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Atypical RNA polymerase subunits required for RNA-directed DNA methylation

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RNA-directed DNA methylation, one of several RNA interference–mediated pathways in the nucleus[1], has been documented in plants[2,3] and in human cells[4,5]. Despite progress in identifying the DNA methyltransferases, histone-modifying enzymes and RNA interference proteins needed for RNA-directed DNA methylation[1], the mechanism remains incompletely understood. We screened for mutants defective in RNA-directed DNA methylation and silencing of a transgene promoter in *Arabidopsis thaliana* and identified three *drd* complementation groups[6]. DRD1 is a SNF2-like protein[6] required for RNA-directed *de novo* methylation. We report here that DRD2 and DRD3 correspond to the second-largest subunit and largest subunit, respectively, of a fourth class of DNA-dependent RNA polymerase (polymerase IV) that is unique to plants. DRD3 is a functionally diversified homolog of NRPD1a or SDE4, identified in a separate screen for mutants defective in post-transcriptional gene silencing[7,8]. The identical DNA methylation patterns observed in all three *drd* mutants suggest that DRD proteins cooperate to create a substrate for RNA-directed *de novo* methylation.

In the two-component α′ promoter silencing system, a target α′ promoter drives expression of a *GFP* reporter gene in seeds[6]. The target α′ promoter is silenced and methylated only in the presence of an unlinked silencer complex, which encodes a hairpin RNA containing α′ promoter sequences (**Fig. 1**). In *drd2* and *drd3* plants, there is heavy loss of non-CG methylation accompanied by residual CG methylation (**Fig. 1** and **Supplementary Fig. 1** online). An identical hybridization pattern was observed in *drd1* mutants[6]. Although symmetrical CG and CNG methylation can be maintained in some cases in the absence of the RNA trigger[9–11], the loss of asymmetrical CNN methylation suggests that there are defects in *de novo* methylation, which needs the continuous presence of RNA[9]. Indeed, DRD1 is required for RNA-directed *de novo* methylation of target promoters[12].

The lack of non-CG methylation of the target α′ promoter in *drd2* and *drd3* mutants does not reflect insufficient production of RNA

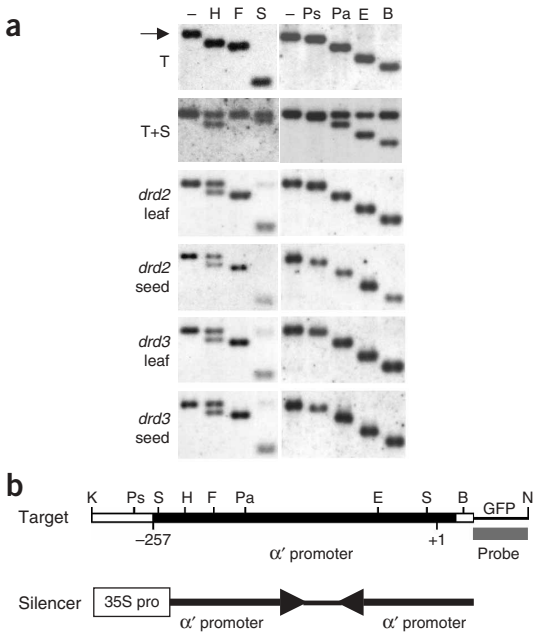

Figure 1 Non-CG methylation is lost in *drd* mutants. (**a**) Methylation of the target α′ promoter was analyzed by digestion with restriction enzymes sensitive to non-CG methylation (*Fnu*4HI, F; *Scr*F1, S; *Pst*I, Ps; *Pag*I, Pa; *Eco*T22I, E; *Bam*HI, B) and CG methylation (*Hpy*CH4IV, H), after a double digestion with methylation-insensitive (–) *Kpn*I (K) and *Nde*I (N). The small arrow at the left indicates the methylated fragment. Results for the unmethylated target gene (T) and the methylated target gene in the presence of the silencer (T+S) are from leaf DNA. Identical patterns were observed with seed DNA[6]. Residual CG methylation in leaf and seed DNA of *drd2* and *drd3* mutants is exemplified by the persistent double band after digestion with H. (**b**) The black bar represents the 257-bp region of the target α′ promoter that is homologous to the hairpin RNA encoded by the silencer complex, which contains an α′ promoter inverted repeat that is transcribed by the constitutive 35S promoter. +1, transcription start site. Part of the *GFP* coding region (gray bar) was used to probe the blots.

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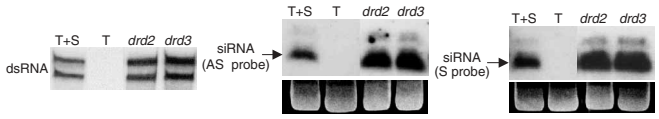

Figure 2 α′ promoter dsRNA and siRNAs accumulate in *drd* mutants. Similar amounts of α′ promoter dsRNA were detected in *drd2* and *drd3* mutants and in wild-type plants containing the target and silencer complexes (T+S) but not the α′ promoter–*GFP* target gene alone (T). Levels of both sense (S) and antisense (AS) siRNAs (~21–24 nucleotides) were slightly elevated, for unknown reasons, in *drd2* and *drd3* mutants compared with wild-type plants. Ethidium bromide staining of the major RNA on the gel is shown as a loading control. The position of a 23-nucleotide DNA oligonucleotide is indicated by the arrow.

signals. Neither the amount of α′ promoter double-stranded RNA (dsRNA) nor the amount of short interfering RNA (siRNA) processing products is diminished in *drd2* and *drd3* plants (**Fig. 2**).

We mapped the *drd3* mutation to an interval of ~80 kb on chromosome 2. Sequencing of candidate genes in this region detected mutations in an area annotated to encode two proteins: At2g40030 and At2g40040 (**Supplementary Methods** online). Subsequent analysis of the cDNA sequence showed that DRD3 is a single protein, 1,976 amino acids in length, comprising the previously annotated At2g40030 and At2g40040 (**Fig. 3a**). We sequenced nine *drd3* alleles; seven of the mutations introduce premature stop codons (**Fig. 3b**).

DRD3 is an atypical subunit of RNA polymerase that is unique to plants. All eukaryotes examined so far have distinct genes encoding the largest and second-largest subunits of DNA-dependent RNA polymerases I, II and III. In addition, the *A. thaliana* genome contains pairs of genes encoding a fourth class of the largest and second-largest subunits (refs. 13,14 and The Arabidopsis Information Resource). Whereas the two second-largest subunits of putative polymerase IV (Pol IV) resemble those of RNA polymerase II (Pol II) from other eukaryotes, the largest subunits of Pol IV differ more substantially from those of conventional eukaryotic Pol II (ref. 14). *DRD3* is one of two *A. thaliana* genes encoding the largest subunit of Pol IV. The second gene is SILENCING-DEFECTIVE 4 (*SDE4*; At1g63020)[8]. SDE4 was identified in an independent screen for mutants impaired in post-transcriptional gene silencing[7]. SDE4 is needed for DNA methylation; for accumulation of siRNA from several endogenous DNA repeats, including the SINE element *AtSN1* and 5S rDNA arrays[8,14,15]; and for DNA methylation of an *FWA* transgene[16]. As the largest subunit of Pol IV, SDE4 was renamed NRPD1a (refs. 8,14). Accordingly, DRD3 corresponds to NRPD1b, and the nine *drd3* alleles reported here (*drd3-1* through *drd3-9*) were renamed *nrpd1b-1* through *nrpd1b-9*. Only *nrpd1b* alleles were recovered in our screen, which identified mutants defective in hairpin RNA-directed promoter methylation and presumed transcriptional gene silencing. By contrast, *nrpd1a* mutants were identified in a post-transcriptional gene silencing system requiring an RNA-dependent RNA polymerase to produce or amplify the RNA trigger[7,8]. These differences imply some functional divergence between the two NRPD1 homologs.

We mapped the *drd2* mutation to a region on chromosome 3 that contains genes encoding both of the second-largest subunits of Pol IV: At3g23780 and At3g18090 (The Arabidopsis Information Resource and **Supplementary Methods** online). Only At3g23780 is expressed[14]. In view of this information and the discovery that *NRPD1b* encodes the largest subunit of Pol IV, we sequenced the gene encoding At3g23780 and successfully identified mutations. We sequenced 12 *drd2* alleles (*drd2-1* through *drd2-12*; **Fig. 3c**). In a separate study, a T-DNA insertion mutant of the gene encoding At3g23780 had a silencing-defective phenotype resembling that of *nrpd1a* mutants[8]. As the second-largest subunit of Pol IV, At3g23780 was named NRPD2a and the T-DNA insertion mutant was designated *nrpd2a-1* (ref. 8). A recent analysis of two additional T-DNA insertion mutants, *nrpd2a-2* and *nrpd2a-3*, detected hypomethylation of 5S rDNA repeats and *AtSN1* and elimination of the corresponding siRNAs[14]. The *drd2* alleles we identified were renamed *nrpd2a-4* through *nrpd2a-15*. Notably, there seems to be

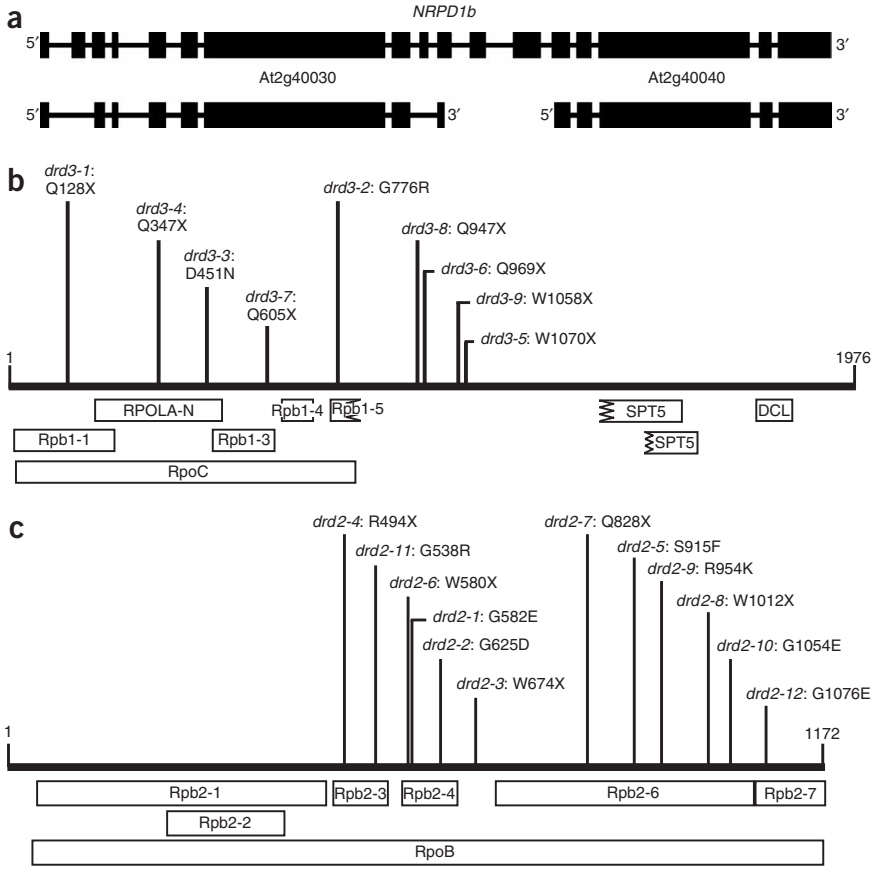

Figure 3 Structure of the gene *NRPD1b* and positions of point mutations. *NRPD1b*, which comprises the previously annotated At2g40030 and At2g40040, contains 17 exons and 16 introns and encodes a protein of 1,976 amino acids (**a**). Positions of point mutations in alleles of *nrpd1b* (**b**) and *nrpd2a* (**c**). Conserved domains were identified in an RPSBLAST search. The DCL motif in NRPD1b was identified in a BLASTP search (see also **Supplementary Methods** and **Supplementary Fig. 2** online). X, stop codon.

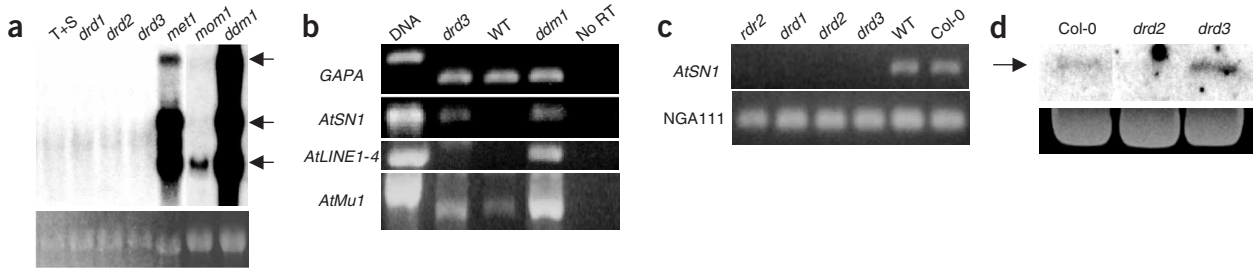

Figure 4 Transposon reactivation in *drd* mutants. (**a**) Northern-blot analysis of an *Athila*-retrotransposon-related sequence[18], which is strongly reactivated in DNA hypomethylation mutants (such as *ddm1* and *met1*; arrows) and weakly reactivated in *mom1* mutants (which are defective in a new protein encoding part of the SNF2 domain[29]) but not in *nrpd1b* (*drd3*), *nrpd2a* (*drd2*) or *drd1* plants. T+S, wild-type plants containing the target and silencer complexes. (**b**) RT-PCR detected reactivation of *AtSN1* and *AtMu1* in *nrpd1b* (*drd3*) mutants; reactivation in *ddm1* mutants is shown as a positive control[19]. Similar results were obtained for *nrpd2a* (*drd2*) and *drd1* plants (not shown). GAPA, constitutively expressed control; RT, reverse transcriptase; WT, wild-type. (**c**) *AtSN1* contains three *Hae*III sites (**Supplementary Fig. 4** online), at least one of which loses methylation in the three *drd* mutants, as indicated by the disappearance of the amplification product observed in wild-type plants containing the target and silencer (WT) and in untransformed plants (Col-0). *rdr2* is shown as a control for loss of *AtSN1* methylation[8,15]. Microsatellite marker locus NGA111, which lacks *Hae*III restriction sites, was used as a control. (**d**) AtSN1 siRNAs accumulate in wild-type (Col-0) and in *nrpd1b* (*drd3*) plants but are reduced in *nrpd2a* (*drd2*) mutants. Ethidium bromide staining of the major RNA on the gel is shown as a loading control. The arrow indicates the position of a 23-nucleotide DNA oligonucleotide.

only one functional homolog of the second-largest subunit of Pol IV, in contrast to the apparent functional diversification of the largest subunit.

We investigated other possible targets of the DRD protein-dependent pathway, in addition to the target α′ promoter. Like *drd1* plants[6], centromeric repeats in *nrpd1b* or *nrpd2a* mutants do not lose methylation (data not shown). This is in contrast to mutants defective in DDM1, a SNF2-like chromatin remodeling protein, which sustain heavy losses of methylation in centromeric repeats[6,17]. We analyzed the expression of several transposons by northern blotting or RT-PCR. Neither the *Athila*-retrotransposon-related sequence[18] nor *AtLINE1-4* was reactivated in *nrpd1b, nrpd2a* or *drd1* plants, although both were stimulated in *ddm1* mutants (**Fig. 4a**,**b** and data not shown). Two of the transposons tested, *AtSN1* and the DNA transposon *AtMu1*, showed increased expression both in *ddm1* (ref. 19) and in *nrpd1b, nrpd2a* and *drd1* mutants (**Fig. 4b** and data not shown). The targets of the DRD protein-dependent silencing pathway therefore seem to overlap only partially with those of DDM1 (ref. 19).

The reactivation of *AtSN1* in *nrpd1b*, *nrpd2a* and *drd1* plants was accompanied by reduced DNA methylation (**Fig. 4c**), indicating that this transposon-like sequence was a common target of NRPD1b and NRPD1a (refs. 8,15). But the loss of *AtSN1* methylation in *nrpd1b* mutants was not accompanied by a loss of *AtSN1* siRNAs (**Fig. 4d**). By contrast, *AtSN1* siRNAs were eliminated in *nrpd1a* and *nrpd2a* plants[8,15]; we confirmed this result in an *nrpd2a* mutant (**Fig. 4d**). We obtained similar findings for 5S rDNA repeats. Although CG and non-CG methylation were reduced to equal extents in *nrpd1b*, *nrpd2a* and *drd1* mutants (**Fig. 5a**,**b**), 5S siRNAs were still present in *nprdb1* and *drd1* plants and failed to accumulate only in *nrpd2a* mutants[8,14] (**Fig. 5c**). We observed the same pattern of accumulation with siRNAs originating from a third endogenous locus, Cluster 55 (**Fig. 5d**).

The results from both the endogenous and transgenic siRNAs and corresponding DNA targets that we studied support the contention that the two largest subunits of Pol IV, NRPD1a and NRPD1b, have nonredundant functions in RNA-directed DNA methylation (**Fig. 6**).

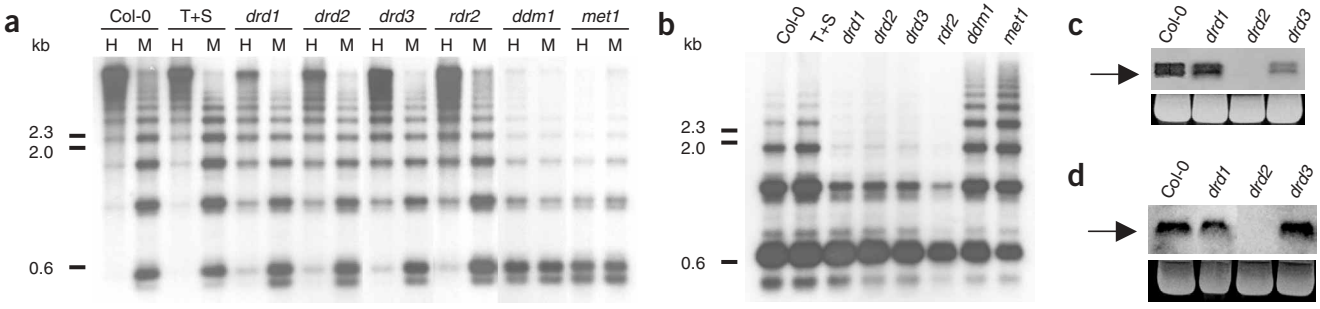

Figure 5 DNA methylation and siRNA accumulation from 5S rDNA repeats. (**a**) Digestion with *Hpa*II (H) reports on CG methylation and *Msp*II (M) reports on CNG methylation of 5S repeats in the Col-0 ecotype[14]. Slightly increased digestion is seen with *Hpa*II and *Msp*II in *drd1*, *nrpd2a* (*drd2*) and *nrpd1b* (*drd3*) mutants compared with wild-type plants (Col-0 and wild-type plants containing the target and silencer complexes, T+S), indicating partial loss of CG and CNG methylation. Increased cleavage is observed with *Hpa*II and *Msp*II in *ddm1* and *met1* mutants, which are defective in global CG methylation[17]. (**b**) *Hae*III digestion reports on CNN methylation of 5S repeats in Col-0 (ref. 14). Losses of CNN methylation are seen in *drd1*, *nrpd2a* (*drd2*) and *nrpd1b* (*drd3*) mutants, but not in *ddm1* or *met1* plants. T+S, wild-type plants containing the target and silencer complexes. 5S rDNA siRNAs (**c**) and siRNAs originating from Cluster 55 (**d**) are detectable in wild-type plants (Col-0) and in *nrpd1b* (*drd3*) and *drd1* mutants, but not in *nrpd2a* (*drd2*) plants. The major RNA species on the ethidium bromide–stained gel is shown as a loading control. The arrows indicate the position of a 24-nucleotide (**c**) or 21-nucleotide (**d**) DNA oligonucleotide.

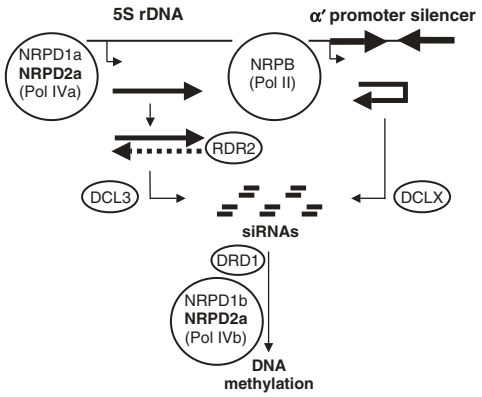

Figure 6 Distinct Pol IV complexes act in RNA-directed DNA methylation. Functional diversification of Pol IV complexes is specified by unique largest subunits: NRPD1a (Pol IVa) and NRPD1b (Pol IVb). Transcripts synthesized by Pol IVa from endogenous loci, such as 5S rDNA, are converted to dsRNA by the RNA-dependent RNA polymerase RDR2 and processed to siRNAs by DCL3 (ref. 8). By contrast, a hairpin RNA is generated in a single step by Pol II–dependent transcription of the α′ promoter inverted repeat, bypassing a requirement for Pol IVa and RDR2. An unidentified Dicer activity (DCLX) processes the hairpin RNA to siRNAs. Pol IVb and the SNF2-like protein DRD1 are required downstream of siRNA formation to induce RNA-directed DNA methylation of target loci by an unknown mechanism. Both Pol IV complexes share a common second-largest subunit NRPD2a (bold). The proposed dual role of NRPD2a is supported by its mutant phenotypes, which suggest a role in both siRNA production (5S rDNA) and DNA methylation downstream of siRNA formation (α′ promoter silencing system).

Whereas NRPD1a is involved in generating siRNAs[8,14–16], NRPD1b acts downstream of this step, as evidenced by the continued presence of α′ promoter siRNAs and endogenous siRNAs in *nrpd1b* mutants. Target DNA sequences nevertheless lost methylation in *nrpd1b* plants, implicating NRPD1b in signaling between siRNAs and DNA. By contrast, the second-largest subunit (NRPD2a) is necessary at both stages of the pathway, siRNA production and DNA methylation, as indicated by the strong reduction of *AtSN1* and 5S siRNAs and the decreased methylation of the cognate target DNAs in *nrpd2a* plants. α′ promoter siRNAs were still present in *nrpd2b* mutants, even though methylation of the target α′ promoter was reduced in these plants, presumably because the synthesis of the hairpin RNA precursor is independent of Pol IV and RNA-dependent RNA polymerase activity (**Fig. 6**).

Comparisons of the amino acid sequences and predicted secondary structures do not uncover any functional motifs or disruptions thereof that could provide obvious explanations for the different roles of NRPD1a and NRPD1b (**Supplementary Fig. 2** online). The RNA polymerase functional domains in the N-terminal two-thirds of both NRPD1 homologs are relatively well conserved, whereas differences are more pronounced in regions predicted from the yeast Rpb1 sequence to interact with other RNA polymerase subunits (*e.g.*, Rpb2 and Rpb9). Thus, the functional diversification of NRPD1a and NRPD1b might be due to their differential interaction with other proteins. The two NRPD1 homologs diverge in their C-terminal regions, except for the common presence of DCL motifs (defective chloroplasts and leaves), which might be important for RNA recognition or utilization (**Supplementary Methods** online). The extended C-terminal region of NRPD1b shows some fragmentary similarity to Spt5 proteins (**Fig. 3b**). Spt5 can repress transcription elongation by Pol II (ref. 20). Conceivably, if this region of NRPD1b has Spt5 activity, it could hinder transcriptional elongation by Pol IVb, helping to create an open DNA structure that facilitates *de novo* cytosine methylation at the siRNA-targeted site (**Supplementary Fig. 3** online).

Forward genetic screens indicate that an increasing number of proteins needed for RNA-directed DNA methylation are plant-specific. These plant proteins include the SNF2-like protein DRD1 (ref. 6) and the Pol IV subunits described here: NRPD2a, NRPD1b and the NRPD1b homolog NRPD1a (refs. 8,14). Other proteins contributing to RNA-directed DNA methylation that are not present in mammals include SGS3, a coiled-coil protein of unknown function[21]; the RNA-dependent RNA polymerases RDR2 (refs. 8,16,22) and RDR6 or SGS2 (ref. 21); and HEN1 (ref. 23), which methylates microRNAs[24]. Moreover, the DCL motif, which is present in NRPD1b, NRPD1a and their rice homologs (**Supplementary Methods** online), is unique to the plant kingdom[25,26]. Thus, if RNA-directed DNA methylation occurs regularly in mammals[4,5], its mechanism or protein machinery differ from those identified in plants.

The functions of the RNA-directed DNA methylation pathway studied here remain to be clarified. The observation that silent transposons are not generally reactivated in *drd* mutants suggests that the functions of the DRD protein-dependent pathway might extend beyond transposon control and silencing of repetitive elements. Well-defined transgene systems have been indispensable for carrying out mutant screens to identify proteins required for RNA-directed DNA methylation. It should now be possible to ascertain the natural roles and targets of this process by using genomics approaches to analyze changes in gene expression and DNA methylation patterns in mutant plants.

METHODS

Plant material. We used plants carrying the *drd2-1* and *drd3-1* alleles for all expression, methylation and small RNA analyses.

Methylation analyses. We analyzed methylation of the target α′ promoter with seed and leaf DNA using methylation-sensitive restriction enzymes and bisulfite sequencing as described previously[6]. We initially monitored silencing of the α′ promoter–*GFP* reporter gene in seeds, but RNA-directed DNA methylation of the target α′ promoter is already detected in leaves, owing to the constitutive activity of the 35S promoter transcribing α′ promoter dsRNA. We used the following methylation-sensitive restriction enzymes (recognition sites given in parentheses; 'm' indicates sensitivity to methylation): *Pst*I (^{m}CTG^{m}CAG); *Scr*F1 (C^{m}CNGG); *Hpy*CH4IV (A^{m}CGT); *Fnu*4HI (G^{m}C^{m}NG^{m}C, if N is C); *Pag*I (T^{m}CATGA); *Eco*T22I (ATG^{m}CAT); and *Bam*HI (GGAT^{m}C^{m}C).

We carried out methylation of *AtSN1* and PCR analysis as described previously[15] (**Supplementary Fig. 4** online). As a PCR control, we amplified the microsatellite marker locus NGA111, which lacks *Hae*III restriction sites.

We amplified the probe to detect 5S rDNA repeats on DNA blots by PCR from genomic plant DNA using 5S-F and 5S-R primers (**Supplementary Table 1** online). For hybridization, we labeled the DNA fragment with random primers and [α-^{32}P]dATP using the Megaprime DNA Labelling System (Amersham Biosciences).

RNA analysis. We detected α′ promoter dsRNA using RNase One, which degrades single-stranded but not dsRNA, in accordance with a published protocol[27]. The two bands visible in the dsRNA blots (**Fig. 2**) are probably due to RNase One cleavage at a mismatch resulting from a PCR-induced point mutation in one half of the α′ promoter inverted repeat (data not shown). We isolated α′ promoter siRNAs from rosette leaves and analyzed them by northern blotting as described previously[6,9].

We analyzed endogenous siRNAs by northern-blot hybridization using established procedures[8,22]. Primer information for specific probes is given in **Supplementary Table 1** online. We used riboprobes to detect *AtSN1* and Cluster 55. We used an end-labeled oligonucleotide to detect 5S rDNA siRNA 1003.

***NRPD1b* cDNA sequence.** We sequenced *NRPD1b* cDNA prepared using a First Strand cDNA Synthesis Kit (Fermentas) with 5 μg of total RNA as recommended by the manufacturer. We amplified the *NRPD1b* gene fragment by PCR and purified it with the QIAquick PCR purification kit (Qiagen, purchased from VWR International GmbH). The purified fragment was sequenced by VBC-GENOMICS Bioscience Research GmbH using primers listed in **Supplementary Table 1** online (DRD3-1 to DRD3-12). The region that is annotated as GBGE290 (3′; an expressed-sequence tag clone for the At2g40040 gene) was not sequenced.

Transposon expression analysis. For northern-blot analysis, we extracted total RNA from rosette leaves, separated it by electrophoresis and blotted it as described[9]. We amplified an *Athila*-specific probe by PCR from genomic *A. thaliana* DNA using primers described in **Supplementary Table 1** online (GS-F and GS-R). We subcloned the amplified fragment into pGEM-T Easy vector (Promega GmbH) and verified its identity by DNA sequencing. We used random-primed labeled fragment (Amersham Biosciences) for hybridization of 15 μg of total *A. thaliana* RNA, carried out as described previously[9]. For RT-PCR analysis, we isolated total RNA from leaf tissue using RNeasy Plant Mini Kit (Qiagen, purchased from VWR International GmbH) in accordance with the manufacturer's instructions. We treated RNA with DNaseI for 30 min and then incubated it at 70 °C for 15 min to inactivate DNase. We produced first-strand cDNA with reverse transcriptase (M-MuLV, 200 U μl^{-1}) in the presence of an RNase inhibitor (40 U μl^{-1}) using an oligo-dT primer (20 pmol; all components of a First Strand cDNA Synthesis Kit purchased from Fermentas GmbH) at 42 °C. RT-PCR conditions were as follows: 94 °C for 1 min, followed by 30 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 30 s, except for *AtSN1*, for which we carried out 40 cycles. We normalized the cDNA input per reaction by RT-PCR analysis of GAPA (glyceraldehyde 3-phosphate dehydrogenase A; At3g26650) using primers described in **Supplementary Table 1** online (GAPA-F and GAPA-R) with 23 PCR cycles. We used primers to detect transcripts from repetitive elements for the *AtMu1* element primers spanning intron 1 (ref. 28); for the locus *AtSN1*, as reported previously[15]; and for *AtLINE1-4* (At2g01840). Primers are described in **Supplementary Table 1** online (AtLINE1-4-F and AtLINE1-4-R).

URL. The Arabidopsis Information Resource is available at http://www.arabidopsis.org/.

GenBank accession number. *NRPD1b* sequence, AY927744.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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