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Zoya Avramova
University of Nebraska–Lincoln, zavramova2@unl.edu

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Zoya Avramova

School of Biological Sciences, University of Nebraska–Lincoln, Lincoln, Nebraska, USA

The Plant Epigenome

Genomes are defined by their primary sequence, which provides the genetic blueprint of a species. Eukaryotic DNA functions within the context of chromatin, which provides additional layers of gene regulation referred to as "epigenetic." The commonly found definition of epigenetics is that of a "study of heritable changes in genome function that occur without a change in DNA sequence" (Ref. 1 and ref. therein). However, evidence that neuronal gene-expression states are also regulated by epigenetic mechanisms, despite evidence that neuronal cells do not divide, has opened space for a broader unifying definition that keeps "the sense of prevailing usage but avoids constraints imposed by stringently required heritability" [1].

Epigenetic mechanisms regulate developmental programs, stress responses and adaptation, senescence, disease, and various patterns of non-Mendelian inheritance. The totipotency of plant cells, in addition to the ability of plants to withstand biotic, abiotic, and genome stresses, such as changes in chromosome number and massive presence of transposable elements, reflects the plasticity of plant genomes and makes them an excellent system to study epigenetic phenomena. Genome plasticity is determined by the EPIGENOME. DNA methylation and histone modification profiles define epigenomes of animals and plants. The main molecular mechanisms operating in epigenetic phenomena are DNA methylation, histone modifications, and RNA-based mechanisms, often referred to as "the three pillars of epigenetics" [2]. Recent advances in genome research technologies, deep sequencing analysis in particular, have led to an explosion of studies and novel results that are reshaping our views. Noncoding RNAs (ncRNAs) are emerging as central players responsible for the establishment, maintenance, and regulation of plant genome epigenetic structure [3].

At the molecular level, a unifying view of epigenetics postulates that DNA methylation and histone modification patterns provide "information" instructing genome function.

Following this information, the chromatin remodelers (the ATPase-containing machines) reposition (the nucleosomes modulating thus the access of Polymerase II (Pol II) to genes. NcRNAs (small silencing RNAs, in particular) are the molecular mechanism integrating numerous seemingly disparate cellular events [4] (Fig. 16.1). Longstanding questions about the molecular basis of pluripotency, tumorigenesis, apoptosis, position effect variegation (PEV), paramutation, imprinting, and cell identity are finding answers in small RNAs.

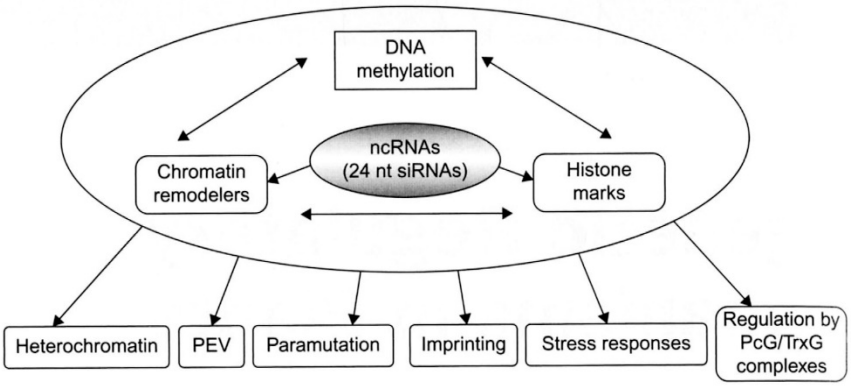

Figure 16.1. The main factors involved in epigenetic mechanisms regulating various plant processes. The noncoding small 24 nt siRNAs are the integrating function capable of mediating the activities of the chromatin remodeling machines, the histone modifying enzymes, and the DNA methylating mechanisms, as suggested by Costa [3].

Plants respond to internal and external stresses by altering expression of specific genes involved in the response. A significant fraction of plant genomes is made of repeated DNA sequences and transposable elements (TE) which, if activated, may cause genome malfunction and instability. NcRNAs are involved in coordinating genome function and in keeping TEs silent. RNA-mediated silencing is an evolutionarily conserved mechanism through which double-stranded RNAs (dsRNAs) induce inactivation of cognate sequences. Once established silent chromatin states can be propagated even in the absence of the initial cues. High-resolution analysis of the *Arabidopsis* exosome revealed an astonishing world of ncRNAs including a novel class of plant RNAs matching the 5′-end of Pol II transcripts (upstream noncoding transcripts) resembling the CUTs (cryptic untranslated transcripts) in yeast and the P-associated short transcripts in mammals [5]. Among the transcripts, many represented precursors for the ~24-nt siRNAs. The latter, referred to as heterochromatic siRNAs, guide RNA-dependent DNA methylation (RdDM) and histone H3 Lysine9 (H3K9) methylation of transposons and heterochromatin-associated repetitive sequences in *Arabidopsis* [6-10].

A number of recent reviews have focused on specific aspects of ncRNAs biogenesis, and on the similarities, differences, and the crosstalk between cellular pathways involving ncRNAs, as well as on their kingdom-specific roles in organismal functions and epigenetic

phenomena [4,10–12]. Here, a few plant-specific epigenetic mechanisms including silencing of TEs, heterochromatin formation, and genome reprogramming, as well as the phenomena of paramutation and imprinting, will be overviewed through the prism of the small heterochromatic 24nt siRNAs (Fig. 16.1). The involvement of the PcG/TrxG-related activities in plant development and the presence of dual silencing/activating (H3K27me3/H3K4me3) marks at developmental genes in *Arabidopsis* will be briefly discussed. Because of space limitations, the newly emerging topic of the epigenetic regulation of plant responses to biotic and abiotic stresses will not be covered. However, recently published results and reviews provide insights into epigenetic mechanisms operating in plants under stress [13–16], suggesting that environmental factors may increase genomic flexibility even in successive, untreated generations, increasing, thus, the potential for adaptation [16].

The Small RNAs in Epigenetic Regulation of Silencing in Plants

About a decade ago, Fire and Mello (1998) established that in *C. elegans*, dsRNA (termed RNA interference, RNAi) can trigger gene silencing [17]. The next year Hamilton and Baulcombe (1999) discovered that a critical step in the dsRNA silencing function is its conversion into small interfering RNAs (siRNAs) by the nuclease activity of a dsRNA-specific (RNase III family) ribonuclease (Dicer) [18]. These findings had an enormous impact on the entire field of molecular biology as they outlined unifying features for seemingly disparate processes, like homologous gene silencing, cosuppression, defense against viral infections, transposon-silencing, DNA methylation, heterochromatin formation, paramutation, nucleolar dominance, and imprinting. Small RNA-mediated silencing mechanisms may operate at different levels: at the transcriptional level (through chromatin) and at the posttranscriptional and translation levels (through mRNA degradation). It is important to emphasize that the minimal machinery executing the various types of silencing is built by similar, albeit highly specific, activities.

The steps common for all RNAi-involving pathways constitute: (i) formation of a dsRNA; (ii) its processing by a Dicer (DCL) enzyme to shorter (20–30 nucleotides) dsRNA duplexes (bearing a 5′ phosphate and 3′ hydroxyl group with two-nucleotide overhangs at the 3′ ends); (iii) binding of the small RNA duplexes to a protein from the Argonaute (AGO) family; and (iv) targeting of the RNA-induced complex to mRNA (or DNA) guided by the strand complementary to the small dsRNA, called the guide.

Upon their formation, the two-nucleotide 3′ overhangs of the liberated small RNA duplex are methylated by a specific methyltransferase HUA ENHANCER1 (HEN1) protecting the small RNA from polyuridylation and degradation [19,20]. Methylation of the 24-nt siRNAs generated by the RDRl-DCL3-AG04 pathway (see further below) might be taking place in the Cajal bodies. Colocalization of the 24-nt siRNAs with AGO4, Pol IVa, RDR2, and DCL3 in the Cajal bodies indicated that multiple steps in siRNA biogenesis were coupled in vivo [21].

The small RNAs are grouped in two categories based on the mode of their biogenesis: microRNAs, miRNAs, and small interfering RNAs, siRNAs. miRNAs are generated from single-stranded RNA transcripts (transcribed from *MIR* genes) and have the ability to fold

back onto themselves to produce imperfectly double-stranded stem loop precursor structures [see Refs 22–25]. siRNAs are processed from long, perfectly double-stranded RNA. siRNAs can be both exogenous and endogenous in origin and provide an epigenetic component of chromatin structure, gene silencing, and resistance against biotic and abiotic stresses.

In plants, several classes of siRNAs derived from distinct loci are: *cis*-acting siRNAs (*casi*RNAs), representing the most abundant endogenously produced siRNAs in plants; *trans*-acting siRNAs (*tasi*RNAs), generated by the convergence of the miRNA and siRNA pathways in plants; and natural antisense transcript-derived siRNAs (*natsi*RNAs), produced in response to stress. *natsi*RNAs are generated from a pair of convergently transcribed RNAs: typically, one transcript is expressed constitutively, whereas the complementary RNA is transcribed only when the plant is subjected to stress [11–14,26,27].

Chromatin-Based Epigenetic Mechanisms Operating in Transcriptional Gene Silencing (TGS)

Despite the fundamental similarities found in all eukaryotes using small RNA regulatory mechanisms, kingdom and species-specific characteristics have evolved to satisfy unique needs. For example, plant cells have evolved pathways upstream of Dicer and downstream of AGO to recognize repeated DNAs and methylated sequences. Instead of cleaving mRNA, AGO4-siRNA complexes recruit chromatin-modifying enzymes. Some plant-exclusive features of chromatin-based epigenetic mechanisms operating at transcriptional gene Silencing (TGS) will be discussed. The main components of these mechanisms were identified by forward genetic screen analysis of *Arabidopsis* mutants impaired in TGS. These include DCL3 (DICER3), AGO4 (ARGONAUTE4), Pol IV (RNA-POLYMERASE IV), Pol V, RDR2 (RNA DEPENDENT RNA POLYMERASE2), DRD1 (DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1) and DRM2 (DOMAINS REARRANGED METHYLTRANSFERASE2). Together, these activities control the accumulation of endogenous 24-nt siRNAs [28]. Several of the proteins involved in the biogenesis of the 24-nt siRNAs are genetically redundant, whereas others have specialized roles.

The Dicers

The sources for dsRNA are variable and dsRNAs can serve as precursors of different classes of small RNAs. Nonetheless, the specific enzyme activity degrading dsRNA, DCL (Dicer), is an ancestrally conserved function of the RNAi machinery [23]. Most animals encode a single Dicer (insects encode two) but in *Arabidopsis* the *DICER-LIKE* genes have proliferated to four (*DCL1–DCL4*). The founding member (*DCL1*) of the plant-specific RNase III-like endonuclease family was identified in a mutant line, carpel factory (caf), displaying floral meristem and organ morphogenesis defects [29]. DCL1 cleaves endogenous dsRNAs to produce both siRNAs and miRNAs; DCL2 and DCL4 process dsRNA precursor into 21- and 22-nt siRNAs and upon combining with AGO proteins guide degradation of homologous RNA in posttranscriptional gene silencing (PTGS) [30–32]. DCL3 is the nuclease involved in all known chromatin-dependent TGS events in plants producing the 24-nt siRNAs (heterochromatic siRNAs) that bind AGO4.

The ARGONAUTE (AGO) proteins were named after the characteristic squid-like phenotype of *ago1* mutant *Arabidopsis* plants. A founding member of the *AGO* gene family, *AGO1* plays pleiotropic roles in embryonic development, cell differentiation, maintenance of stem identity, and organ polarity [33]. ARGONAUTE (AGO) proteins are the integral players in all known small RNA-directed regulatory pathways. The AGO family members segregate into three sister groups based on their phylogenetic relatedness and capacity to bind a particular class of small RNAs. Members of Group 1 (called the AGO proteins) bind to both miRNAs and siRNAs; Group 2 members (the PIWI proteins) interact with piRNAs, and Group 3 members (described only in *C. elegans*) bind to secondary siRNAs. Plants encode only AGO (Group 1) proteins [23,34].

All ARGONAUTE proteins carry conserved PAZ, MID, and PIWI domains at the C-terminal and variable N-terminal domains. The PAZ domain recognizes and binds the 3′ end of small RNAs, the MID domain binds to the 5′ phosphate of small RNAs, and the PIWI domain adopts a folded structure similar to that of RNaseH enzymes exhibiting endonuclease (slicer) activity [35]. The PIWI domains specifically interact with GW (glycine-tryptophan) repeat-containing partner proteins [36].

Among the ten *Arabidopsis* AGO proteins [23,34] slicer activity has been demonstrated for AGO1, AGO4, and AGO7. Only AGO4 and AGO6 operate in the DCL3-siRNAs TGS pathway and may be partially redundant [9,37,38]. The roles of the other AGO proteins are less clear. Although AGO9 and AGO8 belong in the same sister group as AGO4, mutations in AGO9 and AGO8 did not display obvious developmental defects or aberrant small RNA levels [34,39].

Some processes upstream of DCL and downstream of AGO involve unique plant specific proteins. For example, RNA-dependent polymerases (RDRs), Chromomethylase3 (CMT3) and RNA polymerases, Pol IV and Pol V, participate in the process of RNA-directed DNA methylation (RdDM).

RNA-Dependent RNA polymerases (RDRs)

These enzymes produce dsRNAs used as substrates for DCLs in various small RNA pathways. Studied initially in plant antiviral defense (reviewed in Ref. 4) the RDRs participate in a number of endogenous functions beyond cellular defense. Heterochromatin structure, gene expression, and silencing of transposable element involve RDRs. These enzymes may initiate the RNAi pathway by producing the trigger dsRNA or may enhance the RNAi response by amplifying the amount of dsRNA.

In *Arabidopsis*, RDR2 generates dsRNA from single-stranded transcripts either by de novo second-strand synthesis from "aberrant" RNA templates (presumably lacking a 5′ cap or a polyA tail) or by using siRNAs as primers to synthesize RNA complementary to the target mRNA. DCL3 cleaves the dsRNAs to generate the 24-nt siRNAs. Hundreds of thousands of RDR2-DCL3-dependent 24-nt siRNAs mapping to heterochromatic regions containing DNA repeats, transposons, or silent euchromatin (the heterochromatic siRNAs) have been identified in *Arabidopsis thaliana*, rice, and tomato [40].

Many species outside plants and fungi do not have an RDR despite utilizing the RNAi machinery [23]. Animals, with the notable exception of *C. elegans*, do not have *RDR* genes, and *S. cerevisiae* also does not carry *RDR* genes; it is noted that this species is devoid of the

RNAi mechanism altogether. Absence of RDR activity in these species indicates that long dsRNA can derive from various sources, such as simultaneous sense and antisense transcription by Pol II or single-stranded RNA transcribed by RNA Pol II from inverted repeats and can form double-stranded hairpin RNAs after mono-directional transcription. *Arabidopsis* and rice have six identifiable RDRs, three of which (RDR3a, RDR3b, and RDR3c) form a distinct phylogenic clade for which no function has been established. The other three, RDR1, RDR2, and RDR6 have direct orthologs in many plant species and contain the catalytic DLDGD motif; all three function upstream of DCL closely linked to both DCL and AGO [41]. Transcriptional silencing of transposons and repeats in the nucleus typically involves DCL3 and AGO4 downstream of RDR2 [8–10]. The accumulation of RDR2-dependent siRNAs is linked to DNA methylation in RNA-directed DNA methylation (RdDM).

RNA-Directed DNA Methylation (RdDM) of Plant Genomes

The first indication that RNA could direct methylation of DNA came from observations that viroid RNA injected in plant cells caused de novo cytosine methylation of homologous genomic DNA sequences [42]. In plants, the RNA-directed DNA methylation (RdDM) pathway controls the establishment of DNA methylation at three sequence contexts (CG, CHG, and CHH) [43]. Three DNA methyltransferases cooperate to establish the genome methylation profile: CHROMOMETHYLASE3 (CMT3) and DOMAINS REARRANGED METHYLTRANFERASE2 (DRM2) produce de novo cytosine methylation, whereas the maintenance methylase, MET1, controls the symmetrical CG methylation on both DNA strands [44].

The *CMT3*-like genes, specific to the plant kingdom, encode methyltransferase proteins containing a chromodomain [45]. Through the chromodomain CMT3 binds dimethylated lysine 9 on histone H3 (H3K9me2) and together with SUVH4 (the activity that establishes the H3K9me2 mark, known also as KRYPTONITE, KYP) CMT3 generates a feedforward loop maintaining CHG methylation [46]. siRNAs guide CMT3 to sequences targeted for non-CG methylations and loss of *CMT3* function causes a large decrease in CHG methylation (and to a lesser extent in CHH) [47–50].

The *DRM* genes share homology with the mammalian *Dnmt3* genes encoding de novo rnethyltransferases [51]. At CG sites, the *DRM* genes are required for the establishment, but not for the maintenance, of preexisting CG methylation. DRM is guided to the targeted sequences by siRNAs and may act redundantly with CMT3 to establish and maintain CHG and CHH methylations (reviewed in Ref. 46).

Symmetric (CG) methylation is achieved by MET1 with the help of DECREASED DNA METHYLATION1 (DDM1) and VARIANT IN METHYLATION1 (VIM1). DDM1 is a chromatin-remodeling factor from the SNF2-family of ATPases. VIM1 is an unconventional rnethylcytosine-binding protein that is enriched at methylated genomic loci and at chromocenters. It binds to hemimethylated CG through the SRA (SET- and RING-associated) domain. Binding of SRA stabilizes the interaction and prevents sliding [52]. VIM1 can also bind to histones, and it was suggested that VIM1 participates in methylated DNA-nucleosome interactions to maintain centromeric heterochromatin [53]. Loss of *MET1* or *DDM1* causes massive genome demethylation, transposon reactivation, and stochastic developmental defects [54,55]. Some results suggested that, once lost, CG methylation in plants could not

be restored with fidelity [56]. However, the robust and specific restoration of the CG-methylation observed for the *Arabidopsis* centromeric repeats and transposons mediated by RNAi reported recently [57] challenged this view (see further below).

Although sounding paradoxical, siRNAs may also guide DNA demethylating activities [58,59]. The DNA glycosylase-lyase protein REPRESSOR OF SILENCING 1 (ROS1) can remove methylated residues and screens for suppressors of *ros1* mutations have identified RdDM factors; the protein ROS3 may guide demethylation by ROS1 [60]. The interplay between siRNA-directed DNA methylation and demethylation pathways might be required for the balance between the two epigenetic states.

High-resolution mapping of cytosine methylation in *Arabidopsis* confirmed previous reports that DNA in the centromeric regions and in repeat sequences was highly methylated [55,61,62]. In addition, it revealed unexpected patterns in the coding regions: less than 5% of genes are methylated at their promoters but about 30% are methylated in their open reading frames [63,64]. Unlike their mammalian counterparts, plant introns are almost completely devoid of TEs, and clusters of dense CG methylation are accumulated at exons but deficient in introns [65]. These patterns were found in highly transcribed and in constitutively expressed genes, whereas genes displaying lower-level and tissue-specific expression patterns had methylated promoters [62]. These DNA methylation profiles contrast with the distribution of methylated cytosines in mammalian genomes where the CG islands in gene promoters are hypomethylated [66].

The gene body methylation in plants is almost exclusively restricted to CG, in marked contrast to the methylation of CG, CHG, and CHH sites typically seen at repeated sequences. Gene body methylation may result from two conflicting activities: one imposing it at CG sites, and one preventing extension to CHG sites. Importantly, the latter activity is not targeted toward silent transposable elements and is likely coupled to transcription elongation, suggesting that CHG methylation hinders this step [67]. According to a model, transcription of genes by Pol II attracts in its wake the maintenance DNA methyltransferase MET1 as well as a H3K9 methyltransferase activity. Gene transcription could also recruit the JmjC-domain containing histone demethylase, IBM1, which by demethylating H3K9 would prevent its recognition by the chromodomain CHG methyltransferase CMT3. Thus, targeting of DNA methylation seems to differ significantly for genes and TEs, despite the fact that many factors are shared by these two processes [68].

Pol IV and Pol V

In *Arabidopsis*, the RdDM machinery involves two plant-specific RNA polymerases, Pol IV and Pol V. Their largest subunits (NRPD1 and NRPE1, respectively) are related to the largest subunit of Pol II (RPB 1) but Pol IV and Pol V function exclusively in the RNA-driven silencing pathway.

Pol V can generate uncapped and nonpolyadenylated transcripts from noncoding sequence that are targeted by RdDM. Pol V transcripts originate from intergenic noncoding regions triggering the siRNA-pathway. The subsequent chromatin modifications established via the siRNA-directed machinery impede transcription of adjacent regions by Pol II and Pol III [69]. In an *nrpe1* mutant, Pol V-generated transcripts disappear and methylation is lost, allowing uni- and/or bi-directional transcription by Pol II and Pol III. These

findings suggest a unique mode for chromatin-based gene silencing based on Pol V generated transcripts [70] (see Fig. 16.2). The model is supported by the pervasive intergenic transcription found in eukaryotic genomes [71].

Pol IV uses the genomic DNA as a template to produce a single-stranded RNA transcript, which is then converted to dsRNA (by RDR2) to be used as a substrate by DCL3. Endogenous loci producing the 24-nt class of chromatin-targeting RNAs are dependent on Pol IV and on RDR2 [72,73]. Pol IV may directly transcribe a methylated DNA template, producing an aberrant (improperly processed or terminated) RNA that is copied by RDR2 to dsRNA precursors of siRNAs that trigger methylation [74,75] (Fig. 16.2A).

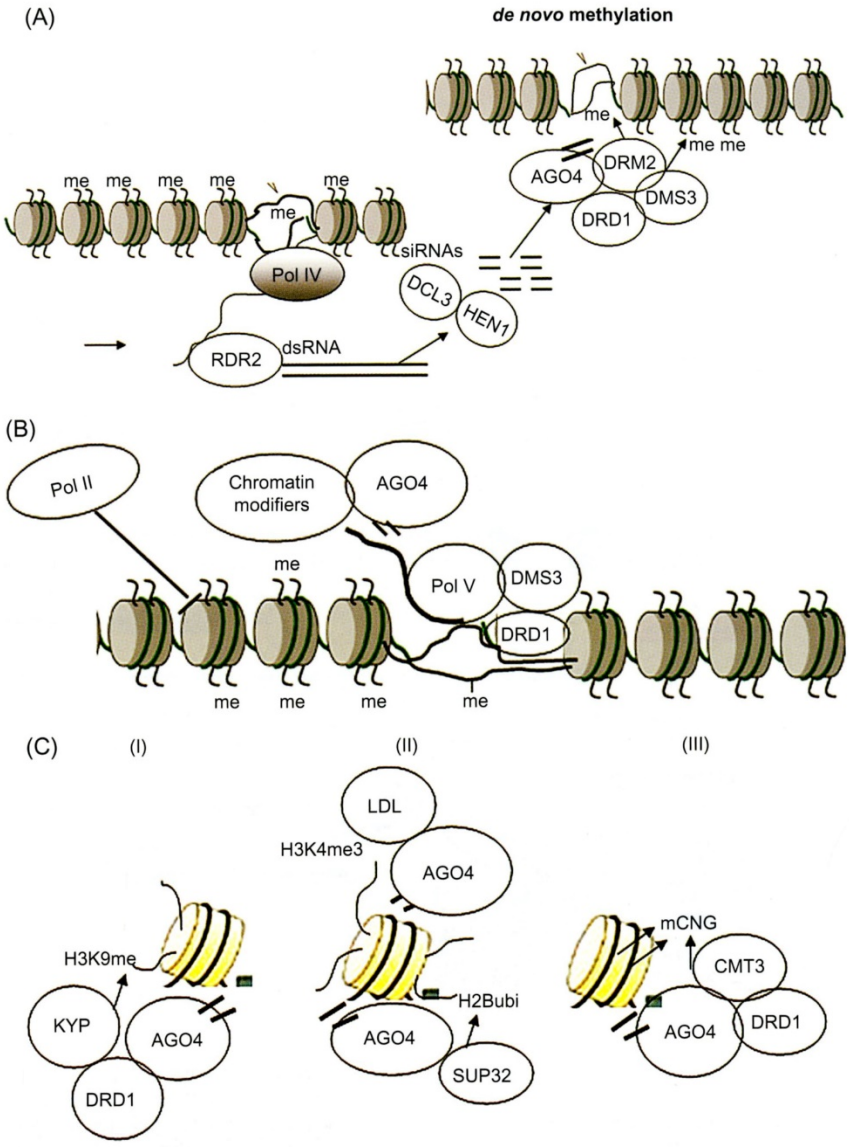

Figure 16.2. AGO4-siRNA complexes involved in chromatin modifications. (A) A model for de novo DNA methylation involving Pol IV transcription as suggested by Matzke et al. [80]. The role of Pol IV is to produce single-stranded RNA transcripts to be used as substrates by RDR2. Pol IV may transcribe from methylated DNA (as illustrated): DRM2 establishes new methyl groups at DNA sequences complementary to the small RNA loaded onto AGO4. The single-stranded RNA produced from methylated DNA by Pol IV is used as a template for a dsRNA synthesis by RDR2 triggering the 24 nt siRNA pathway. dsRNA is processed by DCL3 and HEN1 into small 3′-end methylated siRNAs. The 24 nt siRNAs guide the AGO4 complex containing DRM2/DRD1/DMS3 to homologous genomic sequences. DRD1, a putative SNF2-like chromatin remodeler, and DMS3, an SMC-hinge domain-containing protein are accessory subunits of the complex [80]. (B) A model for spreading of silent chromatin and inhibiting Pol II activity through Pol V transcription, according to Wierzbicki et al. [85]. siRNAs and Pol V transcripts are produced by two independent pathways that collaborate to silence genes and to block Pol II activity. Pol V transcribes noncoding sequences enabled by DRD1 and DMS3. AGO4-siRNA complexes originated in a separate pathway recognize target loci by pairing with Pol V generated transcripts (see text). AGO4 also recruits DNA and histone modifiers (see panel C) to generate heterochromatin. The mechanism of recruiting chromatin modifiers is not clear. (C) AGO4-siRNA complexes in histone modifications and in DNA methylation establishing and propagating silenced chromatin. Once at a target locus, AGO4 and SIRNA complexes might recruit several different chromatin-modifying enzymes to effect gene silencing. The order of action of these chromatin-modifying enzymes is not known, and their relative importance for gene silencing might be locus-specific. (I) Establishing the Silencing H3K9me2 mark: SUVH4/KYP cooperates with the AGO4 complex to establish H3K9me2 according to [8–10,37,93). (II) Removal of activating marks: LDL enzyme brought about by the AGO4-siRNA complex demethylates H3K4me3; deubiquitination of ubiquitinated H2B (H2Bubi) by the ubiquitinase SUP32 recruited and targeted by AGO4-siRNA [94]. (III) Establishing the CNG methylation: guided by homologous RNAs, AGO4 recruits the DNA methyltransferase CMT3 to produce CNG methylation at target loci [43,46,48].

Subunits and Partners of Pol IV and Pol V

The N-terminal portions of NRPD1, NRPE1, and RPB1 containing the catalytic domains are conserved in Pol II, Pol IV, and Pol V. By contrast, their C-terminal domains *(CTDs)* differ in a very significant way: the CTD of RPB 1 contains a reiterated heptapeptide recruiting proteins that process nascent RNA and catalyze histone modifications associated with Pol II transcription [76]. The NRPE1 of Pol V contains the WG/GW repeats, which can specifically interact with AGO4 [38]. Thus, the unique CTDs in the largest subunits of Pol II and Pol V attract different factors for different transcription functions [75].

Pol IV and Pol V share the same second largest subunit (NRPD2/NRPE2) and a smaller subunit, RDM2, similar in sequence to the Pol II subunit RPB4 [77]. Despite similarity in amino acid sequence, RDM2 has evolved as a Pol IV and Pol V specific subunit that does not function in Pol II complexes. Furthermore, the subunit differences have been localized to regions important for template entry and RNA exit points [78] suggesting that Pol IV and Pol V are evolutionarily derivatives of Pol II specialized for generating and/or using noncoding RNAs for chromatin-based gene silencing [70,75,79–81]. As a result, plants have

remarkably diversified their transcriptional machinery, enhancing their ability to modulate the epigenetic states of their genomes during transcription.

The SNF2-like ATPase nucleosome-remodeling factor DEFECTIVE IN RNA-DIRECTED DNA METHYLATION (DRD1) functions with Pol IV and Pol V complexes [75, 82]. The cloning of a maize SNF2 ATPase protein (RMR1) related to DRD1 showed that a chromatin remodeling activity was required for paramutation at the *purple plant* (*pl1*) gene locus [83] (see further below).

DEFECTIVE IN MERISTEM SILENCING 3 (DMS3) is a protein consisting solely of the hinge domain region found in the structural maintenance of chromosomes (SMC) proteins, known to be involved in chromosome architecture [84]. DMS3 and DRD1 are involved in the assembly of Pol V initiation complex [85].

How Are AGO and siRNAs Targeted to Specific Chromatin Sites?

How repeated sequences are selected for siRNA production and how the complex is recruited to the DNA to be silenced is still unclear. Lack of biochemically tractable initiation of silencing functional assays does not allow following the AGO-siRNA targeting steps in the context of intact chromatin. However, recent studies have provided groundbreaking insights into the sequence of the events. In *Arabidopsis*, targeting of DRM2 depends on AGO4-bound siRNAs as a guide [6,9,39]. An RdDM effector, KTF1, with similarity to the transcription elongation factor SPT5, contains a C-terminal extension rich in GW/WG repeats. KTF1 colocalizes with ARGONAUTE 4 (AGO4) in nuclear foci and binds AGO4 and RNA transcripts. Thus, KTF1 acts as an adaptor binding transcripts generated by Pol V and as a recruiter for AGO4-bound siRNAs to form an RdDM effector complex [86]. Chromatin-targeting siRNAs might base pair directly with unwound genomic DNA or could bind to a locus by RNA-RNA interaction with a nascent single-stranded transcript. Base pairing between AGO4-associated siRNAs and nascent Pol V transcripts has been observed indeed, supporting a recent model according to which AGO4 is recruited to target loci by Pol V transcription [85]. Furthermore, siRNAs and Pol V transcripts are produced by two independent pathways that collaborate to promote heterochromatin formation and gene silencing [85]. In one pathway, Pol IV, RDR2, and DCL3 produce 24-nt siRNAs that associate with AGO4; in a separate pathway, DRD1 and DMS3 enable Pol V transcription of noncoding sequences at target loci. siRNA-AGO4 complexes are guided to target loci by interacting with Pol V transcripts. The proposed direct interaction between AGO4 and Pol V [70] has not been detected in vivo suggesting that AGO4 recruitment to chromatin is primarily an RNA-mediated process although protein-protein interactions are not excluded [85]. Interestingly, in *S. pombe*, heterochromatin formation is achieved by the RITS complex guided to chromatin via associations with nascent Pol II transcripts [85a], suggesting that plants and yeast use similar RNA guidance mechanisms for recruiting Argonaute-containing transcriptional silencing complexes to target loci. Apparently, the unique RNA polymerase, Pol V, has evolved in plants for the specialized role of generating noncoding RNAs that can serve as scaffolds for Argonaute recruitment [85] (Fig. 16.2B).

Furthermore, AGO4 can function through two separable mechanisms: by recruiting components that signal DNA methylation independently of its catalytic activity and/or by

the catalytic activity required for the generation of secondary siRNAs reinforcing its repressive effects [87].

The *SINE*-related tandem repeat in the promoter of *FLOWERING WAGENINGEN* (*FWA*) gene provides an example for the involvement of repeated sequence in the production of siRNA. The siRNAs, then, recruit RdDM to an unmethylated *FWA* promoter in trans to silence *FWA* in vegetative tissues. However, the tandem repeat is dispensable, as an *FWA* locus without repeats could also recruit siRNA-producing proteins [88,89]. Likewise, many transposons in heterochromatin do not contain tandem repeats, suggesting that additional signals guide RdDM to silent heterochromatin. Furthermore, repeat-independent siRNA production could also result from aberrant RNA processing of very highly transcribed transgenes (a phenomenon termed sense post-transcriptional gene silencing (S-PTGS) [90]). Additional signals include DNA methylation, modified histone marks, and proteins that "read" the marks and recruit the siRNA complex by interaction with its components. For example, the two SRA domain factors (SUVH9 and SUVH2) bind methylated cytosines and are essential both for de novo and for maintenance DNA methylations: SUVH9 preferentially binds asymmetric, while SUVH2 preferentially binds symmetrically, CG methylated sites [91]. The methylcytosine binding domain proteins MBD6 and MBDl0 act in conjunction with RdDM to effect large-scale silencing of ribosomal DNA loci in the phenomenon of nucleolar dominance [92].

Non-CG methylation of developmental genes can be readily restored after it is lost, suggesting that targeting signals exist and remain in cells in the absence of DNA methyltransferase [44]. For the CNG methylation at the *SUP* locus controlled by CMT3, this signal may come from H3K9me2 (established by SUVH4/KYP) [50,93]; at other loci, only the H3K9me2 or a combination of pathways involving siRNAs may be used, whereas at the *Ta3* locus, CMT3 can propagate CNG methylation without siRNAs or DRD1 [44,88]. Histone H3 lysine4 (H3K4) demethylation helps de novo DNA methylation of an *FWA* transgene and histone H2B deubiquitination regulates gene silencing via siRNAs [94] (Fig. 16.2C).

Transposable Elements (TEs) and Heterochromatin

DNA methylation is an epigenetic mark associated with silencing of TEs constituting about 45% and 75% of the human and maize genomes, respectively [95]. Their potential to transpose may cause significant damage to the host genome. Consequently, eukaryotes have evolved mechanisms, including epigenetic RNAi-mediated chromatin modifications, to respond to the genome invaders and to suppress their activity. Among the earliest recognized mechanisms for keeping TEs silent is their sequestration in domains of constitutive heterochromatin. DNA methylation, histone modifications associated with silenced states, and condensed chromatin packing are features characteristic of heterochromatin found at the centromeres and the telomeres of eukaryotic chromosomes. The accumulation of TEs in regions essential for genome integrity suggests that silencing of TEs activity is critical for proper chromosome function. On the other hand, employment of TEs in essential cellular functions indicates that eukaryotes have "learned" not only to silence but also to take advantage of their presence. The DNA of centromeric heterochromatin in its condensed state is essential for the recruitment of the cohesin complex mediating sister-chromatid

cohesion [96]. TEs are also a source of genetic and epigenetic material that can be utilized by the host to control chromatin structure, gene activity, phenotypic diversity, paramutation, and imprinting.

Presence of endogenous centromeric repeats is important but not sufficient to guarantee kinetochore formation [reviewed in Ref. 97]. The establishment and maintenance of centromeric chromatin (characterized by the centromere-specific histone H3 variant, CENP-A, as the key determinant of centromere identity and the location of kinetochores) is epigenetically regulated. In the fission yeast *S. pombe*, the centromeric heterochromatin is maintained by noncoding transcripts from the outer (pericentromeric) repeats. These transcripts are processed into small interfering RNAs (siRNA) targeted to homologous sequences; together with recruited heterochromatin proteins, siRNAs are essential for the establishment of CENP-A centromeric heterochromatin. The Argonaute-associated proteins, Chp 1 and Tas3, and the Suv39 and HP1 homologs, Clr4 and Swi6HP1, are required for establishing the centromeric heterochromatin [98]. Once assembled, CENP-A chromatin is propagated by epigenetic means in the absence of heterochromatin. These studies identified an important, potentially conserved, role for RNAi in directing centromere and kinetochore formation [98].

In *Arabidopsis*, the heterochromatin is located mainly at the centromeres, represented by retrotransposons interspersed among arrays of satellite repeats, at the pericentromeric regions composed mainly of DNA transposons, and in the knobs representing jumbled TE islands triggering RNAi-mediated silencing, perhaps through read-through transcription (AGI, 2000). The 180 base pairs centromeric methylated satellite repeats are found in thousands of tandem copies. However, in mutants lacking either MET1, or the histone deacetylase HDA6/SIL1, or the chromatin remodeling ATPase DDM1, the silenced TEs and satellite repeats were reactivated causing decondensation of centromeres. Silencing lost in *met1* or *hda6* was reestablished in backcrosses to wild type, but silencing lost in RNAi mutants and *ddm1* was not. 24-nt siRNAs corresponding to the centromeric sequences were retained in *met1* and *hda6*, but not in *ddm1*, suggesting that siRNAs are guiding DDM1 for the silencing of centromeric DNA [7,46,99]. It should be noted that *S. pombe* lacks DNA methylation and plants do not have homologs of Argonaute-associated proteins Chp1 and Tas3 suggesting a convergent evolution of the siRNAs chromatin targeting pathways in *S. pombe* and in plants.

In grasses, a *Ty3*/*gypsy* class of centromere-specific retrotransposons are conserved and highly enriched in domains associated with CENP-A and with the flanking centromeric satellite DNAs [100]. These elements are actively transcribed in maize, suggesting that deposition of centromeric histones might be a transcription-coupled event [101]. Transcribed repeats silence the retrotransposons and transcripts from retrotransposons help silencing the repeats suggesting roles for siRNAs in the evolution of centromeres [102].

In addition to DNA methylation, constitutive heterochromatin in *Arabidopsis* is marked by "repressive" histone modifications, including histone H3 dimethylation at Lys9 (H3K9me2) and methylation at Lys27 (H3K27me). H3K9me2 overlaps almost exclusively with transposons and other repeats, while H3K27me3 is associated mostly with inactive euchromatin. Nontranscribed genes may be associated with H3K27me3, H3K4me1, and H3K4me2 [55,103–108]. H3K9me2 and H3K27me1 are mediated by SUVH2, SUVH4 (KYP),

SUVH5, and SUVH6, and *ARABIDOPSIS* TRITHORAX-RELATED PROTEIN 5 (ATXR5) and ATXR6 [109,110] (for a review on plant heterochromatin proteins see Ref. 110 and ref. therein).

Little is known about histone methylation in large-genome plants, which make up the bulk of the angiosperms. Combining high cytological resolution of maize pachytene chromosomes, three-dimensional light microscopy, and the ability to quantify staining patterns relative to cytological features, Shi and Dawe [111] reported that each methylation state identified different regions of the epigenome: H3K27me2 marks classical heterochromatin, H3K4me2 is limited to areas clearly demarcating the euchromatic gene space, while H3K9me2 and H3K27me3 occur in euchromatic domains; H3K9me3 is associated with centromeres and H4K20me2/3 is nearly or completely absent in maize. H3K9me2 appears excluded from repeats and associating with genes but does not overlap with either H3K27me3 or H3K4me2 [111]. Apparently, the presumed epigenetic code has the capacity to evolve along with changes in genome structure.

CG methylation provides distinct and direct information for a specific subset of histone methylation marks illustrating a crosstalk between DNA methylation and histone modifications (Fig. 16.1). CG methylation characteristic of heterochromatin specifically prevented H3K27 trimethylation but H3K27 mono- and dimethylation label silent heterochromatin independently of DNA methylation [108].

Position Effect Variegation (PEV)

Heterochromatin can epigenetically influence the expression of nearby genes causing variegated phenotypes in genetically identical cells. The PEV phenomenon, described by H. Muller in 1938, is illustrated by the *Drosophila* gene, *white*, which shows unstable expression when moved in close proximity to heterochromatin. In plants, variegated gene expression has been reported in *Oenothera blandina* after X-ray chromosomal disruptions and translocations [112,113]. A phenomenon similar to PEV is observed when transgenes are inserted into heterochromatic regions in fission yeast [114] and proximity to TEs might be responsible for the silencing effects. Furthermore, gene screens for suppressors/enhancers of PEV have identified the same factors as those involved in TE silencing and heterochromatin structure, including the RNAi-mediated silencing implicating siRNAs in PEV [115].

Heterochromatin can spread linearly to about 10 kb until it encounters a boundary. TEs can function as nucleation centers for facultative heterochromatin as well as insulators [116]. RdDM spreading is associated with the production of secondary siRNAs, which originate outside the region targeted by primary siRNAs [74,80]. An interesting example is that the spreading of methylation and siRNAs from a *LINE* element into the adjacent gene (*BONSAI*) is dependent on the chromatin remodeler DDM1 and a histone demethylase (INCREASE IN BONSAI METHYLATION1, IBM1) [54,117] but is not mediated by DRM2 or other components of the RNAi-dependent de novo methylation machinery [67].

Epigenetic Variations and Plant Evolution

Because of their polymorphic locations and abilities to spread epigenetic marks, TE can influence transcription of nearby genes or cause readthrough, which would be subjected to silencing. Thus, TEs can produce phenotypic variability by forming epialleles that are metastable in nature and have variegated expression that resembles PEV. Epialleles are formed due to the proximity of a gene to a TE, and are regulated by the epigenetic mechanisms that the TE recruits. Natural epigenetic variation can originate from polymorphisms in transposon insertions and repeats, as illustrated by the siRNA-producing loci and DNA methylation in different *Arabidopsis* species and ecotypes of *A. thaliana* [118]. A Mutator element in the first intron of the floral repressor FLOWERING LOCUS C (FLC) in the ecotype Landsberg erecta (Ler) but not Columbia (Col) is a target of siRNAs that induce histone modifications and flowering time differences [119]. A HAT transposon in the promoter of *FLC* produces abundant siRNAs in Ler but not in Col, resulting in hypermethylation of the promoter only in Ler [120]. These results illustrated the role of the siRNAs-silencing pathway on the evolution of flowering time strategies and speciation. *FWA* epigenetic mutants cause a heritable late-flowering phenotype due to ectopic expression of the *FWA* gene in vegetative tissue. The expression level is heritable but variable within the examined 21 *Arabidopsis* accessions that have two direct repeats at the promoter [89].

A naturally occurring mutant of *Linaria vulgaris* displaying a strikingly different flower phenotype with radial symmetry instead of bilateral, results from a different expression of the *Lcyc* gene. The *Lcyc* gene controlling flower symmetry is extensively methylated and transcriptionally silent in the radial flower mutant. The modification cosegregates with the mutant phenotype, is heritable, and occasionally reverts phenotypically during somatic development, correlating with demethylation of *Lcyc* and restoration of gene expression [121]. A spontaneous epimutation was identified at the *Colorless nonripening*, *Cnr*, locus, a component in the regulatory network controlling tomato fruit ripening [122]. Mounting evidence is suggesting that epialleles and epigenetic mutations might play a more significant role in plant developmental processes, in the generation of natural variation and plant evolution, than has hitherto been suspected. The consequences of transgenerational epigenetic effects driven by *cis-* and *trans*-acting effects, chromatin modifications, RNA-mediated pathways, and regulatory networks modulating differential expression of homologous genes in polyploids might also facilitate adaptive evolution of polyploid plants and domestication of crops [16,123,124]. Some epialleles may undergo paramutation, a *trans*-interaction between alleles that induce heritable expression change in one allele.

Paramutation

Paramutation is one of the best-known examples of non-Mendelian inheritance arising from allelic interactions that lead to meiotically heritable gene silencing. Because changes in gene expression are not associated with changes in DNA sequence, paramutation is a classic example of an epigenetic phenomenon. Among the best-studied examples are the four maize loci, *Pl1*, *p1*, *r1*, and *b1*, involved in the anthocyanin synthesis pathway. *B'* and *Pl'* display strong paramutation strengths, while *r1* and *p1* are variable [125,126]. All encode transcription factors that increase pigmentation in the plant and a paramutation event

at any of these loci is reflected by a decrease in coloration, providing an easily tractable system to study the phenomenon [127]. The allele that is capable of affecting (silencing) expression from the homologous locus is referred to as the paramutagenic allele and is usually expressed at low levels; by contrast, the affected (paramutable) allele is highly expressed. The paramutable *B-I* allele is transcribed at about a 20-fold higher level (providing for the strong red coloration of plants) than the paramutagenic *B′* allele found in colorless plants [128]. Combining the two alleles (in *B-I*/*B′* heterozygote) results in silencing of *B-I* transcription with 100% penetrance. The most remarkable feature, however, is that the silenced *B-I* allele acquires paramutagenic capability itself, effectively silencing transcription from other homologous alleles in subsequent generations—secondary paramutation ("the vampire" effect).

The molecular basis of paramutation has been a long-standing enigma but recent studies have provided breakthrough insights. Fine structural analysis revealed that an array of seven direct tandem 853-base repeats located ~100 kb upstream of *b1* are necessary for the paramutagenicity of *B′* and the ability of *B-1* to undergo paramutation [129,130]. The sequences are present as a single copy in neutral *b1* alleles, while alleles with three repeats show partial paramutational ability [129–132]. Thereby, the presence and the numbers, as well as the organization of the repeats (as observed at the *p1* locus, [133]), are essential for paramutation.

A critical question is how the silencing information is communicated. Some maize mutants deficient in the establishment and/or maintenance of paramutation at distinct loci are defective in genes encoding RdDM factors: a genetic suppressor of paramutation, *Mediator of Paramutation1* (*MOP1*), encodes the maize ortholog of RDR2; *Required to Maintain Repression*, *RMR1*, encodes an SNF2-like putative chromatin-remodeling factor related to DRD1 and CLSYl, and RMR6 is the largest subunit of Pol IV (RPD1). All of these factors are required for siRNA production, for DNA methylation at the silent epialleles, and for paramutation at the *purple plant* (*pl1*) locus [83,134]. MOP1 is needed for paramutations at all four loci [126]. The *mop1* gene is also required for silencing transgenes and *Mutator*-like transposons in maize [134]. The role of the chromatin remodeler RMR1 is not exactly known, but it may act as a cofactor for Pol IV and, thus, be involved in interactions between siRNAs and chromatin [80].

RMR6, the largest subunit of the maize Pol IV (ZmRPD1), is required for both paramutation and for normal maize development [135]. ZmRPD1 is essential for accumulating the majority of 24-nt siRNAs, indicating that it operates at repetitive DNAs. However, the biochemical function of ZmRPD1 remains unclear as it failed to produce detectable RNA transcripts for genomic regions represented by those siRNAs [133]. Furthermore, the primary polymerase for repetitive DNAs, including hypermethylated and repressed transposons in maize, seems to be Pol II [83] and not Pol IV, as in *Arabidopsis*. Transposon sequences proximal to Pol II templates could interfere with Pol II-dependent RNA synthesis, resulting in the production of abnormal Pol II transcripts, which could trigger the siRNA pathway [133].

Although essential for paramutation, the RNAi machinery is not the only factor. For example, the 853 nt repeats are transcribed from both *B′* and *B-I* loci indicating that siRNAs alone are not sufficient to establish paramutation. Recruitment of siRNA machinery to a

locus is not always sufficient for the RNA-directed DNA methylation either. For example, differences between the silent (*FWA*) and the unmethylated (*fwa-I*) epialleles in *Arabidopsis* were not accounted for by siRNA production: the repeat-derived siRNAs accumulate equally in plants with wild-type *FWA*, and those with *fwa-1* and an introduced transgene can silence an unmethylated *fwa-1* endogenous gene [88,89].

How interallelic transfer of epigenetic information is achieved remains to be determined. Some models propose *trans*-communication between epialleles, including physical pairing of alleles and transfer of silencing complexes, histone modifiers, nucleosome repositioning factors, and DNA-methylating activities on the paramutable locus [126]. In some cases, acquisition of DNA methylation accompanies *trans*-inactivation of paramutable alleles as found for the maize *r1*, *p1*, the petunia *A* loci, and *FWA* [126,136,137]. However, cytosine methylation is not the causative factor for establishing the *B′* silent state, despite *B-I* and *B′* alleles having different methylation profiles [129,130]. Thereby, other factors contribute to the epigenetic states and to the ability of certain epialleles to influence homologous sequences both in *cis* and in *trans*. Such factors could be chromosomal location (PEV), ploidy, environmental factors, and histone modifications. For example, the tomato *sulf* locus mapping near heterochromatin experiences silencing effects dependent on ploidy [138] and inactivation of an active transgene *HPT* locus in *Arabidopsis* is observed in a tetraploid but not diploid background [139].

Imprinting, Genome Resetting, and Reprogramming

Imprinting

Differential expression of alleles of the same gene depending on the parent-of-origin (gene imprinting) is thought to have evolved independently in mammals and in flowering plants [140]. Imprinting occurs in the nourishing tissues, the placenta of mammals, and the endosperm of plants. The formation of the endosperm, a process involving a double fertilization of the central cell (CC) by the pollen, is a defining feature of flowering plants. Pollen contains three nuclei: the vegetative nucleus (VN) is in the cell developing into the pollen tube; as it grows, the pollen tube transports the two sperm nuclei to the ovules. One of the sperm nuclei fertilizes the egg, and the third nucleus fertilizes the diploid central cell to form the triploid endosperm. After fertilization, the proliferation of the endosperm ensures nutritional resources for the embryo. In humans, mutations of imprinted genes are associated with developmental disorders and diseases; mutations in plant imprinted genes lead to defective reproduction and loss of viability [142].

Ten imprinted genes are currently recognized in *Arabidopsis*: two encode Polycomb group proteins (*MEDEA*, *MEA*, and *FERTILIZATION INDEPENDENT SEED2*, *FIS2*), an RNA-binding protein (*MATERNALLY EXPRESSED PAB C-TERMINAL*, *MPC*), two encode transcription factors (*FWA* and *PHERES1*, *PHE1*), and four genes encode class IV homeodomain transcription factors [141–144]. Although imprinted in the endosperm, the *PHE1* gene is set apart from *MEA*, *FIS2*, *FWA*, and *MPC* because *PHE1* is expressed from the paternal allele [145,146]. Assuming that genes with endosperm-preferred expression are less methylated at 5′ sequences in the endosperm than in the embryo and that less

methylated genes in the endosperm exhibit endosperm-preferred expression, it is estimated that there are ~50 imprinted genes in *Arabidopsis* encoding mainly transcription factors and chromatin-related functions [144].

In mammals, imprinting is reflected by differential methylation of specific sequences in the gametes [147]. In *Arabidopsis*, imprinting is usually due to differences in the epigenetic marks (histone and DNA methylation) on alleles in the central cell, which are maintained in the endosperm [148]. The 5-methylcytosine DNA glycosylase *DEMETER* (*DME*) is expressed only in the CC before fertilization and demethylates the maternal alleles of imprinted genes, establishing methylation asymmetry between embryo and endosperm [144,148]. A subset of Pol IV-dependent siRNAs specifically expressed from the maternal chromosomes was shown to accumulate in the maternal gametophyte and to persist during seed development, linking genomic imprinting with RNA silencing mechanisms [149].

Bulk methylation in wild-type endosperm is lower in all sequence contexts compared with the embryo [150]. Genome-wide maize endosperm has 13% less 5-methylcytosine than embryos or leaves [151] and an imprinted gene is less methylated in the CC than in the egg cell or sperm [152]. Transposable elements are more heavily methylated than protein-coding genes, and genes are more methylated within their bodies than at their 5′ and 3′ ends. The reduced CG methylation at repeats and gene-bodies in *Arabidopsis* wild type endosperm was partially restored to levels found in other tissues in the *dme* endosperm, indicating that the CG demethylation is specific to maternal sequences [144]. In contrast to CG, methylations of CHG and CHH were reduced in *dme* endosperm suggesting that DME activity is necessary for up-regulating RNAi-mediated methylation in endosperm and for activating the TEs by demethylating them.

Importantly, the genome-wide CG demethylation of the maternal endosperm genome is accompanied by similarly extensive CHH hypermethylation of the TEs in the embryo revealing that siRNA accumulation in the CC contributes to enhanced methylation and silencing of elements in the egg cell (and later in the embryo). This could happen through siRNA transport, which could be the original force behind the evolution of the central cell demethylation. Thus, RNAi drives a substantial reconfiguration of the methylation landscape in the seed, suggesting that imprinting in plants evolved from targeted methylation of TEs to reinforce transposon silencing in the embryo [144,148].

Genome Resetting and Reprogramming in the Male Gametophyte

A strikingly similar process occurs in the *Arabidopsis* male gametophyte where reactivation of the TEs in the pollen vegetative nucleus (VN) reinforces silencing of the TEs in reproductive sperm cells [153]. Apparently similar mechanisms operate in germ lines for detecting aberrant RNAs and for silencing TE in the embryo [148,149,153]. However, in pollen, TEs are reactivated and transpose, but only in the VN, which accompanies the sperm cells but does not provide DNA to the fertilized zygote. In the gametes, the mutagenic activity of TEs is epigenetically suppressed by siRNAs, preventing transmission to the next generation. The expression of the small RNAs coincides with down-regulation of the heterochromatin remodeler *DDM1* and of many TE siRNAs. An unknown DNA demethylase active only in the VN may act to selectively remove DNA methylation from some TEs.

The TEs are transiently activated in a coordinated fashion and down-regulation of the genes responsible for TEs silencing is confined to the VN of pollen. A silenced TE is transiently reactivated in maize pollen as well [153], and TE expression has been noted in the pollen of rice [154], indicating that the reactivation of silenced TEs in pollen is conserved among flowering plants. In contrast, the TEs in terminally differentiated senescing leaf cells are not coordinately reactivated, suggesting that TE activation in the pollen VN represents a cell type–specific epigenetic reprogramming that has evolved for a function. Changes in histone H4 acetylation and in histone variants consistent with reactivation of TEs and loss of heterochromatin observed in the pollen from both *Lilium* and *Arabidopsis* [155–157] support a genome-wide reprogramming taking place in pollen.

To answer the question of why epigenetic reactivation of TEs is needed in the VN of pollen, Slotkin and coauthors [153] suggest that the relative position of the VN next to the sperm cells is important for the silencing of TEs in the next generation. Interestingly, transposon-related siRNAs (21 nt long from the *Athila* retrotransposon family) are generated and accumulate in pollen and sperm [153]. The authors propose that these 21-nucleotide siRNAs, originating in the VN, travel to the adjacent sperm cells to reinforce silencing. Thus, only those transposons with the potential to be expressed (because they were expressed in the vegetative nucleus) would be targeted by siRNAs in sperm nuclei. Although new transposition events occur in pollen, they are not inherited because the transposon activation occurs in the VN, which does not contribute DNA to the zygote akin to the TE reactivation that takes place in the endosperm. During reprogramming in *Drosophila* and mouse germlines, epigenetic marks are first lost and then robustly reset each generation, resulting in transient TE expression [158,159]. Subsequent remethylation and silencing in sperm depends on the sperm-specific piRNA [160]. Movement of signals (small RNAs) from germline companion cells into germ cells conserved in insect nurse cells is consistent with speculation that the evolution of the sperm-companion vegetative cells interactions has promoted TE silencing in angiosperms. In contrast to the model suggesting that imprinted genes in the endosperm have evolved from TE-silencing mechanisms (see above), the authors conclude that the molecular mechanisms involved in the permanent silencing of foreign DNA have evolved from mechanisms required for the successful development of an embryo.

Genome Reprogramming during Flowering

Plant developmental and environmental responses involve reprogramming at specific genome loci so the normal program of plant development is reiterated in each generation. Epigenetic repression of *FLOWERING LOCUS* C (*FLC*) in winter-annual ecotypes of *Arabidopsis* by prolonged cold (vernalization) ensures that plants flower in spring and not during winter. Flowering is induced by the photoperiod (day length) and/or temperature, which stimulate *FLOWERING LOCUS T* (*FT*). FLC represses the activity of *FT* to prevent flowering [161].

The activities of both paternally and maternally derived *FLC* reporter genes are reset after vernalization, but the timing of their initial expression differs. The paternal gene copy is active during early gametogenesis and in the single-celled zygote, whereas the maternal

copy is not expressed until the early multicellular embryo stage. In the progeny, the paternally derived *FLC* gene is expressed in the single-celled zygote and through embryo development, but not in the fertilized CC, which generates the endosperm. *FLC* activity during late embryo development is a prerequisite for the repressive action of *FLC* on the flowering of the adult plant [162].

Positioned at the convergence node of at least four distinct pathways that block transition from the vegetative to the reproductive stage, *FLC* gene is repressed by low temperature through changes in *FLC* chromatin. Chromatin-based mechanisms involve histone modifications [163–166], replacement by histone variants [167–169], and a Pol IV-dependent antisense RNA initiating in the 3′-region of the gene [170].

Transcription from the *AP1* locus, required for the initiation of flowering and the reprogramming of the *AP1* locus to an actively transcribed state, is accompanied by a change of the chromatin structure at the *AP1* promoter. It involves removal of a nucleosome from the transcription start site and dynamic repositioning of the TSS-nucleosome in a process that is developmentally regulated [165].

Restoring Lost Methylation at Heterochromatin

Given the importance of TEs for the formation of heterochromatin for centromere function and the role of DNA methylation in keeping the TEs silent, it is critical that cells maintain TE methylation levels throughout cell divisions and developmental transitions. It may be expected then that mechanisms guarding cells against accidental loss of heterochromatic CG methylation exist. Indeed, a novel corrective mechanism for restoring lost methylation from regions that need to remain silent was recently revealed [57]. Loss of CG DNA methylation during gametophyte generations was restored through the RNAi machinery in a robust and specific CG-remethylation of the *Arabidopsis* centromeric repeats and transposons. Methylation was RDR2 dependent, was guided by 24-nt siRNAs corresponding to transposons and repetitive DNA sequences, and did not spread to adjacent sequences. Furthermore, the RNAi machinery is crucial for discriminating remethylatable from non-remethylatable sequences and is resilencing only transposons activated in the *ddm1* mutants. Clearly, ability to remethylate plays a protective role against the deleterious effects of active transposable elements. Apparently, it is advantageous for cells to maintain ongoing production of siRNAs from repeated DNAs, either as a backup for CG DNA methylation or to ensure rapid initiation of silencing at new transposon insertions [171].

Polycomb Group (PcG) and Trithorax Group (TrxG) Complexes in Plants

In animals, expression states of homeotic genes (active or silent) are maintained and faithfully propagated throughout development by the counteracting activities of the PcG/TrxG complexes (Ref. 172 and ref. therein). In contrast to animals, plant organs (leaves, flowers) and seeds originate from the same undifferentiated meristem active throughout the life cycle. Although differentiation and organogenesis are not fixed in embryogenesis, PcG/TrxG homologs play roles in plant development as well. In plants, as in animals, development of a wrong organ at a wrong place (homeosis) is a consequence of a mutation

of a homeotic gene. Unlike their animal counterparts, plant homeotic genes are not clustered and belong to the MADS-box family of transcription factors but nonetheless, the PcG/TrxG complexes similarly regulate their expression by modifying their nucleosomes. Like their metazoan counterparts, the *Arabidopsis* PcG complexes establish H3K27me3 through the biochemical activity of Enhancer of zeste (EZ)-related proteins [173–175], while Trithorax family members specifically tri-methylate histone H3K4 [176–178]. In both plant and metazoan chromatins, the H3K27me3 and H3K4me3 modifications are associated with transcriptionally silenced and active gene states, respectively.

At animal genes, two classes of PcG repressor complexes, PRC2 and PRC1, participate in the transcription-resistant chromatin structure [172]. PRC2 catalyzes the H3K27 trimethylation resulting in the recruitment of PRC1, which maintains suppression by catalyzing H2A monoubiquitination [179].

In *Arabidopsis*, PRC2 complexes are conserved both structurally and functionally and, like their animal counterparts, form 600 kDa complexes involved in development, flowering, and imprinting [174,177]. The *Arabidopsis* H3K27me3 modifying mechanisms have proliferated to three *EZ* homologs (CURLY LEAVES, CLF, SWINGER, SWN, and MEDEA, MEA) forming at least three distinct PRC2 complexes containing proteins homologous to the metazoan PRC2 components: EMBRYONIC FLOWER1 (EMF2), VERNALIZATION2 (VRN2), FERTILIZATION INDEPENDENT SEED2 (FIS2) corresponding to SU(Z)12, and two WD-40 proteins (FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA1 (MSI1)) corresponding to ESC and P55, respectively. The different PRC2 complexes may have distinct functions as MEA expression is limited to the female gametophyte and embryo development, while CLF and SWN are expressed in adult plants [180].

Genes for proteins homologous to the PRC1 complex have not been identified in *Arabidopsis* but a functionally similar complex might be formed by the LIKE-HETEROCHROMATIN PROTEIN1 (LHP1), two RING-domain containing proteins, and a plant-specific protein, EMBRYONIC FLOWER1 (EMF1) [181]. LHP1 localizes at chromatin domains rich in H3K27me3 [103,105] and EMF1 is involved in the H3K27 tri-methylation [182].

The H3K4me3 mark of actively transcribed genes is established by the methyl transferase activity of the trithorax protein, which functions in a complex (COMPASS) conserved in yeast, *Drosophila*, and humans [183]. Genetic, biochemical, and molecular characteristics of the *ARABIDOPSIS HOMOLOG* OF *TRITHORAX1*, *ATX1*, have defined it as the plant counterpart of animal trithorax factors [106,176] but a plant COMPASS complex has not been identified yet. A WDR5-related protein capable of binding ATX1 was reported recently [184]. The evolution and function of *Trithorax*-related genes in plants, as well as the role of ATX1 in maintaining normal levels of homeotic gene expression during plant development and transition to flowering, for organ identity, and for biotic and abiotic response mechanisms in *Arabidopsis*, was recently reviewed [178].

Antagonistic PcG/TrxG Functions in Arabidopsis

The flower homeotic gene *AGAMUS*, *AG*, is silent in young seedlings and in vegetative tissues, but its correct expression during flowering is critical for flower organ development and identity. Expression of *AG* is suppressed by the *Arabidopsis* homolog of EZ, CLF, and

up-regulated by the homolog of trithorax, ATX1 [173,176], supporting the idea that CLF and ATX1 have counteracting activities at the *AG* locus. Interestingly, loss of both ATX1 and CLF functions in *atx1*$^{-/-}$*clf*$^{-/-}$ mutants rescued the single-mutant phenotypes, suggesting that the Trx-like and the EZ-like plant homologs counterbalance each other at the shared locus [107]. Partial normalization of axial-skeletal transformations in mice was also observed when *Mll* (a human homolog of trithorax) and *BMI*-1 (a PcG component) were simultaneously deleted [185]. The molecular basis of this remarkable shift toward wild-type phenotypes in the double *atx1*/*clf* mutants was related to the partial restoration of the histone marks on the *AG* nucleosomes that were lost in the single mutants. Restored patterns, however, were not identical with the initial patterns, an observation that could account for the variability and instability of phenotypes often seen in epigenetic mutants. At the molecular level, both H3K4me3 and H3K27me3 marks were required for the normal suppression of *AG* in leaves [107], perhaps establishing a chromatin state similar to the bivalent states of embryonic stem cell chromatin [186]. Contrary to the expectation that absent *ATX1* and *CLF* functions would erase the H3K4me3 and H3K27me3 marks, there was a partial restoration of the marks on the *AG*-nucleosomes in the double-mutant chromatin. The results suggested that in the absence of both ATX1 and CLF their roles could be undertaken by a different pair of modifiers supporting a model in which the PcG and TrxG-complexes form specific pairs to generate simultaneously present H3K4me3 and H3K27me3 marks. ATX1 and CLF physically interact, providing a mechanistic basis for the observed effects [107].

Bivalent Chromatin Marks

Simultaneously present H3K4me3 and H3K27me3 marks found at silent genes in embryonic stem cells has suggested that coexisting "activating" and "silencing" nucleosomal modifications establish a bivalent chromatin state at loci "poised" for transcription later in development [186]. In addition to pluripotent cells, K4me3-K27me3 colocalization is functional in more differentiated cells as well [187]. Dual H3K4me3-H3K27me3 marks labeling the nonexpressing *AG* locus in young seedling chromatin might similarly reflect a bivalent chromatin state to be expressed at a later developmental stage [107]. Coexisting H3K4me3 and H3K27me3 marks were found at two other loci (*FLC* and *AP1*) involved in the transition to flowering [165], suggesting that bivalent chromatin states might be a general characteristic of developmentally regulated genes in plants. The distribution patterns of the two antagonistic marks at promoters and at downstream gene-body nucleosomes are different. Presence of H3K4me3 and H3K27me3 on downstream nucleosomes remained unchanged throughout developmental transitions, independently of the transcriptional activity of *AG*, *AP1*, or *FLC*. In stark contrast, the H3K4me3/H3K27me3 profile at 5′-TSS nucleosomes changed dynamically, reflecting changes in transcription [165]. "Activating" H3K4me3 and "silencing" H3K27me3 modifications coexist at 5′-end nucleosomes of both the transcriptionally active *FLC*- and the silent *AG* genes, while highly transcribed *AP1* displays neither of the two marks, suggesting that distinct mechanisms "read" and operate at each locus even for genes belonging to the same MADS-box family.

Regulation of Imprinted Genes by PcG

PcG proteins directly regulate the silencing of the paternal allele in the male gametophyte of the imprinted gene *MEA* [188] and reduce the level of biparental *MEA* expression in vegetative tissues [189]. Thus, the PcG component, MEA, participates in both maternally and paternally derived PcG complexes to silence the paternal *MEA* allele in the endosperm. Polycomb group proteins and DNA methylation also regulate the *PHE1* gene imprinting. The paternal copy of *PHE1* is preferentially expressed while the maternal allele is silenced in the *Arabidopsis* endosperm [145] where the Polycomb group complex (MEA, FIE, FIS2) silences the maternal *PHE1* allele after fertilization. Loss of DNA methylation at a site 3′ of the maternal *PHE1* allele is required for its silencing by the PcG proteins, suggesting that DME might demethylate the maternal *PHE1* allele in the central cell [146], triggering the siRNA silencing pathway.

Chromatin Remodeling

Ultimately, it is the structure of chromatin that provides the permissive or restrictive environment for the transcriptional machinery exercising, thus, epigenetic control over a gene's expression. How chromatin structure (chromatin remodeling, involving assembly/disassembly, or repositioning of nucleosomes) drives or responds to changes in gene expression is a subject of intensive studies. Revealing how chromatin remodeling, epigenetic marks (DNA methylation and histone modifications), and small RNAs are related mechanistically to achieve coordinated genome-wide control is among the most complex matters. Studies in animal systems are leading the way and have provided significant insights into the crosstalk of these factors at the molecular level. The nucleosome chaperones (CAF-l, NAP1, and HIRA) and chromatin remodelers (the SWI/SNF, RSC, ISWI, INO80, SWR1, and Mi-2/CHD) are specialized multiprotein machines regulating access to nucleosomal DNA by altering the structure, composition, and positioning of nucleosomes. ATP-dependent activities can remodel chromatin by either mobilizing nucleosomes on DNA or by exchanging one histone variant for another, within the nucleosome [190].

Components of the remodeling machinery are conserved throughout eukaryotes. In *Arabidopsis*, homologs of individual components of the nucleosome chaperone complexes, CAF-1, NAP1, and HIRA [191–194], of the CHD-type proteins PICKLE (PKL) and MOM1 [195,196], and of the ATP-dependent SWI/SNF remodelers [197–201] have been identified and shown to influence gene expression and plant development [200–204]. Most of the plant Snf2 proteins carry similar function as their yeast and animal homologs but some have been adapted for functions occurring only in plants. Forty-one members of the *Arabidopsis* Snf2 family fall into 19 distinct subfamilies, reflecting the expansion of the SWI/SNF ATPase regulatory repertoire, while preserving essential ancestral functions [200,204]. DDM1, CLSY, and DRD1 are plant-specific ATPase activities involved in genome-wide DNA methylation, transposon silencing, and Pol IV-Pol V functions tightly coupled and guided by the 24-nt siRNAs, as discussed above. In contrast to yeast, *Drosophila*, and mammals, isolation and biochemical characterization of a full-size complex of plant origin has yet to be achieved for any of the chromatin-remodeling activities. However, homologs for the core subunits of the SWR1 chromatin remodeling complex have been characterized

in *Arabidopsis* and shown to be functionally related to those described in yeast and human [205]. The SWR1-like chromatin-remodeling complex also contains a plant-specific protein, SEF, which genetically and physically interacts with the ATPase subunit counterpart, PIE1, and together with the ARP6 homolog (ESD) control gene expression at the chromatin level [206].

Conclusions and Perspectives

- TEs are the major component of heterochromatin at the centromeres and telomeres. Regulation of the TEs activity is required for proper chromosome function, and epigenetic mechanisms in plants are largely oriented toward repressing TEs. Histone and DNA modifications are common epigenetic tools but they may be used in kingdom and species-specific ways. For example, in *Arabidopsis* CG, CHC, and CHH methylations are present mainly in repeats, whereas the gene bodies are CG methylated [62–65]. It remains to be seen how general these DNA methylation patterns would be for other plants. In plants, the activities establishing the epigenetic chromatin marks are largely dependent on guidance by the small heterochromatic 24-nt siRNAs.
- Findings of siRNAs in all three eukaryotic kingdoms indicate that the siRNA machinery was present in the last common ancestor of plants, animals, and fungi [23]. Although the machinery might be ancient, the siRNA have diversified over time to acquire specialized roles. Unique plant-specific proteins function upstream of DCL3 and downstream of AGO4 to produce 24-nt siRNAs that guide DNA methylation and heterochromatin formation. Instead of cleaving mRNA, AGO4-siRNA complexes recruit chromatin-modifying enzymes. Some of the proteins involved in the biogenesis of the 24-nt siRNAs are genetically redundant, whereas others have specialized roles. The diversification of RNA silencing pathways in plants reflects the intricate ways evolved by the sessile organisms to cope with biotic, abiotic, and genome stresses.
- Pol IV and Pol V transcription complexes have evolved as evolutionary derivatives of the Pol II mechanism specialized for generating and/or using noncoding RNAs for chromatin-based gene silencing. Elucidation of their function helped solve the paradox that transcription of DNA sequences that are silent at the chromatin level is required for the assembly of chromatin in a silent state nonpermissive for transcription by Pol II and Pol III. Thus, plants have enhanced their ability to modulate the epigenetic states of their genomes by remarkably diversifying their transcriptional machinery [85].
- As the ancestral role of chromatin-targeted siRNAs is the genome-wide suppression of repeated DNA, the number of endogenous genes that are controlled by this system might be small in *Arabidopsis*. However, in species with large genomes, like maize, the epigenetic control of TEs by chromatin-targeted RNAi has a much more important role regulating developmental genes [133,135].
- In addition to silencing TEs, flowering plants have evolved intricate ways to implement siRNA pathways in the regulation of pollen and embryo development through gene imprinting. In a highly specific RNAi-targeted process, transposon activation and

siRNA accumulation in the central cell contribute to enhanced methylation and silencing of repetitive elements in the egg (and later the embryo). The model viewing imprinted genes not as exceptional sequences specifically targeted for demethylation in the central cell but rather as part of a process that reshapes DNA methylation of the entire maternal genome in the endosperm [148,149] is consistent with the hypothesis that imprinting arose as a byproduct of silencing the invading foreign DNA [207]. Transposon-silencing mechanisms might have been co-opted for the regulation of ribosome biogenesis and nucleolar dominance in interspecies crosses as well [208].

- Transient TE reactivation occurring in the pollen is limited to the VN and is signaling TE silencing in the neighboring sperm preventing, *via* siRNAs, transposon activation in the embryo. It was proposed that the molecular mechanisms involved in the permanent silencing of foreign DNA have evolved from mechanisms required for the successful development of an embryo [153]. This model proposes that epigenetic silencing of TEs has evolved from a developmental process in stark contrast to the models for the origin of gene imprinting in the endosperm and the nucleolar dominance which suggested that the TEs silencing mechanism has been co-opted for developmental and nucleolar functions.
- In addition to organismal development, epigenetic control of TEs has had a role in genome evolution. Epialleles are formed due to the proximity of a gene to a TE, and the regulatory complexes that the TE recruits. Natural epigenetic variation can originate from polymorphisms in transposon insertions and repeats, as illustrated by different *Arabidopsis* species and ecotypes of *A. thaliana* [95,118–120]. The consequences of transgenerational epigenetic effects for speciation and adaptive evolution are increasingly attracting attention [16,120–124].
- As in animals, plant developmental processes are regulated by antagonistic PcG/TrxG-related activities. Dual histone methylations (H3K27me3 and H3K4me3) mark silent genes in animal stem cells and nondifferentiated cells establishing a bivalent chromatin state at loci poised for transcription later in development. Dual activating/silencing marks found at developmentally regulated *Arabidopsis* genes illustrate features of the epigenetic "code" conserved in animal and plant kingdoms despite differences in the developmental patterns. Coexisting H3K4me3 and H3K27me3 may form a distinct bimodular "syllable" in the histone "code" that conveys specific "meaning" at different genes [165].
- DNA and histone modifications are linked with chromatin remodeling and nucleosome positioning. Although individual components of the remodeling machinery are conserved throughout eukaryotes, still very little is known about this mechanism in plants as full-size remodeling complexes have not been isolated and characterized biochemically. Given the existence of plant-specific histone modifications and DNA methylations, one might expect that interactions with the nucleosome remodeling machinery might be plant-specific as well.

- Uncovering features of plant-specific "dialects" in the epigenetic language "written" by the histone and DNA modifications, finding plant-unique ways of employing the enormously complex small RNAs mechanisms, and establishing correlations between chromatin-driven genome reprogramming processes in plants, would continue to be among the most challenging, fascinating, and revealing endeavors of contemporary molecular biology research.

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