Signaling and Communication in Plants

Gideon Grafi
Nir Ohad *Editors*

Epigenetic Memory and Control in Plants

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Signaling and Communication in Plants

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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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