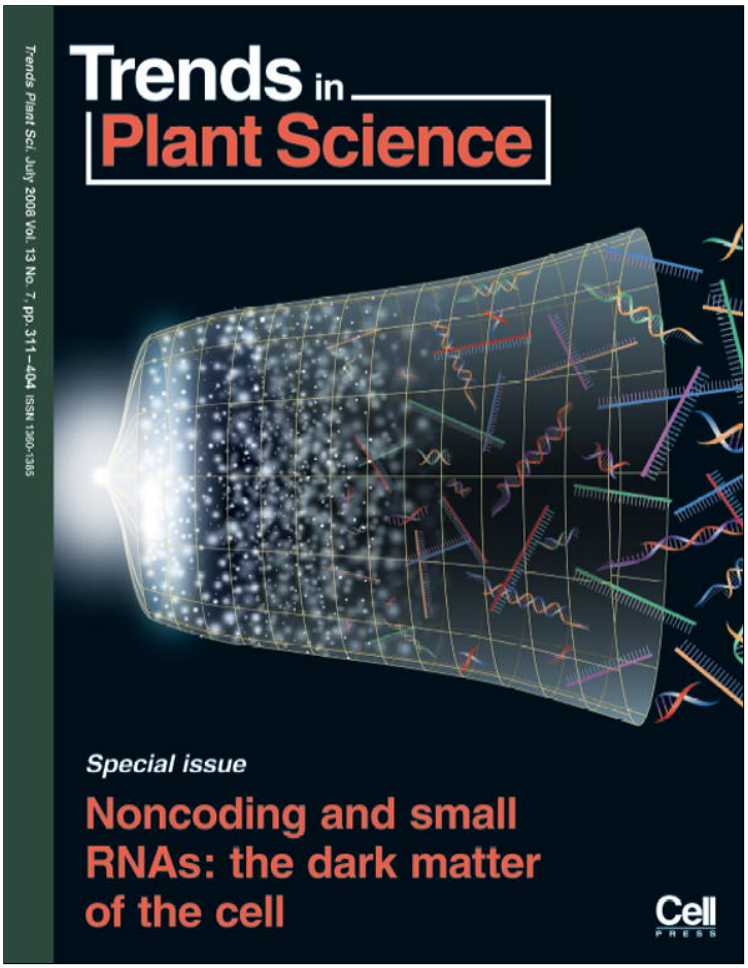

Review

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Special Issue: Noncoding and small RNAs

Inputs and outputs for chromatin-targeted RNAi

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Plant gene silencing is targeted to transposons and repeated sequences by small RNAs from the RNA interference (RNAi) pathway. Like classical RNAi, RNA-directed chromatin silencing involves the cleavage of double-stranded RNA by Dicer endonucleases to create small interfering RNAs (siRNAs), which bind to the Argonaute protein. The production of double-stranded RNA (dsRNA) must be carefully controlled to prevent inappropriate silencing. A plant-specific RNA polymerase IV (Pol IV) initiates siRNA production at silent heterochromatin, but Pol IV-independent mechanisms for making dsRNA also exist. Downstream of siRNA biogenesis, multiple chromatin marks might be targeted by Argonaute–siRNA complexes, yet mechanisms of chromatin modification remain poorly understood. Genomic studies of siRNA target loci promise to reveal novel biological functions for chromatin-targeted RNAi.

Establishment of plant gene silencing is guided by small RNAs

A substantial fraction of plant genomes, like almost all eukaryote genomes, is composed of transposable elements and repeated DNA sequences. Stable genome maintenance requires that cells suppress transposon mobility and prevent recombination between homologous repeats. Transcriptional gene silencing is mediated by epigenetic chromatin modifications: cytosine DNA methylation, a variety of post-translational histone modifications, and changes in nucleosome density and positioning. An enduring question in gene silencing research is how cells initially distinguish repeated DNA from endogenous genes, thereby restricting transcriptional silencing to its intended target sequences. Once established, many silent chromatin states can be propagated even in the absence of the initial guidance cues [1].

Early suggestions that RNA could target specific sequences for chromatin modification came from plant experiments with viroids (tiny RNA viruses that do not encode proteins), transgenic RNA viruses, and inverted repeat transgenes that create double-stranded RNA (dsRNA) [2–4]. In each case, the presence of dsRNA was associated with DNA methylation of homologous sequences. Subsequent reverse genetic studies in *Arabidopsis thaliana* confirmed that the establishment of plant gene silencing is guided by small interfering RNAs (siRNAs), which are generated by the RNA interference (RNAi) pathway [1,5]. The tandem repeat-containing gene *FWA* is efficiently methylated and silenced after transformation into wildtype *Arabidopsis* [6,7]. Mutations in several genes encoding RNAi proteins caused an inability to silence transgenic *FWA*, phenocopying *domains rearranged methylase2* (*drm2*) mutants, which lack an essential *de novo* DNA methyltransferase enzyme. In maize (*Zea mays*), paramutation is a homology-dependent gene silencing process in which 'paramutagenic' alleles can induce chromatin-level silencing at susceptible target loci (see review by Jay B. Hollick in this issue). Importantly, paramutation at the tandem-repeat-containing *b1* gene of maize depends on the RNAi protein MODIFIER OF PARAMUTATION 1 (MOP1) [8]. The *Arabidopsis* MOP1 ortholog RNA-DEPENDENT RNA POLYMERASE2 (RDR2) is required for *FWA de novo* silencing, indicating mechanistic similarity between chromatin silencing processes in monocots and eudicots [5].

Two catalytic steps in siRNA biogenesis are shared between plant chromatin-targeting RNAi and the RNAi pathways involving mRNA cleavage that are found in a wide range of eukaryotes [9]. First, dsRNA is cleaved by the RNaseIII nuclease Dicer to produce short 21–24-nucleotide (nt) siRNAs. There are four Dicer proteins in *Arabidopsis* (DCL1–DCL4), but DCL3 is the major enzyme required to produce the 24-nt siRNAs most commonly implicated in transcriptional silencing. Phylogenetic analysis shows that Dicer isoforms arose early in plant evolution, so DCL3 orthologs in other plants probably participate in chromatin silencing [10]. By analogy to other Dicers, DCL3 probably acts in a complex with a DRB family dsRNA-binding

Glossary

***De novo* DNA methylation**: DNA methylation that is initiated on previously unmethylated genomic loci.
Deep sequencing of small RNAs: use of novel short read sequencing technologies to characterize large libraries of cloned small RNAs.
DRB family protein: RNAi protein that contains two dsRNA-binding domains and that forms a complex with the Dicer nuclease. For example, *Arabidopsis* HYL1 binds to the Dicer protein DCL1.
FWA: *Arabidopsis thaliana* gene encoding a putative transcription factor. *FWA* contains a tandem repeat in its promoter and 5′UTR, and has been used as a model for understanding silencing of repeat-containing genes because it is readily silenced when transformed into *Arabidopsis*.
RRM domain: the RNA recognition motif (RRM) is found in proteins that are known or hypothesized to bind single-stranded RNA.
SNF2-family ATPases: a large family of proteins that use the energy of ATP to alter DNA–protein interactions. Many of these enzymes are chromatin remodellers or helicases.

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protein and the siRNA methylase HEN1 [11–13] (see article in this issue by Xuemei Chen on siRNA modification). Which of the five *Arabidopsis* DRB paralogs forms a complex with DCL3 is currently unknown.

Second, siRNAs bind to the effector protein Argonaute, a structural homolog of RNaseH, forming a functional Argonaute–siRNA complex. AGO4 is the principal chromatin-targeting Argonaute in *Arabidopsis*, but AGO6 acts redundantly with AGO4 and AGO9 might also act in chromatin silencing because the three proteins form a clade within the *Arabidopsis* Argonaute family [14,15]. Despite these fundamental similarities with post-transcriptional RNAi, novel ways for plant cells to recognize repeated DNAs have evolved upstream of Dicer. Transcriptional silencing by RNAi also involves evolutionary innovation because Argonaute and siRNA complexes must recruit chromatin-modifying enzymes instead of cleaving mRNA. Multiple synergistic chromatin modifications are likely to participate in RNA-directed transcriptional silencing. This review focuses on such elaboration of RNAi processes upstream of Dicer and downstream of Argonaute. Many of these mechanisms have not been described outside plants.

A pathway for the synthesis of endogenous siRNAs

To silence transposons and other repeated DNAs by RNAi, cells must generate dsRNA from these sequences as a substrate for Dicer (Figure 1). Genetic and bioinformatic studies converged to identify a plant-specific DNA-dependent RNA polymerase IV (Pol IV) as the first enzyme in a hypothetical pathway that synthesizes endogenous siRNAs (see article by Craig Pikaard on Pol IV in this issue) [16–19]. Pol IV has two isoforms that contain different large subunits, either NRPD1A or NRPD1B. Although the catalytic activity of either isoform remains to be verified, one model postulates that the Pol IV that contains NRPD1A uses genomic DNA as a template to produce a single-stranded RNA transcript, which is then converted to dsRNA by RNA-DEPENDENT RNA POLYMERASE2 (RDR2) in *Arabidopsis* and by the RDR2 ortholog MOP1 in maize [5,8,20]. Compelling evidence that Pol IV and RDR2 cooperate to produce DCL3 substrates comes from deep-sequencing experiments in *Arabidopsis*, which show that *nrpd1a, nrpd1b* and *rdr2* mutants have nearly identical defects in the synthesis of 24-nt siRNAs on a genome-wide scale (nearly all endogenous loci producing the 24-nt class of chromatin-targeting RNAs are dependent on Pol IV and on RDR2) [21,22]. Cytological co-localization of these proteins with DCL3 suggests that multiple steps in siRNA biogenesis are coupled *in vivo* [23,24].

Additional proteins that are involved in siRNA production by Pol IV and RDR2 have been found from an unexpected source: genetic screens assaying the systemic spreading of post-transcriptional RNAi. In one such assay, the endogenous phytoene desaturase gene (*PDS*) is silenced in response to a transgene that expresses an inverted repeat of the *PDS* gene from the vasculature-specific *SUC2* promoter (similar results have been reported with other assays that test intercellular spreading of post-transcriptional RNAi) [25–27]. *nrpd1a* and *rdr2* mutants are defective for spreading of silencing, yet this phenotype is not shared by *dcl3* and *ago4*. Hence, the spreading of post-transcriptional silencing has different genetic requirements from chromatin-directed silencing.

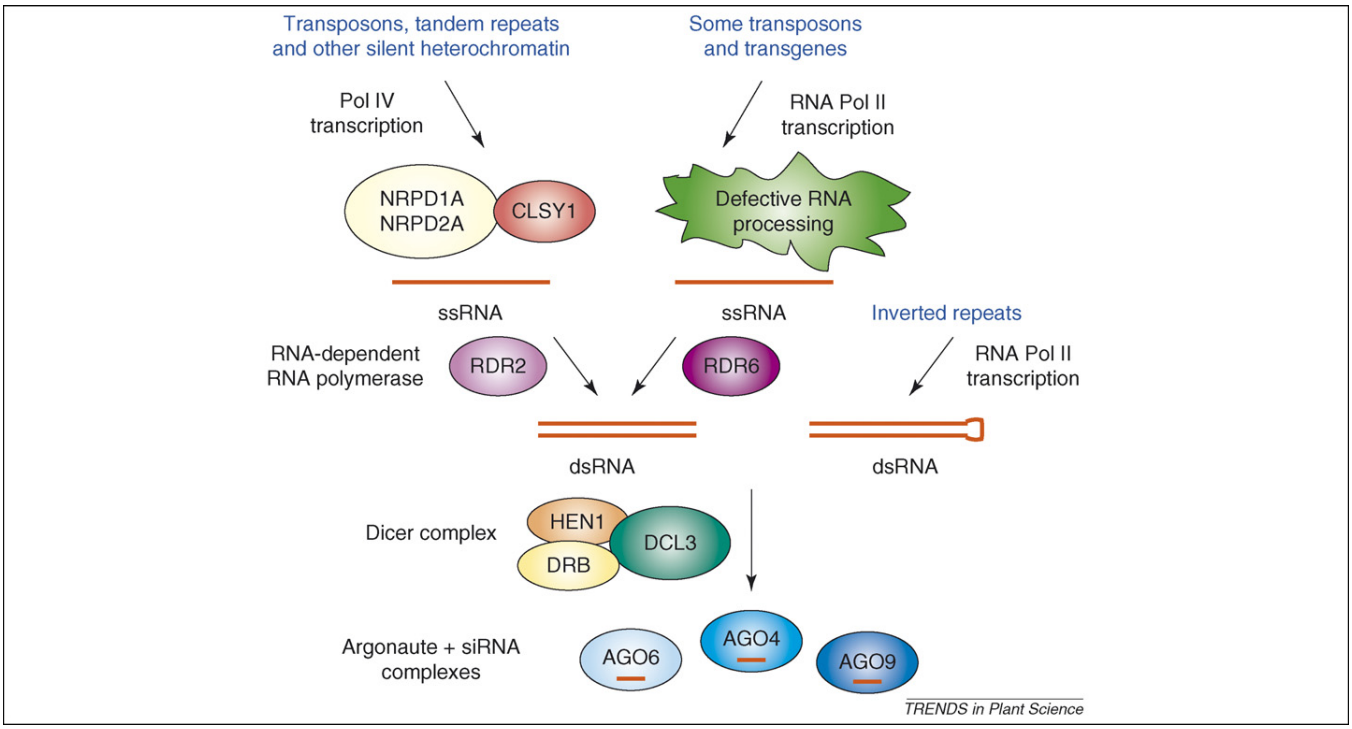

Figure 1. siRNA production from repeated DNA sequences. Multiple mechanisms can initiate siRNA biogenesis from transposons, tandem repeats, high-copy repeat sequences, and inverted repeats. Pol IV is attracted to transposons and to tandem repeats, potentially generating single-stranded RNA. Pol IV-derived single-stranded RNA and single-stranded RNA that is defectively processed from RNA Pol II transcripts can be converted to dsRNA by RNA-dependent RNA polymerases (RDR2 and RDR6). Inverted repeats can form double-stranded hairpin RNAs after mono-directional transcription. dsRNA is a substrate for the chromatin-targeting Dicer DCL3 and for other Dicer enzymes. RNA species are shown in red. See text for details about individual protein components.

It is hypothesized that the siRNAs that traffic between cells are made from Pol IV and RDR2-dependent dsRNA, but are cleaved by DCL4 instead of DCL3 and are bound to different Argonaute proteins. A SNF2-family ATPase and putative chromatin remodeling protein, CLSY1, is required for siRNA generation, presumably in conjunction with Pol IV and RDR2 [27]. If Pol IV and RDR2 always require CLSY1, the protein should also function in the synthesis of chromatin-targeting siRNAs. *clsy1* mutants show mislocalization of RDR2 (and to a lesser extent Pol IV), implying that appropriate chromatin structure is important for maintaining stable siRNA production. The RRM domain RNA-binding proteins FCA and FPA were also identified from the *SUC-PDS* screen; these proteins are better known as members of the autonomous pathway that controls the flowering-time regulator *FLC* [28]. Although the *fca-9 fpa-7* double mutant has reduced *SUC-PDS* derived siRNAs, much like *nrpd1a* and *rdr2* mutants, it shows no change in the levels of several endogenous siRNAs. Further studies are required to determine where in the genome CLSY1, FCA and FPA are needed for endogenous siRNA production.

How are repeated sequences selected for siRNA production?

Small RNAs target epigenetic chromatin modifications to *bona fide* silencing targets, so selection of genomic loci for siRNA production is crucial for plants to initiate heterochromatin formation accurately without inappropriately silencing endogenous genes (Figure 1). The question of how Pol IV and RDR2 are recruited to repeated DNAs is not fully answered. Hints about how some transposons are recognized have come from studies of the *Arabidopsis FWA* gene. A version of *FWA* that contains a single copy of the promoter tandem repeats is not silenced upon transformation, despite containing all the nucleotide sequences found in the endogenous gene [29]. Additionally, crossing the *nrpd1a* and *rdr2* mutants, both of which lack *FWA* siRNAs, allows recruitment of siRNA production to the repeat-containing *FWA* locus in F_1 individuals. Thus, tandem repeat character could be a signal to begin siRNA production.

Many transposons that populate silent heterochromatin do not contain tandem repeats, so other mechanisms must be capable of loading Pol IV and RDR2 at high-copy repeated sequences. Recruitment of siRNA biogenesis on a genome-wide scale has been studied by deep sequencing of siRNAs from an F_1 plant generated by crossing a *nrpd1a nrpd1b* mutant with *nrpd2a nrpd2b* [22]. The mutant parental plants in this cross lack almost all 24-nt siRNAs because their Pol IV subunits are missing, yet the F_1 regains siRNAs efficiently throughout the genome when fully functional Pol IV is restored by complementation of recessive mutations. DNA methylation or another mark of pre-existing gene silencing might recruit Pol IV and RDR2 to silent heterochromatin. Correlative evidence supporting this idea comes from the observation that 90% of 24-nt siRNA clusters co-localize with DNA methylation when whole-genome DNA methylation datasets are compared to data from deep sequencing experiments [22]. *FWA* does not require DNA methylation to recruit Pol IV, RDR2 and DCL3, as demonstrated by the fact that an unmethylated *fwa* epiallele retains wildtype levels of siRNA production [29]. Nevertheless, a locus without tandem repeats might depend on DNA methylation to recruit siRNA-producing proteins.

A minority of siRNA-generating loci in the *Arabidopsis* genome are Pol IV- and RDR2-independent, indicating that alternative mechanisms for producing chromatin-targeted siRNAs do exist [22,30]. One class of Pol IV- and RDR2-independent loci contains inverted repeats, which can form dsRNA after mono-directional transcription creates an mRNA capable of forming a hairpin. The *Mu Killer* locus of maize is an inverted repeat sequence formed by a partial duplication of a *Mutator* (*MuDR*) family transposon [31]. *Mu Killer* produces siRNAs that silence the expression of the *MuDR* genes *mudrA* and *mudrB* throughout the maize genome [31,32]. (Silencing of both transposon open reading frames [ORFs] indicates that the chromatin state induced by *Mu Killer*-derived siRNAs can spread, because the inverted repeat does not contain *mudrB* sequences.) Both siRNA production and the initiation of *MuDR* silencing are independent of the maize RDR2 ortholog MOP1, presumably because hairpin formation bypasses the requirement for synthesis of dsRNA by an RNA-dependent RNA polymerase [33]. Wildtype maize plants do produce MOP1-dependent siRNAs from *MuDR* transposons [33]. These siRNAs lack the ability of *Mu Killer*-derived siRNAs to initiate silencing of active *MuDR* transposons, however, and it has been suggested that they instead maintain silencing at *MuDR* copies that have previously been heterochromatinized. The existence of the naturally occurring *Mu Killer* locus indicates that inverted repeat formation is a mechanism by which endogenous transposons can trigger plant chromatin silencing. Silencing of transposons (and potentially endogenous genes) by homologous inverted repeats is likely to be a much more prevalent phenomenon in large plant genomes that contain a high proportion of repetitive DNA.

Repeat-independent siRNA production might occur at transposons and sequence repeats through aberrant RNA processing during transcription [34,35]. Incorrectly processed RNA transcripts lead to RNAi, possibly by recruiting RNA-dependent RNA polymerases such as *Arabidopsis* RDR6 to produce dsRNA as a Dicer substrate [36,37]. This mechanism could underlie the silencing of very highly transcribed transgenes (a phenomenon termed sense post-transcriptional gene silencing [S-PTGS]), but such silencing might also occur at transposon promoters or via read-through transcription from endogenous genes. S-PTGS produces 21- or 22-nt siRNAs that target mRNA cleavage [38]. However, these classes of siRNA can cause DNA methylation of homologous sequences, and there is higher-than-expected genome-wide correlation of 21- and 22-nt siRNA clusters with DNA methylation [22,39]. Furthermore, dsRNAs from aberrant RNA processing might be cleaved by DCL3 at some frequency, leading to AGO4 loading and chromatin targeting.

In summary, there are many possible mechanisms by which chromatin-targeting siRNAs could be produced from transposons and repeated DNAs (Figure 1). Some take advantage of the tandem and inverted-repeat character of transposons, others of their high copy number. These

siRNA-producing pathways might act redundantly at a given genomic locus.

Which chromatin modifications are targeted by siRNAs?

mRNA cleavage can be reconstituted *in vitro* with purified Argonaute and siRNAs. By contrast, the mechanism by which Argonaute and siRNA complexes target chromatin-modifying enzymes is poorly understood. One barrier to understanding is that chromatin-level silencing takes place in the context of intact chromosomes and is difficult to assay biochemically. AGO4 and siRNA complexes must initiate transcriptional silencing, because they provide sequence specificity. However, several chromatin modifications occur in response to siRNA targeting, and unraveling their order of action might be difficult (Figure 2). In addition, the silencing mechanisms described below could have different degrees of functional importance, depending on the locus in question.

Cytosine DNA methylation is a well-characterized epigenetic mark that underlies homology-dependent gene silencing. Establishment of DNA methylation in *Arabidopsis* depends completely on the *de novo* DNA methyltransferase DRM2, which might always utilize Argonaute-bound siRNAs as a guide (there are two DRM proteins in *Arabidopsis*, but the *drm2* single mutant is completely defective for *de novo* DNA methylation) [6,40]. Unlike the *ago4* mutant, *drm2* is completely unable to establish silencing directed by inverted repeats [40]. Residual silencing activity in *ago4* could reflect redundancy among Argonaute proteins. Redundancy in RNAi pathways is further complicated by the finding that inverted repeats produce siRNAs independently of Pol IV and RDR2 [40,41].

DRM2 is particularly active on the asymmetric CHH sequences that are methylated at siRNA-targeted repeat loci (and conspicuously unmethylated at sites of DNA methylation within genes that depend only on the MET1 DNA methyltransferase) [42]. However, DNA methylation by a second DNA methyltransferase, CHROMOMETHYLASE3 (CMT3), is also directed in the CNG sequence context by AGO4 and siRNAs. This is shown by the fact that *ago4* mutants are defective for silencing of *SUPERMAN* (*SUP*) in *clk-st*, an *Arabidopsis* line in which endogenous *SUP* is silenced in part by a transgenic inverted repeat at a different locus (similar results are seen with inverted-repeat-driven silencing of the *PAI* genes) [43,44]. *cmt3* mutants have a stronger defect on *clk-st*/*SUP* and *PAI*, but CMT3 can also be guided by the KRYPTONITE/SUVH4 histone H3 lysine 9 (H3K9) methyltransferase [45,46]. The *drm2* mutant does not have a *SUP*-silencing defect in *clk-st*, showing that AGO4 and siRNAs direct CMT3 rather than DRM2 to this locus. AGO4 and siRNA complexes could recruit KRYPTONITE/SUVH4 and thus facilitate CMT3 activity, or might target CMT3 in a histone H3K9-methylation-independent way.

Post-transcriptional histone modifications other than histone H3K9 methylation also control siRNA-directed transcriptional silencing. Histone H3 lysine 4 (H3K4) methylation is associated with active genes, and demethylation of H3K4 by the LSD1 protein leads to gene silencing in mammals and in *Schizosaccharomyces pombe* [47,48]. *Arabidopsis ldl1 ldl2* mutants lacking two homologs of LSD1 fail to silence an *FWA* transgene in a manner similar to *drm2* and *rdr2* mutants [49]. This shows that histone H3K4 demethylation helps to establish DNA methylation (*de novo* DNA methylation in the mutant is slightly higher than in *rdr2* and *drm2*, but much lower than in wildtype). Although the *ldl1 ldl2* mutant has reduced maintenance of CG methylation at endogenous *FWA*, the effect of the mutations is much greater at a newly transformed *FWA* transgene. Thus, histone H3K4 demethylation is specifically required for AGO4 and siRNAs to target DRM2. Histone H2B de-ubiquitination has also been found to regulate RNA-directed gene silencing [50]. Decreased DNA methylation and histone H3K9 dimethylation were seen at an siRNA-targeted transgene, *RD29A-luc*, when the H2B deubiquitinating enzyme *SUP32*/*UBP26* was mutated, although loss of DNA methylation was more subtle at endogenous transposons. Double-mutant analysis combining the *sup32*/*ubp26* mutation with other

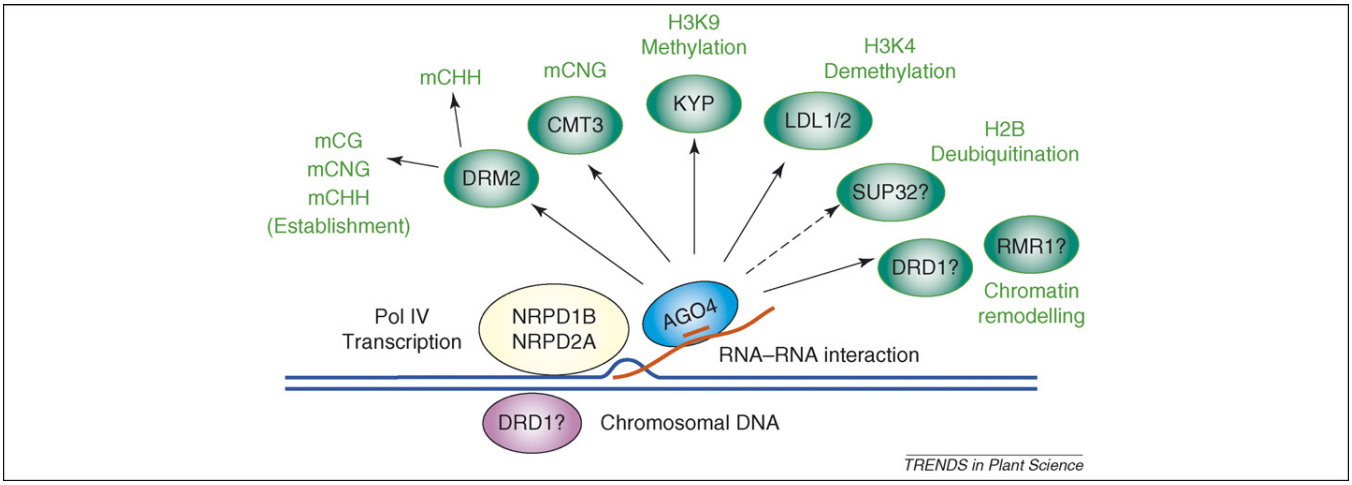

Figure 2. Chromatin modifications downstream of Argonaute and siRNAs. AGO4 and siRNAs probably bind to chromatin through RNA–RNA association with a nascent transcript produced by the NRPD1B-containing isoform of Pol IV (RNA species are shown in red, whereas chromosomal DNA is in blue). Once at a target locus, AGO4 and siRNA complexes might recruit several different chromatin-modifying enzymes (green) to effect gene silencing. The order of action of these chromatin-modifying enzymes is not known, and their relative importance for gene silencing might be locus-specific. The putative chromatin-remodeling enzyme DRD1 could alter chromatin structure downstream of AGO4 and siRNAs, or might facilitate transcription by Pol IV.

RNAi mutations will allow H2B deubiquitination to be placed more precisely into existing chromatin-targeting RNAi pathways.

Nucleosome positioning and chromatin structure play a role in siRNA-directed gene silencing, as shown by the *drd1* mutant, which lacks a putative SNF2-related chromatin-remodeling ATPase [51]. DRD1 is required for *de novo* DNA methylation of the *FWA* transgene by DRM2, and for DNA methylation of the targets of inverted repeat-derived siRNAs [52,53]. (In the latter context, the *drd1–6* mutation has a more severe effect than *ago4–1* on the establishment of silencing.) DRD1 also mediates CMT3-dependent methylation of CNG sequences at solo long terminal repeat transposons (solo LTRs) and at the short interspersed nuclear element (SINE) transposon AtSN1, perhaps because CMT3 is targeted by RNAi at such loci. Notably, siRNA levels at *FWA* and at solo LTRs are normal in *drd1*, so DRD1 must act downstream of siRNA biogenesis [52,54].

The recent cloning of a maize paramutation mutant showed that an additional SNF2 ATPase protein, RMR1, is required for both siRNA production and full DNA methylation at silent *Pl1-Rhoades* epialleles of the *purple plant* (*pl1*) gene [55]. Bisulfite sequencing studies of *rmr1* will give a clearer picture of how the phenotype of this mutant resembles that of *mop1*/*rdr2*. It is not clear how widespread RMR1 function will be in siRNA-directed chromatin silencing: the protein controls the transcription of *Pl1-Rhoades* in an unusual post-transcriptional manner. Nonetheless, the genome-wide activity of siRNA-directed DNA methylation pathways in plants could require several chromatin-remodeling factors.

How do Argonaute and siRNAs target chromatin modifications?

Chromatin-targeting siRNAs in plants might base pair directly with unwound genomic DNA, or could bind to particular loci by RNA–RNA interaction with a nascent single-stranded transcript (Figure 2). That the latter model is correct is suggested by prevailing evidence, particularly by analogy to *S. pombe* in which point mutations in the RNaseH domain of Argonaute that abolish catalytic activity were found to prevent chromatin targeting [56]. This result indicates that cleavage of nascent mRNA is probably part of the mechanism by which Argonaute attracts the Clr4 histone H3K9 methyltransferase. Confusingly, *ago4* catalytic point mutations reduce RNA-directed DNA methylation at some but not all loci [57]. Targeting of chromatin by siRNAs in *S. pombe* and in plants might represent convergent evolution; some of the Argonaute-associated proteins involved in yeast chromatin-directed RNAi (such as Chp1 and Tas3) are not found in plant genomes [58] and *S. pombe* lacks cytosine DNA methylation. However, the role of the second, NRPD1B-containing, isoform of Pol IV provides evidence that AGO4 and siRNA complexes bind to nascent transcripts in plants [17,19]. *nrpd1b* mutants contain normal levels of LTR- and *FWA*-derived siRNAs, yet are completely defective for siRNA-directed DNA methylation [29,54]. As *nrpd1b* mutant plants are fully capable of producing siRNAs, NRPD1b-type Pol IV could be required to synthesize a single-stranded transcript that is bound by AGO4 and siRNA.

We do not know which enzymatic activities associate directly with AGO4 and siRNAs. To discover the order in which chromatin modifications occur at a locus where silencing is initiated, researchers must develop biochemically tractable establishment-of-silencing assays. The role of putative chromatin-remodeling factors in siRNA-directed epigenetic gene silencing might be especially opaque. We do not have functional assays for DRD1 or RMR1, and their activity might well be modulated by histone modifications. Another possibility is that DRD1 acts as a co-factor for NRPD1b-type Pol IV (Figure 2), in which case it could be required for all transactions between siRNAs and chromatin.

Feedback between different epigenetic modifications could play a role in propagating endogenous siRNA synthesis. Pol IV and RDR2 are preferentially found at loci containing DNA methylation, suggesting that silencing might enhance siRNA production [21,22]. Furthermore, the functional properties of siRNAs can be modulated by chromatin state even if overall siRNA levels remain constant. As an example, siRNAs produced from unmethylated *fwa* epigenetic alleles are unable to facilitate efficient *de novo* DNA methylation of an incoming transgene, unlike those produced from methylated *FWA* alleles [29].

Biological functions of siRNA-directed chromatin modifications

The gene-silencing defect of *Arabidopsis* RNAi pathway mutations is mild compared to that of the *methyltransferase1* (*met1*) mutant, which lacks DNA methylation in the CG sequence context. This shows that siRNA-targeting is often dispensable for silencing once this mechanism has been established. However, siRNA-directed methylation by the DRM2 DNA methyltransferase might be important for reinforcing MET1 activity. If methylation is lost from a particular CG, MET1 might not be able to replace it because its preferred substrate is hemimethylated DNA created by replication of a symmetrically methylated CG (Figure 3; [59]). The presence of siRNAs and active DRM2 could thus compensate for occasional errors in

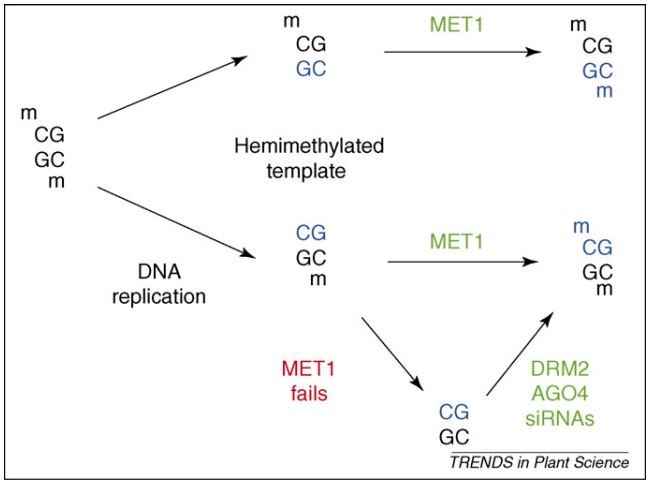

Figure 3. siRNA-directed DNA methylation as a backup for maintenance DNA methylation. The MET1 DNA methyltransferase is thought to act on its preferred substrate, hemimethylated CG dinucleotides, after DNA replication. If maintenance DNA methylation by MET1 fails, targeting of DRM2 by Argonaute and siRNAs might be required to propagate DNA methylation patterns.

the maintenance of DNA methylation. *drm2 met1* double mutants are extremely sick, perhaps because their loss of CG DNA methylation during gametophyte generations is much more severe than that in *met1* single mutants [60–62]. Even if their role in maintaining gene silencing is minor, endogenous siRNAs directed against major transposon families could allow plants to rapidly silence mobile transposons and newly expanded repeat families.

It is clearly advantageous for cells to maintain ongoing production of siRNAs from repeated DNAs, either as a backup for CG DNA methylation or to ensure rapid initiation of silencing at new transposon insertions. Interestingly, silencing of *Arabidopsis* ribosomal DNA (rDNA) arrays requires siRNA-directed DNA methylation, and even CG DNA methylation is siRNA-dependent in these repeats [63]. The rDNA has thus co-opted transposon silencing mechanisms to regulate ribosome biogenesis (and nucleolar dominance in interspecies crosses). Another potential biological role for RNA-directed heterochromatin formation is in centromere function. *S. pombe* RNAi mutants fail to maintain centromere gene silencing and have both defects in *de novo* kinetochore assembly and reduced loading of cohesin complexes that hold sister chromatids together (cohesin is enriched at centromeres in wildtype cells) [64,65]. This role might be minor in plants because centromere gene silencing depends much more on CG DNA methylation, which is largely siRNA-independent [1,18,20,66,67].

Genome-wide suppression of repeated DNA is clearly the ancestral role of chromatin-targeted siRNA in plants, and the number of endogenous genes that are controlled by this system might be quite small. *Arabidopsis* chromatin-pathway RNAi mutants have near-wildtype phenotypes, but plants with larger genomes are more likely to have intergenic repeat sequences that could control endogenous gene expression. Maize *mop1* mutants have stochastic developmental abnormalities that suggest that chromatin-targeted RNAi has a much more important role in controlling gene expression in this plant species [20,68,69].

Future prospects

The discovery of genes whose DNA methylation depends on chromatin-targeted RNAi will be accelerated by short-read DNA sequencing of sodium bisulfite-treated DNA (BS-Seq), a technique that can analyze DNA methylation at the single-nucleotide level on a genome-wide scale [70]. Bisulfite converts cytosine, but not methylcytosine, to uracil. Unlike chromatin immunoprecipitation methods, BS-Seq at sufficient depth can detect low levels of CNG and CHH DNA methylation [70–72]. These RNA-directed modifications were previously impossible to distinguish from CG DNA methylation in a high-throughput fashion, but it is now feasible to identify all targets of siRNA-directed DNA methylation in mutant plant lines. Applying BS-Seq to rice and maize chromatin-pathway RNAi mutants generated by TILLING or by insertional mutagenesis should unveil further biological roles of siRNA-directed gene silencing.

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