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Small RNA-mediated epigenetic modifications in plants

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Epigenetic modifications in plants can be directed and mediated by small RNAs (sRNAs). This regulation is composed of a highly interactive network of sRNA-directed DNA methylation, histone, and chromatin modifications, all of which control transcription. Identification and functional characterization of components of the siRNA-directed DNA methylation pathway have provided insights into epigenetic pathways that form heterochromatin and into chromatin-based pathways for gene silencing, paramutation, genetic imprinting, and epigenetic reprogramming. Next-generation sequencing technologies have facilitated new discoveries and have helped create a basic blueprint of the plant epigenome. As the multiple layers of epigenetic regulation in plants are dissected, a more comprehensive understanding of the biological importance of epigenetic marks and states has been developed.

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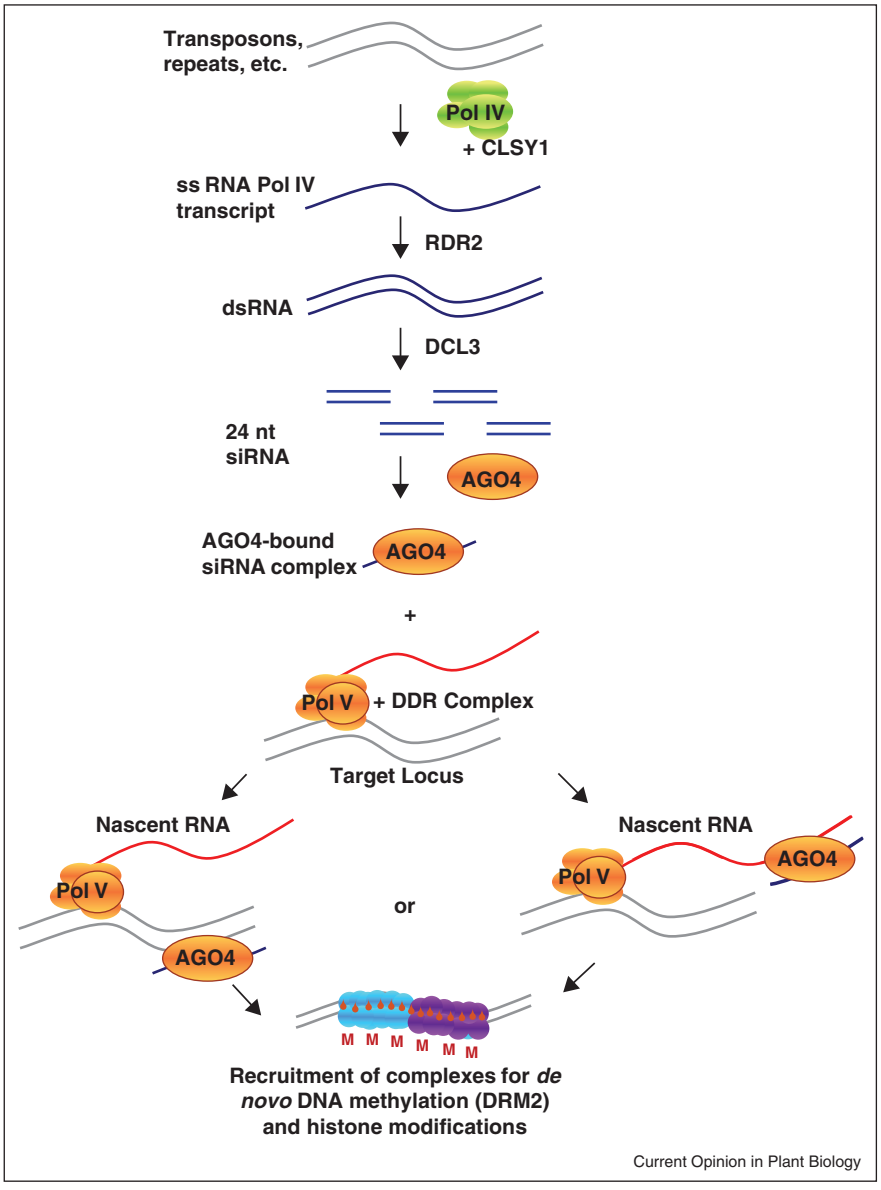
Introduction

Small RNAs (sRNAs) are now known to be a core component of a signaling network that mediates epigenetic modifications in plants. Epigenetic regulation can be mediated through a dynamic interplay between sRNAs, DNA methylation, and histone modifications, which together modulate transcriptional silencing of DNA. Regulatory sRNAs are short (approximately 20–24 nt in length), noncoding RNAs produced through the RNA interference (RNAi) pathway that involves the plant-specific DNA-dependent RNA polymerases Pol IV and Pol V [1,2], the RNA-dependent RNA polymerase RDR2 [3,4], the double-stranded RNA endonuclease DICER-LIKE3 (DCL3) [4,5], and at least two Argonautes, AGO4 and AGO6 [6–9].

sRNAs 21 nt in length are typically microRNAs (miRNAs) or trans-acting small interfering (ta-siRNAs), both of which are involved in post-transcriptional silencing. Small interfering RNAs (siRNAs) typically 24 nt in length are involved in heterochromatin formation and transcriptional gene silencing by guiding sequence-specific DNA and histone methylation through a pathway termed RNA-directed DNA methylation (RdDM) [1,10,11,12••,13]. Targeted RdDM begins with siRNAs produced by the RNAi pathway. At different steps, this pathway utilizes both of the RNA polymerases Pol IV and Pol V. RNA polymerase IV acts upstream of Pol V, functioning in a complex with CLASSY1 (CLSY), a SNF2-like chromatin remodeling factor [14] and RDR2, which copies single-stranded RNA (ssRNA) into double-stranded RNA (dsRNA). The dsRNA molecules are cleaved by DCL3 [4,15] into 24 nt heterochromatic siRNAs that are recruited by an effector complex containing either AGO4 or AGO6 to help guide chromatin modifications to homologous DNA sequences [6–10,16]. Pol V acts downstream in a complex termed DDR [17••] composed of DEFECTIVE IN RNA DIRECTED DNA METHYLATION1 (DRD1), another SNF2-like chromatin remodeling factor [18], DEFECTIVE IN MERISTEM SILENCING 3 (DMS3), a structural-maintenance-of-chromosomes hinge domain-containing protein [19] and RNA-DIRECTED DNA METHYLATION 1 (RDM1), a novel protein [17••]. Pol V with the DDR complex functions to amplify and reinforce siRNA production and to mediate *de novo* methylation at the target sites of siRNAs [1,13]. Pol V, with the above-mentioned accessory factors, is believed to transcribe genomic sequences that have been targeted to interact with siRNAs [1]. The AGO4-bound siRNA complex can either interact with a nascent Pol V-derived RNA or the target DNA to facilitate recruitment of effectors of *de novo* DNA methylation and histone modifying complexes to the target loci [1,20–22,23••].

The Pol IV-mediated production of siRNAs described above reflects primary RdDM (1° RdDM), and the siRNAs produced by Pol IV form the most abundant class of sRNAs (Figure 1). The siRNAs produced at this stage can be amplified by a turnover mechanism in which Pol IV transcribes the methylated DNA template, thereby producing an aberrant or perhaps atypically processed RNA that can be copied by RDR2 leading to the production of additional 1° siRNAs that can trigger methylation at the target region (Figure 2) [11,24,25]. Another important aspect of RdDM utilizes 2° siRNAs to trigger the spreading of methylation into areas adjacent and beyond the 1° siRNA-targeted sites [19,23••]. It is possible that some of the Pol IV 1° sRNAs may act in *trans* at distal, related sites, to direct 2° RdDM in a Pol V-dependent manner.

Figure 1

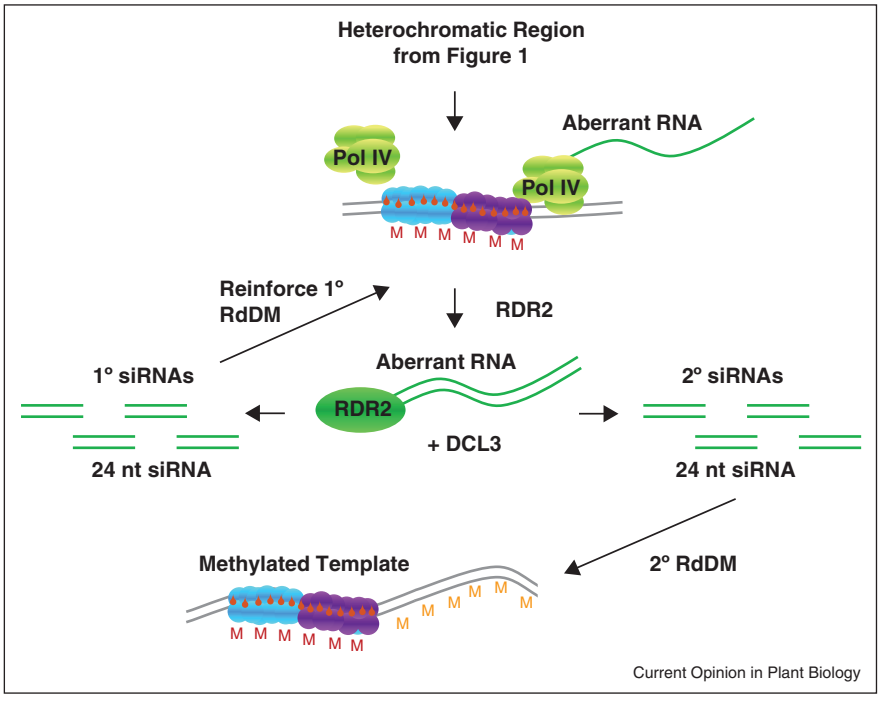


Heterochromatin formation via *de novo* DNA methylation and the recruitment of histone modifying enzymes. Data suggest that RNA polymerase IV transcribes transposons and other genomic regions, recruits RDR2 to make a double-stranded RNA that is cleaved by DCL3 into 24 nt sRNAs. These are loaded into an AGO4 complex, and this complex is then either recruited to function with the Pol V-DDR complex, or the AGO4 complex associates with Pol V-derived nascent transcripts. The activity of these proteins recruits *de novo* DNA methyltransferases including DRM2, as well as other chromatin remodeling enzymes.

For 2° RdDM, Pol IV is believed to transcribe a methylated target template and the downstream sequence. The result is an aberrant RNA that gets copied and cleaved by RDR2 and DCL3, respectively, to produce 2° siRNAs that induce methylation downstream of the target site [23••] (Figure 2). In primary RdDM, the synthesis and amplification of 1° siRNAs target and reinforce methylation at the original siRNA generating locus. Whereas, in 2° RdDM, 2° siRNAs are produced to facilitate the spreading of methylation adjacent to the region of primary RdDM. Notably, the establishment and maintenance of 1° RdDM is independent of 2° RdDM [23••].

The progress made in identifying the machinery associated with siRNA biogenesis and siRNA-directed DNA methylation in plants has also revealed a fairly complex repertoire of RNA-mediated epigenetic regulatory mechanisms. The contribution of sRNAs is discussed here, with an emphasis on the epigenetic aspects of sRNAs in

Figure 2



RNA polymerase IV-dependent production of small RNAs (1° siRNAs) reinforces existing heterochromatic regions by primary RNA-directed DNA methylation (1° RdDM). Primary RdDM can lead to the production of 2° siRNAs which trigger the spreading of methylation into adjacent regions, resulting in 2° RdDM. Secondary RdDM results from a Pol IV-derived aberrant RNA transcribed from methylated target templates. Some of these sRNAs may act in *trans* to direct 2° RdDM in a Pol V-dependent manner.

the context of RdDM, heterochromatin formation and chromatin-based gene silencing, RNA-mediated chromatin silencing in paramutation, development, genetic imprinting, and heritable epigenetic changes by way of mobile siRNAs.

Small RNA-directed DNA methylation

DNA methylation is one of the most well-studied epigenetic modifications. In plants, methylation can occur at any cytosine and in three different sequence contexts. 'Symmetric methylation' corresponds to CG and CHG sites, while 'asymmetric' methylation corresponds to CHH sites [26]; in each case, the H represents A, C, or T. As described above, the recruitment of the siRNA-producing machinery is the first step in the *de novo* DNA methylation of cytosines, and the second step is the targeted siRNA-directed DNA methylation at the homologous DNA region. There are multiple DNA methyltransferases involved in the establishment and maintenance of RdDM, including DOMAINS REARRANGED METHYLTRANSFERASE 1 and 2 (DRM1 and DRM2), which establish CHH methylation, CHROMOMETHYLASE 3 (CMT3), which establishes CHG methylation [27–30] and METHYLTRANSFERASE 1 (MET1), which maintains CG methylation [30,31]. Epigenetic regulation through demethylation is also important since sRNAs are impacted upon loss of methylation [32••,33,34]. DNA demethylation in plants is known to result from the activity of the DNA glycosylase/lyase proteins REPRESSOR OF SILENCING 1 and 3 (ROS1 and ROS3), DEMETER (DME) and DME-like (DML) [33–35].

A recently identified regulator of RdDM, RNA-DIRECTED DNA METHYLATION 1 (RDM1) was shown to be associated with the accumulation of 24 nt siRNAs, DNA methylation, and silencing at target loci [12••]. RDM1 was found to encode a protein that can bind single-stranded, methylated DNA. In addition, RDM1 was shown to associate with RNA polymerase II, AGO4 and DRM2, which makes it a strong candidate for being a part of the AGO4-effector complex of RdDM [12••]. RDM1 was found to copurify with DRD1 and DMS3, forming the DDR complex (DRD1–DMS3–RDM1) [17••]. Gao *et al.* [12••] proposed that the single-stranded, methyl-DNA-binding activity of RDM1 could facilitate AGO4 targeting. Additionally, Gao *et al.* [12••] showed that RDM1 and Pol V are colocalized in the perinucleolar processing center and that RDM1 is required for Pol V transcripts. It appears that RDM1 may also function to recruit Pol V to RdDM target sites. Thus RDM1 may play a central role in the complex linking transcription and sRNAs with methylated DNA.

The genomic landscape of sRNAs, methylation and chromatin

A number of recent studies have provided a novel genome-wide view of cytosine methylation and snapshots of the state of chromatin in plant genomes. The data from these studies provide insights into how these genomic characteristics are impacted by siRNAs, influencing the activity or silencing of the sRNA target sites. Marks of silencing in the plants' genome landscape are particularly acute in regions enriched in transposons, retroelements, pericentromeric regions, and rRNA genes.

A genome-wide, high-resolution map of the transcriptome, small RNA transcriptome (smRNAome) and cytosine methylome in *Arabidopsis* revealed a strong correlation between sRNAs and DNA methylation [32••,36••]. Lister *et al.* [32••] showed that there was a 25-fold greater chance of identifying a methylcytosine at an sRNA-producing locus than finding a methylcytosine at a non-sRNA locus, consistent with the outcome of 1° RdDM in which siRNAs are driving DNA methylation and vice versa. Overall, siRNA-directed DNA methylation covers about 30% of the *Arabidopsis* genome. Notably, two-thirds of methylated loci are not associated with sRNAs, so this apparently reflects substantial genomic cytosine methylation independent of sRNAs; or it may indicate substantial sRNA-mediated RdDM in developmental stages not assayed in their experiments. To further elucidate the connection between sRNAs and DNA methylation, Lister *et al.* [32••] performed deep sequencing of the smRNAome from DNA methyltransferase mutants *met1* and the triple-mutant *ddc* (*drm1 drm2 cmt3*), as well as the demethylation triple-mutant *rdd* (*ros1 dml2 dml3*). Evidence of sRNAs directing DNA methylation was demonstrated by the abundant methylation that was dependent on MET1, DRM1, DRM2, and CMT3 and the overlap with sRNA-generating regions from five tasiRNA generating loci. Lister *et al.* [32••] also showed that without demethylase activity, in the *rdd* triple mutant, the DNA near ta-siRNAs is targeted for *de novo* methylation, as demonstrated by an increase in DNA methylation near these loci. The sRNA population was altered as a result of the disruption of methylase and demethylase activities. For example, regions of the genome with reduced DNA methylation also had a lower abundance of sRNAs in the methyltransferase mutants, while in the absence of demethylase activity in *rdd*, there was a higher density of sRNAs. Thus methylation and demethylation both function to modulate sRNA levels. Additional studies will be needed to examine the extent to which sRNAs may facilitate the balance between methylation and demethylation. This will provide further insights into how epigenomic plasticity is maintained and regulated.

Zemech *et al.* [37•] conducted a large study that quantified genomic levels of methylation in plants (*Arabidopsis*, rice, chlorella, *Selaginella moellendorffii*, and *Physcomitrella patens*), seven animals, and five fungi with the intent to gather evolutionary insights into the methylation landscape of these genomes. Like observations made in *Arabidopsis* [30,32••,36••], their study also found that gene body methylation is conserved between plants and animals. A general trend observed in rice was that the genes most likely to be methylated are modestly expressed, whereas the genes least likely to be methylated are at the extremes of transcriptional activity [37•]. Overall, the methylation patterns in rice closely resemble those in *Arabidopsis* [30,38] but the early diverging land plants, *S. moellendorffii* and *P. patens*, do not have heavily methylated genes. Transposons and repeats were uniformly methylated in all of the plant types [37•].

Heterochromatin is typically composed of transposons, retrotransposons, and other repetitive elements that are maintained in the transcriptionally silent state usually attributed to methylation or post-translational histone modifications [39,40]. In plants, the population of sRNAs is quite large and diverse [41–44]. A large portion of sRNAs originate from repeats and transposons; these serve the very important role of silencing transposons and other repeat elements, representing an epigenetic 'architecture' of plant genomes. Multiple studies have shown the impact on chromatin from a loss of RdDM pathway components, specifically, these studies have examined the impact of mutations in Pol IV, Pol V, RDR2, DRM2, AGO4, and DCL3 [8,11,45–48]. In many of these studies, the focus was on 5S rDNA, and observations at these loci showed a reduction in DNA methylation, a reduction or elimination of 5S-derived siRNAs, derepression of 5S rDNA genes, changes in chromatin compaction, and differential silencing in the rDNA arrays — all of which were either Pol IV-mediated or Pol V-mediated effects [8,11,45–49]. Pontes *et al.* [50] reported a connection between siRNA-directed methylation and the effect on heterochromatin organization in chromocenters. In their study, *pol V* and *drd1* mutants exhibited decondensation of pericentromeric repeats and depletion of histone H3 lysine 9 dimethylation (H3K9me2) at chromocenters [50]. Separately, Cantu *et al.* [51] examined the methylation pattern of the wheat epigenome and observed a large number of sRNAs matching transposable elements (TEs). The wheat genome is composed of more than 80% TEs, so the epigenetic silencing mediated by sRNAs serves an extremely important role of suppressing the mutagenic activity of TEs [51]. With extensive, whole-genome datasets now available for DNA methylation and histone modifications, it is possible to identify heterochromatin from its marks rather than the presence of specific repeats; thus one important area of research will be to better define the characteristics for definably heterochromatic regions in plant genomes that lack repetitive characteristics, and to determine why some repeat elements lack marks of heterochromatin.

Small RNAs and paramutation

The epigenetic phenomenon of paramutation was described first in maize. Alleles of the same gene, which have the same sequence but different functional states, can have an allelic interaction in which the silent, paramutagenic allele transfers its silent state to the previously active allele [52,53]. The previously active allele will retain the silenced state which is meiotically heritable. Alleman *et al.* [54•] and Sidorenko *et al.* [55,56] utilized forward genetic screens that identified mutants deficient in the establishment/maintenance of paramutation and affect plant pigmentation. Their screens and subsequent screens from other labs [57,58•,59,60] have identified multiple mutants defective in paramutation. These include mutants in MEDIATOR OF PARAMUTATION 1 (MOP1) and REQUIRED TO MAINTAIN REPRESSION 1 (RMR1). Subsequent mutants have been named in accordance with the respective lab's naming methodology (or renamed *post hoc* if the cloned gene matches something previously known). The link between paramutation and RdDM was first demonstrated with the mutants *mop1*, the maize ortholog of *Arabidopsis RDR2* [54•,56,61], and *rmr6*, the maize ortholog of a *Arabidopsis* Pol IV subunit [58•]; both mutants showed a reduction of 24-nt siRNA levels, loss of siRNA-directed DNA methylation and derepression of transposons. The maize gene *RMR1* [57], encodes a SNF2-like chromatin remodeling factor, and is related to *Arabidopsis* DRD1 [13,18] and CLSY1 [13,14]. Stonaker *et al.* [62] and Sidorenko *et al.* [55] also identified paramutation mutants that are paralogs of NRPD2/NRPE2, the shared second largest subunit of Pol IV and Pol V in *Arabidopsis*; these mutants were denoted *rmr7* and *mop2*, respectively, and RNA gels indicate that both mutants display a strong reduction in 24 nt siRNAs.

In addition to the sRNA phenotype of maize mutants with compromised paramutation, developmental phenotypes have also been observed in many of these mutants. Both *mop1* and *rmr6* display a severe phenotype, whereas the mutants in the *Arabidopsis* orthologs are far less severe. As in *Arabidopsis*, there is a delay in flowering time but the morphological abnormalities consist of a range of effects, that is, shorter stature, spindly barren stalks, and aberrant development that leads to feminized tassels [63]. The phenotype of the maize mutants may be attributable to reactivation of previously silenced transposons [64]. The allelic maize mutants *rmr7* and *mop2* are also developmentally impaired but not to the same extent of *mop1* and *rmr6* [55,62]. This may be due to partial redundancy among maize *NRPD2/NRPE2*-like genes. The gross defects observed in maize that are not observed in *Arabidopsis* may reflect fundamental differences in the function, and a greater need for constraint on the plasticity of the maize genome considering that it has a higher TE content.

Mobile small RNAs

sRNAs that function non-cell autonomously have been implicated in a number of processes that range from developmental patterning to epigenetic reprogramming and inheritance [65•,66,67••,68••,69,70]. sRNAs are capable of moving from cell-to-cell to carry a short-range signal specifying leaf and root developmental patterns [65•,66]. Molnar *et al.* [67••] utilized grafting experiments with mutants in sRNA biogenesis to show that mobile 24 nt sRNAs can direct DNA methylation in the genome of the recipient cell. In this study, the mobile sRNA was synthesized and the signal was shown to move from the shoot into the root to guide DNA methylation. Notably, the mobility was found to be influenced by factors such as genomic locus or origin of the sRNA and the cell type in which the sRNAs accumulate. Another recent example of epigenetic restructuring by way of a heritable silencing signal was shown in developing pollen [68••]. In the pollen vegetative nucleus (VN), TEs are preferentially transcribed [68••]. The chromatin remodeling factor DECREASE IN DNA METHYLATION 1 (DDM1) is a major regulator of TE activity in *Arabidopsis* and it regulates DNA methylation, 24 nt siRNA production and TE silencing [71]. DDM1 accumulates in sperm cells (SCs) but not in the VN. Slotkin *et al.* [68••] were able to show that this diminished DDM1 activity stimulates TE transcription and activity specifically in the non-germline VN. The increase in TE transcripts does not result in inherited transposition effects since the VN does not contribute DNA to the embryo; rather, the TE transcripts stimulate the production of sRNAs via post-transcriptional gene silencing mechanisms [68••]. These sRNAs can be mobilized to suppress transposons and protect the germline SC. Remarkably, parallel events occur during female gametogenesis; a study by Mosher *et al.* [69] identified high levels of Pol IV-dependent (p4)-siRNAs in the endosperm of developing seed that are dependent on maternal expression of genes for biogenesis of p4-siRNAs. The p4-siRNAs may reinforce the silencing of transposons in the female gametophyte. More recent work has demonstrated both this and the role of AGO9 in interactions with TE-derived siRNAs in somatic companion cells that move to the female gametophyte [70]. Thus, development of the gametes and zygote depends on specific epigenetic reprogramming events that may serve as a defense mechanism to prevent the incursion of transposons at a critical phase in the plant life cycle. We should also note that there are striking parallels found in both plant and animal gametogenesis; the evidence for this is synthesized in a recent review by Bourc'his and Voinnet [72].

Conclusion

The plasticity of the plant epigenome appears to be influenced by a variety of biological processes that utilize sRNAs. At the most basic level, sRNAs function as regulators of gene expression through their influence

on DNA methylation, histone and chromatin states, and gene silencing. However, specialization of sRNA activities has resulted in a diversity of functions, as these molecules have been implicated in paramutation, genetic imprinting and epigenetic reprogramming, and more recently in cell-to-cell movement for transmitting epigenetic information. We may still be in the beginning stages of comprehending the complexity of sRNA-mediated epigenetic phenomena. There are still gaps in our knowledge about the machinery involved in sRNA biosynthesis and about the regulation of sRNA-controlled methylation and heterochromatin formation. Future experiments are likely to address questions about the natural epigenetic variation, hybrid genetics, and epigenomic responses to stress and environmental factors. For example, stress-induced sRNAs have been shown to be involved in events related to physiology and development [73–76]. A better understanding of selection for genetic imprinting will require insights into the genetic variation in a population and the influence of natural selection within these populations. Detailed genomic studies of many genotypes will be needed because genetic incompatibility in hybrids is often related to changes in chromatin integrity that may occur from disruptive patterns in DNA methylation and imprinting, heterochromatin formation, TE mobilization, and other factors. As the techniques continue to improve for genome-wide and ultimately perhaps tissue-type-specific or cell-type-specific chromatin and sRNA analysis, we can continue to define and refine the conceptual framework for the molecular mechanisms underlying plasticity in the epigenome and its reprogramming.

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