNA (Spitale et al. 2011). In addition, it was recently shown in animals that the PRC2 preferentially targets stalled promoters of coding and noncoding transcripts (Enderle et al. 2011). Possibly, both the *FLC* sense and *COLDAIR* transcripts correspond to such stalled transcripts in the cold and act redundantly to nucleate H3K27me3.

Once plants experience warmer temperatures after vernalization, H3K27me3 increases more strongly throughout the entire *FLC* locus (see Fig. 3b) (Finnegan and Dennis 2007; Angel et al. 2011). Finnegan and Dennis speculated that increased cell division rates after plants resume growth in warm temperatures are required for the H3K27me3 spreading, which was not observed in fully mature leaves. Also VRN5, which spreads across the locus in the post-vernalization phase, could be implicated in spreading (De Lucia et al. 2008).

The propensity of H3K27me3 to spread from a nucleation region has been mechanistically studied in animals and is explained by an autocatalytic positive feedback loop (see Fig. 1, right branch). It was shown that the PRC2 is recruited to nucleosomes modified at H3K27me3 through a direct recognition by the EED/ESC core component (Hansen et al. 2008; Margueron et al. 2009). Importantly, interaction of modified nucleosomes with the PRC2 leads to an allosteric activation of the HMTase, which can explain the spreading of H3K27me3 to adjacent nucleosomes (Margueron et al. 2009).

3.1.5 Mathematical Modeling of the Cell Autonomous Bistable Chromatin Switch

Two recent studies implemented a modeling approach to bridge the gap between correlative experimental observations and a plausible causative molecular model of the mechanism of the bistable switch at *FLC* (Angel et al. 2011; Satake and Iwasa 2012). Both models were strongly inspired by pioneering work performed in yeast, which consisted of modeling cell autonomously inherited bistable chromatin states (Dodd et al. 2007). To model the vernalization response at *FLC*, the models postulated three nucleosomal states corresponding to an active modified state (A), a neutral, unmodified state (U), and a repressed modified state (M). The A state could represent a combination of histone modifications that were shown to correlate with high *FLC* expression, such as H3K4me3, H3K36me2, and various histone acetylations (He 2009); the M state corresponds to the H3K27me3-modified state. Note that modeling explains how the locus can bistably switch from an A- to M-dominated state but not how these states actually control transcription, which is still an open question.

In the models, the A and M state are mutually exclusive so that a directed interchange must transit by U. Experimental evidence from studies with Drosophila PRC2 justifies this stipulation, as Drosophila PRC2 cannot add H3K27me3 on H3 tails that are already modified by the active H3K4me3 mark (Schmitges et al. 2011). The assumption may be a simplification because each nucleosome contains two H3 tails that could be modified independently thus resulting in a bivalent nucleosomal unit. Bivalent chromatin regions that are modified with activating and repressing histone modifications have been identified in animals and plants, and it has been speculated that bivalent chromatin corresponds to a poised, not yet determined, epigenetic state (Bernstein et al. 2006; Roudier et al. 2011, Grafi et al. 2011). A caveat of most studies is that they do not distinguish between truly bivalent nucleosomes and a readout resulting from a mixed contribution within a population of cells.

A and M modifications further antagonize each other within each locus, which corresponds to the recruitment of chromatin-associated complexes by one modification leading to the removal of the opposing modification. Such recruited chromatin complexes could include histone deacetylases (HDAC) or histone demethylases (HDM). Notably, the model relies on the presence of an H3K27me3-specific HDM, which has only recently been identified in plants as the Jumonji domain protein

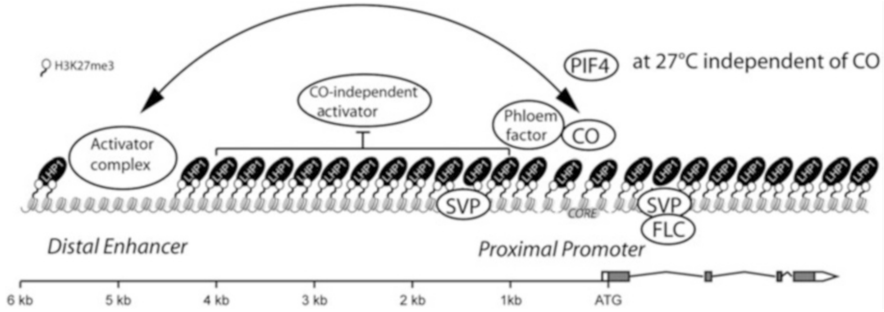

Fig. 4 Regulation of *FT* by chromatin structure and transcription factors. *FT* is controlled by regulatory elements located at the proximal promoter and a distal enhancer. The locus is widely covered by H3K27me3 and bound by the PRC1 component LHP1. The distal enhancer is located upstream of the H3K27me3-modified region. CO binds to *COREs* located at the proximal promoter. A cross talk between CO and transcription factors binding to the distal enhancer could require the formation of a chromatin loop. Without chromatin-mediated repression, *FT* is expressed independent of CO presumably because other transcription factors can access hidden *cis*-elements within the promoter. The transcriptional repressors FLC and SVP bind *FT* despite the presence of H3K27me3. At elevated ambient temperatures, PIF4 can bind *FT*'s proximal promoter and cause CO-independent activation. An unknown phloem-specific obligatory cofactor is postulated because *FT* is not ectopically expressed even if H3K27me3 is completely removed from the locus

RELATIVE OF EARLY FLOWERING (REF) 6 (Lu et al. 2011). Last, both, A and M, are controlled by positive feedback loops. For H3K27me3, this corresponds to the spreading mechanism described above, where the H3K27me3 modification recruits its own writer, the PRC2 complex (see Fig. 1, right branch).

A stochastic transit from A to U or M to U can also be supported by nucleosome exchange, which occurs as replacement at a basal rate in mitotically quiescent cells and at an increased rate during replication. Simulations of the core model showed that the actual transition from A to M (and reverse) occurs very rapidly, relatively independent of a variation of parameters. In contrast, variation in some parameters changed the resistance of A or M towards the transition state.

In their theoretical approach, Satake and colleagues showed that only systems that were highly stable for A and M allowed for a long-term memory of vernalization (Satake and Iwasa 2012). Angel et al. (2011) combined their modeling approach with new experimental data and showed that the slow gradients of stable *FLC* downregulation that are observed during vernalization are explained by cell autonomous bistable switching. The model was corroborated by demonstrating that cells expressing a *FLC:GUS* reporter in roots switched stochastically during the cold to a transcription "OFF" state. As the system is composed of many single bistable components, a gradual response of the entire seedling is observed (Angel et al. 2011).

The mathematical model by Angel et al. also pinpointed the relevance of the H3K27me3-rich nucleation region in the response to cold. Experimental data showed that the increase of H3K27me3 at the *FLC* 5′ region is quantitatively

correlated to the duration of the cold period. The H3K27me3 response saturates after ca. 4 weeks, which corresponds to a duration required for maintained *FLC* expression and for full *VIN3* induction (see Fig. 3b) (Angel et al. 2011). Only a model that postulated free crosstalk between nucleosomes at the nucleation site and across the locus independent of their distance was bistable, whereas a model that did only allow interactions between neighboring modifications failed the criterion.

3.1.6 H3K27me3 Modification and Transcription

H3K27me3 is correlated with stable *FLC* repression, but it is unclear how the repression is functionally achieved. H3K27me3 recruits other proteins such as LIKE-HETEROCHROMATIN PROTEIN 1 (LHP1), which features a chromodomain that directly binds the modification (Turck et al. 2007; Zhang et al. 2007b). Plants that carry mutations in *LHP1* are vernalization defective because they are unable to fully repress *FLC* in VE III and upregulate the gene during the post-vernalization phase (Mylne et al. 2006; Sung et al. 2006a). It is yet unclear if the *lhp1* mutation affects H3K27me3 levels and spreading at *FLC* or represents a downstream event. On a more general scale, *lhp1* mutants do not show alterations in H3K27me3 modification levels across all target regions at chromosome 4 (Turck et al. 2007).

LHP1 appears to be an integral part of a plant PRC1, together with members of two closely related but distinct RING-proteins, which are each encoded by small gene families (see Fig. 1 and Table 1) (Sanchez-Pulido et al. 2008; Xu and Shen 2008; Bratzel et al. 2010). In plants and animals, the RING finger proteins ubiquitinate a lysine residue within the globular domain of H2A (Bratzel et al. 2010). In animals, H2A ubiquitination has been correlated with chromatin compaction conferring target gene repression by interference with transcription initiation (Dellino et al. 2004; Stock et al. 2007) although chromatin compaction by RING finger proteins was also shown to occur independent of H2A ubiquitination (Eskeland et al. 2010). So far, the study of H2A ubiquitination in plants has been hampered by the fact that the region surrounding the target lysine residue is not fully conserved between plants and animals, which precludes the use of antibodies developed for animals to study this modification.

3.2 Preconditions for High FLC Expression

A functional copy of *FRI* is required for high levels of *FLC* prior vernalization. In early genetic studies in *Drosophila melanogaster*, the trxG mutations were defined by their antagonistic effect on PcG mutations. Cloning and further characterization showed that many trxG proteins are chromatin components that actively promote transcription. Transcriptome comparisons between plants possessing functional and mutated *FRI* and *FLC* genes established that only a small number of genes are

directly controlled by FRI, which makes it an unlikely general component of chromatin regulation (Schmid et al. 2003). However, a connection between FRI, high *FLC* expression, and activating histone modifications such as H3K4me3, H3K36me3, H2Bub1, and H2A.Z has been suggested by genetic analysis of mutants that show reduced *FLC* expression in presence of active FRI (Farrona et al. 2008; He 2009; Jarillo et al. 2009; Kim et al. 2009). Recently it was shown that a complex composed of FRI and 4 associated proteins directly binds to the *FLC* promoter to orchestrate transcription by recruiting several chromatin-modifying complexes (Choi et al. 2011). To fully appreciate the data, a brief introduction to the transcriptional cycle and its interconnection with chromatin modifications seems worthwhile.

3.2.1 Chromatin Features Implicated in Transcription Initiation and Elongation

Detailed molecular studies performed in animals and yeast have lead to the concept of the chromatin transcription cycle that can be broken down to several discrete steps: (1) recruitment of the pre-initiation complex (PIC), (2) transcription initiation, (3) transcription elongation, and (4) transcription termination (see Fig. 1, left branch) (Weake and Workman 2010; Kornberg 2007; Schuettengruber et al. 2011). The PIC assembles at transcriptional start sites requires the formation of a local nucleosome-free region (NFRs) to accommodate its large size. PIC comprises RNA polymerase II (RNAPII) and many accessory factors, including the general transcription factors (GTFs) TFIIA–TFIIH. The large multi-subunit MEDIATOR complex provides an interface between sequence-specific DNA-binding transcription factors and chromatin-remodeling enzymes acting to facilitate PIC assembly.

NFRs were observed at many eukaryotic transcription start sites and are often flanked by nucleosomes that contain variant H2A.Z in place of the canonical H2A (Raisner et al. 2005). A chromatin-remodeling complex called SWR1C is responsible for the exchange of assembled H2A.Z-containing nucleosome (Mizuguchi et al. 2004). The precise function of the variant forms is still unclear, but some data suggest that these nucleosomes are more tightly linked to the DNA, which argues for a function in reducing the sliding of canonical nucleosomes at the flanks of nucleosome-free regions (Marques et al. 2010). H2A.Z is not a typical “active” chromatin mark as it does not directly correlate with transcriptional activation presumably because highly transcribed genes tend to lose nucleosomes located downstream of the transcription start site including those containing H2A.Z (Raisner et al. 2005). Rather H2A.Z is a precondition for transcription and assembly of PIC and may be particularly required for transcription if other chromatin features work against maintenance of an NFR.

Once the PIC is assembled, the helicase component of TFIIH unwinds the DNA at the transcription start site to initiate transcription (Kornberg 2007; Shandilya and Roberts 2012). Transcription initiation is accompanied by the phosphorylation of Serine 5 (S5) residue in the “YSPTSPS” motif, which is repeated many times in the

carboxy-terminal tail (CTD) of RNAPII. The residue is phosphorylated by CDK7, a component of TFIIH. S5 phosphorylation participates in releasing RNAPII from the promoter and recruits, together with other factors, the RNAPII Associated Factor 1 Complex (PAF1C). PAF1C, in turn, is an assembly platform for other complexes such as histone chaperones and of a ubiquitination complex composed of RAD6 and Bre1 (Belotserkovskaya et al. 2003). RAD6 (an E2 ubiquitin-conjugating enzyme) and Bre1 (an E3 ubiquitin ligase) catalyze the mono ubiquitination of H2B (H2Bub1). RAD6/Bre1 can also be recruited directly to transcription start sites by DNA-binding transcription factors. The H2Bub1 serves as a mark for the recruitment of COMPASS (Complex Proteins Associated with Set1) containing the trithorax-related SET1 HMTase that catalyzes H3K4me3. H2Bub1 and H3K4me3 accumulate mostly around the transcription start site and are hallmarks of transcription initiation. During a step called promoter clearance, the GTFs dissociate form RNAPII, which transcribes a short stretch into the gene. H2Bub1 seems to be required for promoter clearance. In contrast, in yeast H2Bub1 interferes with transcription elongation, and the recruitment of SET2 HMTases that catalyze H3K36 di- and trimethylation stimulates transcriptional elongation (Henry et al. 2003). A second type of phosphorylation in the repeated motif at the CTD targets Serine 2 residue and also participates in recruiting H3K36me2 and H3K36me3 HMTases (Drogat and Hermand 2012).

Different hypotheses have been brought forward to explain the role of H3K36 methylation. Most importantly, H3K36me3 plays a role in preventing erroneous transcriptional initiation within the relatively open chromatin structure present during transcription (Lee et al. 2007). Recently, a role in defining exon/intron boundaries and an impact on alternatively splicing has been suggested (Wagner and Carpenter 2012).

3.2.2 FLC Exemplifies the Evolutionary Conservation of the Interplay Between Chromatin and Transcription

The mechanistic studies recapitulated above have mostly been performed in animals and yeast, though studies on *FLC* regulation in plants point towards a general conservation of the underlying mechanisms (Farrona et al. 2008; He 2009). Among the mutants that showed early flowering in the presence of FRI or vernalization insensitive early flowering in absence or presence of FRI are genes that were shown to encode for homologs of SWR1 complex (SWR1C) and PAF1 complex (PAF1C) components. PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1 (PIE1) encodes for the ATP-dependent chromatin-remodeling activity related to SWR1 of the SWR1C (Noh and Amasino 2003; Choi et al. 2007). PIE1 physically interacts with three other proteins that were identified based on *Arabidopsis* flowering time mutants and that are homologs of yeast SWR1C: ACTIN-RELATED PROTEIN 6 (ARP6)/EARLY IN SHORT DAYS 1 (ESD1)/SUPPRESSOR OF FRIGIDA 3(SUF3), SWC6/SERRATED LEAVES AND EARLY FLOWERING (SEF), and SWC2 (Choi et al. 2005; Deal et al. 2005;

Martin-Trillo et al. 2006; March-Diaz et al. 2007). The flowering phenotype of mutants in either gene is dependent on *FLC* but also on its close relatives MADS FACTOR AFFECTING FLOWERING (MAF) 4 and MAF5. The PIE/SWR1 complex interacts with H2A.Z which is encoded by four genes in *Arabidopsis* (Deal et al. 2007) and stacked mutants with severely reduced expression levels of the H2A.Z variants phenotypically resemble SWR1C mutants (Choi et al. 2007; Deal et al. 2007).

Four out of five PAF1C components were identified in genetic screens as EARLY FLOWERING (ELF) or VERNALIZAITION INSENSITIVE (VIP) (Zhang et al. 2003; He et al. 2004; Oh et al. 2004). Deletion of PAF1C components causes other developmental abnormalities as expected for mutations that affect such a general transcriptional component, but it is still remarkable that these mutations have only limited effect on plant development in general. Single mutants are likely to cause a complete loss of the entire PAF1C function as they behave strictly nonadditive in the genetic analysis. This includes the *vip3* mutant, which encodes for a gene product not reported in animals. Since VIP3 protein physically interacts with other PAF1C components it seems to be a plant-specific component of the PAF1C (Zhang et al. 2003; Oh et al. 2004).

Genome-wide profiling of the effect of PAF1C mutants on epigenetic chromatin marks showed no global effects on the H3K4me3 and H3K36me2 marks whose specific HMTases are assumed to be directly or indirectly recruited by PAF1C (Oh et al. 2008). However, a shift in the distribution of these epigenetic marks at target genes was observed. The H3K4me3 modification, which usually shows a distinct peak at transcriptional start sites, shifted in the 3′ direction, whereas the H3K36me2 modification, usually mostly enriched in the 3′ half of transcribed genes, shifted towards the 5′ end. Interestingly, the genes most misexpressed in PAF1C mutants were doubly marked by the active H3K4me3 and the repressive H3K27me3 mark. At *FLC*, the H3K27me3 mark, which is in general mostly enriched over transcribed regions, spread into the promoter region and increased in level throughout the gene body in PAF1C mutants (Oh et al. 2008). Parafibronectin/CDC73 is the only component of the animal and yeast PAF1C that was not isolated in forward genetic screens, but a homologous gene *PLANT HOMOLOGOUS TO PARAFIBROMIN (PHP)/CDC73* exists in *Arabidopsis*. Reverse genetic analysis of *php/cdc73* mutants pointed towards a more specialized role of this PAF1C component (Park et al. 2010; Yu and Michaels 2010). Only a subset of genes affected in other PAF1C mutants was misregulated in *php* mutant plants and the subset was even stronger enriched for H3K27me3 targets including *FLC* (Park et al. 2010). Taken together, it occurs that in plants PAF1C is not a requirement for transcription but rather plays a role in fine-tuning of gene expression and chromatin modifications. This role is particularly visible for genes that are also marked by H3K27me3 indicating that the plant PAF1C could play an antagonistic role to the PcG complexes in molecular memories.

In animals, the PAF1C participates in recruiting the enzymes that catalyze H2Bub1. *Arabidopsis* mutants that are affected in either the ubiquitin-conjugating enzymes UBC1 or UBC2 or in both ubiquitin ligases HUB1 and 2 show reduced

levels of *FLC* and a loss of H2Bub1 at the *FLC* locus (Cao et al. 2008; Gu et al. 2009; Xu et al. 2009). The mutants also show reduced levels of H3K4me3 at the 5′of *FLC*, indicating that H2Bub1 recruits a COMPASS-related complex in plants as it does in yeast and animals. As for PAF1C, loss of H2Bub1 does not abolishe global H3K4me3, again arguing for a more gene-specific effect in plants than in yeast, where both functions are a prerequisite for SET1 function (Wood et al. 2003; Gu et al. 2009). A possible explanation for this could be found in the greater diversity and partial redundancy of genes encoding for H3K4 directed HMTases. Besides the closest TRX relatives in *Arabidopsis*, ARABIDOPSIS TRITHORAX 1 (ATX1)/SET DOMAIN GROUP 27 (SDG27) and ATX2, also ATX RELATED 7 (ATXR7)/SDG2 and EARLY FLOWERING IN SHORT DAYS (EFS)/SDG8 have been implicated as H3K4 HMTases (Kim et al. 2005; Saleh et al. 2008b; Guo et al. 2010). ATX1/SDG27, ATXR3/SDG2, and EFS/SDG8 show less *FLC* expression and reduced levels of H3K4me3 at the locus (Pien et al. 2008; Saleh et al. 2008a; Yun et al. 2012) although there is some controversy for EFS/SDG8, which has been suggested to catalyze methylation of H3K36 instead of H3K4 (Zhao et al. 2005; Xu et al. 2008; Ko et al. 2010).

In addition to one or several H3K4-directed HMTases, the COMPASS core complex is composed of three components all of which are functionally conserved in *Arabidopsis* (Jiang et al. 2011). In contrast to deletions in the PAF1C, loss-of-function mutants of COMPASS components that are encoded by single copy genes are embryo lethal. Viable knock-down mutants corroborate the role of COMPASS in *FLC* expression and show reduced levels of H3K4me3 around the *FLC* TSS (Jiang et al. 2011). The mutants show other developmental abnormalities indicating that COMPASS function is crucial for the expression of many genes beside *FLC*.

The *Arabidopsis* UBIQUITIN PROTEASE 26 (UBP26) can cleave mono-ubiquitin from H2B (Sridhar et al. 2007). A T-DNA insertion in the accession C24 showed increased global H2Bub1 levels and increased occupancy of H2Bub1 across the *FLC* transcribed region (Schmitz et al. 2009). Although H2Bub1 plays a positive role in transcription initiation, *FLC* levels are decreased in *ubp26* mutants (Schmitz et al. 2009). *FLC* H3K4me3 levels are unaffected by the *ubp26* mutation, but H3K36me3 levels are considerably decreased. The data suggest that in plants as in yeast H2Bub1 has to undergo a cycle of ubiquitination and deubiquitination during transcription (Weake and Workman 2008). Mutations of UBP26 in other accessions than C24 showed much stronger phenotypes and mostly did not survive late embryogenesis (Schmitz et al. 2009). The reason for this natural variation in sensitivity to loss of *UBP26* gene is currently unknown, but the strong effects observed in some accessions point towards the general importance for H2Bub cycling in the transcriptional regulation of many genes. At *FLC*, loss of H3K36me3 caused by a lack of H2Bub1 deubiquitination led to an increase of H3K27me3 across the locus (Schmitz et al. 2009).

As mentioned above, EFS/SDG8 HMTase seems to have a dual catalytic specificity towards H3K4 and H3K36. Loss of *EFS* results in early flowering and reduced *FLC* expression (Kim et al. 2005). In the *FRI* background, *efs* mutants show a reduction of H3K4me3 at the TSS and of H3K36me3 in the gene body (Ko et al. 2010).

EFS interacts with the FRI complex at the *FLC* promoter, which suggests that EFS can be directly recruited independently of COMPASS (Choi et al. 2011). Interestingly EFS seems also to play a role in recruiting FRI to the *FLC* locus as association of FRI with the *FLC* promoter is lost in *efs* mutants (Ko et al. 2010).

3.3 Resetting of FLC

A resetting of the epigenetic memory of *FLC* is required to allow every generation of *Arabidopsis* to make their own winter experience. Although *FLC* expression is detected in floral buds, the actual resetting occurs during fertilization and early embryo development (Sheldon et al. 2008; Choi et al. 2009). The analysis of transgenic plants that express a *GUS* reporter gene controlled by *FLC* genomic regulatory regions in different mutant backgrounds suggested three distinct stages in the resetting process (Choi et al. 2009). The first stage corresponds to the first 3 days after pollination during which *FLC* expression is fully silenced; the second stage corresponds to the embryo heart stage, when *FLC* is activated in the embryo but not the endosperm. The third stage basically starts at the torpedo stage until the adult plant experiences vernalization and reflects the maintenance of high expression of *FLC*. Interestingly, *FLC* activation during stage II is not dependent on FRI and its interacting partner SUPPRESSOR OF FRIGIDA 4 (SUF4). FRI and SUF4 are only required in stage III. In contrast, other factors that suppress the effect of FRI on FLC expression in adult plants are important for stage II activation, including components of the PAF1C, SWR1C, and HMTases related to the trxG (Yun et al. 2011).

3.4 Regulation of PEP1 in the Perennial Arabis alpina: A Forgetful FLC

The *FLC* gene is not found in all plant species that respond to vernalization indicating that the mechanisms regulating the response to winter have evolved independently in different plant families. Even within the Brassicaceae, where the participation of *FLC* homologs in the vernalization response seems conserved (Lin et al. 2005; Kim et al. 2007; Okazaki et al. 2007; Yuan et al. 2009; Zhao et al. 2010), the molecular response has evolved differently to accommodate differences in lifestyle between related species that grow as annuals or perennials (Albani and Coupland 2010). Annual species such as *A. thaliana* flower once and then undergo senescence, whereas perennial species flower repeatedly. Perennials do not flower perpetually but show a seasonal flowering pattern and produce flowers only during a defined time period after initiation. They show a differentiated transition to flowering so that only a subset of meristems commit to flowering whereas others

remain vegetative and support plant growth and flowering in the following season. In consequence, perennial plants that respond to vernalization and therefore have a memory of winter must reset their memory once a sufficient number of meristems are committed to flowering (Albani and Coupland 2010). *Arabis alpina*, a close relative of *Arabidopsis*, shows a perennial growth habit and most accessions have an obligate vernalization requirement (Koch et al. 2006). An important difference between vernalization responsive biannual *A. thaliana* accessions and the perennial *A. alpina* is the time point of floral meristem establishment. Flower meristems in *Arabidopsis* form after the return to warm temperatures, particularly fast if plants are transferred to LD growth conditions. In contrast, in *A. alpina*, the flower meristems are formed during the cold period, which usually falls into SD growth conditions, but the meristems are arrested until an increased ambient temperature is perceived (Wang et al. 2009).

An induced mutagenesis study in *A. alpina* identified *PERPETUAL FLOWERING 1* (*PEP1*), the ortholog of *Arabidopsis FLC*, as regulator of vernalization and seasonal flowering (Wang et al. 2009). Plants that carry either induced or naturally occurring mutations in *PEP1* flower without vernalization and are also affected in their seasonal control of flowering indicating that these processes share regulatory components. *PEP1* transcripts are gradually downregulated during prolonged cold, but in contrast to the situation observed for *FLC*, *PEP1* repression is instable as the gene is upregulated after the return of *A. alpina* plants to warmer growth temperatures (Wang et al. 2009). In contrast to *FLC*, H3K27me3 increases across the entire locus of *PEP1* during the cold and not only at a nucleation region (Wang et al. 2009). Furthermore, the reduction of H3K27me3 across the *PEP1* locus after a return to warm is diametrically opposite to the increase seen at *FLC* and may distinguish perennial from annual species in the Brassicaceae family.

In their mathematical model, Satake et al. proposed that the difference between perennial and annuals depends on the rate at which histones turn from an activated to a repressed state after the return to warm temperatures (Satake and Iwasa 2012). Angel et al. did not model perennial plants but realized that the number of nucleosomes in the system was an important factor of bistability (Angel et al. 2011). Both observations provide interesting angels for future molecular work in this new model species.

4 Regulation of Photoperiodic Flowering by Chromatin Structure

PcG-mediated repression is not only involved in repression of *FLC* but also crucial for the photoperiod-dependent regulation of *FT* in *Arabidopsis*. So far, aspects of bistable (or epigenetic) gene expression have not been discovered for *FT*. Rather, the role of PcG complexes in the regulation of *FT* exemplifies that chromatin-mediated regulation of gene expression is not restricted to the formation of molecular memories.

FT is induced immediately in response to LD and this induction is reversed after a return to SD growth conditions (Corbesier et al. 2007). Even in LD, *FT* expression is diurnal as it follows the circadian activity profile of its upstream activator, CONSTANS (CO) (see Fig. 2). If a period of 3–5 LDs is interrupted by a return to SDs, the plants are committed to flowering despite the loss of *FT* expression indicating that other molecular memories play a role in the apical meristem (Adrian et al. 2009).

Plants that carry mutations in the PcG components *EMBRYONIC FLOWER 2* (*EMF2*), *CLF* or *LHP1* show upregulation of *FT* independent of day length and consequently, early flowering (Goodrich et al. 1997; Kotake et al. 2003; Farrona et al. 2008). The H3K27me3 mark spreads widely across the *FT* locus including its up- and downstream regions (Adrian et al. 2010). At the promoter, the mark extends to approximately 5 kb upstream of the TSS, and it has been shown that the region located upstream is required for *FT* induction in LDs by CO (see Fig. 4). Apparently absence and presence of H3K27me3 defines the boundary for a distal enhancer. In *lhp1* and *clf* mutants, the distal enhancer is not required to induce *FT*, but the induction also loses its dependency on the upstream activator CO presumably because other transcription factors can now access their binding sites, which are usually buried within the condensed chromatin (see Fig. 4) (Adrian et al. 2010; Farrona et al. 2011).

Despite the requirement of the distal enhancer for CO-dependent regulation of *FT*, experimental evidence suggests that the proximal promoter is the direct entry point of control by CO. Until recently it was unclear whether CO was a canonical transcription factor as DNA binding had not been demonstrated. Tiwari et al. (2010) showed in vitro binding of CO to *constans responsive elements* (*CORE*s) that are found in several copies within the proximal *FT* promoter. An overlapping set of elements were shown to affect *FT* induction by CO in the context of a promoter that included the distal enhancer in stably transformed transgenic plants (Adrian et al. 2010). Interestingly, in transient assays carried out either in transformed protoplasts or particle bombarded leaves, induction by CO can be achieved with the *FT* proximal promoter alone (Adrian et al. 2010; Tiwari et al. 2010). It is tempting to speculate that absence of repressive chromatin features in transiently transfected DNA alters the requirements for CO to control *FT*.

FT is expressed specifically in phloem companion cells at the minor veins of *Arabidopsis* rosette leaves (Takada and Goto 2003). Genome-wide analysis of H3K27me3 target genes in seedlings has established that ca. 15 % of the *Arabidopsis* coding genes are associated with the mark (Zhang et al. 2007a). Many H3K27me3 positive genes are expressed in a tissue-/organ-specific pattern, which seems to point towards a key function of PcG-mediated regulation in controlling tissue specificity of gene expression. Despite this presumed role of PcG complexes, *FT* expression is restricted to phloem companion cells in *lhp1* and *clf* mutants (Adrian et al. 2010; Farrona et al. 2011). Mutants that carry homozygous loss-of-function alleles of *CLF* and its homologue *SWN* show

a complete absence of H3K27me3 (Lafos et al. 2011). Double *clf swn* mutants show very severe developmental abnormalities after germination and develop into calli (Chanvivattana et al. 2004). Surprisingly, in these structures, *FT* is downregulated despite the absence of the repressive H3K27me3 mark (Farrona et al. 2011). The data indicate that tissue specificity of *FT* expression is not directly controlled by the PcG pathway but requires additional obligatory cofactor(s) (see Fig. 4).

Chromatin-mediated regulation may be implicated in other aspects of *FT* regulation. First, *FT* is most strongly expressed in cauline leaves and floral organs, where expression does not require the distal enhancer or activation by the photoperiod pathway (Adrian et al. 2010). It is interesting to speculate that the chromatin structure at *FT* is less repressive in petals and sepals, therefore providing an opportunity to activate the gene by *cis*-elements that are otherwise deeply buried in the H3K27me3 covered region. So far, however, there is no indication of a function for *FT* expression in flower organs and no data that show a loss of H3K27me3 at *FT* in flower organs.

Regulation of *FT* by ambient temperature may also implicate chromatin aspects. *FT* expression is increased and decreased by growth in high and low ambient temperatures, respectively (Blazquez et al. 2003; Lee et al. 2007). Reduction of *FT* expression at low temperatures was shown to be dependent on the MADS domain transcription factor SHORT VEGETATIVE PHASE (SVP) that directly binds to two *CArG* boxes present at the *FT* locus (Lee et al. 2007). One binding site is located in the first intron of *FT* and overlaps with a binding site identified for FLC (Searle et al. 2006). Indeed FLC and SVP are able to form a complex and cooperatively repress *FT* (Li et al. 2008). Note that both proteins seem to be able to access *FT* without interference from H3K27me3 and the PRC1 component LHP1. Concerning the temperature effect of SVP on *FT* expression, it is still unclear by which mechanism the repressive effect of SVP on *FT* is potentiated at lower temperatures since the protein seems not altered between conditions. A temperature-dependent impact of SVP on chromatin-mediated repression is still an open possibility. Kumar and Wigge (2010) reported a reduction of H2A.Z across many loci at high temperatures and a loss of temperature-dependent gene regulation in SWR1C mutants. They suggested that H2A.Z is involved in the perception of temperature in *Arabidopsis*. Possibly, H2A.Z or other chromatin components act as thermosensor at *FT* to either alter SVP binding or modulate the transcriptional readout of the transcriptional repressor.

If *Arabidopsis* seedlings are grown at 27°C, *FT* is expressed at sufficient levels in SD to promote early flowering (Balasubramanian et al. 2006). This induction of *FT* is mainly dependent of the bHLH transcription factor PHYTOCHROM INTERACTING FACTOR (PIF) 4, which directly binds to the *FT* proximal promoter and stimulates expression independently from CO (Kumar et al. 2012). High temperature increases PIF4 accumulation, but in addition, PIF4 binding to *FT* is promoted by a loss of H2A.Z, again suggesting that chromatin is a regulatory component of *FT* expression.

5 Conclusions

Many chromatin modifications are an integral part of basic transcription, because they assist the initiating, elongating, and terminating RNAPII during the process. For specific genes, the same modifications can be embedded in the transcription regulatory network because the specific chromatin structure at these genes changes the role of chromatin modifications from an assisting to an essential one. In a particular scenario, chromatin modifications can be crucial to achieve bistable gene expression and thereby enable long-term molecular memories. The regulation of *Arabidopsis FLC* by PcG complexes in response to vernalization is an example of such chromatin-mediated bistability. Its homologue *PEP1* in the perennial relative *Arabis alpina* demonstrates that subtle differences in bistability can engender dramatic changes in lifestyle. For *FLC* and *PEP1*, the term epigenetic chromatin modification fully applies for H3K27me3, but it should be handled with care. Other examples, illustrated by *Arabidopsis FT* regulation, indicate that the same mark plays a modulating role and does not result in bistability. In the *FT* scenario, gene expression follows the direction given by transcription factors and repressive chromatin plays a more passive role as structural component.

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Hormonal Signaling in Plants and Animals: An Epigenetics Viewpoint

Andrzej Jerzmanowski and Rafal Archacki

Abstract In the past 10 years, enormous progress has been made in elucidating the nature of plant hormone receptors. This has allowed much greater insight into the mechanisms underlying hormone-mediated effects on the level of gene expression, particularly for hormones whose main receptors are localized in the nucleus. Surprisingly, and in contrast to the case for intensively studied nuclear hormone receptors in animals, very little is known about the contribution of chromatin-based epigenetic mechanisms in conveying and integrating responses to plant hormones. Here, we examine the similarities and differences between plant and animal nuclear receptor systems with the aim of revealing analogies that could help identify possible intersections between plant hormone signaling and epigenetic mechanisms.

1 Introduction

There is surprisingly little to say about the subject of this chapter encapsulated in the title, particularly if one expects an extended analysis of the relevant literature. The current state of research in this area may reflect difficulties in formulating questions that could probe potential intersections of epigenetics and plant hormonal signaling. As in many other areas of plant biology, it may be profitable to look carefully for both homologies and analogies in the animal world.

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Cell-to-cell communication occurred early in the evolution of unicellular life and has continued to evolve in all subsequent forms of life on Earth. The ever-increasing sophistication of these systems has accompanied most of the so-called Major Evolutionary Transitions that have occurred over the last two billion years, particularly the transition between unicellular and multicellular life (Maynard-Smith and Szathmáry 1995). In multicellular organisms, cell-to-cell communication systems play a fundamental role in coordinating cellular activities underlying development and growth, with cues coming from both the external and internal environment.

In animal physiology, the traditional classification of signaling systems into three general categories, endocrine, paracrine, and autocrine, is based on the criterion of distance over which the signaling molecule is transported to exert its effect. The term "hormonal system" is reserved for endocrine or long-distance signaling. Hormones are defined as chemical agents produced and secreted by highly specialized endocrine tissues and transported by the circulatory system to distant targets in the body, upon which they act. The classic example of endocrine signaling is the production, distribution via the bloodstream, and effects on target tissues of substances such as thyroxine and triiodothyronine, estrogens and androgens, or insulin and glucagon. In vertebrates, there are over 50 different substances that fulfill the criteria of a hormone. The term "paracrine signaling" is used to describe phenomena like the action of neurotransmitters at synapses between neuronal cells, or the effects of cytokines that are released by certain cells during an inflammatory reaction and interact with the receptors of nearby target immune cells to trigger specific responses. The signaling here is also highly specific but is strictly targeted to the local area. Finally, the term "autocrine signaling" is used when an agent (a chemical messenger) that is secreted outside a cell binds to transmembrane receptors (autocrine receptors) on the same cell and triggers specific responses. A typical case is the increased proliferation of a T lymphocyte of the immune system in response to its own interleukin-2 growth factor. Similar autocrine stimulation is often responsible for the uncontrolled proliferation of cancer cells.

How do plant hormones (phytohormones, also known as growth regulators) fit into the aforementioned three-layer cell-to-cell communication system described in animals? Phytohormones comprise five classical types: auxins, cytokinins, gibberellins (GA), ethylene, and abscisic acid (ABA), plus several other types that have been described in recent decades, the most important of which are the brassinosteroids, jasmonates, salicylates, and stringolactones (Williams 2011). While some of these chemical messengers can be transported by the plant vasculature (and in the case of auxins, by an elaborate cell-to-cell transport system) and act over long distances, they also affect cells in close proximity, being translocated by different means, as well as the very cells from which they originate. Thus, there appears to be no exclusive specialization of this group of messengers for long-distance signaling, because they can act at any distance. Similarly to their animal counterparts, phytohormones exhibit biological activity at very low concentrations (10^{-6} to 10^{-5} mol/L) and their activity is dose dependent. However, unlike animal hormones that are highly specific in their effects, those of phytohormones are astonishingly broad and therefore extremely complex. In addition, it is generally

assumed that most plant cells can produce and respond to most phytohormones. The above comparison strongly suggests that there is something fundamentally different between the strategies for using signaling molecules in animals and plants.

2 Current View of Hormonal Regulation in Plants

2.1 Plant vs. Animal Receptors

In animals, the hormone receptors are compatible with the physicochemical properties of hormone signals, in particular their ability to cross plasma membranes. Hydrophilic, water-soluble peptide and glycoprotein hormones bind to the extracellular domain of transmembrane proteins that belong to the family of so-called seven-spanning receptors (from seven transmembrane helices). Many of these receptors interact via their intracellular domain with heterotrimeric G proteins. These are GTP-binding regulators capable of initiating the signal transduction pathway in the cell through activating effector enzymes (like adenylate cyclase or phospholipase C), which results in the appearance of second messengers (cAMP, inositol triphosphate, diacylglycerol, Ca^{2+}). This in turn leads to activation or inactivation of downstream enzymes and regulatory proteins, usually with the involvement of protein phosphorylation and dephosphorylation. In contrast, lipophilic compounds like steroid and thyroid hormones, retinoids, and vitamin D can pass freely through plasma membranes and so do not need outer-membrane receptors. Instead, they bind directly to their intracellular receptors, which carry DNA-binding domains and, upon activation by ligand binding, act as transcription factors. These intracellular receptors are localized either directly in the nucleus (e.g., receptors for vitamin D, retinoids, and thyroid hormones) or in the cytosol (e.g., receptors for steroid hormones—cortisol, estrogen, and testosterone). In the latter case, the receptor's basic inactive state is maintained by its association with a heat-shock protein chaperone. Upon binding of a steroid compound, the receptor is released from this complex and translocated into the nucleus where it dimerizes and binds to a DNA sequence known as a Hormone Response Element (HRE), usually located in the enhancer region of steroid-dependent genes (reviewed in Aranda and Pascual 2001).

The nature of plant hormone receptors remained a mystery for many years. The breakthrough came with discoveries made during the last two decades and the major mechanisms responsible for phytohormone reception have now been mostly elucidated. The emerging picture reflects both fundamental differences and some common themes with the receptor systems functioning in animals. Three major plant hormones, the cytokinins, ethylene, and brassinosteroids, have transmembrane receptors. Cytokinins use so-called two-component membrane receptors, well known in prokaryotes but absent in animals. These receptors act by phosphorylation of a histidine kinase transmitter domain, a modification that is induced by a signaling molecule upon its binding to the histidine kinase input domain.

This phosphorylation is then transmitted to an intracellular receiver domain of a response regulator, resulting in activation of the receiver's output domain and initiation of the signaling pathway in the cytosol. Ethylene receptors represent a modification of the two-component system differing from a canonical form by the absence of a phospho-relay to a response regulator. Brassinosteroids (BRs) use a plasma membrane-associated Leucine-Rich-Repeat Receptor-Like Kinase (LRR-RLK), also present in animals. The BR ligand binds directly to the extracellular LRR motif of the kinase, stimulating autophosphorylation of its internal domain (reviewed in Bishopp et al. 2006).

Perhaps the biggest surprise was the discovery of similarly functioning intracellular receptor systems responsible for the perception of auxins, gibberellins, and jasmonates. In all three cases, the physiological response to hormone is blocked by central repressors and these hormones use F-box ubiquitin ligase proteins (auxins and jasmonates) or a protein associating with the F-box ubiquitin ligase (gibberellins) as a receptor (reviewed by Chow and McCourt 2006; Santner and Estelle 2009; Spartz and Gray 2008). One common functional feature is a conformational change in the receptor that occurs upon hormone binding, which enables recruitment of the hormone response repressor to the proper SCF (SKIP/CULLIN/F-BOX) complex for ubiquitination and subsequent destruction by the 26S proteasome.

The most recent breakthrough in plant hormone perception studies is the discovery of the ABA receptor system, also referred to as the "ABA signalosome" (reviewed by Umezawa et al. 2010). This turned out to be a soluble intracellular receptor, not directly associated with the 26S proteasome system. It is composed of three major elements: the PYR/PYL/RCAR ABA-binding proteins, a type 2C protein phosphatase PP2C, and a SNF1-related protein kinase SnRK2. In the absence of ABA, PP2C directly interacts with SnRK2 and its multiple phosphorylation sites are maintained in a dephosphorylated state. The presence of ABA leads to SnRK2 phosphorylation and subsequent activation. PYR/PYL/RCAR binds to the hormone, which enables it to interact with PP2C and inhibit its dephosphorylating activity. This releases SnRK2 from negative control by PP2C, which leads to its phosphorylation and then the subsequent activation (also by phosphorylation) of transcription factors mediating the effects of ABA.

2.2 Cross talk Between Plant Hormones

The prevailing view is that the organismal complexity of plants is lower than that of animals. Plants are composed of fewer cell types that are less differentiated and less integrated than the cells comprising highly specialized animal tissues. With regard to the membrane receptor-mediated perception of hormonal signals, animals rely mostly on specialization, i.e., cells of individual tissues have different receptors, which make them sensitive to particular types of hormones. The integration of different tissues of animals is achieved by a centrally controlled neural/endocrine system that is responsible for collecting information and maintaining body homeostasis. Plants lack such a central integrator and must rely on a much higher degree of integration occurring at the level of individual cells. Plant cells have developed an intricate and highly flexible

system of reading and integrating signals from both neighboring cells and the environment. While there is little direct data describing the interactions of different hormones in a single cell, it is generally assumed that most plant cells are simultaneously exposed to two or more hormones, which they can recognize and act upon. The integrated physiological effect of these different messengers strongly depends on the localization of the cell in the plant, the stage of development, and the environmental conditions (Depuydt and Hardtke 2011).

In general terms, the interdependence or cross talk of plant hormones is best illustrated by the effects of mutations in one hormone pathway on plant responses to other hormones. For example, mutants in the auxin signaling pathway or auxin transport strongly influence responses to ethylene and ABA. This need not necessarily result from cross talk occurring at the single cell level, but may reflect a disturbance in the general responsiveness of the plant. Hormones strongly affect each other's synthesis, catabolism, and perception, by regulating the respective genes at the transcriptional level (Stepanova et al. 2007; Dugardeyn et al. 2008). On the other hand, data showing that ethylene and ABA stabilize central repressors of GA signaling, the DELLA proteins, thus counteracting the effect of gibberellins, or that multiple mutants in *Arabidopsis* DELLAs are resistant to ABA-mediated inhibition of growth, suggest that individual cells may indeed be targets of different hormones acting simultaneously and that the effects of these hormones are somehow integrated (Weiss and Ori 2007; Achard et al. 2003, 2006). A similar conclusion can be drawn from the known antagonistic effects of auxins and cytokinins during development (Nordstrom et al. 2004).

Cross talk between different pathways often indicates that they share a common module. However, apart from the auxin and BR pathways, which share the signaling elements ARF2 and BIN2 (Vert et al. 2008), no such common elements have been identified so far within the response pathways of other hormones, although direct protein-protein interactions between key regulatory proteins in GA and JA and BR pathways have been recently reported (Hou et al. 2010; Bai et al. 2012; Gallego-Bartolome et al. 2012). It has therefore been suggested that integration among plant hormones occurs mainly at the gene network level (Jaillais and Chory 2010; Rodrigo et al. 2011). In the following part of this review, we will discuss plausible models for such integration and their possible links with chromatin mechanisms, based on known analogies to animal systems and some recent experimental data obtained from plant studies.

3 Lessons from Animals

3.1 The Chromatin Environment as a Potential Space for Signal Integration

The mechanisms underlying the modulation of chromatin structure, including posttranslational modifications of histones, DNA methylation, histone variant exchange, active chromatin remodeling, and targeting of chromatin *loci* by small

RNAs, are vital for the functioning of the epigenetic system that enables mitotic and sometimes transgenerational inheritance of gene expression patterns. However, not all chromatin changes are necessarily inherited. Such changes very often accompany transient up- or downregulation of transcriptional activity in classic signal transduction pathways required to maintain a cell's homeostasis rather than its differentiation status, and may be short-lasting (Talbert and Henikoff 2006; Carlberg and Seuter 2010). It is therefore important to distinguish between heritable (truly epigenetic) and non-heritable effects of chromatin modifications. One example of the latter may be hormone nuclear receptors and their interactions with chromatin modifiers that we will discuss later in this chapter.

Lehner et al. (2006) systematically mapped genetic interactions in *Caenorhabditis elegans* using RNA interference. They identified a class of six highly connected "hub" genes with the characteristic properties of buffers of genetic variation. The buffering capacity of these hubs was recognized due to the enhancement of the phenotypic consequences of mutations in many unrelated genes following their inactivation. In particular, the hub genes were found to interact with components of multiple signaling pathways. Interestingly, all six hub genes turned out to be elements of the chromatin remodeling machinery. This discovery illustrates the capacity of chromatin to serve as a modulator and integrator of different signaling pathways in the cell. Not surprisingly, in multicellular eukaryotes, null mutations in genes encoding components of the chromatin modification system are very often either embryo-lethal or they severely hamper growth and development (Hargreaves and Crabtree 2011). This confirms the strong correlation between these hubs and viability, sometimes described as the "centrality–lethality rule" (Jeong et al. 2001).

In order for a protein to serve as a hub it must be highly connected, i.e., capable of interactions with a large number of protein partners implicated in the majority of key cellular processes. This is why disruption of a hub leads to such catastrophic consequences for the entire cellular physiology. If the hub is placed at the crossroads of several cellular signaling pathways and the downstream effector system (like the chromatin machinery controlling the accessibility of DNA to transcription regulators), it may act as a checkpoint and integrator of the flow of chemical information to and from DNA. The concept of a "hub" is derived from analysis of the topologies of regulatory networks and does not directly translate to physical structures acting in the cell. However, in many cases, proteins acting in well-interconnected and functionally adjacent networks tend to be involved in the formation of more or less tight physical complexes (Zotenko et al. 2008). Importantly, hubs are conserved among different species. They are often duplicated or represented by slightly different and partially redundant variants, which greatly increases the robustness of biological networks and their tolerance to mutations (Kafri et al. 2008).

We will illustrate the concept of the hub function using the example of SWI/SNF complexes. The evolutionarily conserved SWI/SNF (from SWItching mating type/Sucrose Non-Fermenting) family belongs to a wider class of ATP-dependent chromatin remodelers that also includes the ISWI, CHD, and INO80 families.

SWI/SNF are large (up to 2 MDa) multi-protein complexes comprising 8–10 subunits, including a single catalytic SNF2-type ATPase. The complexes are capable of modifying DNA–histone interactions through the DNA-translocase activity of the ATPase, leading to chromatin remodeling and changes in the accessibility of DNA sequences to trans-acting factors. In multicellular eukaryotes, SWI/SNF complexes have been implicated in numerous activities that require access to DNA, including DNA replication, repair, chromosomal stability, centromere function, and, of course, gene expression (Clapier and Cairns 2009). These complexes have been shown to be mostly involved in transcriptional activation, but there are also numerous reports implicating SWI/SNF in repression (Trotter and Archer 2008). In order to better understand the chromatin-wide functions of these complexes, Euskirchen et al. (2011) recently used ChIP-seq to globally map regions that bind SWI/SNF ATPase and two major core components (homologs of subunits SNF5 and SWI3) of the human SWI/SNF. They found that SWI/SNF in chromatin characteristically overlaps regions like enhancers and promoters that require tight control of nucleosome occupancy. It is believed that the targeting of SWI/SNF to particular genomic sites, and its activity (e.g., in either transcriptional activation or repression), is largely dependent on the subunit composition of the complex and its transient interactions with different regulatory proteins. This is consistent with analysis by mass spectrometry of immunoprecipitated SWI/SNF complexes (Euskirchen et al. 2011), which showed that they co-purify with an extensive panel of proteins (over 100 in total) involved in key processes regulating chromosome structure, nucleosome positioning, and chromatin assembly, and through these interactions, directly affect the cell cycle and differentiation. Moreover, analysis of the network of overrepresented pathways identified using ChIP-seq for SWI/SNF core components revealed several in which SWI/SNF chromatin remodeling performs critical roles. Together, these analyses indicate that SWI/SNF complexes, through combinatorial assemblies of core subunits and interacting partners, can facilitate cross talk between genomic events and an array of fundamental cellular processes.

3.2 Nuclear Hormone Receptors: Key Players in Dynamic Combinatorial Networks Linking Hormone Signaling with Chromatin

As mentioned above, animal nuclear receptors (NRs) comprise a large family of ligand-activated transcription factors that share elements of protein structure (conserved DNA-binding and ligand-binding domains) and have a common mode of operation (reviewed in Aranda and Pascual 2001). Humans have close to 50 different NRs that control both development and the metabolism of the body. The ligand-binding domain (LBD) is critical for linking the ability of NRs to bind DNA via the DNA-binding domain (DBD), with inputs flowing from the

cellular environment. The DBD is comprised of a type-II zinc finger motif and is responsible for targeting the receptors to their cognate hormone response elements (HRE). It binds DNA as a dimer, with each monomer recognizing a six-base-pair DNA sequence element. The LBD is responsible for several different activities including binding of the low-molecular weight ligand, homo- or heterodimerization, interaction with heat-shock protein (cytosolic NRs), and activation or repression of transcription. The binding of a hormone ligand triggers all subsequent events by inducing a conformational change in the LBD that alters its affinity for other proteins. The fundamental property of NRs that enables them to act as regulated switches of gene transcription is their ability to interact with a network of co-regulatory proteins. This depends on the specific activation function 2 (AF-2) domain located in the C-terminal part of the LBD, which undergoes dramatic rearrangement upon ligand binding, resulting in the formation of a new binding surface for co-activators. In cooperation with the AF-1 activation domain located in the N-terminal part of the LBD, the newly created interacting surfaces of AF-2 enable the recruitment of a series of adaptor proteins, which in turn recruit chromatin-modifying complexes, such as histone acetyltransferases containing CBP/p300 and p/CAF, histone arginine methyltransferases containing CARM1 or PRMT1, and SWI/SNF and other ATP-dependent chromatin remodelers (Aranda and Pascual 2001). Upon assembling around a promoter, this complicated network of chromatin modulators is bound by a Mediator complex, which provides a direct link to the basal transcriptional apparatus. The current concept of the function of this complicated system, based on the results of ChIP and FRAP analyses, is that of a "transcriptional clock" enabling the sequential recruitment of co-activators (Carlberg and Seuter 2010). Recent experimental data suggest that the whole system is highly dynamic and subject to rapid posttranslational modification of its components by phosphorylation, methylation, acetylation, and ubiquitination (reviewed by Rochette-Egly 2005).

3.3 SWI/SNF Function at Promoters of Hormone Response Genes

As stated above, hormone binding by a nuclear receptor enables its interaction with co-activators, one of which is the ATP-dependent chromatin remodeling complex, SWI/SNF. Genes regulated by steroid hormones were found to be significant in vivo targets of regulation by SWI/SNF complexes (Zraly et al. 2006; Belandia and Parker 2003). It is now generally agreed that SWI/SNF binding plays a critical role in determining the dynamics of nucleosomes at promoters of genes targeted by NRs. For example, the expression of human SWI/SNF in SWI/SNF-deficient mouse cells was found to greatly increase the accessibility of the Glucocorticoid Response Element sequence normally protected by a single nucleosome (Nuc B) positioned on the Mouse Mammary Tumor Virus (MMTV) promoter (reviewed in Hebbar and Archer 2003). Furthermore, interactions of the glucocorticoid receptor (GR) with

its cognate sequences in promoters were shown to be invariably associated with localized modulation of the chromatin structure (John et al. 2008). Surprisingly, the use of dominant negative mutants in chromatin remodeling revealed that these structural transitions (reflected by DNase I hypersensitivity) were in many cases independent of SWI/SNF (John et al. 2008). From these studies it was concluded that GR action throughout the mammalian genome is universally associated with the reorganization of positioned nucleosomes, in a process that may involve various remodeling activities. While some of the remodelers may be recruited by GRs in a manner typical for the recruitment of co-activators by transcription factors, others can be associated with the target site prior to any GR interaction. It is possible that the pre-association of the remodeling machineries with particular hormone NR sites may be critical in determining the tissue-specific responses to steroid hormones. With regard to SWI/SNF, it was shown that these complexes cycle dynamically between chromatin-associated and dissociated states, in a manner that is dependent on the hormone and a functional ATPase domain of the BRM (or BRG1) catalytic subunit (Johnson et al. 2008). These results provide direct proof of a functional link between local chromatin remodeling by SWI/SNF, and transcriptional activation at the MMTV promoter. Moreover, SWI/SNF remodeling activity mediates cyclic binding and unbinding of GR to positioned nucleosomes. The nature of GR–nucleosome interactions is therefore transient and highly dynamic. The frequency of GR recruitment to its cognate sequence probably depends on many factors, one of the most important being the availability of SWI/SNF complexes (Mellor 2006).

The recent development of the microarray-based ChIP-on-chip and ChIP-seq approaches has enabled rapid mapping of nucleosome positions over large segments of chromosomal DNA, which has given unprecedented insight into chromatin dynamics related to transcription. Some recent results using these approaches have provided completely novel information about the functional role of nucleosome movements in promoter regions. The nucleosome position map of actively transcribed genes shows two highly characteristic 5′ and 3′, 150–200 bp "nucleosome depleted regions" (NDRs), positioned upstream of the transcription start site (TSS) and downstream of the transcription termination site (TTS). These two NDRs form clear structural demarcation points of the transcriptional units. The 5′ NDRs are flanked by two tightly bound positioned nucleosomes: the "+1" located downstream of the NDR, overlapping the TSS, and the "−1" placed at a characteristic distance upstream of the NDR. These two nucleosomes generally contain the histone H2A.Z variant instead of H2A, which makes them more labile compared with canonical nucleosomes. The first few nucleosomes located downstream of the +1 position show rather strong phasing that becomes more fuzzy for those located further from the TSS (reviewed in Arya et al. 2010).

It has been demonstrated that transcription factor binding sites usually colocalize with the 5′NDRs, which makes the accessibility of these regions absolutely critical for the cellular response at the gene expression level to the occurrence of or changes in the concentration of transcription factors. While the establishment of NDRs is partly dependent on the affinities of specific DNA sequences for histone octamers

(Brogaard et al. 2012), chromatin remodeling, particularly of the ISWI and SWI/SNF type, has also been shown to play an active role *in vivo* in establishing and maintaining fully functional 5′ NDRs reviewed in (Iyer, 2012). Accordingly, yeast mutants in the SWI/SNF showed altered chromatin structure of promoters leading to impaired activation and repression of different genes including those responding to environmental stimuli (Bryant et al. 2008; Shivaswamy and Iyer 2008).

The recent data are consistent with the interpretation that SWI/SNF complexes (and probably other types of remodelers, particularly those with histone chaperone activity) function primarily to overcome sequence-dependent effects during nucleosome positioning. Accordingly, the absence of active SWI/SNF-mediated remodeling results in some promoters acquiring excess nucleosomes, which hampers transcriptional activation (Tolkunov et al. 2011). Another important factor influencing nucleosome occupancy at promoters could be the availability of different interaction partners of SWI/SNF, which can affect its efficiency in remodeling, association with histone modification complexes, and stability. It is possible that through these interactions, the cell can integrate different pathways affecting SWI/SNF activity with the nuclear hormone receptor-dependent responses.

A surprising twist in the story linking steroid hormone receptor function with structural features of chromatin came in the recent report of Pham et al. (2011), who examined genome-wide nucleosome occupancy at human promoters upon GR activation by dexamethasone. The most prominent effect of GR activation was rapid increased in nucleosome occupancy at existing nucleosome peaks within 2 kb of the TSS in most Pol II genes, which was a genome-wide phenomenon, occurring both on genes regulated and not regulated by GR. Surprisingly, the BRG1-containing SWI/SNF complex was important for both high nucleosome occupancy after GR activation and low nucleosome occupancy in the absence of GR activation. Irrespective of the biological meaning of this phenomenon, these data confirm the essential role of chromatin remodeling in structural transitions at promoters that accompany responses to hormone signals.

3.4 Central Role of the Ubiquitin–Proteasome System in Controlling the Dynamics of NR-Associated Complexes

In the past decade, the ubiquitin–proteasome system (UPS) has emerged as a central constituent of the hormonal regulatory network in both animals and plants (see below for a discussion of the role of the UPS in plant signaling). Basically, the UPS acts in two stages: in stage 1 a protein is tagged for degradation by the three-step enzymatically catalyzed addition of ubiquitin polypeptides, and in stage 2 the tagged protein is recognized by the 26S proteasome, a large multi-subunit protease complex, which proteolytically degrades it to small peptides, releasing the ubiquitin tag for further use. The enzymatic components that link chains of ubiquitin to proteins consist of the E1 ubiquitin-activating enzyme, a certain number of

E2 ubiquitin-carrier or conjugating enzymes, and a large number of critical E3 ubiquitin-protein ligases that provide specificity to the system by recognizing protein substrates and directly coupling them to activated ubiquitin. The results of global genome-wide chromatin immunoprecipitation with antibodies recognizing constituents of the 26S proteasome in yeast, surprisingly revealed that the majority of ca. 6,400 yeast genes are associated with proteasomal proteins. A more detailed characterization of these associations showed that the 26S proteasome or its 19 and 20S subcomplexes are broadly involved in DNA-centered activities, particularly RNA polymerase II-dependent transcription (Sikder et al. 2006). It also became apparent that ubiquitination and 26S proteasome-mediated degradation are common among proteins participating in the functioning of nuclear receptors. Ubiquitination and degradation in response to the ligand were observed for NRs, different co-activators and co-repressors, as well as components of the general transcriptional apparatus. The prevailing view is that this widespread proteasome-mediated degradation serves to remove resident co-activators and co-repressors to clear the stage ready for the next complement required to adjust the cellular machinery to newly occurring signal (Nicolaides et al. 2010; Keppler et al. 2011).

Several recent biochemical studies have shown that animal SWI/SNF complexes are also direct targets of regulation by 26S proteasome degradation (Chen and Archer 2005; Sohn et al. 2007; Keppler and Archer 2010). Among the different subunits of mammalian SWI/SNF (called BAF–Brg-1 Associated Factors), BAF57 is a direct interaction partner of estrogen receptor alpha (Belandia et al. 2002) and the androgen receptor (Link et al. 2005), which facilitates recruitment of the SWI/SNF complex. The recruitment of SWI/SNF to the GR occurs through interaction with BAF60a (Hsiao et al. 2003), which also mediates interaction between p53 and the SWI/SNF complex. BAF155, a homolog of SWI3 and a key scaffolding subunit of the complex, has been shown to play a central role in controlling and maintaining the stoichiometry of SWI/SNF subunits in the cell. One way in which it fulfills this function is through controlling the interactions of other subunits with TRIP12, the E3 ubiquitin ligase responsible for the ubiquitination of BAF57 (Keppler and Archer 2010). Another recent study demonstrated that the catalytic activity of the 26S proteasome limits the inducible association of SWI/SNF with promoters of SWI/SNF-regulated inflammatory genes through proteasomal degradation of the ATPase subunit Brg1 (Cullen et al. 2009). Thus, proteasomal degradation is critical not only for maintaining the stoichiometry of subunits during SWI/SNF formation, but also for regulating the extent of chromatin remodeling performed by the complex.

The general picture of animal ligand-inducible nuclear receptor functioning that emerges from the studies outlined above is that of a complex network of elements in which NRs serve as adaptors linking *cis*-regulatory regions of genes with chromatin-modifying complexes and the RNA Pol II machinery. All these elements are connected via a common ubiquitin–proteasome regulatory system that acts as a functional matrix through which different elements can be fine-tuned and coordinated. It is obvious that this system is also subject to other controls, like those exerted by posttranslational modification (e.g., phosphorylation) of its components.

4 Chromatin Mechanisms and Hormonal Signaling in Plants: A Story That Has Barely Begun

4.1 The Hypothetical Plant Nuclear Receptor

It is clear that among plant hormonal signaling pathways the auxin, jasmonate, and gibberellin systems represent the closest analogs to hormone-induced nuclear receptors of animals. Both act in the nucleus, involve the activation of nuclear proteins (or proteins transported to the nucleus) via conformational change induced by binding of small-molecular weight ligands, and work by directly affecting transcription. Moreover, both systems are functionally and physically connected to the ubiquitin targeting and conjugation module of the 26S proteasome protein degradation system. The major difference is that plants do not possess an obvious equivalent of a canonical animal nuclear receptor, i.e., a protein with the properties of both a typical transcription factor (DNA-binding domain) and a ligand receptor (ligand-binding domain). We are of the opinion that hormonal signaling systems of plants and animals are not linked by a common evolutionary origin, but are an example of convergence, whereby the same or a similar biological trait is achieved by unrelated mechanisms. It is therefore of little use to look for sequence homologies in order to reveal which element of the plant hormone receptor system is indeed the equivalent of the animal NR. Instead, one should apply an analogy/ convergence rule to determine the functional equivalence between particular components of animal and plant systems.

What are the conditions that the NR has to fulfill in order to play its role as an adaptor between the external biochemical environment and DNA sequence elements? It must undergo conformational changes following binding of a ligand and then specifically recognize a cognate DNA sequence to activate or repress transcription. While the F-box protein receptors of auxins and jasmonates, TIR1 and COI1, respectively, or the GID1 receptor of gibberellins fulfill the first requirement, they do not by themselves bind to DNA. DNA binding at hormone-specific *cis*-acting sequences (also termed Hormone Response Element—HRE) is the function of *bona fide* transcription factors: ARFs in the auxin pathway, TFs specific for jasmonic pathway-responsive genes, and specific TFs, like the basic-helix-loop-helix (bHLH) transcription factors in the GA pathway (Hauvermale et al. 2012). At low hormone levels, these factors are associated with repressors that block their function: AUX/IAA, JAZ, and DELLA proteins, respectively. The repressors must be bound by the ligand-activated protein partners (TIR1, COl1, or GID1, respectively) in order to release TFs, which may then act at the transcriptional level. Therefore, functional plant nuclear receptors, equivalent to animal NRs, appear to be modular in nature and consist of three elements: (1) a sequence-specific transcription factor (TF), (2) a TF-associated inactivation factor blocking the interaction of the TF with DNA and/or the transcriptional machinery, and (3) a ligand-responsive factor. These modular nuclear receptors, like the canonical NRs of animals, are subject to constant surveillance by the ubiquitin-targeting system, to

which they are in fact physically linked via F-box-containing or F-box-interacting elements. To look for further analogies with animal NRs, we must now turn to the plant SWI/SNF chromatin remodeling machinery.

4.2 Plant Chromatin Remodeling and Its (Potential) Connections to Hormonal Signaling

Plant genomes encode 41 proteins belonging to the Snf2 family of ATPases that are capable of mediating chromatin remodeling using energy derived from ATP hydrolysis. This large group contains representatives of the major subfamilies occurring in yeast and animals as well as plant-specific subfamilies (Knizewski et al. 2008). Here, we will concentrate on the plant SWI/SNF and CHD subfamilies, which have been most thoroughly characterized.

The SWI/SNF subfamily is represented in *Arabidopsis* by four ATPases: SYD, BRM, CHR12, and CHR23. *Arabidopsis* also has homologs of all the other major core subunits of the SWI/SNF complex (Jerzmanowski 2007). Studies on insertional mutants in different subunits of SWI/SNF suggest that they take part in both the control of developmental programs and in responses to environmental cues. The best described are the functions in development of the BRM and SYD ATPases, and the four different SWI3 homologs: SWI3A, SWI3B, SWI3C, and SWI3D (Bezhani et al. 2007; Hurtado et al. 2006; Tang et al. 2008; Sarnowski et al. 2005). Three lines of evidence link plant SWI/SNF functions with hormone signaling:

1. Transcriptional profiling of *brm* and *syd* Arabidopsis lines has revealed disturbances in many genes involved in hormone signaling pathways, particularly those of auxins and gibberellins (Bezhani et al. 2007). Furthermore, transcriptional profiles of *brm* and *ga1-3*, a mutant defective in GA synthesis, show significant overlap (over 40 % of the differentially expressed genes in *brm* and *ga1-3*, compared with wild-type plants) (Archacki et al. 2013), suggesting that the hormone pathway and SWI/SNF remodeling frequently converge on common gene targets. Consistently, BRM was shown to act as activator directly associating with promoters of *GIBBERELLIN 3-OXIDASE 1* (*GA3ox1*) and *SCARECROW-LIKE 3* (*SCL3*), two genes involved in GA biosynthesis and signaling, respectively (Archacki et al. 2013).
2. SYD ATPase is directly recruited to promoters of *MYC2* and *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) genes involved in jasmonate and ethylene pathways (Walley et al. 2008), and BRM regulates *ABA INSENSITIVE 3* (*ABI3*) and *ABA INSENSITIVE 5* (*ABI5*) genes involved in ABA pathway (Han et al. 2012).
3. One of the SWI/SNF core subunits, SWI3B, was shown to interact with PP2C phosphatase (Saez et al. 2008), a key negative regulator of ABA-dependent genes and a target of the ABA-bound PYR/PYL/RCAR receptor (see above), suggesting that SWI/SNF may directly modulate the ABA response at the receptor level.

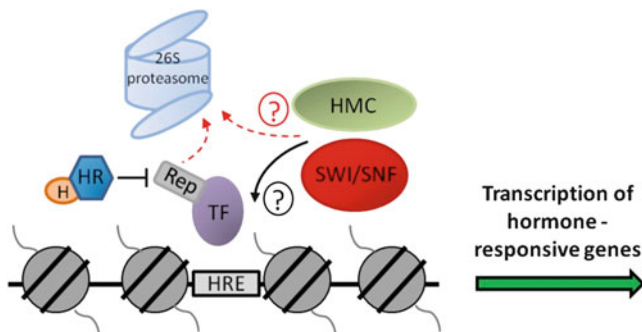

Fig. 1 Hypothetical involvement of chromatin-based mechanisms in regulation of hormone-dependent gene expression in plants. By analogy to animal systems, a complex functional plant nuclear receptor, consisting of hormone receptor protein (HR), repressor (Rep), and transcription factor (TF), cooperates (either directly via protein–protein interactions or indirectly) with different factors that modify chromatin, including chromatin remodeling complexes (for example SWI/SNF) and histone-modifying complexes (HMC). Binding of hormone (H) to the receptor triggers degradation of the repressor (Rep) protein by the 26S proteasome system. It is currently unknown whether chromatin remodeling complexes or histone-modifying complexes are targets for ubiquitin–proteasome regulatory system in plants. HRE hormone response element

In plants, similarly to animals, SWI/SNF remodelers seem to be instrumental in controlling the chromatin state at promoters to which they are recruited by specific transcription factors. It was recently shown that two activators, LEAFY (LFY) and SEPALLATA3 (SEP3), interact with SWI/SNF to recruit the complex to the promoters of the homeotic genes AP3 and AG, respectively, during flower development (Wu et al. 2012). The same scenario might occur over many hormone-regulated loci (Fig. 1). If this is the case, is such a mechanism employed exclusively for local gene-specific regulation or, as has been shown for animals, is it connected to a wider network that can be controlled in a systemic way, e.g., by factors affecting the abundance of currently available remodelers? In this respect, it would be especially interesting to know if plant SWI/SNF remodelers are regulated at the protein level by the 26S proteasome system, which, as has been amply demonstrated, is critical for plant hormone signaling.

Interestingly, another plant chromatin remodeler PICKLE (PKL), a member of the CHD (chromodomain)-containing Snf2 ATPase family, which is involved in the repression of seed-specific traits in germinating seedlings, was shown to be related to GA signaling. The *pkl* phenotype is enhanced by inhibition of GA biosynthesis, suggesting that GA acts in a parallel pathway to repress expression of seed-associated traits (reviewed by Zhang and Ogas 2009). Interestingly, PKL also seems to contribute to GA signaling during post-germinative growth. Moreover, adult *pkl* plants display the characteristic phenotypic traits of GA signaling mutants, like increased levels of active GAs and lowered responsiveness to externally applied GAs, consistent with the role of PKL in the GA signaling pathway during the post-germinative phase of growth (Henderson et al. 2004). Microarray analyses have revealed that PKL and GA have common targets (over 30 % overlap among

seed genes repressed by each). However, the analysis of epistasis with respect to these common targets suggests that they are controlled by PKL and GA via parallel pathways rather than a linear pathway (Zhang et al. 2008). In addition to the link between SWI/SNF and phytohormones described above, this is yet another example of chromatin modification and a hormonal signaling pathway converging on the same genes.

It is highly plausible that the activity of chromatin remodelers at many plant hormone-controlled promoters is accompanied by different histone modifications, having either positive or negative effects on transcription, as has been shown for animal thyroid hormone receptor-regulated TSH promoters (Wang et al. 2010). Such modifications have the potential to be transmitted mitotically, offering the possibility of stabilization of the hormone-induced transcriptional state. However, there are few reports about involvement of histone modifications in regulation of plant hormone-dependent genes (Yu et al. 2008; Fukazawa et al. 2010; Cho et al. 2012). The global dynamic changes in histone modifications during hormone-mediated induction have yet to be characterized.

5 Conclusions

Here we have reviewed the current concepts of hormone-induced nuclear receptor functioning in animals with the aim of identifying common themes with the emerging, albeit still less detailed, picture of the functioning of plant hormonal pathways. Our major focus was the chromatin (epigenetic) level of regulation and how and to what extent the chromatin modification apparatus may participate in the functioning and possibly also the integration of different plant hormone signaling pathways.

While many basic mechanisms of chromatin modification, e.g., ATP-dependent nucleosome remodeling, can be considered generic as they are able to affect any region of the genome, they can also act highly selectively due to their association with sequence-specific recruiting factors, mostly transcription factors. This is best illustrated by studies on GR receptors in animals, which use chromatin remodelers as co-activators. Here, the chromatin modification system is not a typical element of a linear hormone signaling pathway with a demonstrable epistatic functional relationship with the other elements. Rather, it represents a branching out of the system from the level of the nuclear receptor to that of the chromatin machinery, which can also act independently of hormone signaling and be affected by many other regulatory pathways. This makes the eventual target of hormone signaling open to other influences that may be exerted in parallel through the connection of chromatin modifications to many different external and internal cues. The relationship between hormone signaling and chromatin modifications is therefore likely to be based on networking rather than on straightforward interactions between subsequent steps in a linear pathway. This may be the reason why there is very limited number of reports linking mechanistically hormone signaling with epigenetic regulation (Zhu 2010).

However, besides the characterization of the process by which particular hormone receptors interact with the chromatin machinery, there is another aspect of

epigenetic regulation of hormonal signaling. That is the potential integrator role in the hormone/chromatin co-regulatory network of the proteasomal protein level control system. This has recently emerged as an important mechanism responsible for functional connections of different processes occurring in the nucleus. How does it operate? What are the roles of protein–protein interactions in the large multi-subunit megacomplexes and of protein modifications (e.g., phosphorylation) in directing particular proteins for destruction and in preventing the destruction of others? The demonstrated connection between the animal SWI/SNF chromatin modification system and both nuclear hormone receptors and the proteasomal targeting system makes the SWI/SNF complexes an ideal focus point for studying such questions. Plants, with their specific hormonal signaling, which depends to a much greater extent than in animals on integration at the single cell level, would be a particularly suitable model for these studies.

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Epigenetic Signalling During the Life of Seeds

Martijn van Zanten, Yongxiu Liu, and Wim J.J. Soppe

Abstract Seeds are essential for reproduction and dispersal of most plant species and constitute a major human food source. The life of a seed is characterised by two major phase transitions, from embryogenesis to seed maturation and from dry seed to germination. These different stages are characterised by specific transcriptomes and require silencing and activation of diverse sets of genes. In addition, fully mature seeds contain very small nuclei with highly compacted chromatin, which is established during seed maturation. These unique characteristics require extensive epigenetic signalling mechanisms to tightly coordinate the phase transitions and control chromatin accessibility. This chapter gives an overview of our present knowledge of the role of epigenetics in the life of seeds, focussing on the later stages of seed development, dormancy and germination in *Arabidopsis thaliana*.

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1 Introduction

Seeds are essential for reproduction and dispersal of most plant species and constitute a major human food source. Dry seeds represent a unique phase in the plant life cycle and are characterised by very low metabolic activities and absence of growth and development. Due to the evolution of this quiescence phase, seed plants were able to inhabit new environments that are not supportive for plant growth during all seasons of the year. Although the size and structure of seeds are highly variable among plant species, they typically consist of an embryonic plant with food reserves contained by protective tissues and generally have very low moisture contents. These properties make seeds highly resistant against various abiotic stresses and enable them to survive long periods of unfavourable environmental conditions (Linkies et al. 2010).

In addition to their unique physiology, seeds have a complex structure that consists of three different components, each with its own genotype. In diploid plants, the diploid embryo is formed after fertilisation and contains an equal contribution of the parental and maternal genome. The endosperm is also a fertilisation product but is triploid and contains two maternal and one paternal genome equivalent. Finally, the diploid testa contains only the maternal tissue. The balanced growth and development of a seed requires communication between these different tissues and attuning of their genetic programmes (Ohto et al. 2007).

Seed development starts after fertilisation and can be divided into two main phases: embryo development and seed maturation (Fig. 1; phases 1 and 2). The length of the seed development phase and the morphology of seeds greatly differ among plant species. Especially the fate of the endosperm is highly variable, ranging from constituting the major part of a seed to a complete absence. The endosperm of the model plant *Arabidopsis thaliana* consists of a single cell layer (Linkies et al. 2010). At the end of the embryo development phase, the gross morphology of the seed has been established and the endosperm and seed coat have attained their final shape. Nevertheless, these seeds are not prepared yet to survive outside the mother plant and still have a high moisture level. Seeds dehydrate and become desiccation tolerant during the seed maturation phase. In addition, storage compounds accumulate and dormancy is induced (Vicente-Carbajosa and Carbonero 2005). Dormancy is defined as the inability of a seed to germinate under favourable environmental conditions and evolved to survive temporal favourable conditions during seasons that are unfavourable for plant growth (Holdsworth et al. 2008). When the seeds have shed from the mother plant, seed dormancy is gradually released during after-ripening or can be broken by imbibition at species-specific temperatures (Graeber et al. 2012).

When seeds are stored under dry conditions, they are metabolically quiet and only show minor changes in transcript levels (Finch-Savage et al. 2007). However under most natural conditions, seeds do not encounter a dry environment but frequently experience imbibition for extended periods of time. This increases their metabolic activity. Seeds in the soil continuously trace environmental conditions to select the optimal moment for germination. This coincides with major changes in transcript levels of dormancy and germination-related genes (Footitt et al. 2011). When seed

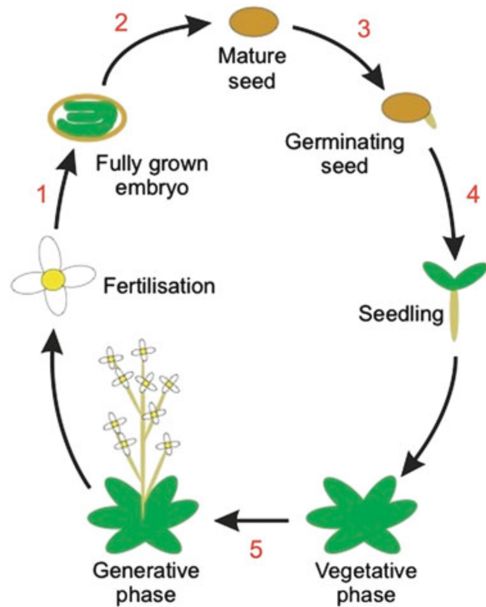

Fig. 1 Overview of the life cycle of *Arabidopsis*. *Numbers* indicate the major phase transitions during the life cycle. (1) Embryogenesis; (2) Seed maturation; (3) Germination; (4) Shift from heterotrophic to photoautotrophic growth and (5) Floral induction

dormancy has been released and favourable environmental conditions are met, seeds enter their final phase changes, germinate (Fig. 1; phase 3) and become photo-autotrophic (Fig. 1; phase 4).

The phytohormones abscisic acid (ABA) and gibberellin (GA) play central roles in the regulation of the different phase changes during the seed life. ABA is a positive regulator of seed maturation, including induction of dormancy, and negatively regulates germination. On the other hand, GA promotes germination and has a negative influence on various ABA responses (Kucera et al. 2005). ABA action during seed maturation is closely connected with the expression of four master transcriptional regulators; *LEAFY COTYLEDON 1* (*LEC1*), *LEC2*, *FUSCA 3* (*FUS3*) and *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) (Holdsworth et al. 2008; To et al. 2006). These central regulators are downregulated during imbibition and remain repressed throughout plant development until a new generation has started again.

The phase transitions during the life of seeds require large-scale reprogramming of the genome, as evidenced by microarray experiments in *Arabidopsis* (Cadman et al. 2006; Nakabayashi et al. 2005). Phase transitions are often associated with major changes in chromatin structure and involve tight and dynamic transcriptional control imposed by epigenetic modifications. This ensures proper progress through the developmental programmes (Exner and Hennig 2008; Fransz and De Jong 2011; see also chapter "Environment-induced chromatin reorganization and plant acclimation"). Therefore, it is not surprising that several of the mutants with known defects in the seed life harbour mutations in genes controlling chromatin organisation. Accordingly, loss-of-function mutations in genes required for DNA methylation resulted in improperly developed embryos and reduced viability (Xiao et al. 2006).

Epigenetic regulation of gene expression and maintenance of genome stability are thus crucial during this early phase of the plant life cycle. This chapter will give an overview of the role of epigenetics in controlling the life of seeds. Epigenetic signalling during imprinting, which is important in the early phases of embryo development, is reviewed by Huh and Rim (see chapter “DNA demethylation and gene imprinting in flowering plants”). Aspects of chromatin structure and function during seed maturation, seed dehydration, and during germination are described by Fransz (Chapter “Environment-induced chromatin reorganization and plant acclimation”).

In this chapter, we extensively discuss the later stages of seed development, seed dormancy and early germination. The next sections will describe the different phases of the life of a seed and the role of epigenetic signalling mechanisms in controlling these phases. We will focus our overview on the model plant *Arabidopsis*, which has been at the centre of epigenetic research.

2 Seed Maturation

2.1 Dynamic Changes in Nuclear Architecture and Chromatin Organisation During Seed Maturation

It was recently shown that the size of embryonic cotyledon nuclei strongly reduces during seed maturation in *Arabidopsis*; nuclear size is recovered upon germination. Consistent with the small nuclear size, the chromatin in embryos of ripe seeds is highly condensed (van Zanten et al. 2011). Increased chromatin compaction is often associated with decreased transcriptional activity (Fransz and de Jong 2011; Exner and Hennig 2008). However, at the end of seed maturation, although nuclei are small and the chromatin is highly condensed, the overall transcript levels are similar to those in other tissues (van Zanten et al. 2012). Part of these transcripts could have been stored and produced earlier during seed development. However, several maturation-related genes show their highest expression levels at the end of seed maturation, which suggests the existence of specific mechanisms that enable efficient transcription despite the dense chromatin (Liu et al. 2011). Genetic studies have uncovered a number of loss-of-function mutations of chromatin-related genes that lead to ectopic expression of embryo-associated genes. Characterization of these mutants has emphasised the importance of chromatin structure in the seed-specific transcriptional programmes and will be discussed in this section.

2.2 Control of Seed Maturation by Four Key Transcriptional Regulators

As mentioned in the introduction, the LEC1, LEC2, FUS3 and ABI3 transcription factors function as master regulators of seed maturation in *Arabidopsis* (Gutierrez et al.

2007; Suzuki and McCarty 2008). A redundant regulatory network among these four proteins controls seed maturation progress through direct interactions with regulatory elements present in the promoters of maturation-related genes (Reidt et al. 2000; To et al. 2006). ABI3, FUS3 and LEC2 are related B3-binding domain transcription factors (Giraudat et al. 1992; Luerssen et al. 1998; Stone et al. 2001), and LEC1 is a HAP3 subunit of the CCAAT-binding transcription factor (Lotan et al. 1998). The *LEC* genes are expressed early in embryogenesis and promote growth arrest of the embryo by inhibiting cell division, whereas *ABI3* acts later in seed development to promote dormancy and desiccation (Raz et al. 2001). Ectopic expression of *LEC1* or *LEC2* is sufficient to confer embryonic traits to vegetative organs (Lotan et al. 1998; Stone et al. 2001; Santos-Mendoza et al. 2005). Overexpression of *ABI3* and *FUS3* results in the ectopic expression of some seed maturation genes, such as *SEED STORAGE ALBUMIN 3* and *CRUCIFERIN C*, in vegetative tissues in an ABA-dependent manner (Parcy et al. 1994; Kagaya et al. 2005). Absence of LEC1, LEC2, FUS3 or ABI3 function alters accumulation of seed storage reserves and causes reduced dormancy. In addition, the loss of some of these factors can lead to a decrease in desiccation tolerance and chlorophyll degradation, as well as reduced ABA sensitivity upon germination (Brocard-Gifford et al. 2003; Gazzarrini et al. 2004; Kagaya et al. 2005; Vicente-Carbajosa and Carbonero 2005; To et al. 2006; Stone et al. 2008).

Little is known about the transcriptional and epigenetic regulation of the *LEC1/LEC2//ABI3/FUS3* master regulators themselves during seed maturation. In the last years, genome-wide analyses of DNA methylation and histone modifications have increased our understanding of the epigenome (Zhang et al. 2006, 2007a; Zhang and Ogas 2009; Turck et al. 2007; Charron et al. 2009; Roudier et al. 2011). These genome-wide analyses evidently included seed-related genes. Their "histone code" in *Arabidopsis* seedlings is summarised in Table 1. However, these epigenomic maps did not include a lot of information about *LEC1/LEC2/ABI3/FUS3* transcription and only the histone H3 lysine 4 dimethylation (H3K4me2) marker for active transcription could be linked to their activity. In addition, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) and histone H3 lysine 27 trimethylation (H3K27me3) are seemingly involved in the repression of *LEC1/LEC2/ABI3/FUS3* transcription in seedlings (Zhang et al. 2007a, b; Charron et al. 2009; Table 1; Fig. 2). It will be necessary to check additional chromatin markers to generate fine epigenomic maps of master regulators of seed maturation. Previous analyses have not been carried out on maturing or dry seeds and do not reflect the epigenetic state of this developmental stage. Chromatin immunoprecipitation (ChIP) experiments with maturing or dry seed have not been successfully performed yet, despite attempts of several labs. This is probably due to the highly compacted state of the chromatin in dry seeds, which likely hampers efficient access of antibodies after chromatin shearing (van Zanten et al. 2011).

2.3 Repression of Germination Genes During Seed Maturation

During seed maturation and in the dormant mature seed, germination is repressed even under favourable environmental conditions. The DELLA protein REPRESSOR OF

Table 1 List of genes that function in seed maturation and their association with epigenetic marks or proteins in seedlings

Epigenetic marker or protein	Effect[a]	*LEC1*	*LEC2*	*FUS3*	*ABI3*	*GAI*	*RGA*	*RGL2*	*SPT*	*PIL5*	References
DNA methylation	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	Zhang et al. (2006), Roudier et al. (2011)
LHP1	–	D	D	D	D	ND	ND	ND	D	ND	Zhang et al. (2007b), Turck et al. (2007)
H3K27me3	–	D	D	D	D	D	D	ND	ND	D	Zhang et al. (2007a), Charron et al. (2009)
H3K27me1	+/–	ND	ND	ND	ND	ND	ND	ND	ND	ND	Roudier et al. (2011)
H3K4me2	+	D	D	D	D	D	D	D	D	D	Roudier et al. (2011)
H3K4me3	+	ND	ND	ND	ND	D	D	D	D	D	Roudier et al. (2011)
H3K9me3	+	ND	ND	ND	ND	D	ND	ND	ND	D	Charron et al. (2009)
H3K36me3	+	ND	ND	ND	ND	D	D	D	D	D	Roudier et al. (2011)
H3K9ac	+	ND	ND	ND	ND	D	D	D	ND	D	Charron et al. (2009)
H3K27ac	+	ND	ND	ND	ND	D	D	ND	ND	ND	Charron et al. (2009)
H2Bub	+	ND	ND	ND	ND	ND	ND	ND	D	ND	Roudier et al. (2011)

[a](+) transcription activating mark, (–) transcription suppressing mark

ND not detected, *D* detected

Table adapted from Zhang and Ogas (2009)

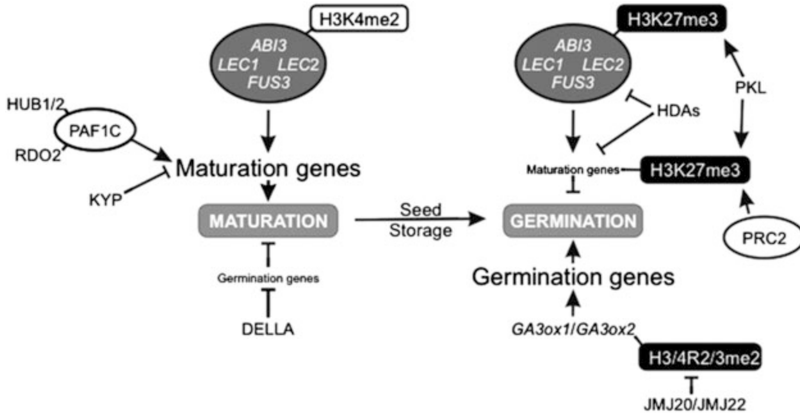

Fig. 2 A model of epigenetic signalling during seed maturation and germination in *Arabidopsis*. Seed maturation is characterised by high expression levels of maturation-related genes and a suppression of germination-related genes, whereas the reverse is true during germination. The relations between the epigenetic regulators and their targets are described in the main text. *White circles* indicate protein complexes involved in epigenetic regulation and *grey circles* represent the four master regulators of seed maturation. Repressive histone marks are shown in *black boxes* and activating marks in *white boxes* that are connected to their target genes

GA1-3 –LIKE2 (RGL2) plays a key role in this by stimulating ABA biosynthesis and ABI5 activity (Piskurewicz et al. 2008). In darkness, two other DELLA proteins, GA-INSENSITIVE (GAI) and REPRESSOR OF GA1-3 (RGA), are required to repress germination in addition to RGL2 (Cao et al. 2005; Fig. 2). Moreover, two basic helix-loop-helix (bHLH) transcription factors, SPATULA (SPT) and PHYTOCHROME INTERACTING FACTOR 3–LIKE 5 (PIL5), are involved in the repression of seed germination by influencing GA signalling (Penfield et al. 2005; Oh et al. 2004, 2006, 2007). SPT is a light-stable repressor of seed germination and mediates germination responses to low temperature. In addition, SPT is required in dormant seeds to maintain the repression of the GA biosynthetic gene GA3-oxidase. PIL5 represses seed germination and GA3-oxidase expression in the dark. PIL5 appears to repress GA responses and seed germination by stimulating expression of *RGA* and *GAI* through direct binding to their promoters (Oh et al. 2007).

Overall, the present data indicate that seed germination during the maturation phase is mainly repressed by inhibition of GA signalling. The above-mentioned epigenomic maps indicate that H3K4me3, H3K36me3 and histone H3 lysine 9 acetylation (H3K9ac) are involved in the transcriptional activation of the three DELLA factors and the two transcription factors *SPT* and *PIL5* (Table 1). H3K27me3 is involved in the repression of *GAI*, *RGA* and *PIL5* transcription. However, empirical evidence about the role of epigenetic signalling in the transcriptional regulation of *SPT*, *PIL5* and DELLA regulatory genes during seed maturation is still lacking.

2.4 *The Role of Histone Modifications in the Control of Seed Maturation*

Altered seed dormancy levels can often be traced back to a misregulation in seed maturation. Seed dormancy is an easily quantifiable trait, and genetic screens for altered dormancy levels led to the identification of several seed maturation genes encoding chromatin remodelling proteins. *HISTONE MONOUBIQUITINATION 1* (*HUB1*) and *HUB2* encode two C3HC4 RING finger proteins with homology to the histone modifying enzyme BRE1 in other species (Liu et al. 2007a). This protein is required for monoubiquitination of histone H2B, which is associated with actively transcribed genes (Hwang et al. 2003). The *hub1* and *hub2* mutants fail to monoubiquitinate histone H2B, resulting in reduced seed dormancy. Several dormancy-related genes such as *DELAY OF GERMINATION 1* (*DOG1*) and *1-CYSTEINE PEROXIREDOXIN 1* (*PER1*) show reduced transcript levels in *hub1* mutant seeds (Liu et al. 2007a).

The function of histone H2B monoubiquitination in plants is not completely understood and its relation with other histone modifications is still in debate. H2B ubiquitination is dynamically regulated during transcription elongation and research in yeast suggested that both ubiquitination and de-ubiquitination are required for this process (Henry et al. 2003). It has been shown in human cell lines that the HUB1 homologue BRE1 binds with the polymerase II-associated factor 1 complex (PAF1C). This complex modulates the local structure of chromatin during transcription elongation and affects methylation of histone H3 at respectively K4 and K36, which are activating epigenetic marks for transcription (Saunders et al. 2006; Kim et al. 2009). Interestingly, the isolation and genetic analysis of additional factors associated with PAF1C, like REDUCED DORMANCY 2 (RDO2) in *Arabidopsis*, confirmed the important role of transcription elongation factors in seed maturation (Liu et al. 2011). *RDO2* encodes the transcription elongation factor S-II (TFIIS). TFIIS factors can enhance transcription elongation by promoting cleavage and reactivation of nascent transcripts whose elongation is blocked under suboptimal conditions that could for instance occur in desiccated embryos. Mutations in RDO2 and other PAF1C-associated factors, such as VERNALIZATION INDEPENDENT 4 (VIP4), VIP5, EARLY FLOWERING 7 (ELF7), ELF8 and ARABIDOPSIS TRITHORAX-RELATED 7 (ATXR7), cause a reduced seed dormancy phenotype (Grasser et al. 2009; Liu et al. 2011) and several dormancy-related genes such as *DOG1* and *FLOWERING LOCUS C* (*FLC*) are downregulated in the *rdo2* mutant (Liu et al. 2011; Mortensen et al. 2011; Fig. 2). Upregulation of PAF1C-associated genes at the end of seed maturation, together with the reduced dormancy phenotypes of their mutants, indicates that they might be especially important in this phase. Likely, PAF1C-associated factors counteract the negative effects of the increased chromatin compaction on gene expression during seed maturation.

Recently, a role for another histone modification in seed dormancy was demonstrated. Mutations in the *KRYPTONITE* (*KYP*)/*SU*(*VAR*)*3-9 HOMOLOG 4* (*SUVH4*) gene, encoding the histone methyltransferase for H3K9me2 (Jackson et al. 2002), cause increased seed dormancy. In accordance, *KYP/SUVH4*-overexpressing

Arabidopsis plants show decreased dormancy (Zheng et al. 2012). This indicates that this repressive chromatin mark influences seed maturation (Fig. 2). Potential direct targets of *KYP/SUVH4* are the seed maturation genes *ABI3* and *DOG1*, which are upregulated in the *kyp-2* mutant.

2.5 Post-transcriptional Regulation of Seed Maturation

The effect of post-transcriptional regulation mechanisms on seed maturation is largely unknown. Nevertheless, *Arabidopsis* embryos of the *dicer-like 1–15* (*dcl1-15*) mutant show faster maturation than those of the wild type (Willmann et al. 2011). DCL1 encodes an enzyme responsible for the biosynthesis of microRNAs and partially functions by repressing the maturation regulators *LEC2* and *FUSCA3* during early embryogenesis. This heterochronic phenotype indicates that microRNAs are important regulators of the timing of the maturation programme.

3 Seed Storage

Dry seeds represent an intermediate state between seed maturation and germination and exhibit exceptional characteristics in comparison with the rest of the plant's life cycle as exemplified by very low humidity contents that are often well below 10 % (Baud et al. 2002). These low humidity levels prevent active metabolism. Changes in chromatin modifications require enzymatic processes and therefore active epigenetic signalling is unlikely to occur in the dry seed. However, stored seeds do show gradual changes in traits whose effects only become evident when they are imbibed under favourable environmental conditions, enabling germination. These processes are after-ripening and ageing. After-ripening causes a gradual release of seed dormancy, enabling seeds to germinate under favourable conditions after extended storage. Prolonged ageing of seeds, however, can lead to reduced viability caused by increasing damage of structures and molecules in the seed (Bailly et al. 2008).

Dry seeds contain a large amount of stored transcripts that have been generated during seed maturation (Nakabayashi et al. 2005). Many of these transcripts will be translated upon imbibition and have a role in germination (Rajjou et al. 2012). Interestingly, transcriptome studies in *Arabidopsis*, barley (*Hordeum vulgare*) and *Nicotiana plumbaginifolia* suggested that changes occur in the mRNA population during storage (Bove et al. 2005; Finch-Savage et al. 2007; Leymarie et al. 2007). These changes mainly constitute reductions in transcript abundance, probably caused by mRNA degradation. The altering transcriptome during seed storage likely influences the timing, ability, and speed of seedling establishment.

The mechanism of seed after-ripening is largely unknown, although evidence has been obtained for a role of oxidation (Oracz et al. 2007). Interestingly, not only proteins but also mRNAs become oxidised during dry storage of seeds. Oxidation of mRNAs leads to changes in their properties. For instance, oxidised mRNA can be more sensitive to degradation or less efficiently translated (Bazin et al. 2011). This is

not an epigenetic phenomenon stricto sensu, because these changes at the mRNA level are not inherited through mitosis or meiosis. However, they do occur without changes in the DNA sequence, they are influenced by environmental conditions like storage conditions, and they happen over very long time periods, even up to hundreds of years in some species.

As mentioned before, extended seed storage is detrimental because seeds gradually lose their viability. An important contributor to this is the accumulation of DNA damage. This can initially be repaired during imbibition and it has been shown that specific DNA ligases are involved in the repair of double strand breaks in seeds (Waterworth et al. 2010). However, extended storage of seeds under suboptimal conditions causes such severe DNA damage that it cannot be fully repaired anymore, leading to lethality of the embryo.

Under natural conditions, most seeds are frequently and extendedly imbibed. During imbibition seeds become metabolically active and it was recently shown in *Arabidopsis* that the transcript levels of several genes alter during the annual seasons in imbibed seeds (Footitt et al. 2011). Such gradual changes in expression levels are likely associated with altered chromatin modifications and epigenetic signalling mechanisms may play a role.

4 Seed Germination

4.1 Chromatin and Epigenetic Control of the Phase Transition from Seed to Seedling

The process by which plant embryos contained in seeds change into established seedlings can be roughly divided into two separate stages. The first is germination, which is defined as the protrusion of the radicle (embryonic root) through surrounding seed tissues (Finch-Savage and Leubner-Metzger 2006; Holdsworth et al. 2008). Next, the germinated seedling progresses from heterotrophic to photoautotrophic growth constituting a shift from dependency on seed storage reserves to active photosynthesis.

It has already been proposed over two decades ago that the change in the transcriptional programme from maturation to germination takes place during early imbibition (Comai and Harada 1990). This highly coordinated change in gene activity is essential for germination and requires regulation at the epigenetic level. In this section, we describe the epigenetic signalling and chromatin remodelling processes that are involved in the transition from seed to seedling. The role of the phytohormone ABA, which generally antagonises germination, will be highlighted as this appears to occur via chromatin and epigenetic modifications and is relatively well studied in this context. Although the functions of chromatin remodelling and epigenetic signalling during germination are increasingly well understood (Daszkowska-Golec 2011; Chinnusamy et al. 2008; Zhang and Ogas 2009; North et al. 2010), their contribution to seedling establishment and initiation of photoautotrophic growth remains poorly studied.

4.2 Dynamic Changes in Chromatin Compaction and Nuclear Size Characterise Germination

Several authors reported that the highly compacted seed chromatin becomes loosened during germination (see chapter "Environment-induced chromatin reorganization and plant acclimation"). For example, wheat (*Triticum aestivum*) seeds showed progressive increase in template activity for RNA synthesis as well as enhanced susceptibility to DNaseII endonuclease treatment during germination (Sugita and Sasaki 1982).

A high level of chromatin compaction in dry seeds was also found in *Arabidopsis*. Measurement of the Relative Heterochromatin Fraction (RHF) (Soppe et al. 2002; Tessadori et al. 2009) demonstrated that the heterochromatic fraction was 0.2 (van Zanten et al. 2011), whereas young vegetative leaves have an RHF typically between 0.08 and 0.14 and differentiated leaves between 0.11 and 0.16 (Tessadori et al. 2004). The chromatin compaction in *Arabidopsis* seeds quickly decreases upon imbibition and already after 2 days, the RHF reduced to ~0.08 (van Zanten et al. 2011). Moreover, in contrast to dry seeds, young seedlings lack condensed chromocenters (Mathieu et al. 2003; van Zanten et al. 2011). Chromocenters are nuclear domains of intensely stained (compact) DNA generally associated with major repetitive elements in the genome including centromeric repeats, transposable elements and ribosomal DNA (Tessadori et al. 2004; Fransz et al. 2006; see also Fig. 1 in chapter "Environment-induced chromatin reorganization and plant acclimation"). The heterochromatic DNA methylation mark, 5-methylcytosine (5-mC), is generally condensed at chromocenters in dry seeds (van Zanten et al. 2011) and adult leaves (Mathieu et al. 2003; Soppe et al. 2002). Fluorescence in situ hybridization studies revealed that 5-mC targeted sequences, as well as (peri)centromeric repeats and 5S rDNA repeats, become temporarily dispersed over the nucleus during seed imbibition/germination (van Zanten et al. 2011). Four days after germination, the nuclei of established seedlings formed conspicuous chromocenters again, comparable to the level observed in 3-week-old leaf nuclei (Mathieu et al. 2003).

As mentioned above, besides having condensed chromatin, seed nuclei are particularly small as shown for the species *Phaseolus vulgaris* L (Kater 1927), *Phaseolus lunatus*, *Zea mays* (Middendorf 1939) and *Arabidopsis* (van Zanten et al. 2011). This is probably an adaptation associated with desiccation tolerance to withstand long periods of drought. In *Arabidopsis* seeds, the re-establishment of nuclear size required germination because imbibition alone was not sufficient to increase nuclear size (van Zanten et al. 2011). Genetic and cytogenetic experiments demonstrated that the changes in chromatin compaction and nuclear size are temporally separated and occur independently. The reduction in size during seed maturation precedes the increase in chromatin compaction. Moreover, the *little nuclei 1* (*linc1*) *linc2* double mutant, which has constitutive small nuclei in all tissues, displayed similar dynamic chromatin condensation and decondensation events as wild-type seeds (van Zanten et al. 2011, 2012).

Based on the existing literature on chromatin dynamics, it can be concluded that the phase transition from embryo to seedling occurs at the transcriptional level during the process of germination. This was also proposed by Comai and Harada (1990) based on

the timing of transcriptional activities of germination-specific genes in *Brassica napus* L. Germinating pea (*Pisum sativum*) seeds have less histones per unit DNA than seedlings, which corresponds with the notion that the genome is reactivated through structural loosening of chromatin during germination (Grellet et al. 1977; Sugita and Sasaki 1982). In further support of the coincidence of major changes in transcriptional activity and chromatin restructuring, Mathieu et al. (2003) reported that the 5S rDNA resides in pre-chromocenters in 2-day-old seedlings and shows low transcript levels. Transcription was re-established only in 4-day-old seedlings when chromocenters had reformed, from which chromatin loops emanate containing 5S rDNA genes enriched with activating H3K9ac and H3K4me epigenetic marks. The exact function of the temporary and strong reduction in chromatin compaction in the transition from dry seed to established seedlings is not yet fully understood, but it is tempting to speculate that it is associated with a major change in the transcriptional programme. This suggests that epigenetic mechanisms may play a role.

4.3 The Role of Transcription Suppressing Epigenetic Factors During Seed Germination

Transcriptional reprogramming during germination requires repression of embryonic properties and activation of genes involved in the advance into photoautotrophic growth. Several independent papers demonstrated roles for chromatin-remodelling factors and epigenetic signalling mechanisms in these processes.

Seedlings of mutants lacking the CHD3 class SWI/SNF chromatin-remodelling factor PICKLE (PKL) display embryonic properties, i.e. "pickle" roots, with a swollen and greenish distal root tip and accumulation of neutral lipid bodies (Ogas et al. 1997, 1999). Thus, PKL is involved in repression of embryonic traits during germination. PKL transcript is absent in dry seeds and is initiated upon seed imbibition (Henderson et al. 2004; Li et al. 2005). The *pkl* phenotype can be repressed by GA application (Ogas et al. 1997) and is strongly enhanced by inhibitors of GA biosynthesis (Ogas et al. 1999). Further analysis revealed that PKL and GA both play a substantial, but partly independent role in repression of the seed maturation transcriptional programme during germination and PKL promotes GA signalling (Henderson et al. 2004; Zhang et al. 2008). In accordance with a role as repressor of embryonic traits, *pkl* mutants display high transcript levels of the central seed maturation regulators *LEC1*, *LEC2* and *FUS3* upon seed imbibition, while these factors are repressed in wild-type seeds (Ogas et al. 1999; Rider et al. 2003).

Unfavourable environmental conditions during the early stages of germination can lead to a return to the seed maturation transcriptional programme. ABI3 and ABI5 are required for growth arrest under osmotic stresses at the early phase of germination. Their degradation enables germination and ABA-induced expression of these transcription factors leads to inhibition of germination. PKL represses the expression of *ABI3* and *ABI5* in an ABA-dependent manner, as *pkl* mutants showed high *ABI3* and *ABI5* transcript levels after ABA treatment and hypersensitivity to ABA-mediated repression of germination (Perruc et al. 2007). Thus, PKL acts as a negative regulator

of ABA signalling during seed germination. Interestingly, this occurs by mediating H3K9 and H3K27 dimethylation at the *ABI3* and *ABI5* loci. The abundance of these epigenetic silencing marks were reduced in *pkl* mutants compared to the corresponding wild types, which was further enhanced by the application of ABA (Perruc et al. 2007). Moreover, H3K27me3 levels at the *LEC1* and *LEC2* loci, and other PKL target genes, were decreased in germinating *pkl* seeds (Zhang et al. 2008). This indicates that PKL is involved in maintaining this silencing mark (Fig. 2). A direct role for PKL in determining levels of H3K27me3 at these repressed loci during germination was recently demonstrated (Zhang et al. 2012a). Taken together, PKL controls ABA-mediated repression of germination by affecting the abundance of silencing epigenetic histone marks associated with the *ABI3* and *ABI5* loci. Although DNA methylation was not affected at these loci, DNA methylation effects were found on the *LEC1* promoter consistent with PKL-mediated silencing of *LEC1* during germination (Zhang et al. 2012a).

The process of epigenetic silencing during germination also involves the Polycomb Repressive Complex 2 (PRC2), which plays a role in repression of flowering in young seedlings by mediating H3K27me3. Mutants in FERTILIZATION INDEPENDENT ENDOSPERM (FIE), an essential component of the PRC2 complex, displayed genome-wide abolishment of H3K27me3 and exhibited increased seed dormancy and germination defects (Bouyer et al. 2011). Therefore, it was concluded that PRC2 is required for termination of the embryonic transcriptional programme to promote the phase transition from embryo to autotrophic seedling (Fig. 2). PRC2 sustains the balance between ABA and GA responsiveness via H3K27me3-mediated inhibition of positive ABA and negative GA regulators in maturing seeds. Moreover, the seed dormancy regulator *DOG1* is repressed through PRC2-catalysed H3K27-trimethylation at its locus (Bouyer et al. 2011). This may prime the seed for germination after the seed maturation programme has been "closed" at the epigenetic level.

Additional histone modifications that are believed to cause transcriptional repression are dimethylation of H3 arginine 2 (H3R2me2) and H4 arginine 3 (H4R3me2) because of their negative correlation with the level of H3K4me3 in human cells (Guccione et al. 2007). It was recently shown that the *Arabidopsis* histone arginine demethylases JMJ20 and JMJ22 are positive regulators of light-stimulated seed germination. Activation of phytochrome B leads to de-repression of JMJ20 and JMJ22. This causes removal of the repressive dimethylation of H3R2 and H4R3 at the GA3ox1 and GA3ox2 genes resulting in increased expression levels and the promotion of germination (Cho et al. 2012; Fig. 2). This finding represents a nice example of the integration of environmental factors in epigenetic signalling within the seed.

An indication that DNA methylation is involved in germination control comes from a study of the microRNA miR402, which has the 5-mC DNA glycosylase *DEMETER-LIKE protein 3* (*DML3*) as target gene (Sunkar and Zhu 2004). Overexpression of miR402 under high salt, dehydration or cold stress conditions resulted in accelerated germination, associated with cleavage of *DML3* mRNA. This likely maintains high 5-mC level at loci that prevent seed germination (Kim et al. 2010). However, the role of DNA methylation in germination is not straightforward because a rapid decrease in

DNA methylation was found in endosperm nuclei of *Silene latifolia* upon germination (Zluvova et al. 2001).

Finally, ABA-mediated control of germination also seems to involve chromatin remodelling at the nucleosome level. The phosphatase type 2C, HYPERSENSITIVE TO ABA1 (HAB1), interacts with SWI3B, a SWI/SNF chromatin-remodeler (Saez et al. 2008). The relevance of this interaction for germination was confirmed using *swi3b* mutants, which are less sensitive to ABA-mediated repression of germination. Presumably, ABA inhibits HAB1 function, which in turn releases inhibition of a SWI3B-containing SWI/SNF chromatin remodelling complex involved in the transcriptional activation of ABA responsive genes, by modulating the nucleosome structure at these loci (Saez et al. 2008).

In contrast to germination, chromatin control and epigenetic regulation of the switch from heterotrophic to photoautotrophic growth in seedlings are less well understood. Only one study found a role for chromatin remodelling in this process so far. Gutzat et al. (2011) showed that during embryogenesis and early germination, both knock-down RETINOBLASTOMA-RELATED protein (RBR) lines and RBR overexpression lines were indistinguishable from wild type. However, seedlings with reduced RBR expression became developmentally arrested in the G1 phase of the cell cycle after germination. Consequently, the embryonic phase was maintained in young mutant plantlets. Interestingly, sucrose could relieve this cell cycle arrest and induced expression of late embryonic genes in the knock-down lines, but not in the wild type. Thus, RBR controls the switch from heterotrophic embryonic development to photoautotrophic growth by suppressing sucrose-inducible embryonic traits in seedlings. Gutzat et al. (2011) demonstrated that this occurs via H3K27me3-mediated gene silencing 3–7 days after germination, at the moment when autotrophy is initiated. This process likely also involves the earlier mentioned PRC2 complex.

4.4 The Role of Transcription Promoting Epigenetic Factors During Seed Germination

Histone acetylation generally activates transcription. Seed germination requires concurrent silencing of embryonic traits and activation of genes associated with seedling establishment and growth. This could explain the simultaneous increase in expression of both histone acetylases (HAT) and histone deacetylases (HDAC/HD) that has been observed during germination in maize (*Zea mays*). However, the balance between these two is probably favouring HATs because global acetylation levels gradually increased during germination (Zhang et al. 2011). Interestingly, both addition of ABA and the HDAC inhibitor Trichostatin-A (TSA) delayed germination in maize. ABA repressed the expression of HATs and HDACs and delayed the increase in H3K9 acetylation levels during germination. In contrast, treatment with TSA increased global histone acetylation levels. The similar phenotypes of ABA and TSA during imbibition could be explained by their mutual effect on prevention of downregulation of *Viviparous1* (*VP1*), which is the maize ortholog of the *Arabidopsis ABI3* gene.

ABA activates *VP1* expression by selective induction of histone acetylation at the *VP1* promoter region. Thus ABA (and TSA) probably controls germination by affecting *VP1* expression in maize via HDAC- and HAT-mediated control of H3 acetylation levels at the *VP1* promoter (Zhang et al. 2011). Increased acetylation levels were also observed at the ribosomal DNA (rDNA) gene promoter region during germination in maize. This increase was accompanied by a decondensation of rDNA sequences and increased rRNA transcript levels. Application of ABA during germination inhibited all of these processes (Zhang et al. 2012b).

In *Arabidopsis*, HDA6 and HDA19 act redundantly to repress embryonic properties after germination (Tanaka et al. 2008). Treatment of the *hda6* mutant with TSA resulted in growth arrest directly after germination. This growth arrest can be partially explained by the enhanced expression of the seed maturation regulators *LEC1*, *FUS3* and *ABI3*, since it could be overcome by their respective mutants (Fig. 2). ABA and GA application or their pharmacological inhibition did not affect growth arrest, indicating that the effects of *hda6* are not a result of altered hormone levels or sensitivity. Interestingly, the *hda6 hda19* double mutant fully arrested growth, did not show cotyledon greening and expansion, and exhibited embryo-like structures after germination. In contrast to *hda6*, the *hda19* single mutant did not show growth arrest with supplemented TSA (Tanaka et al. 2008). Thus, *HDA6* appears to be a key factor in the repression of embryonic properties after germination that acts redundantly with *HDA19*. Interestingly, prolonged culturing of a *pkl* mutant with reduced *HDA6* transcript (*HDA6*::RNAi *pkl*) showed embryo-like structures on leaves, which was not the case in the *HDA6*::RNAi nor in *pkl* single mutants. Thus, PKL and HDA6 have a partial redundant function. Overall, it was concluded that HDA6 and HDA19 act independently, but additional to PKL in the repression of the embryonic genetic programme (Tanaka et al. 2008). In agreement, loss-of-PKL did not affect acetylation levels of seed-specific genes (Zhang et al. 2008). Interestingly, HDA6 was identified to act downstream of the microRNAs in DCL1-mediated repression of the maturation regulators *LEC2* and *FUSCA3* (Willmann et al. 2011) (see also sections above). This may indicate that microRNAs (and DCL1) also play a role in the phase transition from seed-to-seedling.

Similar to other HDACs, *AtHD2C* is repressed by ABA. Enhanced expression of this HDAC results in early germination due to ABA hyposensitivity and enhanced stress tolerance, including improved germination under drought and salt stress (Sridha and Wu 2006). Genes from the Late Embryogenesis Abundant (LEA) class were induced by *AtHD2C* expression (Sridha and Wu 2006) and by treatment with TSA in imbibed seeds (Tai et al. 2005). A mutant analysis of all four members of the HD2 family of histone deacetylase confirmed the role of histone acetylation in seed dormancy (Colville et al. 2011). This study also showed reduced germination in *hd2c* mutants, but in contrast *hd2a* null mutants showed enhanced germination. These different germination rates indicate a complex regulation of histone acetylation during seed maturation, which was also evident from the recent observation that HD2C physically interacts with HDA6 and binds to histone H3. The ABA-responsive genes *ABI1* and *ABI2* showed decreased expression in the *hd2c* and *hda6* mutants, associated with a decrease in H3K9 dimethylation and an increase in H3K9K14 acetylation at these loci (Luo et al. 2012).

Tai et al. (2005) reported the occurrence of a transient deacetylation event during early seed germination. Application of TSA 1 day after imbibition resulted in reduced repression of LEA genes. However, when the HDAC inhibitor was applied at day three after germination, no effect on transcription was found. The authors concluded therefore that deacetylation on the first day of imbibition is critical in the epigenetic control of gene expression during seed germination.

Finally, HDAC-mediated effects on seed germination may also occur indirectly. For example, Song et al. (2005) reported interaction of HDA19 with the global co-repressor of transcription, AtSin3, which enhances the transcriptional repression of the APETALA2/EREBP-type transcription factor AtERF7. AtERF7 and AtSin3 RNA interference lines showed hypersensitivity to ABA during seed germination.

Apart from acetylation, additional transcription activating histone marks are likely to be involved in gene activation during seed germination, among them H3K4me3. A transcriptional network modelling study in *Arabidopsis* identified the *EARLY FLOWERING IN SHORT DAYS* (*EFS*) gene as a phase transition regulator during seed germination (Bassel et al. 2011). *EFS* has previously been identified as a histone H3 methyltransferase involved in H3 lysine 4 trimethylation of *FLOWERING LOCUS C* (Kim et al. 2005). A role for *EFS* in the regulation of germination seems likely, since *efs* mutant seeds show various seed phenotypes including precocious germination (Bassel et al. 2011).

4.5 The Role of Post-Transcriptional Regulation in the Control of Seed Germination

As mentioned already above, small RNAs regulate seed maturation and germination. Overexpression of miR160 (35S:MIR160), for instance, resulted in reduced sensitivity to ABA during germination and decreased seed dormancy (Liu et al. 2007b). The Auxin Response Factors (ARFs) *ARF10*, *ARF16* and *ARF17* are targeted by miR160 in *Arabidopsis*. Transgenic plants expressing a miR160-resistant form of *ARF10* (*mARF10*), which has silent mutations in the miRNA target site, showed developmental defects including ABA sensitivity of seed germination.

Genetic studies on increased dormancy and/or ABA hypersensitivity during germination have also identified mutations resulting in disruption of RNA capping (*ABA-hypersensitive 1* (*abh1*)), microRNA biosynthesis (*hyponastic leaves 1* (*hyl1*)), mRNA splicing, export and degradation (*supersensitive to ABA and drought 1* (*sad1*)), and degradation of polyadenylated RNA (*ABA hypersensitive germination 2* (*ahg2*)) (Han et al. 2004; Hugouvieux et al. 2001; Lu and Feodoroff 2000; Xiong et al. 2001; Nishimura et al. 2005). These genes encode factors functioning in RNA processing, indicating that mechanisms controlling mRNA processing control dormancy and/or ABA hypersensitivity at germination. Together, these results clearly indicate a crucial role for RNA processing and siRNA/microRNA in seed maturation and germination. However, very little is known about the posttranscriptional regulation of specific seed maturation-related genes.

5 Conclusions and Outlook

In this chapter we presented an overview of epigenetic regulators that influence the main processes that occur in seeds, focussing on seed maturation, dormancy and germination in the model plant *Arabidopsis*. Figure 2 summarises the main epigenetic processes during these stages, whereas Table 2 gives a detailed list of the different modifying genes and epigenetic regulators that have been described in this chapter.

Seed maturation prepares the embryo to survive in the dry seed after it has been shed from the mother plant. This preparation requires active transcription of genes related to storage reserves accumulation, desiccation tolerance and dormancy induction up to the very end of the maturation phase. The chromatin at the end of seed maturation is highly condensed while transcript levels still equal those observed in leaf tissue. This implicates that epigenetic mechanisms likely play important roles in the processes that maintain gene expression. Successful germination requires the downregulation of maturation genes and the upregulation of genes involved in the germination programme and later those involved in autotrophic growth.

Our knowledge of epigenetic signalling in seeds is still fragmentary, but genetic studies have revealed important roles for various histone modifications (Table 1), RNA elongation and posttranscriptional RNA processing factors in the regulation of seed maturation. Germination is characterised by major epigenetic changes, in which the plant hormone ABA seems to act as a major regulator. Interestingly, many seed maturation genes contain both repressive and permissive chromatin marks in seedlings (Table 1). This combination of marks is known as bivalent state and has been observed in diverse organisms including mouse, rice and *Arabidopsis* (Bernstein et al. 2006; He et al. 2010; Roudier et al. 2011). Genes containing this combination of chromatin marks are primed for transcription, which is postponed until the arrival of developmental or environmental signals dictating either transcription or repression (Lan et al. 2008).

Most of our present understanding of epigenetic signalling in seeds has been obtained by molecular genetic approaches, using lines with reduced or enhanced expression of specific genes that are involved in epigenetic signalling. However, loss-of-function mutations in the majority of genes with a role in epigenetic signalling do not cause obvious seed phenotypes, which could be due to the functional redundancy of these regulators (van Zanten and Soppe, unpublished results). This suggests that this approach will not lead to a full understanding of epigenetic signalling during the seed's life.

Obtaining a more complete understanding of the epigenetic mechanisms underlying seed maturation will require new approaches. We expect that a detailed analysis of chromatin modifications during the different phases of the seed's life for several key maturation and germination genes will reveal the major epigenetic changes occurring in the seed. These chromatin modifications could subsequently be analysed at a genome-wide level and their regulation and effects on target genes could be studied in detail once efficient protocols for ChiP in seed tissues become available. A full

Table 2 List of chromatin modifying genes and epigenetic regulators that function in seed maturation and germination as presented in this chapter, with AGI code, in order of appearance

Abbreviation	Full name	AGI code	Protein	Function in seed biology	References
HUB1	*HISTONE MONOUBIQUITINATION 1*	At2g44950	C3HC4 RING finger protein required for H2B monoubiquitination; homology to BRE1 histone modifier in other species	Maintenance of transcription during late seed maturation; positive regulator of seed dormancy	Liu et al. (2007a, b)
HUB2	*HISTONE MONOUBIQUITINATION 2*	At1g55250	C3HC4 RING finger protein required for H2B monoubiquitination; homology to BRE1 histone modifier in other species	Maintenance of transcription during late seed maturation; positive regulator of seed dormancy	Liu et al. (2011)
RDO2	*REDUCED DORMANCY 2*	At2g38560	TFIIS transcription elongation factor	Maintenance of transcription during late seed maturation; positive regulator of seed dormancy	Liu et al. (2011)
VIP4	*VERNALIZATION INDEPENDENT 4*	At5g61150	Component of PAF1C complex; homology to the yeast protein Leo1	Maintenance of transcription during late seed maturation; positive regulator of seed dormancy	Liu et al. (2011)
VIP5	*VERNALIZATION INDEPENDENT 5*	At1g61040	Component of PAF1C complex; homology to the yeast protein Rtf1	Maintenance of transcription during late seed maturation; positive regulator of seed dormancy	Liu et al. (2011)
ELF7	*EARLY FLOWERING 7*	At1g79730	Component of PAF1C complex; homology to the yeast protein PAF1	Maintenance of transcription during late seed maturation; positive regulator of seed dormancy	Liu et al. (2011)
ELF8	*EARLY FLOWERING 8*	At2g06210	Component of PAF1C complex; homology to the yeast protein CTR9	Maintenance of transcription during late seed maturation; positive regulator of seed dormancy	Liu et al. (2011)
ATXR7	*ARABIDOPSIS TRITHORAX-RELATED 7*	At5g42400	*Arabidopsis* homologue of SET1 methyl transferase	Maintenance of transcription during late seed maturation; positive regulator of seed dormancy	Liu et al. (2011)

KYP	*KRYPTONITE*	At5g13960	SU(VAR)3-9 HOMOLOG 4 (SUVH4) H3K9me2 methyltransferase	Seed maturation; positive regulator of seed dormancy	Zheng et al. (2012)
DCL1	*DICER-LIKE 1*	At1g01040	Enzyme responsible for the biosynthesis of microRNAs	Controls the progress of seed maturation by repressing the maturation regulators during early embryogenesis	Willmann et al. (2011)
PKL	*PICKLE*	At2g25170	CHD3 class SWI/SNF chromatin-remodelling factor	Required for repression of embryonic properties in seedlings during germination	Perruc et al. (2007)
FIE	*FERTILIZATION INDEPENDENT ENDOSPERM*	At3g20740	Component of the polycomb repressive complex 2	Required for termination of the embryonic transcriptional program to promote the phase transition from embryo to seedling	Bouyer et al. (2011)
JMJ20	*JUMONJI C DOMAIN-CONTAINING PROTEIN 20*	At5g63080	*Arabidopsis* histone arginine demethylases	Positive regulator of light stimulated seed germination	Cho et al. (2012)
JMJ22	*JUMONJI C DOMAIN-CONTAINING PROTEIN 21*	At5g06550	*Arabidopsis* histone arginine demethylases	Positive regulator of light stimulated seed germination	Cho et al. (2012)
DML3	*DEMETER-LIKE PROTEIN 3*	At4g34060	5-mC DNA glycosylase	Repressor of germination	Kim et al. (2010)
HAB1	*HYPERSENSITIVE TO ABA1*	At1g72770	Phosphatase type 2C	Controls sensitivity to ABA during germination via interaction with SWI3B by modulation of nucleosome structure	Saez et al. (2008)
RBR	*RETINOBLASTOMA-RELATED*	At3g12280	Plant homolog of the tumour suppressor Retinoblastoma (pRb) regulator of cell cycle	Control of the progression from seedling heterotrophic to photoautotrophic growth by affecting H3K27me3-mediated gene silencing	Gutzat et al. (2011)

(continued)

Table 2 (continued)

Abbreviation	Full name	AGI code	Protein	Function in seed biology	References
HDA6	*HISTONE DEACETYLASE 6*	At5g63110	RPD3-type histone deacetylase	Repression of embryonic properties after germination	Tanaka et al. (2008)
HDA19	*HISTONE DEACETYLASE 19*	At4g38130	RPD3-type histone deacetylase	Repression of embryonic properties after germination	Tanaka et al. (2008)
HD2A	*HISTONE DEACETYLASE 2A*	At3g44750	Histone deacetylases	Negative regulator of germination	Colville et al. (2011)
HD2C	*HISTONE DEACETYLASE 2C*	At5g03740	Histone deacetylase	Positive regulator of germination	Sridha and Wu (2006), Colville et al. (2011)
EFS	*EARLY FLOWERING IN SHORT DAYS*	At1g77300	Histone H3 methyltransferase involved in H3 lysine 4 trimethylation	Repressor of germination	Bassel et al. (2011)

understanding of epigenetic signalling during the seed's life will also require the development of fine epigenomic maps of the different phases of the seed life.

The interrelations between the various chromatin modification factors in the seed, although likely to be largely similar to those in other tissues, are only beginning to be understood. It is intriguing that chromatin modifications often seem to be selective and that the expression of some genes is strongly affected by the absence of a chromatin modification factor, whereas those of many others seem not. This suggests the presence of many additional regulators besides the known direct chromatin modifying proteins. It is a future challenge to identify these regulators and unravel the underlying signalling networks.

Developmental processes in seeds are influenced by environmental signals, for instance light and temperature. Environmental signalling is crucial for the proper timing of seed processes, especially in determining the moment of germination. This raises the intriguing question to what degree the environment influences epigenetic signalling in seeds. In this respect, it is very interesting that the environment experienced by the plant embryo can determine properties of the later adult life stages, independent of the immediate effects on the embryo (Rubio de Casas et al. 2012). It is an attractive idea that these influences are mediated by epigenetic mechanisms.

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Epigenetic Control of Cell Division

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Abstract Posttranslational modification (PTM) of histone tails plays a critical role in the dynamic of chromatin and chromosomes. However, emerging evidence suggests that individual histone modifications do not reliably predict a single functional output [Sims and Reinberg (Nat Rev Mol Cell Biol 9:815–820, 2008)]. In plants, the cell cycle-dependent phosphorylation of histone H3 has been described best; it is hyperphosphorylated at serines 10/28 and at threonines 3/11/32 during both mitosis and meiosis in patterns that are specifically coordinated in both space and time. Although this posttranslational modification is highly conserved, data show that the chromosomal distribution of individual modifications can differ between groups of eukaryotes. We describe the function of plant Aurora and Haspin kinases which have the capacity to phosphorylate H3 and discuss the cross talk between phosphorylation and other PTMs.

1 Introduction

Cell cycle progression occurs in a unidirectional manner and requires the precise coordination of important processes of DNA replication, chromosome segregation, cell division, and cell growth. Transition of decondensed interphase chromatin to the condensed metaphase chromatin during cell division is the most obvious dynamic change in the chromatin. Combinatorial histone modifications together with DNA methylation are well-defined epigenetic mechanisms that control the chromatin structures and mediate signaling for cellular processes. Phosphorylation of histone H3 is an outstanding event in cell division and is associated with chromosome condensation and segregation in eukaryotes. In plants, the cell

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cycle-dependent phosphorylation of histone H3 has been well described; it is hyperphosphorylated at serines 10/28 and at threonines 3/11 during both mitosis and meiosis. In this chapter, we review the cell cycle-dependent histone phosphorylations and their known kinases in plant species. In addition, the cross talk between histone H3 phosphorylations and other posttranslational modifications is described.

2 Posttranslational Histone Modifications

Histones are subjected to a variety of posttranslational modifications (PTMs), including acetylation, phosphorylation, ADP-ribosylation, methylation, and ubiquitination (Espino et al. 2005; Fuchs et al. 2006; Ito 2007; Jenuwein and Allis 2001). In histone H3, the sites of these posttranslational modifications are mainly clustered within the first 40 amino acids of the N-terminal domain (Earley et al. 2007; Lee et al. 2007; Metzger et al. 2008; Wozniak et al. 2007). Such modifications are required for interactions with specific protein domains, such as bromodomains, which interact with histone containing acetylated lysine residues (Chua et al. 2005); chromodomains, which bind to lysine-methylated histone tails (Li et al. 2007); or 14-3-3 proteins, which interact with phosphorylated histone H3 (Macdonald et al. 2005; Winter et al. 2008).

2.1 Cell Cycle-Dependent Phosphorylation of Histone H3

Phosphorylation of histone H3 is the best analyzed cell cycle-dependent PTM. Early observations in several eukaryotes have shown that the level of histone H3 phosphorylation, which is minimal in interphase, increases during mitosis (Gurtley et al. 1975; Hendzel et al. 1997). With the development of antibodies specific for histone H3 phosphorylated at S10, Hendzel et al. (1997) were able to show, in vivo, a precise temporal and spatial pattern of H3S10 phosphorylation in mammalian cells. They found that S10 phosphorylation of H3 is initiated in late G2 and then spreads throughout the chromatin as it undergoes condensation, up to the end of mitosis. However, although the process of PTMs is highly conserved, its significance and chromosomal distribution may differ to some extent among different species groups (Fuchs et al. 2006; Houben et al. 2007a, b; Loidl 2004). Although histones and their PTMs are highly conserved, recent data show that chromosomal distribution patterns of PTMs are not always conserved. For instance, in mammals the cell cycle-dependent phosphorylation of H3 at serines10/28 that originates in the pericentromere (Goto et al. 1999) and spreads throughout the chromosomes during the G2–M transition phase is most likely to be interlinked with the initiation of chromosome condensation (Van Hooser et al. 1998). In yeast, on the other hand, phosphorylation of S10 in H3 is not required for cell-cycle progression, where phosphorylation of histone H2B might replace the function of H3 phosphorylation

(Hsu et al. 2000). In plants, the distribution of phosphorylated histone H3 at S10 (Houben et al. 1999; Kaszas and Cande 2000; Kurihara et al. 2005; Manzanero et al. 2000), and S28 (Gernand et al. 2003; Zhang et al. 2005) correlates with the position of the pericentromere during mitosis and meiosis II (Fig. 1a, b). Analysis of dicentric chromosomes revealed hyperphosphorylated H3S10 only at the functional centromere (Fu et al. 2012; Han et al. 2006; Houben et al. 1999). Indicating that H3S10 phosphorylation is an epigenetic mark for active (peri)centromeres.

High-resolution scanning electron microscopy (Fig. 1e–g) supports the view that during the progression of mitosis, phosphorylation of histone H3 at S10 accumulates in the pericentromeric chromatin at metaphase (Schroeder-Reiter et al. 2003). The immunosignal gap represents the core centromere, characterized by parallel chromatin fibers, reduced DNA, and enriched protein amounts compared to the chromosome arms (Wanner and Formanek 2000). Within the core centromere, histone H3 is replaced by the evolutionarily conserved centromere-specific histone H3-variant CENP-A (called CENH3 in plants; HTR12 in *Arabidopsis*) (Earnshaw and Rothfield 1985). This agrees with the observation that CENH3 immunofluorescence signals are restricted to subdomains in the primary constriction and the immediately bordering pericentric region (Houben et al. 2007a, b; Schroeder-Reiter et al. 2012).

In contrast to monocentric mitotic chromosomes, the holocentric chromosomes of the genus *Luzula* (Gernand et al. 2003; Nagaki et al. 2005) and of *Rhynchospora tenuis* (Guerra et al. 2006) were labeled along the entire length of the chromosomes during mitosis with anti-H3S10ph (Fig. 1c) and anti-H3S28ph (Fig. 1d). Consistently, phosphorylation of histone H3S10/S28 occurs where sister chromatids cohere until the onset of anaphase; in polycentric chromosomes, cohesion occurs along the entire chromatid arms while in monocentrics only at a single chromosome region. Interestingly, in monocentric plants, the distribution of S10 and S28 phosphorylation during meiosis varies between the two meiotic divisions (Gernand et al. 2003; Kaszas and Cande 2000; Manzanero et al. 2000; Manzanero et al. 2002). During the meiosis I, the chromosomes are highly phosphorylated throughout their entire length, while in the meiosis II, the H3S10 phosphorylation is restricted to the pericentromeric regions, as in mitotic chromosomes (Fig. 1h, i). At the same time, single chromatids resulting from equational division of univalent chromosomes show no H3S10 phosphorylation at second meiosis. Irrespective of their low level of H3 phosphorylation, however, prematurely separated chromatids show normal condensation, and their kinetochores interact with the microtubules. These findings led to the hypothesis that in plants, pericentromeric H3 phosphorylation at serines 10 and 28 is required for cohesion of sister chromatids during metaphase I and for sister chromatid pericentromeres during mitosis and metaphase II, respectively (Gernand et al. 2003; Manzanero et al. 2000). This hypothesis was further supported by the observation that in a maize mutant (*afdI*) defective in sister chromatid cohesion, the univalents at metaphase I showed strong phosphorylation only at the pericentromeric regions (Kaszas and Cande 2000). The fact that histone H3S10 becomes dephosphorylated at interkinesis and phosphorylated again during

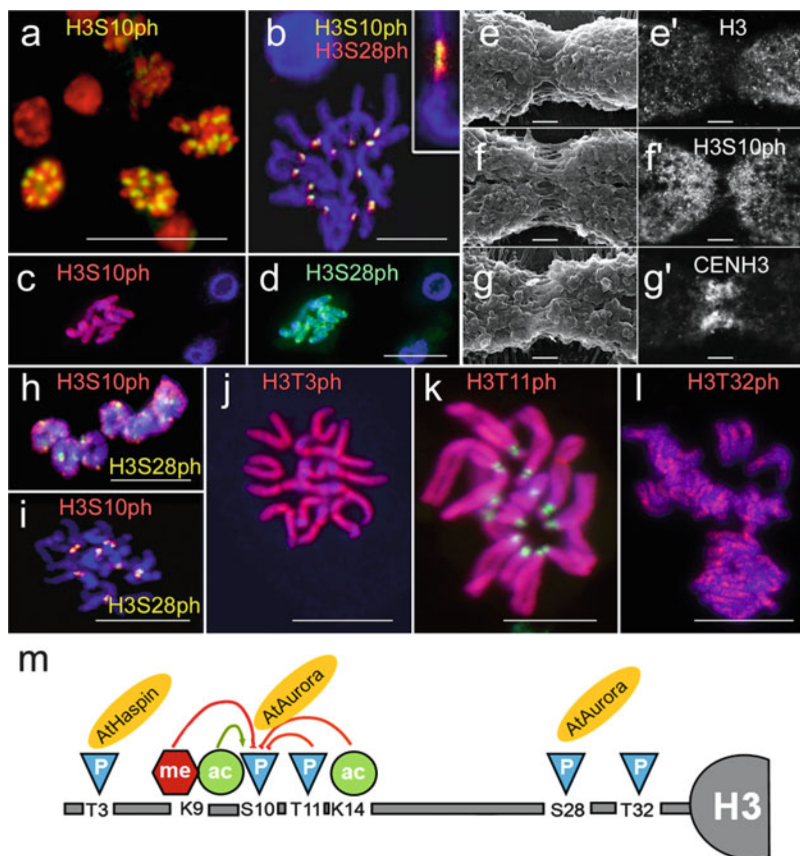

Fig. 1 Cell cycle-regulated histone H3 phosphorylation in plants. (**a**) *Arabidopsis thaliana*, (**b**, **e**–**g**, **j**) *Hordeum vulgare*, (**c**, **d**) *Luzula luzuloides*, (**h**, **i**) *Secale cereale*, (**k**) *Vicia faba*, and (**l**) *Triticum aestivum*. (**a**, **b**). On monocentric chromosomes, the pericentromeric regions show H3S10ph (*red*), whereas H3S28ph (*yellow*) is confined to the central part of the pericentromeric region (*inset*). The polycentric chromosomes of *L. luzuloides* are entirely labeled with H3 phosphorylated at S10 (in *red*) (**c**) and S28 (in *green*) (**d**). (**e**–**g**) Scanning electron micrographs of barley metaphase chromosomes labeled with antibodies against H3 (**e**), H3S10ph (**f**), and CENH3 (**g**). The conventional scanning electron image shows chromosome topography, whereas the backscattered electron signal (**e**′–**g**′) monitors the immuno signals (Houben et al. 2007a, b; Schroeder-Reiter et al. 2003). (**h**, **i**) Distribution of phosphorylated H3S10 (*red*) and H3S28 (*green*) differs between the first and second meiotic division. At the first division (**h**), entire *S. cereale* chromosomes are labeled with H3S10 (*red*), whereas at second division (**i**), H3S10ph is confined to the pericentromeric regions (Gernand et al. 2003). H3T3ph (*red*) (**j**), H3T11ph (*red*) (**k**), and H3T32ph (**l**) correlate with condensation of metaphase chromosomes (Caperta et al. 2008). (**k**) H3S28ph (*yellow*) is confined to the pericentromeric region. Interphase nuclei display no detectable H3 phosphorylation. *Size bars* in (**a**–**d**) and (**h**–**l**) show 10 μm; *size bars* in (**e**–**g**) indicate 0.5 μm. (**m**) A scheme on the histone H3 N-terminal-terminal positions undergoing phosphorylation in a cell cycle-regulated way in plants. Corresponding kinases and cross regulations of histone H3S10 phosphorylation via AtAurora1 and posttranslational histone in *cis* modifications are indicated (Demidov et al. 2009)

prophase II indicates that histone H3 phosphorylation is reversible, and it can occur independent of the DNA replication process.

Compared to S10 phosphorylation, there are fewer reports addressing the phosphorylation of other amino acid residues within the histone H3 tail. Phosphorylation at threonines 3 and 11 in plants occurs along entire chromosome arms (Fig. 1j, k) and correlates with the condensation of mitotic and meiotic chromosomes (Ashtiyani et al. 2011; Houben et al. 2007a, b). H3T32 is also phosphorylated during mitosis of plants (Fig. 1l), with a distribution pattern similar to that of H3T11ph (Caperta et al. 2008). Phosphorylation at H3T32 also occurs in animal cells (Tamada et al. 2006), although its distribution pattern in mitosis and meiosis is not yet described.

In mammals, phosphorylation of T3 and T11 is most abundant in the centromere (Dai et al. 2005; Polioudaki et al. 2004; Preuss et al. 2003) where it may serve as a recognition code for kinetochore assembly. In contrast, in mammals, phosphorylation of S10/28 has a likely function in chromosome condensation. The coincidence of H3 phosphorylation (at serines 10/28) with chromosome condensation in animal cells had led to the proposal of a causal relationship (Goto et al. 1999; Hendzel et al. 1997). However, since this correlation does not exist in plants and other organisms, this proposal has been modified to the "production ready label" hypothesis (Hans and Dimitrov 2001; Prigent and Dimitrov 2003) suggesting that H3 phosphorylation during cell division serves as a "mark" that chromosomes are ready for separation. With the finding that in plant cells phosphorylation at T3/11/32, rather than S10/28, correlates with chromosome condensation, the original proposal by Hendzel et al. (1997) may well be valid due to species-specific differences in the biological significance of the histone code.

The distribution pattern of histone H3 phosphorylation has been artificially altered by the application of phosphatase and kinase inhibitors. Cantharidin, a natural compound isolated from the blister beetle, is a potent inhibitor of protein serine/threonine phosphatases especially PP2A and PP4. Application of Cantharidin increases histone H3S10/28 phosphorylation along chromosome arms from prophase to telophase in plants (Gernand et al. 2003; Manzanero et al. 2002). Unlike the situation in mammals, where phosphatase inhibitors induced premature chromosome condensation and stimulate H3 phosphorylation in interphase nuclei (Ajiro et al. 1996), no such severe effect of interphase histone H3 phosphorylation could be found in plants. The H3S10 phosphorylation pattern, after in vitro treatment with Cantharidin, resembles that of the chromosomes at first meiotic division in plants. It could be that the phosphorylation of the pericentric chromatin, and of chromosome arms, is controlled by different kinases. Alternatively, there might only be one kinase whose activity along the chromosome is regulated differently in mitosis and meiosis. Following phosphatase inhibitor treatment, the observed meta/anaphase cells appeared normal, with the exception of very rare lagging of chromatids at anaphase, and a delayed transition from metaphase to anaphase (Manzanero and Houben, unpublished). It should be noted that after treatment of seedlings with Cantharidin, the transcription of approximately 10 % of the 24,000 genes of *Arabidopsis* were changed significantly (Bajsa et al. 2011). Hence, a direct

or indirect interaction between Cantharidin treatment and histone H3 phosphatase inhibition is possible.

Inhibition of histone deacetylation by trichostatin A (TSA) in *Nicotiana sylvestris* protoplasts during mitosis induces the accumulation of metaphase cells and reduces H3S10ph at anaphase and telophase (Li et al. 2005), which suggests a direct or indirect interaction between H3 phosphorylation and acetylation. For threonine 11 of histone H3, a different response to Cantharidin has been reported (Houben et al. 2005). T11 became phosphorylated during interphase, but this phosphorylation was restricted to pericentromeric regions. It is likely that phosphorylation takes place in interphase as well but remains undetected because the rate of dephosphorylation exceeds that of phosphorylation. Alternatively, the kinase phosphorylating T11 may be inactivated by dephosphorylation during interphase. Thus, inhibition of phosphatase activity could result in activation of this kinase. Since Cantharidin affects a number of different PP2A- and PP1-type phosphatases (MacKintosh and MacKintosh 1994), the specific enzyme involved cannot be deduced. It could be, however, that the artificially induced H3T11 phosphorylation of the pericentric interphase chromatin and the phosphorylation of the chromosome arms are controlled by different kinases/phosphatases. A reduced level of H3S10/28 phosphorylation and an aberrant segregation of mitotic chromosomes have been revealed by varying the Aurora kinase activity of cultured tobacco cells and of *Arabidopsis* seedlings with the ATP-competitive Aurora inhibitor Hesperadin and inhibitor II (Demidov et al. 2009; Kurihara et al. 2006). Hesperadin, which has been developed as potential anticancer drug (Hauf et al. 2003), is a small molecule that inhibits Aurora B activity. Because the ATP-binding sites of Aurora B are conserved between humans and *Arabidopsis*, Hesperadin potentially inhibits plant Aurora kinases in the same manner as ATP-competitive inhibitors in animals (Kurihara et al. 2006). Since chromatids were normally condensed without H3S10/S28 phosphorylation and a high frequency of lagging anaphase chromosomes in Hesperadin-treated plant cells was found, it is likely that H3S10/S28 phosphorylation is required for the dissociation of mitotic sister chromatid cohesion in plants. Localization and level of histone H3T11 phosphorylation was unchanged in Hesperadin-treated *Arabidopsis* cells, suggesting that Hesperadin specifically inhibits AtAurora in vivo. Moreover, Hesperadin did not influence the chromosomal distribution of the euchromatin marker H3K4me2 and the heterochromatin marker H3K9me2 (Demidov et al. 2009).

The histone H3 family contains several evolutionarily conserved members. Histone H3.3, which differs in sequence in few amino acids from the canonical H3.1, was shown to play a likely role in the transcriptional activation of genes (Stroud et al. 2012). In metazoan histone, H3.3 contains a serine to alanine replacement at amino acid position 31, which is phosphorylated during mitosis (Hake et al. 2005). In contrast to phosphorylated H3S10/S28, H3.3S31ph is localized immediately adjacent to centromeres arguing for a unique function for the phosphorylated H3.3 that is distinct from its suspected role in gene activation. Phosphorylation of H3.3S31 also occurs during mitosis of the urochordate *Oikopleura dioica*, suggesting this histone modification and its function in mitosis

is already present at the invertebrate-vertebrate transition (Schulmeister et al. 2007). *Arabidopsis* histone H3.3 differs from H3.1 by 4-aa sites: amino acids 31, 41, 87, and 90 (Shi et al. 2011). However, unlike in metazoan in *Arabidopsis*, alanine is replaced by threonine at position 31. Whether also in other plants a cell cycle-dependent phosphorylation of H3.3 occurs is not known.

The centromere-specific histone H3 variant CENH3, which is one of the best analyzed component of active centromeres also undergoes PTMs. In human serine 7 of CENH3 is phosphorylated by Aurora B in a temporal pattern that is similar to H3S10 (Zeitlin et al. 2001). Notably, in plants, CENH3S50 and H3S28 are phosphorylated with almost identical kinetics (Zhang et al. 2005). The temporal coordination of CENH3S50 and H3S28 phosphorylation in maize (Zhang et al. 2005) suggests that one factor regulating centromere-mediated cohesin accumulation is a histone kinase, which binds first at CENH3 and then diffuses outwards over histone H3 to define the boundaries of the pericentromeric domains.

The primary function of the cell cycle-dependent histone H3 phosphorylation may be to identify different domains of the chromosomes and to mark their progress through the cell cycle (Prigent and Dimitrov 2003). Older models proposed that histone modifications may directly influence either the structure or the folding dynamics of nucleosomal arrays, but there is little evidence supporting such models (Peterson and Laniel 2004). It seems likely that histone modifications control the binding of chromatin proteins to the nucleosomes. For example, some chromodomain proteins bind to methylated lysines, whereas bromodomain-containing proteins specifically bind to acetylated lysines (reviewed in Fischle et al. 2003). Interactions of H3S10ph with 14–3–3 proteins is required for transcriptional activation (Winter et al. 2008). Also phosphorylation of H3S10 in *S. cerevisiae* facilitates the sequential acetylation of lysine 14 by directly enhancing the binding of the GCN5 acetyltransferase (Clements et al. 2003). The reversible cycle of histone phosphorylation/dephosphorylation may govern the capacity of chromatin-binding proteins to bind methylated lysines and to re-release these binding factors at the appropriate stage of the cell cycle. Evidence exists that in mammals, phosphorylation of H3S10 is responsible for the dissociation of the methyl H3K9-binding protein HP1 during mitosis (Fischle et al. 2005; Hirota et al. 2005). Whether a similar binary "methyl/phos switch" exists in plants remains to be studied. However, the finding that the subnuclear localization of HP1-like protein was unaffected in *Arabidopsis* mutants displaying a significant reduction in H3K9 methylation raises some doubt about the role of H3K9 methylation in HP1 recruitment to chromocenters in plants (Zemach et al. 2006).

2.2 Cell Cycle-Dependent Phosphorylation of H2A

Histone H2A of metazoa undergoes phosphorylation at the conserved site T119 particularly in centromeres during mitosis. The Aurora B complex of *Drosophila* is

required for this phosphorylation, while Polo kinase suppresses phosphorylation of T119 on chromosome arms by the nucleosomal histone kinase-1 (NHK-1) (Brittle et al. 2007). Inactivation of Cdc2 kinase is required for loss of centromeric phosphorylation at the metaphase–anaphase transition. Therefore, these mitotic kinases together control the temporal and spatial pattern of H2A phosphorylation at centromeres (Brittle et al. 2007). In fission yeast compelling evidence exists that the mitotic checkpoint protein kinase Bub1 (budding uninhibited by benzimidazole 1) phosphorylates S121 of histone H2A (equivalent to *Drosophila* H2AT119) promoting the recruitment of Shugoshin and Aurora B to the centromere (Kawashima et al. 2010; Wang et al. 2011). Bub1 was originally characterized as a conserved component of the spindle assembly checkpoint (Hoyt et al. 1991). Bub1 creates a mark for Shugoshin localization and the correct partitioning of chromosomes (Kawashima et al. 2010).

For plants, Green et al. (1990) provided the first biochemical evidence that histone H2A undergoes phosphorylation. Recently, Dong and Han (2012) identified six members of the H2A gene family in maize that contain a threonine in the evolutionarily conserved LPKKT-domain (position T133), which is undergoing phosphorylation in a cell cycle-dependent manner. During mitosis, H2AT133 phosphorylation becomes strong in metaphase and is specific to centromere regions but drops during late anaphase and telophase. From meiosis I to meiosis II, phosphorylation of H2A at T133 persists at the centromeric regions. Immunostaining of a dicentric maize chromosome revealed that only the active centromere is marked by this histone modification. Yet it is unknown whether in plants H2A serves as a substrate for Bub1-like protein kinase.

In yeast and in metazoan, histone H2AX, a ubiquitous variant of the H2A histone family, is rapidly phosphorylated at its C-terminal S139 on either side of a DNA double-strand break at a distance of 1–50 kb by specific kinases such as ATM (ataxia telangiectasia mutated) and ATR (ATM- and RAD3-related), which are activated by DNA damage and upon replication stress, respectively. Phosphorylated H2AX (also called γH2AX) reversibly triggers the accumulation of components involved in DNA recombination repair and in cell cycle checkpoint activation including histone acetyltransferase and cohesin (for review, see Redon et al. 2002; Thiriet and Hayes 2005). Interactions of the phosphorylated S139 of histone H2AX with the tandem BRCT (BRCA1 C-terminus) repeats of the DNA damage checkpoint protein MDC1 have been reported (Stucki et al. 2005). In plants, phosphorylation of H2AX is induced by gamma irradiation at only one-third the rate observed in yeast and mammals (Friesner et al. 2005). An increased number of H2AX foci in late S/G2- and M-phase cells were found after hydroxyurea- and aphidicolin-induced DNA replication stress in *Vicia faba* (Rybaczek et al. 2007). H2AX phos-specific antibodies have been also used to visualize meiotic double-strand breaks in higher eukaryotes including plants (Sanchez-Moran et al. 2007).

2.3 Cell Cycle-Dependent Phosphorylation of Histone H4

Enhanced mitotic phosphorylation of histone H4 and H2A at their respective serine 1 residue has been detected in *Caenorhabditis elegans*, *Drosophila*, and mammals (Barber et al. 2004). Because the amino-terminal sequence of H4 is largely conserved throughout evolution, it is likely that also plants share a comparable cell cycle-dependent phosphorylation. In animals the onset, duration, and subcellular localization of H4S1/H2AS1 phosphorylation are similar to H3 phosphorylation on S10 and S28, suggesting that these modification events may together participate in the condensation or segregation of mitotic chromosomes. In addition, a lower level of H4S1/H2AS1 phosphorylation was found in early S-phase cells, possibly on newly deposited histones on replicating DNA (Barber et al. 2004).

2.4 Cell Cycle-Dependent Phosphorylation of Histone H1

The linker histone H1 is essential for the higher-order structure of chromatin. In dividing micronuclei of *Tetrahymena thermophila*, histone H1 is extensively phosphorylated in vivo by the protein kinase-A (PKA) (Sweet et al. 1997). A similar kinase recognition consensus sequence is also present in the C-terminal part of many members of the *Arabidopsis* H1 family. Aurora B-dependent phosphorylation of H1.4 at position S27 is most intensive at metaphase in dividing human cells (Hergeth et al. 2011). Like for histone H3S10, phosphorylation of H1.4 at S27 prevents binding of the HP1. In *Arabidopsis*, a similar Aurora recognition site might exist although with a replacement of serine by threonine. The cell cycle-dependent phosphorylation of human H1.4 at S35 leads to the dissociation of H1 from nucleosomes, resulting in a change in the packing density of chromatin (Chu et al. 2011). It is likely that in plants, this process is different, as the same sequence motif does not exist in histone H1.

3 Kinases Involved in Cell Cycle-Dependent Histone Phosphorylation

Several kinases implicated in histone H3 phosphorylation during mitosis in mammalian cells, Aurora B for H3S10 and H3S28 and Haspin for H3T3, appear to have orthologs in a wide range of eukaryotes. On the other hand, also lineages-specific kinases exist like the metazoan-specific histone kinase Dlk/ZIP (Preuss et al. 2003).

3.1 Aurora Kinases

Although H3S10 can be phosphorylated by multiple kinases under different conditions, Aurora is the most important kinase that phosphorylates this residue in vivo during normal mitosis (Xu et al. 2009). Genetic and biochemical data indicate that members of the Aurora kinase family, in particular, Ipl1p of *S. cerevisiae* and the B-type Aurora of *C. elegans*, *Drosophila*, and mammals, can control cell cycle-regulated histone H3 phosphorylation at serines 10 and 28, as opposed to the activity of type 1 phosphatase PP1 (Glc7p in budding yeast and nematodes) (Crosio et al. 2002; Giet and Glover 2001; Goto et al. 2002; Hsu et al. 2000). PP1 is also associated with mitotic chromosomes in vertebrates (Murnion et al. 2001). These findings suggest that PP1 could also be the phosphatase for H3S10 in higher organisms.

Studies of the intracellular localization of Aurora kinases in mitotic cells revealed an association with mitotic structures. Members of this family are overexpressed in a variety of cancers (Bischoff et al. 1998; Sen et al. 2002; Tatsuka et al. 1998), suggesting a crucial role in cell proliferation. Three *Arabidopsis* Aurora protein kinases were characterized, AtAurora1 (At4g32830), AtAurora2 (At2g25880), and AtAurora3 (At2g45490), which share high amino acid identities with the S/T kinase domain of yeast Ipl1 and animal Auroras. Structure and expression of AtAurora1 and AtAurora2 suggest that these genes arose by a recent gene duplication, whereas the diversification of plant Aurora kinase variants predates the origin of land plants. The transcripts and proteins of all three kinases are most abundant in tissues containing dividing cells. Intracellular localization of green fluorescent protein-tagged AtAurora kinases revealed an AtAurora-type-specific association mainly with dynamic mitotic structures, such as microtubule spindles and centromeres, and with the emerging cell plate of dividing tobacco BY-2 cells. Immunolabeling using *Arabidopsis* Aurora antibodies yielded specific signals at the centromeres that are coincident with histone H3 that is phosphorylated at S10 during mitosis. An in vitro kinase assay demonstrated that AtAurora1 preferentially phosphorylates histone H3 at S10 and S28 (Demidov et al. 2005; Kawabe et al. 2005).

Aurora-like kinases play key roles in chromosome segregation and cytokinesis in yeast, plant, and animal systems. For example, in plants, AtAurora1 and AtAurora2 are essential for correct cell division orientation and functionally diverged from AtAurora3, because it cannot complement the phenotype of the AtAurora1/2 mutants (Van Damme et al. 2011). AtAurora1-RNAi plants demonstrate cell division defects, arrested apical meristematic development, and ectopic meristem formation (Petrovska et al. 2012). Aurora functions before cytokinesis, since modulation of AtAurora1 expression by the Dbox of CycB1;1 abolishes the accumulation of AUR1-GFP at the forming cell plate. Further, it restores main root length and lateral root density to wild-type levels in AtAurora1/2 mutants (Van Damme et al. 2011). Interestingly, in addition to their role in cell division, AtAurora kinases are required for the regulation of endocycles, and plants with

downregulated Aurora kinases showed higher levels of endoreduplication (Petrovska et al. 2012).

Phosphorylation of histone H3 by Aurora kinases should not be considered separately from other PTMs. A combination of PTM marks on one histone and their physiological effects on each other is an important aspect of studying the function of chromatin. Cross talk between different modifications presumably helps to fine-tune control of different high-complexity processes in chromatin. In human, Aurora B phosphorylates histone H3S10 and releases heterochromatin protein 1 (HP1) from methylated histone H3K9 during mitosis (Fischle et al. 2005). Inhibition or depletion of the mitotic kinase Aurora B causes retention of HP1 proteins on mitotic chromosomes, suggesting that H3S10 phosphorylation is necessary for the dissociation of HP1 from chromatin in M phase (Hirota et al. 2005). These data suggest that binary marking by K9 methylation and S10 phosphorylation (the so-called methyl/phos switch) is required for mitosis.

Phosphorylation of H3S10 can enhance acetylation of histone H3K14 (Lo et al. 2000) or abolish acetylation of H3K9 by Gcn5 in yeast (Edmondson et al. 2002) and inhibit methylation at K9 by Suv39h in human cells (Rea et al. 2000). For plants, there is only sparse information on the cross regulation between H3S10 phosphorylation and posttranslational modifications of neighboring amino acid positions (Fig. 1m). We found that in vitro phosphorylation of H3S10 by AtAurora1 is strongly increased by K9 acetylation and decreased by K14 acetylation and T11 phosphorylation. However, S10 phosphorylation activity is almost unaltered by mono-, di-, or trimethylation of K9. An interference of H3K9 dimethylation by SUVR4 occurs by a preexisting phosphorylation at S10 (Demidov et al. 2009).

3.2 Haspin Kinases

Haspin kinase was first discovered in male germ cells of mice (Tanaka et al. 1999). It phosphorylates histone H3 at T3 which is the only identified substrate for mammalian Haspin so far (rather than itself). Although Haspin kinase is most strongly expressed in testis, it also ubiquitously appears at lower level in proliferating somatic cells. Mammalian Haspin is localized to the nucleus in interphase and revealed a cell cycle-dependent association with condensed chromosomes and centrosomes throughout mitosis (Dai and Higgins 2005; Dai et al. 2005; Tanaka et al. 1999). The ectopic expression of mammalian Haspin led to an increased level of H3T3 phosphorylation, delayed mitosis, and reduced proliferation. In contrast, depletion of Haspin by RNA interference (RNAi) resulted in a reduced level of mitotic H3T3 phosphorylation and prevented normal chromosome alignment at metaphase (Dai et al. 2005). As the chromosome alignment defects in Haspin-depleted cells are similar to those caused by depletion of cohesin Scc1 (Dai et al. 2009), the observed defects are likely due to premature loss of sister chromatid cohesion (Dai et al. 2006). Accordantly, in *Schizosaccharomyces pombe*, Haspin phosphorylates histone H3 at T3 and colocalizes with cohesin by interacting with

Pds5, a cohesin-binding protein. In contrast, the budding yeast Haspin homologues (Alk1 and Alk2) did not reveal in vitro kinase activity towards histone H3 (Nespoli et al. 2006). Furthermore, phosphorylation of histone H3T3 by Haspin is necessary for recruitment of the chromosome passenger complex components, Aurora B and Survivin, to the inner centromere in human and fission yeast (Kelly et al. 2010; Wang et al. 2010; Yamagishi et al. 2010).

The first Haspin homologue in plants was characterized in *Arabidopsis* (AtHaspin, At1g09450), which phosphorylates in vitro histone H3 at T3 (Ashtiyani et al. 2011; Kurihara et al. 2011) and at T11 (Kurihara et al. 2011). Microscopical detection of GFP-tagged AtHaspin revealed that during interphase, AtHaspin was mainly localized in the cytoplasm and at the nuclear periphery. However, during mitosis and after nuclear envelope breakdown, AtHaspin was detected on the condensed metaphase chromosomes (Kurihara et al. 2011). The highest expression of AtHaspin was observed in tissues with high level of cell proliferation like shoot apical meristems and flower buds. However, at lower level, it transcribed in all other differentiated tissues (Ashtiyani et al. 2011; Kurihara et al. 2011). Complete inactivation of AtHaspin results in alteration in division plane orientation and aberrant cell division in early embryos, which cause embryo lethality (Ashtiyani et al. 2011). Reduction of AtHaspin by RNAi caused reduction of H3T3 phosphorylation level and reduced chromatin condensation in interphase nuclei; however, no significant changes were detected in sister chromatid cohesin as observed in human. At the whole plant level, altered expression of the AtHaspin induced pleiotropic phenotypes with defects in floral organs and vascular tissue, reduced fertility, and modified adventitious shoot apical meristems that then gave rise to plants with multi-rosettes and multi-shoots (Ashtiyani et al. 2011). The observed growth phenotypes in AtHaspin mutants are hard to explain by a mitotic function of AtHapsin only.

The in vitro Haspin activity towards histone H3 is strongly reduced by modifications of adjacent amino acids such as H3R2me2, H3K4me3, and H3K4ac in human (Eswaran et al. 2009; Han et al. 2011) and H3R2me2, H3K4me3, and H3K4ac/H3T6ph in *Arabidopsis* (Karimi-Ashtiyani and Houben 2012). The observed cross talk between Haspin-driven histone H3T3 phosphorylation and neighboring amino acids and regulation of Haspin activity by adjacent non-cell cycle-dependent histone modifications is in agreement with the probable additional non-mitotic function for Haspin kinases.

3.3 NIMA Kinases

NIMA (never in mitosis) kinase is a serine/threonine kinase first discovered in the fungus *Aspergillus nidulans*. NIMA kinase regulates the entry into mitosis and chromatin condensation through phosphorylation of histone H3S10 (Osmani et al. 1991). In mammals, 11 NIMA-related kinases (Neks) have been described (O'Connell et al. 2003). Human Nek2 has the closest sequence similarity with

fungal NIMA (Fry 2002). The first indication of the existence of NIMA-related genes in plants was found in *Antirrhinum majus* (Zhang et al. 1996). The first plant Nek to be isolated was the tomato SPAK, involved in the regulation of shoot architecture and flowering (Pnueli et al. 2001). Nine NIMA-related kinases were identified in poplar (PNeks), six in rice (OsNeks), and seven in *A. thaliana* (AtNeks) (Vigneault et al. 2007). However, there are no indications that any plant NIMA-like kinase characterized so far is being involved in histone phosphorylation (Agueci et al. 2012; Cloutier et al. 2005; Motose et al. 2008; Vigneault et al. 2007). The potential kinase(s) involved in phosphorylation of H3T11 in plants remain to be identified. In mammals, Dlk/ZIP kinase seems to be a likely candidate since it phosphorylates H3 at T11 in vitro and its association with centromeres parallels precisely the appearance of T11 phosphorylation (Preuss et al. 2003). A BLAST search of the *Arabidopsis* databank for homologues of DAP kinase family members (Kogel et al. 2001) only revealed protein kinases with low similarity.

The finding of different kinases with H3 substrate specificity suggests that various kinases could function as mitotic H3 kinases in different organisms. It is also conceivable that within any single organism, many kinases can phosphorylate histones during cell division. As for mitotic cells, certain residues of H3 are phosphorylated in interphase cells of both metazoan and plants. However, many of the responsible kinases seem to have evolved independently in different lineages and target specific subsets of genes [reviewed in see (Cerutti and Casas-Mollano 2009)]. Histone phosphorylation, like other PTMs, has also been linked to the activation of transcription, apoptosis, DNA damage repair, and even sex chromosome dosage compensation [reviewed in (Banerjee and Chakravarti 2011; Cerutti and Casas-Mollano 2009; Johansen and Johansen 2006; Loury and Sassone-Corsi 2004; Prigent and Dimitrov 2003; Sanchez Mde et al. 2008; Xu et al. 2009)]; although our knowledge of its involvement in these processes in plants is limited.

4 Cell Cycle-Dependent Acetylation and Methylation of Histones

An overall reduction in histone acetylation during mitosis consistent with the repressed transcriptional activity has been reported for metazoan (reviewed in Xu et al. 2009). A similar tendency has been reported for plants. Indirect immunostaining of mitotic *Vicia faba* cells with antibodies directed against acetylated isoforms of histone H4 (acetylated at lysines 5, 8, 12, 16, and H4 tetraacetylated) revealed a cell cycle-dependent alteration of the acetylation level. Inhibition of deacetylase in vivo by Trichostatin A provided evidence of a high level of acetylation at lysine positions 5, 12, and 16, at or immediately after replication, which becomes reduced before mitosis when deacetylase is not inhibited experimentally (Belyaev et al. 1997). Also, in tissue culture tobacco cells, acetylation of H4 and H3 was dramatically reduced during mitosis in a

stage-specific manner; while deacetylation of H4 commenced at prophase and persisted up to telophase, histone H3 remained acetylated up to metaphase but was deacetylated at anaphase and telophase (Li et al. 2005). In barley at prometaphase, centromeric acetylation at H4K5 decreased suddenly. Subtelomeric acetylation at H4K16 was detected throughout the cell cycle, although it was reduced at metaphase (Wako et al. 2002). A metaphase-specific histone acetylation of the nucleolus organizing regions (NORs) was observed in *Vicia faba* (Houben et al. 1996; Jasencakova et al. 2000) and also in barley (Jasencakova et al. 2001; Wako et al. 2002). On the other hand, increased H4K5 acetylation at the NORs was not observed in *Silene* species, *Allium cepa*, and *Nicotiana tabacum* (Vyskot et al. 1999). Likely, strong histone acetylation in the NORs at metaphase enables these chromosome domains to avoid condensation in preparation for the onset of transcription starting at mitotic telophase (Wako et al. 2002). The mechanisms by which specific residues or chromosomal loci are selectively acetylated or deacetylated during cell divison are unclear. A possible relationship between histone H4 acetylation, DNA methylation, and histone H3 dimethylation at K9 during mitosis has been proposed by Yang et al. (2010). This assumption is based on the observation that the treatment of maize seedlings with trichostatin A resulted in increased H4 acetylation accompanied by the decondensation of interphase chromatin and a decrease in both global H3K9 dimethylation and DNA methylation during mitosis. On the other hand, treatment with 5-azacytidine caused chromatin decondensation and mediated an increase in H4 acetylation, in addition to reduced DNA methylation and H3K9 dimethylation during interphase and mitosis. The information on other cell cycle-dependent PTMs (e.g., ubiquitination, sumoylation) is limited, and the direct link of these modifications with mitosis and meiosis remains to be explored in plants.

5 Outlook

Complementary approaches, combining biochemical, genetic, and cell biological approaches, will be necessary to decipher the meaning of the diverse combinations of cell cycle-interrelated histone modifications. To identify all histone variants and PTMs, the complete profile of histone modifications occurring during mitosis and meioses should be identified by advanced mass spectrometry. Mass spectrometry also has advantages over single modification antibody analysis as it permits for simultaneous detection of multiple modifications on the same peptide (DiMaggio and Garcia 2010; Garcia et al. 2005). Considering plant development-specific-interrelated chromatin modifications, novel methods for the characterization of cell type-specific PTMs are required (Lafos et al. 2011; Lafos and Schubert 2009).

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Small RNA-Mediated Control of Development in Plants

Angel Emilio Martínez de Alba, Jean-Sébastien Parent, and Hervé Vaucheret

Abstract In the last decade, it became clear that RNA–RNA interactions are extremely important in the regulation of gene expression. Indeed, although a large portion of most eukaryotic genome is actively transcribed into RNA, only a small fraction encodes proteins. In many cases, long non-protein-coding RNAs are processed into small RNAs, 20–30 nucleotides in length, which regulate gene expression through base pairing with complementary sense RNA. Such mechanisms fine tune regulate the expression of genes during development and serve as a flexible, sequence specific, source of regulation that promotes adaptability in response to biotic and abiotic stresses. In this chapter, we review studies that uncovered the mode of action of the different classes of small RNAs during the development of plants.

1 Introduction

The tremendous interest in the structure–function studies of RNA lies in the progressive understanding that RNA is much more actively involved in the direct control of gene expression than initially anticipated. Indeed, for a long period of time, RNA has only been considered as a mere intermediate carrier of genetic information. The main factor that determined this paradigm shift was the identification of small non-protein-coding RNAs, 20–30 nucleotides (nt) in length, which mediate transcriptional and post-transcriptional silencing processes. These small RNAs confer specificity to a set of pathways collectively termed "RNA silencing". RNA silencing likely is an ancient eukaryotic process involved in sequence-specific control of invading nucleic acids. However, RNA silencing does not only maintain the quiescence of viruses and transposons but also controls the expression of many

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genes pertaining to development and patterning in eukaryotic organisms found in all kingdoms of life. Because of their small size, small RNAs have not been recognized as developmental regulatory molecules until recently, and biologists are still recovering from the shock that such an ancient and fundamental mechanism has remained overlooked for so long. As much as the discovery of epigenetic regulations and transposable elements has altered our views on gene expression regulation, the breakthrough of the discovery of RNA silencing in the 1990s has sparked a renaissance in our recognition of RNA as an additional, key player in this process.

2 Small RNA Biogenesis

Small RNAs are the generic sequence-specificity determinants of RNA silencing. Most of them are produced by specific cleavage of perfectly or imperfectly double-stranded (ds) RNA molecules by DICER or DICER-LIKE (DCL) proteins, which belong to the RNase III family of dsRNA specific endonucleases (Chapman and Carrington 2007; Ghildiyal and Zamore 2009; Voinnet 2009). Following their production, small RNAs are sorted into specific ARGONAUTE (AGO) family proteins (Hutvagner and Simard 2008; Tolia and Joshua-Tor 2007; Vaucheret 2008). AGO proteins contain a domain called PAZ, which binds to the 3′ end of small RNAs, a domain called MID, which binds to the 5′ end of small RNAs, and a domain called PIWI, which carries an RNaseH-like motif that exhibits RNA cleavage activity (Tolia and Joshua-Tor 2007). AGO proteins function as the core of RNA silencing complexes. Small RNAs guide AGO proteins to their target through complementary base pairing. Then, AGO (often associated with other proteins) silences these targets through RNA cleavage, translational interference, or chromatin modifications (Bartel 2009; Brodersen and Voinnet 2009; Fabian et al. 2010).

Plant small RNAs can be divided into two main classes: microRNAs (miRNA) and small interfering RNAs (siRNA), which comprise several subclasses. Although they are closely related biochemically, siRNAs and miRNAs differ in their mode of biogenesis (Brodersen and Voinnet 2006; Vaucheret 2006). The model plant species *Arabidopsis thaliana* contains four DCL genes that exhibit specialized functions in small RNA biogenesis. DCL1 processes imperfectly paired miRNA precursors, whereas DCL2, DCL3 and DCL4 process mostly perfectly paired siRNA precursors. What makes a dsRNA a particularly attractive substrate for DCL2, DCL3 or DCL4 remains unclear. However, when dsRNA is produced by an RNA-DEPENDENT RNA POLYMERASE (RDR), the DCL specificity could rely on the specific relationship existing between RDRs and DCLs. In *Arabidopsis*, it is known that DCL4 mostly processes endogenous RDR6-derived dsRNA (Gasciolli et al. 2005), whereas DCL3 generally processes endogenous RDR2-derived dsRNA (Xie et al. 2004). In contrast, DCL2 is thought to process RDR-independent dsRNA produced by endogenous inverted repeats, although this type

of dsRNA substrate is often processed nonexclusively by DCL2, DCL3 and/or DCL4 (Dunoyer et al. 2010). DCL2, DCL3 and DCL4 can substitute to each other when one is missing (Gasciolli et al. 2005) and only when DCL2, DCL3 and DCL4 are missing can DCL1 process some siRNAs in addition to miRNAs (Bouché et al. 2006).

2.1 miRNAs

miRNAs are processed from long single-stranded (ss) primary transcripts (pri-miRNA) that are transcribed from specific non-protein-coding *MIR* genes by DNA-DEPENDENT RNA POLYMERASE II (Pol II). Like regular protein-coding mRNAs, pri-miRNAs are capped at their 5′ end, may contain introns, and are poly-adenylated at their 3′ end (Jones-Rhoades et al. 2006). One of the defining features of *MIR* transcripts is their intramolecular sequence complementarity, which forms a characteristic fold-back stem–loop structure. The RNA precursor undergoes two processing events within the nucleus that liberates a 21-nt mature miRNA/miRNA passenger strand (miRNA*) duplex with two-nucleotide 3′ overhangs (Park et al. 2002; Reinhart et al. 2002; Xie et al. 2003; Kurihara and Watanabe 2004; Kurihara et al. 2006). In most cases, DCL1 first catalyses cleavage at the loop-distal side, and subsequently on the loop-proximal side, of the miRNA/miRNA* region, in a sequential manner (Kurihara and Watanabe 2004), but examples of reverse order have been described (Mateos et al. 2010; Song et al. 2010; Werner et al. 2010). The nuclear maturation and processing of pri-miRNA is orchestrated by DCL1 but requires the additional activity of interacting partners, including the CAP-BINDING PROTEINs CBP20 and CBP80/ABH1 (Gregory et al. 2008; Kim et al. 2008; Laubinger et al. 2008), the C2H2-zinc finger protein SERRATE (SE) (Lobbes et al. 2006; Yang et al. 2006), the double-stranded RNA-binding protein HYPONASTIC LEAVES 1 (HYL1) (Han et al. 2004) and the RNA-binding protein DAWDLE (Yu et al. 2008). To protect the miRNA/miRNA* duplex from degradation, its 3′ ends are methylated by the methyltransferase HUA ENHANCER 1 (HEN1) (Li et al. 2005), and exported out of the nucleus by HASTY (HST) (Park et al. 2005), although it is not yet known which of these two steps occurs first. Upon export, the two strands of the miRNA/miRNA* duplex are separated and the miRNA strand, also called guide strand, is bound by an AGO family protein. The miRNA governs AGO protein for target transcripts recognition through sequence complementarity to the loaded miRNA. Thus, miRNAs act in *trans* upon the mRNAs of other genes to modulate their expression spatially and temporally. In the majority of studied cases, targeting leads to slicing of at least a fraction of total target RNAs (Mallory et al. 2008), although it is clear that repression of targets involves both degradative and nondegradative mechanisms (Voinnet 2009) (Figs. 1 and 2).

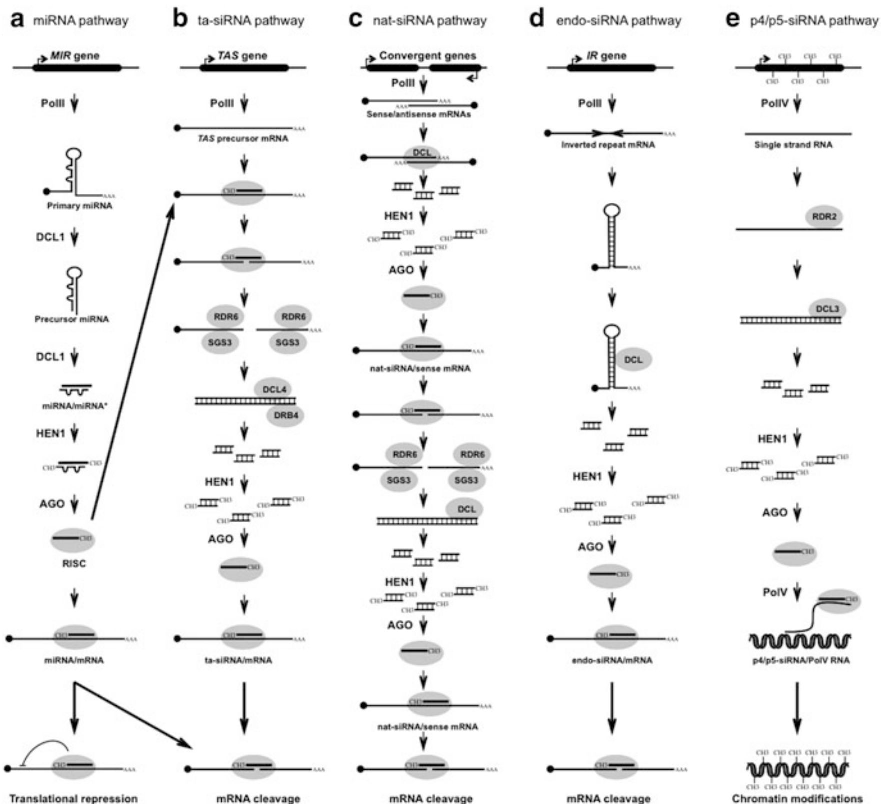

Fig. 1 Endogenous small RNA-mediated silencing. (**a**) miRNA pathway. miRNAs are generated by transcription of non-protein-coding *MIR* genes by RNA Pol II into a single-stranded RNA that folds back to form a hairpin structure, called primary miRNA. The RNA precursor is generally processed from the free-end opposite to the loop by DCL1 to yield 21-nt miRNAs duplexes. The miRNA/miRNA* duplexes are methylated at their 3′ ends by HEN1 and transferred to the cytoplasm by a HASTY-dependent export system. The miRNA guide strand is selected and incorporated into the RNA-induced silencing complex (RISC) containing an AGO protein (usually AGO1). The RISC is recruited to the target gene on the basis of sequence complementarity, and AGO represses gene expression by either RNA cleavage or translational repression. (**b**) ta-siRNA pathway. Non-protein-coding *TAS* genes are transcribed by PolII. After initial miRNA-mediated cleavage of the *TAS* precursor, the resulting fragments act as templates for the formation of long dsRNA by concerted action of RDR6 and SGS3. The resulting dsRNA is transported to the nucleus where it is processed by DCL4 and its partner DRB4 into phased 21-nt duplexes, which are then methylated by HEN1. The ta-siRNAs are incorporated into a RISC containing AGO protein and regulate the expression of complementary target mRNAs. (**c**) nat-siRNA pathway. Natural antisense transcripts produced by Pol II form dsRNA within their overlapping regions. The dsRNAs are processed by a DCL into siRNAs that target one of the transcripts through an unidentified AGO protein containing RISC complex. RDR6-SGS3, together with Pol IV, forms an amplification loop to generate more nat-siRNAs, which reinforce the cleavage of the target transcript. (**d**) endo-siRNA pathway. Long inverted-repeat transcripts produced by Pol II fold back to form dsRNAs with perfect or near-perfect complementarity. These hairpins are processed by DCL2, DCL3 and/or DCL4 into siRNAs duplexes. Following methylation by HEN1, one strand is loaded onto an

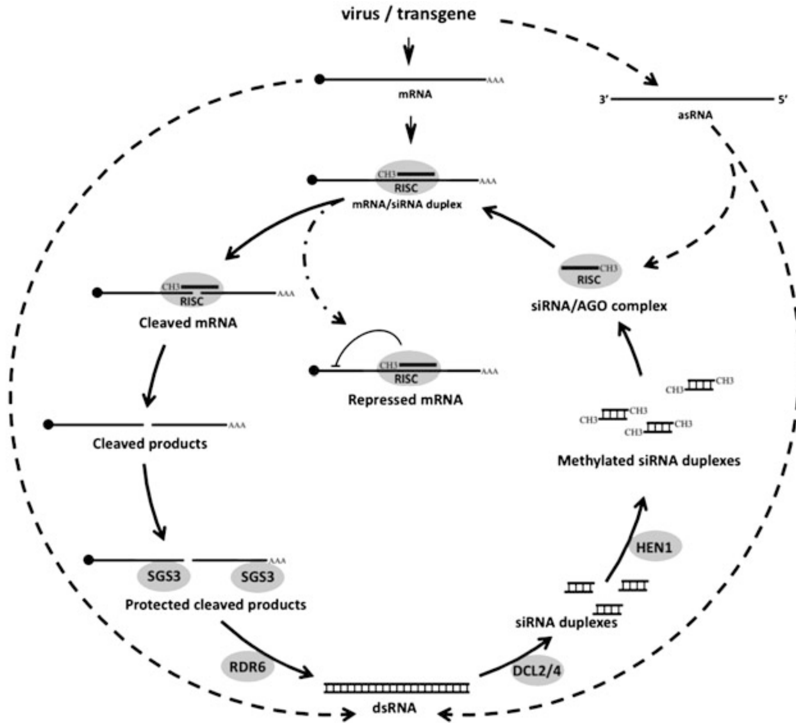

Fig. 2 PTGS of invading nucleic acids. The exact mode of production of exogenous primary siRNAs remains unknown. They could result from the dicing of dsRNA formed by the annealing of sense and antisense RNAs derived from exogenous nucleic acid, or by direct trimming of antisense RNAs (dashed lines). When incorporated in the RISC complex, 21–22-nt antisense primary siRNAs guides AGO proteins to sense mRNA throughout sequence homology, leading to RNA cleavage. The resulting cleavage products are stabilized by SGS3 and used as template by RDR6 to synthesize dsRNAs. These long dsRNAs are then processed by DCL2 and DCL4 into secondary siRNAs that are loaded on AGO proteins, leading to RNA cleavage and/or translational inhibition, which complete the silencing of the exogenous nucleic acids

Fig. 1 (continued) AGO protein and guides the complex to the target mRNA through complementarity. (**e**) p4/p5-siRNA pathway. Regions of the genome rich in retroelements, repetitive DNA and methylated DNA are transcribed by Pol IV, resulting in the formation of ssRNA that is converted into dsRNA by the action of RDR2. This dsRNA is processed into predominantly 24-nt long p4/p5-siRNAs by DCL3. These 24-nt siRNAs associate with AGO4 (or AGO6, or AGO9) and nascent transcripts produced by Pol V to form the RNA-induced transcriptional gene silencing (RITS) complex that recruits proteins involved in heterochromatin formation, including DNA methyltransferases DRM2 and CMT3, to the p4/p5-siRNA target loci

2.2 siRNAs

In contrast to miRNAs that are processed from short hairpin structures resulting from the imperfect and partial pairing of long ssRNA molecules, siRNAs derive from long, nearly perfectly paired, dsRNA precursors produced from either the transcription of long inverted repeats, convergent transcription of overlapping genes or the action of cellular or viral RDRs on ssRNAs (Brodersen and Voinnet 2006; Vaucheret 2006). Based upon their origins and functions, *A. thaliana* endogenous siRNAs can be divided into four classes: trans-acting siRNAs (ta-siRNA), natural antisense transcript-derived siRNAs (nat-siRNA), endogenous siRNAs (endo-siRNA) and Pol lV/Pol V siRNAs (p4/p5-siRNA).

2.2.1 ta-siRNAs

ta-siRNAs derive from long transcripts of non-protein-coding *TAS* genes, which contain specific miRNA-binding sites (Allen et al. 2005; Peragine et al. 2004; Vazquez et al. 2004). *TAS* genes are transcribed by Pol II and likely are exported/trafficked by the THO/TREX complex to miRNA/AGO catalytic centres (Jauvion et al. 2010; Yelina et al. 2010). ta-siRNA biogenesis is initiated by miRNA-directed cleavage of *TAS* transcripts, thus signifying the crosstalk among the different small RNA pathways. After RNA precursor cleavage, the RNA-binding SUPPRESSOR OF GENE SILENCING 3 (SGS3) protein stabilizes the cleavage products, which likely prevents degradation and allows recruiting RDR6 which, assisted by the putative RNA export factor SILENCING DEFECTIVE 5 (SDE5), catalyses the synthesis of a second complement RNA strand (Elmayan et al. 2009; Hernandez-Pinzon et al. 2007; Jauvion et al. 2010; Yoshikawa et al. 2005). Such dsRNAs are next processed sequentially by DCL4 (Gasciolli et al. 2005; Xie et al. 2005) assisted by DOUBLE STRAND RNA-BINDING PROTEIN 4 (DRB4) (Nakazawa et al. 2007) to generate a population of 21-nt siRNAs duplexes that, analogous to miRNAs, are methylated by HEN1 (Li et al. 2005). Resulting siRNA duplexes are in phase with the site of precise miRNA-guided cleavage of the primary *TAS* transcript. Thus, the register of the ta-siRNAs, and hence their sequence and subsequently their targets, are determined by the initial cleavage site of the miRNA (Allen et al. 2005; Axtell et al. 2006; Montgomery et al. 2008; Rajagopalan et al. 2006; Vazquez et al. 2004; Vaucheret 2005; Yoshikawa et al. 2005). One strand of the ta-siRNA duplex associates with AGO1 (Allen and Howell 2010) to guide cleavage of target mRNAs. A subset of ta-siRNAs notably regulates the expression of *AUXIN RESPONSE FACTORS* (*ARF*) gene family members to control vegetative phase transition in *Arabidopsis* (Adenot et al. 2006; Fahlgren et al. 2006; Garcia et al. 2006; Hunter et al. 2006; Peragine et al. 2004). ta-siRNAs appear to function in development (much like miRNAs) but they have a unique mode of biogenesis involving components of both miRNA and siRNA pathways (Vaucheret 2005).

2.2.2 nat-siRNAs

nat-siRNAs originate from dsRNA precursors derived from natural antisense transcripts (NAT) which are generated by convergent transcription of two overlapping genes at a given genomic locus (*cis*-NAT genes). nat-siRNAs could also derive from complementary RNAs transcribed from two distinct loci (*trans*-NAT genes) (Wang et al. 2005a). Co-expression of overlapping sense/antisense transcripts could potentially form dsRNAs, which initiate the regulation process. Although it is still unclear how much of these converging transcriptions lead to RNA silencing, a few examples have been reported (Borsani et al. 2005; Katiyar-Agarwal et al. 2006). In both publications, one of the overlapping genes is constitutively expressed, while the stress-induced expression of the other gene governs the formation of the dsRNA. Although dsRNAs are assumed to result from annealing of overlapping sense/antisense transcripts RNA pairs, RNA polymerase IV (Pol IV), RDR6 and SGS3 are required for primary nat-siRNA accumulation in addition to a DCL (Borsani et al. 2005). Primary nat-siRNAs are loaded onto a yet unidentified AGO protein to direct the cleavage of the constitutively expressed complementary transcript. In a second step, the cleaved transcript is converted into dsRNA in a Pol IV-, RDR6- and SGS3-dependent manner (Borsani et al. 2005). Processing of this dsRNA by a DCL produces a phased array of 21-nt nat-siRNAs species, which further promote the silencing of the constitutive expressed transcripts (Borsani et al. 2005). The second step is mechanistically similar to the biogenesis of ta-siRNAs. Most probably, nat-siRNAs are methylated by HEN1 to protect them from degradation, as the RNA methyltransferase mutant *hen1* reduces the level of nat-siRNA accumulation (Katiyar-Agarwal et al. 2006). The nat-siRNA pathway appears to function as a plant adaptive protection mechanism in response to either abiotic or biotic stress (Borsani et al. 2005; Katiyar-Agarwal et al. 2006).

2.2.3 Endo-siRNAs

Inverted-repeats of longer length than miRNA precursors are found throughout the *Arabidopsis* genome. Some of them generate siRNAs referred to as endo-siRNAs (Dunoyer et al. 2010). Like pri-miRNAs, endo-siRNA precursors fold back to form dsRNA molecules with perfect or near-perfect complementarity, which likely makes them substrates of DCL2, DCL3 and DCL4 instead of DCL1. This explains the variety of lengths (21-, 22- and/or 24-nt) encountered in this family of siRNAs. The biological role of these small RNA molecules is still unknown, but it has been proposed that they could be used in adaptation to the environment and also trans-generational memory (Dunoyer et al. 2010).

2.2.4 p4/p5-siRNAs

The last and, by far, most abundant class of siRNA population, is formed by ~24-nt siRNAs which mostly derive from transposons and DNA repeats (Kasschau et al. 2007). Their production can be separated in two genetically distinct steps that have been precisely studied in *Arabidopsis* (Daxinger et al. 2009). At first, the biogenesis of 24-nt siRNAs requires the plant-specific Pol IV, which is recruited to target specific genomic loci by a mechanism that remains largely unknown (Lahmy et al. 2010; Pikaard et al. 2008). Pol IV likely generates ssRNA transcripts (Pikaard et al. 2008) that are transformed into dsRNA by RDR2. CLASSY 1 (CLSY1), a putative chromatin-remodelling factor, is required to assist Pol IV and RDR2 during dsRNA production (Dunoyer et al. 2010; Greenberg et al. 2011; Smith et al. 2007). dsRNAs are then processed by DCL3 into 24-nt primary siRNAs that are then methylated on their 3′ ends by HEN1 (Li et al. 2005). One strand of the 24-nt duplex is loaded into either AGO4, AGO6 or AGO9 effector proteins, which cleave nascent transcripts produced by a second plant-specific RNA polymerase V (Pol V) (Haag and Pikaard 2011). Pol V attracts DNA methyltransferases, chromatin-remodelling proteins and histone modification enzymes, causing transcriptional silencing at the locus of origin (Duran-Figueroa and Vielle-Calzada 2010; Havecker et al. 2010; Henderson and Jacobsen 2007; Law and Jacobsen 2010; Matzke et al. 2009; Olmedo-Monfil et al. 2010; Zaratiegui et al. 2007).

3 Modes of Action

A common set of enzymatic activities (RDRs, DCLs and AGOs) and mechanisms (synthesis of dsRNA, dicing into small RNA, and small RNA-directed RNA cleavage) are shared between TGS and PTGS pathways. However, the two processes have different outcome. Indeed, 24-nt siRNAs mediate transcriptional gene silencing (TGS) through DNA methylation and chromatin modifications while 21- and 22-nt siRNAs and miRNAs mediate post-transcriptional gene silencing (PTGS) through RNA cleavage and translational inhibition (Ding and Voinnet 2007; Mallory and Vaucheret 2009; Voinnet 2009). The distinct activity of 24-nt vs. 21–22-nt small RNAs is mostly based on the selectivity of AGO proteins towards small RNAs. Indeed, after excision from the dsRNA precursor, one strand of the small RNA duplex is eliminated (the passenger strand), whereas the other strand (the guide strand) is selectively sorted to one or more AGO proteins according to the 5′ nucleotide or other sequence/structural elements of the small RNA (Mi et al. 2008; Montgomery et al. 2008; Takeda et al. 2008; Zhu et al. 2011). Base pairing between the small RNA guide strand and the target RNA determines the locus specificity, whereas the association of AGO to other proteins determines the type of silencing.

Silencing heritability also varies between TGS and PTGS. Indeed, TGS leads to chromatin modifications, which sometimes are inherited, thus maintaining silencing over multiple generations, even after the initial trigger has been eliminated. On the contrary, during PTGS, RNA degradation is achieved with no epigenetic incidence, i.e., there is no direct effect on the transcription rate of the corresponding gene, even when DNA methylation is triggered in the body of the silenced (trans) gene, and no heritability of the silent state after elimination of the trigger.

3.1 Transcriptional Level

In plants, RNA-directed DNA methylation (RdDM) was first observed in tobacco plants infected with a circular RNA pathogen known as viroid. Under viroid infection, RNA–RNA replication takes place and integrated transgenic DNA sequences homologous to the viroid become methylated (Wassenegger et al. 1994). Further investigations showed that the constitutive expression of a dsRNA results in *de novo* DNA methylation of the homologous DNA sequences. If the dsRNA is homologous to transcribed regions, DNA methylation does not impair transcription. However, if the dsRNA is homologous to promoter regions, DNA methylation leads to transcriptional silencing (Mette et al. 2000; Sijen et al. 2001). Importantly, production of siRNAs matching the promoter sequence provided the first evidence for siRNA-dependent mechanisms role in RdDM (Matzke et al. 2004; Mette et al. 2000). *Arabidopsis* presents DNA methylation throughout its genome but it is enriched in repetitive heterochromatic domains and also found in euchromatic regions, typically at dispersed transposons and related sequences. (Chan et al. 2005; Zhang et al. 2006; Zilberman et al. 2007). p4/p5-siRNAs provoke transcriptional gene silencing, epigenetic modifications and RdMD at transposons and DNA repeats (Matzke et al. 2007; Slotkin and Martienssen 2007). RdDM induces *de novo* methylation of cytosines in all sequence contexts (CG, CHG, CHH, where H is A, T or C) at the region of siRNA–DNA sequence homology. Maintenance of CG methylation is carried out by the DNA METHYLTRANSFERASE 1 (MET1) (Cokus et al. 2008; Lister et al. 2008). In parallel, most CHG and CHH methylations are maintained by the DNA cytosine methyltransferases DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2), and CHROMOMETHYLASE3 (CMT3), a plant specific DNA methyltransferase (Cao and Jacobsen 2002). On the contrary, *de novo* methylation in all nucleotide contexts (CG, CHG and CHH) is catalysed mostly by only one DNA methyltransferase, DRM2 (Cao et al. 2003; Matzke et al. 2009). Gene body methylation does not affect transcription but may silence cryptic promoters and/or reflect maintenance methylation following ancient RdDM events (Zilberman 2008). At some RdDM loci, siRNAs are not detected in absence of DRM2, suggesting that a positive feedback loop is required for efficient siRNA-dependent de novo methylation and gene silencing (Cao et al. 2003). *Arabidopsis* encodes ten AGO proteins (Vaucheret 2008). Not surprisingly, a partial redundancy between AGO proteins has been observed in RdDM. Indeed, AGO6 was found to act, in certain cases, redundantly

with AGO4 to guide DNA methylation and transcriptional gene silencing (Zheng et al. 2007). The function of some AGO proteins function remains unknown, therefore it is possible that more AGO proteins play a role in RdDM. Pol V interaction with AGO4 has been proven to be essential for RdDM (El-Shami et al. 2007), suggesting that Pol V and AGO4 tightly cooperates in siRNA-directed de novo DNA methylation. Similarly, the RNA "slicer" activity of AGO4 is required for RdDM of some loci (Qi et al. 2006), most probably for the production of secondary siRNAs involved in guiding the downstream spreading of DNA methylation and concurrent transcriptional silencing with the help of Pol V.

3.2 Post-transcriptional Level

The production of transgenic plants exhibiting reliable expression of a transgene conferring a desirable trait is one of the major challenges of modern plant biology. PTGS was originally described as an undesired outcome occurring while attempting to highly express the introduced transgene. Indeed, in several cases, plants that were co-suppressed for both the transgene and the homologous endogenous gene(s) were identified (Napoli et al. 1990; Smith et al. 1990; van der Krol et al. 1990). Subsequent investigations led to the characterization of dsRNA as the sequence-specific molecule inducing PTGS (Fire et al. 1998; Waterhouse et al. 1998) and to the identification of 21–22-nt small RNAs as effectors of PTGS (Hamilton and Baulcombe 1999).

The discovery that several classes of endogenous small RNA exist and the growing number of reports have revealed the extent of PTGS phenomena and the diversity of silencing mechanisms as well as the mechanistic differences among the various PTGS pathways. miRNAs function in trans by guiding an RNA-silencing complex to target mRNAs derived from unlinked loci. miRNAs mediate cleavage when they exhibit extensive complementarity with target mRNAs. Because most plant miRNAs bind perfectly or near-perfectly to their target, cleavage is considered as the main process for miRNA-mediated gene regulation in plants (Rhoades et al. 2002; Schwab et al. 2005). However, translational inhibition has also been observed in some cases (Aukerman and Sakai 2003; Brodersen et al. 2008; Chen 2004; Gandikota et al. 2007). The rules governing the choice between cleavage and translational inhibition are still not understood. On the other hand, 21- and 22-nt siRNAs act either *in cis* or *in trans* because they exhibit complementarity to RNA transcribed from their locus of origin in addition to RNA transcribed from homologous unlinked loci.

Apart from the size of the small RNA molecule, the identity of the AGO partner also seems to have an effect on the outcome upon target recognition. Analysis of small RNAs cloned after specific AGO pull-down experiments revealed distinct features for different AGO proteins (Mi et al. 2008; Montgomery et al. 2008; Takeda et al. 2008; Zhu et al. 2011). AGO4, AGO6 and AGO9 proteins mostly

associate with 24-nt siRNAs, whereas AGO1, AGO2, AGO5, AGO7 and AGO10 mostly associate with 21–22-nt molecules. AGO7 and AGO10 show an almost exclusive association with miR390 and miR165/166, respectively, whereas AGO1, AGO2 and AGO5 associate with small RNAs that exhibit a uridine, an adenosine and a cytosine at their 5′ end, respectively. Therefore, loading of a small RNA onto an AGO protein seems to follow a very tight selective process that deeply impacts the silencing outcome.

4 Examples of Regulatory Circuits Involving Coordinated Action of the Pathways

Small RNAs can regulate various aspects of plant development or stress response programmes. Here, we described several examples showing that the integration of various silencing mechanisms is necessary to ensure proper developmental programmes.

4.1 Leaf Development

Several miRNAs and ta-siRNAs participate in the control of leaves patterning and development in various plant species (Jung et al. 2009). Genes encoding the homeodomain-leucine zipper (HD-ZIP) class III transcription factors, which control meristem development (Prigge et al. 2005) are targets of miR165/166 (Rubio-Somoza and Weigel 2011). Five TEOSINTE BRANCHED/CYCLOIDEA/PROLIFERATING CELL FACTOR (TCP) transcription factors, which regulate leaf development, are targets of miR319 and over-expression of miR319 resulted in jaw-D phenotypes, including uneven, curved leaf shape and curvature (Palatnik et al. 2003). *ARF* genes, which code for transcription factors that regulate auxin signalling, are targeted by several miRNAs. ARFs are a class of plant-specific DNA-binding proteins, which control auxin-regulated transcription and function in plant development, particularly on root and shoot development. There are 23 *ARF* genes in *Arabidopsis* and at least 8 of them are targeted by miRNAs or ta-siRNAs. *ARF6* and *ARF8* play a role in gynoecium and stamen development and are targets of miR167 (Wu et al. 2006). Regulation of *ARF10*, *ARF16* and *ARF17* genes is achieved by miR160-guided cleavage, allowing proper phyllotaxis in the rosette (Liu et al. 2007; Mallory et al. 2005; Wang et al. 2005b). CUP-SHAPPED COTYLEDON1 (CUC1) and CUC2 regulate separation of the organs by restricting cell proliferation and are regulated by miR164-mediated cleavage (Laufs et al. 2004; Mallory et al. 2004). The *CUC1* and *CUC2* genes belong to the NAC-domain transcription factors family and miR164 has been shown to target another four *NAC* family genes (*NAC1* (*At1g01010*), *ORE1* (*At5g39610*), *At5g07680* and *At5g61430*).

The *NAC* family genes function in various developmental processes, including lateral root development and organ boundary formation in shoot meristem and flower development (Guo et al. 2005; Mallory et al. 2004; Sieber et al. 2007).

Plants have further adapted RNA silencing to regulate protein-coding genes through the use of ta-siRNAs (Allen et al. 2005; Peragine et al. 2004; Vazquez et al. 2004). ta-siRNAs are endogenous siRNAs that, like miRNAs, regulate genes different from those from which they originate and thus act *in trans*. In *Arabidopsis*, a capped and polyadenylated transcript from a *TAS3* locus is channelled into the RNA silencing pathway by a cleavage event triggered by miR390. The cleaved *TAS3* transcripts are then copied into dsRNAs by RDR6 and the dsRNAs are converted to siRNAs by DCL4. The *ARF* derived ta-siRNAs (ta-siARF) regulate their target mRNAs (*ARF3* and *ARF4*) in the same manner as miRNAs do. ta-siARFs are the only ta-siRNAs for which a role in plant development is known.

4.2 Flowering Time

Several miRNAs and p4/p5-siRNAs participate in the control of flowering time. APETALA2 (AP2) and AP2-like transcript levels are not affected by the overproduction of miR72, but the AP2 protein level is reduced, thus regulating the transition from vegetative growth to reproductive growth and floral development (Chen 2004). Although cleavage of target mRNAs is also directed by miR172, the mRNA levels remain unchanged due to a feedback regulation (Schwab et al. 2005). Similarly, OLIGOURIDYLATE-binding PROTEIN 1b (UBP1b) and SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 3 (SPL3), the targets of miR854 and miR156/157, respectively, are also regulated through translational repression (Arteaga-Vazquez et al. 2006; Gandikota et al. 2007) thus suggesting a widespread coexistence of translational repression and mRNA cleavage (Brodersen et al. 2008).

The *FLOWERING WAGENINGEN* (*FWA*) gene is a well-studied example of a developmental gene regulated by transcriptional repression through DNA methylation. *FWA* encodes a homeodomain-containing transcription factor that represses flowering. *FWA* gene expression is subjected to imprinting as it is solely expressed from its maternal gene, in the central cell and the endosperm tissue. Thus, the gene is expressed in endosperm and repressed in other tissues and such repressed state is associated with methylation of a gene upstream region (Soppe et al. 2000). Two tandem direct repeats present in *FWA* promoter seem to be necessary for repression and appear to attract DNA methylation (Lippman et al. 2004). Hypomethylation in this area induced by mutations in the chromatin-remodelling protein *DECREASE IN DNA METHYLATION 1* (*DDM1*) and *MET1* genes leads to an ectopic expression and a late-flowering phenotype (Soppe et al. 2000). After introduction into the *Arabidopsis* genome, extra copies of the *FWA* gene are methylated and silenced. Consequently, the transformation of wild-type plants by a *FWA* transgene does not lead to late-flowering phenotype because endogenous and transgenic copies are

both inactive. In contrast to wild-type plants, which methylate and silence transgenic *FWA* copies, RdDM mutants lacking *de novo* methylation express the transgene and exhibit a late-flowering phenotype. Using this approach, a range of genes necessary for *de novo* methylation has been revealed (Chan et al. 2004). Intriguingly, efficient *de novo* methylation of *FWA* transgenic copies requires the endogenous *FWA* gene to be methylated (Chan et al. 2006). In addition, it was shown that siRNAs are formed on direct repeats of the 5′-area of *FWA* gene (Lippman et al. 2004). The level of *FWA* siRNAs appears to be unchanged whether the *FWA* gene is methylated or not, indicating that *FWA* siRNAs are necessary to establish *FWA* DNA methylation but not sufficient to maintain silencing.

4.3 Pathogen Response

Several miRNAs and siRNAs participate in pathogen responses. In *Arabidopsis*, miR393 was the first small RNA identified in defence against bacteria (Navarro et al. 2006). Accumulation of miR393 is induced by Flg22, a bacterial flagellin-derived pathogen-associated molecular pattern (PAMP). miR393 negatively regulates auxin signalling by targeting the messenger RNAs of auxin receptors TRANSPORT INHIBITOR RESPONSE 1 (TIR1), AUXIN SIGNALING F-BOX PROTEIN 2 (AFB2) and AFB3. Over-expressing Myc-AFB1 (which is not targeted by miR393) in *tir1-1* background, resulted in enhanced susceptibility and disease symptoms after infection by the virulent *Pseudomonas syringae* pv. tomato strain DC3000 (Pst DC3000). Conversely, over-expression of miR393a from a strong constitutive promoter resulted in lower levels of *TIR1* mRNA and restricted bacterial growth. Interestingly, miR393* was recently shown to participate in bacterial pathogen response in association with AGO2. Indeed, miR393*, which has a 5′A, associates with AGO2 and regulates genes involved in effector-triggered immunity, while miR393, which has a 5′U and associates with AGO1 to target genes involved in PAMP-triggered immunity (Zhang et al. 2011).

Apart from miR393 and miR393*, two other miRNAs, miR160 and miR167, were also upregulated after infection. These miRNAs target members of the ARF family of transcription factors also involved in auxin signalling (Rhoades et al. 2002). Thus, multiple components of the auxin signalling pathway seem suppressed upon Pseudomonas infection, supporting the previous suggestion that, besides its many roles in plant development, auxin is a negative regulator of plant defence (Robert-Seilaniantz et al. 2007). Another miRNA, miR825, predicted to target remorins (zinc finger homeobox proteins) and frataxin-related proteins, was also found upregulated during Pst hrcC infection (Fahlgren et al. 2007).

The interaction between plants and *Agrobacterium tumefaciens* is of general interest because of the widespread use of this pathogen for transferring genes into plant genomes. An oncogenic strain of Agrobacterium was shown to induce miR393 at the infected zones of tobacco, whereas a disarmed strain was unable to do so (Pruss et al. 2008) suggesting that some T-DNA-encoded factors are sensed

by the host to induce miR393 and, perhaps, to promote defence, as seen with Pseudomonas. A possible counter–counter-defensive strategy from the pathogen in this particular interaction was evidenced by the fact that tumour growth is itself promoted by auxin pathway de-repression. Interestingly, in tumours induced during virulent Agrobacterium infection of *Arabidopsis*, the levels of miR393 and miR167 are significantly reduced. Moreover, roots and stems of miRNA-deficient mutants, *dcl1* and *hen1*, are immune to Agrobacterium infection (Dunoyer et al. 2006). Globally, these results point to the complexity underlying the mechanisms by which auxin signalling pathways appear to be modulated by miRNAs during bacterial infections.

Similarly to miRNAs, siRNAs were also recently found to contribute to plant antibacterial immunity. The bacterial pathogen-induced nat-siRNAs enhance the host defence response by repressing a putative negative regulator of the disease resistance pathway (Katiyar-Agarwal et al. 2006). It therefore seems that nat-siRNAs can be involved in cellular responses to pathogen attacks. The first plant-endogenous nat-siRNA identified as being involved in plant immunity was nat-siRNAATGB2 (Katiyar-Agarwal et al. 2006). Elicitation of *Arabidopsis* carrying the resistance gene *RPS2* with *P. syringae* (avrRpt2) induces nat-siRNAATGB2 (Katiyar-Agarwal et al. 2006). More recently, a novel class of endogenous siRNAs—long siRNAs (lsiRNAs)—that are 30–40 nt long have been shown to contribute to host defence response to bacterial pathogens (Katiyar-Agarwal et al. 2007). One such lsiRNA, AtlsiRNA, is induced by infection with *P. syringae* (avrRpt2). Moreover, knockout mutant of the AtlsiRNA-1 predicted target displayed enhanced resistance to bacteria (Katiyar-Agarwal et al. 2007). It has been proposed that AtlsiRNA-1 employs a unique mechanism to degrade target mRNA by decapping followed by 5′–3′ exoribonuclease-mediated decay (Katiyar-Agarwal et al. 2007).

4.4 Antiviral Response

While it is well established that virus-derived siRNAs play a direct role in the plant antiviral defence response by being turned back onto the pathogen's genome, there is now indication that host-encoded, as opposed to parasite-encoded, small RNAs might also be involved in such a response. Indeed, two miRNAs, bra-miR158 and bra-miR1885, have been shown to be significantly upregulated during *Brassica rapa* infection by *Turnip mosaic virus* (TuMV) (He et al. 2008). However, this response was highly specific to TuMV infection as similar experiments performed on *B. rapa* and *B. napus* with *Cucumber mosaic virus*, *Tobacco mosaic virus* or the fungal pathogen *Sclerotinia sclerotiorum* showed no induction of either miRNA (He et al. 2008). Interestingly, the putative target for bra-miR1885 is predicted as a member of the TIR-NUCLEOTIDE-BINDING SITE DOMAINS (NBS)-C-terminal LEUCINE-RICH REPEATS (LRR) class of disease-resistant proteins (He et al. 2008). It is therefore possible that the reported induction reflects a pathogen's

attempt to modulate its host resistance pathway rather than a bona fide plant defence response.

Plant small RNAs, including miRNAs and siRNAs, seem to be key regulatory components of the plant defence machinery against pathogens. Apparently, and upon detection of pathogen-related molecules, plant cells undergo changes in small RNA profiles that mediate the establishment of a specific defence response (Katiyar-Agarwal and Jin 2010; Ruiz-Ferrer and Voinnet 2009). Despite the fact that plants enclose several hundred miRNAs and a huge number of siRNAs only in a few cases the latest have been described to be involved in plant immunity.

4.5 Homeostasis of the System

In addition to regulating development or stress response, miRNAs regulate the functioning of their own pathway through negative feedback loops that control *DCL1* and *AGO1* genes expression. Indeed, many small RNAs are expressed in a tissue-specific manner and, most probably, AGO1 and DCL1 regulatory loops serve cells to fine-tune their optimal levels for proper cellular function therefore providing a precise and accurate response to either endogenous or environmental stimuli.

DCL1 is essential for the biogenesis of miRNAs, and is under a strict control through regulatory loops involving two miRNAs. One loop requires the miR838-excision from the *DCL1* pre-mRNA (Rajagopalan et al. 2006), whereas the second involves miR162-guided cleavage of mature *DCL1* mRNA (Xie et al. 2003).

AGO1 expression also is firmly regulated due to its essential role in plant development. AGO1 homeostasis is achieved by several regulatory loops, which allow miRNA and siRNA pathways to correctly function. *AGO1* expression is tightly regulated through the miRNA pathway by miR168-guided cleavage of *AGO1* mRNA (Vaucheret et al. 2004), miR168 preferential stabilization by AGO1 (Vaucheret et al. 2006) and translational repression of *AGO1* by AGO10 (Mallory et al. 2009). An additional regulatory layer involves *AGO1* mRNA cleavage, followed by the generation of *AGO1* siRNAs through the siRNA pathway, which likely contribute to the regulation of *AGO1* mRNA level (Mallory and Vaucheret 2009). In agreement with the central role that miR168 plays in AGO1 regulation, expression of a miR168-resistant version of AGO1 leads to severe developmental defects and the eventual death of the plant (Vaucheret et al. 2004). In addition, factors that also regulate the AGO1 protein level or activity have been recently characterized. AGO1 is positively regulated by the cyclophilin protein SQUINT (SQN) (Smith et al. 2009) and negatively regulated by the F-box protein FBW2 (Earley et al. 2010).

Similar to AGO1, AGO2 has been identified as a target of miRNA gene silencing (Allen et al. 2005). The miR403 target site was identified within the 3′UTR of the AGO2 transcript. Therefore, AGO1 is also involved in the regulation of AGO2 as it binds to miR403, which leads to the cleavage of *AGO2* messenger (Allen et al. 2005).

In perfect agreement with such AGO1-mediated control of AGO2, upregulation of AGO2 was reported when the *SERRATE* gene was mutated (Lobbes et al. 2006), a background that has been shown to disturb the miRNA biogenesis pathway (Lobbes et al. 2006;Yang et al. 2006). Interestingly, this regulation was shown to be important for virus resistance. Indeed, pathogens usually target AGO1 to lower the plant defence. Consequently, *AGO2* downregulation is impaired, leading to an over-accumulation of AGO2 (Harvey et al. 2011). The accumulated AGO2 proteins can then bind to virus-derived siRNAs and contribute to plant defence. Accordingly, *ago2* mutants are more sensitive to virus infections, and *ago1 ago2* double mutants exhibit higher levels of viral RNA than single *ago1* or *ago2* mutants (Wang et al. 2011).

5 Conclusions

Plants are essential for life on earth because they produce oxygen and chemicals from sunlight and, of course, are used as food resource by a large variety of organisms, including bacteria, fungi, nematodes, insects and mammals. Given their sessile condition, the development of plants is dramatically influenced by the environment. When exposed to biotic or abiotic stresses, they trigger a set of mechanisms and developmental processes for shaping the body to adapt to the unfavourable environment. However, although the global structure of a plant can be highly variable, its detailed organization on a small scale is not. Like any organ of an animal, any plant organ is precisely specified. It possesses a determinate structure, in contrast with the indeterminate pattern of branching and sprouting of the plant as a whole. The internal organization of a plant raises essentially the same problems in the genetic control of pattern formation as it does in animal development, and they are solved in similar ways. Since the discovery of small RNAs, growing evidences have revealed the importance of base pairing between complementary sense mRNA and antisense small RNAs for fine tuning gene expression during almost every phase of development and in response to environmental changes.

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DNA Demethylation and Gene Imprinting in Flowering Plants

Jin Hoe Huh and Hyun Jung Rim

Abstract Gene imprinting is the monoallelic gene expression in a parent-of-origin-dependent manner that results from differential epigenetic states of the parental alleles. It is important for plant reproduction, in particular, the development of endosperm that provides nutrients to the embryo in flowering plants. With a few exceptions, all known plant gene imprinting occurs in the endosperm. The distinctive mechanisms of gene imprinting in the endosperm involve DNA demethylation and histone modifications. Notably, regulation of many imprinted genes begins prior to fertilization of the central cell member of the female gametophyte, where active DNA demethylation, the process which removes DNA methylation independently of DNA replication, is initiated by a plant-specific DNA demethylase. Recent genome-wide studies revealed the "imprintome"—the whole set of imprinted genes—in *Arabidopsis thaliana* endosperm. From the evolutionary point of view, lines of evidence suggest that both double fertilization and gene imprinting might have coevolved in flowering plants for their reproductive success.

1 Introduction

Chromatin provides a wealth of biological information required for growth and development of multicellular organisms. In eukaryotes expression of genetic information stored in the genome is modulated by different chromatin states. DNA methylation and histone modifications are two major epigenetic mechanisms to control the chromatin structure without changing the underlying DNA sequences. The presence or absence of such covalent modifications determines the transcriptional activity, and therefore, unlimited number of different transcriptional

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programs can exist in a cell by varying the structure of "epigenome"—the global landscape of epigenetic modifications throughout the genome, which can dynamically change during development. During specific developmental stages, a set of genes are actively transcribed while others are transiently held in a repressed state depending on the chromatin structure. It is thus conceivable that epigenetic gene regulation involving DNA methylation and histone modifications is responsible for diversifying the transcriptional profile in eukaryotes, providing great flexibility in response to different developmental cues. At the same time, robust transcriptional regulation is required to maintain the characteristics and functions of a specific cell type, and for this purpose, a distinct epigenomic profile serves as a cellular memory associated with cell identity. Therefore, aberrant changes in epigenome structure often result in dysregulation of global gene transcription due to memory failure, which sometimes leads to the development of disease and/or cancer.

It is believed that histone modifications serve as short-term epigenetic memory that can be maintained within a few cell divisions, whereas DNA methylation induces long-term silencing over many cell divisions. DNA methylation is a simple but crucial modification that has a profound effect on gene activity. DNA methylation is one of the crucial epigenetic modifications implicated in diverse biological processes, including gene imprinting, X-chromosome inactivation, and transposon silencing. In particular, extensive studies on the mechanisms of gene imprinting in mammals and plants have revealed the molecular basis of DNA demethylation, the process by which DNA methylation is removed. In this chapter, we will describe the essential features of gene imprinting and epigenetic processes behind, and introduce recent progress in this exciting field.

2 Gene Imprinting

Gene imprinting describes a phenomena when two alleles at the same locus are differentially expressed in a parent-of-origin specific manner within the same cell. Imprinting is thought to have evolved independently in angiosperms and mammals. The evolution and mechanisms of plant gene imprinting are intimately tied to their distinct reproductive strategies (Haig and Westoby 1989). Because there is no change in DNA sequence, there must be a change in epigenetic state that is associated with differential expression of the alleles at the imprinted locus. It is estimated that there are approximately 100 or more imprinted genes in mammals (Barlow 2011; Li and Sasaki 2011), and a large subset of those genes are crucial for placental development. The endosperm of plants functions analogously to the placenta and is currently the major source of imprinting in plants except a few instances. Imprinting can be classified into two groups: (1) genes that are both expressed, but have a parent-of-origin effect on allelic expression levels, and (2) monoallelic expression, where their parent-of-origins affect the on or off transcription states. The latter has been more extensively studied and has a direct impact on

endosperm and seed development (Gehring et al. 2009b; Huh et al. 2008; Kohler et al. 2012; Raissig et al. 2011).

The establishment of imprinting is due to the differences in epigenetic marks set on the alleles derived from the parental gametes. Two major epigenetic modifications that participate in the control of gene imprinting are DNA methylation and histone methylation. DNA methylation or methylation of cytosine residues (5-methylcytosine; 5mC) usually has repressive effects on gene expression as does the methylation of lysine-27 on histone 3 (H3K27). DNA can be target for methylation, and once it is established, DNA methylation is maintained through cell division by METHYLTRANSFERASE1 (MET1), a homologue of the mammalian Dnmt1 (Law and Jacobsen 2010). H3K27 methylation on the other hand is a general function of PcG complexes, but how PcG complexes target sites for repression is still elusive.

Imprinted genes in mammals are usually found in clusters that are under the control of an imprinting control region (ICR), and it is the methylation or lack thereof in the ICR that determines the expression of sex-specific genes in the cluster (Barlow 2011). In plants, imprinted genes appear to be controlled on an individual level by the presence or lack of methylation at specific sites in or around a gene. In addition to DNA methylation, H3K27 methylation mediated by PcG complex is also needed for imprinting of some loci in both mammals and plants, even in the absence of DNA methylation. In mammals, other histone modifications are also found coincidently with DNA methylation, such as H3K9 and H4K20 methylation (Barlow 2011).

One of the major differences between mammals and plants is in the establishment of maternal and paternal gamete-specific epigenetic patterns. In mammals, during primordial germ cell proliferation early in development, the genome is globally demethylated and subsequent de novo methylation during spermatogenesis and oogenesis reestablishes the gamete-sex-specific methylation patterns (Wu and Zhang 2010). DNA demethylation is critical in plants to erase methylation marks and establishes allele-specific transcription (Huh et al. 2008; Wu and Zhang 2010; Zhu 2009). Because endosperm of plants does not contribute genetic materials to the next generation, reestablishment of epigenetic patterns is not necessary, indicating a one-way control of endosperm gene imprinting.

3 Reproductive Strategies of Flowering Plants

Gene imprinting occurs primarily in the mammalian placenta and the angiosperm endosperm, tissues that nourish the developing embryo, and is a process that is critical for reproductive success. Therefore, it is necessary to understand the unique features and mechanisms for germ cell specification and reproduction processes in flowering plants.

3.1 Germ Cells

Unlike animal germ cells that directly give rise to male and female gametes, plant germ cells are committed first to producing multicellular haploid structures—the gametophytes. Thus, the flowering plant life cycle alternates between multicellular diploid (sporophyte) and haploid (gametophyte) generations.

Primordial germ cells are the embryonic precursors of gametes. In mammals, primordial germ cells are established far in advance of male and female gamete differentiation, and far from the site of developing reproductive organs (Hayashi et al. 2007). During embryogenesis, they migrate to the sites of developing ovaries or testes. Mammalian germ cells in an ovary enter meiosis to produce the primary oocyte, and germ cells in the testicular primordia are dormant and later at puberty they enter meiosis to develop into sperm. By contrast, plant germ cells are not specified until late in sporophyte generation, during floral organogenesis, when the floral meristem produces floral organ initials (sepals, petals, stamens, carpels) (Lohmann and Weigel 2002). Sepals and petals are vegetative organs, whereas stamens and carpels are the male and female reproductive organs, respectively. Plant germ cells develop at the site of the male and female organ initials *per se* due to the absence of cell migration observed in mammals.

3.2 Female Gametogenesis

The heart of plant reproduction is the ovule (Skinner et al. 2004), which emerges from meristematic cells in the carpel floral organ. The ovule is comprised of three structures: a nucellar region that generates the female gametophyte (Yadegari and Drews 2004), integuments, which are layers of cells that surround and protect the female gametophyte, and a funiculus, which contains the vascular system that is connected to the maternal plant. Within the nucellar region, an archesporial cell, the plant version of a female primordial germ cell, is formed. The archesporial cell differentiates to form the megaspore mother cell, the terminal cell type of the sporophyte generation, which undergoes meiosis resulting in the formation of four haploid megaspores (Fig. 1). In the majority of flowering plants, including *Arabidopsis thaliana*, only one megaspore survives while the other three go through programmed cell death. The functional haploid megaspore undergoes three mitoses to form the multicellular haploid female gametophyte, initially a coenocyte with eight nuclei; cell walls partition the female gametophyte into distinct cells: egg, central, synergid, and antipodal. During cellularization, two nuclei migrate to the center of the female gametophyte, fuse, and are enclosed by cell walls to form a diploid central cell. The central cell is adjacent to the haploid egg cell, which is flanked by two haploid synergid cells. Two sperms carried by the pollen tube enter the ovule and fertilize the egg and central cells to form the embryo

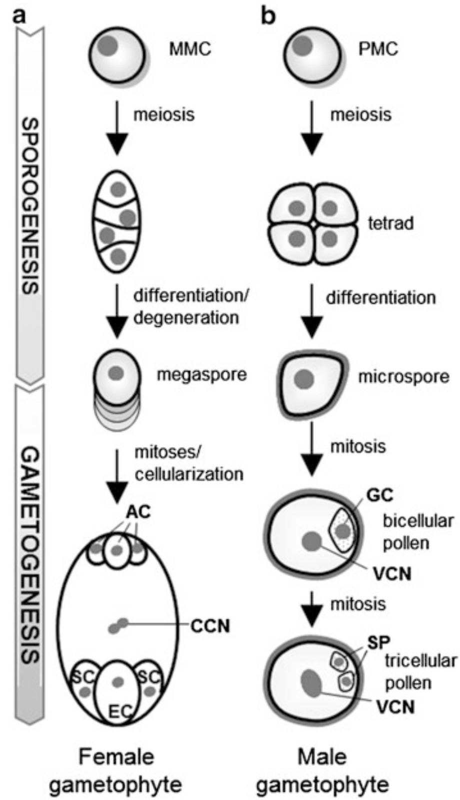

Fig. 1 Male and female sporogenesis and gametogenesis in flowering plants. (**a**) Megaspore and female gametophyte development. *AC* antipodal cell, *CCN* central cell nucleus, *EC* egg cell, *MMC* megaspore mother cell, *SC* synergid cell. (**b**) Microspore and male gametophyte development. *GC* generative cell, *PMC* pollen mother cell, *SP* sperm cell, *VCN* vegetative cell nucleus. Adapted from the figure by Huh et al. (2008)

and endosperm, respectively. The function of antipodal cells is unknown, which undergo programmed cell death in *Arabidopsis* (Yadegari and Drews 2004).

The genes and mechanisms that control ovule and female gametophyte development are being elucidated by molecular analysis of mutations that result in infertility, as well as genome-wide expression profiling of the *Arabidopsis* female gametophyte (Dresselhaus 2006; Skinner et al. 2004; Yadegari and Drews 2004). As described below, epigenetic processes (DNA methylation/demethylation and histone modifications) occur in the central cell, which is crucial for the establishment and maintenance of gene imprinting in the endosperm.

3.3 Male Gametogenesis

The male germ line arises from somatic cells in the stamen, the male reproductive floral organ. During early stamen development, archesporial cells are initiated and differentiate into pollen mother cells (Fig. 1). Each pollen mother cell undergoes meiosis to produce a tetrad of haploid microspores. All microspores survive and

undergo two mitoses to form the multicellular haploid male gametophyte, pollen. The initial asymmetric mitotic division produces bicellular pollen with a small generative cell encased in the vegetative cell. The generative cell undergoes another round of mitosis to produce two haploid male gametes (sperm). The vegetative cell produces the pollen tube that carries the two sperm cells to the ovule and female gametophyte where they fertilize the egg and central cells.

Chromatin remodeling and transcriptional regulation play an important role in development of male gametes (Okada et al. 2005, 2007). Transcriptional repression of male germline-specific genes in non-germ cells is crucial for spatial and temporal control of male germline development (Haerizadeh et al. 2006). Therefore, it is plausible that in plants germline-specific gene activity induces a gamete-specific chromatin state, and accompanied chromatin remodeling processes including histone modifications distinguish a germline chromatin structure from that of non-germ cells in plants. As described below, chromatin states, which have been shaped differently in the male and female gametophytes, are transmitted upon fertilization to the next sporophyte generation. These differentially established epigenetic marks, created before fertilization, are responsible for gene imprinting in the endosperm.

3.4 Double Fertilization

Pollen is released from stamen and germinates a pollen tube on specialized stigma cells of the carpel. The pollen tube, which is formed by the vegetative cell and carries two sperm cells, grows within a transmitting tract to the ovules. The pollen tube penetrates a synergid cell in the female gametophyte and releases the two sperm cells that migrate and fertilize the egg and central cells to form the diploid embryo and triploid endosperm, respectively. The female gametophyte secretes signaling molecules, some of which might be small, secreted proteins that guide the pollen tube and mediate sperm cell discharge and transport to the egg and central cells (Dresselhaus 2006).

The formation of endosperm by double fertilization is a defining characteristic of the more than 250,000 species of flowering plants, called angiosperms, which have seeds that are covered and protected from the environment. Nonflowering seed plants, called gymnosperms (e.g., conifers with naked seeds exposed to the environment), have a single fertilization event, and a large multicellular female gametophyte acquires nutrients from the parent plant and nutritionally supports the embryo. By contrast, in angiosperms, nutritional support of the embryo is primarily provided by the endosperm.

The origin and rapid evolution of the dominant angiosperms with endosperm formed by double fertilization have long been mysterious. Over 100 years ago it was discovered that the endosperm is a product of double fertilization. However, the evolutionary origin of double fertilization and endosperm is still under debate, and the examination on the female gametophytes of basal angiosperms reveals an

extensive degree of developmental and structural lability (Friedman 2006). Endosperm might be derived from a supernumerary embryo that acquired an embryo-nourishing function. Alternatively, the female gametophyte, greatly reduced in cell number in modern angiosperms, may have been sexualized by fertilization of the central (Friedman 2001). As described in Sect. 6, several studies shed light on this mystery and appear to support the latter hypothesis (Nowack et al. 2007).

3.5 Embryogenesis

After fertilization, the embryo establishes a basic body plan through coordinated cell divisions and expansions (Le et al. 2007). In *Arabidopsis* and most angiosperms, the zygote undergoes an asymmetric division to form a small apical cell and a larger basal cell. The small apical cell acquires an embryonic fate, while the large basal cell is primarily committed to producing a suspensor, which connects the embryo to the ovule, and is a conduit for nutrients during the very early stages of embryogenesis. Along the apical–basal axis the embryo generates a shoot apical meristem, cotyledon leaves that also function as in nutrient storage, a hypocotyl, root, and root apical meristem. The shoot and root apical meristems, similar to animal stem cells, are undifferentiated cells that have the properties of self-renewal and multiple differentiation potential and are responsible for generating all of the organs (leaf, shoot, root, flowers) of the adult plant. In addition, a radial pattern is established consisting of a concentric arrangement of epidermis, subepidermis, and a central vascular cylinder.

3.6 Endosperm Supports Embryo Development

The endosperm and embryo, which are genetically identical with the exception that the endosperm has an extra maternal genome, have dramatically different patterns of development (Berger et al. 2007; Brown and Lemmon 2007). However, the difference in ploidy is not solely responsible for their distinct developmental pathways, as triploid *Arabidopsis* embryos develop into morphologically normal adult plants. It is likely that the distinct developmental pathways of the embryo and the endosperm are due to differential genetic and epigenetic programming of the egg and central cells.

Fertilization of the central cell produces a primary endosperm nucleus surrounded by cytoplasm, which proliferates rapidly to form a syncytium of nuclei that are positioned by nuclear-based radial microtubules. Three developmental domains are formed along the apical/basal axis of the endosperm; a micropylar domain that surrounds the embryo, a central domain composed of a thin layer of cells, and a chalazal domain located above maternal tissue sitting atop a vascular system. Further proliferation accompanied by cellularization occurs in a wave

along the apical/basal axis, yet the chalazal endosperm remains syncytial, and forms a basal haustorial portion that penetrates the maternal tissue. The highly differentiated ultrastructure of the chalazal endosperm, intimately associated with specialized maternal cells above a vascular system, suggests an important role in transporting maternal resources into the developing endosperm (Brown et al. 2003; Nguyen et al. 2000).

Besides importing nutrients from maternal tissue, the endosperm synthesizes copious reserves of starch, protein, and lipids. In dicotyledonous seeds, which have two cotyledon leaves (e.g., legumes), the developing embryo absorbs the nutritive content from the endosperm. In monocotyledonous seeds, which have a single cotyledon leaf (e.g., grains), the endosperm persists, comprises the bulk of the seed, and is broken down and absorbed by the embryo soon after germination. Not only does the embryo depend heavily upon resources provided by endosperm. Indeed, two-thirds of human caloric intake is derived from the endosperm in angiosperm seeds. The embryo and endosperm are surrounded by a seed coat, which is derived from the integuments of the ovule. The seed coat protects the embryo and endosperm and also transfers nutrients from the maternal plant.

3.7 Endosperm in Parthenogenic Plants

Over 400 flowering plant species are capable of producing seed asexually, leading to parthenogenic embryo development, by a process termed apomixis (Bicknell and Koltunow 2004). Although multiple developmental mechanisms exist, in all cases a cell is generated that undergoes embryogenesis without meiosis or fertilization, and an endosperm is produced that supports the development of the parthenogenic embryo. It is notable that the central cell is fertilized to form a sexual endosperm in most apomictic species. This underscores the importance of biparental endosperm in angiosperm reproduction. As described below, gene imprinting may be a reason why a sexually derived endosperm is nearly indispensable.

4 Epigenetic Components of Plant Gene Imprinting

Gene imprinting is the differential expression of maternal and paternal derived alleles and has evolved independently in mammal and flowering plant lineages. Research over the last decade has revealed that epigenetic marks (DNA methylation and histone modification) and their regulators (histone methyltransferases, DNA methyltransferases, DNA demethylating DNA glycosylases) establish and maintain plant gene imprinting (Gehring et al. 2009b; Huh et al. 2008; Kohler et al. 2012; Raissig et al. 2011). In Sects. 4.1 and 4.2, the two major regulators of gene imprinting—histone modifications and DNA methylation—will be described. Detailed DNA demethylation mechanisms will be discussed in Sect. 4.3.

4.1 Histone Modifications by Polycomb Group Proteins

In animals, Polycomb group (PcG) proteins silence gene expression by directing the posttranslational modification of histones (Schuettengruber et al. 2007; Schwartz and Pirrotta 2007). PcG genes, discovered in *Drosophila melanogaster* as repressors of homeotic genes, play an important role in the control of cell proliferation, stem cell identity, cancer, gene imprinting, and X inactivation. Three PcG complexes, PRC1, PRC2, and PhoRC, work together to silence genes by methylating specific lysines on histone H3, and by interpreting these histone marks. A simple stepwise model for PcG proteins has a component of the PhoRC complex binding to DNA motifs and recruiting a PRC2 complex to the locus. The PRC1 complex is then recruited to the methylated H3K27 mark placed by PRC2. The structure and function of the PRC2 complex are highly conserved during evolution. In Drosphila, Enhancer of zeste (E(Z)), a SET-domain polypeptide, methylates H3K27, a histone modification associated with gene silencing. E(Z) functions in a complex with Supressor of zeste 12 (SU(Z)12), a C2H2 zinc-finger protein, and two WD-40 proteins, Extra sec comb (ESC) and P55.

Flowering plants have genes encoding proteins in the PRC2 complex that regulate developmental processes, including the response of the shoot apical meristem to environmental cues that promote the generation of a reproductive floral meristem, regulation of homeotic genes that control flower organ identity, the maternal control of seed viability, and gene imprinting (Calonje and Sung 2006; Pien and Grossniklaus 2007). However, until recently it was thought that flowering plants do not have genes encoding proteins in the PRC1 complex, and it is thought that either H3K27 methylation represses transcription directly or other proteins replace PRC1 (Sung et al. 2006). The orthologs in *Arabidopsis* that have a profound effect on cellular programming of gene imprinting are the SET-domain Polycomb group protein MEDEA (MEA) (Grossniklaus et al. 1998; Kiyosue et al. 1999), the C2H2 zinc-finger protein FERTILIZATION INDEPENDENT SEED2 (FIS2) (Luo et al. 1999), and two WD-40 proteins FERTILIZATON INDEPENDENT ENDOSPERM (FIE) (Ohad et al. 1999) and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) (Guitton et al. 2004; Kohler et al. 2003a). These plant PRC2 components, like their animal counterparts, form a 600 kDa complex (Chanvivattana et al. 2004; Kohler et al. 2003b) and are necessary for H3K27 methylation at their target loci (Gehring et al. 2006; Makarevich et al. 2006).

4.2 DNA Methylation

In mammals, DNA methylation (5mC) in the symmetric CpG sequence context is an abundant epigenetic modification (Klose and Bird 2006; Law and Jacobsen 2010). DNA methylation regulates gene imprinting and X-chromosome inactivation and silences transposons and retrotransposons. Aberrant promoter DNA

methylation is associated with gene silencing and plays a critical role in disease and cancer development (Baylin and Ohm 2006).

The de novo DNA methyltransferases, DNMT3a and DNMT3b, methylate cytosine at previously unmethylated sites. Patterns of symmetric CpG methylation are maintained after DNA replication by the maintenance DNA methyltransferase DNMT1, which methylates cytosine in the newly synthesized DNA strands. In mammals, the DNA methylation marks are erased and reset each generation during gametogenesis and embryogenesis (Reik 2007; Wu and Zhang 2010). DNA methylation prevents gene transcription by multiple mechanisms: blocking the access of transcription factors to DNA or recruiting methyl-CpG binding proteins, which form complexes with histone deacetylases, histone methyltransferases, or chromatin remodeling proteins and promote repressive chromatin structure.

As in mammals, DNA methylation in flowering plants regulates gene imprinting and silences transposons, retrotransposons, and repeated sequences (Gehring and Henikoff 2007; Henderson and Jacobsen 2007; Matzke et al. 2007). In *Arabidopsis*, orthologs of DNMT1 and DNMT3 DNA methyltransferases, METHYLTRANSFERASE1 (MET1) and DOMAINS REARRANGED METHYLTRANSFERASE (DRM) family enzymes, maintain and establish de novo DNA methylation, respectively. However, several properties of plant DNA methylation are distinct. In addition to methylation in the CpG sequence context, plant DNA methylation is present at CHG and CHH (H = A, C, or T) sequence contexts that are maintained, in part, by plant-specific DNA methyltransferases including CHROMOMETHYLASE 3 (CMT3). Also, to a great extent, small RNAs generated by RNA-interference pathways guide the placement of non-CpG DNA methylation in plants, which is known as an RNA-directed DNA methylation (RdDM) pathway (Law and Jacobsen 2010). Notably, unlike mammals, genome DNA methylation is not reset each generation, which has profound implications on the mechanisms that plants use to regulate gene imprinting.

4.3 DNA Demethylation

DNA demethylation may occur by passive or active mechanisms (Wu and Zhang 2010). Passive DNA demethylation is when 5-methylcytosine is replaced with cytosine in a replication-dependent manner in the absence of maintenance DNA methylation. In contrast, active DNA demethylation is a process by which DNA methylation is removed independently of DNA replication. Recent progress in this field revealed that active DNA demethylation is responsible for many developmental processes and can be achieved by diverse pathways.

4.3.1 Possible DNA Demethylation Pathways

There are lines of evidence demonstrating that active DNA demethylation occurs in developing mammalian embryos (Wu and Zhang 2010). Shortly after a sperm fertilizes an egg, rapid loss of DNA methylation takes place only to the paternal

genome before the completion of the first cell division (Gehring et al. 2009b; Wu and Zhang 2010). Even though such demethylation occurs globally, some genomic regions are still resistant to a wave of genome-wide demethylation which include imprinting control regions (ICRs) and some restrotroansposable elements, and centric and pericentric heterochromatin. In subsequent rounds of cell division, the maternal genome goes through gradual, passive demethylation in a replication-dependent manner due probably to the exclusion of maternally derived DNMT1 activity from the nucleus (Carlson et al. 1992). Global DNA demethylation also occurs in the primordial germ cells (PGCs) of early embryos in the absence of apparent cell division while migrating to the genital ridge (Hajkova et al. 2002). Since these initial findings, many researchers have searched for the enzymes responsible for direct removal of DNA methylation. There are several mechanisms proposed for DNA demethylation: (1) direct removal of the methyl group of 5mC, (2) replacement of 5mC with unmethylated C via the base excision repair (BER) pathway (Fig. 2), (3) deamination of 5mC to T followed by the BER of T•G mismatch, (4) removal of the 5mC-containing patch through the nucleotide excision repair (NER) pathway, and (5) hydroxylation/oxidation of 5mC (Fig. 3; Bhutani et al. 2011; Wu and Zhang 2010). As a carbon–carbon bond between a methyl group and a carbon at 5 position of cytosine is too strong to be enzymatically broken from the thermodynamic view point, the first mechanism is very unlikely to occur (Bird 2002). Instead, a multistep DNA repair process is proposed to remove DNA methylation (Gehring et al. 2009b; Bhutani et al. 2011; Law and Jacobsen 2010; Wu and Zhang 2010). As discussed below, particularly in animals, active DNA demethylation appears to primarily occur through indirect 5mC excision involving deamination or hydroxylation/oxidation processes followed by the BER pathway.

4.3.2 DNA Demethylation in Animals

From a biochemical point of view, one of the most plausible DNA demethylation pathways—direct removal of 5mC—is unlikely to operate in mammals. Rather, recent studies suggest that 5mC is enzymatically converted to an intermediate base, thymine or 5-hydroxymethylcytosine (5hmC), by two different families of proteins. The AID/APOBEC family proteins can deaminate 5mC to thymine leading to the formation of T•G mismatch (Fig. 3; Law and Jacobsen 2010; Wu and Zhang 2010; Zhu 2009). Alternatively, 5mC can be hydroxylated by the ten-eleven translocation (TET) family proteins to form 5hmC, which can be either converted again to 5-hydroxymethyluracil (5hmU) by AID/APOBECs or further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by TETs (Fig. 3; Cortellino et al. 2011; Guo et al. 2011; Tahiliani et al. 2009; Williams et al. 2011; Xu et al. 2011). These intermediates (i.e., thymine, 5hmU, or 5caC) can be excised by the family of DNA glycosylases such as thymine DNA glycosylase (TDG) and single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1) (Fig. 3). It

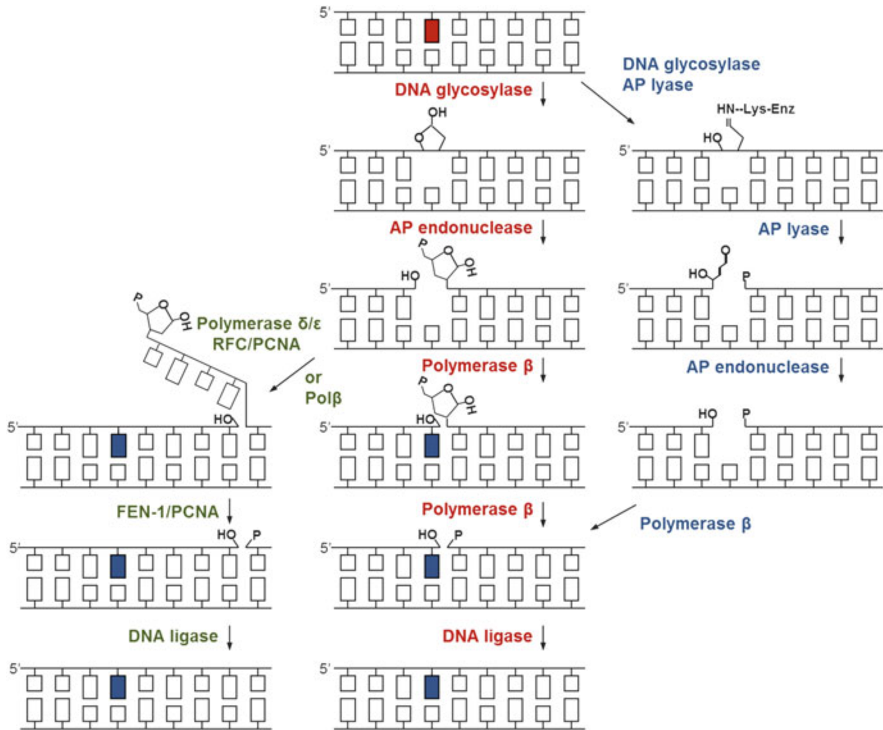

Fig. 2 Overview of base excision repair (BER) pathways. The short-patch BER pathway shown in the *center* is initiated by excision of an inappropriate base (*red*) by a DNA glycosylase generating an apurinic or apyrimidinic (AP) site. On the *right* is the short-patch BER pathway involving bifunctional DNA glycosylase/AP lyase. These two pathways require AP endonuclease, Pol β, and DNA ligase to restore the original sequence (*blue*). On the *left* is the long-patch repair pathway, in which 2–6 bases past the AP site are repaired with aid of RFC, PCNA, FEN-1, and DNA ligase. Adapted from the figure by Schärer and Jiricny (2001)

was also proposed that the growth arrest and DNA-damage-inducible protein 45 alpha (Gadd45a) promotes NER, which eventually replaces 5mC with cytosine (Barreto et al. 2007). Taken together, current models suggest that DNA demethylation in animals does not involve direct removal of 5mC from the genome. Instead, the pathways of active DNA demethylation begin with chemical modifications of 5mC to another base followed by DNA repair.

4.3.3 DNA Demethylation in Plants

In contrast to animals, the direct excision of 5mC by specific DNA glycosylase family proteins based on DNA repair appears to be a major pathway for active DNA demethylation in plants. DNA glycosylase proteins generally function in the BER pathway and excise modified, damaged, or mispaired bases from DNA (Fig. 2;

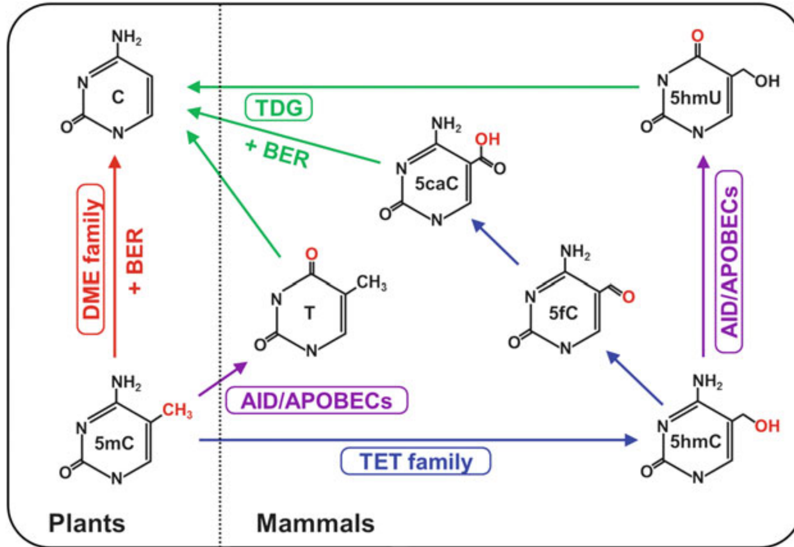

Fig. 3 Active DNA demethylation pathways. In plants (*left panel*) DME family proteins—DME, ROS1, DML2, and DML3—function as 5mC DNA glycosylase to directly excise 5mC and initiate the BER pathway replacing 5mC with unmethylated cytosine. In animals (*right panel*) no 5mC DNA glycosylases have been identified. Instead, three enzyme families have been implicated in active DNA demethylation via the BER pathway. (1) 5mC can be hydroxylated by the ten-eleven translocation (TET) family of enzymes (*blue*) to form 5hmC or further oxidized to 5fC and 5caC. (2) 5mC (or 5hmC) can be deaminated by the AID/APOBEC family members (purple) to form 5 mU or 5hmU. (3) Replacement of these intermediates (5 mU, 5hmU, or 5caC) is initiated by the UDG family of DNA glycosylases (*green*) like TDG or SMUG1, resulting in cytosine replacement and DNA demethylation. Modified from the figure by Bhutani et al. (2011)

David et al. 2007). DNA glycosylases remove the target base by cleaving the N-glycosylic bond, creating an abasic site, whereas the lyase activity nicks the DNA. An AP endonuclease generates a 3′-hydroxyl used by a DNA repair polymerase that inserts the proper nucleotide. A DNA ligase seals the nick to complete the repair process. In *Arabidopsis*, the DEMETER (DME) family of DNA glycosylases is required for active DNA demethylation. The DME gene is the founding member of this family of genes and was initially found to be necessary for gene imprinting in endosperm (Choi et al. 2002), and the following biochemical study suggests that DME is an authentic DNA demethylase that has long been sought by many researchers (Gehring et al. 2006). In addition to DME, three other DME family members of DNA glycosylases are present in the *Arabidopsis* genome—Repressor of Silencing 1 (ROS1), DEMETER-like 2 (DML2), and DEMETER-like 3 (DML3) (Choi et al. 2002; Penterman et al. 2007). ROS1 is required to maintain the expression of a transgene and its homologous endogenous gene (Gong et al. 2002). Both DML2 and DML3 appear to have functional redundancy as *ros1/dml2/dml3* triple mutants do not display any significant abnormalities in phenotype (Penterman et al. 2007). The DME family of DNA

glycosylases excises 5mC in vitro and in vivo (Agius et al. 2006; Gehring et al. 2006; Morales-Ruiz et al. 2006), which is eventually replaced by unmethylated cytosine through the BER pathway (Figs. 2 and 3). The DME family enzymes are bifunctional DNA glycosylases with additional lyase activity (Gehring et al. 2006; Morales-Ruiz et al. 2006; Agius et al. 2006).

Recombinant DME family proteins, purified from *Escherichia coli*, were shown to have base excision activity against methylated but not unmethylated DNA substrates (Gehring et al. 2006; Morales-Ruiz et al. 2006; Penterman et al. 2007). Complementation tests demonstrated that both lysine and aspartic acid residues in the catalytic glycosylase domain were necessary for 5mC excision (Agius et al. 2006; Choi et al. 2004; Gehring et al. 2006). This suggests that DME family proteins have a conserved structure and catalytic mechanisms that are common to many DNA glycosylases present in bacteria to humans (Mok et al. 2010). The DME family proteins are able to excise 5mC from any sequence context—CG, CHG, or CHH—even though there is a debate over preferred sequence context (Agius et al. 2006; Gehring et al. 2006; Morales-Ruiz et al. 2006).

As DNA methylation commonly occurs at symmetric CG sequences, uncontrolled active DNA demethylation by the DME family enzymes might cause the formation of double-strand breaks (DSBs) due to lyase activity forming a strand nick, which are critical to the genome stability. The enzyme kinetics study demonstrated that DME preferred hemimethylated over fully methylated DNA by twofold (Gehring et al. 2006), suggesting that the DSB formation is intrinsically inhibited so that the genome stability can be secured during the course of DNA demethylation. Thus, removal of both 5mCs at symmetric CG sites should occur sequentially by removing one 5mC on the strand with the other 5mC on the opposite strand being held for excision until the first excision repair is completed.

This active DNA demethylation regulates gene imprinting (Choi et al. 2002; Gehring et al. 2006; Jullien et al. 2006a; Kinoshita et al. 2004) and protects the genome from accumulating inappropriate DNA methylation (Zhu et al. 2007; Penterman et al. 2007). Differential de novo DNA methylation plays an important role for gene imprinting during embryogenesis in mammals, whereas allele-specific active DNA demethylation is more critical to establish gene imprinting in plants. The detailed mechanism of gene imprinting in plants will be discussed in Sect. 5.

It is still elusive whether alternative DNA demethylation pathways also exist in plants as proposed in animals. So far, there is no clear evidence for the presence of 5hmC or its implication in gene regulation in plants, which is thought to be an important 5mC derivative subjected to further modification for base excision-mediated DNA demethylation. Therefore, it would be of great interest to reveal the existence of 5hmC or the corresponding enzyme activity that might mediate indirect removal of 5mC as in animals. A recent study reported that 5hmC might be present in the *Arabidopsis* genome (Yao et al. 2012), even though the level of 5hmC was estimated to be extremely low. In addition, no putative homologs of the mammalian TET proteins or the 5mC hydroxylation activity have been identified in plants, suggesting that DNA demethylation via the conversion of 5mC to 5hmC is still elusive. The conversion of 5mC to 5hmC might change its binding affinity to

methyl-binding proteins without being removed. Also, it might facilitate passive DNA demethylation because 5hmC are not recognized well by the maintenance DNA methyltransferases. Previous studies reported that DME and ROS1 were active for both 5mC and thymine that were paired with guanine even though the former was preferred (Agius et al. 2006; Gehring et al. 2006; Morales-Ruiz et al. 2006). Therefore, it is conceivable that, as proposed in mammals, thymine which has been converted from 5mC via oxidative deamination can be removed by DME family proteins or by authentic thymine DNA glycosylases present in plants.

4.3.4 Targeting of DNA Demethylases

Most DNA glycosylases recognize and remove damaged or modified bases from DNA, which are usually present at a very low frequency in the genome. Therefore, it is a formidable challenge for typical DNA glycosylases to accurately find lesions among a vast number of normal bases (David et al. 2007). However, DME family proteins encounter the opposite situation—5mC is highly abundant in the genome, and, therefore, it must either remove a large number of targets or the targets for removal must be selectively chosen (Lister et al. 2008; Penterman et al. 2007). In contrast to the well-documented global DNA demethylation in mammals, there is no strong evidence for abrupt genome-wide demethylation in plants (Zhu 2009). The known DNA demethylases in *Arabidopsis* do not seem to function in global demethylation because *dme*, *ros1*, or *ros1/dml2/dml3* mutants displayed only a fraction of genomic regions affected in the methylation status (Lister et al. 2008; Penterman et al. 2007; Zhu et al. 2007). Therefore, it is highly conceivable that a certain mechanism(s) responsible for targeting the DNA demethylases may exist and that some proteins or molecules may guide them. One possible mechanism of targeting involves small RNAs (Zheng et al. 2008; Zhu 2009). Similar to the RdDM pathway, sequence-specific active DNA demethylation might be guided by small RNAs. A loss-of-function mutation in *ROS3*, which encodes an RNA recognition motif (RRM) protein that binds to small RNAs, causes DNA hypermethylation and transcriptional gene silencing at multiple loci (Zheng et al. 2008).

A recent study reported that histone H1.2 is a DME-interacting protein (DIP) and that *MEA*, *FWA*, and *FIS2* imprinting required histone H1 for DME regulation (Rea et al. 2012). This suggests that histone H1 is involved in DME-mediated DNA demethylation and gene regulation at imprinted loci. Considering histone H1 is a linker histone present between nucleosomes, it is possible that H1 allows DNA binding proteins including DME to access their target regions by modifying the chromatin structure.

Another study also proposed that a chromatin state is important for DNA demethylation and gene imprinting. Ikeda et al. (2011) showed that STRUCTURE SPECIFIC RECOGNITION PROTEIN 1 (SSRP1), a high mobility group (HMG) domain containing nonhistone chromosomal protein, plays a crucial role in DME-mediated DNA demethylation and gene imprinting in *Arabidopsis*. In the absence of functional SSRP1, DME cannot remove DNA methylation at its target loci such

as *FWA*, *MEA*, and *FIS2* (Ikeda et al. 2011). SSRP1 is a component of the FACT (facilitates chromatin transcription/transaction) histone chaperone complex (Duroux et al. 2004; Lolas et al. 2010; Orphanides et al. 1999). The complex contributes to the remodeling of chromatin by displacing histones H2A and H2B, thereby influencing the initiation of transcription, transcription elongation, DNA replication, and DNA repair (Formosa 2008). Even though the physical interaction of DME with SSRP1 is still unclear, it is evident that chromatin configuration and DNA demethylation are tightly linked so as to facilitate gene activation.

5 Cellular Programming of Plant Gene Imprinting

More than 50 imprinted genes have been identified so far in *Arabidopsis* and maize (Table 1; Gehring et al. 2009a; Hsieh et al. 2011; Kohler et al. 2012; Raissig et al. 2011; Wolff et al. 2011). Here we focus on the mechanisms of gene imprinting that have been substantially studied in *Arabidopsis* endosperm. Some of the imprinted gene products are PcG components, and in particular, they participate in the maintenance of imprinting in endosperm after fertilization.

5.1 Establishment of Gene Imprinting by DNA Demethylation

From genetic studies it was demonstrated that imprinting of *MEA* and *FIS2* involves MET1-mediated DNA methylation (Jullien et al. 2006b; Xiao et al. 2003). Further studies revealed that there exists differential DNA methylation between the paternal and maternal alleles of *MEA* and *FIS2* (Gehring et al. 2006; Jullien et al. 2006a). Maternal alleles of these imprinted genes are hypomethylated, whereas the paternal alleles are hypermethylated in the endosperm. Therefore, it was speculated that differential expression between the two parental alleles was determined by the status of DNA methylation that has been epigenetically inherited from the gametes. Unlike mammals, however, imprinting of these genes is not triggered by paternal-specific de novo methylation during gametogenesis, but the default state of these imprinted genes is more likely to be MET1-dependent methylation and transcriptional silencing. Thus, a maternal-specific activator(s) should exist and release the default silencing to activate gene expression only in the female gametophyte. On the contrary, in the male gametophyte, the paternal allele would remain silent because a maternal-specific activator(s) is absent there.

And then what is the maternal-specific activator(s) in the female gametophyte? Is DNA methylation a primary silencing mark that can be removed directly or indirectly by the activator(s)? DME has been identified as a transcriptional activator that induces *MEA* expression in the central cell (Choi et al. 2002). *DME* is a parent-of-origin effect gene because only the maternal *DME* is required for seed viability. *DME* expression is confined to the central cell and its expression disappears after

Table 1 List of imprinted genes in *Arabidopsis* and maize

M/P[a]	Gene		GeneID	Product	Function	References
Arabidopsis thaliana						
M	*FERTILIZATION-INDEPENDENT SEED2*	*FIS2*	AT2G35670	Zinc-finger transcription factor	PcG silencing	Chaudhury et al. (1997), Jullien et al. (2006a)
M	*MEDEA*	*MEA*	AT1G02580	PcG SET-domain protein	PcG silencing/H3K27 methylation	Grossniklaus et al. (1998), Kinoshita et al. (1999)
M	*FLOWERING WAGENINGEN*	*FWA*	AT4G25530	HD-ZIP transcription factor	Unknown	Jullien et al. (2006a), Soppe et al. (2000)
M	*FORMIN HOMOLOGY 5*	*FH5*	AT5G54650	Formin homolog	Endosperm cellularization	Gerald et al. (2009), Ingouff et al. (2005)
M	*MATERNALLY EXPRESSED PABC-TERMINAL*	*MPC*	AT3G19350	Poly(A) binding C-terminal domain	Unknown	Tiwari et al. (2008)
M	*SUPPRESSOR OF DRM1 DRM2 CMT3*	*SDC*	AT2G17690	F-box domain containing protein	Unknown	Gehring et al. (2009a), Hsieh et al. (2011)
M	*MATERNAL EFFECT EMBRYO ARREST 27*	*MEE27*	AT2G34880	JmjC domain protein	Embryo development ending in seed dormancy	Gehring et al. (2009a), Hsieh et al. (2011)
M	*JAGGED LATERAL ORGANS*	*JLO*	AT4G00220	Lateral organ boundary domain protein	Auxin transport	Gehring et al. (2009a), Hsieh et al. (2011)
M	*ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR/APETALA2*	*ERF/AP2*	AT4G31060	ERF/AP2 transcription factor	DNA binding	Gehring et al. (2009a), Hsieh et al. (2011)
M	*ETHYLENE INSENSITIVE 2*	*EIN2*	AT5G03280	Membrane protein	Ethylene signal transduction	Gehring et al. (2009a), Hsieh et al. (2011)
M	*MTO 1 RESPONDING UP 1*	*MRU1*	AT5G35490	Unknown	Unknown	Gehring et al. (2009a), Hsieh et al. (2011)
M	*C-MYB-LIKE TRANSCRIPTION FACTOR 3R-2*	*MYB3R2*	AT4G00540	MYB transcription factor	Unknown	Gehring et al. (2009a)
M	*HOMEODOMAIN GLABROUS 3*	*HDG3*	AT2G32370		Unknown	Gehring et al. (2009a)

(continued)

Table 1 (continued)

M/P[a]	Gene		GeneID	Product	Function	References
				HD-ZIP transcription factor		
M	*HOMEODOMAIN GLABROUS 8*	*HDG8*	AT3G03260	HD-ZIP transcription factor	Unknown	Gehring et al. (2009a)
M	*HOMEODOMAIN GLABROUS 9*	*HDG9*	AT5G17320	HD-ZIP transcription factor	Unknown	Gehring et al. (2009a)
M	*DSRNA-BINDING PROTEIN 2*	*DRB2*	AT2G28380	Hyl1-like protein	Double-stranded RNA binding	Hsieh et al. (2011)
M	*B LYMPHOMA MO-MLV INSERTION REGION 1C*	*BMI1C*	AT3G23060	PRC1-like complex component	H2A monoubiquitination	Bratzel et al. (2012), Wolff et al. (2011)
M	*SKP1-LIKE 8*	*SK8*	AT3G21830	E3 ligase component	Ubiquitin-mediated proteolysis	Wolff et al. (2011)
M	*AGAMOUS-LIKE 36*	*AGL36*	AT5G26650	MADS-box transcription factor	Unknown	Wolff et al. (2011)
P	*PHERES1*	*PHE1*	AT1G65330	MADS-box transcription factor	Unknown	Kohler et al. (2003b, 2005), Makarevich et al. (2008)
P	*SU(VAR)3-9 HOMOLOG 7*	*SUVH7*	AT1G17770	SET-domain protein	H3K9 methylation	Gehring et al. (2009a), Hsieh et al. (2011)
P	*YUCCA 10*	*YUC10*	AT1G48910	Flavin monooxygenase	Auxin biogenesis	Gehring et al. (2009a), Hsieh et al. (2011)
P	*VARIANT IN METHYLATION 5*	*VIM5*	AT1G57800	Zinc-finger transcription factor	MET1 cofactor	Gehring et al. (2009a), Hsieh et al. (2011)
P	*HOMEODOMAIN GLABROUS 3*	*HDG3*	AT2G32370	HD-ZIP transcription factor	Cotyledon development	Gehring et al. (2009a), Hsieh et al. (2011)
P	*AGAMOUS-LIKE 92*	*AGL92*	AT1G31640	MADS-box transcription factor	Endosperm cellularization	Hsieh et al. (2011)
P	*DUO1-ACTIVATED F-BOX 1*	*DAF1*	AT3G62230	Male germline-specific transcription factor	Pollen sperm cell differentiation	Wolff et al. (2011)

P	*PICKLE RELATED 2*	*PKR2*	AT4G31900	Chromo domain protein	Chromatin assembly or disassembly	Wolff et al. (2011)
P	*NUCLEAR FACTOR Y, SUBUNIT C7*	*NF-YC7*	AT5G50470	Transcription factor CBF/NF-Y/ archaeal histone	Histone folding	Wolff et al. (2011)
P	–	–	AT5G62110	Homeodomain-like protein	Unknown	Gehring et al. (2009a)
P	–	–	AT1G60410	F-box domain protein	Unknown	Gehring et al. (2009a), Hsieh et al. (2011)
P	–	–	AT2G21930	F-box associated domain protein	Unknown	Hsieh et al. (2011)
P	–	–	AT1G60400	F-box/RNI-like superfamily protein	Unknown	Wolff et al. (2011)
P	–	–	AT3G50720	Protein kinase superfamily protein	Unknown	Wolff et al. (2011)
P	–	–	AT2G36560	Unknown	Unknown	Hsieh et al. (2011)
P	–	–	AT1G49290	Unknown	Unknown	Wolff et al. (2011)
P	–	–	AT3G49770	Unknown	Unknown	Wolff et al. (2011)
P	–	–	AT5G54350	Unknown	Unknown	Wolff et al. (2011)
Zea mays						
Locus-specific imprinted genes						
M	*Fertilization-independent endosperm1*	*Fie1*	–	WD-40 repeat protein	Unknown	Danilevskaya et al. (2003), Gutiérrez-Marcos et al. (2006)
M	*Fertilization-independent endosperm2*	*Fie2*	–	WD-40 repeat protein	Unknown	Danilevskaya et al. (2003), Gutiérrez-Marcos et al. (2006)
M	*No-apical-meristem-related protein*	*Nrp*	–	NAM family transcription factor	Unknown	Guo et al. (2003), Haun and Springer (2008)

(continued)

Table 1 (continued)

M/P[a]	Gene		GeneID	Product	Function	References
M	*Maternally expressed gene1*	*Meg1*	–	Small cys-rich glycosylated peptide	Possible structural role in basal endosperm transfer region	Gutierrez-Marcos et al. (2004)
M	*Maize E(z)-like gene1*	*Mez1*	–	PcG SET-domain protein	Unknown	Haun et al. (2007), Haun and Springer (2008)
M	*Maternally expressed in embryo 1*	*Mee1*	–	Unknown	Unknown	Jahnke and Scholten (2009)
P	*Paternally expressed gene1*	*Peg1*	–	Unknown	Unknown	Gutierrez-Marcos et al. (2003)
Allele-specific imprinted genes						
–	–	*R1 gene*	–	Myc-like transcription factor	Anthocyanin pigmentation	Kermicle (1970), Ludwig et al. (1989)
–	–	*Dzr-1*	–	Not known	Zein regulation (posttranscriptional)	Chaudhuri and Messing (1994)
–	–	*Zein*	–	Zein protein	Storage	Lund et al. (1995a)
–	–	*α-Tubulin*	–	Tubulin homolog	Cytoskeleton	Lund et al. (1995b)

[a]Allellic expression according to the parent of origin
M maternally expressed genes, *P* Paternally expressed genes

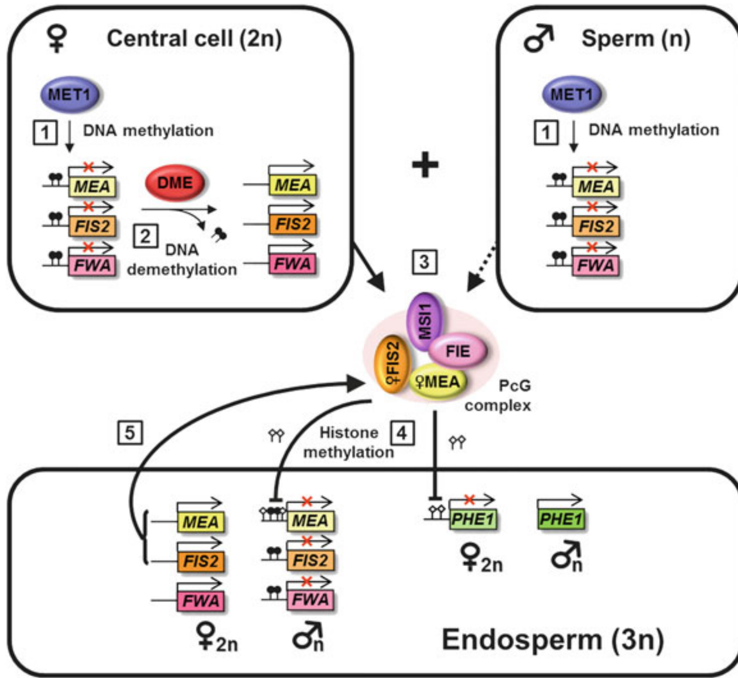

Fig. 4 Model of endosperm gene imprinting during seed development. (1) Both paternal and maternal alleles of imprinted genes are methylated by MET1 as a default state in the central cell and sperm, respectively. (2) DME, 5mC DNA glycosylase in the central cell, demethylates and activates *MEA*, *FIS2*, and *FWA* alleles. (3) Upon fertilization, maternally expressed but paternally silenced MEA and FIS2 participate in a PcG complex. (4) In turn, the PcG complex represses its targets such as paternal *MEA* and maternal *PHE1* through histone modifications involving H3K27 methylation. (5) Maternal *MEA* and *FIS2* not repressed by the PcG complex are continuously expressed replenishing the PcG complex, which forms an autoregulatory feedback loop. Silencing of paternal *FIS2* and *FWA* appears to be solely dependent upon DNA methylation which is inherited from the gametes

fertilization, whereas maternal *MEA* allele expression persists in the endosperm (Choi et al. 2002). Ovules carrying mutant *dme* do not express *MEA*, and as a result, the seeds eventually abort. The finding that the *met1* mutation could suppress *dme* seed abortion by restoring *MEA* expression suggests that DME and MET1 antagonistically regulate *MEA* (Xiao et al. 2003). The current model suggests that DME removes DNA methylation at the maternal *MEA* allele in the central cell and that the hypomethylated maternal *MEA* is exclusively expressed in early endosperm while the methylated paternal *MEA* remains transcriptionally silent (Fig. 4).

DME is responsible for DNA demethylation and transcriptional activation of the maternal *MEA* in endosperm (Gehring et al. 2006). As described in Sect. 4.3.3, *DME* encodes a DNA glycosylase that specifically excises 5mC from DNA (Gehring et al. 2006; Morales-Ruiz et al. 2006). Bisulfite sequencing analysis revealed that only the paternal *MEA* was methylated in the wild-type endosperm,

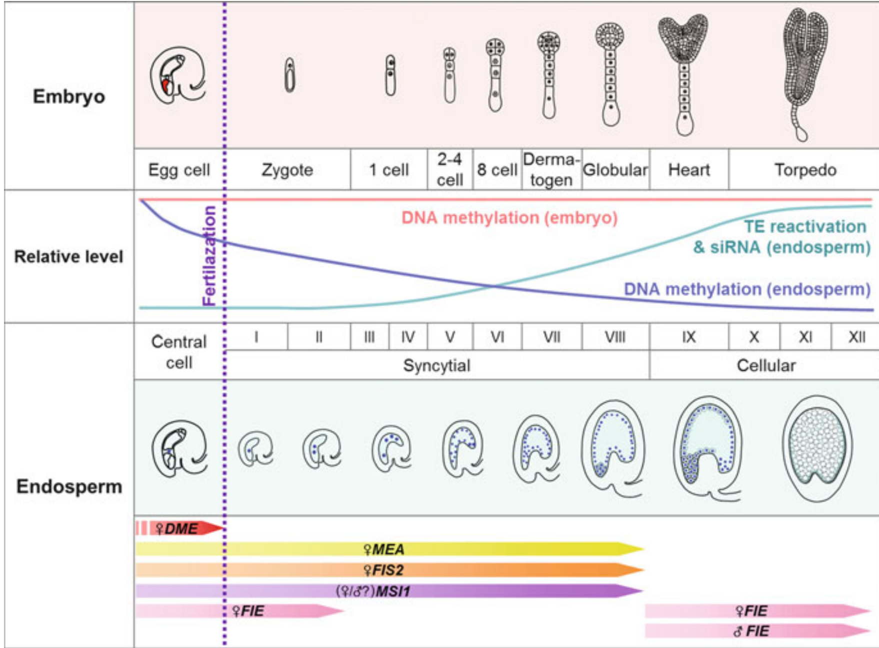

Fig. 5 Changes in DNA methylation and siRNA levels and expression patterns of PcG components in developing endosperm. DNA methylation in the central cell decreases due to DME DNA demethylase that is expressed only prior to fertilization. After fertilization, the level of DNA methylation in endosperm gradually decreases because the MET1 activity is downregulated (Jullien et al. 2008). By contrast, the level of siRNA increases in the endosperm due to RNA polymerase IV-dependent siRNA biogenesis (Mosher et al. 2009). At the same time, transposable elements are reactivated in the endosperm because of global hypomethylation which may also boost siRNA production. The level of DNA methylation in the embryo is relatively unchanged because the DNA demethylation machinery does not appear to operate as in the endosperm. Gene imprinting in endosperm is also mediated by the PcG complex. *MEA* and *FIS2*, two of the four components that consist of the PcG complex, are maternally expressed in early endosperm, whereas *FIE* displays the biphasic expression pattern (Yadegari et al. 2000)

whereas both parental alleles were methylated in *dme* mutant endosperm (Gehring et al. 2006). This finding unambiguously demonstrates that active DNA demethylation occurs because expression of *DME* and its demethylation function take place in a nondividing mature central cell. In addition, a passive DNA demethylation process by downregulation of MET1 activity may be partly responsible for global hypomethylation and endosperm gene imprinting (Fig. 5; Jullien et al. 2008). DME is also required for the maternal activation of two other imprinted genes *FIS2* and *FWA*, and their DNA methylation/demethylation pattern in both parental alleles is very similar to that of *MEA* (Jullien et al. 2006a; Kinoshita et al. 2004).

Therefore, imprinting of *MEA*, *FIS2*, and *FWA* in the endosperm is initiated and established by DME-mediated active DNA demethylation in the central cell, while the paternal alleles remain methylated and silenced (Fig. 4). The methylation state

of each allele is likely to persist via epigenetic mechanisms throughout nuclear divisions during early endosperm development. The on/off switch of DNA methylation is sufficient for the establishment and maintenance of both *FIS2* and *FWA* imprinting (Jullien et al. 2006a). By contrast, *MEA* imprinting requires an additional autoregulatory mechanism, which is discussed below.

5.2 Maintenance of Gene Imprinting by PcG Silencing

Both *MEA* and *FIS2* are imprinted in the endosperm. MEA is homologous to *Drosophila* E(z) whose SET domain has methyltransferase activity on H3K27 (Grossniklaus et al. 1998; Kiyosue et al. 1999). FIS2 is a zinc-finger transcription factor homologous to *Drosophila* Su(z)12 (Luo et al. 1999). The FIS class gene products, MEA, FIS2, and FIE, appear to function in a large PcG complex along with additional components such as MSI1 and retinoblastoma-related protein RBR1 (Ebel et al. 2004; Kohler et al. 2003a; Yadegari et al. 2000). This multimeric PcG complex is predicted to repress gene transcription via histone modification and chromatin remodeling, and the established patterns are stably propagated through mitotic cell cycles. This PcG complex is thought to negatively regulate endosperm cell proliferation because autonomous central cell divisions occur in *mea*, *fis2*, or *fie* mutants in the absence of fertilization (Chaudhury et al. 1997; Grossniklaus et al. 1998; Kiyosue et al. 1999; Ohad et al. 1996).

The differential methylation states of maternal and paternal *MEA* alleles are mitotically inherited to the endosperm after fertilization. However, DNA methylation is not directly involved in the maintenance of paternal *MEA* silencing because even the hypomethylated paternal *MEA* allele contributed by *met1* mutants is not expressed in the endosperm (Gehring et al. 2006). Rather, the FIS–PcG complex containing MEA itself appears to keep the silenced paternal *MEA* in a repressed state (Baroux et al. 2006; Gehring et al. 2006; Jullien et al. 2006a). Disruption of the FIS–PcG complex causes loss of *MEA* imprinting as silencing of the paternal allele is released. In addition, MEA is physically associated with the *MEA* promoter sequence (Baroux et al. 2006). These findings propose a self-imprinting mechanism of MEA, where maternally expressed MEA replenishes the FIS–PcG complex, and in turn, the complex keeps repressing the silenced paternal *MEA* allele (Figs. 4 and 5) (Gehring et al. 2006; Huh et al. 2008).

PHERES1 (*PHE1*) is another imprinted gene in the *Arabidopsis* endosperm (Kohler et al. 2003b). Whereas *MEA*, *FIS2*, and *FWA* are maternally expressed, paternal *PHE1* expression predominates in the endosperm, while the maternal *PHE1* is silent or very weakly expressed (Kohler et al. 2005). The silenced maternal *PHE1* allele is a direct target of the FIS–PcG complex (Kohler et al. 2005). In *mea* mutant seeds, for example, silencing of the maternal *PHE1* is released leading to biallelic expression (Kohler et al. 2005). Unlike other imprinted genes, however, the role of DNA methylation in *PHE1* imprinting is questionable. Rather, histone modification via the FIS–PcG complex likely both establishes and maintains the

silencing of the paternal *PHE1* (Fig. 4). Thus, in support of the parental conflict theory, the *Arabidopsis* endosperm has sets of maternal-silenced and paternal-silenced genes.

Notably, MEA is required for H3K27 methylation, one of the epigenetic silencing marks, at the silenced paternal *MEA* and the maternal *PHE1* alleles (Gehring et al. 2006; Jullien et al. 2006a; Makarevich et al. 2006). Silencing of the paternal *MEA* is released in the *mea* mutant endosperm accompanied with loss of H3K27 methylation (Gehring et al. 2006). Repression of the *PHE1* allele is also associated with H3K27 methylation (Makarevich et al. 2006). A mutation in the catalytic center of the MEA SET domain abolishes *PHE1* repression, suggesting that histone methyltransferase activity of MEA is necessary for its function in PcG silencing and gene imprinting (Makarevich et al. 2006).

These data support the model that all four FIS-class proteins form a core FIS–PcG complex functioning similarly to PRC2 to repress central cell proliferation until fertilization (Huh et al. 2008; Kohler et al. 2012). Thus identifying FIS-PcG target genes in the central cell is of great importance as they are likely required for endosperm proliferation upon fertilization.

6 Origins of Endosperm Imprinting

The endosperm is an unusual tissue. It is one of the two double fertilization products and thus can be considered as a separate organism from the embryo. What is unique to the endosperm is that it is fertilized but does not transmit any genetic information to the next generation. Its single purpose appears to be altruistic, sacrificing itself to ensure the success of its embryo sibling. Uncovering the endosperm evolutionary origin could provide insight into the mechanisms of female gametophyte and seed development. Because imprinting in plants appears to be primarily restricted to the endosperm, understanding the evolutionary forces that drive imprinting will ultimately gain insight into endosperm origins.

Is gene imprinting an integral feature of seed development that cannot be uncoupled? It was reported that seeds can be produced without double fertilization by bypassing genomic imprinting (Nowack et al. 2007). Mutants for *CDKA;1* which encodes a Cdc2/Cdc28 homologue produce pollen with only one sperm (Iwakawa et al. 2006; Nowack et al. 2006). This mutant pollen with a single sperm preferentially fertilizes the egg cell while the central cell remains unfertilized. Embryos from the eggs fertilized with *cdka;1* mutant pollen abort about 3 days after pollination and only a few of unfertilized central cell divisions occur (Nowack et al. 2006). This finding suggests that a positive signal is generated from a developing embryo to initiate central cell proliferation even in the absence of fertilization. Strikingly, disruption of the PcG complex which is required for the maintenance of endosperm

gene imprinting allows single-fertilized seeds to develop with unfertilized homodiploid endosperm (Nowack et al. 2007). When PcG mutants such as *mea*, *fis2*, and *fie* are pollinated with *cdka;1* pollen, viable seeds can be produced albeit the seed size is smaller than wild type (Nowack et al. 2007). This strongly suggests that genomic imprinting in the endosperm is not necessary for seed development under certain circumstances and that an unfertilized diploid central cell in the female gametophyte has a full potential to develop functional endosperm without paternal contribution. These results support the hypothesis that during the evolution of plants, the multicellular gymnosperm female gametophyte was reduced to the central cell in the angiosperm female gametophyte, and that fertilization of the central cell is a trigger that activates the development of multicellular endosperm (Nowack et al. 2007).

7 Genome-Wide Imprintome Analysis

Genome-wide DNA methylation analysis revealed that the endosperm genome is relatively hypomathylated than in the embryo and that DME is likely responsible for endosperm hypomethylation because the *dme* mutant endosperm displayed a higher level of DNA methylation (Gehring et al. 2009a; Hsieh et al. 2009). Interestingly, many imprinted genes in endosperm were differentially methylated between the embryo and endosperm (Gehring et al. 2009a). Although DNA methylation is one important mechanism responsible for gene imprinting, DNA methylation alone is not sufficient for all imprinted gene expression. Recent studies revealed a collection of imprinted genes—“imprintome”—in the *Arabidopsis* endosperm by genome-wide transcriptome analysis (Hsieh et al. 2011; Wolff et al. 2011). In these studies, F1 hybrid seeds were generated by reciprocal crosses between two different ecotypes and subjected to deep sequencing for endosperm RNAs. Using single nucleotide polymorphisms (SNPs) to measure allele-specific expression levels, novel imprinted genes could be identified for maternal or paternal-specific expression. Consistent with the current model of gene imprinting based on differential DNA methylation, many of new imprinted genes are likely regulated by allele-specific DNA demethylation (Gehring et al. 2009a; Hsieh et al. 2011). In addition, the contribution of PRC2 complex is evident because imprinting of several genes was abolished in the PRC2 mutant endosperm (Hsieh et al. 2011). However, a few imprinted genes are likely regulated by unknown mechanisms because their monoallelic expression in endosperm was not affected by *met1*, *dme*, or *fie* mutation (Hsieh et al. 2011). Even though a number of novel imprinted genes were identified recently, there could be more yet to be identified due partly to insufficient SNPs available between the parents or to different genetic backgrounds used in the previous studies (Hsieh et al. 2011; Wolff et al. 2011).

8 Conclusions

Two epigenetic modifications—DNA methylation and histone modifications—are dynamically regulated for endosperm gene imprinting. Initiation of gene imprinting requires active DNA demethylation by DME in the female gametophyte for maternal-specific gene expression. Asymmetric methylation patterns between the two parental alleles are inherited to the endosperm after fertilization and results in parent-of-origin-specific gene expression. This allele-specific epigenetic status is maintained and fortified by the PcG complex, which, in turn, autoregulates its own components. Such epigenetic regulation and imprinting are vital to proper endosperm development and seed viability since mutations in the components of this regulatory circuit produce unviable seeds.

Nevertheless, loss of imprinting (i.e., gain of biallelic expression) does not always compromise seed development. When the paternal genome is derived from *met1* mutants, *FIS2* and *FWA* are biparentally expressed in the endosperm producing viable seeds (Jullien et al. 2006a). Fertilization of a *fis* mutant ovule with *cdka;1* pollen produces viable seeds with homodiploid endosperm in the absence of paternal genome contribution, thus bypassing the requirement of gene imprinting (Nowack et al. 2007). That the diploid condition is sufficient for a viable seed is evident by the presence of biparental diploid endosperm in *Nuphar polysepalum*, a basal angiosperm (Williams and Friedman 2002). Therefore it is reasonable to speculate that endosperms of most flowering plants might have evolved a unique imprinting mechanism to ensure that fertilization of the central cell takes place and that the male contributes to the production of healthy endosperm for the next generation. Thus, in flowering plants gene imprinting may function to prevent parthenogenic seed development (Luo et al. 2000).

In the past decade, extensive studies have been carried out to understand the molecular mechanisms of plant gene imprinting. These efforts culminated in the identification of DNA demethylase, one of the key epigenetic regulators that many researchers have been searching for. Therefore, gene imprinting has served well as a model system to study epigenetic gene regulation in plants, and with no doubt, further understanding on the functions of imprinted genes will provide valuable insight into the evolutionary consequence of gene imprinting.

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Transgenerational Epigenetic Inheritance in Plants

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Abstract Epigenetics is broadly defined as the heritable change in gene expression without base sequence alteration. Heritable epigenetic changes commonly occur from cell to cell in an individual organism during development. Whether or not they occur from individual to individual, or across generation, has long been a matter of argument, but recent surveys suggest it to be positive. One of the underlying mechanisms is thought to be DNA methylation. Many studies have suggested that phenotype and DNA methylation patterns simultaneously change upon environmental stresses and are occasionally transmitted to the progeny. Here, we filtered each case through three conditions: phenotypic changes, i.e., acquired characters are beneficial for the organism; inheritance extends, at least, over three generations; and responsible genes are identified. Few cases fulfill these conditions demonstrating the cause–effect relationship between methylation of causative genes and phenotypic changes. Nevertheless the findings indicate that, under certain circumstances, acquired traits are heritable over generations and may play critical roles in evolution.

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1 Lamarck Meets Epigenetics

Until two or three decades ago, the majority of biologists were negative about the idea of "inheritance of acquired characters." The idea was first proposed by the French naturalist, Jean Baptiste de Lamarck (1744–1829), who introduced two laws of evolution—the law of use/disuse and the law of inheritance of acquired traits (Lamarck 1809). Since then, much has been said for and against it, but the theory was finally declined after the 1930s. The reasons were multifarious: conflict with the contemporaries such as Cuvier (Burkhardt 1970), counter theory of the natural selection by Darwin (1872), experimental misconduct by, for example, Weissman and Pavlov (Koestler 1971; Razran 1958), misinterpretation of data performed by Kammerer (Kammerer 1923; Koestler 1971; Vargas 2009), political abuse by Lysenko (Soyfer 2001), and others. However, the main reason for declining the theory might have been the lack of scientific knowledge to logically explain the observed phenomena, which have implied the "Lamarckian inheritance." Indeed, botanists and plant breeders have long recognized that altered properties during the growth were occasionally transmitted to the offspring.

The word "epigenetics" was first introduced by Waddington in 1942 (Waddington 1942). Since the 1980s, the theory of epigenetics has become the highlight of developmental biology and genetics (Holliday 2006). Epigenetics was broadly defined as the "change in gene expression without base sequence alteration" (Riggs and Porter 1996). This is frequently found during somatic cell differentiation in animal cells, typically occurring in clonal expansion of a single cell, leading to a diversity of cell types (Holliday 1993). An epigenetically acquired trait within an organism is transmissible from cell to cell, and commonly observed during ontogeny (Reik et al. 2001). This alteration is usually erased when germ cells are formed, precluding inheritance of epigenetically acquired traits by the next generation.

In spite of this common idea, some studies pointed out that epigenetically acquired traits upon environmental stresses were sometimes transmitted to the offspring. A well-known example is the effect of diet during pregnancy. Women subjected to poor diet during the World War II in the Netherlands gave birth to low weight babies, and this was also found in their offspring, who had never met diet restriction (Susser and Stein 1994). Studies on plants also suggested that epigenetically acquired traits were sexually transmitted under certain circumstances (Jablonka and Raz 2009). An example is the effect of nutrient on flax (*Linum usitatissimum*) and tobacco (*Nicotiana rustica*) plants. Grown under nutrient-rich condition, the mature plants exhibited heavier weight than those grown under normal condition. This trait was inherited by the progeny (Durrant 1962; Hill 1965). However, since appropriate explanation on molecular basis was not available, these observations have not drawn much attention until the 1990s.

Apart from the epigenetic studies, the presence of modified nucleotides in DNA was documented in the mid-twentieth century. The 5-methylcytosine was first proposed to be present in DNA in 1925 (Johnson and Coghill 1925), and isolated

from eukaryotic DNA, including mammals, fish, and maize, in 1950 (Wyatt 1950). Further studies indicated that modification of DNA by cytosine methylation is a post-replicative and reversible event (reviewed in Bird 2002 and references therein). It was thought to best explain the molecular basis of flexible and reversible gene control system (Riggs and Porter 1996; Bird 2002). To our knowledge, pioneer papers describing its biological roles in eukaryotes were published in the 1970s. In 1973, the possibility of 5-methylcytosine being involved in gene regulation was suggested (Scarano 1973). In 1975, its possible roles were proposed in restriction–modification of cytoplasmic genes (Sager and Kitchin 1975), in X-chromosome inactivation (Riggs 1975), and in cellular development (Holliday and Pugh 1975). The epigenetic theory could explain various puzzling phenomena such as non-Mendelian inheritance and genomic imprinting. As it became popular and widely accepted, DNA methylation was considered to be one of the most probable molecular mechanisms (Riggs and Porter 1996; Bird 2002; Scarano et al. 2005). Note that studies on epigenetics and DNA methylation have independently developed, and met almost a half century later. This situation resembles that between heredity and nucleic acid chemistry, which merged one century later (the 1960s) since Mendel and Miescher established each concept (the 1860s) (Portugal and Cohen 1980).

2 Definition

No concrete definition appears to be available for the word “epigenetics” (Richards et al. 2010). A recent idea is that epigenetics is “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence” (Richards et al. 2010). In this article, we essentially adopt this definition, but exclude the transmission within the cell lineage, or within the individual body.

“Transgeneration” needs a clear definition. “Transgenerational inheritance” is often used to indicate transmission of characters to the progeny or to the offspring. The words “progeny” and “offspring” indicate the descendant of an individual. They usually include the next direct generation. From the point of cell biology, this needs a caution (Skinner 2008). When a gestating female (F0) is exposed to toxicants or radiation, F1 generation embryo and F2 generation germ-line are directly affected, and when adult (F0) is exposed, F1 generation germ-line is affected. Thus, the generation which is not directly exposed is F3 in the former case and F2 in the latter case (Skinner 2008). Cautious handling was suggested for F1 and F2 studies, referred to as transgenerational (Skinner 2008). This argument might be true for plants as well, and suggests that careful analyses of at least F3 and later generations are necessary to claim “transgenerational inheritance of acquired traits.” In this article, the “transgeneration” means the transmission of traits over three generations.

DNA methylation also needs a definition. In addition to the major four nucleosides (adenine, thymine, guanine, and cytosine), natural DNA contains modified nucleosides such as 5-methylcytosine, N6-methyladenine, and 5-hydroxymethylcytosine as the minor components (Stacey 1965; Kappler 1971). In eukaryotic DNA, 5-methylcytosine is prevailing, comprising up to 4 and 30 % of total cytosine residues in mammals and plants, respectively (Kappler 1971; Finnegan et al. 1998). The location of 5-methylcytosine also differs between mammalian and plant DNAs, being found only in CpG in the former, but also in CpHpG and CpHpH (H is A, T, or C) in the latter. Both CpG and CpHpG sequences are symmetric, with methylation transmitted through meiosis by the action of maintenance methyltransferases specific for each sequence. Methylation of asymmetric CpHpH is usually considered not to be heritable, being reestablished in every generation (Jones et al. 2001). Due to the abundance and possible biological function, methylation of cytosine residues is commonly referred as DNA methylation (or simply methylation). We follow this nomenclature in this article.

The aim of the present article is to attempt to classify data related to transgenerational inheritance of acquired traits. To this end, we took account of three conditions to evaluate published experimental data. First, we will restrict the meaning of the "acquired traits" to the characters that are beneficial or at least not detrimental for the organism, so that the change could ultimately contribute to evolution. Second, we will consider cases clearly indicating a stable transmission up to at least F3 generation as suggested by Skinner (2008). Third, in order to assure the "cause–effect" relationship between an acquired trait and a causative gene function, we will review cases in which altered phenotype is clearly correlated with altered expression of the corresponding gene. We realize that few cases are available, which satisfy the above-mentioned conditions. And yet many reports are suggestive and positive for the present topic, and readers are strongly recommended to refer to these papers and excellent review articles (Jablonka and Lamb 1989; Agrawal et al. 1999; Rapp and Wendel 2005; Holliday 2006; Richards 2006; Henderson and Jacobsen 2007; Chinnusamy and Zhu 2009; Jablonka and Raz 2009; Haring et al. 2010; Lang-Mladek et al. 2010; Franklin and Mansuy 2010; Hauser et al. 2011; Gertz et al. 2011; Lauria and Rossi 2011).

3 Cases of Lamarckian Inheritance

Lamarck proposed that, when an organ (of animal) is frequently used in response to environmental conditions, its form changes to best fit the situation (the first law of evolution; use/disuse of organs). The changed trait is transmitted to the offspring (the second law of evolution; inheritance of acquired characters). Lamarck gave several examples including the long neck of the giraffe, strong claws of the sloth, and hind legs of the kangaroo, all cases not being accepted by modern view of evolution. However, special regard should be paid to the fact that, in the eighteenth century, biological knowledge on organisms was limited. We should appreciate

Table. 1 Stable heritable traits that change by environmental and chemical factors

Organism	Trait	Environmental factor	Generations	References
Flax	Increased blanching/ weight	Nutrient	F6	Durrant (1962)
Arabidopsis	Homologous recombination	UV-C, pathogen	F4	Molinier et al. (2006)
Rat	Increased tumor formation	Endocrine disrupters	F4	Anway et al. (2006)
Rat	Increased ovarian disease	Endocrine disrupters	F3	Nilsson et al. (2012)
Rat	Increased stress response	Endocrine disrupter	F3	Crews et al. (2012)
Rat	Decreased fertility	Endocrine disrupters	F4	Anway et al. (2005)
Mouse	Decreased fertility	Toxicant (dioxin)	F4	Bruner-Tran and Osteen (2011)
Human	Low birth weight	Nutrient	F2	Susser and Stein (1994)
Rat	Low birth and brain weight	Nutrient	F2	Zamenhof et al. (1971)
Maize	Seed color	Spontaneous	Many	Chandler et al. (2000)
Snapdragon	Flower color	Spontaneous	Many	Lönnig and Saedler (1997)
Pea	Winkled seed	Spontaneous	Many	Bhattacharyya et al. (1990)

that, within available information, Lamarck constituted the idea of heredity and evolution.

Now we may be able to modernize his idea. “Living organisms alter traits depending upon environmental conditions, and, under certain circumstances, changed traits are transmitted to the offspring.” This idea implies that changed traits are beneficial for the organisms so that they are stably inherited through many generations. To date, a considerable amount of reports has been available, describing “transgenerational inheritance of acquired characters” (Jablonka and Raz 2009; Hauser et al. 2011; Paszkowski and Grossniklaus 2011). Clear examples, however, are surprisingly few. In plants, many characters have been reported to change upon abiotic (temperature, light, nutrient, mutagen) and biotic (pathogen and herbivore) treatments (Paszkowski and Grossniklaus 2011; Hauser et al. 2011). Most observed traits are detrimental and such plants will possibly not survive under natural conditions. In many cases, transgeneration was examined only up to F2 (Hauser et al. 2011). In mammals, the situation appears to be the same. Within such limitation, we searched reports, and selected several examples (Table 1).

A notable case was the experiments with flax performed in the early 1960s. When wild-type flax (*L. usitatissimum*) was grown under nutrient-rich condition with ammonia, phosphate, and potassium, the mature plants exhibited a threefold heavier weight in comparison with those grown under non-nutrient-rich condition. This trait was stably transmitted to the progeny over six generations, irrespective of

the culture condition employed thereafter (Durrant 1962) (Table 1). Unfortunately, further experiments to confirm and extend the observation into later generations were apparently not performed, but, to our knowledge, this report is one of the reliable surveys in the field. Among numerous experiments with *Arabidopsis*, perhaps the clearest case is the stable inheritance of increased frequency of homologous recombination induced by treatments with pathogen (bacterial flagellin) and UV-C irradiation to the parental plant. Whole experiments were artificially conducted in a laboratory, but the situation could happen under natural condition, and if so, acquired genomic flexibility is possibly beneficial for adaptation (Molinier et al. 2006) (Table 1).

In contrast to the above, most cases reported from mammals indicate acquired traits to be detrimental. This may be due to the fact that worsening of the environmental conditions often deteriorates health, which is easily and clearly observed. Many experiments were performed with experimental animals including rats and mice by conditioning toxicants, nutrients, and stresses, all being naturally available in the human society. For example, commonly used agricultural chemicals such as vinclozolin (fungicide) and methoxychlor (pesticide) were found to be endocrine disrupters functioning as antiandrogenic and estrogenic compounds, respectively. Rats exposed to these chemicals exhibited decreased fertility and increased tumor formation. The trait was inherited up to F4 generation (Anway et al. 2005, 2006) (Table 1). A recent study showed that a female rat treated with vinclozolin developed an excess response to stress in the offspring for three generations, suggesting detrimental effects on behavior, or psychological activity (Crews et al. 2012) (Table 1). Women who were exposed to severe food restriction during the World War II gave birth to low weight babies, and this was transmitted to grandchildren (Table 1, Susser and Stein 1994). This observation was confirmed by experiments using rats, showing not only low birth weight but also low brain weight (Table 1, Zamenhof et al. 1971).

In plants, stable alteration in morphology (e.g., pigmentation) and physiology (flowering time, metabolic pathways) has been known. Typical cases were observed in maize, snapdragon, and pea (Table 1). The change, however, appears to have spontaneously occurred, and it is difficult to identify how, when, and where the specified change happened.

4 Molecular Background

Molecular events, which caused trait-change in organisms depicted in Table 1, have intensively been examined. In the case of flax (Table 1), the original authors have not studied the underlying mechanism, but later, researchers suggested that gene duplication might have played a critical role (Cullis 2005). In the case of several other examples (Table 1), involvement of DNA methylation was strongly suggested. Genomic DNA from F1 generation of vinclozolin-treated parental rat was screened for methylation polymorphism by PCR, and 25 differentially

expressed regions were identified. Among several genes analyzed, one encoding cytokine-inducible SH2-protein was shown to have altered methylation patterns, either hyper- or hypomethyaltion, which were transmitted to F2 and F3 (Anway et al. 2005). Similarly, trans-generationally altered methylation pattern was observed in rats with high frequency of ovarian disease upon treatment with endocrine disruptors (Nilsson et al. 2012; Bruner-Tran and Osteen 2011). These experiments clearly indicate a close association of aberrant phenotypes with changed DNA methylation patterns through generations.

In the case of spontaneous alteration of traits (Table 1), different mechanisms were found including paramutation and transposon insertion. Paramutation is defined as "heritable change in gene expression induced by allele interactions" (Chandler et al. 2000), and this is shown in phenotypic change in pigmentation of maize seeds (Table 1). Paramutation occurs through changes in chromatin structure, leading to a high frequency of homologous recombination (Chandler et al. 2000; Stam and Mittelsten Scheid 2005). Insertion of transposons, or transposable elements, into genomic sequence generates all kind of mutations that could lead, at least partly, to inactivating gene functions (Lönnig and Saedler 2002). Such a loss-of-function mechanism often links to regressive evolution, which is not always disadvantage for organisms. Some possible examples include the inactivation of metabolic pathways for toxic heavy metal absorption, or for conversion of nontoxic precursors into toxic compounds resulting in tolerance against harmful environment (Lönnig and Saedler 2002). For breeding of cultivated plants, loss of function is also helpful as seen in flower color, leaf size, and seed structure (Lönnig and Saedler 1997; Bhattacharyya et al. 1990) (Table 1).

Both paramutation and transposon insertion are stably inherited by the offspring, although causative environmental factors have not necessarily been identified in many cases. Several surveys, however, showed that environmental stresses affected genomic organization (Walbot 1992; Chandler et al. 2000). For example, the frequency of homologous recombination increased in experimental plants including *Arabidopsis* and tobacco by pathogen infection (Lucht et al. 2002), heat shock (Lebel et al. 1993), and osmotic pressure (Puchta et al. 1995). Transposon activity has also been reported to increase in maize upon pathogen infection (Mottinger et al. 1984) and UV irradiation (Walbot 1992). Activation of transposons is not always beneficial for the host. Far from that, the majority of them is quiescent, or silenced in genome to prevent their jumping and hindering normal gene functions (Wong and Choo 2004; Tran et al. 2005). Indeed, 80–90 % of wheat DNA is supposed to be derived from transposon-like sequences (Cantu et al. 2010). Molecular analyses have indicated that transposons and their footprints are heavily methylated, resulting in complete inactivation (Cantu et al. 2010; Tran et al. 2005; Lisch 2009). Control of frequency of paramutation was also suggested to be associated with methylation of the loci (Meyer et al. 1993; Haring et al. 2010; Walker and Panavas 2001). Such methylation patterns are stable, and demethylation was thought to be necessary to reactivate transposons and paramutations (Meyer et al. 1993; Lisch 2009; Lönnig and Saedler 2002). Thus, methylation

status of DNA appears to broadly play critical roles in direct and indirect activation of genes to respond to environmental stresses.

5 Epimutation of Causative Genes

DNA methylation is one of the most important molecular bases for flexible gene expression to cope with environmental stresses and to acquire transgenerational characters. Many studies have basically supported this idea, but few described clear correlation between altered traits and causative gene functions (Paszkowski and Grossniklaus 2011). We will briefly outline some cases (Table 2) and discuss further two representative observations: toadflax and rice.

A well-known case is the study on toadflax (*Linaria vulgaris*) (Table 2). The wild-type plant forms asymmetric bilateral flowers (zygomorphic), whereas a mutant forms symmetric radial flowers. The Lcyc gene toadflax, which is responsible for flower development, was found to be heavily methylated in the mutant, but not in the wild-type plant. It was concluded that radial form is the result of epimutation due to gene inactivation by DNA methylation (Cubas et al. 1999).

A tomato mutant exhibiting yellow fruits was first identified among commercial tomatoes in 1993, and was named colorless non-ripening (*Cnr*) (Thompson et al. 1999) (Table 2). Molecular analysis identified a gene encoding an SBP-box transcription factor within the Cnr region and showed that *Cnr* phenotype is derived from inactivation of *SBP* by hypermethylation, which occurred spontaneously and naturally. Three revertants showing normal phenotype appeared from 3,000 *Cnr* mutants, indicating stable but reversible nature of epimutation (Manning et al. 2006). The *Cnr* fruits fail to ripen, showing inhibition of softening, yellow skin, and nonpigmented pericarp. These traits are perhaps not beneficial for tomatoes to maintain species and will not prevail in the population.

During vernalization studies, late flowering mutants under a long-day condition were produced by mutagen treatments (EMS and irradiation) (Koornneef et al. 1991); *FWA* (*Flowering Wageningen*) was one of the mutated genes (Table 2). The *fwa* mutant constitutively expressed the *FWA* gene, while wild-type plants did not. *FWA* encodes a homeodomain-containing transcription factor, and is inactive during vegetative growth due to methylation of promoter regions, containing a transposon-derived DNA sequence (Soppe et al. 2000). *FWA* is transcriptionally reactivated in female ovule by demethylation, suggesting that the methylation system is utilized as a molecular switch for *FWA* expression in an individual plant. This differs from other cases, in which heritable and stable methylation change itself is associated with phenotypic changes. In this context, whether or not such an imprinting system is regarded as an "acquired trait" remains to be discussed.

Melons belong to Cucurbitaceae family, which is composed of more than 950 species in the world. They reproduce unisexually with male and female flowers on different plants (diecious) or on the same plant (monoecious). Sex determination of

Table. 2 Inheritance of morphological traits associated with changes in DNA methylation pattern of specific genes

Plant	Morphology	Cause	Gene	Regulation	Methylation	Generation	Advantage	References
Toadflax	Flower symmetry	Spontaneous	*Lcyc*	Down	Hyper	F2<	–	Cubas et al. (1999)
Tomato	Fruit ripening	Spontaneous	*Cnr* (*SBP*)	Down	Hyper	F3<[a]	–	Manning et al. (2006)
Arabidopsis	Flowering time	Spontaneous	*FWA*	Down	Hyper	Many	–	Soppe et al. (2000)
Melon	Sex determination	Spontaneous	*CwWIP1*	Down	Hyper	Many	+	Martin et al. (2009)
Mimulus	Tricome number	Herbivore	*Mixta*-like	Down	Hyper?	F6/F1[b]	+	Scoville et al. (2011)
Rice	Disease resistance	5azadC	*Xa21*-like	Up	Hypo	F10<	+	Akimoto et al. (2007)

[a]Despite the failure in normal ripening, the mutant can be selfed and at least F3 generation was available
[b]Phenotype was confirmed in up to F6, and gene expression was examined in F1

flowers was found to be controlled by a single gene, *CmWIP1*, encoding a zinc-finger transcription factor (Martin et al. 2009) (Table 2). Transcriptionally active *CmWIP1* leads to production of male flowers, and its inactivation leads to female flowers. Sequencing of gynoecious plant (forming only female flowers) revealed the presence of a transposon of *hAT* family (*Gyno-hAT*) at 1.3 kb upstream of *CmWIP1* promoter region. Male flowers did not contain this transposon. Subsequent methylation mapping revealed heavy methylation not only at the *Gyno-hAT* locus but also at its neighboring regions spanning into promoter of *CmWIP1*. Bisulfite sequencing confirmed a 97 % of methylation to be on CG context. Female plants (gynoecious) occasionally produce reverted male-like flowers, in which *CmWIP1* promoter was partially demethylated. Hence, expression of *CmWIP1* in gynoecious plant is repressed by methylation, resulting in suppression of development of male organs. Observation suggested that transposon-induced epigenetic change could positively contribute to plant reproduction and that such an acquired trait was beneficial and have persisted through generations.

When leaves were injured by herbivores, the number of trichomes increased in other leaves to prevent further damage (Holeski 2007) as observed in yellow monkey-flower (*Mimulus guttatus*) (Table 2); when a leaf was artificially injured by punching, trichome density increased in neighboring leaves. The increased density was observed in the offspring, which never experienced injury. This was associated with decreased expression of *Mixta*-like gene encoding a Myb transcription factor, which negatively controls trichome development. A preliminary experiment with azacytidine, which erased the parental memory, implied an involvement of methylation in this system (Scoville et al. 2011).

All experiments described above suggest that "acquired traits" were obtained through inactivation of relevant genes by hypermethylation. A contrary case was obtained with the study on rice (Table 2). When rice seeds were treated with 5-azadeoxycitidine, mature plants acquired resistance trait against bacterial blight disease (Akimoto et al. 2007). Wild-type plants were highly susceptible to the disease. One of the affected genes was found to be *Xa21G*, which encodes a disease-resistant protein (R-protein). *Xa21G* was fully methylated and transcriptionaly silent in the wild type, while it was demethylated and transcriptionaly activated in the mutant. The difference in disease response and *Xa21G* methylation status between mutant and wild-type plants was faithfully maintained for at least ten generations.

6 Loss of Function by Epimutation

The experiment with toadflax (*L. vulgaris*) was notable as the first clear example of transgenerational epigenetic inheritance (Table 2). It suggested that mutation has spontaneously occurred by methylation and has been maintained over 250 years since its discovery by Linnaeus. Some caution, however, is necessary to correctly interpret the data.

A naturally growing mutant with radial flowers was first found in Sweden in 1742 and described by Linnaeus in 1744 as Peloria, which means “monster” in Greek (Gustafsson 1979). The plant attracted much attention as a study material on floral evolution and was said to have had grown at the original area until the 1950s. There is an argument as to whether these rediscovered peloric plants originated from a single lineage, or after extinction, a similar mutation appeared frequently and spontaneously (Theissen 2000). A gene controlling floral symmetry was identified from snapdragon (*Antirrhinum majus*), named as *cycloidea* (*Cyc*) (Luo et al. 1996), and molecular characterization showed its product to be a transcription factor for *RADIALIS* (*RAD*) gene (Costa et al. 2005). Due to historical interests, an ortholog of *Cyc* was isolated from toadflax (*L. vulgaris*), named *Lcyc*, and examined whether the gene structure differed between Peloria and wild-type plants (Cubas et al. 1999). Results indicated that, while sequence of *Lcyc* was identical between the two, methylation status was different. Upon digestion with a pair of restriction endonucleases, *Sau*3A (methylation-sensitive) and *Mbo*I (methylation-insensitive), five GATC sites in *Lcyc* locus were found to be methylated at the last cytosine residue in peloric *Linaria* plants, but not in wild-type plants. Occasionally, observed revertants showed normal asymmetric flower structure, and demethylation at the *Lcyc* locus. Results suggested that transgenerational peloric phenotype was derived from inactivation of *Lcyc* by heavy methylation and therefore that such an epimutation may play a significant role in evolution (Cubas et al. 1999).

Some comments are available on the work. First, the origin of peloric *Linaria* plants used in this experiment is not clear. Since plants described by Linnaeus have apparently not been maintained until today, observed characters, both phenotype and methylation, are not assured to be transgenerational over 250 years. Second, confirmation experiments are desirable to correctly define the cause–effect relationship between phenotype expression and *Lcyc* methylation. The original report analyzed methylation at only few sites in the *Lcyc* locus from F2 generation. This is improved by direct bisulfite sequencing at *Lcyc* and neighboring regions in DNAs from all available generations (F0 to F2, and further). Quantification of *Lcyc* transcripts is also necessary to estimate the gene activity through generations. Third, peloria phenotype is not necessarily beneficial for the species. Toadflax is a self-incompatible plant, and pollination by insects is indispensable. Radial flowers often hamper efficient pollination by bees and bumblebees, resulting in low seed production (Lönnig and Saedler 1997; Kalisz and Purugganan 2004). In this sense, the “acquired peloric trait” may not be a successful example of evolution.

7 Gain of Function by Epimutation

DNA methylation inhibitors, 5-azacytidine and 5-azadeoxycytidine, are frequently used in epigenetic research to induce methylation mutants or epimutation. Their effect in inducing demethylation accompanied by phenotypic alterations is distinct (Sano 2010). However, identification of cause–effect relationship between

hypomethylated gene(s) and altered phenotype often meets difficulties, because of a global demethylation, which happens not only at the gene of interest but also at many other unidentified genes possibly involved in phenotype expression. In the case of rice study (Table 2), disease-resistant phenotype was fortunately found to be directly linked with a specific gene, as it functions under the gene-for-gene mechanism (Keen 1990, see the following section). Several comments are necessary for this finding.

Germinated rice seeds (*Oryza sativa* ssp. *japonica*) were treated with 5-azadeoxycytidine for 3 days, grown to maturity, and the progeny was cultivated in the field over ten generations. Genomic regions that changed methylation status were screened by the methylation-sensitive amplified polymorphism (MSAP) method, and one clone encoding a disease resistance Xa21-like protein was identified and designated *Xa21G*. In wild-type plants, all cytosines were methylated within its promoter region, whereas in mutant plants, corresponding methylation was completely erased throughout generations. Expression of *Xa21G* was not detectable in the wild type but was constitutively expressed in the mutant. When infected with *Xanthomonas oryzae* pv. *oryzae*, the progeny of mutant was resistant while wild type was highly susceptible (Fig. 1) (Akimoto et al. 2007).

Plants have evolved specific defense systems to protect themselves against attack from a wide range of pathogens (Staskawicz et al. 1995; Heath 2000). Upon pathogen challenge, a necrotic lesion is formed at the site of pathogen entry, thereby preventing further spread of disease. This event is referred to as the hypersensitive response. The hypersensitive response is initiated by recognition of pathogen attack, followed by an oxidative burst, induction of defense-related gene expression, and hypersensitive cell death (Heath 2000). When a plant is infected by a pathogen, it directly recognizes the particular protein (avirulent protein) derived from the pathogen through the corresponding resistance (*R*) gene product (Hammond-Kosack and Jones 1997). This type of hypersensitive response is limited to a particular pathogen and is referred to as the "gene-for-gene" mechanism (Keen 1990). This is typically seen in interaction between rice and bacterial pathogen, *X. oryzae* pv. *oryzae*, which induces bacterial blight disease (Ronald 1997) (Fig. 1). The rice *R*-gene product is Xa21 protein and bacterial avirulent protein (avrXa21) is thought to be a secreted peptide, which acts as a sensor signal molecule (Lee et al. 2008, 2009).

Rice (*O. sativa* ssp. *indica*) genome contains at least eight gene members encoding Xa21-like proteins, among which five were inactive (Wang et al. 1998), one was partially active, and only Xa21 exhibited resistance activity against all seven virulent races of the bacterium (Wang et al. 1996, 1998). However, *Xa21* itself was originated from wild rice, *Oryza longistaminata*, and introduced into cultivated rice, *O. sativa* ssp. *indica* (cv. IR24) by crossing to confer strong resistance (Khush et al. 1991). The other member of cultivated rice, *O. sativa* ssp. *japonica*, does not possess this particular *Xa21* gene, resulting in susceptibility to the disease. And yet *japonica* rice was found to possess *Xa21*-like gene (*Xa21G*), which is potentially able to confer resistance. Under natural condition, however, its expression is totally suppressed, even if infected with the pathogen (*X. oryzae* pv. *oryzae*), and host plants

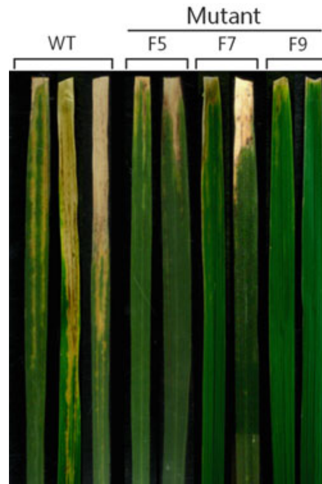

Fig. 1 Transgenerational inheritance of disease resistance acquired by epimutation. Disease response of wild-type and 5-azadeoxycytidine-derived epimutated rice plants. Healthy leaves from 3-month-old plants were inoculated with *Xanthomonas oryzae* pv. *oryzae*, race PR2 by the scissors-dip method (Kauffman et al. 1973) at the position approximately two-thirds from the tip, and incubated at 23 °C for 16 days. Samples include the wild-type (WT) and the progeny of the epimutant, F5, F7, and F9 generations. Representative two or three samples from each plant are shown

develop severe blight disease symptom (Fig. 1). The constitutive suppression was due to stable and heavy methylation at the promoter region regardless of the pathogen attack.

Why was such an important gene driven to silence? One clue is the presence of transposon-related sequences around the *Xa21G* gene. A 646-bp sequence, encoding a gag-pol polyprotein fragment, is located at 4.4 kb downstream of the *Xa21G* locus. Two tandemly arranged small sequences related to *OSTE28* (49 bp) and *pSINE1* (121 bp) are found at 1 kb upstream of the promoter, and two sequences related to *CACTA-Q* (86 bp) and *OSTE24* (121 bp) are located at 300 bp downstream of the stop codon. This feature resembles other *Xa21*-related genes, containing at least two transposon-like sequences in or out the open reading frame (Song et al. 1997). Transposable elements are usually selectively and heavily methylated, thereby being prevented to actively jump over the genome (Hirochika et al. 2000; Zemach et al. 2010). It is conceivable that during inactivation of invading sequences by methylation, genes located at the vicinity were also methylated. Such a "methylation spreading" appears to be rather common, as found in several cases (Table 2): a *COPIA*-like sequence in the *CNR* gene of tomato (Manning et al. 2006), an *hAT* transposon in the *CmWIP1* gene of melon (Martin et al. 2009), and two tandem repeats of *SINE*-derived sequence in the *FWA* gene in *Arabidopsis* (Soppe et al. 2000). This may also be the case with the *Xa21* multigene family members in rice. Indeed, the *pSINE1* transposon-like sequence was found to be heavily methylated in *japonica* rice (Takata et al. 2007).

Another clue is a change in growing condition for rice. Cultivation of rice by humans began ca. 10,000 years ago, and intensive breeding by selection and crossing produced high yield varieties, which possibly lost disease-resistant characters. Under natural condition, plants with such a loss of function might have diminished under natural selection, but cultivation possibly rescued them from extinction. The loss of function, however, is not because the gene is lost, but because the gene is quiescent. This means that the *Xa21G* can be reactivated, whenever the lock is released.

8 Fluctuant Epimutation

The demethylation system is certainly one of the mechanisms involved in gene activation. Two questions then arise: first, does demethylation happen under natural condition? Second, if this occurs, is its pattern heritable?

Active demethylation in vivo has repeatedly been suggested to be a prerequisite for physiological process, development, and stress response in plants and animals (Zhu 2009; Furner and Matzke 2011). Removal of 5-methylcytosine residues from DNA is catalyzed by multiple mechanisms, including DNA glycosidases through the base excision-repair pathway, cytosine deaminases, and conversion into hydroxymethylcytosines (Zhu 2009; Jullien and Berger 2010). Its biochemical aspect is relatively well documented, but physiological aspect, such as how, when, and where demethylation takes place, is not necessarily clear yet. One of the triggers, which induce demethylation, is environmental factors. The methylation pattern of genomic DNA has occasionally been reported to change upon biotic and abiotic stresses in several plant species (Chinnusamy and Zhu 2009). For example, global hypomethylation was induced by a simple mechanical touching in white bryony (Galaud et al. 1993), by low temperature in maize (Steward et al. 2000), and by heavy metals in clover and tobacco plants (Aina et al. 2004; Choi and Sano 2007). In contrast, global hypermethylation was induced by drought in pea (Labra et al. 2002). Hypomethylation was also induced upon pathogen infection in tobacco (Wada et al. 2004) and *Arabidopsis* (Pavet et al. 2006). In some cases, decrease of methylation was observed to take place within several hours after the onset of the stress (Steward et al. 2000; Choi and Sano 2007). Several hypomethylated genes involved in stress responses were found to be transcriptionally activated (Wada et al. 2004; Choi and Sano 2007). A dynamic change of genome-wide methylation was examined by a methylome profiling in *Arabidopsis* exposed to pathogen infection (Dowen et al. 2012). Active reprogramming of methylation, either remethylation or demethylation, occurred in response to pathogen attack. Many regions, which were differentially methylated upon stress, were associated with differentially expressed genes (Dowen et al. 2012). The answer to the first question about natural demethylation is positive.

Whether or not a stress-induced change of methylation is commonly transmitted to the progeny has not clearly been determined. It is generally believed that basic

methylation patterns are inherent to a species, and faithfully maintained over generations (Riggs and Porter 1996; Bird 2002). In other words, even if methylation patterns are variable in somatic cells, those in germ cells do not change, being same as the parental line (Jullien and Berger 2010; Hackett et al. 2012). Although there are some cases showing a transgenerational transmission of changed methylation patterns (Table 1), the majority of stress-induced methylation changes are not heritable. In this context, the answer to the second question about methylation inheritance is partly negative.

However, genome-wide analyses of methylation status revealed that, although overall methylation is relatively stable through many generations, methylation polymorphism does occur at an individual cytosine residue at higher frequency than polymorphism due to nucleotide change (Schmitz et al. 2011). Methylation polymorphism may induce epimutations, but they are not stably inherited over long term (Becker et al. 2011). For example, a differentially methylated region in *Arabidopsis* was demethylated after 31 generations but remethylated in the following generations. Such a fluctuation of DNA methylation may result in a cycle of forward and reverse epimutation (Becker et al. 2011). Importantly, methylation fluctuation appears not to be dependent on external stresses but on spontaneous fluctuations that occur during reproduction. Therefore, the answers to the first and second questions about the inheritance of natural demethylation are now positive.

9 Atavistic Mutants

Atavism is defined as “the reappearance of ancestral characteristics in individual members of a species” (Hall 1995). Genetic information, which was once used but not used now, is not completely lost, but lies quiescent in the genome and can be reactivated. Many examples have been shown in animals such as whale legs and horse toes, but its mechanisms are not clear (Hall 1995; Tomić and Meyer-Rochow 2011). Among few cases reported from plants, the floral symmetry is representative. The ancestral form is thought to be radial symmetry. It evolved into asymmetric form, which is advantageous for pollinators and seed production (Neal et al. 1998). The flower of Snapdragon (*A. majus*) is asymmetric, but often reverts into the radial form. A similar revertant in toadflax (*L. vulgaris*) was shown to be created by epigenetic silencing of a gene involved in flower development (Table 2) (Cubas et al. 1999). Epimutation was proposed to be one of the atavism mechanisms (Tomić and Meyer-Rochow 2011).

Atavistic mutations so far reported are mostly regressive. Since silencing of a gene was necessary for fitness during evolution, its reactivation would negatively affect the survival. Is there any opposite case? A useful gene was accidentally silenced, resulting in disadvantageous phenotype. If so, its reactivation would be beneficial for the survival. The study on rice-blight disease system implies such a case. The disease-resistant gene was locked by excess methylation and unlocked by demethylation. Although a naturally occurring revertant is currently not found,

the case supports the idea that atavisms have positive features and may play a certain role in evolution.

The idea of "transgenerational inheritance of acquired traits" has once been completely declined, and even today, it is considered to be rare or exceptional in biology. The reason might be that we do not have enough examples to support the idea. Various new techniques, including genome-wide methylation mapping (methylome), micro-array screening, biostatistics, and bioinformatics, have now been available to efficiently survey epimutation. If we select proper experimental materials such as atavistic mutants, the number of cases will certainly increase. Then it is conceivable that "transgenerational inheritance of acquired traits" becomes a common phenomenon, and will be assured to play a critical role in evolution.

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