Epigenetic control of plant development: new layers of complexity

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Important aspects of plant development are under epigenetic control, that is, under the control of heritable changes in gene expression that are not associated with alterations in DNA sequence. It is becoming clear that RNA molecules play a key role in epigenetic gene regulation by providing sequence specificity for the targeting of developmentally important genes. RNA-based control of gene expression can be exerted posttranscriptionally by interfering with transcript stability or translation. Moreover, RNA molecules also appear to direct developmentally relevant gene regulation at the transcriptional level by modifying chromatin structure and/or DNA methylation.

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Abbreviations

AG	*AGAMOUS*
AP2	*APETALA2*
CLF	*CURLY LEAF*
CMT3	*CHROMOMETHYLASE3*
DME	*DEMETER*
DRM1	*DOMAINS-REARRANGED METHYLASE1*
Eed	Embryonic ectoderm development
E(z)	Enhancer of Zeste
Esc	Extra sex combs
FIE	*FERTILIZATION-INDEPENDENT ENDOSPERM*
FIS2	*FERTILIZATION-INDEPENDENT SEED2*
FLC	*FLOWERING LOCUS C*
H3K27	histone 3 lysine 27
H3K9	histone 3 lysine 9
HP1	HETEROCHROMATIN PROTEIN1
KYP	*KRYPTONITE*
LHP1	*LIKE HP1*
MEA	*MEDEA*
MET1	*DNA METHYLTRANSFERASE1*
MET1as	MET1 antisense
miR	microRNA
PcG	*Polycomb* group
PHB	*PHABULOSA*
PHE	*PHERES*
PHV	*PHAVOLUTA*
PTGS	posttranscriptional gene silencing
5′RACE	5′ rapid amplification of cDNA ends
RdDM	RNA-dependent DNA methylation
REV	*REVOLUTA*
ROS1	*REPRESSOR OF SILENCING1*
TFL2	*TERMINAL FLOWER2*
TGS	transcriptional gene silencing
vrn1	*vernalization1*

Introduction

The accurate regulation of gene expression in space and time is fundamental for development. The spatial and temporal expression profiles of many genes are controlled genetically by specific DNA sequences. Moreover, many aspects of development involve epigenetic regulation: mitotically and/or meiotically heritable yet reversible changes in gene expression without changes in DNA sequence. Many epigenetic changes depend on the recognition of sequence homology at the DNA or RNA level. This recognition can lead to transcriptional gene silencing (TGS), which is associated with DNA methylation and/or chromatin modifications, or to posttranscriptional gene silencing (PTGS), either by sequence specific RNA degradation or by inhibition of translation. Mechanistic aspects of PTGS and TGS have been the subjects of several recent reviews (e.g. [1•,2,3•,4–6]) and are not discussed here. We focus on developmental aspects that are controlled by PTGS or TGS regulatory mechanisms.

Small RNAs mark silent genes

The discovery [7] and cloning [8–14] of a plethora of small regulatory RNAs that are associated with PTGS in plants — and the analogous RNA interference phenomenon in animals — have provided a clue as to which genes may be regulated by small RNAs [15•]. Historically, small RNAs are grouped into three classes (reviewed in [16]): small temporal RNAs (stRNA) [17], small interfering RNAs (siRNA) [18], and microRNAs (miRNA) [8–10]. However, such a classification may be misleading, or based on criteria that are too narrow [14,19–22]. An additional ambiguous term, shRNA, is used to describe either 'short heterochromatic RNA' [23] or 'short hairpin RNA' [24]. To prevent confusion, we refer to these RNAs collectively as 'small RNAs', encompassing all of the classes mentioned above.

A key feature of many small RNAs is that their transcription and/or processing is controlled in time and space [8–11,14,25]. Furthermore, almost 70% of the small RNAs analyzed by Rhoades and co-workers [15•] were predicted to have transcription factors as targets, whereas only 6% of all protein-coding genes in *Arabidopsis* are transcription factors. Taken together, these observations suggest that small RNAs have a regulatory function in plant development. Although the regulation of endogenous mRNAs by small RNAs has been shown experimentally [12,26••,27],

it remains difficult to associate these small-RNA-mediated effects with developmental phenotypes.

Posttranscriptional effects mediated by small RNAs

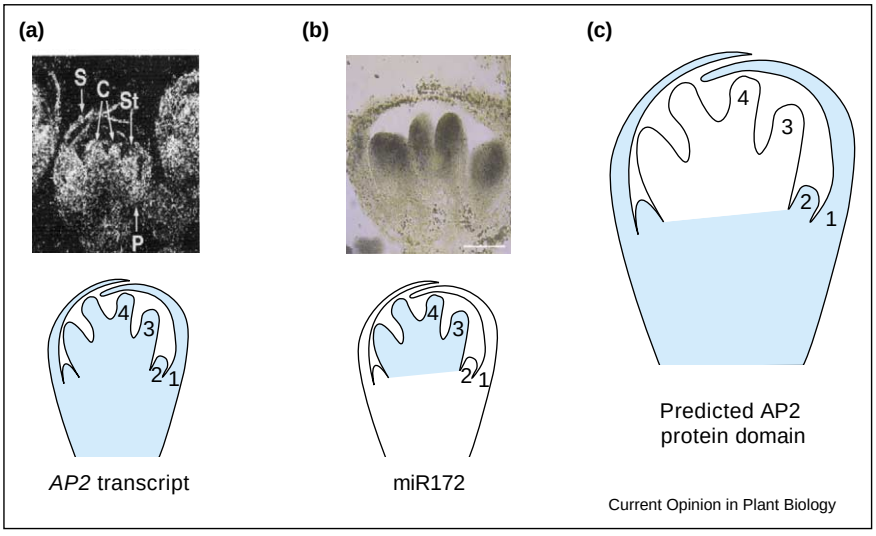
A re-examination of mutants that have been obtained by activation tagging has recently shed light on why it is difficult to find phenotypes that result from the perturbation of small RNA regulation: small RNA-encoding loci rather than protein-coding genes were overexpressed in these mutants. For example, the *Arabidopsis* gain-of-function mutant *jaw-D* has defects in leaf shape and curvature [28]. In this mutant, the small RNA miR–JAW is strongly upregulated, causing RNA cleavage of at least five members of the TCP transcription factor family [29]. To demonstrate that miR–JAW is responsible for cleavage of the TCP transcripts, Palatnik *et al.* [30••] created mutations in two TCP genes that altered their miR-JAW target sequence without affecting the corresponding amino-acid sequence. When introduced into *jaw-D* plants, these mutant transcripts not only were resistant to cleavage by miR–JAW but also rescued the *jaw-D* phenotype at least partially. Activation of miR–JAW is therefore responsible for the cleavage of TCP transcripts and for the phenotype of *jaw-D* mutants [30••]. It is worth noting that the miR–JAW locus has a homolog in the *Arabidopsis* genome, miR–J_h, which may also participate in the cleavage of TCP transcripts. Thus, mutations in just one of these homologs, miR–JAW or miR–J_h, may have no phenotypic effects. Similarly, there are five putative TCP targets with possibly redundant functions. The overexpression of small RNA-encoding loci and the expression of transcripts that contain mutated target sites may overcome the problem of genetic redundancy, which seems to be common in developmental processes that are regulated by small RNAs.

Another case of small RNA-mediated regulation of gene expression is illustrated by the class-III homeodomain-leucine zipper (HD-ZIP) genes, which are involved in establishing the adaxial–abaxial polarity of lateral organs. Dominant gain-of-function alleles have been described for three class-III HD-ZIP genes: gain-of-function alleles of *PHABULOSA* (*PHB)* and *PHAVOLUTA* (*PHV*) lead to a dramatic adaxialization of lateral organs [31], whereas gain-of-function alleles of *REVOLUTA* (*REV*) alter leaf development [32] and vascular patterning [33••]. The gain-of-function mutations in these genes are substitutions or small insertions, which all map to a short, highly conserved stretch in a putative sterol/lipid-binding domain (START domain). For *phv* and *phb*, it had been hypothesized that single-amino-acid changes in the START domain render PHV and PHB constitutively active, either by disrupting its ligand binding or by abolishing the need for such binding [31]. However, the discovery of the small RNAs miR165 and miR166, which are complementary to the stretch mutated in *phv*, *phb* and *rev*, suggests that *PHV*, *PHB* and *REV* are regulated by small RNAs. Thus, the gain-of-function phenotypes may be due to the loss of this regulation rather than to changes in protein sequence [15•]. Indeed, a modified *REV* cDNA, in which the putative target site of miR165 and miR166 is altered without affecting the REV protein sequence, phenocopied the *rev* mutation when introduced into wildtype plants. In contrast, an unmodified *REV* cDNA had no effect, demonstrating that the phenotype observed in *rev*, and probably also in *phv* and *phb*, is caused by the loss of small-RNA-mediated regulation [33••]. In the *Arabidopsis* genome, two loci encode miR165 and seven loci encode miR166 [14]. These small RNAs regulate at least three target genes that have partially overlapping functions, indicating that there may be considerable redundancy in this process.

Translational effects mediated by small RNAs

In the case of *REV* regulation described above, a 3′ cleavage product was found in 5′ rapid amplification of cDNA ends (5′ RACE) experiments aimed at determining the 5′ end of RNA species. This suggests that miR165 and miR166 cause the degradation of their target RNAs. Target degradation may not be the main mode of regulation by small RNAs, however, as illustrated for the small RNA miR172. The predicted target of miR172 is a small subfamily of *APETALA2* (*AP2*)-like transcription factor genes that includes the floral homeotic gene *AP2* itself [25]. Kasschau and co-workers [27] found 5′ RACE products of *AP2* and three *AP2*-like genes whose 5′ ends were all located in the centre of complementarity between miR172 and its predicted targets, suggesting that miR172 regulates the *AP2*-like genes by RNA degradation. In contrast, Aukerman and Sakai [34••] reported that the main mode of miR172 action is translational inhibition. They screened an activation-tagged population of *Arabidopsis* for early flowering and found a mutant in which miR172 is upregulated. In addition to early flowering, this mutant showed floral defects that were reminiscent of strong *ap2* alleles, such as the absence of petals and the transformation of sepals to carpels [35]. Immunoblot analyses using an antibody that is specific to AP2 showed that the AP2 protein was dramatically reduced in plants that overexpressed miR172, whereas the transcript levels of *AP2* and those of *AP2*-like target genes were unaffected. This suggests that translational inhibition by miR172 is responsible for the mutant phenotype. To resolve this apparent contradiction, Aukerman and Sakai [34••] performed 5′ RACE experiments and found the RNA cleavage products that had been reported previously by Kasschau *et al.* [27]. However, these cleavage products were not detectable on RNA blots, whereas the full-length RNA was. Taken together, these findings suggest that miR172 regulates its targets primarily by a translational mechanism, and that the small amount of RNA cleavage products may result

Figure 1



Model of how miR172 expression could restrict AP2 protein accumulation in whorls 1 and 2 of the flower meristem. **(a)** Autoradiograph (top) and schematic representation (bottom) of *AP2* mRNA accumulation in stage 7 flower meristems. **(b)** Photograph (top) and schematic representation (bottom) of miR172 accumulation in stage 7 flower meristems as shown in [36••]. **(c)** Proposed expression domain of AP2 protein. Numbers indicate whorls. Note that the expression domains of the miR172 and AP2 protein in (b) and (c) do not overlap but are complementary. Images courtesy of (a) the American Society of Plant Biologists and (b) X Chen. C, carpel; P, petal; S, sepal; St, stamen.

from an overlap between the translational and RNA cleavage pathways [34••].

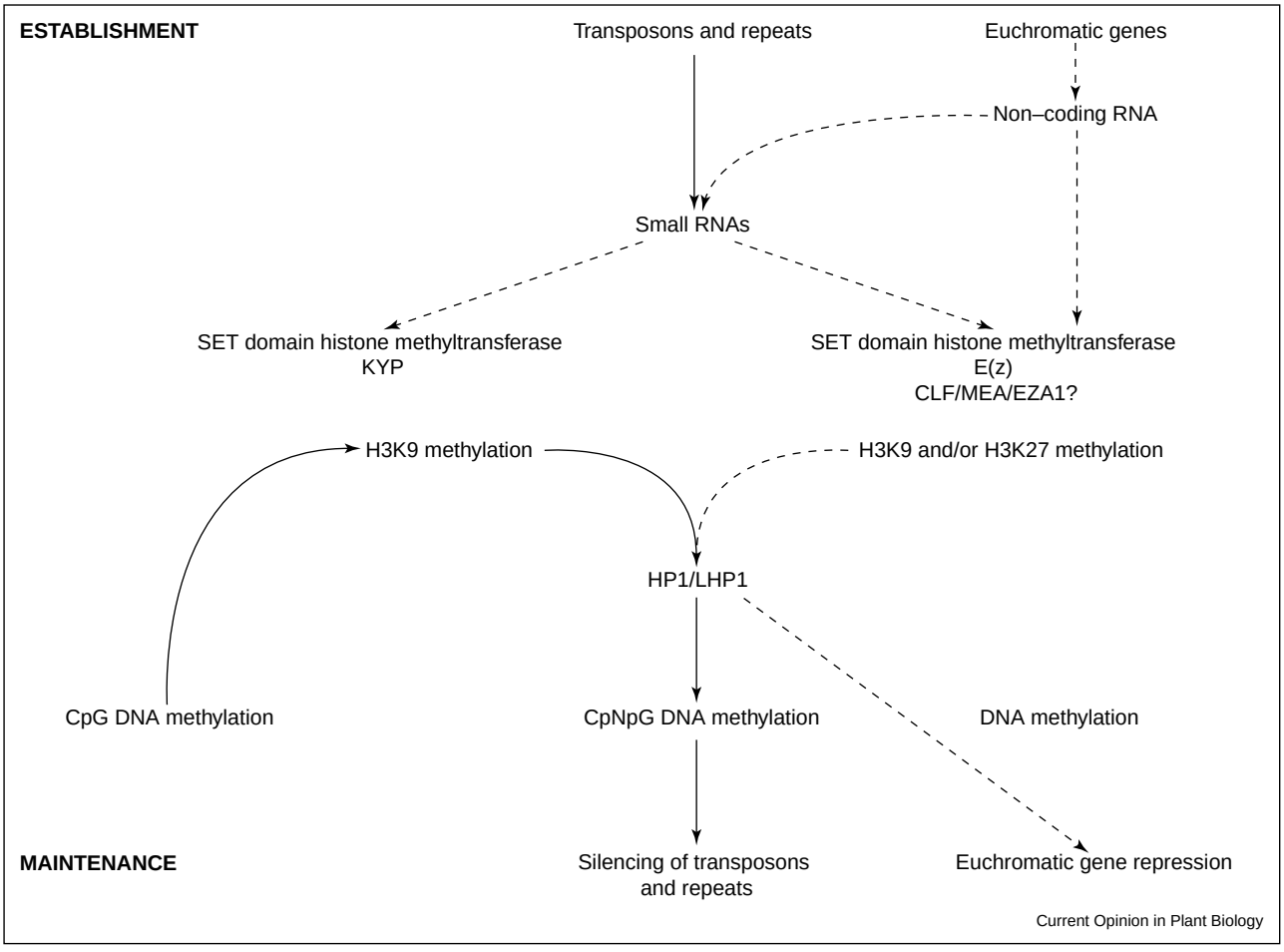
A translational mechanism for miR172 action has also been described by Chen [36••]. *AP2* restricts the expression of another floral homeotic gene, *AGAMOUS* (*AG*), to whorls 3 and 4 of the developing flower [37]. *AP2* transcript is found in all whorls [38], however, indicating that AP2 acts in concert with another unknown factor that is expressed in whorls 1 and 2 to restrict *AG* expression to whorls 3 and 4. Using a modified *in-situ* hybridization procedure, Chen [36••] was able to visualize the expression patterns of miR172 in developing flowers and found that miR172 is expressed only in whorls 3 and 4. This finding suggests that the *AG* expression domain is defined by miR172-mediated suppression of *AP2* translation in whorls 3 and 4 rather than by the expression of a co-factor in whorls 1 and 2 (Figure 1).

Transcriptional control of gene expression involving RNAs

Viroid RNA can trigger DNA methylation, a phenomenon termed RNA-dependent DNA methylation (RdDM) [39]. The deliberate expression of transgenes that produce double-stranded RNA (dsRNA) leads to the methylation and silencing of homologous genes by TGS, if the dsRNA is homologous to the promoter [40,41], or by PTGS, if the homology lies within the coding sequence [41]. Small RNAs are associated with both types of silencing, suggesting that PTGS and TGS are mechanistically related [41]. It was recently shown that the RNA-interference machinery is involved in the establishment of inactive chromatin states in *Schizosaccharomyces pombe* ([24,42]; see Figure 2 for an overview). These findings suggest that transcriptional repression may be initiated or maintained by RNAs. Furthermore, the regulation of some imprinted genes, dosage compensation, and X-inactivation in animals involves non-coding RNAs (reviewed in [43]). Transcriptional repression of the inactive X chromosome depends on *Polycomb* group (PcG) complexes, indicating that PcG repression may also involve RNA [44•,45•].

PcG and *trithorax* group (trxG) proteins, which were first identified in *Drosophila*, mediate the cellular memory of transcriptional states over many cell divisions. There are two PcG repressor complexes in *Drosophila*, the Enhancer of Zeste–Extra sex combs [E(z)–Esc] complex and the *Polycomb* Repressive Complex 1 (PRC1). These complexes are involved in the initiation and long-term memory of PcG repression, respectively (reviewed in [46]). E(z) methylates histone 3 lysine 27 (H3K27), and this histone methylation mark correlates with homeobox gene (HOX) repression ([47•–49•]; Figure 2). Furthermore, the mammalian E(z)–Esc homologs, Embryonic ectoderm development (Eed)–Enx1 and Eed–Ezh2, are transiently recruited during X-chromosome inactivation to methylate histone 3 lysine 9 (H3K9) and/or H3K27 [44•,45•]. H3K9 or H3K27 methylation is recognized by HETEROCHROMATIN PROTEIN1 (HP1), which forms inactive chromatin ([50,51]; Figure 2). Although not yet demon-

Figure 2



Flowchart highlighting key steps in the establishment and maintenance of transcriptional repression. Solid arrows indicate events that are supported by experimental evidence in plants. Dotted arrows indicate events that are suggested to occur in plants or documented in non-plant systems.

strated, it is possible that PcG repression in plants involves RNA, as X inactivation in mammals depends on both non-coding RNAs and PcG complexes.

Target genes of PcG repression in plants

Only complexes of the E(z)–Esc-type are present in plants (reviewed in [52,53]). Mutations in PcG genes cause developmental aberrations, such as improper response to vernalization, early flowering, aberrant floral organ identity, or abortive seed development. Interestingly, PcG target genes in plants encode MADS-domain transcription factors, many of which are functionally but not structurally homologous to homeotic genes in *Drosophila*, which are the main targets of PcG repression (reviewed in [52,53]). As the composition of PcG complexes has been extensively reviewed [52,53], we focus on the regulation of PcG targets in plants and on the possible involvement of RNA and methylation in these processes.

AGAMOUS repression by *CURLY LEAF* and *EMBRYONIC FLOWER*

AG, which encodes a MADS-domain transcription factor that has tight temporal and spatial regulation, is a target of PcG complexes in plants. In *ag* mutants, carpels and stamens are replaced by sepals and petals, and the floral meristems are indeterminate. Plants that overexpress *AG* under the control of the constitutive Cauliflower mosaic virus (CaMV) 35S promoter (35S::AG) flower early, produce a terminal flower, and have perianth organs that are transformed into reproductive organs. Mutations in the PcG genes *CURLY LEAF* (*CLF*), *EMBRYONIC FLOWER1* (*EMF1*) and *EMF2* cause certain phenotypes that are typical of 35S::AG-expressing plants. *AG* is expressed ectopically in *clf*, *emf1* or *emf2* mutants [54–57], suggesting that *AG* is repressed by PcG proteins. In *clf* mutants, the expression of *AG* is initiated correctly in young floral meristems, but *AG* is expressed ectopically in

the outer whorls during later stages of development [55]. It is worth noting that a large intron with enhancer activity is required to maintain the repression of *AG* by *CLF* [58]. This intron is also hypermethylated in plants that have reduced and redistributed DNA methylation caused by antisense repression of *DNA METHYLTRANSFERASE1* (*MET1*); these plants phenocopy *ag* mutants [59]. It will be interesting to investigate whether RNAs are involved in targeting PcG repression and DNA methylation to this intron.

FLOWERING LOCUS C repression by *VERNALIZATION*

The MADS-box gene *FLOWERING LOCUS C* (*FLC*), a major floral repressor, is another target of PcG repression [60]. Upon vernalization (i.e. prolonged exposure to cold temperature), the transcription of *FLC* is repressed, thereby promoting flowering. Vernalization leads to the stable repression of *FLC* long before flowering, suggesting that *FLC* repression is maintained over many mitotic cycles. Two mutants, *vernalization1* (*vrn1*) and *vrn2*, have been isolated in which *FLC* repression is established but not maintained after vernalization [61,62]. The *VRN1* and *VRN2* genes encode PcG genes that are homologous to Suppressor of Zeste12 [Su(Z)12] [61] and an unspecific DNA-binding factor, respectively [62]. Like *AG*, *FLC* contains a large intron that is required for the maintenance of *FLC* repression [63], suggesting that *AG* and *FLC* are repressed by a similar mechanism involving PcG complexes. The intron was found to have a more open chromatin configuration in *vrn2* mutants than in wildtype plants [61].

PHERES repression by the MEA–FIE PcG complex

Recently, targets have also been isolated for the MEA–FIE PcG complex, which contains *MEDEA* (*MEA*) [64], *FERTILIZATION-INDEPENDENT SEED2* (*FIS2*) [65], *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) [66] and *MSI1* [67•], collectively referred to as the *FIS*-class genes. GeneCHIP analysis of *fis* mutants led to the identification of a target gene for *FIS*-class genes, *PHERES* (*PHE*), which is another MADS-box gene [68••]. During seed development, *PHE* expression is initiated shortly after fertilization and then downregulated. In *fis* mutants, *PHE* transcription is initiated correctly but the subsequent repression is compromised. This is reminiscent of the deregulation of PcG target genes in *Drosophila* PcG mutants. *MEA* interacts directly with the promoter sequences of *PHE*, as revealed by chromatin immunoprecipitation assays using αMEA and αFIE antibodies. *PHE* is also repressed in the *decreased DNA methylation1* (*ddm1*) mutant [68••,69], suggesting that *PHE* is regulated by DNA methylation and/or by chromatin remodeling [70,71]. This regulation is reminiscent of the regulation of *AG* in MET1 antisense (MET1as) plants: despite genome-wide hypomethylation, the *AG* gene was hypermethylated and repressed in these plants [59]. Unlike *AG* and *FLC*, *PHE* is intronless. Nevertheless, the establishment of sequence-specific PcG silencing is likely to involve a similar mechanism at each of these three loci.

MADS-box gene repression by *LIKE HETEROCHROMATIN PROTEIN1/TERMINAL FLOWER2*

Arabidopsis has a sole homolog of *HP1*, *LIKE HP1* (*LHP1*) [72], also known as *TERMINAL FLOWER2* (*TFL2*) [73•]. Interestingly, mutants that are deficient in *LHP1/TFL2* have pleiotropic phenotypes, some of which (e.g. a terminal flower) are reminiscent of plants that have derepressed *AG* expression. Indeed, *AG* and other MADS-box genes are derepressed in *tfl2* [73•], suggesting a role for *LHP1/TFL2* in the establishment or maintenance of MADS-box gene repression. Interestingly, heterochromatic genes are not derepressed in *lhp1/tfl2* mutants, suggesting that the main targets of *LHP1/TFL2* are in euchromatic regions [73•].

Is methylation involved in MADS-box gene repression?

LHP1 has been shown to bind to H3K9 methylated histones in *Arabidopsis* [74••] and to interact with *CHROMOMETHYLASE3* (*CMT3*), a DNA methyltransferase that methylates cytosines at CpNpGs [75]. *cmt3* mutants do not show phenotypic aberrations, however, despite their heavy or complete loss of DNA methylation at all of the CpNpG sites investigated. This indicates either that DNA methylation at CpNpG is irrelevant for MADS-box gene repression or that *CMT3* acts redundantly with one of the two other CMT homologs in *Arabidopsis* (http://chromdb.biosci.arizona.edu/). The identification of transposons as the main targets of *CMT3* in genome-wide profiling of DNA methylation in *cmt3* mutants supports the first notion [76]. Conversely, *CMT3* seems to act redundantly with *DOMAINS-REARRANGED METHYLASE1* (*DRM1*) and *DRM2*, two *de novo* DNA methyltransferases [77]: *drm1 drm2 cmt3* triple mutants showed pleiotropic phenotypes [78]. It remains to be determined whether these phenotypes are associated with the derepression of *AG* or with other MADS-box genes.

MET1 acts as the maintenance and *de novo* methyltransferase at CpGs [79]; it is required for both the transmission of epigenetic marks during gametogenesis [80•] and for RdDM (W Aufsatz, M Matzke, personal communication). The role of *MET1* in the repression of MADS-box genes is controversial. Finnegan *et al.* [79] reported that *AG* is derepressed in MET1as, whereas Jacobsen *et al.* [59] found that *AG* was repressed and hypermethylated in similar transgenic lines. These apparently contradicting results may be explained either by ecotype differences or by secondary effects that occurred in the MET1as lines. Indeed, loss of H3K9 methylation was observed in *met1*

mutants [81•,82•], suggesting that CpG methylation guides histone H3K9 methylation. Conversely, mutants that are deficient in *KRYPTONITE* (*KYP*), which encodes a H3K9 methyltransferase, were devoid of H3K9 and CpNpG DNA methylation [74••]. Thus, in this specific-sequence context, histone methylation precedes DNA methylation. It appears unlikely that *KYP* is involved in the repression of MADS-box genes as *kyp* mutants do not show phenotypic abnormalities even after extensive inbreeding [74••]. These findings suggest either that H3K9 methylation is dispensable for MADS-box gene repression or that histone H3K9 methylation at MADS-box target loci is mediated by another of the eight *Arabidopsis KYP* homologs.

Do RNAs guide gene-specific activation?

Genomic imprinting refers to parent-of-origin-dependent gene regulation [83]. For example, only maternally but not paternally inherited *MEA* alleles are active after fertilization [84]. *DEMETER* (*DME*), a transcriptional activator of *MEA* before fertilization, may be involved in this process [85••]. *DME* encodes a DNA glycosylase that has the capacity to nick the promoter sequences of *MEA*. A similar glycosylase gene, *REPRESSOR OF SILENCING1* (*ROS1*), was found to prevent TGS at a repetitive transgene locus despite the presence of small RNAs that were homologous to the promoter sequence of the transgene [86••]. ROS1 specifically nicked methylated CpNpG, but not methylated CpG or unmethylated DNA substrates *in vitro*, suggesting that ROS1 activity was guided by specific DNA-methylation patterns that eventually led to the activation of the transgene. These specific methylation patterns are established by an RdDM mechanism, and so it is tempting to speculate that the activation of certain epigenetically regulated loci, possibly including imprinted genes such as *MEA*, may involve RNAs.

Conclusions

Plant development requires the precise temporal and spatial expression of regulatory genes, which is partly mediated by epigenetic mechanisms at the transcriptional or posttranscriptional level. The precise molecular mechanisms of transcriptional control during plant development are not fully understood. The identification of PcG targets marks an important step in elucidating the underlying mechanisms. However, the expression of PcG target genes is usually limited to a small number of cells, such as meristematic or gametophytic cells, that are embedded in non-expressing tissues. Novel dissection methods, such as laser-capture microscopy [87,88], combined with highly sensitive detection procedures may therefore be required for the analysis of DNA or chromatin modifications at target loci.

It is becoming more and more evident that small RNAs are involved in many epigenetic phenomena and play an important role during plant development by interfering with transcript stability or translation. However, their action has been masked by the genetic redundancy of small-RNA-encoding loci and their target genes. The ectopic expression of small RNAs and the expression of genes that have altered miRNA target sites have proven valuable tools in unraveling the posttranscriptional control of gene expression during development. The application of such new approaches promises to unravel many novel aspects of epigenetic gene regulation during plant development in the near future.

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