A stepwise pathway for biogenesis of 24-nt secondary siRNAs and spreading of DNA methylation

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We used a transgene system to study spreading of RNA-directed DNA methylation (RdDM) during transcriptional gene silencing in *Arabidopsis thaliana*. Forward and reverse genetics approaches using this system delineated a stepwise pathway for the biogenesis of secondary siRNAs and unidirectional spreading of methylation from an upstream enhancer element into downstream sequences. *Trans*-acting, hairpin-derived primary siRNAs induce primary RdDM, independently of an enhancer-associated 'nascent' RNA, at the target enhancer region. Primary RdDM is a key step in the pathway because it attracts the secondary siRNA-generating machinery, including RNA polymerase IV, RNA-dependent RNA polymerase2 and Dicer-like3 (DCL3). These factors act in a turnover pathway involving a nascent RNA, which normally accumulates stably in non-silenced plants, to produce *cis*-acting secondary siRNAs that induce methylation in the downstream region. The identification of DCL3 in a forward genetic screen for silencing-defective mutants demonstrated a strict requirement for 24-nt siRNAs to direct methylation. A similar stepwise process for spreading of DNA methylation may occur in mammalian genomes, which are extensively transcribed in upstream regulatory regions.

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Introduction

Spreading of silent chromatin along a chromosome is a feature of many epigenetic processes in eukaryotes but the

mechanisms, including the potential roles of non-coding RNAs, are still under investigation (Talbert and Henikoff, 2006; Clark, 2007; Pauler *et al*, 2007; Kwon and Workman, 2008). In fission yeast, an RNAi-mediated pathway fosters spreading of histone H3 lysine 9 methylation in heterochromatic DNA repeats (Locke and Martienssen, 2006; Iida *et al*, 2008; Zhang *et al*, 2008). The process of RNA-directed DNA methylation (RdDM) in *Arabidopsis* offers an opportunity to study spreading of cytosine methylation facilitated by the RNAi machinery and siRNAs in euchromatic portions of the genome.

RNA-directed DNA methylation is a small RNA-mediated epigenetic modification that is highly developed in flowering plants (Bei *et al*, 2007). The hallmarks of RdDM include methylation of cytosines in all sequence contexts (CG, CNG, CNN, where N is A, T or C) and restriction of methylation to the region of RNA–DNA sequence homology. The establishment and maintenance of RdDM require for the most part conventional DNA cytosine methyltransferases, histone-modifying enzymes and nuclear-localized RNAi proteins (Matzke *et al*, 2007; Chan, 2008). In addition, several plant-specific proteins are required, most notably subunits of two novel RNA polymerases termed Pol IV and Pol V (Pikaard *et al*, 2008; Wierzbicki *et al*, 2008). Pol IV and Pol V share the same second largest subunit, NRPD2/NRPE2, but are distinguished by their unique largest subunits, NRPD1 and NRPE1, respectively. Pol IV is needed to produce and/or amplify the small RNA trigger, whereas Pol V acts downstream of this step to facilitate *de novo* methylation at the small RNA-targeted site (Pikaard *et al*, 2008). RdDM can silence transposons, but the potential reversibility of this process suggests broader functions in stress responses and plant development (Penterman *et al*, 2007; Chan, 2008; Hollick, 2008).

To identify components of the RdDM machinery that are important for development, we established a two-component transgene silencing system based on an enhancer that is active in shoot and root meristem regions (Kanno *et al*, 2008). A forward genetic screen to recover mutants defective in RdDM of the target enhancer and silencing of a downstream *GFP* reporter gene has identified so far four *dms* (defective in meristem silencing) mutants. These include the previously identified SNF2-like factor DRD1 (DMS1) and the Pol V subunits, NRPE2a (DMS2) and NRPE1 (DMS5) (Kanno *et al*, 2004, 2005). Whether Pol V transcribes extensively in this transgene system or acts primarily to open chromatin at the siRNA-targeted site to expose DNA to cytosine methyltransferases is unknown. A recent study detected Pol V-dependent intergenic transcripts that may interact with siRNAs to induce methylation of homologous endogenous sequences (Wierzbicki *et al*, 2008). DMS3 is a novel structural-maintenance-of-chromosomes hinge domain-containing protein that has an unknown function in RdDM (Kanno *et al*, 2008).

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A distinctive feature of the meristem silencing system is the production of 24-nt secondary siRNAs that are associated with spreading of methylation downstream of the enhancer region originally targeted by primary siRNAs, a phenomenon termed transitivity (Voinnet, 2008). Secondary siRNA biogenesis is correlated with the disappearance of a longer 'nascent' transcript that overlaps with primary siRNAs. To explain these observations, we proposed a hypothetical pathway of secondary siRNA formation involving Pol IV as well as unidentified Argonaute (AGO), RNA-dependent RNA polymerase (RDR) and Dicer-like (DCL) activities (Kanno *et al*, 2008).

Formation of approximately 21–22-nt secondary siRNAs and spreading of methylation within transcribed regions have been observed during post-transcriptional gene silencing (PTGS) in plants and shown to require RDR6 and transcription of the target gene (Vaistij *et al*, 2002; Van Houdt *et al*, 2003; Eamens *et al*, 2008). By contrast, our meristem silencing system provides a well-defined system for analysing the genetic requirements for the biogenesis of 24-nt secondary siRNAs and spreading of methylation (secondary RdDM) within upstream regulatory regions during transcriptional gene silencing (TGS). Here, we report the findings of experiments designed to investigate possible functions for two RNA-dependent RNA polymerases, RDR2 and RDR6, as well as NRPD1 and AGO4 in 24-nt secondary siRNA biogenesis, secondary RdDM and silencing of the *GFP* reporter gene. We also report the identification of a *dcl3* mutant in a forward genetic screen.

Results

Secondary siRNA biogenesis, DNA methylation and 'nascent' RNA accumulation

We used a reverse genetics approach to test several factors proposed to be involved in secondary siRNA biogenesis in our transgene TGS system (Figure 1A–C). RDR2 is needed to produce 24-nt 'heterochromatic' siRNAs (Xie *et al*, 2004); RDR6 is required to generate 21–22-nt secondary siRNAs in the PTGS pathway (Vaistij *et al*, 2002); AGO4 is needed for *de novo* DNA methylation (Chan *et al*, 2004) and for RNA slicing at some target loci (Qi *et al*, 2006); NRPD1, the largest subunit of Pol IV, is required for biogenesis of 24-nt heterochromatic siRNAs (Herr *et al*, 2005; Onodera *et al*, 2005). DCL3 is a dicer activity that produces 24-nt 'heterochromatic' siRNAs (Xie *et al*, 2004) and is discussed later in the context of a forward genetic screen.

We introgressed the *rdr2-1*, *rdr6-1*, *nprpd1-7* and *ago4-1* mutations into the target (T)-silencer (S) line by crossing doubly homozygous plants (T/T;S/S) with the respective homozygous mutant (m/m). The resulting F1 progeny (genotype T/-; S/-; M/m) were self-fertilized to generate a segregating F2 population. To determine whether any of the mutants release *GFP* silencing, F2 seeds were sown on sterile medium and the number of GFP-positive seedlings was determined approximately 20 days after germination. If a mutation releases silencing, approximately one-third of the F2 progeny should be GFP positive; if a mutation does not release silencing, only around 18.5% of the F2 progeny should be GFP positive. With the exception of *ago4-1*, which appeared to partially release *GFP* silencing, none of the mutations alleviated silencing of the *GFP* reporter gene

Figure 1 Transgene silencing system and hypothetical model for spreading of DNA methylation through secondary siRNAs. (A) The target locus (T) contains a meristem-specific enhancer (region targeted for methylation shown in black) placed upstream of a minimal promoter (hatched) and *GFP*-coding region. The unlinked silencer locus (S) contains a transcribed inverted DNA repeat of distal enhancer sequences encoding a hairpin RNA. The hairpin RNA trigger is diced into short interfering (si) RNAs, which induce *de novo* methylation of the target enhancer in *trans*. There is a short tandem repeat in the target enhancer (Kanno *et al*, 2008), which has an unknown function in spreading of methylation and silencing. 35Sp: 35S promoter of cauliflower mosaic virus. (B) Transgenic seedlings containing only the target locus (T) display GFP fluorescence in root and shoot apical meristem regions (RAM and SAM, respectively) (left); in seedlings containing the target and silencer loci (T+S), GFP fluorescence is abolished (right). (C) In the hypothetical model tested in this study, primary siRNAs (blue dashes) originating from DCL processing of the hairpin RNA trigger induce primary RdDM (blue 'm') of the targeted enhancer region (black box) and may guide AGO slicing of an overlapping nascent RNA (black arrow), which is proposed to be transcribed by Pol IV from the methylated DNA template. The Pol IV-generated nascent RNA, or cleavage fragments thereof, provide substrates for RDR; the resulting double-stranded RNA is diced to generate 24-nt secondary siRNAs (red dashes) that induce secondary RdDM (red 'm') in the downstream region (black shade). *GFP* expression is silenced. Primary and secondary RdDM require DRD1, Pol V and DMS3 (Kanno *et al*, 2008) and are presumably catalysed by the *de novo* methyltransferase DRM2 (Cao *et al*, 2003) with the participation of AGO4 (Chan *et al*, 2004).

(Supplementary Table 1). We will first describe experiments with the three mutants that do not release *GFP* silencing: *rdr2*, *rdr6* and *nprpd1*.

To obtain *rdr2*, *rdr6* and *nprpd1* mutant plants and their wild-type siblings for the analysis of secondary siRNAs, DNA methylation and the nascent RNA, we genotyped F3 progeny to obtain the following genotypes: T/T; S/(S);m/m and T/T; S/(S);M/M. We used only F3 progeny that were homozygous

for the target locus to avoid dosage effects on nascent RNA synthesis that might alter the amount of secondary siRNAs independently of any effect of a specific mutation. The silencer locus could be either homozygous or hemizygous, as in both states sufficient primary siRNAs are produced to induce *GFP* silencing.

All wild-type and mutant plants harbouring the silencer locus contained primary siRNAs, which were present in three size classes (21-, 22- and 24-nt), that presumably result from multiple DCL enzymes acting on abundant hairpin RNAs in plants (Figure 2A) (Fusaro *et al*, 2006). By contrast, 24-nt secondary siRNAs were detected in wild-type plants and in the *rdm6* mutant, but not in *rdm2* and *nrpd1* plants (Figure 2B). The genotypes of the *rdm2*, *rdm6* and *nrpd1* mutants were confirmed by testing for endogenous siRNA02, which is dependent on RDR2 and NRPD1 (Figure 2C), and tasiRNA255, which is dependent on RDR6 (Figure 2D).

The absence of the secondary siRNAs in the *rdm2* and *nrpd1* mutants was correlated with a strong reduction of secondary RdDM (Figure 3A and B). By contrast, secondary RdDM was present at wild-type levels in the *rdm6* mutant (Figure 3C), which also contained a wild-type level of secondary siRNAs (Figure 2B). Primary RdDM remained at wild-type levels in the *rdm2* and *nrpd1* mutants (Figure 3A and B) and is apparently sufficient for silencing *GFP* expression, as indicated by the failure of *rdm2* and *nrpd1* mutations, which eliminate secondary siRNAs and secondary RdDM, to release *GFP* silencing (Supplementary Table 1).

The nascent RNA accumulates in the wild-type target line lacking the silencer locus but is greatly reduced or undetectable in *rdm2*, *rdm6* and *nrpd1* mutants and their wild-type siblings that contain silencer-encoded primary siRNAs (Figure 2E). In plants containing primary siRNAs and

secondary siRNAs (wild-type plants and the *rdm6* mutant), the absence of the nascent RNA can be explained by invoking the proposed turnover pathway involving AGO, RDR and DCL proteins (Figure 1C). This explanation does not account for the absence of the nascent RNA in *nrpd1* and *rdm2* mutants, which also lack secondary siRNAs despite wild-type levels of primary siRNAs and primary RdDM. One possibility is that the nascent RNA is cleaved by an AGO protein in the region overlapping with primary siRNAs. However, a 3'-cleavage fragment was not detected with the primers used (Figure 2E).

An alternate explanation is that the nascent RNA is not transcribed in *nrpd1* plants because of impaired Pol IVa function. Interestingly, the nascent RNA can be detected in the non-silenced target line in the *nrpd1* background (Figure 2E), indicating that it is transcribed by an RNA polymerase other than Pol IV—presumably Pol II—in the absence of primary siRNAs and primary RdDM. A hypothetical explanation for the missing nascent RNA in the *rdm2* mutant is that a Pol IV-generated transcript, or cleavage products thereof, is rapidly turned over by nuclear exonucleases, such as XRN2 and XRN3 (Gy *et al*, 2007), unless copied into double-stranded RNA by RDR2.

The effects of the *ago4-1* mutation on *GFP* silencing are complex because reactivation of *GFP* expression was not uniformly observed in both the root and shoot meristem regions of all seedlings. However, when considering seedlings in which reactivation occurred in both meristematic regions, there was a good correspondence between homozygosity for the *ago4-1* mutation and a GFP-positive phenotype (Supplementary Table 1). A contribution of AGO4 to *GFP* silencing is consistent with the substantial loss of primary RdDM in *ago4-1* plants (Figure 3D). The *ago4-1* mutant lacked detectable secondary siRNAs (Figure 2B) and

Figure 2 RNA profiles in *rdm2*, *rdm6*, *nrpd1* and *ago4* mutants. Northern blots of small RNAs: (A) primary siRNAs derived from hairpin RNA trigger; (B) secondary siRNAs; (C) siRNA02; (D) tasiRNA255. The arrowheads at the right indicate 21- and 24-nt-size classes. Ethidium bromide staining of the major RNA on the gels is shown as a loading control. The same blot was reprobed in B–D. (E) RT–PCR detection of nascent RNA; (F) RT–PCR of an actin control; (G) *GFP* expression. The DNA lane is amplified genomic DNA. Abbreviations: T/T;-/-, non-silenced plants containing a homozygous target locus; T/T;S(S), silenced plants containing a homozygous target locus and either a hemizygous or homozygous silencer locus; WT, wild-type transgenic plant; Col-0, wild-type non-transgenic plants. For each of the four mutants tested (small letters), results from wild-type siblings (capital letters) are shown for comparison. The quantitative differences in primary siRNAs (A) are most likely due to the fact that RNA was prepared from pools of F2 or F3 plants that could be either homozygous or hemizygous for the silencer locus.

Figure 3 Analysis of DNA methylation at the targeted enhancer and downstream region by bisulphite sequencing in *rdr2*, *rdr6*, *nrpd1* and *ago4* mutants. The graphs show the percentage of methylation at individual cytosines in mutant plants (top) and their wild-type siblings (bottom). (A) *rdr2/RDR2*; (B) *nrpd1/NRPD1*; (C) *rdr6/RDR6*; (D) *ago4/AGO4*. The black bar represents the enhancer region targeted by primary siRNAs (primary RdDM); the shaded grey bar indicates the downstream region targeted by secondary siRNAs (secondary RdDM). Black lines: CG methylation; blue lines: CNG methylation; red lines: CNN methylation. Residual secondary RdDM in *rdr2* may be due to low levels of 24-nt siRNAs produced by RDR6/DCL3 activities (Gascioli *et al*, 2005; Eamens *et al*, 2008). The results are from at least 10 cloned sequences. Original data are shown in Supplementary Figure 4.

consequently had negligible secondary RdDM (Figure 3D); as expected from the absence of secondary siRNAs, accumulation of the nascent RNA was observed in *ago4-1* plants (Figure 2E).

Identification of a *dcl3*-null mutation in a forward genetic screen

In a forward genetic screen using this transgene TGS system to identify silencing-defective mutants (Kanno *et al*, 2008), we identified one allele of a new complementation group, *dms6*. The *dms6-1* mutant displayed an unusual pattern of accumulation of siRNA1003 (originating from 5S rDNA repeats) resembling that observed in a *dcl3-1* mutant, which contains a T-DNA insertion in the *DCL3* gene (Xie *et al*, 2004). The most striking feature of this pattern is an irregular ladder of RNAs that migrate above the 24-nt siRNA1003 observed in

wild-type plants (Figure 4A). Given the similar patterns of siRNA1003 accumulation observed in *dms6-1* and the known *dcl3-1* mutant, we sequenced the *DCL3* gene in the *dms6-1* mutant and indeed detected a mutation that introduces a premature stop codon at amino acid 130, which is near the N-terminal part of the DCL3 protein (Figure 4, bottom); consequently, *dms6-1*, which is most likely a null allele of *DCL3*, has been renamed *dcl3-5*.

In the *dcl3-5* mutant, 24-nt primary siRNAs derived from the hairpin RNA trigger are eliminated, whereas 21- and 22-nt primary siRNAs are present at slightly elevated levels (Figure 4B), probably through the compensatory action of DCL4 and DCL2, respectively (Fusaro *et al*, 2006). However, despite their increased abundance, the 21- and 22-nt siRNAs are unable to efficiently induce RdDM of the target enhancer region (Figure 5A). The *dcl3-5* mutation also reduces

Figure 4 RNA profile in the *dcl3-5* mutant. Northern blots of small RNAs (A–C; E–J) and RT-PCR detection of the nascent RNA (D). (A) siRNA1003; (B) primary siRNAs; (C) secondary siRNAs; (D) nascent RNA (top) and actin control (bottom); (E) 45S rDNA siRNAs; (F) siRNA02; (G) solo LTR siRNAs; (H) *Tag2* siRNAs; (I) *Sat5* siRNAs. The arrowheads at the left indicate 21- and 24-nt size classes. In panels G–I, the lanes were pieced together from the same gel, as indicated by the dividing white lines. At the bottom of each blot, ethidium bromide staining of the major RNA on the gels is shown as a loading control. Bottom: domain structure of the DCL3 protein (1531 amino acids; Schauer *et al*, 2002) and position of the *dcl3-5* mutation. It is unclear why a *dcl3* mutation was recovered in this mutant screen and not a previous one based on silencing a seed-specific promoter (Kanno *et al*, 2004, 2005), but it may reflect the enhanced sensitivity of the present screen, which uses detection of enhanced GFP in colourless root tips.

methylation of endogenous sequences including a *Tag2* transposon-related sequence (Figure 5B) and 5S rDNA repeats (Supplementary Figure 2).

Secondary siRNAs are undetectable in the *dcl3-5* mutant (Figure 4C); however, it is unlikely that the absence of secondary siRNAs is a direct result of DCL3 deficiency, as initiation of secondary siRNA biogenesis depends on pre-existing primary RdDM, which is greatly reduced in the *dcl3-5* mutant (Figure 5A). Nevertheless, the fact that the secondary siRNAs are 24-nt in length suggests that they are indeed products of DCL3 and able to induce methylation. The absence of secondary siRNAs is consistent with the presence of the nascent RNA in *dcl3-5* plants (Figure 4D).

We tested the accumulation of additional endogenous 24-nt siRNAs in the *dcl3-5* mutant. A pattern of larger (>24-nt) siRNAs, similar to those observed for siRNA1003, as well as faint smaller siRNAs were observed from 45S rDNA repeats (Figure 4E). Other endogenous 24-nt siRNAs that are derived from sequences located in euchromatic chromosome arms, including siRNA02, a retrotransposon solo LTR and the

Tag2 element analysed for methylation are mainly present as 21- and 22-nt size classes in *dcl3-5* plants (Figure 4F–H). Interestingly, siRNAs derived from *SAT5*, a repetitive sequence family comprising several dispersed single copies in euchromatin and a tandem repeat block in the pericentromeric heterochromatin on chromosome 5 (L Daxinger and M Matzke, unpublished data) appear as prominent 21–22-nt siRNAs as well as a ladder of larger RNAs resembling those seen for siRNAs originating from 5S and 45S rDNA (Figure 4I).

Discussion

We have defined a stepwise pathway for biogenesis of 24-nt secondary siRNAs and unidirectional spreading of DNA methylation in *Arabidopsis*. Hairpin-derived primary siRNAs induce primary RdDM at the target enhancer region. This step initiates the Pol IV-RDR2-dependent turnover of a nascent RNA to produce secondary siRNAs, which trigger secondary RdDM in the downstream region (Figure 6).

Figure 5 Analysis of DNA methylation at the targeted enhancer and an endogenous *Tag2* element by bisulphite sequencing in the *dcl3-5* mutant. The graphs show the percentage of methylation at individual cytosines in the *dcl3-5* mutant (top) and wild-type plants (bottom) in the following sequences. **(A)** Targeted enhancer region (black bar) and downstream region (shaded grey bar); **(B)** *Tag2* element. Black lines: CG methylation; blue lines: CNG methylation; red lines: CNN methylation. The results are from eight cloned sequences. Original data are shown in Supplementary Figure 4. The nearly complete loss of asymmetric CNN methylation in both sequences indicates a defect in *de novo* methylation; the residual CG and CNG methylation is probably due to siRNA-independent maintenance of symmetrical methylation (Aufsatz *et al*, 2002).

The stepwise scenario is supported by the observations that primary and secondary siRNA formation, as well as the corresponding RdDM steps, occur sequentially and can be uncoupled genetically.

Our study supports a previous conjecture that primary RdDM is required to initiate 24-nt secondary siRNA formation. This requirement was initially suggested by the absence of secondary siRNAs in *nrpe1* and *drd1* mutants, which still produce primary siRNAs and the overlapping nascent RNA but lack primary RdDM (Kanno *et al*, 2008). The nascent RNA stably accumulates in the absence of primary RdDM in non-silenced plants but, in that instance, is presumably a Pol II transcript (Figure 6) because it is still made in an *nrpd1* mutant. The key function of primary RdDM appears to be in attracting the secondary siRNA-generating machinery, which includes Pol IV and RDR2. The contribution may be direct if primary RdDM provides a template that is preferentially recognized by Pol IV, which has been proposed to transcribe methylated DNA (Herr *et al*, 2005; Onodera *et al*, 2005). In this model, Pol IV would transcribe the nascent RNA from the methylated template (Figure 6). An indirect contribution of primary RdDM is also possible if the nascent RNA needs to be sliced by AGO4 to provide substrates for RDR2. AGO4, which associates with WG/GW repeats in the C-terminal domain of NRPE1 (El-Shami *et al*, 2007), would be brought in together with Pol V during the primary RdDM step. Owing to the partial effect of the *ago4-1* mutation on primary RdDM and the probable redundancy of AGO4 and AGO6 (Zheng *et al*, 2007), we could not discern whether AGO slicing of the nascent RNA is necessary for the biogenesis of secondary siRNAs. This issue can be tested in the future by using *ago4* (and *ago6*) mutants deficient in catalytic PIWI domain but not the siRNA-binding PAZ domain (Qi *et al*, 2006).

Whereas both of the RdDM steps require the same factors (Pol V-DRD1-DMS3-AGO4-DRM2) (Figure 6), the two DCL3-dependent 24-nt siRNA populations differ in their genetic

requirements. *Cis*-acting secondary siRNAs, but not *trans*-acting hairpin-derived primary siRNAs, require Pol IV and RDR2 for their formation. Owing to the strong correlation between the appearance of secondary siRNAs and the disappearance of the nascent RNA, we infer that the nascent RNA is directly or indirectly transcribed by Pol IV and/or RDR2 during secondary siRNA formation. Secondary RdDM occurs only in the presence of secondary siRNAs, which are generated after primary RdDM is induced. Primary RdDM can be established and maintained independently of secondary RdDM, as observed in *rdr2* and *nrpd1* mutants. The dispensability of RDR2 (and RDR6) for primary RdDM differs from the situation in fission yeast, where heterochromatin formation induced in *trans* by hairpin-derived siRNAs nevertheless requires RDR-dependent synthesis of double-stranded RNA in *cis* at the target locus (Iida *et al*, 2008). The basis of this difference is unknown but, as discussed below, it may reflect whether siRNAs interact with target DNA or RNA sequences.

Although the nascent RNA is clearly involved in the formation of secondary siRNAs that induce secondary RdDM, our data suggest that it does not contribute to primary RdDM or *GFP* silencing. The evidence for this is that the nascent RNA is greatly reduced or undetectable in *rdr2* and *nrpd1* mutants that lack secondary siRNAs, yet primary RdDM is present at wild-type levels and the *GFP* reporter gene is silenced in these mutants. Thus the nascent RNA acts only in the non-essential pathway of secondary siRNA biogenesis. This finding, together with the sharp boundary between primary and secondary RdDM, can be used to argue that primary siRNAs induce primary RdDM by interacting directly with the DNA target sequence and not with a nascent 'enhancer-associated' RNA (Han *et al*, 2007). The dispensability of secondary siRNAs and secondary RdDM for *GFP* silencing in our system is consistent with the fact that we have not recovered *nrpd1* or *rdr2* mutants in our forward genetic screen (this study; Kanno *et al*, 2008). Interestingly,

Figure 6 Stepwise pathway for secondary siRNA biogenesis and spreading of methylation. Proteins whose functions have been demonstrated in this study are shown in white letters on dark boxes. Top: in non-silenced plants containing the target locus (T) only, the targeted enhancer region (black box) is unmethylated and the nascent RNA, which normally does not interfere with *GFP* expression, is presumably transcribed by Pol II because it is still detectable in an *nrpd1* mutant. Bottom: in the presence of the silencer locus (S), *GFP* expression is silenced by primary RdDM (blue 'm'), which is induced by hairpin-derived, DCL3-dependent 24-nt primary siRNAs (blue dashes). Primary RdDM attracts the secondary siRNA-generating machinery, which includes Pol IV, RDR2 and DCL3. In this model, Pol IV transcribes the methylated template to produce the nascent RNA, which is copied and diced by RDR2 and DCL3, respectively, to produce 24-nt secondary siRNAs (red dashes) that guide secondary RdDM (red 'm') in the downstream region (black shade). Pol IV may also transcribe the double-stranded RNA to sustain secondary siRNA production once the cycle is initiated (Supplementary Figure 2). In both cases, Pol IV transcribes a precursor of secondary siRNAs. Whether AGO cleavage of the nascent RNA is required to provide substrates for RDR2 is still not known. siRNA-guided *de novo* methylation requires DRD1, DMS3 and Pol V (Kanno *et al*, 2008) and is presumably catalysed by DRM2 (Cao *et al*, 2003).

both *nrpd1* and *nrpe1* mutants were recovered in an independent forward screen using a hairpin RNA to transcriptionally silence a 35S promoter-driven *GFP* transgene (Eamens *et al*, 2008). The unusual requirement for Pol IV in hairpin RNA-triggered silencing may reflect the need to amplify low levels of 35S promoter siRNAs.

RDRs and direction of methylation spreading

During PTGS, spreading of RdDM into transcribed regions is associated with the production of approximately 21–22-nt secondary siRNAs that require RDR6 and transcription of target locus for their biogenesis (Vaistij *et al*, 2002; Eamens *et al*, 2008). The observation that formation of 24-nt secondary siRNAs in our TGS system is unaffected in an *rdr6* mutant but is abolished in an *rdr2* mutant clearly distinguishes the two pathways of secondary siRNA biogenesis. A further difference is that NRPD1 and NRPE1 are dispensable for initiating secondary siRNA formation in a PTGS system that is triggered by a virus (Eamens *et al*, 2008), whereas both of these factors are required for secondary siRNAs and secondary RdDM in our TGS system. Although it is relatively easy to distinguish primary and secondary siRNAs that are derived

from the transgene sequences, it is less clear how to distinguish endogenous primary and secondary siRNAs. On the basis of our results with the transgene system, we suggest that endogenous secondary siRNAs are those that require DRD1/Pol V-mediated DNA methylation, and hence will decrease in abundance in *drd1* and *nrpe1* mutants (Supplementary Figure 3).

Bidirectional spreading has been observed in a PTGS system (Vaistij *et al*, 2002), whereas spreading of methylation in our transgene system occurs in the 3'-direction only (Supplementary Figure 4). Spreading is probably unidirectional because the nascent RNA, which provides a template for RDR2, does not extend upstream of the targeted enhancer region; consistent with this, no secondary siRNAs originating from the upstream region are detectable (T Kanno, L Daxinger, M Matzke, unpublished data). For reasons that are not yet clear, spreading of methylation is not always observed in transgenic plants (Aufsatz *et al*, 2002; Kanno *et al*, 2004, 2005; Vogt *et al*, 2004). Methylation spreading that triggers silencing of endogenous genes in *Arabidopsis* has been reported recently, but the genetic requirements differ somewhat from those needed in our transgene system. Bidirectional spreading of DNA methylation and siRNAs from tandem repeats occurred at the *SDC* locus, but the two phenomena occurred independently and required the CG methyltransferase MET1 and Pol IV-RDR2-DCL3, respectively (Henderson and Jacobsen, 2008). Unidirectional spreading of methylation and 24-nt siRNAs from a LINE retrotransposon into an adjacent *APC* gene was observed in a *ddm1* mutant (Saze and Kakutani, 2007). The number of plant genes that might be regulated by methylation spreading under normal or adverse conditions is not yet known (Woo and Richards, 2008).

Identification of a *dcl3* mutant

Our study is the first to report the identification of a *dcl3* mutation in a forward screen for silencing-defective mutants. The only other EMS-induced point mutation of DCL3 (*dcl3-3*) was isolated as an enhancer of silencing in a transgene PTGS system (Smith *et al*, 2007). In addition to DCL3, which generates 24-nt heterochromatic siRNAs (Xie *et al*, 2004), there are three other DCL activities in *Arabidopsis*: DCL1 produces miRNAs; DCL2 generates 22-nt virus-derived siRNAs and 24-nt stress-related nat-siRNAs; DCL4 generates 21-nt tasiRNAs and siRNAs involved in RNAi (Vazquez, 2006; Voinnet, 2008).

The *dcl3-5* mutation identified in our study demonstrated that hairpin-derived 24-nt siRNAs are essential to induce primary RdDM of the transgene target sequence; 21- and 22-nt siRNAs, even when present at elevated levels in the *dcl3-5* mutant, were unable to compensate for the loss of the 24-nt-size class, perhaps because they are too small or not loaded onto the right effector complex. The strict requirement for hairpin-derived 24-nt siRNAs to induce methylation in our system and in others (Wang *et al*, 2008) contrasts with a previous report that DCL2, DCL3 and DCL4 are partially redundant in *de novo* methylation (Henderson *et al*, 2006). The discrepancy is unlikely to reflect differences in DCL processing of transgenic hairpin RNAs versus RDR-derived double-stranded RNAs because the endogenous *Tag2* element that we studied sustained a heavy loss of methylation in the *dcl3-5* mutant despite the presence of 21–22-nt siRNAs.

Perhaps other locus-specific effects that remain to be identified can account for the differing results.

We observed larger (>24-nt) small RNAs derived from 5S and 45S ribosomal DNA sequences and the *SAT5* repetitive sequence in the *dcl3-5* mutant. Indeed this feature allowed us to select *DCL3* as a candidate gene for the *dms6-1* mutant. By contrast, other endogenous 24-nt siRNAs are present mainly as 21- and 22-nt-size classes in *dcl3* plants, presumably owing to the compensating activities of DCL4 and DCL2, respectively (Xie *et al*, 2004; Gascioli *et al*, 2005; Fusaro *et al*, 2006; Henderson *et al*, 2006; Dunoyer *et al*, 2007). *SAT5* repeats provide an interesting example in which prominent 21- and 22-nt siRNAs are observed in addition to larger (>24-nt) small RNAs. The former may originate from dispersed single copies of *SAT5* that are present in euchromatic regions, whereas the latter might originate from a block of *SAT5* repeats in the pericentromeric region on chromosome 5. The variable accumulation of different size classes of endogenous siRNAs observed in *dcl3* mutants may thus reflect differential accessibility of DCL activities to double-stranded RNAs derived from dispersed versus tandem repeats.

RdDM in mammalian cells?

Mammals methylate their genomes but lack Pol IV subunits, RDRs and 24-nt siRNAs. However, there is evidence that Pol II can transcribe RNA and hence act as an RDR (Lehmann *et al*, 2007; Chang *et al*, 2008). A DMS3-related protein, SmcHD1, is important for murine X chromosome inactivation, which involves non-coding RNAs, DNA methylation and spreading of silencing along the inactive X chromosome (Blewitt *et al*, 2008). Piwi-interacting RNAs (piRNAs) are a larger size class of small RNA (25–30 nt) that guide *de novo* methylation of retrotransposons in the male germ line in mice (Kuramochi-Miyagawa *et al*, 2008). It is thus possible to envision a similar pathway of RdDM and methylation spreading through regions of mammalian genomes that encode overlapping short and long non-coding RNAs (Kapronov *et al*, 2007).

Materials and methods

Nomenclature

In recently updated nomenclature (Wierzbicki *et al*, 2008), Pol IVa and Pol IVb are renamed Pol IV and Pol V, respectively. The largest subunits of Pol IV and Pol V, previously termed NRPD1a and NRPD1b, have been renamed NRPD1 and NRPE1, respectively. The shared second largest subunit, formerly NRPD2a, has the synonymous name NRPD2a/NRPE2a.

Mutants

We used the following *Arabidopsis thaliana* mutants in this study: *rdp2-1* (SAIL_1277H08; Xie *et al*, 2004), *rdp6-1* (Elmayan *et al*, 1998), *nrdp1-7* (formerly *nrdp1a-7*) (Smith *et al*, 2007), *ago4-1* (Zilberman *et al*, 2003) and *dcl3-1* (SALK_005512; Xie *et al*, 2004). All mutants are in the Columbia (Col-0) ecotype, with the exception of *ago4-1*, which is in the Landsberg erecta line. The mutants were genotyped using primers listed in Supplementary Table 2. Genotypes of *rdp2* and *rdp6* plants were confirmed by testing for the presence of siRNA02 and tasiRNA255, respectively (Figure 2). In

addition, the *nrdp1-7* and *ago4-1* mutations were confirmed by sequencing the respective gene in DNA isolated from the genotyped plants.

Small RNA isolation and northern blot analysis

Small RNAs were isolated from pooled 21-day-old seedlings or inflorescences using the mirVana miRNA isolation kit (Ambion) and analysed by northern blot hybridization according to published procedures (Kanno *et al*, 2005; Huettel *et al*, 2006). To obtain enough plants of the proper genotype for *rdp2*, *rdp6* and *nrdp1* mutants and their wild-type siblings, we used plants from the F3 generation. For the *ago4-1* mutant, we used plants of the F2 generation. We used end-labelled oligonucleotides to detect siRNA1003 (from 5S rDNA repeats), siRNAs from 45S rDNA repeats, tasiRNA 255 and siRNA02. We used end-labelled LNA (locked nucleic acid) oligonucleotides to detect siRNAs from *Tag2* and *SAT5*. A riboprobe was used to detect siRNAs from the solo LTR (Huettel *et al*, 2006). Primer information for specific probes is shown in Supplementary Table 2.

Nascent RNA analysis

To detect nascent RNAs, total RNA was isolated from mixed floral inflorescences using Trizol (Invitrogen). A cDNA was synthesized according to the manufacturer's instructions using a First Strand cDNA Synthesis kit (Fermentas) using an oligonucleotide d(T) primer and 1 µg of total RNA. The primers are shown in Supplementary Table 2. These primers detect a portion of the nascent transcript starting directly downstream of the targeted enhancer region and extending to the end of the *GFP* coding region (see the 'green primer' set in Supplementary Figure 1 of Kanno *et al*, 2008).

Bisulphite sequencing

We isolated genomic DNA from rosette leaves using a DNeasy Plant Maxi kit (Qiagen). Bisulphite treatment of DNA was conducted using a EpiTect Bisulphite kit (Qiagen) according to the manufacturer's instructions with several modifications as follows. Genomic DNA was predigested with *HindIII* and 500 ng of the DNA was treated in the reaction solution containing 35 µl of the DNA protection buffer. Following incubation for 2 min at 95°C, eight cycles of these thermocycler conditions were performed: for 1 min at 95°C and for 2 h at 75°C. Primers are shown in Supplementary Table 2. As a control for complete bisulphite conversion, exon 15 from the *PHAVOLUTA* locus that lacks methylated cytosines in the wild type was used (Bao *et al*, 2004; Reinders *et al*, 2008). At least five clones were sequenced from each mutant and wild-type control. Bisulphite conversion of the *PHAVOLUTA* sequence ranged in all cases between 99 and 100%. Original data from the target enhancer in the various mutants and the *Tag2* sequence in *dcl3-5* are shown in Supplementary Figure 4.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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