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Role of *Arabidopsis ARGONAUTE4* in RNA-Directed DNA Methylation Triggered by Inverted Repeats

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Summary

In a number of organisms, transgenes containing transcribed inverted repeats (IRs) that produce hairpin RNA can trigger RNA-mediated silencing, which is associated with 21–24 nucleotide small interfering RNAs (siRNAs) [1]. In plants, IR-driven RNA silencing also causes extensive cytosine methylation of homologous DNA in both the transgene "trigger" and any other homologous DNA sequences—"targets" [2]. Endogenous genomic sequences, including transposable elements and repeated elements, are also subject to RNA-mediated silencing. The RNA silencing gene *ARGONAUTE4* (*AGO4*) is required for maintenance of DNA methylation at several endogenous loci and for the establishment of methylation at the *FWA* gene [3, 4]. Here, we show that mutation of *AGO4* substantially reduces the maintenance of DNA methylation triggered by IR transgenes, but *AGO4* loss-of-function does not block the initiation of DNA methylation by IRs. *AGO4* primarily affects non-CG methylation of the target sequences, while the IR trigger sequences lose methylation in all sequence contexts. Finally, we find that *AGO4* and the *DRM* methyltransferase genes are required for maintenance of siRNAs at a subset of endogenous sequences, but *AGO4* is not required for the accumulation of IR-induced siRNAs or a number of endogenous siRNAs, suggesting that AGO4 may function downstream of siRNA production.

Results and Discussion

Several transgenic RNA silencing systems have been developed to study silencing induced by inverted repeat transgenes. In this study we used two such systems to determine the role of AGO4 in IR-induced, RNA-mediated silencing. The first system we studied utilizes the AP1RNAi transgene to induce posttranscriptional silencing (PTGS) of the endogenous *APETALA1* (*AP1*) gene [5]. The AP1RNAi transgene consists of the 35S promoter driving two copies of a portion of the *AP1* cDNA arranged as an inverted repeat and separated by a non-homologous spacer (Figure 1A). This transgene creates hairpin RNA that causes PTGS of *AP1*, resulting in a weak *ap1* loss-of-function phenotype. Second, we utilized a two-component transgene system (HxK) designed to study IR-induced transcriptional silencing that was developed by the Matzke laboratory [6]. The K target transgene contains the *NPTII* gene driven by the *NOPALINE SYNTHASE* promoter (*NOS*pro). When active, this transgene confers resistance to the antibiotic kanamycin. The H trigger transgene is a constitutively expressed hairpin construct, similar to AP1RNAi, with the stem corresponding to the *NOS*pro sequence (Figure 1B). In plants containing both transgenes (HxK), the H transgene causes extensive CG, CNG (where N = A, T, or C), and asymmetric (not CG or CNG) DNA methylation of the *NOS*pro sequences in the K transgene, leading to transcriptional silencing (TGS) of the *NPTII* gene and kanamycin sensitivity. Thus, the AP1RNAi and HxK systems are similar inverted repeat RNA silencing systems. In the AP1RNAi system, double-stranded RNA and siRNAs are targeted against the mRNA coding region, leading to PTGS, while in the HxK system they are targeted to a promoter, leading to TGS.

AGO4 Controls DNA Methylation Triggered by Inverted Repeat Transgenes

In order to examine the role of *AGO4* in the maintenance of DNA methylation triggered by inverted repeat transgenes, we used bisulfite sequencing to analyze methylation in the AP1RNAi and HxK systems in *ago4-1* mutant plants. In the AP1RNAi system, we observed extensive DNA methylation in all sequence contexts of the endogenous target *AP1* gene (Figure 1C). In *ago4-1* plants, CNG methylation of *AP1* was decreased from 69% to 30%, and asymmetric methylation was decreased from 29% to 4% (Figure 1C). CG methylation was also reduced from 94% to 67%, but the significance of this reduction is difficult to gauge, since only one CG site is present within the region analyzed (Table S1).

Despite the reduction in DNA methylation, we did not observe a detectable loss of *AP1* silencing, consistent with the observation that targeting RNA silencing to transcribed regions leads to posttranscriptional rather than transcriptional repression [7]. We observed that wild-type plants carrying the AP1RNAi transgene have sepals that fail to senesce as the silique matures (Figures 1D and 1E) but have normal petals—a phenotype consistent with the weaker AP1RNAi lines analyzed in the Meyerowitz lab [5]. AP1RNAi *ago4-1* plants had persistent sepals (Figure 1F) but also exhibited extensive lack of petals; bract-like, carpelloid sepals; and secondary flowers (Figure 1G)—phenotypes associated with

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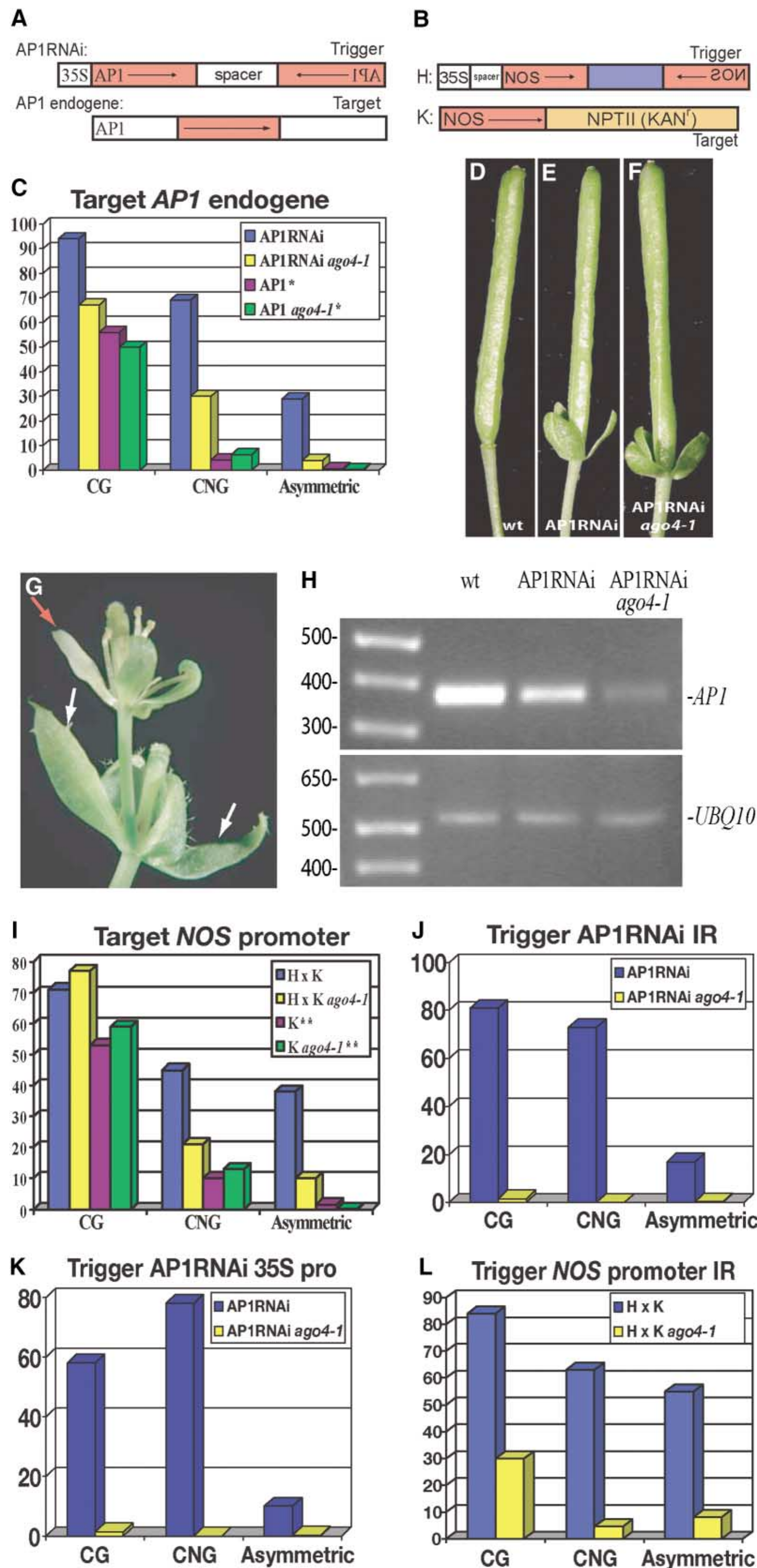

Figure 1. *AGO4* Controls Inverted Repeat-Induced DNA Methylation

(A and B) Schematic representations of (A) AP1RNAi transgene and *AP1* endogene and (B) H and K transgenes.

(C) Bisulfite sequencing results showing the percent methylation present at the *AP1* endogene. *AP1 in the graph legend refers to plants in which the AP1RNAi transgene has been crossed away.

(D–F) Siliques from (D) a wild-type plant, (E) an AP1RNAi plant showing persistent sepals, and (F) an AP1RNAi *ago4-1* plant with persistent sepals.

(G) Primary and secondary flowers from an AP1RNAi *ago4-1* plant. The primary flower has no petals, while the secondary flower has a single petal (red arrow). The white arrows point to bract-like, carpelloid sepals on the primary flower.

(H) RT-PCR of the endogenous *AP1* transcript. The constitutively expressed *UBIQUITIN10* (*UBQ10*) gene was used as a control.

(I–L) Bisulfite sequencing results showing the percent methylation present at (I) the *NOS* promoter sequence present in the K transgene (**K in the graph legend refers to plants in which the H transgene has been crossed away), (J) the *AP1* IR region of the AP1RNAi transgene, (K) the 35S promoter (pro) of the AP1RNAi transgene, and (L) the *NOS* promoter IR region of the H transgene. Detailed data for each graph can be found in Table S1.

stronger *AP1* silencing. We analyzed the levels of *AP1* mRNA in wild-type, AP1RNAi, and AP1RNAi *ago4-1* plants (Figure 1H) and found a reduction in AP1RNAi plants and a further reduction in AP1RNAi *ago4-1*—observations consistent with the phenotypes in these lines. *AGO4* is therefore not required for PTGS of *AP1*,

and loss of AGO4 function in fact results in enhanced silencing in this system.

In agreement with previous reports [2, 8], we detected extensive DNA methylation of the *NOS*pro sequence in the K target transgene in all sequence contexts (Figure 1I). In *ago4-1* plants CNG methylation was reduced from 45% to 21% and asymmetric methylation was reduced from 38% to 10%, while CG methylation was unaffected (Figure 1I). The reduction in non-CG methylation was not sufficient to substantially reactivate the *NPTII* gene within the K transgene, since *ago4-1* plants remained sensitive to kanamycin (data not shown). The abundant remaining CG methylation at the *NOS* promoter of the K transgene (Figure 1I) likely accounts for persistence of TGS in *ago4-1* plants in the HxK system, since previous studies have shown that CG methylation is the primary mechanism of gene silencing at this sequence [2, 8]. These results are consistent with our previous findings that *AGO4* largely affects non-CG methylation at endogenous loci and suggest that *AGO4* may mostly affect transcriptional silencing of loci that have few CG sites, little CG methylation, or both, such as in the *SUPERMAN* gene [3].

We also examined DNA methylation of the trigger inverted repeat sequences present in the AP1RNAi and HxK systems. In wild-type plants we found extensive DNA methylation in all sequence contexts in the IR portion of the AP1RNAi transgene, as well as in the nonrepeated 35S promoter upstream of the AP1 IR (Figures 1J and 1K). We also found heavy methylation in all sequence contexts in the hairpin region of the H transgene in wild-type plants (Figure 1L), but the 35S promoter was essentially unmethylated (Table S1). In *ago4-1* plants we found a virtually complete loss of methylation in all sequence contexts of the AP1RNAi transgene sequences, including the IR and the upstream 35S promoter (Figures 1J and 1K). The *ago4-1* mutation also led to extensive loss of methylation in all sequence contexts at the H transgene IR (CG from 84% to 30%, CNG from 63% to 4.6%, and asymmetric from 55% to 8%; Figure 1L), although the effect was not as severe as at AP1RNAi. Thus, mutation of *AGO4* led to a profound reduction of DNA methylation of both the target and trigger DNA sequences. At the target sequences, we observed mostly a loss of non-CG methylation, while at the trigger sequences we observed a loss of CG and non-CG methylation. The differing effects on CG methylation may be due to the fact that unlike the target loci, which are not transcribed in the tissues analyzed, the trigger loci are strongly and constitutively transcribed from the 35S promoter. Reduction of CG methylation in *ago4-1* has also been observed at the constitutively transcribed 5S rDNA arrays [9]. High levels of transcription may therefore interfere with RNA-independent maintenance of CG methylation, thus requiring reinforcement by AGO4 via RNA targeting.

Trigger-Dependent Methylation that Is AGO4 Independent

Although we observed substantial decreases of non-CG methylation in *ago4-1* plants, some non-CG methylation nevertheless persists, and we have previously shown that the same is true for some endogenous loci such as *SUPERMAN* and the *AtSN1* short interspersed nuclear element [3]. One possibility is that this methylation is maintained in the absence of an active signal, which in the case of IR transgenes is likely to be siRNAs or double-stranded RNA. Alternatively, it is possible that RNA signals can still be communicated between trigger and target loci in *ago4-1* mutants. In order to investigate these possibilities, we crossed away the IR triggers in the AP1RNAi and HxK systems and examined DNA methylation of the target loci. If the *ago4-1* mutation completely disrupts the communication between trigger and target, then crossing away the trigger locus should have the same effect on target methylation as does *ago4-1*. On the other hand, if the target can still sense the trigger in the absence of AGO4, then crossing away the trigger should have a stronger effect on target methylation than *ago4-1*. We found that CG methylation was maintained well in both systems in the absence of a trigger, but CNG methylation was dramatically reduced, and asymmetric methylation was virtually eliminated (Figures 1C and 1I). Significantly, the methylation levels were substantially below those of *ago4-1* plants containing a trigger locus. Furthermore, the *ago4-1* mutation did not further reduce methylation in the absence of a trigger (Figures 1C and 1I). It therefore appears that AGO4 is not involved in trigger-independent maintenance of methylation and that signal from a trigger locus can still reach the target locus in the absence of *AGO4* function, suggesting that there exists an additional, partially redundant pathway directing DNA methylation.

Role of *AGO4* in the Initiation of DNA Methylation

The experiments presented thus far have tested the role of *AGO4* in the maintenance of preexisting DNA methylation, since the methylation had been induced in transgenic systems before the introduction of *ago4-1*. Because *ago4-1* does not completely eliminate maintenance methylation, we can also examine the role of *AGO4* in the initial establishment of DNA methylation. In order to examine this in the AP1RNAi system, we set up a genetic scheme to analyze the effect of *ago4-1* on the establishment of methylation of a naïve *AP1* endogene, which was genetically marked with a Columbia ecotype-specific DNA polymorphism (Figure 2A). Thus, we crossed a plant homozygous for *ago4-1*, the AP1RNAi inverted repeat construct, and the *AP1* gene from the L*er* ecotype to a plant homozygous for *ago4-1* and the unmethylated *AP1* gene from the Columbia ecotype. As a control, a similar cross was set up with plants that were homozygous wild-type for *AGO4*. The F1 progeny of these crosses were allowed to self-pollinate, and F2 plants homozygous for the previously unmethylated Columbia *AP1* allele were identified by using PCR-based molecular markers. These plants were then subjected to bisulfite genomic sequencing (Figure 2A). Similar levels of methylation at CG and CNG sites were found in wild-type and mutant plants, while asymmetric methylation was higher in wild-type than in *ago4-1* plants (Figure 2B). Methylation could thus be established de novo in *ago4-1* plants. Except for asymmetric methylation in *ago4-1*, all methylation levels were below those found

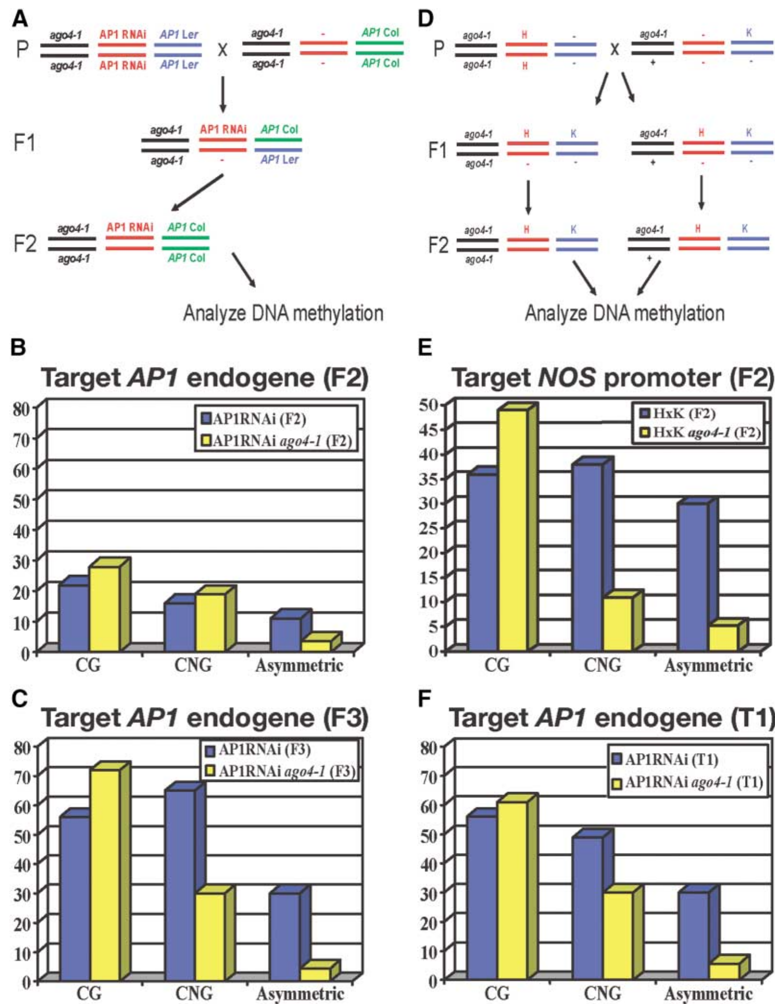

Figure 2. *AGO4* Does Not Block De Novo DNA Methylation Triggered by Inverted Repeats

(A) Genetic crosses performed to examine establishment of DNA methylation at *AP1* in *ago4-1* mutant plants (see text for explanation).

(B and C) Bisulfite sequencing results showing the percent methylation present at the *AP1* endogene in (B) the F2 and (C) the F3 generations of the cross shown in (A).

(D) Genetic crosses performed to examine establishment of DNA methylation at the *NOS* promoter in *ago4-1* or *AGO4* plants (see text for explanation).

(E) Bisulfite sequencing results showing the percent methylation present at the *NOS* promoter sequence of the K transgene in F2 plants of the crosses shown in (D).

(F) Bisulfite sequencing results showing the percent methylation present at the *AP1* endogene in T1-transformed plants. Detailed data for each graph can be found in Table S1.

in the original inbred AP1RNAi plants (Figure 1C), suggesting that establishment of full levels of DNA methylation was not yet complete. We therefore allowed the F2 plants to self-pollinate and examined DNA methylation in the F3 progeny. The methylation levels in these plants were substantially higher than in the F2 plants and closer to the values seen in the original inbred AP1RNAi plants (compare Figure 2C with Figure 1C). These data indicate that at least three plant generations are required for full levels of methylation to be established in the AP1RNAi system and further demonstrate that de novo methylation of the *AP1* endogene can occur in the *ago4-1* mutant.

We also tested the role of *AGO4* in the initiation of DNA methylation in the HxK system. We previously showed that the *drm1 drm2* methyltransferase double mutant completely blocked the establishment of DNA methylation in the HxK system, which normally occurs when the H and K transgenes are first brought together in a cross [8]. To test this with *ago4-1*, we crossed a plant homozygous for *ago4-1* and the H transgene with a plant heterozygous for *ago4-1* and the K transgene (Figure 2D). F1 plants were genotyped for *ago4-1* and the K transgene, and plants containing K were selfed. F2 progeny of plants homozygous for *ago4-1* were genotyped for the presence of H and K, while F2 progeny of plants heterozygous for *ago4-1* were genotyped for the presence of H and K and wild-type *AGO4* (Figure 2D). In this way we could compare HxK plants that were either wild-type or mutant for *AGO4*. We found that DNA methylation was established in both wild-type and *ago4-1* mutant plants, showing that *AGO4* function is not strictly required for de novo methylation in the HxK system (Figure 2E). *AGO4* loss-of-function did significantly impair the maintenance of CNG and asymmetric methylation, consistent with the situation we observed in the inbred lines (compare Figure 2E with Figure 1I).

Since we previously found that *AGO4* is required for transformation-dependent establishment of methylation at *FWA* transgenes [4], we tested whether *ago4-1* would block de novo methylation initiated by transforming plants with the AP1RNAi transgene. Wild-type and *ago4-1* plants were transformed with the AP1RNAi construct to generate new silenced lines. Wild-type plants transformed with AP1RNAi exhibited phenotypes similar to that of the original AP1RNAi line, while *ago4-1* lines showed stronger phenotypes consistent with our earlier observations with the inbred line. DNA methylation levels at the endogenous *AP1* gene were assayed in T1 plants. The *ago4-1* mutation failed to block establish-

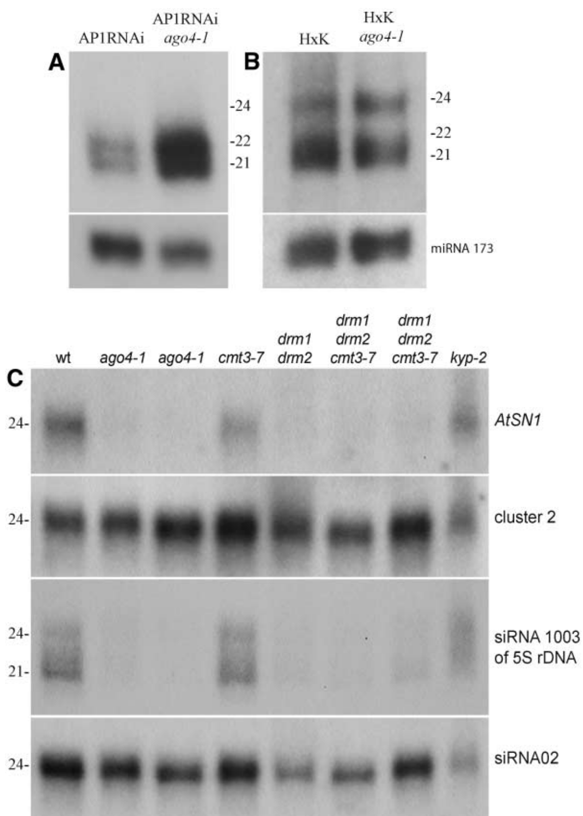

Figure 3. Role of *AGO4* and DNA/Histone Mmethyltransferases in the Steady-State Levels of siRNAs

(A and B) Small RNA Northern blots probed with (A) an *AP1* probe and (B) a *NOS* promoter probe. Each blot was reprobed with a microRNA 173 (miRNA 173) probe as a loading control. The AP1RNAi *ago4-1* lane in (B) was slightly underloaded to emphasize the difference in *AP1* siRNAs.

(C) A small RNA Northern blot probed sequentially with *AtSN1*, cluster 2, 5S rDNA, and siRNA02 probes. Two independent samples of *ago4-1* and *drm1 drm2 cmt3-7* are shown.

ment of DNA methylation, since high levels of methylation were found in both wild-type and mutant plants (Figure 2F). However, *ago4-1* did impair maintenance methylation to about the same extent as in the inbred lines (compare Figure 2F with Figure 1C). Thus, *ago4-1* failed to prevent IR-mediated de novo methylation when the trigger was introduced by either transformation or cross, further reinforcing the conclusion that an AGO4-independent pathway exists that is capable of directing both the establishment and maintenance of DNA methylation.

Effect of *ago4-1* on siRNA Accumulation

Since the silencing systems assayed above are known or predicted to be associated with abundant siRNAs [6], we examined the role of *AGO4* in siRNA accumulation in these systems. We found abundant 21–22 nucleotide *AP1* siRNAs in AP1RNAi plants, which persisted and were, in fact, substantially more abundant in *ago4-1* plants (Figure 3A). We detected 21–22 nt and 24 nt *NOS*-pro siRNAs in HxK plants, the levels of which were unchanged in *ago4-1* plants (Figure 3B). Loss of methylation at the 35S promoter of the AP1RNAi transgene provides a possible explanation for increased levels of *AP1* siRNAs observed in *ago4-1* plants, since decreased methylation is predicted to increase transcription of the hairpin RNA. The increase in siRNA levels also likely accounts for the enhanced *AP1* PTGS phenotype and reduced *AP1* mRNA levels observed in *ago4*-1.

Our previous work showed that siRNAs corresponding to the *AtSN1* locus were undetectable in *ago4-1* plants [3]. To test if the effect of *ago4-1* on endogenous siRNA accumulation was general, we tested three additional endogenous loci that are associated with detectable levels of siRNAs: cluster 2, 5S rDNA, and siRNA02 [9]. Like at *AtSN1*, the siRNAs corresponding to these loci are dependent on the RNA silencing genes *RNA-DEPENDENT RNA POLYMERASE2* (*RDR2*) and *DICER-LIKE3* (*DCL3*) [9]. We found that siRNAs corresponding to 5S rDNA were greatly reduced in *ago4-1* plants, while the levels of siRNAs to cluster 2 and siRNA02 were not substantially altered (Figure 3C). We also found that the *AtSN1* and 5S rDNA siRNAs were greatly reduced in *drm1 drm2* double mutants and *drm1 drm2 cmt3* triple mutants, but these mutants did not substantially alter siRNA levels at cluster 2 or siRNA02 (Figure 3C). This same pattern of siRNA dependence has been observed for the *hen1* mutation [9], a gene involved in microRNA function [10] and gene silencing [11]. The histone lysine 9 methyltransferase mutant, *kryptonite* [12, 13], had no detectable effect on siRNAs from any of the loci tested (Figure 3C).

It is not clear why *ago4-1*, as well as *drm* and *hen1*, affect siRNA accumulation differently at *AtSN1* and 5S rDNA versus cluster 2 and siRNA02. One difference is that *AtSN1* and 5S rDNA are highly repeated loci, whereas the cluster 2 sequence is unique and the siRNA02 sequence is found in only two copies (nucleotides 41347–41368 and 43419–43440 on P1 clone MDA7, chromosome 5). Furthermore, *AtSN1* and 5S rDNA exhibit extensive DNA methylation, which is reduced in *ago4-1* mutant plants [3, 9], while we failed to detect significant methylation at the cluster 2 and siRNA02 loci (data not shown). It seems likely that AGO4 generally functions downstream of the synthesis of siRNAs, since AGO proteins are known components of RNA silencing effector complexes [1]. Thus one possible explanation for the differing effects of *ago4-1* on siRNA accumulation may be the existence of multiple effector complexes that integrate and stabilize siRNAs. The *AtSN1* and 5S rDNA siRNAs may be primarily integrated into an AGO4-containing complex, and therefore may be destabilized and lost in the *ago4-1* mutant. The other siRNAs examined here may be primarily included in a different effector complex, such as the RISC complex that degrades RNA, and are therefore unaffected in *ago4-1*. An alternative explanation, consistent with the loss of siRNAs in *drm* mutants, is that DNA methylation (or another chromatin modification downstream of DNA methylation) may act to positively affect siRNA production at highly repeated loci and may thus be required for siRNA accumulation at *AtSN1* and 5S rDNA. This possibility is also supported by the recent finding that *AtSN1* siRNAs are reduced by mutations in genes responsible for maintenance of CG methylation, the putative chromatin remodeling factor *DDM1*, and the DNA methyltransferase *MET1* [14].

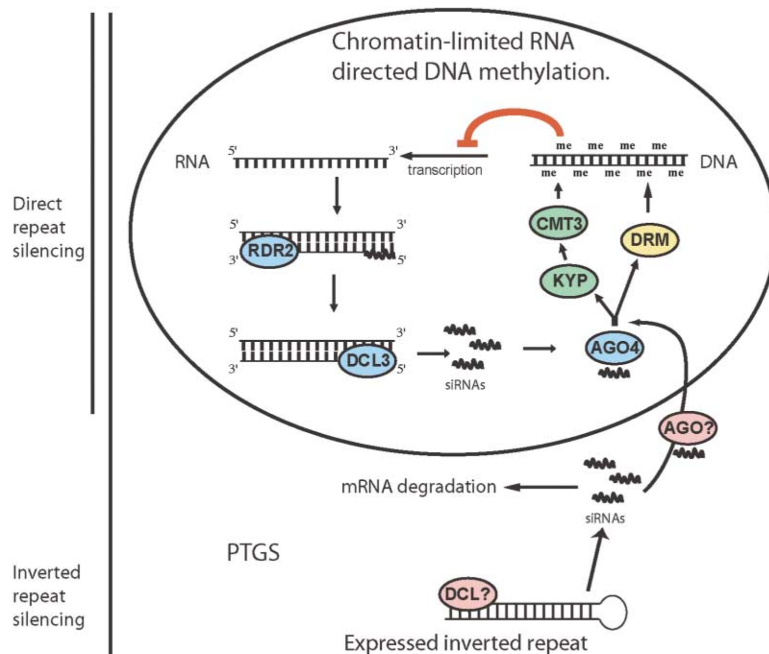

Figure 4. Model for RNA-Directed DNA Methylation in *Arabidopsis*

Diagram inside of the circle represents a hypothesized chromatin-limited siRNA silencing pathway in which siRNAs direct DNA methylation. siRNAs made by RDR2 and DCL3, together with AGO4, target DNA methylation through the action of KYP, CMT3, and DRM proteins. The bottom pathway represents PTGS, in which double-stranded RNA is processed into siRNAs by an unknown DICER protein. These siRNAs are used to degrade target mRNA and contribute to RNA-directed DNA methylation independently of AGO4, possibly through another AGO protein. We propose that RNA-directed DNA methylation driven by inverted repeats utilizes both pathways while direct repeat silencing is limited to the nuclear AGO4-dependent pathway.

Multiple Pathways for the Establishment and Maintenance of RNA-Directed DNA Methylation

We have previously shown that *AGO4*, *DCL3*, *RDR2, SDE4*, and *DRM2* are required for transformation-dependent establishment of methylation at the *FWA* gene [4, 15]. Furthermore, mutation of *AGO4*, *RDR2, SDE4*, or *DRM2* causes complete loss of non-CG methylation at two endogenous loci: *FWA* and *MEA-ISR* (the *dcl3* mutant shows a partial effect). These data suggest that AGO4 is a component of an RNA-mediated silencing pathway that is involved in both the establishment of methylation and the maintenance of non-CG methylation. However, in this study we found that AGO4 does not completely control DNA methylation triggered by inverted repeat silencing constructs. In particular, our finding that non-CG methylation is more extensively eliminated by crossing away the trigger transgene than it is in *ago4-1* implies the presence of an alternative AGO4-independent pathway directing DNA methylation (Figure 4). The observation that *AGO4* is not required for establishment of IR-induced methylation by either transformation or cross further reinforces this conclusion.

A possible explanation for these results is that different pathways control methylation, both establishment and maintenance, at direct repeats versus inverted repeat sequences. In support of this idea, substantial differences are known to exist between how DNA methylation is controlled at loci consisting of tandem direct repeats (*FWA* and *MEA-ISR*) and loci involving IRs (AP1RNAi, HxK, *SUPERMAN/clk-st*, *PAI1-4*, and *AtMu1*). As mentioned above, maintenance of non-CG DNA methylation at *FWA* and *MEA-ISR* is dependent on AGO4/RDR2/DCL3/SDE4 [4], as well as the DRM DNA methyltransferases [16], whereas the DNA methyltransferase CMT3 plays only a minor role [16]. On the other hand, substantial non-CG DNA methylation remains at IR-containing loci tested in *ago4* mutants, and DRM and CMT3 play largely redundant roles at these loci [3, 16]. Furthermore, establishment of methylation at *FWA* is transformation dependent [15], whereas IR-containing loci (AP1RNAi, H, *SUPERMAN*, and *PAI*) can induce DNA methylation of target loci when introduced by a cross [15, 17]. These differences suggest that the only mechanism for targeting methylation to tandem direct repeats may be an RNAi-like system involving AGO4/RDR2/DCL3/SDE4 (Figure 4). An additional pathway, which may or may not be RNA based, appears to target methylation to loci involving IRs. One such pathway could be the PTGS machinery that generates siRNAs that are used primarily to degrade RNA (Figure 4). Interestingly, both pathways converge on the DRM and CMT3 methyltransferases, since *drm1 drm2* mutants block the establishment of methylation both at *FWA* and in the HxK system, and *drm1 drm2 cmt3* triple mutants eliminate all non-CG methylation at both direct and inverted repeats [3, 8, 15, 16].

Supplemental Data

Supplemental Data including Experimental Procedures and a table are available at http://www.current-biology.com/cgi/content/full/14/13/1214/DC1/.

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