

# Epigenetic Regulation in Plants

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## SUMMARY

The study of epigenetics in plants has a long and rich history, from initial descriptions of non-Mendelian gene behaviors to seminal discoveries of chromatin-modifying proteins and RNAs that mediate gene silencing in most eukaryotes, including humans. Genetic screens in the model plant *Arabidopsis* have been particularly rewarding, identifying more than 130 epigenetic regulators thus far. The diversity of epigenetic pathways in plants is remarkable, presumably contributing to the phenotypic plasticity of plant postembryonic development and the ability to survive and reproduce in unpredictable environments.

## Outline

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## OVERVIEW

**Epigenetic Regulation in Plants**

Plants are masters of epigenetic regulation. All of the major epigenetic mechanisms known to occur in eukaryotes are used by plants, with the responsible pathways elaborated to a degree that is unsurpassed in other taxa. DNA methylation occurs in CG, CHG, and CHH sequence contexts in plant genomes, in patterns that reflect a balance between enzyme activities that install, maintain, or remove methylation. As in other eukaryotes, histone-modifying enzymes influence epigenetic states in plants and these enzymes are encoded by comparatively large gene families, allowing for diversified as well as overlapping functions. RNA-mediated gene silencing is accomplished using multiple distinct pathways to combat viruses, tame transposons, orchestrate development, and help organize the genome. The interplay between DNA methylation, histone modification, and noncoding RNAs provides plants with a multilayered and robust epigenetic circuitry.

The prominence of epigenetic regulation in plants reflects their mode of development, lifestyle, and evolutionary history. Unlike growth in mammals, in which organ and tissue formation is largely specified during embryonic development, plants grow by continuously producing new organs from self-sustaining stem cell populations known as meristems. Consequently, postembryonic development in plants is a continu-

ous process shaped by environmental influences resulting in a high degree of phenotypic plasticity. Because plants are unable to escape their surroundings, they are forced to cope with changeable and often unfavorable growth conditions. Epigenetic regulatory mechanisms can facilitate metastable changes in gene activity and fine-tune gene expression patterns, thus enabling plants to survive and reproduce successfully in unpredictable environments. Polyploidization, an increase in the number of sets of chromosomes, is common in plants, amplifying gene families and fostering the functional specialization of duplicated genes, including those involved in epigenetic regulation.

Understanding the epigenetic regulatory machinery of plants has come, in large part, from genetic screens, most notably in *Arabidopsis thaliana*, a member of the mustard family that is highly amenable to genetic analyses and was the first plant species to have its genome sequenced. Crop plants, particularly maize, have also contributed substantially to the discovery of epigenetic phenomena and epigenetic regulatory mechanisms. The study of plant epigenetics and epigenomics has a long and rich history and, in synergy with parallel studies in animal and fungal systems, is contributing significantly to our basic understanding of epigenetic regulation.

## 1 PLANTS AS MODELS FOR EPIGENETIC RESEARCH

### 1.1 Overview

Plant studies have provided numerous seminal contributions to the field of epigenetics. Among them is the distinction between euchromatin and heterochromatin based on cytological analyses (Heitz 1929). The observation in tomato and maize of heritable changes in a gene's expression state on exposure to an allele with an alternative state, a phenomenon known as paramutation, was early evidence for non-Mendelian epigenetic inheritance (reviewed in Arteaga-Vazquez and Chandler 2010) and is now apparent in mammals and flies, as well as plants. Parental imprinting of individual genes, that is, the expression of a single allele of either maternal or paternal origin, which is a process whose misregulation is the basis for multiple genetic disorders in humans (see **Beaudet and Zoghbi 2014**), was first observed in maize (reviewed in Alleman and Doctor 2000). The repeated occurrence of individuals with altered flower symmetry, first described by Carl von Linné in the 18th century as “peloria” (monster) individuals, is now known to be caused by the formation of a silenced epiallele whose DNA sequence is identical to expressed alleles of the gene (Cubas et al. 1999). Epialleles can affect developmental switches; the FWA gene in *Arabidopsis* is an example in which silencing in some natural ecotypes (strains) delays flowering (Soppe et al. 2000). Cytogenetic analyses of metaphase chromosomes in plant hybrids in the 1930s showed that secondary constrictions and other cytogenetic characteristics were reproducibly altered in certain hybrid combinations. This differential production of secondary constrictions was later shown to be caused by the reproducible silencing of one parental set of ribosomal RNA (rRNA) genes, and the selective expression of the other parental set at these loci, an epigenetic phenomenon widespread among eukaryotes and known as nucleolar dominance (reviewed in Preuss and Pikaard 2007). Only the active rRNA genes give rise to secondary constrictions, which are less condensed than flanking regions because of the persistent binding of RNA polymerase I transcription factors throughout the cell cycle. The pioneering work on transposable elements in maize by Barbara McClintock and others in the 1940s revealed numerous links between genetic behavior and epigenetic regulation (reviewed in Lisch 2009). Indeed, extant transposons and degenerate remains of transposons provide a means of establishing new regulatory elements and epigenetic modifications throughout plant genomes.

As transgenic technology in plants such as tobacco, petunia, and *Arabidopsis* became routine in the late 1980s, it became evident that transgene expression was unpredictable (reviewed in Wassenegger 2002; Matzke and Matzke

2004). This led to the realization that homology-dependent gene silencing can occur when transgenes bearing identical sequences to genes already present in the genome are introduced, with an enhanced messenger RNA (mRNA) turnover (posttranscriptional gene silencing, PTGS) or repression of transcription (transcriptional gene silencing, TGS) being the molecular basis for silencing. A striking example of PTGS, termed “cosuppression,” occurred when overexpression of the gene encoding chalcone synthase (*CHS*), a regulator of flower pigmentation, was attempted in petunia, this unexpectedly resulted in variegated or even completely white flowers rather than darker purple flowers. The lack of pigmentation in white sectors was because of coordinated gene silencing of both the *CHS* transgene and the endogenous *CHS* gene (reviewed in Jorgensen et al. 2006). PTGS is a form of RNA interference (RNAi), later discovered in *Caenorhabditis elegans* and other organisms (see Sec. 3).

By the mid-1990s, links between PTGS and virus resistance had been forged. Epigenetic mechanisms involving RNA molecules were shown to naturally protect plants from uncontrolled replication of viruses, which can be both inducers and targets of PTGS. It was soon realized that these mechanisms could be exploited to experimentally down-regulate plant genes by constructing viral vectors containing plant gene sequences thus harnessing the virus-induced gene silencing pathway (VIGS, a topic discussed in more detail in **Baulcombe and Dean 2014** and reviewed in Senthil-Kumar and Mysore 2011). Another mechanism discovered in viroid-infected plants is the RNA-guided specification of DNA methylation (RNA-directed DNA methylation, RdDM) and other heterochromatic marks at homologous DNA regions (Wassenegger et al. 1994). Transgene-induced RdDM is now widely used to methylate and transcriptionally silence promoters and is accomplished by intentionally generating double-stranded RNA (dsRNA) matching promoter sequences (see Sec. 3.4), a strategy that also works in animals (reviewed in **Verdel et al. 2009**). Q1  
Q2

Another pioneering contribution of plant epigenetic research was the application of deep sequencing to conduct genome-wide analyses (Zhang et al. 2006), including the first analysis of genomic methylation at single-base resolution (Cokus et al. 2008; Lister et al. 2008). These studies required the development of bioinformatics tools that have since been applied broadly for biomedical research.

### 1.2 The Epigenomes of Plants and Mammals Are Similar

Despite their obvious morphological differences and long evolutionary separation, the fundamental similarities be-

tween animals and plants are remarkable. These similarities include many aspects of genome and epigenome organization, especially between plants and mammals. For instance, genome size, genome complexity, and the ratio of heterochromatin to euchromatin of most seed plants are comparable to those in mammals. Also plants and mammals make similar use of DNA methylation and histone posttranslational modifications (PTMs) for gene regulation. Overall, a comparison of genome organization and epigenetic regulation in different model systems reveals that there are more common features between plants and mammals than there are within the animal kingdom itself (**Appendix 2**). Consequently, epigenetic mechanisms discovered in plants or mammals are generally relevant to both systems.

### 1.3 Unique Aspects of Plant Development Relevant to Epigenetic Regulation

In considering the similarities and differences between plants and mammals, it is important to consider the special life histories of plants. In mammals, fertilization is achieved by fusion of two haploid cells that are direct products of the preceding meiosis. In contrast, plants have a haploid (gametophyte) growth stage that follows meiosis and precedes fertilization (Fig. 1). The male and female gametophytes are the pollen and embryo sac, respectively, each composed of multiple cells that are produced by mitotic divisions of the initial haploid meiotic products. In haploid gametophytes, which are genetically and metabolically active, loss of genetic or epigenetic information cannot be compensated for by information on homologous chromosomes, hence, deleterious mutations in essential genes are selected against. Unlike mammals, there is no evidence for a massive erasure of epigenetic marks during plant gametogenesis. Instead, repressive epigenetic marks in plant sperm and egg cells appear to be reinforced by specific *trans*-silencing RNAs produced in neighboring nuclei. This might explain how epigenetic changes are often transmissible through meiosis in plants.

Another distinctive feature of plants relative to animals is the lack of a defined germline set aside early in embryogenesis. Instead, germ cells are produced late in plant development after stem cells in the shoot undergo a transition from producing progenitor cells of vegetative organs to producing daughter cells that give rise to the floral organs in which meiosis and gametogenesis occur (Fig. 1). Thus, epigenetic modifications acquired by meristem cells in response to the plant's interactions with its environment have the potential to be conveyed to the germ cells.

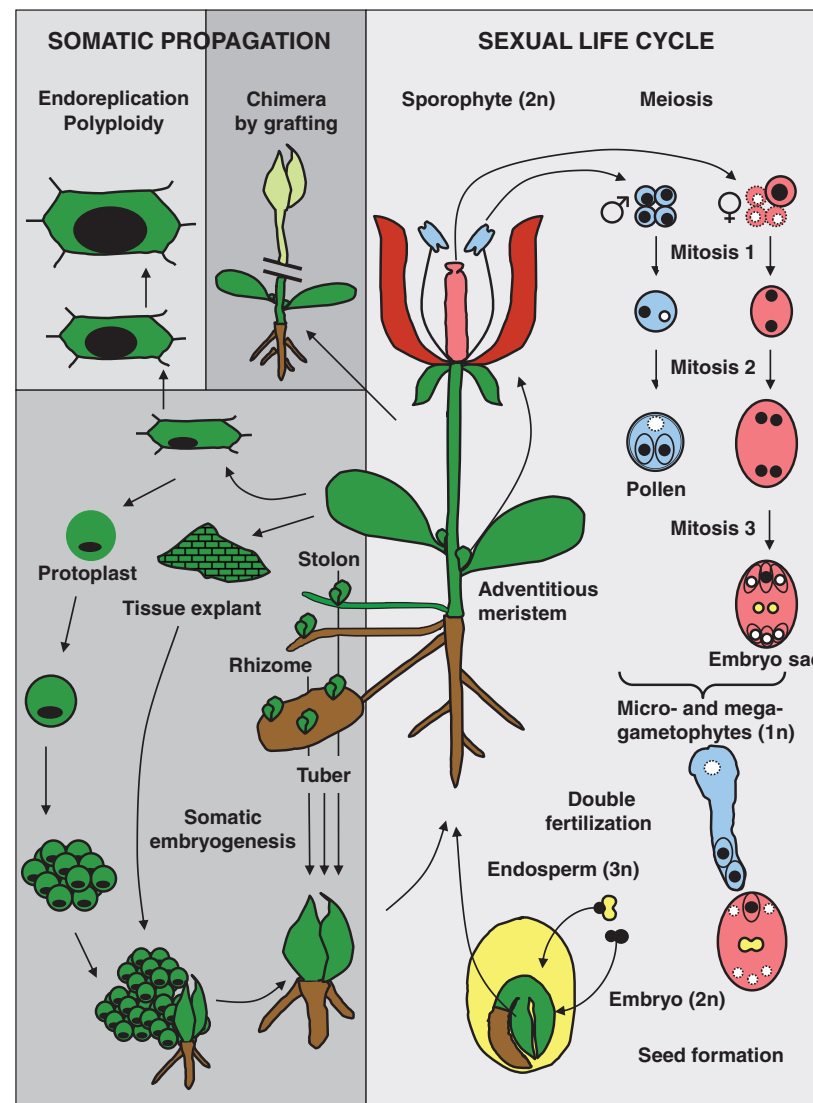
In addition to the clusters of stem cells at the apex of the shoot and root, known as apical meristems, plants have

lateral meristems. These include the stem cells that give rise to new xylem and phloem each year in perennials, resulting in the characteristic growth rings of tree trunks, as well as buds that are located at the base of each leaf and that can sprout to form vegetative organs or flowers. Many plants also have specialized organs, such as underground rhizomes, tubers, or bulbs that have stem cells capable of giving rise to shoots that form independent, new plants. These mechanisms of vegetative or clonal propagation are common in plants and are often more successful than seed dissemination as a strategy for colonizing a favorable location. Importantly, mitotically transmissible epigenetic states can be perpetuated among clones resulting from vegetative propagation.

In tissue culture, some differentiated plant somatic cells can be reprogrammed to form somatic embryos that develop into plants without a requirement for fertilization. As a result, cloning via somatic embryogenesis is routine for many plant species. However, among what should be genetically uniform clones, a surprising amount of phenotypic variability is observed. This so-called “somaclonal variation” has a strong epigenetic basis and is potentially useful for plant breeding and selection of adaptive traits (reviewed in Miguel and Marum 2011).

Another plant-specific feature is the existence of plasmodesmata, which are cytoplasmic connections between cells allowing metabolites, proteins, RNAs, and even viruses to pass. Plant shoots can be cut and grafted as scions onto genetically different rootstocks (Fig. 1). This results in chimeras in which roots and shoots are genetically different. Diffusible epigenetic signals move through plasmodesmata and the vascular system and can be transmitted between roots to shoots in grafted plants (see Sec. 3.6.2 and **Dunoyer et al. 2013**). In this way, the epigenetic state of stem cells and gametes can potentially be modified by signals emanating from distant organs of the plant.

Plants have a high tolerance for polyploidy (the multiplication of the whole chromosome complement) compared with mammals. In fact, most flowering plants having undergone polyploidization at least once in their evolutionary histories. Polyploid crops include wheat, cotton, potato, peanut, sugarcane, coffee, canola, and tobacco. The prevalence of polyploidy in plants suggests that it offers certain fitness advantages, such as built-in hybrid vigor or resistance to the effects of deleterious mutations, whereas allowing duplicated genes to potentially acquire beneficial mutations. The formation of polyploids is often associated with significant genomic and epigenetic changes (reviewed in Jackson and Chen 2010). Some of these changes occur within one or a few generations and can contribute to rapid adaptation and evolution in plants. Even diploid plants contain polyploid cells as a result of



**Figure 1.** Specialties of the plant life cycle. Plants can propagate sexually (gametogenesis, fertilization, and seed formation; *right*) as well as somatically (vegetative sprigs, de- and re-differentiation, or somatic embryogenesis; *left*). The body of higher plants with roots, stem, leaves, and flowers is the diploid sporophyte. During meiosis, the chromosome number is reduced by half. Whereas in animals the meiotic products become the gametes without further division and fuse directly to produce the diploid embryo, plants form haploid male or female gametophytes via two or three mitotic divisions to form pollen or embryo sacs, respectively. The pollen grain ultimately contains one vegetative (white) and two generative (black) nuclei. The two generative nuclei fertilize the egg cell (black) and the central cell, the latter having a diploid nucleus derived from fusion of the two polar nuclei (yellow). This double fertilization gives rise to a diploid embryo and a triploid endosperm, the latter providing a nutrient source for the developing embryo. After seed germination, the embryo will grow into a new sporophyte. Most plants also have the potential for vegetative propagation through activation of lateral meristems, outgrowth of specialized organs (tubers, rhizomes or stolons), amplification in tissue culture, or regeneration from individual somatic cells after removal of the cell wall (protoplasts). Endoreduplication is frequent in plants, producing polyploid cells or tissues. Plants can be grafted to produce chimeras.

endoreplication, with endopolyploidy common in cells of the leaf, seed, and other plant organs. By contrast, endopolyploidy in mammalian cells is less common, but does occur, for instance, in muscle and liver cells.

#### 1.4 Genetic Attributes of Plants as Model Systems for Epigenetics

Plants can be efficiently mutagenized by chemical or physical (e.g., radiation) treatments, random insertion of trans-



genes, or mobilization of transposable elements. Moreover, in plants that self-pollinate, such as *A. thaliana*, homozygous mutants can be easily identified among the thousands of progeny from a single mutagenized plant, without the need for laborious outcrossing or backcrossing procedures. Screens for mutations in epigenetic regulators are typically based on the recovery of expression of silenced marker genes, which are generally engineered transgenes. The ability to easily generate transgenic plants has thus greatly benefitted epigenetic research. In addition to such forward genetic approaches, reverse genetic approaches that ablate gene function are also possible. This has been facilitated by insertion mutants or the use of transgene-induced RNAi to knockout or knockdown the expression of candidate genes, such as genes homologous to epigenetic regulators identified in other organisms (Table 1).

Gene families encoding epigenetic modifiers can differ significantly in the number of family members between plants and mammals. In many cases, having large families of plant chromatin-modifying activities results in partial functional redundancies such that mutations in individual family members are less severe than in mammals. This can be useful if complete loss-of-function is lethal, thereby making mutants unavailable for study.

Nonessential genes in pathways that determine coloration of plant tissues have proven useful for identifying mutants affecting their epigenetic control (Fig. 2A–E). Other epigenetic modifier mutants cause morphological defects that are often tolerated in plants without lethal consequences (Fig. 2F). Thousands of individual plants can be screened for changes in the expression of endogenous or transgenic traits (Fig. 2G). Once specific epigenetic mutants are characterized, suppressor screens are often successful for identifying interacting components or alternative pathways, a strategy that has also been used in *Drosophila* (Elgin and Reuter 2013) and mouse (Blewitt and Whitelaw 2013).

*A. thaliana* has emerged as a leading model system for plant epigenetics research because of the availability of comprehensive collections of insertion mutations in nearly every gene, facile mutagenesis, extensive natural variation, complete genome sequence information for numerous accessions, and a wealth of genome-wide expression, and chromatin-modification data. *Arabidopsis* is thus the focus of this article, and Table 1 summarizes the epigenetic modifiers for which functional information is available. This information has been obtained through a combination of forward- and reverse-genetic screens, biochemical assays, or homologies with known epigenetic modifiers in other systems. For more detailed information about individual genes in *Arabidopsis*, the reader is referred to TAIR, The *Arabidopsis* Information Resource (<http://www.arabidopsis.org>).

## 2 MOLECULAR COMPONENTS OF CHROMATIN IN PLANTS

More than 130 genes encoding proteins involved in epigenetic regulation in plants have been identified (Table 1). The epigenetic modifiers known to date can roughly be sorted into five groups according to their proven or presumptive functions as discussed **below**. This list is clearly **Q3** incomplete as new activities continue to be identified in ongoing research, but provides a framework for understanding plant epigenetics.

### 2.1 Regulators of DNA Modification

5-methylcytosine (5mC) is a hallmark of epigenetic gene silencing and heterochromatin in both plants and mammals (the latter of which is discussed in Li and Zhang 2014; see also **Appendix 2**; reviewed in Furner and Matzke 2011; Meyer 2011). Whereas in nuclei of differentiated mammalian cells, 5mC is found nearly exclusively at CG sites (often referred to as CpG sites in mammals), plants methylate cytosines within CG, CHG, or CHH motifs (in which H is A, T, or C). Mammalian promoters are often present within methylation-free CG-rich regions known as CpG islands, but CpG islands are not easily distinguishable in plants. Nevertheless, cytosine methylation is nonrandomly distributed in plants, occurring primarily in repetitive regions of the genome that are enriched in transposable elements, centromeric repeats or silent 5S, and 45S rRNA gene repeats. Cytosine methylation also occurs at some differentially regulated promoters and within the protein-coding regions of highly expressed genes (Zilberman et al. 2007). The latter gene body methylation is evolutionarily conserved, occurring in animal species as diverse as humans and honeybees (Feng et al. 2010). The significance of gene body CG methylation is not yet clear, but its enrichment within exons suggests a potential role in pre-mRNA splicing.

#### 2.1.1 DNA Methyltransferases

DNA methyltransferases catalyze cytosine methylation de novo at previously unmethylated cytosines or maintain preexisting cytosine methylation patterns (illustrated in **Fig. 2** in Li and Zhang 2014). In plants, de novo methylation is directed by small interfering RNAs (siRNAs) and occurs on cytosines present within CG, CHG, or CHH motifs (see Sec. 3.5). Maintenance methylation perpetuates preexisting methylation patterns, especially at CG or CHG motifs, which are known as symmetrical sites because each DNA strand reads CG or CHG in the 5' to 3' direction. This symmetry provides a basis for cytosine methylation patterns on mother strands to be imparted to daughter strands

**Table 1.** Components of epigenetic regulation in the model plant genus *Arabidopsis thaliana*

Gene or mutant acronym <sup>a</sup>	Gene or mutant name	Confirmed or putative function of protein
<b>DNA modification</b>		
<i>CMT3</i>	Chromomethyltransferase	DNA methyltransferase (mainly CHG)
<i>DME</i>	Demeter	DNA glycosylase-domain protein, cytosine demethylation
<i>DML2,3</i>	Demeter-like proteins	DNA glycosylase-domain protein, cytosine demethylation
<i>DNMT2</i>	DNA methyltransferase	Interaction with HD2
<i>DRM1</i>	Domains-rearranged methyltransferase	DNA methyltransferase (de novo, CHH)
<i>DRM2</i>	Domains-rearranged methyltransferase	DNA methyltransferase
<i>HOG1</i>	Homology-dependent gene silencing	S-adenosyl-L-homocysteine hydrolase
<i>MBD10</i>	Methylcytosine-binding domain protein	Methylcytosine-binding protein
<i>MBD6</i>	Methylcytosine-binding domain protein	Methylcytosine-binding protein
<i>MET1, DDM2</i>	Methyltransferase, decreased DNA methylation	DNA methyltransferase (CG)
<i>ROS1</i>	Repressor of silencing	DNA glycosylase-domain protein, cytosine demethylation
<i>ROS3</i>	Repressor of silencing	DNA glycosylase-domain protein, cytosine demethylation
<i>VIM1,-2,-3</i>	Variant in methylation	Methylcytosine-binding proteins
<i>ZDP</i>	Zinc finger DNA 3'-phosphoesterase	3'-phosphatase
<b>Histone modification</b>		
<i>ATX1</i>	<i>Arabidopsis</i> homolog of trithorax	Histone methyltransferase
<i>ATXR3/SDG2</i>	<i>Arabidopsis</i> homolog of trithorax, SET domain group	Histone methyltransferase
<i>ATXR5,-6,-7</i>	<i>Arabidopsis</i> trithorax-related proteins	Histone methyltransferase (ATXR5,-6: H3K27me1)
<i>EFS/SDG8/ASHH2</i>	Early flowering in short days, SET domain group, ASH1 homolog	Histone methyltransferase
<i>ELF6</i>	Early flowering	Histone demethylase (H3K4me1,-2,-3)
<i>FLD, LDL1,-2</i>	Flowering locus D, LDS1-like	Histone demethylases
<i>HAC1,-5,-12</i>	Histone acetyltransferase CBP-like	Histone acetyltransferase
<i>HAG1,-2</i>	Histone acetyltransferase GCN5-like	Histone acetyltransferase
<i>HAM1,-2</i>	Histone acetyltransferase Myst-like	Histone acetyltransferase
<i>HD2a-d; HDT1,-2,-3,-4</i>	Histone deacetylase	Atypical histone deacetylase
<i>HDA1</i>	Histone deacetylase	Histone deacetylase
<i>HDA6, SIL1, AXE1</i>	Histone deacetylase, modifier of silencing, auxin-gene repression, RNA-mediated transcriptional silencing	Histone deacetylase
<i>RTS1</i>		
<i>HUB1,-2</i>	E3 ubiquitin ligase	H2B monoubiquitination
<i>IBM1</i>	Increase in bonsai methylation	Histone demethylase
<i>JMJ14</i>	<i>A. thaliana</i> jumonji	Histone demethylase (H3K4me1,-2,-3)
<i>MEE27</i>	Maternal effect embryo arrest	Histone demethylase
<i>OTLD1</i>	Otubain-like deubiquitinase	H2B deubiquitination
<i>REF6</i>	Relative of early flowering	Histone demethylase
<i>SUP32/UBP26</i>	Suppressor of ros, ubiquitin protease	H2B deubiquitination
<i>SUVH2</i>	Su(Var)3-9 homolog	H3K9 methyltransferase
<i>SUVH4, KYP1</i>	Su(Var)3-9 homolog, kryptonite	H3K9 methyltransferase
<i>SUVH5,-6</i>	Su(Var)3-9 homolog	Recognition of DNA methylation (SUVH5)
<i>UBC1,-2</i>	E2 ubiquitin-conjugating enzyme	H2B monoubiquitination
<i>ULT1</i>	Ultrapetala	Regulator of histone methylation, ATX1 interactor
<b>Polycomb-group proteins and interacting components</b>		
<i>AtBMI1a,-b,-c</i>	B cell-specific Mo-MLV integration site 1	PRC1 subunit
<i>AtCYP71</i>	Cyclophilin	LHP1 and H3 interaction
<i>AtRING1a,-b</i>	RING finger protein	PRC1 subunit
<i>CLF</i>	Curly leaf	Polycomb-group protein (E(z))
<i>CUL4</i>	Cullin protein	Subunit of E3 ligase complex, MSI1 interactor
<i>DDB1</i>	Damaged DNA-binding	Subunit of E3 ligase complex, MSI1 interactor

Continued

**Table 1.** *Continued*

Gene or mutant acronym <sup>a</sup>	Gene or mutant name	Confirmed or putative function of protein
<i>EMF2</i>	Embryonic flower	Polycomb-group protein (Su(z)12)
<i>FIE, FIS3</i>	Fertilization-independent endosperm, fertilization-independent seeds	Polycomb-group protein (Esc)
<i>FIS2</i>	Fertilization-independent seeds	Polycomb-group proteins (Su(z)12)
<i>LHP1/TFL2</i>	Like heterochromatin protein, terminal flower	PRC1 subunit, formation of repressive chromatin
<i>LIF2</i>	LHP1-interacting factor	RNA processing, LHP1 interaction
<i>MEA, FIS1</i>	Medea, fertilization-independent seeds	Polycomb-group protein (E(z))
<i>MSI1</i>	Multicopy suppressor of IRA homolog	Polycomb-group protein (p55)
<i>MSI4/FVE</i>	Multicopy suppressor of IRA homolog	Polycomb-group protein (p55), Cul4-DDB1 and PCR2 interactor
<i>MSI5</i>	Multicopy suppressor of IRA homolog	Polycomb-group protein (p55), interactor of HDA6, FLC silencing
<i>RBR</i>	Retinoblastoma-related protein	PRC2 regulator during development
<i>SWN</i>	Swinger	Polycomb-group protein (E(z))
<i>VEL1</i>	Vernalization-like	Homeodomain protein
<i>VIL2</i>	Vin3-like	Homeodomain protein
<i>VIN3</i>	Vernalization-insensitive	Homeodomain protein
<i>VRN2</i>	Vernalization	Polycomb-group protein (Su(z)12)
<i>VRN5</i>	Vernalization	Homeodomain protein
<b>Chromatin formation or chromatin remodeling</b>		
<i>ARP4,-5</i>	Actin-related protein	Subunits of INO80 complex
<i>AtASF1a,-b</i>	<i>Arabidopsis</i> antisilencing factor	Histone chaperone H3/H4
<i>AtCHR12</i>	<i>Arabidopsis</i> chromatin remodeling	SNF2/Brahma-type protein
<i>AtNAP1 1-4</i>	<i>Arabidopsis</i> nucleosome assembly protein	Histone chaperone H2A/H2B
<i>AtSWI3 A-D</i>	<i>Arabidopsis</i> homolog of SWI3	Subunits of SWITCH/SUCROSE NONFERMENTABLE (SWI/SNF)-remodeling complexes
<i>AtSWP73 A,B</i>	<i>Arabidopsis</i> homolog of SWP73	Subunit of SWI/SNF-remodeling complex
<i>BRM</i>	Brahma	SWI2/SNF2 ATPase family protein
<i>BRU1/MGO3/TSK</i>	Brushy, Mgoun, Tonsoku	Uncharacterized protein
<i>BSH</i>	Bushy	Subunit of SWI/SNF-remodeling complexes
<i>CHR11</i>	Chromatin-remodeling protein	ISWI-like chromatin remodeling protein
<i>CLSY1</i>	Classy	SWI2/SNF2 family ATPase
<i>DDM1, SOM</i>	Decreased DNA methylation, somniferous	SWI2/SNF2 family ATPase
<i>DMS3/IDN1</i>	Defective in meristem silencing, involved in de novo	Structural-maintenance-of-chromosomes protein; required for RNA Pol V transcription
<i>DMS11</i>	Defective in meristem silencing	GHLK ATPase, interaction with DMS3
<i>DRD1</i>	Defective in RNA-directed DNA methylation	SWI2/SNF2 ATPase; required for RNA Pol V transcription
<i>FAS1</i>	Fasciated	Chromatin assembly factor subunit H3/H4
<i>FAS2</i>	Fasciated	Chromatin assembly factor subunit H3/H4
<i>HIRA</i>	Histone regulator A	Histone chaperone H3/H4
<i>INO80</i>	Inositol-requiring	Subunits of INO80 complex
<i>MGO1</i>	Mgoun	DNA topoisomerase
<i>MOM1</i>	Morpheus' molecule	Genetic interaction with RNA Pol V
<i>MSI1</i>	Multicopy suppressor of IRA homolog	Chromatin assembly factor subunit, H3/H4
<i>NRP1,-2</i>	NAP (nucleosome assembly protein) -related protein	Histone chaperone H2A/H2B
<i>PIE</i>	Photoperiod-independent early flowering	ATPase subunit of SWR1
<i>PKL</i>	Pickle	CHD3 chromatin remodeling factor
<i>RPA2</i>	Replication protein A	Single-stranded DNA-binding protein
<i>SEF/SWC6</i>	Serrated and early flowering, SWR1 complex	Subunit of SWR1
<i>SPD</i>	Splayed	SWI2/SNF2 family ATPase
<i>SPT16</i>	Suppressor of Ty insertion-like	Histone chaperone H2A/H2B

*Continued*



Table 1. Continued

Gene or mutant acronym <sup>a</sup>	Gene or mutant name	Confirmed or putative function of protein
<i>SSRP1</i>	Structure-specific recognition protein	high mobility group (HMG) protein, histone chaperone subunit
<i>SUF3/ESD1/ARP6</i>	Suppressor of Frigida, early in short days, actin-related protein	Subunit of SWR2, H2A.Z deposition
<i>TSL</i>	Tousled	Phosphorylation of ASF1
<b>RNA silencing</b>		
<i>ABH1/CBP80</i>	ABA-hypersensitive, cap-binding complex	microRNAs (miRNA) processing
<i>AGO1</i>	Argonaute	PAZ-PIWI domain protein, translational repression
<i>AGO10, PNH/ZLL</i>	Argonaute, Pinhead, Zwillie	PAZ-PIWI domain protein, translational repression
<i>AGO4</i>	Argonaute	PAZ-PIWI domain protein, siRNA-binding
<i>AGO6</i>	Argonaute	PAZ-PIWI domain protein, siRNA-binding
<i>AGO7, ZIP</i>	Argonaute, Zippy	PAZ-PIWI domain protein
<i>AGO9</i>	Argonaute	PAZ-PIWI domain protein, female gametophyte formation, siRNA binding
<i>AtNUC-11</i>	Nucleolin-like	rRNA gene regulation
<i>DCL1, CAF1, SIN1, EMB76, SUS1</i>	Dicer-like, Carpel factory, short integuments, embryo-defective, suspensor	RNAse III (dsRNAse)
<i>DCL2</i>	Dicer-like	RNAse III (dsRNAse)
<i>DCL3</i>	Dicer-like	RNAse III (dsRNAse), siRNA generation
<i>DCL4, SMD</i>	Dicer-like	RNAse III (dsRNAse)
<i>DDL</i>	Dawdle	FHA domain protein, miRNA processing
<i>DRB1</i>	Double-stranded RNA-binding protein	Dicer 1-interacting protein, miRNA strand selection
<i>DRB2, -4</i>	Double-stranded RNA-binding protein	Dicer-interacting proteins, siRNA processing
<i>ESD7</i>	Early in short days	Subunit of DNA polymerase epsilon
<i>FCA</i>	Flowering time	RRM-domain protein
<i>FDM1-5</i>	Factor of DNA methylation	dsRNA-binding protein (FDM1)
<i>FPA</i>	Flowering time	RRM-domain protein
<i>FRY1</i>	Fierly	Nucleotidase
<i>HEN1</i>	HUA enhancer	dsRNA-binding, RNA methyltransferase
<i>HST</i>	Hasty	miRNA export receptor
<i>HYL1</i>	Hyponastic leaves	Nuclear dsRNA-binding protein
<i>IDN2/RDM12</i>	Involved in de novo, RNA-directed DNA methylation	dsRNA-binding protein
<i>KTF1/RDM3/SPT5-1</i>	KOW domain-containing transcription factor, suppressor of Ty insertion-like	Part of RdDM effector complex
<i>NRP(A/B/C/D)5</i>	Nuclear RNA polymerases I, II, III, IV	5th subunit common to Pols I–IV
<i>NRP(B/D/E)3a, -3b</i>	Nuclear RNA polymerases II, IV, and V	Alternative 3rd subunits of Pols II, IV, or V
<i>NRP(B/D/E)9a, -9b</i>	Nuclear RNA polymerases II, IV, and V	Alternative 9th subunits of Pols II, IV, and V
<i>NRP(D/E)2, DRD2</i>	Nuclear RNA polymerases IV and V; defective in RNA-directed DNA methylation 2	2nd subunit of Pol IV and Pol V
<i>NRP(D/E)4</i>	Nuclear RNA polymerases IV and V	4th subunit of RNA Pol IV and RNA Pol V
<i>NRP(D/E)5b</i>	Nuclear RNA Pol IV and V	Alternative 5th subunit of RNA Pol IV (and RNA Pol V likely)
<i>NRPD1, SDE4</i>	Nuclear RNA polymerase IV, silencing defective 4	Pol IV largest subunit
<i>NRPD7a</i>	Nuclear RNA Pol IV	Major 7th subunit of RNA Pol IV
<i>NRPE1, DRD3</i>	Nuclear RNA Pol V, defective in RNA-directed DNA methylation	RNA Pol V largest subunit
<i>NRPE5</i>	Nuclear RNA Pol V	5th subunit of RNA Pol V
<i>NRPE7/NRPD7b</i>	Nuclear RNA Pol V	Major 7th subunit of RNA Pol V; alternative seventh subunit of RNA Pol IV
<i>RDM1</i>	RNA-directed DNA methylation	Component of DDR complex required for RNA Pol V transcription
<i>RDM12</i>	RNA-directed DNA methylation	Coiled-coil protein
<i>RDM4/DMS4</i>	RNA-directed DNA methylation, defective in meristem silencing	IWR1-like regulator of multisubunit RNA polymerase assembly
<i>RDR1</i>	RNA-dependent RNA polymerase	RNA-dependent RNA polymerase

Continued

Table 1. Continued

Gene or mutant acronym <sup>a</sup>	Gene or mutant name	Confirmed or putative function of protein
<i>RDR2</i>	RNA-dependent RNA polymerase	RNA-dependent RNA polymerase
<i>RDR6</i> , <i>SDE1</i> , <i>SGS2</i>	RNA-dependent RNA polymerase, silencing defective, suppressor of gene silencing	RNA-dependent RNA polymerase
<i>SDE3</i> , -5	Silencing-defective	RNA helicase
<i>SE</i>	Serrate	Zn finger protein, miRNA processing
<i>SGS3</i>	Suppressor of gene silencing	Coiled-coil protein
<i>SHH1</i> / <i>DTF1</i>	Sawadee homeodomain homolog, DNA-binding transcription factor	Homeodomain protein
<i>SR45</i>	Arginine/serine-rich	Splicing factor
<i>WEX</i>	Werner syndrome-like exonuclease	RNase D exonuclease
<i>XRN2</i> , -3	XRN homolog	Exoribonuclease
<i>XRN4</i> / <i>EIN5</i>	XRN homolog, ethylene insensitive	Exoribonuclease, small RNA processing

<sup>a</sup> Different acronyms in row one refer to the same gene.

after each round of DNA replication. Maintenance methylation also occurs following DNA repair, allowing newly synthesized DNA to be methylated based on the methylation pattern of the nonrepaired strand.

Three families of DNA methyltransferase are conserved among eukaryotes and are present in plants: homologs of mammalian *Dnmt1*, *Dnmt2*, and *Dnmt3* (Fig. 4 in Li and Zhang 2014). METHYLTRANSFERASE 1 (*MET1*), the plant homolog of mammalian *Dnmt1*, is the major CG maintenance methyltransferase and may also contribute to CG de novo methylation. DNMT2 homologs are also present in plants and possess transfer RNA (tRNA) methylase activity, like mammalian DNMT2 (Goll et al. 2006); however, no catalytic activity on DNA has been observed for these enzymes. The plant DOMAINS-REARRANGED METHYLTRANSFERASEs (*DRM*) and their mammalian homologs, the *Dnmt3* group, are predominantly de novo methyltransferases. DRM proteins have the domains of their amino- and carboxy-terminal halves arranged in reverse order when compared with *Dnmt3* (domains VI–X are followed by I–V). DRM2 catalyzes methylation of cytosines in all sequence contexts and is the prominent cytosine methyltransferase in the RNA-directed DNA methylation pathway (see Sec. 3.5.2).

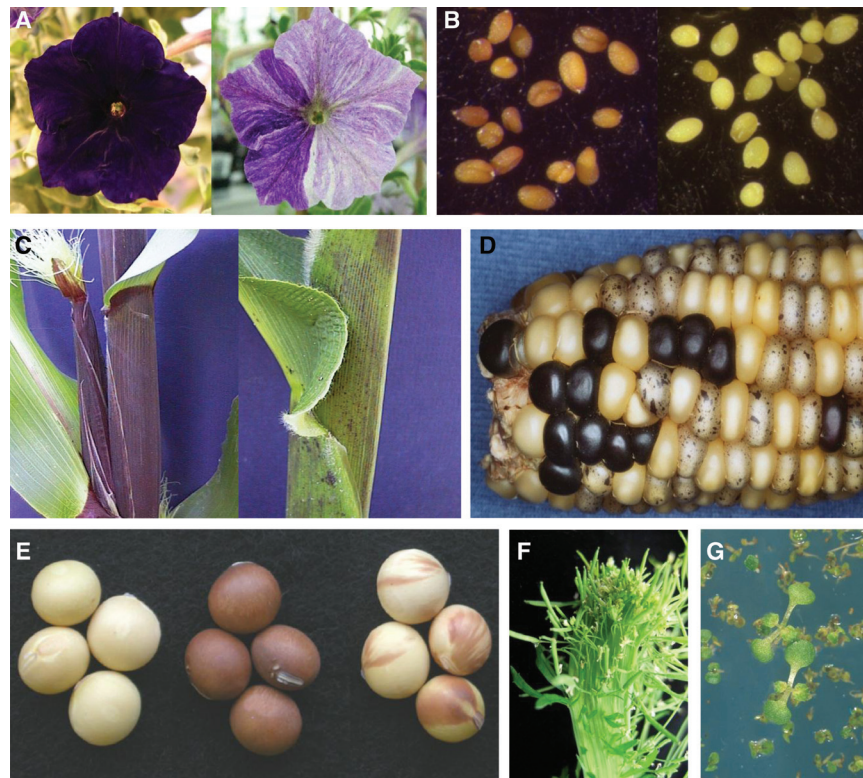
Unlike mammals, plants have a unique family of cytosine methyltransferases wherein the defining characteristic is the presence of a chromodomain that binds to methylated histones. CHROMOMETHYLASE 3 (*CMT3*) is the enzyme primarily responsible for CHG maintenance methylation. *CMT3*'s chromodomain binds histone H3 that is dimethylated on lysine 9 (H3K9me2). CHG methylation, in turn, provides a binding site for the MET- AND RING-ASSOCIATED (SRA) domain of the H3K9 methyltransferase *SUVH4*, leading to H3K9 dimethylation. Thus, *CMT3* and *SUVH4* constitute a self-reinforcing loop in which

repressive DNA methylation and histone modification marks specify one another to maintain an epigenetic state (reviewed in Law and Jacobsen 2010). CHROMOMETHYLASE 2 (*CMT2*) plays a role in maintaining CHH methylation in specific genomic contexts, such as the central regions of large transposable elements, presumably via crosstalk with histone modifications like its paralog, *CMT3* (Zemach et al. 2013).

In contrast to mammals in which *dnmt1* and *dnmt3* mutants die during embryonic development, or shortly after birth, *met1*, *cmt2*, *cmt3*, and *drm* mutants are viable, even in combination with one another. As a result, the role of DNA methylation in a variety of processes can be studied in plants, including vegetative and reproductive development, gametogenesis, fertilization, and the cross-talk between DNA methylation and histone modification (as in the case of *CMT3* described above; reviewed in Furner and Matzke 2011; Meyer 2011).

2.1.2 Cytosine Demethylation and DNA Glycosylases

In spite of mechanisms allowing DNA methylation to be maintained, cytosine methylation can be lost. Passive loss occurs when methylation fails to be maintained during replication or following DNA repair. Active demethylation can also occur via enzymatic activity. Active demethylation in animals was shown decades ago, but the exact players and mechanisms are still debated (reviewed in Gehring et al. 2009; Niehrs 2009). In *Arabidopsis*, active demethylation is catalyzed by REPRESSOR OF SILENCING 1 (*ROS1*), DEMETER (*DME*), or DEMETER-LIKE proteins (*DML2*, -3). These are large proteins that contain DNA glycosylase domains. *ROS1* displays nicking activity on methylated DNA, resulting in the removal and replacement of methylated cytosines via a pathway related to base exci-



**Figure 2.** Assays for epigenetic control in plants. Genes determining coloration of plant tissue allow easy and inexpensive gene expression readout in vivo. (A) Expression of the dihydroflavonol reductase gene (DFR) gene is required for dark purple petunia flowers, whereas silencing of the DFR promoter gives rise to variegated, light coloration. (B) Seeds from *Arabidopsis* expressing the chalcone synthase gene (CHS) have dark seed coats, whereas silencing of CHS after integration of a homologous transgene results in yellow seeds. (C) Maize plants with the B-I gene have purple pigmentation, in contrast to green plants with a paramutagenic, inactive B' allele whose DNA sequence is identical. (D) Maize ear, segregating a transposon insertion (Spm) in the B-Peru gene required for anthocyanin pigment. Purple kernels represent revertants in which the Spm element excised from the gene in the germ line. The heavily spotted kernels contain the Spm element in the active form that induces frequent somatic excision sectors during kernel development. The kernels with rare, small purple sectors represent kernels in which the Spm element has been epigenetically silenced. (E) The dark color of soybeans (middle) is extinguished in cultivated varieties (left) because of natural posttranscriptional silencing of the CHS gene and can be partially reversed by infection of the parental plant with a virus possessing a PTGS suppressor protein, producing a mottled pattern (right). (F) Epigenetic regulation can become manifest also in plant morphology; reduced function of a chromatin assembly factor subunit leads to a “fasciated” *Arabidopsis* stem. (G) Release of silencing from a transgenic resistance marker in *Arabidopsis* can be scored by growth on selective medium. (A, Courtesy of Jan Kooter; B, courtesy of Ian Furner; C, reprinted from Chandler et al. 2000, with kind permission from Springer Science and Business Media; D, courtesy of Vicki Chandler; E, reprinted, with permission, from Senda et al. 2004, © American Society of Plant Biologists.)

Q5

sion repair (Agius et al. 2006). The demethylation process requires the ZINC FINGER DNA 3'PHOSPHOESTERASE (ZDP), which is thought to remove the 3' phosphate at the nick site, thereby generating a 3' hydroxyl that allows DNA ligase to seal the gap (Martinez-Macías et al. 2012). ROS1 is expressed constitutively, potentially contributing to loss of DNA methylation in nondividing cells at all stages of development. ROS1 is thought to be guided to its sites of action by its association with ROS3, an RNA-binding protein, suggesting that RNA may play a role in guiding demethylation as well as in guiding de novo methylation in the

RNA-directed DNA methylation pathway (Zheng et al. 2008). The DNA glycosylase DME is especially important in the gametophytes, where it helps erase 5mC patterns that otherwise silence a specific subset of genes (Choi et al. 2002; Schoft et al. 2011). Mutations eliminating the CG methyltransferase MET1 suppress *dme* mutants, suggesting that DME is required primarily for demethylation of CG dinucleotides (Xiao et al. 2003). The DME paralogs, DML2 and -3, affect cytosine methylation in CG, CHG, and CHH



contexts at specific genomic sites (Ortega-Galisteo et al. 2008).

Collectively, the functions of the multiple demethylating enzymes in *Arabidopsis* indicate that reversibility of DNA methylation is critical for the regulation of specific genes during development. There is also growing evidence that cytosine methylation can be rapidly lost in other ways (reviewed in Reinders and Paszkowski 2009). Spontaneous changes in cytosine methylation can occur sporadically among isogenic populations (Becker et al. 2011; Schmitz et al. 2011), thereby creating or derepressing epialleles that may influence fitness and natural selection (Roux et al. 2011).

### 2.1.3 Methylcytosine-Binding Proteins

METHYL-C-BINDING DOMAIN (MBD) proteins, which share sequence similarity with mammalian proteins such as MeCP2, are epigenetic “reader” modules thought to help transduce DNA methylation patterns into altered transcriptional activity (see **Figs. 8 and 9 in Li and Zhang 2014**). Plants have more MBD-containing genes (13 in *Arabidopsis*) than mammals. However, outside the conserved MBD domain, there is little homology with mammalian MBDs (Springer and Kaeppler 2005). Only three members of the *Arabidopsis* family are known to bind specifically to methylated DNA, and these are missing in monocotyledonous plants such as maize, wheat, and rice (reviewed in Grafi et al. 2007). Other protein domains also confer 5mC binding. For instance, the SRA domain of SUVH4 (an H3K9 methyltransferase) binds to methylated CHG sites providing a mechanistic link between repressive histone and DNA modifications as mentioned in Section 2.1.1. Likewise, the SRA domains of VIM proteins (orthologs of UHRF1 in mammals) bind to hemimethylated DNA and recruit MET1/DNMT1 to modify the new daughter strand following replication (Woo et al. 2007, 2008).

### 2.1.4 Proteins Required for Methyl Group Donor Synthesis

Most methylating enzymes require the cofactor, S-adenosyl-methionine (SAM, or AdoMet) as the methyl group donor. A key enzyme in SAM biosynthesis, S-ADENOSYL-L-HOMOCYSTEINE HYDROLASE, regulates SAM levels by removing substrate inhibition. Mutations in the *Arabidopsis* gene encoding this enzyme result in decreased DNA methylation and release of transcriptional silencing, especially from genes in pericentromeric heterochromatin (Rocha et al. 2005; Jordan et al. 2007). The mutations also affect histone methylation, further impacting epigenetic regulation (Baubec et al. 2010).

## 2.2 Histone-Modifying Enzymes and Histone Variants

As in other organisms, plants contain several histone variants and enzymes that posttranslationally modify histones and influence gene regulation (introduced in **Allis et al. 2014**). Application of chromatin immunoprecipitation followed by deep sequencing has given insight into the genome-wide distribution of histone variants and histones bearing different posttranslational modifications (reviewed in Roudier et al. 2009), including the coincidence or mutual exclusion of specific chromatin marks (Roudier et al. 2011). Histone-modifying enzymes are often encoded by large gene families in plants (reviewed in Berr et al. 2011; Deal and Henikoff 2011; Lauria and Rossi 2011).

### 2.2.1 Histone Deacetylases and Histone Acetyltransferases

Histone acetylation is an epigenetic mark generally associated with active chromatin and transcription, whereas transcriptionally nonactive sequences usually lack acetylation. The functions of histone acetyltransferase (abbreviated to HAT) “writer” activities can be reversed by histone deacetylase (HDAC) “eraser” enzymes that allow for the reversibility of acetylation as an epigenetic mark. The structure and function of these genes is extensively discussed in **Marmorstein and Zhou (2014)** and **Seto and Yoshida (2014)**. Plants have multiple gene family members for both HATs and HDACs (Pandey et al. 2002; Chen and Tian 2007). The members of the HAT family can be classified into five different subfamilies based on their structure and different substrate specificities (Earley et al. 2007). The GCN5-homolog, HAG1, for instance specifically methylates H3K14, as in yeast and mammals, and regulates several developmental processes in *Arabidopsis* (reviewed in Servet et al. 2010). We currently know of plant homologs to three of the five HAT subfamilies (see Table 1). Plants also have homologs of the histone deacetylases that are highly conserved throughout all eukaryotes, catalyzing the removal of histone acetylation to generate more repressive chromatin. There is a plant-specific family of putative histone deacetylases, named the HD2 or HDT family, which is implicated in gene silencing, but definitive HDAC activity has not yet been shown for recombinant HDT proteins. Genetic screens have so far identified only two HDACs that function as epigenetic regulators: *HDA1* and *HDA6* (Table 1). *HDA6* plays a role in maintaining CG and CHG methylation, interacting with the DNA methyltransferase MET1, and is involved in transgene and transposon silencing, rRNA gene repression, and nucleolar dominance. Although deficiency mutants have only subtle morphological defects, *HDA6* is involved in seed maturation, flowering time con-

tol, and stress responses (reviewed in Aufsatz et al. 2007; Kim et al. 2012). Pleiotropic morphological changes are apparent on reduced expression and/or overexpression of HDA1 and HD2A/HD1, but their functions are not clear. Dissection of HAT and HDAC function is somewhat complicated by their redundancy and their potential operation in the context of large multiprotein complexes.

### 2.2.2 Histone Methyltransferases and Histone Demethylases

Like acetylation, histone methylation is a potentially reversible mark. Histone lysine methyltransferases (HKMTs) installing this modification are usually characterized by the presence of a SET domain (SU(VAR)/E(Z)/TRX; discussed extensively in Cheng 2014). Depending on the specific histone lysine that is methylated, HKMTs can promote or inhibit transcription. Some SET domain proteins are members of the Polycomb group (PcG) or trithorax group (TrxG) that maintain transcriptionally repressed or active states, respectively, of specific genes during plant and animal development (see Sec. 2.3 and Grossniklaus and Paro 2014). Other SET domain proteins of the Su(var)3-9 family participate in maintaining condensed heterochromatin, silencing transposons, or controlling DNA replication.

The *Arabidopsis* genome encodes 49 SET domain proteins, grouped into four conserved families: E(Z), ASH1, TRX, and SU(VAR)3-9-related proteins (Pontvianne et al. 2010). The latter and largest group is mostly, but not exclusively, responsible for H3K9 methylation. KRYPTONITE/SUPPRESSOR OF VARIATION 3-9 homolog 4 (KYP/SUVH4) and SUVH2, -5, and -6 catalyze H3K9me1 and -me2 installation. As described for SUVH4, their SRA domain, which binds 5mC, contributes to their cooperation with CMT3 to maintain CHG methylation and H3K9me2 at silenced loci. H3K9me3 is likely accomplished by the more distantly related SUVH proteins, and in contrast to animals in which it is a prominent heterochromatic mark, in plants it is found at expressed euchromatic genes. The TRX family member ARABIDOPSIS TRITHORAX 1 (ATX1) activates floral homeotic genes, presumably by means of its ability to catalyze histone H3 lysine 4 (H3K4) methylation. ATXR5 and ATXR6 monomethylate H3K27, a mark of repressed heterochromatin. Overreplication of heterochromatin occurs in *atxr5 atxr6* double mutants, pointing to a role in replication control (Jacob et al. 2010). Mutants lacking ATXR3 have reduced H3K4me2/3 and severe developmental defects, partly because of reduced cell size. ASH1 HOMOLOG 2 (ASHH2), a member of the ASH1 group, catalyzes H3K36me2/3, thereby reinforcing active chromatin marks at target loci, including several pathogen re-

sistance genes. Genes of the E(z) group are relevant to Polycomb regulation (as discussed in Sec. 2.3).

There are two classes of proteins that can demethylate histone tails at different positions. The **lysine-specific histone demethylase-LIKE** (LDM) proteins act via amine oxidation, and the JUMONJI-C-DOMAIN (JmjC) proteins act via hydroxylation (discussed extensively in Cheng 2014; see also Shi and Tsukada 2013). *Arabidopsis* has four LDM and 21 JmjC genes in several subgroups (reviewed in Chen and Tian 2007; Liu et al. 2010). The LDM proteins and some JmjC proteins are involved in flowering time control, have specific target genes, and can act in cooperation with transcription factors. The JmjC protein, INCREASE IN BONSAI METHYLATION (IBM1), counteracts H3K9 methylation and CHG DNA methylation. Developmental defects due to loss of IBM1 are consequently suppressed in double mutations with *suvh4* and *cmt3*. Several enzymes potentially changing histone methylation marks are still uncharacterized, and antagonism or cooperativity, as well as target specificity are likely to result in a complex pattern of writing and reading these marks.

### 2.2.3 Enzymes for Other Histone Modifications

One of the other important histone PTMs, in addition to acetylation and methylation, is phosphorylation (reviewed in Houben et al. 2007). Histone phosphorylation is involved in DNA repair ( $\gamma$ H2AX) and the regulation of chromosome segregation and cell division (Aurora kinases). Histone phosphorylation can be influenced by other epigenetic histone marks, as is the case for phosphorylation of H3S10, which is influenced by the modification state of the adjacent lysine, H3K9. ADP-ribosylation of histones occurs in plants, but has not been studied extensively. Ubiquitination of histones H2A and/or H2B also has important regulatory functions in animals and plants (reviewed in Berr et al. 2011; Bycroft 2011) and can recruit or exclude other modifying enzymes. Proteins that install or remove ubiquitination have been characterized and influence cell cycle, development, and pathogen resistance.

### 2.2.4 Histone Variants, Linker Histones, and Nonhistone Proteins

Histones are among the most conserved proteins across eukaryotic taxa and are encoded by highly redundant gene families. Like animals, plants have developed structurally and functionally distinct classes of H2A and H3 histone variants (discussed in Henikoff and Smith 2014). The physical properties of the variants play an important role in their dynamic association with DNA (reviewed in Ingouff and Berger 2010; Deal and Henikoff 2011).



Phosphorylation of the H2AX variant marks the sites of DNA damage and is believed to recruit DNA repair proteins. H2A.Z is a variant found mainly around the transcriptional start site of genes, likely regulating transcription, and is mutually exclusive with DNA methylation (Zilberman et al. 2008). Its incorporation requires the activity of a SWR1 chromatin-remodeling complex. On heat stress, H2A.Z-containing nucleosomes dissociate from the DNA, accompanied by changes in gene expression (Kumar and Wigge 2010). The CenH3 variant of histone H3 specifies the nucleosomes of centromeric regions and is required for kinetochore assembly, microtubule attachment, and chromosome segregation during cell division. Histone variant H3.3, which differs from the canonical H3 subunit at only a few amino acids, is found predominantly at regulatory regions and expressed genes. It is incorporated into chromatin in a replication-independent manner, involving special chaperones and remodeling complexes. However, the mechanisms responsible for histone variant replacement outside of replication are not well understood in plants.

In addition to the four histones that form nucleosome core particles, the compaction of DNA and its accessibility to interacting proteins is also determined by linker histones, particularly histone H1. Linker histones display substantial diversity, and their functional specialization is suggested by the expression of stress-inducible variants. Down-regulating specific linker histones is sometimes compensated for by the up-regulation of other variants, but can also result in DNA hypomethylation and pleiotropic phenotypic defects (reviewed in Jerzmanowski 2007).

As in other eukaryotes, plants have nonhistone chromosomal proteins that may contribute to epigenetic regulation, including HMG proteins. The best characterized and most diverse subgroup of these proteins in plants is the HMGB family, whose members differ in expression level, pattern, localization, and interaction with DNA and other proteins. Mutation and ectopic expression of individual family members indicate their partial subfunctionalization and a role in development and stress response (reviewed in Pedersen and Grasser 2010). One HMG protein, STRUCTURE-SPECIFIC RECOGNITION PROTEIN (SSRP1), is indirectly involved in DNA demethylation of imprinted genes in the central cell of the female gametophyte (Ikeda et al. 2011).

Cohesin complexes secure sister chromatid alignment before their separation during mitotic and meiotic anaphase, and are also involved in DNA repair, spindle attachment, chromosome condensation, and regulation of DNA accessibility. Cohesin structure, assembly, and removal appear to be highly conserved, but some family members in plants may have specialized functions (reviewed in Yuan

et al. 2011). For instance, DEFECTIVE IN MERISTEM SILENCING3/INVOLVED IN DE NOVO (DMS3/IDN1) is a protein related to the hinge domain region of cohesins and condensins and is necessary for DNA-dependent RNA polymerase V (RNA Pol V) transcription in the establishment of RdDM (see Sec. 3.5.2).

Proteins such as REPLICATION PROTEIN A2 (RPA2) or REPLICATION FACTOR C1 have been identified in mutant screens for epigenetic regulators (Elmayan et al. 2005; Kapoor et al. 2005). Enhanced phenotypes of chromatin mutants when combined with loss-of-function mutants of the topoisomerase homolog MGOUN (MGO) also indicate a role for this protein, especially in connection with stem cell and meristem maintenance (Graf et al. 2010). It can be expected that many other nonhistone proteins interacting with DNA will also turn out to be direct or indirect epigenetic regulators.

### 2.3 Polycomb Proteins and Interacting Components

PcG group proteins were initially identified as master regulators and suppressors of homeotic genes in *Drosophila*. In balance with the activating TrxG proteins, PcGs determine cell proliferation and cell identity (see PcG proteins in Grossniklaus and Paro 2014 and TrxG proteins in Kingston and Tamkun 2014).

POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) in plants is the more conserved of two different types of PcG complexes and is responsible for trimethylation of histone H3 at lysine 27 (H3K27me3), as in animals. Each subunit in the *Drosophila* PRC2 complex has several paralogous equivalents in *Arabidopsis* (see Fig. 3 in Grossniklaus and Paro 2014). MEDEA (MEA), CURLY LEAF (CLF), and SWINGER (SWN) corresponding to the *Drosophila* protein E(Z); FERTILIZATION-INDEPENDENT SEEDS 2 (FIS2), VERNALIZATION 2 (VRN2), and EMBRYONIC FLOWER 2 (EMF2) corresponding to Su(z)12; FERTILIZATION-INDEPENDENT ENDOSPERM (FIE) corresponding to Esc; and MULTICOPY SUPPRESSOR OF IRA HOMOLOG 1–5 corresponding to p55 (reviewed in Koehler and Hennig 2010). This diversification of PRC2 component proteins is thought to be associated with the evolutionary expansion of land plants (reviewed in Butenko and Ohad 2011). Plant PRC2 components are required at different developmental stages and function at specific, but sometimes overlapping, subsets of genes. For many functions, plant PRC2 interacts with additional proteins or specific RNA transcripts, as in the control of flowering time (discussed in Baulcombe and Dean 2014). PcG proteins themselves are under strict regulatory control, in part via DNA demethylation. At least two PcG genes (MEA and FIS2) are imprinted genes, meaning that they are differ-

entially expressed depending on the parent from which they were inherited (reviewed in Raissig et al. 2011). Although imprinting evolved independently in plants and mammals (Table 1), DNA methylation and PcG proteins are key components in both cases.

PRC1, the other PcG protein complex, is more dissimilar in *Arabidopsis* compared with animals, but has related functions. Like the *Drosophila* protein POLYCOMB, the *Arabidopsis*-LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) binds to H3K27me<sub>3</sub> and might “read” and “translate” this modification into additional and more repressive chromatin configurations, such as by interaction with the H2A ubiquitinating enzymes, AtRING 1, -2, and AtBMI1, also homologs of PRC1 components.

Subunits of both PRC core complexes interact with other proteins that could modulate specificity, but the means by which PcG complexes are recruited to specific target genes in plants is still not clear. Importantly, the consensus sequences for Polycomb or Trithorax responsive elements determined in animals have not been identified in plants.

## 2.4 Nucleosome-Organizing Proteins

Replication, transcription, recombination, and repair require transient or lasting changes in the positioning of nucleosomes and their association with DNA. Therefore, dynamic processes at the chromatin level are not restricted to reversible DNA or protein modifications, but can include changes in nucleosome occupancy, nucleosome composition, and the accessibility of the DNA to other proteins.

### 2.4.1 Chromatin-Remodeling Complexes

Relocation or dissociation of nucleosomes can be accomplished by chromatin-remodeling ATPases such as the SWI/SNF complex first identified in yeast and named according to the processes affected in the mutants (see **Becker and Workman 2013**). Plants have several such complexes (reviewed in Jerzmanowski 2007). Genetic screens have provided functional information for only a handful of putative chromatin remodelers, the first identified being DECREASE IN DNA METHYLATION 1 (DDM1).

Loss-of-DDM1-function results in genome-wide reduction of DNA methylation and H3K9me<sub>2</sub>, transcriptional activation of repetitive elements, and dysregulation of numerous genes. As a result, *ddm1* mutants display severe developmental and morphological defects that increase in severity with successive generations. The gradual reduced fitness of *ddm1* mutants is attributable to the accumulation of epimutations and insertional mutations caused by reactivated transposons. Part of the epigenetic information, in the form of DNA methylation, is irrevers-

ibly lost in *ddm1* mutants, yet backcrosses with wild-type plants can restore the pattern of modification at some loci as a result of de novo methylation (Teixeira et al. 2009). Like the SWI2/SNF2 ATPase protein, DDM1 displays ATP-dependent nucleosome repositioning activity in vitro (Brzeski and Jerzmanowski 2003). The loss of cytosine methylation that occurs in *ddm1* mutants does not occur in mutants that lack both DDM1 and linker histone H1 (Zemach et al. 2013) indicating that DDM1 is needed for the maintenance methylation machinery to be able to access nucleosomal DNA that is highly packaged, involving both core and linker histones. DDM1's ortholog in mammals, LYMPHOID-SPECIFIC HELICASE, is similarly important for global CpG methylation and development.

Two members of the SWI2/SNF2 family in *Arabidopsis*, DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) and CLASSY 1 (CLSY1) are unique to the plant kingdom and have specialized roles in RNA-directed DNA methylation (see Sec. 3.5.2). Four other SWI2/SNF2 proteins, BRAHMA (BRM), SPLAYED (SPD), and MINUSCULE 1 and 2, are involved in the control of similar, but not identical, subsets of genes involved in hormone responses and stem cell maintenance (Sang et al. 2012).

Besides the ATPases, the other three core subunits of SWI/SNF remodelers are represented in plants, including several SWI3 family members (AtSWI3 A-D), one SNF5 homolog (BSH), and two SWP73 homologs (reviewed in Jerzmanowski 2007). Their roles in plants have not been studied extensively, but SWI3 has been shown to interact with RNA binding proteins involved in RNA-directed DNA methylation and to influence the spacing of nucleosomes at silenced loci (Zhu et al. 2012).

Subunits of other remodeling complexes include CHROMATIN-REMODELING PROTEIN 11 (CHR11; an ISWI complex protein), PICKLE (PKL; a CHD3 complex protein), and INOSITOL-REQUIRING 80 and ACTIN-RELATED PROTEINS 4 AND 5 (INO80, ARP4, ARP5; INO80 complex proteins). Mutants defective for these activities are characterized by developmental abnormalities and impaired DNA repair.

MORPHEUS' MOLECULE 1 (MOM1) is a plant-specific epigenetic regulator that is related to SWI2/SNF2 by virtue of a homologous, but incomplete, ATPase domain and is also related to CHD3. The lack of *mom1*, unlike *ddm1* or *pkl*, does not result in morphological defects. Its exact mode of action is unknown, yet *mom1* mutants are characterized by having an intermediate chromatin state at a pool of target genes that it silences, which partially overlap with those of the RdDM pathway (reviewed in Habu 2010).

The SWR1 complex C plays an important epigenetic role in the deposition of H2A.Z at transcription start sites (and the antagonistic exclusion of DNA methylation; see

**Q8 Sec. 2.4.1).** The *Arabidopsis* SWR1 complex consists of a core containing the ATPase subunit PHOTOPERIOD-INDEPENDENT EARLY FLOWERING (PIE), SWR1 COMPLEX 6 (SWC6), and ACTIN-RELATED PROTEIN 6 (ARP6). How this complex is directed to specific target genes is not clear, but its function is essential for developmental decisions and stress responsiveness.

### 2.4.2 Chromatin Assembly Factors

Whereas SWI/SNF and other remodeling complexes act on nucleosomes that are already associated with DNA, other activities are required for assembling core histones into new nucleosomes after replication, reestablishing chromatin after repair or recombination-associated DNA synthesis, or for the exchange of histones in connection with transcriptional processes. These functions are exerted by histone chaperones, which are mostly acidic proteins that interact with each other and specific canonical or variant histones (see **Almouzni and Cedar 2014** for more detail).

Plants have three chaperones for loading H3/H4 tetramers and three for adding H2A/H2B dimers (reviewed in Zhu et al. 2011b). The CHROMATIN ASSEMBLY FACTOR 1 (CAF-1) complex helps to bring H3/H4 tetramers to the replication fork. Mutations in genes encoding the two larger CAF-1 subunits in *Arabidopsis* (*fas1*, *fas2*) cause characteristic morphological anomalies (fasciation, Fig. 2F), deficiencies in DNA repair, reductions in rRNA gene copy numbers, and derepression of repetitive elements. This indicates that correct nucleosome deposition is essential for development, genome stability, and epigenetic control. As would be expected, the lack of CAF-1 subunits does not interfere with maintenance of DNA methylation, but it can lead to the erasure of other histone-based epigenetic marks. Reduced levels of the third CAF-1 component, MSI1, does not cause fasciation, but lead to distorted seed development and several morphological changes, probably caused by its multiple roles, including a role within the PRC2 complex. There are two other H3/H4 chaperones that function independently of replication, but also in cooperation with CAF-1: HISTONE REGULATOR A (HIRA), for which there is one *Arabidopsis* homolog and ANTISILENCING FUNCTION 1 (ASF1), which has two *Arabidopsis* homologs.

Histones H2A and H2B are assumed to be installed by NUCLEOSOME ASSEMBLY PROTEIN 1 (NAP1), NAP1-RELATED PROTEIN (NRP), and FACILITATES CHROMATIN TRANSCRIPTION (FACT) proteins. In *Arabidopsis*, there are a number of homologs to these proteins, four NAP1, two NRP, and two FACT homologs, with conserved sequences to the yeast or animal counterparts (reviewed in Zhu et al. 2011b).

Some chaperone subunits can be regulated by post-translational modification, as indicated by the phenotype of TOUSLED (TSL) mutants, which lack an ASF1 kinase. A mutant of the *Arabidopsis* protein named BRUSHY (BRU) has a similar phenotype to plant CAF-1 *fas* mutants—that is, sensitivity to DNA damage, interference with TGS, and developmental defects. Although BRU has no homology with any known histone chaperones, the additional dysregulation of PcG-controlled genes at specific genomic clusters, likely indicates BRU's importance for maintaining different epigenetic states (Ohno et al. 2011).

It is striking that most mutations in genes for histone chaperones affect the organization of the meristems, the maintenance of stem cells, or processes in the differentiation zone adjacent to the stem cell niche within the meristem. This might reflect the requirement for correct histone and nucleosome deposition to perpetuate stem cell identities in plants.

## 3 MOLECULAR COMPONENTS OF RNA-MEDIATED GENE SILENCING PATHWAYS

As discussed in the preceding sections, cytosine methylation and posttranslational histone modifications are important aspects of epigenetic gene regulation in plants, helping to establish or maintain gene “on” or “off” states at the level of transcription. Epigenetic regulation also occurs posttranscriptionally through targeted mRNA degradation or translational inhibition. This PTGS can thus act to control the temporal or spatial distribution of developmentally important mRNAs and serve as a potent defense against invaders that include viruses, microbial pathogens, and transgenes (Ruiz-Ferrer and Voinnet 2009; Vazquez et al. 2010).

A common feature of transcriptional and posttranscriptional silencing mechanisms in plants is the involvement of small RNAs, specifically miRNAs or siRNAs. The biogenesis of these small RNAs in plants shares similarities with their biogenesis in other eukaryotes, suggesting that RNA-based silencing mechanisms, involving mi- and siRNA, have a common ancient origin (Chapman and Carrington 2007; Shabalina and Koonin 2008). However, duplication and subfunctionalization of genes involved in miRNA- or siRNA-mediated processes in plants has led to the evolution of multiple pathways that are specialized to accomplish specific tasks (Herr 2005; Baulcombe 2006; Xie and Qi 2008; Chen 2009; Vazquez et al. 2010). These include (1) a pathway for the biogenesis of miRNAs that are complementary to target sequences, resulting in the down-regulation of individual transcribed mRNAs (Sec. 3.2); (2) a pathway in which an miRNA initiates the production of secondary *trans*-acting siRNAs that down-regulate multi-

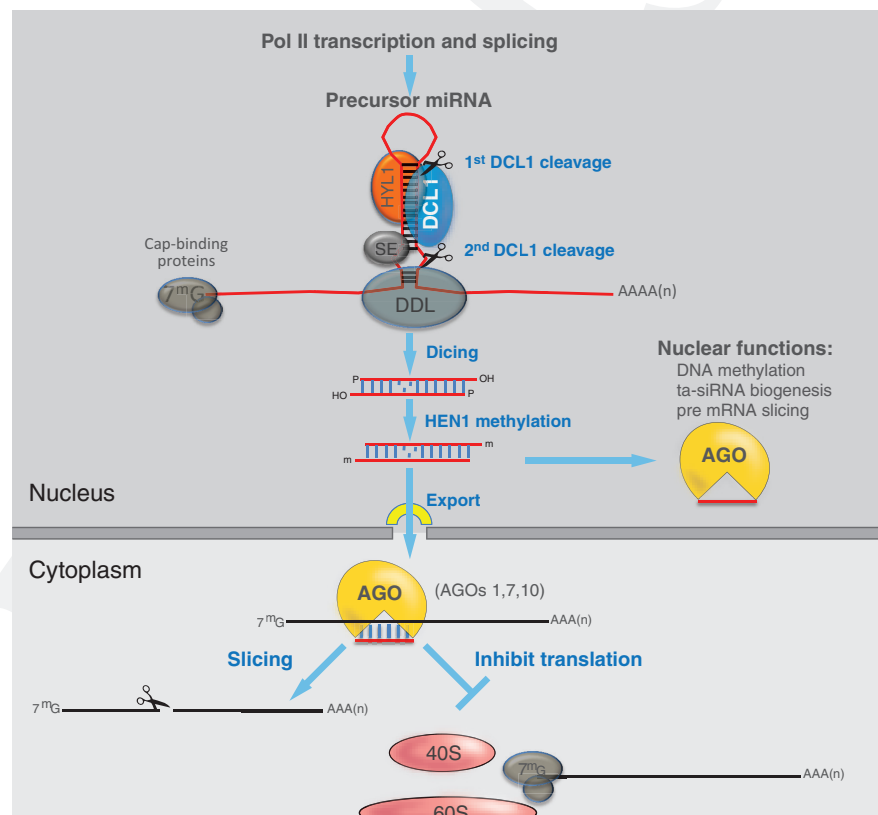
ple targets with no complementarity to the initiating miRNA; (3) a pathway for siRNA-mediated degradation of invading viral RNAs or transgene RNAs (Sec. 3.4); and (4) a pathway for siRNA-mediated DNA methylation and transcriptional silencing of transposons, viruses, and specific genes (Sec. 3.5). Collectively, these pathways provide plants with an arsenal of RNA-mediated silencing capabilities that is unrivaled by other eukaryotes.

### 3.1 Common Features of miRNA and siRNA Biogenesis and Silencing

miRNAs and siRNAs share a number of common features in plants, as in other eukaryotes (Bartel 2004; Voinnet 2009; Axtell et al. 2011). Both are produced from dsRNA precursors by the action of RNaseIII-related Dicer (DCL) endonucleases. Resulting small RNAs are then incorporated into a multiprotein RNA-induced silencing complex (RISC) that has, at its core, a member of the Argonaute (AGO)

protein family (Czech and Hannon 2011). The AGO protein binds the 3' end of the small RNA via its PAZ domain and uses the small RNA to base-pair with complementary target RNAs (Joshua-Tor 2006). As a consequence, the target RNA can be cleaved by the AGO protein's PIWI domain, or translation can be blocked without cleavage of the associated RNA, or chromatin-modifying machinery can be recruited to transcriptionally silence the locus. The different outcomes depend on both the class of small RNA involved and the particular AGO protein partner (see also Martienssen and Moazed 2014).

The double-stranded precursors of miRNAs or siRNAs are generated in several ways. In the case of miRNAs, DNA-dependent RNA polymerase II (RNA Pol II) transcripts with extensive self-complementarity fold back on themselves to form stem-loop structures with imperfect double-stranded stems that can be diced by DCL1 (Fig. 3). In the case of siRNAs, the double-stranded precursors can be generated via convergent, bidirectional transcription by a



**Figure 3.** miRNA biogenesis and modes of action. Capped and polyadenylated transcripts of nuclear RNA Pol II that form imperfect hairpins serve as the precursors for miRNAs. DCL1 cleaves these precursors with the help of the HYL1, DDL1, and SE nucleic acid-binding proteins. Diced products are end-methylated by HEN1 and exported to the cytoplasm in a process involving the exportin 5 homolog, HST. In association with AGO1 or a related AGO protein, such as AGO10, the miRNA guides the cleavage or translational inhibition of complementary mRNAs. Nuclear functions in which miRNAs are implicated include specific cases of cytosine methylation, *trans*-acting siRNA (tasiRNA) production or pre-mRNA degradation.



DNA-dependent RNA polymerase such as RNA Pol II, thereby generating transcripts that overlap and base-pair. Alternatively, RNA transcripts can be used as templates for an RNA-dependent RNA polymerase (RdRP), to generate a complementary strand.

Diversification of the core machinery responsible for siRNA biogenesis and function underlies the evolution of the different small RNA silencing pathways in plants (Vazquez et al. 2010; Xie and Qi 2008). Like fission yeast (*S. pombe*) and nematodes (*C. elegans*), but unlike mammals or *Drosophila*, plants make use of RNA-dependent RNA polymerases for the production of dsRNA. Six distinct RdRPs are encoded by the *Arabidopsis* genome. Likewise, *Arabidopsis* has four distinct Dicer endonucleases, DICER-LIKE (DCL) 1 through 4, whereas mammals, fission yeast and nematodes have only one Dicer, and fruit flies have two. The plant Dicers generate small RNAs with different sizes: miRNAs of (mostly) 21 nt (DCL1), or siRNAs that are 21 nt (DCL4), 22 nt (DCL2), or 23–24 nt (DCL3). The different sized siRNAs have distinct, yet partially overlapping functions, based on their associations with a diversified AGO protein family that has 10 members in *Arabidopsis* (Vaucheret 2008). As discussed **below**, the RdRPs, Dicers, and AGO proteins are used in a variety of permutations to accomplish diverse small RNA-mediated silencing phenomena.

### 3.2 miRNA Biogenesis and Function

miRNAs are critical for plant and animal development, helping limit the functions of complementary mRNAs to a specific subset of cells by bringing about their posttranscriptional degradation or translational repression in adjacent cells (Carrington and Ambros 2003; Ambros 2004; Bartel 2009; Cuperus et al. 2011). miRNAs silence gene expression by base-pairing to target mRNAs in the context of a multiprotein RISC complex, facilitating mRNA cleavage referred to as “slicing,” or the inhibition of mRNA translation (Fig. 3). The importance of miRNAs in plant development is underscored by the fact that many of the genes involved in miRNA biogenesis and function were initially identified in genetic screens for mutants affecting development. Developmental processes requiring miRNAs include stem cell maintenance and differentiation, specification of organ polarity, vascular development, floral patterning, hormone signaling, and responsiveness to environmental stresses (reviewed in Rubio-Somoza and Weigel 2011; Khraiweh et al. 2012).

In plants, as in nematodes, loci encoding miRNAs were initially discovered as genes not encoding a protein, but required for the negative regulation of developmentally important mRNAs (Ambros 2004; Chen 2005). We now know

that the transcripts of these noncoding RNA loci are processed into small (micro) RNAs with perfect or near-perfect complementarity to the down-regulated mRNAs. miRNAs with mismatches relative to their target mRNAs tend to repress translation by binding stably to the mRNA, hence, interfering with the translation machinery. By contrast, miRNAs with perfect complementarity to their target mRNAs tend to induce mRNA cleavage through slicing by the PIWI domain of the associated AGO protein. In animals, in which miRNAs rarely match their target mRNAs perfectly, translation inhibition is the most prevalent form of miRNA action (Ambros 2004; Bartel 2004). In plants, perfect or near-perfect complementarity of plant miRNAs to their target mRNAs is the norm, thereby favoring mRNA slicing (Jones-Rhoades et al. 2006; Axtell et al. 2011; Cuperus et al. 2011). However, these are tendencies, not rules, such that some plant miRNAs and siRNAs block translation, and some mammalian miRNAs direct mRNA slicing.

miRNAs are transcribed by DNA-dependent RNA Pol II, sometimes as separate miRNA genes and sometimes as sequences present within long noncoding RNAs or in introns of protein-coding genes. As in animals, plant miRNAs originate from 70 to more than 600-bp-long precursor RNAs that fold back to form imperfect hairpin or stem-loop structures (Bartel 2004; Jones-Rhoades et al. 2006; Cuperus et al. 2011). **In mammals, the RNase III-related endonuclease, Drosha, cleaves the initial pre-miRNA stem-loop precursor on the loop-proximal side of the stem, aided by the dsRNA binding protein, Pasha. Alternatively, in the case of intron-encoded miRNAs, or “mirtrons,” splicing and lariat debranching enzymes generate the initial pre-miRNA (Okamura et al. 2007; Westholm and Lai 2011). The resulting partially processed pre-RNA is then transported to the cytoplasm by an Exportin 5-mediated pathway to undergo further processing. In the cytoplasm, Dicer cuts the stem at a site 22 nt from the Drosha cleavage site, thereby generating a duplex that includes the mature miRNA, or miR, and the complementary passenger strand, miR\* (Du and Zamore 2005; Carthew and Sontheimer 2009). Plants, however, lack Drosha and Pasha orthologs and are thought to generate miRNAs entirely within the nucleus, with DCL1 responsible for both cleavages of the stem-loop precursor RNA, generating miR/miR\* duplexes that are typically 21-nt long (Fig. 3). DCL1 processing is assisted by the RNA-binding proteins HYPONASTIC LEAVES 1 (HYL1; also known as DRB1) and DAWDLE (DDL), as well as a zinc-finger protein, SERRATE (SE). The miR/miR\* duplex is then methylated by HUA ENHANCER 1 (HEN1) at the 3'-terminal ribose of each RNA strand, thereby protecting the small RNAs from uridylation and increasing their stability. Most methylated miR/miR\* duplexes are then thought to be exported from**



the nucleus to the cytoplasm in a process involving HASTY (HST), a homolog of mammalian Exportin 5. However, the abundance of specific miRNAs is differentially affected in *hst* mutants, suggesting variation in the trafficking of different miRNAs. The miR strand is loaded onto AGO1, or a related AGO family member, including AGO10 or AGO7, and the resulting AGO-RISC complex carries out slicing or translational inhibition of mRNAs complementary to the miRNA (reviewed in Chen 2009; Poethig 2009; Axtell et al. 2011; Cuperus et al. 2011).

Comparative analyses of angiosperms (flowering plants), gymnosperms, and more primitive plants, such as ferns and mosses, have identified a number of highly conserved miRNAs that are critical for meristem function and development (Axtell et al. 2007; Axtell and Bowman 2008). Although the coding sequences of the mRNAs targeted by these miRNAs have changed during evolution, the miRNA-binding sites within the mRNAs are nearly invariant, indicating that the need for such complementarity has constrained the evolution of the matching miRNA and mRNA sequences for at least 400 million years.

AGO1 is the founding member of the AGO protein family and is the principal mRNA slicer in *Arabidopsis* for DCL1, DCL2, and DCL4-generated small RNAs (Baumberger and Baulcombe 2005). AGO1 was identified as an *Arabidopsis* mutant defective in leaf development before the discovery of miRNAs. The name “Argonaute” was inspired by the phenotype of *ago1* mutants, whose narrow, filamentous leaves gave the plants the appearance of a small octopus of the genus *Argonauta*. *ago1* hypomorphic mutants display severe shoot apical meristem defects, as do *ago10* mutants. The latter are also known as pinhead (*pnh*) mutants (see Table 1) because of the absence of lateral organ formation at the shoot apex. These dramatic apical meristem phenotypes derive from defects in stem cell maintenance and cell lineage specification (reviewed in Kidner and Martienssen 2005; Vaucheret 2008).

Approximately 50% of the known miRNA targets in *Arabidopsis* are transcription factors, many of which modulate meristem formation and identity. Other miRNAs target mRNAs encoding F-box proteins that are involved in the ubiquitin-mediated turnover of proteins in developmentally important signaling pathways. The biogenesis of some miRNAs is induced by specific environmental stresses, leading to down-regulation of complementary mRNAs during adaptive stress responses (Axtell and Bowman 2008; Poethig 2009; Cuperus et al. 2011).

An interesting regulatory strategy in plants is the use of decoys to sequester specific miRNAs from key targets. For instance, one function attributed to AGO10 is the sequestration of miR166/165 so as to make the miRNAs unavailable to AGO1 (Zhu et al. 2011a). In other cases, mRNAs

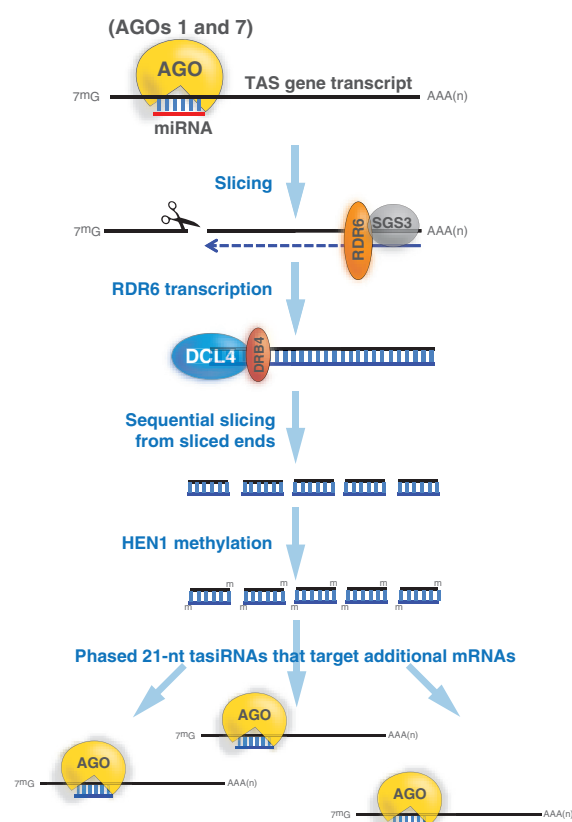
with imperfect complementarity to miRNAs serve as decoys, or target mimics, that tie up miRNA-AGO RISC complexes such that other mRNA targets escape inactivation (Franco-Zorrilla et al. 2007). Interestingly, AGO1 and DCL1 are themselves targeted by miRNAs, allowing for negative feedback of miRNA biogenesis.

Mature miRNAs are detected in both the nucleus and cytoplasm, suggesting that miRNAs may have functions in both cellular compartments. Evidence that miRNAs can direct DNA cytosine methylation was among the earliest clues that miRNAs can regulate nuclear activities (Bao et al. 2004). There is also evidence that posttranscriptional silencing can be mediated by small RNA-guided cleavage of intronic sequences (Hoffer et al. 2011). Collectively, these observations suggest that pre-mRNAs have the potential to be targeted by miRNA or siRNA RISCs before mRNA maturation or export to the cytoplasm. Whether dedicated machinery exists for RISC trafficking from the cytoplasm to the nucleus, or whether RISC assembly can occur in both compartments, is not clear.

### 3.3 Trans-Acting siRNAs

Most miRNAs directly regulate specific mRNAs. However, plants have evolved another pathway in which miRNAs initiate the production of secondary siRNAs that then target mRNAs (Fig. 4). This so-called tasiRNA pathway gets its name because the secondary siRNAs act on loci that are distinct from the loci that give rise to the siRNAs (reviewed in Chen 2009; Chuck et al. 2009; Allen and Howell 2010).

In *Arabidopsis*, there are four families of TAS genes, loci that generate tasiRNAs: TAS 1–4. TAS1 and TAS2 are both targeted by miR173 in association with AGO1, TAS4 is targeted by miR828-AGO1, and TAS3 is targeted by miR390-AGO7 (Montgomery et al. 2008). TAS loci generate long noncoding RNAs that are cleaved by the miRNA-AGO-RISC complex, triggering the conversion of the 3' cleavage product into dsRNA through the action of RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) and its partner, SUPPRESSOR OF GENE SILENCING 3 (SGS3). The dsRNA is then diced by DCL4 into 21-nt tasiRNAs that are phased with respect to the original miRNA cleavage site, such that tasiRNA sequences have defined sequences, much like miRNAs, and function like miRNAs to direct the slicing of downstream complementary target mRNAs (Fig. 4). The tasiRNAs produced by the TAS3 locus target several Auxin response factor mRNAs, thereby affecting developmental transitions that are regulated by the plant hormone auxin. By modification of auxin responses, tasiRNAs contribute to the transition of the meristem from a juvenile phase, with vegetative organs only, to an adult phase capable of responding to signals promoting flowering (Poethig et al. 2006).



**Figure 4.** tasiRNA biogenesis and function. Specific miRNAs, associated with AGO1 or AGO7, target and cleave the transcripts of telomere-associated satellite (TAS) gene loci, ultimately giving rise to tasiRNAs. In the case of AGO1, initiating miRNAs that are 22 nt in length induce the recruitment of the RNA-dependent RNA polymerase, RDR6 (and its partner SGS3), to the 3' cleaved fragment resulting in transcription of the complementary strand to generate dsRNA. Dicing by DCL4, aided by the dsRNA-binding protein, DRB4, results in the production of 21-nt tasiRNAs that are phased with respect to the miRNA-cleaved end. These tasiRNAs, in turn, target specific complementary RNAs in association with AGO1, thus behaving like target-specific miRNAs.

### 3.4 siRNA-Mediated Silencing as a Genome Defense Strategy

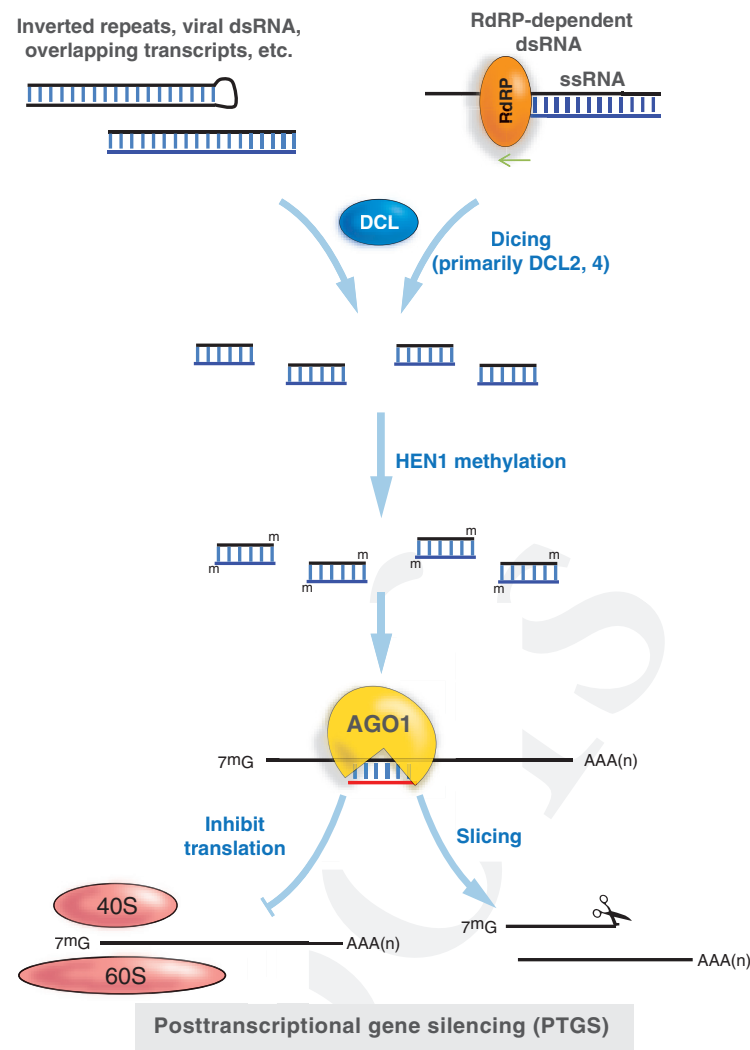
Multifunctional siRNA-AGO complexes and/or dicer endonucleases have evolved as useful components in the defense against viral and microbial pathogen attack (Martienssen and Moazed 2014). Most plant viruses are RNA viruses whose dsRNA forms are subject to DCL-catalyzed dicing and degradation. For DNA viruses, such as geminiviruses or Cauliflower Mosaic Virus, the viral genome can be the target of siRNA-mediated DNA methylation, or siRNAs can facilitate the degradation or inactivation of viral transcripts, resulting in transcriptional silencing and decreased viral RNA synthesis. Defense against foreign or invasive nucleic acids thus provides the most likely explanation for the evolution of small RNA-

mediated RNAi pathways in eukaryotes and for the diversification of RNA-silencing capabilities in plants (Ding 2010). These include the ability to silence genes at a transcriptional and/or posttranscriptional level and an ability to spread defensive small RNAs to cells distant from the site of initial infection, thereby arming the plant against a spreading virus, enabling it to mount a systemic defense (illustrated in Fig. 1 of Dunoyer et al. 2013).

Most plant viruses are single-stranded RNA viruses whose replication by endogenous or viral encoded RNA-dependent RNA Polymerase transcription produces dsRNA intermediates (Wassenegger and Krczal 2006) that can be diced into 21–24-nt siRNAs, primarily by DCLs 2, -3, and -4 (Fig. 5) (Vazquez et al. 2010). Other plant viruses have dsRNA genomes providing direct substrates for Dicer cleavage. Single-stranded DNA viruses, such as geminiviruses, replicate via double-stranded DNA intermediates and generate RNA transcripts from both strands of the DNA. These RNAs can overlap and base-pair resulting in dsRNA that can be diced. Alternatively, long viral RNA transcripts might fold back on themselves into structures with some dsRNA regions that might be diced. Thus, antiviral RNAi responses are not limited only to dsRNAs produced by the virus itself.

Transgenes introduced into plant genomes can fall victim to silencing pathways that probably evolved for antiviral defense, as illustrated in Figure 5. This occurs because transgenes often integrate in multiple copies, and in inverted orientation relative to one another. Thus, readthrough transcripts that extend from one transgene into an adjacent transgene integrated in the opposite orientation produce RNA with regions of self-complementarity, or inverted repeats, resulting in dsRNA that can be diced. A transgene might also integrate into a chromosome adjacent to a strong promoter pointed in the opposite orientation relative to the transgene's promoter, resulting in convergent bidirectional transcription and dsRNA formation.

PTGS of transgenes and VIGS involve the production of 21–24-nt siRNAs generated by DCLs 2, -3, and -4 (Fig. 5). In general, the 21- and 22-nt siRNAs generated by DCL4 are thought to guide mRNA cleavage in association with AGO1. Following endonucleolytic cleavage by a 21-nt siRNA-AGO1 complex, the severed 3' segment of the mRNA lacking a 7-methylguanosine cap at the 5' end is degraded in the 5' to 3' direction by the exonuclease, AtXRN4 (Rymarquis et al. 2011). The 5' fragment is probably degraded by the exosome in a 3' to 5' direction. An intriguing property of 22-nt small RNAs, which are less abundant than 21-nt siRNAs, is that their association with AGO1 brings about mRNA cleavage that is coupled to the recruitment of RDR6, similar to the action of the 22-nt miRNAs that give rise to tasiRNAs. As a result, the 3' AGO cleavage



**Figure 5.** PTGS induced by viruses or transgenes. Double-stranded precursors of siRNAs that bring about PTGS can be dsRNA viral genomes or replication intermediates, dsRNAs formed by overlapping transcripts from adjacent transcription units, or inverted repeats resulting from tandem transgenes that integrate in opposite orientations. Alternatively, single-stranded RNAs can be made double-stranded by the action of RNA-dependent RNA polymerases, such as RDR6 or RDR1. Dicing can then occur by DCLs 2, -3 or -4, with 22-nt DCL2 products and 21-nt DCL4 products being primarily associated with PTGS. On association with AGO1, the siRNAs target complementary RNAs for degradation or translational inhibition.

fragment is converted into a dsRNA, which is then diced by DCL4 to generate 21-nt secondary siRNAs (Chen et al. 2010; Cuperus et al. 2010; Manavella et al. 2012).

siRNAs that are generated by DCL3 associate primarily with AGO4, or the related AGO6 or AGO9 proteins (Havemaker et al. 2010), and can direct RNA slicing of viral or transgene-derived RNAs. However, these 24-nt siRNA-AGO RISC complexes have the added property of directing epigenetic modifications to homologous DNA sequences (see Sec. 3.5.1). The combination of mRNA degradation, production of secondary siRNAs, and transcriptional silencing constitutes a potent response to an invading nucle-

ic acid. However, it is not surprising that viruses have evolved counter-measures, encoding proteins that suppress RNA silencing (discussed in **Baulcombe and Dean 2014**), in an evolutionary arms race between pathogen and host (reviewed in Ding and Voinnet 2007; Bivalkar-Mehla et al. 2011).

### 3.5 RNA-Directed DNA Methylation and Heterochromatin Formation

RNA-mediated transcriptional silencing of transposons, retroviruses, and other genomic repeats is well documented

in diverse eukaryotes, including plants, mammals, fission yeast, and fruit flies. In fission yeast and fruit flies, which do not use DNA methylation as part of their gene silencing toolkit, small RNAs mediate changes in histone modification to bring about transcriptional silencing (detailed in Elgin and Reuter 2013 and Allshire and Ekwall 2014). However, in plants and mammals, which methylate their DNA, cytosine methylation and repressive histone modifications are partners in the silencing of repetitive elements and the specification of heterochromatin. In plants, the silencing of facultative heterochromatin, which includes retrotransposons and other expressed repeats such as excess rRNA genes, involves 24-nt siRNAs that direct the de novo cytosine methylation of corresponding genomic sequences (Matzke et al. 2009; Law and Jacobsen 2010; Haag and Pikaard 2011; Zhang and Zhu 2011). In the male germline of mammals, small RNAs similarly associate with proteins of the PIWI subfamily of AGO proteins (piRNAs) and are implicated in directing histone modifications and de novo cytosine methylation of transposons (Klattenhoff and Theurkauf 2008; He et al. 2011; Pillai and Chuma 2012).

### 3.5.1 RdDM

RdDM was first observed in tobacco plants infected with viroids (Wassenegger et al. 1994). Viroids are plant pathogens consisting solely of a circular noncoding RNA only several hundred nucleotides in length. Replicating viroids were found to trigger de novo methylation of viroid cDNAs that had been integrated as transgenes into the tobacco genome. RNA viruses were similarly found to elicit the methylation of homologous DNA sequences in the nuclear genome. Moreover, by expressing transgenes engineered to express dsRNAs homologous to promoter sequences, cytosine methylation was shown to be directed to the promoter, resulting in homology-dependent transcriptional silencing of the corresponding gene (Mette et al. 2000).

RdDM is typified by the methylation of cytosines primarily within the region of RNA-DNA sequence identity, although RNA-mediated spreading to other sequences can occur (see Sec. 3.6). RdDM establishes methylation in all sequence contexts (see Sec. 2.1), but CHH methylation is a specific hallmark of RdDM. This is because symmetrical CG and CHG methylation can be perpetuated at every round of replication by maintenance methylation, but perpetuation of CHH methylation at many silenced loci requires continuous, RNA-guided de novo methylation in every cell cycle (Law and Jacobsen 2010).

Combined data from forward and reverse genetic screens have revealed many of the key molecular components needed for RNA-directed DNA methylation and TGS (see Secs. 2.1 and 2.2). These genetic screens have relied on

reporter transgenes or endogenous genes having a visible phenotype when silenced, allowing mutants that release (or prevent) silencing to be readily identified.

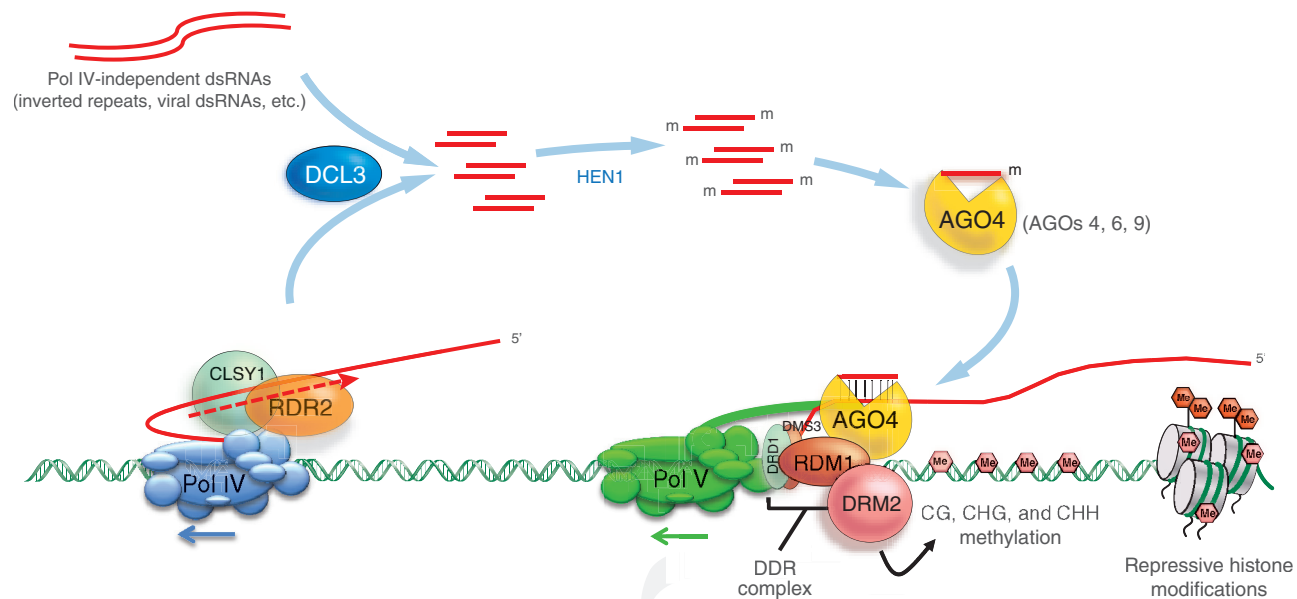
### 3.5.2 Plant-Specific Machinery for RNA-Directed DNA Methylation

As explained previously (Sec. 2.1.1), de novo methylation during RdDM is catalyzed by the DRM class of DNA methyltransferases. The full details of how DRM2 is recruited to DNA are not yet clear. However, a number of key activities have been identified, revealing a critical role for noncoding RNAs and siRNAs to allow the DNA methylation machinery to hone in on its target sites (Fig. 6).

All known eukaryotes have three highly conserved nuclear multisubunit DNA-dependent RNA polymerases that are essential for viability: RNA Pol I, II, and III. Interestingly, plants have evolved two additional nuclear DNA-dependent RNA polymerases—RNA Pol IV (in the early literature referred to as RNA Pol IVa) and RNA Pol V (previously referred to as RNA Pol IVb)—that play important roles in RdDM in *Arabidopsis* (Haag and Pikaard 2011). It is clear that RNA Pols IV and V evolved as specialized forms of RNA Pol II, a process that began in green algae before the emergence of land plants (Luo and Hall 2007; Tucker et al. 2010). *Arabidopsis* RNA Pols II, IV, and V each have 12 core subunits, approximately half of which are common to all three polymerases and encoded by the same genes (Ream et al. 2009). Subunits that are unique to RNA Pols IV or V are encoded by genes that arose via the duplication of RNA Pol II subunit genes, followed by subfunctionalization. For some subunits, there are two or more variants (Ream et al. 2009; Law et al. 2011). These alternative subunits can confer different capabilities to the enzymes, suggesting that there are multiple functional subtypes of RNA Pols II, IV, and V (Tan et al. 2012).

RNA Pol IV and Pol V have distinct roles in the RdDM pathway (reviewed in Matzke et al. 2009; Lahmy et al. 2010; Law and Jacobsen 2010; Haag and Pikaard 2011; Zhang and Zhu 2011). RNA Pol IV is thought to act early in the pathway because it colocalizes with genetic loci that give rise to abundant 24-nt siRNAs and is required for their biogenesis. Moreover, the loss of RNA Pol IV activity causes other proteins of the RdDM pathway to mislocalize. Genetic and biochemical evidence suggest that RNA Pol IV transcripts serve as templates for RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), thereby generating dsRNAs. RNA Pol IV and RDR2 physically interact in vivo, with evidence that their activities are mechanistically coupled, allowing for the channeling of precursor RNAs for 24-nt siRNA biogenesis (Haag et al. 2012). CLSY1, a putative ATP-dependent chromatin remodeler in the SWI2/SNF2 helicase





**Figure 6.** RdDM. Retrotransposons, viruses, transgenes, or repetitive genes are subject to silencing by RdDM. RNA Pol IV is thought to generate single-stranded RNAs (*bottom left*) that serve as templates for the RNA-dependent RNA polymerase, RDR2. Resulting dsRNAs are diced by DCL3 to generate 24-nt siRNA duplexes that are then methylated by HEN1 and loaded onto AGO4, or its closely related family members, AGO6 or AGO9. dsRNA derived from inverted repeat transgenes or viruses (*top left*) can bypass the need for RNA Pol IV and RDR2. AGO-siRNA complexes are recruited to their sites of action by binding to transcripts generated by RNA Pol V, as well as by physical interactions with the carboxy-terminal domain of the RNA Pol V largest subunit. At some loci, RNA Pol II is thought to substitute for RNA Pol V for the production of scaffold transcripts to which AGO-siRNA complexes bind. The DDR complex (DRD1, DMS3, and RDM1) enables RNA Pol V transcription. The RDM1 subunit of the DDR complex also interacts with AGO4 and the de novo cytosine methyltransferase, DRM2, thus potentially serving as a bridge that recruits DRM2 to sites of RNA Pol V transcription.

domain protein family, is thought to assist the RNA Pol IV–RDR2 transcription complex. As a result, nuclear localization patterns of RNA Pol IV and RDR2 are perturbed in *clsy1* mutants.

dsRNAs that result from the collaboration of RNA Pol IV with RDR2 are cleaved into 24-nt duplexes by DICER-LIKE 3 (DCL3), and HEN1 methylates the three ends of the siRNAs on their 2' hydroxyl groups, helping to stabilize the siRNAs in the same way as for miRNAs (see Sec. 3.2). One strand of the duplex is then loaded into AGO4, or the closely related 24-nt siRNA-binding proteins, AGO6 or AGO9. Recruiting the resulting siRNA-AGO complexes is the function of RNA Pol V, whose RNA transcripts have been detected at a number of loci that are subjected to RdDM. These RNAs are dependent on the RNA Pol V active site and can be chemically crosslinked to RNA Pol V, suggesting that they are direct RNA Pol V transcripts. RNA Pol V transcription does not require siRNA biogenesis, but is dependent on the putative chromatin remodeler, DRD1, DMS3 (a protein that shares homology with the hinge domain regions of structural maintenance of chromosomes proteins, such as cohesins and condensins; Wier-

bicki et al. 2008; Wierzbicki et al. 2009) and REQUIRED FOR DNA METHYLATION1 (RDM1), a single-stranded DNA-binding protein with a preference for binding methylated DNA in vitro. DRD1, DMS3, and RDM1 physically associate within a multifunctional complex (DDR) that copurifies with RNA Pol V (Law and Jacobsen 2010; Zhang and Zhu 2011). Whether the DDR complex recruits RNA Pol V to target loci or mediates RNA Pol V transcriptional elongation is still unclear.

Based on chemical crosslinking and immunoprecipitation studies, current models suggest that AGO4-RISC complexes are recruited to target loci by siRNA base-pairing to RNA Pol V transcripts (Wierzbicki et al. 2008; Wierzbicki et al. 2009). In this way, RNA Pol V transcripts are thought to serve as scaffolds for recruiting the chromatin-modifying machinery into proximity of the chromatin to be modified. AGO4 also binds a tryptophan (W) and glycine (G)-rich region within the carboxy-terminal domain of the RNA Pol V largest subunit, further contributing to RISC assembly at RNA Pol V-transcribed loci (Lahmy et al. 2010).

Two AGO4-interacting proteins are important for RdDM: INVOLVED IN DE NOVO 2 (IDN2/RDM12), a



protein that binds dsRNAs with 5' overhangs, possibly siRNAs base-paired with RNA Pol V transcripts, and KOW DOMAIN-CONTAINING TRANSCRIPTION FACTOR (KTF1, also known as RDM3 or SPT5-LIKE) that shares similarity with the yeast RNA Pol II transcription factor, SPT5 (Lahmy et al. 2010; Law and Jacobsen 2010; Zhang and Zhu 2011). In vitro, KTF1 binds RNA and has a WG/GW-rich domain that facilitates interactions with AGO4. KTF1 might therefore play a role in the recruitment of AGO4 to RNA Pol V transcripts, promoting AGO4 slicing of RNA Pol V transcripts as well as recruitment of downstream silencing components.

At present, it is not clear how DRM2 DNA methyltransferase is recruited to Pol V-transcribed loci, but the RDM1 protein of the DDR complex might serve as a bridge between AGO4 and DRM2 (Zhang and Zhu 2011). RDM1 also interacts with RNA Pol II, which can substitute for RNA Pol V in the production of scaffold transcripts at some loci and is also implicated in the recruitment of RNA Pol IV and Pol V at some loci (Zheng et al. 2009). RDM1 might therefore be a key protein that can mediate DRM2 recruitment in both RNA Pol V- and Pol II-dependent pathways.

A classic epigenetic phenomena, first discovered in maize, is paramutation, the heritable alteration of gene activity that can occur when certain active and silenced alleles are brought together within the same nucleus. Maize genes wherein mutation disrupts paramutation include: MAINTENANCE OF PARAMUTATION 1 (MOP1; the ortholog of *Arabidopsis* RDR2); REQUIRED TO MAINTAIN REPRESSION 6 (RMR6; the ortholog of the *Arabidopsis* RNA Pol IV largest subunit, NRPD1); RMR1, a putative chromatin remodeling ATPase related to CLSY1 and DRD1; and MOP2/RMR7, one of three homologs of the second-largest subunit of *Arabidopsis* RNA Pol IV and Pol V (Arteaga-Vazquez and Chandler 2010; Erhard and Hollick 2011). These findings clearly implicate proteins of the RdDM pathway in paramutation yet it remains unclear whether RNA-directed cytosine methylation occurs and is the cause of gene silencing.

### 3.5.3 Repressive Histone Modifications Associated with Transcriptional Gene Silencing

At loci subjected to RdDM, the methylated DNA is wrapped by histones that bear posttranslational modifications typical of heterochromatin. Such a condensed chromatin state is refractive to transcription by RNA Pol I, II, or III. Several of the chromatin-modifying enzymes involved in establishing or maintaining heterochromatin (see Sec. 2.2) have been identified in genetic screens looking for mutants that interfere with RdDM. These include histone

H3K9 and H3K27 methyltransferases, the broad-specificity histone deacetylase HDA6, UBIQUITIN PROTEASE 26 (UBP26 or SUP32), which is required to deubiquitinate histone H2B, and JM14, a JumonjiC domain-containing protein that demethylates H3K4me3, a modification typical of actively transcribed loci. Collectively, these activities are indicative of a silencing process that includes the establishment of chromatin marks typical of the silenced state (reviewed in Matzke et al. 2009; Lahmy et al. 2010; Law and Jacobsen 2010; Haag and Pikaard 2011; Zhang and Zhu 2011).

### 3.5.4 RNA-Mediated Silencing of Endogenous Genes

Most RNA-directed DNA methylation and TGS is focused on retrotransposons and repetitive genetic elements that are remnants of transposons, presumably as a means of transposon taming to keep their proliferation in check (Zaratiegui et al. 2007). One consequence is that a number of regulatory genes are affected by their proximity to transposons or repetitive elements. For example, transposon-derived repeats in the promoter of the *Arabidopsis* flowering time gene FWA are targeted by RdDM, thus silencing the gene. Because many plant genes have transposon insertions in the vicinity of promoters or in introns, this mode of regulation is likely to be common in the plant kingdom. Indeed, Barbara McClintock's hypothesis that transposons act as controlling elements regulating adjacent host genes (McClintock 1950) has been confirmed in multiple cases.

Not all repetitive sequences subjected to RdDM share a connection with transposons or are likely candidates for being targeted by machinery adapted for genome defense. Notable examples are the repetitive 5S rRNA genes transcribed by RNA Pol III and the 45S ribosomal RNA genes transcribed by RNA Pol I (Layat et al. 2012). These genes are essential for ribosome synthesis and are clustered in long tandem arrays at several loci, each having hundreds of gene copies. RdDM appears to help shut down excess rRNA genes such that their effective dosage can be regulated according to the cellular demand for ribosomes and protein synthesis (Preuss et al. 2008).

### 3.6 Amplification and Spreading of siRNA-Mediated Silencing

An important aspect of siRNA-mediated silencing in plants is that initial siRNA production can trigger additional, secondary siRNAs. Moreover, siRNAs can move from cell to cell or be transported to other organs, thereby amplifying siRNA-mediated silencing ultimately executed at the tran-

scriptional or posttranscriptional levels (see also Dunoyer et al. 2013; Baulcombe and Dean 2013).

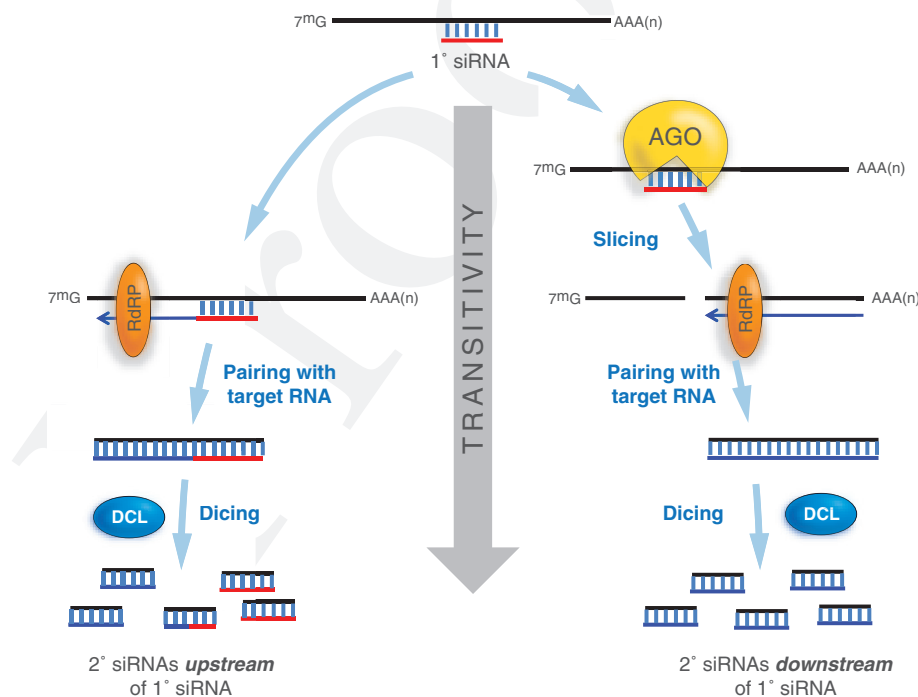
### 3.6.1 Transitivity

Transitivity is a term used to describe the generation of secondary siRNAs that are induced by a primary siRNA trigger. For instance, two adjacent genes that point toward one another can generate transcripts that overlap in their 3' regions and can base-pair. Dicing of the resulting dsRNA in the region of overlap can generate primary siRNAs that can then base-pair with a complementary long mRNA (or other long RNA) sequence and prime transcription by RNA-dependent RNA polymerase, particularly RDR6. The result is a dsRNA that extends from the siRNA primer to the 5' end of the RNA template. Subsequent dicing of these dsRNAs generates secondary RNAs corresponding to upstream (5') regions (left side of Fig. 7).

Q4 Secondary siRNAs can also be generated in the region 3' of a primary siRNA (or miRNA) trigger, as described above for *trans*-acting siRNAs, which are secondary siRNAs whose biogenesis is triggered by an miRNA. In this case, siRNA or miRNA-mediated cleavage of an RNA transcript facilitates the use of the uncapped 3' fragment as a template for prim-

er-independent RDR6 transcription (right side of Fig. 7). The mechanistic details of how this occurs are not yet clear, but RDR6 must initiate transcription from the 3' end of the RNA fragment, a site that is distal from the siRNA or miRNA cleavage site. This process is specifically triggered by 22-nt siRNAs or 22-nt miRNAs that associate with AGO1 (Chen et al. 2010; Cuperus et al. 2010; Manavella et al. 2012), but not by 21-nt siRNA-AGO1 complexes. Although 22-nt miRNAs are not common, they can be produced by DCL1 from dsRNA hairpin precursors that have an extra nucleotide in the miR strand relative to the 21-nt miR\* strand, forming a bulge that does not inhibit dicing. Presumably, a change in AGO1 conformation, or its association with other required proteins, occurs on binding 22-nt siRNAs, and results in the recruitment of RDR6. The resulting dsRNA is then diced by DCL4, with the help of the dsRNA binding protein DRB4, generating 21-nt secondary siRNAs that correspond to the region downstream from the primary siRNA or miRNA trigger.

Overall, the ability to produce secondary siRNAs both upstream of and downstream from the region complementary to the primary siRNA(s) magnifies the RNA silencing response, thus strengthening the plant's resistance to an invading virus or nucleic acid (Brodersen and Voinnet 2006).



**Figure 7.** Transitivity: amplification and spreading of secondary siRNAs from the site of a primary siRNA target. Primary siRNAs are thought to prime RNA-dependent RNA polymerase activity, resulting in dsRNAs extending toward the 5' end of the target RNA (*left*). Subsequent dicing gives rise to secondary siRNAs in the region upstream of the initiating primary siRNA. 22-nt siRNAs also have the property of recruiting RDR6 to the 3' fragment following AGO slicing (*right*), resulting in dsRNA and secondary siRNA biogenesis in the region downstream from the initiating, primary siRNA.

### 3.6.2 Non-Cell-Autonomous Silencing

Small RNAs can bring about posttranscriptional or transcriptional silencing in neighboring cells or even in cells in distant organs. For short-range movement, RNAs travel from their cell of origin into neighboring cells through plasmodesmata. This conclusion is supported by the fact that guard cells, which form the openings (stomata) for gas exchange in leaves, lack plasmodesmata and are excluded from receiving silencing signals from adjacent cells (Voinet et al. 1998). Long-range movement is a consequence of RNA loading into phloem cells, allowing their transport through the vascular system followed by their unloading and cell-to-cell spread in recipient tissues. This was shown in grafting experiments involving transgenes, mutants, and deep sequencing of small RNAs (Dunoyer et al. 2013).

Evidence that mobile RNAs are important transmitters of information between plant cells extends to the male and female gametophytes. In pollen, there is evidence that small RNAs produced by the vegetative cell can direct silencing in the two sperm cells. The hypothesis is that derepression of transposable elements in the vegetative cell allows for the biogenesis of siRNAs that then move to the sperm cells to reinforce the silencing of the corresponding transposons. In this way, epigenetic programming in sperm cells, in which transposition would result in frequent deleterious mutations, is guaranteed via the activity of the vegetative cell, which does not contribute genomic information to the next generation through fertilization (Slotkin et al. 2009). Likewise, abundant RNA Pol IV-dependent siRNAs of maternal origin accumulate in embryos and developing seeds and may play roles in the epigenetic reprogramming of paternal chromosomes (Mosher et al. 2009).

## 4 OUTLOOK

The past decade has witnessed an explosion of information concerning the proteins, RNAs, and chemical modifications that contribute to epigenetic control. However, there is much to learn about the nature of epigenetic inheritance and the role of epigenetics as a source of variation contributing to fitness and natural selection. Much of what we know thus far has come from the analysis of mutants generated using harsh mutagenesis treatments and comparison of these mutants, under laboratory conditions, to nonmutagenized reference plants referred to as being “wild type.” Breeders have long known that wild-type plants collected from different geographical origins are themselves a rich source of genetic diversity. Natural variation among ecotypes—strains or races of a species that are adapted to specific niches—reflects naturally occurring nucleotide sequence changes, recombination, or transposi-

tion events, gains, or losses of DNA sequences, or hybridization events. In plants with a high degree of inbreeding such as *A. thaliana*, individual ecotypes display a high degree of genetic homogeneity. However, the genetic diversity within the species, revealed by genomic comparisons of available ecotypes, is remarkable (<http://1001genomes.org>; Ossowski et al. 2010). It can be expected that the degree of epigenetic variation between ecotypes is also substantial, allowing an opportunity to explore whether epigenetic adaptations contribute to plant form, survival, and performance under different conditions (Becker et al. 2011; Richards 2011; Schmitz et al. 2011). Important agricultural challenges and questions, such as understanding the molecular basis for genotype by environment interactions or heterosis (hybrid vigor), are likely to have answers rooted in epigenetic regulatory mechanisms.

Understanding what is genetic and what is epigenetic is not a simple matter. For instance, there is clear evidence for genetic changes that induce switches in epigenetic states (Durand et al. 2012), such as mutations that allow read-through transcripts to bring about the silencing of adjacent genes, including tumor suppressor genes in humans. Conversely, epigenetically controlled accessibility of DNA is likely to influence the probability of genetic rearrangements caused by recombination or transposon mobilization (reviewed in Magori and Citovsky 2011; Mirouze and Paszkowski 2011). Understanding the degree to which genetically programmed expression of DNA-binding transcription factors combines with epigenetic mechanisms that limit enhancer or promoter specificity, RNA polymerase processivity or splice-site selection, is a major challenge for the future.

Our understanding of how environmental conditions such as photoperiod or temperature provoke changes in RNA- or chromatin-based transcriptional regulation is still in its infancy. The majority of these changes lasts only as long as the trigger is present and are therefore not thought to be epigenetic. However, some environmental conditions induce altered chromatin and gene expression states that persist even after a return to the original environmental condition, as in the case of vernalization (Baulcombe and Dean 2014) in which plants “remember” their experience of winter to flower the following spring. There is also the possibility that environmentally or pathogen-induced epigenetic states might be transmitted to progeny if the changes occur in meristems and can be maintained through meiosis. So far, there is only rudimentary evidence for transmission and inheritance of adaptive epigenetic states, as opposed to DNA sequence-based inheritance (Paszkowski and Grossniklaus 2011; Pecinka and Mittelsten Scheid 2012). Nevertheless, with our growing insight into epigenetic regulation and the transmission of mobile



small RNAs influencing chromatin states, such neo-Lamarckian possibilities warrant careful consideration.

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## WWW RESOURCES

<http://www.arabidopsis.leeds.ac.uk/act/coexpalyser.php> *Arabidopsis* coexpression mining  
<http://www.arabidopsis.org/index.jsp> TAIR

- <http://asrp.cgrb.oregonstate.edu> *Arabidopsis* small RNA
- <http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi> Gene expression in *Arabidopsis*
- <http://www.chromdb.org> Chromatin genes
- <http://www.cymate.org/> Bisulfite sequence analysis
- <http://epigara.biologie.ens.fr/cgi-bin/gbrowse/a2e/> *Arabidopsis* epigenetics EPIGARA
- [http://epigenome.rutgers.edu/cgi-bin/gb2/gbrowse/Histone\\_modifications](http://epigenome.rutgers.edu/cgi-bin/gb2/gbrowse/Histone_modifications) *Arabidopsis* histone modifications Rutgers
- <http://www.erapg.org/publicpage.m?key=everyone&trail=/everyone> European plant genomic research
- <https://www.genevestigator.com/gv/> Gene expression in several organisms
- <http://genomes.mcdb.ucla.edu/AthBSseq/> *Arabidopsis* methylome of silencing mutants
- <http://katahdin.mssm.edu/kismeth/revpage.pl> Bisulfite sequence analysis
- [https://www.mcdb.ucla.edu/Research/Jacobsen/LabWebSite/P\\_EpigenomicsData.shtml](https://www.mcdb.ucla.edu/Research/Jacobsen/LabWebSite/P_EpigenomicsData.shtml) *Arabidopsis* histone modifications UCLA
- <http://mpss.udel.edu> MPSS (Massively parallel signature sequencing)
- <http://neomorph.salk.edu/epigenome/epigenome.html> *Arabidopsis* epigenome
- <https://www.plant-epigenome.org> EPIC (Epigenomics of Plants International Consortium)
- <http://signal.salk.edu/cgi-bin/methylome> *Arabidopsis* methylome
- <http://1001genomes.org> *Arabidopsis* genetic variation

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### Queries

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- Q1 Please check change to 3.4: There is no Section 3.4.1 in the article.
- Q2 Verdel et al. (2009) is cited in the text, but is not included in the reference list. Please provide full bibliographic reference.
- Q3 In what section "below?"
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## Epigenetic Regulation in Plants

Craig S. Pikaard and Ortrun Mittelsten Scheid

*TOC Blurb:* All major eukaryotic epigenetic mechanisms are used by plants. These may contribute to their phenotypic plasticity and their ability to survive and reproduce in unpredictable environments.