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Endogenous targets of RNA-directed DNA methylation and Pol IV in *Arabidopsis*

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DRD1 is a SWI/SNF-like protein that cooperates with a plant-specific RNA polymerase, Pol IVb, to facilitate RNA-directed *de novo* methylation and silencing of homologous DNA. Screens to identify endogenous targets of this pathway in *Arabidopsis* revealed intergenic regions and plant genes located primarily in euchromatin. Many putative targets are near retrotransposon LTRs or other intergenic sequences that encode short RNAs, which might epigenetically regulate adjacent genes. Consistent with this, derepression of a solo LTR in *drd1* and pol IVb mutants was accompanied by reduced cytosine methylation and transcriptional upregulation of neighboring sequences. The solo LTR and several other LTRs that flank reactivated targets are associated with euchromatic histone modifications but little or no H3K9 dimethylation, a hallmark of constitutive heterochromatin. By contrast, LTRs of retrotransposons that remain silent in the mutants despite reduced cytosine methylation lack euchromatic marks and have H3K9 dimethylation. We propose that DRD1 and Pol IVb establish a basal level of silencing that can potentially be reversed in euchromatin, and further reinforced in heterochromatin by other proteins that induce more stable modifications.

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Introduction

RNA-directed DNA methylation, which is one of several RNAi-mediated pathways in the nucleus, induces *de novo* cytosine (C) methylation within a region of RNA–DNA sequence complementarity (Matzke and Birchler, 2005; Wassenegger, 2005). RNA-directed DNA methylation has been documented most thoroughly in plants, where it has been shown that promoter-directed double-stranded RNAs can elicit transcriptional gene silencing and promoter methylation in diverse species (Mathieu and Bender, 2004; Matzke *et al*, 2004; Cigan *et al*, 2005). In mammalian cells, promoter-directed short RNAs can trigger transcriptional gene silencing (Morris, 2005; Weinberg *et al*, 2006), but this is not always accompanied by detectable DNA methylation (Ting *et al*, 2005).

In plants, RNA induces methylation of not only CG dinucleotides, which are the primary sites of methylation in mammals, but also cytosines in other sequence contexts (CNG and CNN, where N is A, T or C). The more complex pattern increases the versatility of cytosine methylation in plants compared to other organisms that methylate their DNA. For example, plant sequences deficient in CG dinucleotides but enriched in non-CG nucleotide groups can potentially be silenced by RNA-directed DNA methylation. Furthermore, the differential inheritance of cytosine methylation in different sequence contexts during DNA replication leads to variable reversibility: whereas symmetric CG and CNG methylation can be perpetuated by DNA methyltransferases that recognize a hemimethylated substrate, asymmetric CNN methylation is not efficiently maintained and requires the continuous presence of the RNA trigger (Jones *et al*, 2001; Aufsatz *et al*, 2002). Thus, CNN methylation is a readily reversible repressive mark that is lost in dividing cells if the inducing RNA is withdrawn.

Genetic approaches in *Arabidopsis thaliana* have demonstrated that establishment and maintenance of RNA-directed DNA methylation require for the most part conserved DNA methyltransferases and histone-modifying enzymes (Chan *et al*, 2005). Recently, however, several plant-specific proteins that are essential for this process have been identified. A forward screen for mutants defective in silencing and methylation of the transgene α' promoter retrieved three *drd* complementation groups (defective in RNA-directed DNA methylation). DRD1 is a plant-specific, putative SWI/SNF-like chromatin remodelling protein, which presumably allows RNA-directed DNA methylation to take place in a chromatin context (Kanno *et al*, 2004, 2005a). DRD2 and DRD3 are, respectively, the second largest subunit and the largest subunit of a novel, plant-specific RNA polymerase termed Pol IV. These proteins have been renamed: DRD2 is NRPD2a and DRD3 is NRPD1b (Kanno *et al*, 2005b). NRPD2a is the only functional second largest subunit of Pol IV in *Arabidopsis*. In addition to NRPD1b, however, there is another largest subunit, NRPD1a, which was originally identified as SDE4 in a screen for silencing defective mutants (Dalmay *et al*, 2000). Current models propose two functionally distinct Pol IV complexes that are specified by their unique largest subunits. Pol IVa (containing NRPD1a and NRPD2a) is required for the production and/or amplification of short RNAs (Herr *et al*, 2005; Onodera *et al*, 2005). By contrast, Pol IVb (containing NRPD1b and NRPD2a) acts downstream of short RNA formation to convert RNA signals into chromatin modifications by an unknown mechanism (Kanno *et al*, 2005b; Pontier *et al*, 2005).

The *drd1* and pol IVb mutants were recovered by screening for release of transgene silencing, which did not reveal

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endogenous DNA targets of these proteins. Initial attempts to identify the targets of DRD1 in *Arabidopsis* focused on sequences that are reactivated in mutants defective in DDM1 (decrease in DNA methylation), a plant SWI/SNF-like protein that has a mammalian homolog, Lsh (Jeddeloh *et al*, 1999; Dennis *et al*, 2001). Several types of tandemly repeated sequences that are packaged into heterochromatin—such as 180 bp centromeric repeat arrays and pericentromeric *Athila* retrotransposons—are reactivated and/or lose CG methylation in *ddm1* mutants but not in *drd1* plants (Vongs *et al*, 1993; Lippman *et al*, 2003; Kanno *et al*, 2004, 2005b). The 5S rDNA repeats lose primarily CG methylation in *ddm1* mutants but asymmetric CNN methylation in *drd1* mutants (Kanno *et al*, 2005b). This accords with the methylation pattern of the transgene α′ promoter, which lost CNN methylation but retained CG methylation when reactivated in *drd1* and pol IVb mutants (Kanno *et al*, 2004, 2005a, b).

These experiments suggested that DRD1 does not play a major role in assembling tandem repeats into heterochromatin containing CG methylation, but instead is important for CNN methylation of target sequences. Asymmetric CNN methylation can be considered a measure of *de novo* methylation because it is not efficiently maintained during DNA replication unless the RNA trigger is continually present. Indeed, DRD1 is essential for RNA-directed *de novo* methylation of target DNA; surprisingly, however, DRD1 is also needed for full erasure of CG methylation following removal of the trigger RNA. These findings led to the proposal that DRD1 facilitates dynamic regulation of DNA methylation (Kanno *et al*, 2005a).

Understanding the natural roles of DRD1 and Pol IVb in plants requires additional information about the endogenous DNA targets that are methylated and silenced by these proteins. Moreover, a characterization of histone modifications that accompany DRD1/Pol IVb-dependent DNA methylation would help to integrate this silencing pathway into the framework of other epigenetic processes. We report here the results of experiments designed to investigate these questions in *Arabidopsis*.

Results

Identification of DRD1 target sequences

Suppression subtractive hybridization (SSH) and cDNA-amplified fragment length polymorphism (AFLP) were used to identify transcripts that differentially accumulate in a *drd1* mutant compared to wild-type plants. These techniques revealed transcripts from plant genes involved in metabolism, photosynthesis, and protein synthesis, as well as several intergenic regions; cases of upregulation and downregulation in the *drd1* mutant were observed (Supplementary Table I; Supplementary Figure 1).

Two sequences representing transcripts that are more abundant in the *drd1* mutant were identified in both the SSH and cDNA-AFLP analyses: an intergenic (*IG*) region together with adjacent sequences from the 3′ end of a truncated non-LTR retrotransposon (*LINE*) (At5g27845) and a gene encoding the ribosomal protein RPL18C (At5g27850). Interestingly, the *RPL18C* gene and the truncated *LINE* sequence are adjacent to each other, in the same orientation, on chromosome 5 (Figure 1).

Real-time RT–PCR confirmed the increased levels of *IG/LINE* and *RPL18C* transcripts in *drd1* plants. Included in this experiment were mutants defective in NRPD2a and NRPD1b (referred to hereafter as pol IVb mutants), NRPD1a (the largest subunit of Pol IVa), RNA-dependent RNA polymerase 2 (RDR2), which is thought to generate double-stranded RNA from single-stranded templates produced by Pol IVa activity, and MET1, a DNA methyltransferase specific for CG dinucleotides. The *IG/LINE* transcript was undetectable in wild-type plants, whereas a strong induction was observed in *drd1*, pol IVb, *nrpd1a*, and *rdr2* mutants. By contrast, negligible activation occurred in the *met1* mutant (Figure 2). The *RPL18C* transcript was present at a low level in wild-type plants but was enhanced in the *drd1* mutant and to a lesser extent in the other mutants tested (Figure 2).

5′ and 3′ RACE revealed that the *IG/LINE* transcript was actually in antisense orientation, initiating in the intergenic region and terminating in the 3′ end of the truncated *LINE*

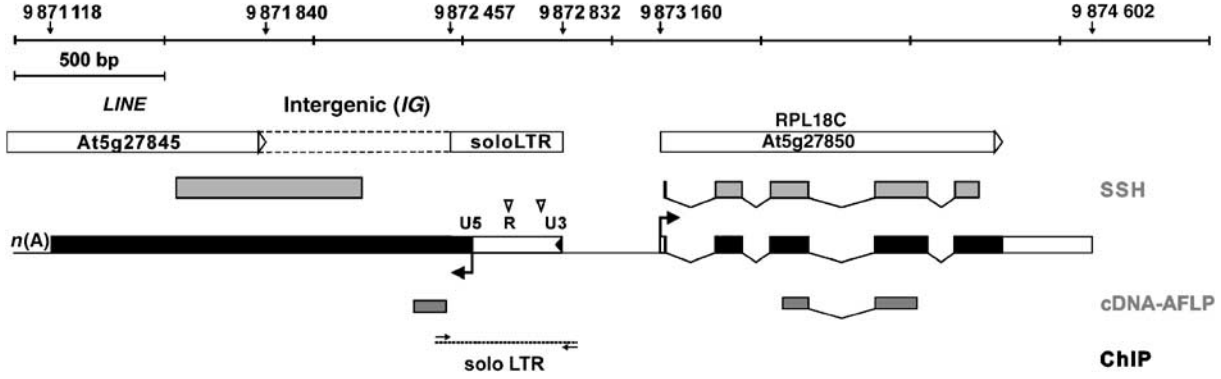

Figure 1 Bidirectional influence of a derepressed solo LTR in a *drd1* mutant. Two transcripts were identified in both the SSH and cDNA-AFLP analyses: one matches a gene encoding the ribosomal protein RPL18C and the second contains part of the *IG* region together with the 3′ end of a truncated long interspersed element (*LINE*, a non-LTR retrotransposon) (gray boxes, SSH and cDNA-AFLP). The truncated *LINE* and the *RPL18C* gene are in the same orientation (horizontal white arrowheads) on chromosome 5 (coordinates at the top). The full-length transcripts (black bars), transcription start and termination sites, and transcript orientations (black arrows) were determined by 5′ and 3′ RACE. The sense *RPL18C* transcript initiates at the annotated start site whereas the antisense *IG/LINE* transcript initiates in the solo LTR, which served as the founding sequence element for a previously uncharacterized *Copia*-like retrotransposon family that we have named LTRCO (LTR/*Copia*) (Supplementary Table II). The antisense *IG/LINE* transcript terminates at a fortuitous polyadenylation site in the bottom DNA strand. Vertical white arrowheads in the solo LTR (which contains canonical U3, R, and U5 regions) indicate positions homologous to short RNAs identified in multiple parallel signature sequencing (Lu *et al*, 2005). The solo LTR was analyzed for histone modifications by ChIP (fine dotted line; primer positions as small arrows).

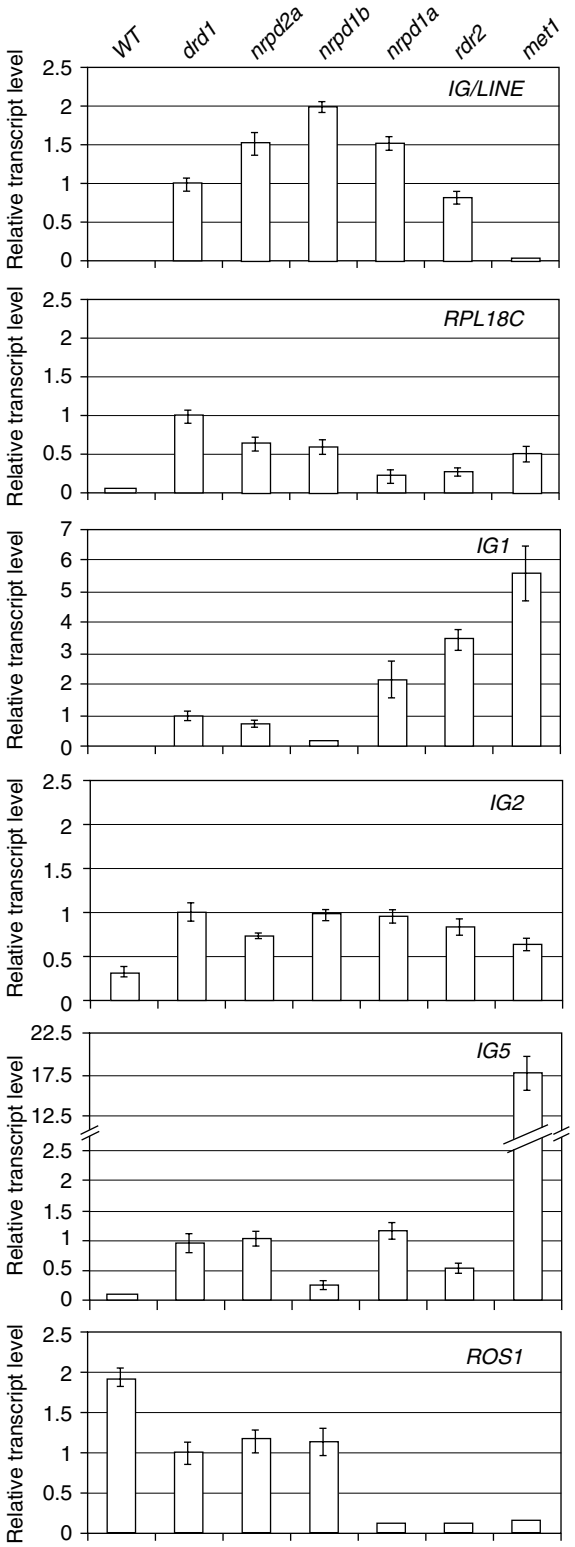

Figure 2 Differential expression of DRD1 targets. Real-time RT–PCR was used to validate transcripts that are upregulated (*IG/LINE*, *RPL18C*, *IG1*, *IG2*, *IG5*) or downregulated (*ROS1*) in the *drd1* mutant as identified in SSH and/or cDNA-AFLP screens (Supplementary Table I). Relative transcript levels are shown in the indicated mutants: *drd1*, *nrpd2a* and *nrpd1b* (referred to collectively in the text as pol IVb mutants), *nrpd1a*, *rdr2*, and *met1*.

element. The *RPL18C* transcript was in the sense orientation and initiated at the annotated transcription start site (Figure 1). These results suggested that the intergenic region, which is 1319 bp, contains a regulatory element that is derepressed in the *drd1* and pol IVb mutants, thereby influencing the expression of the adjacent sequences in a bidirectional manner. Indeed, BLAST-N and CENSOR searches (Jurka *et al*, 2005) using the 1319 bp intergenic region as a query revealed a dispersed repetitive sequence, which we identified as a previously unannotated solo LTR, 376 bp in length, derived from a small, previously uncharacterized *Copia*-like retrotransposon family that we named LTRCO (Supplementary Table II). The derepressed solo LTR in the *drd1* and pol IVb mutants was presumably acting in one direction as a promoter for the antisense transcript of the *IG/LINE* sequence and in the opposite direction as an enhancer for transcription of the *RPL18C* sense RNA (Figure 1).

To study epigenetic changes accompanying derepression of the solo LTR, we analyzed cytosine methylation in wild-type and mutant plants. *Hpa*II and *Msp*I (reporting on CG and CNG methylation, respectively; Supplementary Figure 2A) cut the solo LTR fragment more efficiently in *drd1* and pol IVb mutants than in wild-type plants; however, neither enzyme digested to completion, indicating retention of some CNG methylation in the mutants (Figure 3, solo LTR). Evidence for primarily CNG (and not CG) methylation was also suggested by minimal digestion with *Hpa*II and *Msp*I in a *met1* mutant, which should reduce specifically CG methylation. By contrast, *Dde*I and *Alu*I, which report on CNN methylation, cleaved almost completely the solo LTR fragment, indicating considerable loss of asymmetric methylation at one or both sites for these enzymes (Figure 3, solo LTR).

Loss of CNN methylation from the solo LTR in the *drd1* and pol IVb mutants might reflect a lack of the corresponding short RNA. However, this cannot be the explanation for the *drd1* and *nrpd1b* mutants, in which wild-type levels of LTR short RNAs were observed. By contrast, LTR short RNAs were not detected in *nrpd2a*, *nrpd1a*, or *rdr2* mutants (Figure 4). These results are consistent with the role of Pol IVa (containing NRPD1a and NRPD2a) and RDR2 in producing or amplifying the short RNA trigger, whereas Pol IVb (containing NRPD1b and NRPD2a) acts downstream of this step to use short RNAs to guide DNA cytosine methylation.

Histone modifications were analyzed by chromatin immunoprecipitation (ChIP) using antibodies against modifications typical of *Arabidopsis* heterochromatin (histone H3 lysine 9 dimethylation (H3K9me2)) or preferentially heterochromatin (histone H3 lysine 27 monomethylation (H3K27me)), and euchromatin (histone H3 lysine 4 trimethylation (H3K4me3) and acetylated H3 (acetyl-H3)) (Fuchs *et al*, 2006). The antibodies reacted as expected with the control sequences for heterochromatin (Figure 5A) and transcriptionally active euchromatin (Figure 5B and C). Similar patterns were observed for the control sequences in all genotypes tested: wild-type plants that contained the transgene α′ promoter target gene alone (ST), wild-type plants that contained the transgene α′ promoter target gene plus the silencer locus (DT), and the DT line in a *drd1* or pol IVb mutant background (Figure 5A–C, all panels).

We first investigated histone modifications associated with the 270 bp transgene α′ promoter, which was the original target sequence used to identify the *drd1* and pol IVb mutants. In the absence of the silencer locus (ST line), the active transgene α′ promoter is associated with the two euchromatic marks tested, H3K4me3 and acetyl-H3, as well

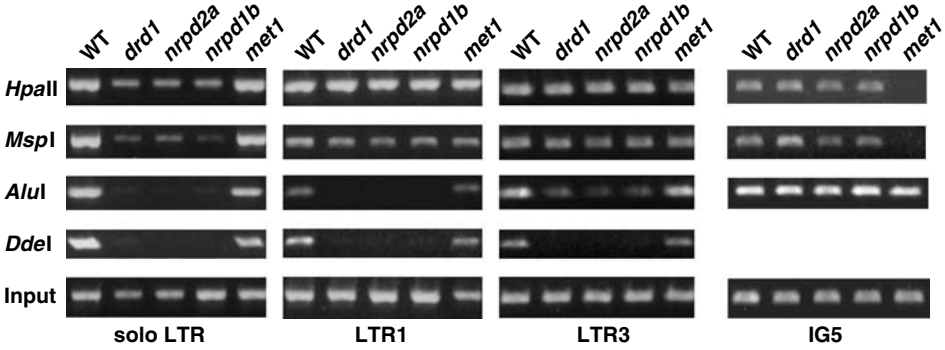

Figure 3 DNA methylation analysis. Cytosine methylation of the solo LTR, LTR1, LTR3 (all from the LTRCO family) and the *Copia* LTR acting as promoter for *IG5* (Supplementary Figure 4C) in *drd1*, pol IVb (*nrpd2a* and *nrpd1b*), and *met1* mutants was studied using enzymes sensitive to CG/CNG methylation (*Hpa*II), CNG methylation (*Msp*I), and CNN methylation (*Alu*I and *Dde*I). The solo LTR, LTR1, and LTR3 all have a single site for *Hpa*II/*Msp*I, but differ somewhat in the number of sites for *Dde*I and *Alu*I (Supplementary Figure 2A). Disappearance or reduced levels of a fragment after digestion with a given enzyme indicates loss of methylation at that site. The bottom panels show undigested input DNA.

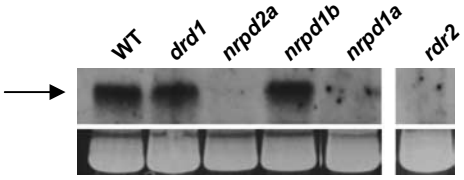

Figure 4 Accumulation of LTR-derived short RNAs. LTR short RNAs from the LTRCO family of elements are detectable in wild-type plants and in *drd1* and *nrpd1b* mutants, but not in *nrpd2a*, *nrpd1a*, and *rdr2* mutants. The arrow indicates the position of a 24-nucleotide DNA oligonucleotide. Ethidium bromide staining of the major RNA species is shown as a loading control.

as some H3K27me (Figure 5D, ST panel). These modifications persisted in the presence of the silencer (DT line), when the α′ promoter is repressed and methylated at cytosines in all sequence contexts (Kanno *et al*, 2004, 2005b), but there was negligible H3K9me2 in the silent state (Figure 5D, DT panel). Little change in the pattern of histone modifications was observed when the transgene α′ promoter was reactivated in the *drd1* and pol IVb mutants (Figure 5D, *drd1*, *nrpd2a*, *nrpd1b* panels).

Similar results were obtained for the solo LTR. In wild-type plants, where the solo LTR is repressed, the histone modification pattern was essentially the same as that observed with the silent transgene α′ promoter: the two euchromatic marks (H3K4me3 and some acetyl-H3) as well as H3K27me were detected but not H3K9me2 (Figure 5E, DT panel). In contrast to the transgene α′ promoter, the solo LTR appeared to lose H3K27 methylation when derepressed in the mutants. Similarly to the transgene α′ promoter, however, the solo LTR maintained the euchromatic marks when derepressed; indeed, acetyl-H3 appeared to increase in the mutants (Figure 5E, *drd1*, *nrpd2a*, *nrpd1b* panels).

In addition to the solo LTR, there are six other more or less intact members of the LTRCO retrotransposon family in the *Arabidopsis* genome (Supplementary Table II). There was no evidence from the SSH and cDNA-AFLP analyses that any of these were reactivated in the *drd1* mutant. In addition, we failed to detect transcription initiating from the left LTR of two intact elements (LTRCO1 and LTRCO3) on chromosome 1 and chromosome 3, respectively, in *drd1* and pol IVb mutants (Supplementary Figure 3A). To examine the reasons for this, we analyzed epigenetic modifications associated with the left LTRs of LTRCO1 and LTRCO3 (designated LTR1 and LTR3; Supplementary Figure 2B) in wild-type and mutant plants. Similarly to the solo LTR, LTR1 lost most if not all asymmetric CNN methylation in *drd1* and pol IVb mutants, where nearly complete cleavage with *Alu*I and *Dde*I was observed (Figure 3, LTR1). In the case of LTR3, some residual methylation was observed at one or more sites recognized by *Alu*I but loss of methylation appeared complete at one or more of the *Dde*I sites (Figure 3, LTR3). The loss of CNN methylation in the mutants is noteworthy because it demonstrates that LTR1 and LTR3 are indeed targets of the DRD1 and Pol IVb pathway. In contrast to the solo LTR, however, which only partially retained CNG methylation at the single *Hpa*II/*Msp*I site in the *drd1* and pol IVb mutants, LTR1 and LTR3 more persistently retained CNG methylation at this site, as indicated by negligible digestion with both *Hpa*II and *Msp*I in these mutants (Figure 3, LTR1 and LTR3).

The ChIP analysis revealed two additional epigenetic differences between LTR1 and LTR3 as compared to the solo LTR and the transgene α′ promoter (Figure 5F and G): (1) LTR1 and LTR3 were deficient in the euchromatic marks, H3K4me3 and acetyl-H3 and (2) LTR1 and LTR3 were associated with not only H3K27me but also H3K9me2. The patterns of histone modification were similar in wild-type plants (DT) and in the mutants (Figure 5F and G, *drd1*, *nrpd2a*, *nrpd1b* panels).

Other upregulated target sequences

We extended our analysis by examining three intergenic regions—termed *IG1*, *IG2*, *IG5*—which were identified as upregulated in the *drd1* mutant by cDNA-AFLP analysis (Supplementary Table I, Supplementary Figure 1). The *IG1* transcript partially matches an unannotated intergenic sequence that encodes 19 short RNAs of both sense and antisense polarity. The *IG2* transcript matches a region upstream and in opposite orientation of a truncated *Athila* solo LTR. The *IG5* transcript initiates in the 3′ LTR of a *Copia*-like retrotransposon (Supplementary Figures 4A–C). Both LTRs encode short RNAs (Lu *et al*, 2005).

Real-time RT–PCR verified the increased accumulation of *IG1*, *IG2*, and *IG5* transcripts in the *drd1* mutant relative to wild-type plants, as well as varying degrees of reactivation in

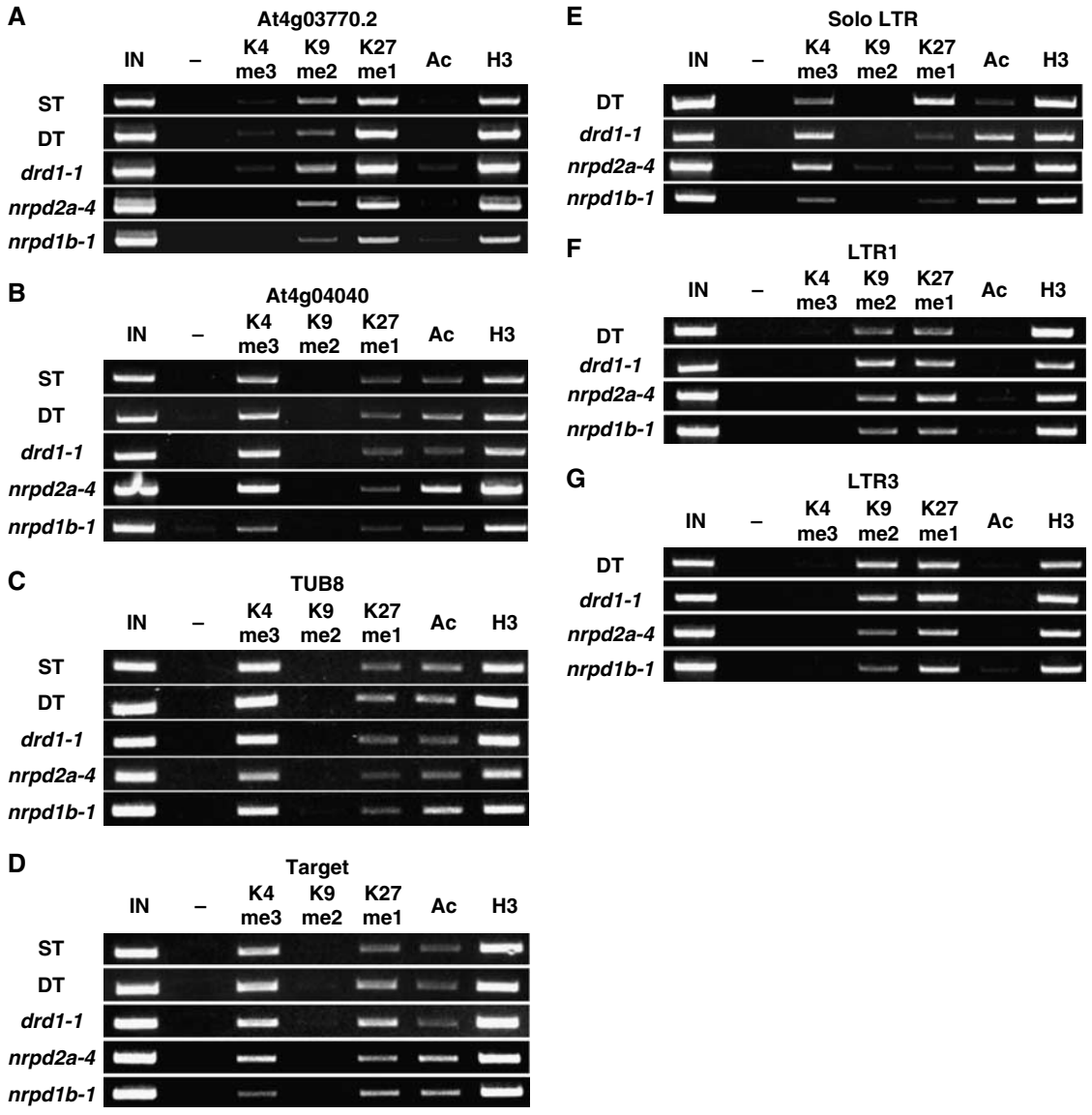

Figure 5 ChIP analysis of reactivated and silent DNA regions in *drd1* and pol IVb mutants. (**A**) A heterochromatin control (At4g03770, a *Gypsy*-like retrotransposon) reacts with antibodies to H3K9me2 and H3K27me; (**B, C**) controls for euchromatin (At4g04040, a putative phosphofructokinase beta subunit and At5g23860, the tubulin protein TUB8) react with antibodies to H3K4me3 and acetylated H3, and weakly with H3K27me (Ac). The transgene α′ target promoter (**D**) and solo LTR (**E**) are derepressed in the *drd1* and pol IVb (*nrpd2a* and *nrpd1b*) mutants. These sequences are enriched in the euchromatic marks (H3K4me3 and acetyl H3) and have H3K27me but little or no H3K9me2. For the transgene α′ promoter, the modifications are similar in wild-type (ST, DT) and mutant backgrounds. The solo LTR loses H3K27me and gains acetyl-H3 in the mutants. By contrast, LTR1 (**F**) and LTR3 (**G**), which remain silenced in *drd1* and pol IVb mutants, are associated with H3K9me2 and enriched in H3K27me. The last lane in each panel shows a control using an antibody to unmodified histone H3. ST, transgenic line containing only the transgene α′ promoter-driven target gene; DT, transgenic line containing the silenced transgene α′ promoter-driven target gene and the unlinked silencer inverted repeat (Kanno *et al*, 2004, 2005b).

pol IVb, *nrpd1a*, and *rdr2* mutants (Figure 2). For *IG1* and *IG5*, however, transcription was higher in a *met1* mutant than in the other mutants tested. This contrasts to the *IG/LINE* antisense transcript, which remained nearly undetectable in *met1* plants (Figure 2). The differing extents of reactivation in *drd1* and pol IVb mutants versus the *met1* mutant may reflect the CG content of target sequences: the upstream regions of *IG1* and *IG5* have a relatively high number of CG dinucleotides (Supplementary Figure 4A and C) compared to the solo LTR that drives expression of the *IG/LINE* antisense transcript (Supplementary Figure 2A). Indeed, upregulation of the *IG5* transcript in the *met1* mutant is accompanied by loss of CG methylation from the associated LTR, as indicated by nearly complete digestion with *Hpa*II and *Msp*I (Figure 3, IG5). Interestingly, the *IG1* and *IG5* transcripts were only weakly induced in the *nrpd1b* mutant compared to *IG2* and *IG/LINE* (Figure 2). This suggests that silencing of *IG1* and *IG5*, in contrast to *IG/LINE*, relies more on CG methylation than on CNN methylation, which requires NRPD1b. *IG2* represents an intermediate case where reactivation was roughly the same in *drd1*, pol IVb, *nrpd1a*, *rdr2*, and *met1* mutants. ChIP analysis indicated that the bidirectionally transcribed region upstream of *IG1*, as well as the *Athila* and *Copia* LTRs upstream of *IG2* and *IG5*, respectively, have

euchromatic histone modifications and H3K27me; only the *Copia* LTR that promotes transcription of *IG5*, which is most strongly induced in the *met1* mutant, has in addition detectable H3K9me2 (Supplementary Figure 5).

Two genes encoding extensin proteins (At1g21310 and At1g76930) were cloned multiple times in the SSH analysis, suggesting that they are conspicuous targets of DRD1-dependent silencing (Supplementary Table I). Real-time RT–PCR confirmed the upregulation of these genes in the *drd1* mutant (Supplementary Figure 3B). However, a consistent increase in expression was not observed in pol IVb, *nrpd1a*, and *rdr2* mutants, suggesting that DRD1 downregulates the two extensin genes in a silencing pathway that may be independent of Pol IV.

Downregulated target sequences in the drd1 mutant

ROS1 (At2g36490), a DNA glycosylase-like protein involved in active demethylation of CG dinucleotides (Kapoor *et al*, 2005), was identified as a candidate for downregulation in the *drd1* mutant (Supplementary Table I). Real-time RT–PCR confirmed that the abundance of *ROS1* transcripts was approximately halved in *drd1* and pol IVb mutants and decreased even more in *nrpd1a*, *rdr2*, and *met1* mutants (Figure 2). This result was notable given the proposed dual role of DRD1 in facilitating not only RNA-directed *de novo* methylation but also full erasure of CG methylation (Kanno *et al*, 2005a).

Discussion

The experiments reported here begin to identify the endogenous targets of DRD1/Pol IVb-dependent DNA methylation and silencing in the *Arabidopsis* genome, and the histone modifications associated with this pathway. Although our screens are not exhaustive, a general pattern has nevertheless emerged. A number of upregulated target regions contain transposons or unannotated sequences in their 5′ flanking regions that encode short RNAs, which could act with DRD1 and Pol IVb to mediate DNA methylation and silencing. Indeed, it is likely that most sequences in the genome that are complementary to available short RNAs can be targets of DRD1/Pol IVb-dependent DNA methylation, which is characterized by methylation of cytosines in all sequence contexts. However, only a subset of these sequences is reactivated in *drd1* mutants. The strength of reactivation depends on several factors including the chromatin state, genomic environment, and sequence composition of a given target sequence.

The upregulated transcripts we identified in the *drd1* mutant represent plant genes and intergenic regions located primarily in euchromatin; however, the actual targets of the DRD1/Pol IVb silencing machinery are probably short RNA-encoding regulatory elements located in the 5′ flanking regions of these sequences. The solo LTR of the LTRCO family provides a good example of a transposon-derived target sequence that acts as a bidirectional promoter/enhancer for transcription of flanking sequences (antisense *IG/LINE* and sense *RPL18C*). In addition, the three *IG* targets are all adjacent to a short RNA-encoding sequence. In the case of *IG2* and *IG5*, this sequence is a retrotransposon LTR. For *IG2*, the truncated *Athila* solo LTR might be acting as an enhancer because it is in opposite orientation to the *IG2* transcript, whereas *IG5* transcription initiates within the *Copia* LTR, which is apparently acting as a promoter. The apparent absence of transposons in the 5′ flanking regions of some target genes (Supplementary Table I) might reflect incomplete annotation of the *Arabidopsis* genome; for example, the solo LTR of the *Copia*-type LTRCO family had not been identified before this study. Alternatively, as illustrated by *IG1*, a 5′ flanking region might be transposon-free but bidirectionally transcribed, producing overlapping sense and antisense transcripts that can anneal to form double-stranded RNA that is processed to short RNAs. Some targets, such as the two extensin genes studied here, encode short RNAs in exons and/or introns and might be silenced by a DRD1-mediated pathway that is independent of Pol IVb. Further analysis of individual putative targets and delineation of their promoter regions is required to determine whether DRD1/Pol IVb-mediated epigenetic regulation always relies on short RNAs complementary to upstream regulatory regions. A previous study demonstrated that the promoter of the *FWA* gene in *Arabidopsis* contains a transposon-derived direct repeat that is the target of short RNA-mediated silencing involving a different SWI/SNF factor (DDM1), CG methylation, and H3K9 methylation (Lippman *et al*, 2004). These findings and ours suggest that *Arabidopsis* has at least two pathways that use short RNAs to induce transcriptional gene silencing associated with distinct epigenetic modifications.

The contribution of histone modifications to the DRD1/Pol IVb-dependent silencing pathway is particularly clear when comparing the solo LTR with LTR1 and LTR3 (all from the LTRCO family). Even though these LTRs have similar DNA sequences, they respond differently in *drd1* and pol IVb mutants. All three appear to be targets of DRD1/Pol IVb-dependent DNA methylation because each loses CNN methylation, which relies on the RNA trigger and the DRD1/Pol IVb machinery, in the *drd1* and pol IVb mutants. Only the solo LTR, however, is significantly derepressed in the mutants. An important factor is probably the chromatin state. Whereas the solo LTR is characterized by persistent euchromatic histone modifications and negligible H3K9me2, LTR1 and LTR3 lack euchromatic marks and are modified by H3K9me2. Moreover, LTR1 and LTR3 are embedded in pericentromeric heterochromatin of *Arabidopsis* chromosomes, whereas the solo LTR resides at the boundary between pericentromeric heterochromatin and euchromatin (Supplementary Figure 1).

Similar observations were made with other target sequences. The transgene α′ promoter and many other candidate targets of DRD1, including the intergenic region *IG1*, are located in euchromatic chromosome arms (Supplementary Figure 1). In addition, the transgene α′ promoter as well as the bidirectionally transcribed sequence upstream of *IG1* and the *Athila* LTR enhancing *IG2* transcription are characterized by euchromatic histone modifications and little or no H3K9me2. Of the targets analyzed in detail, only the *Copia* LTR driving transcription of *IG5* has notable H3K9me2 but this is nevertheless accompanied by euchromatic marks. *IG5* is also highly dependent on CG methylation for silencing, as evidenced by stronger reactivation in *met1* than in *drd1* mutants.

In summary, even though all sequences analyzed in detail in this study are targets for DRD1/Pol IVb-dependent DNA cytosine methylation, they are reactivated to differing extents in *drd1* and pol IVb mutants. Sequences that are

strongly upregulated in *drd1* and pol IVb mutants have a predominantly euchromatic character and reside in gene-rich regions, whereas sequences that are weakly reactivated or remain silent in the *drd1* and pol IVb mutants tend to be located in transposon-rich constitutive heterochromatin containing H3K9 dimethylation. The degree of reactivation in the mutants may also depend to some extent on the number of CG dinucleotides: sequences with a lower CG content (such as the solo LTR) reactivate more fully in *drd1* mutants than in *met1* mutants, whereas the converse is true for sequences with a higher CG content (upstream regions of *IG1* and *IG5*).

In view of these results, we propose a two-part model in which DRD1 and Pol IVb establish a basal level of potentially reversible silencing, which involves primarily cytosine methylation at DNA sequences that are homologous to the RNA trigger. For targets in gene-rich euchromatin, which lack H3K9me2, silencing can potentially be reversed. By contrast, targets in transposon-rich heterochromatin are subject to further modifications, including H3K9me2 and persistent CNG methylation, which foster more stable silencing (Figure 6).

An important contributor to the potential reversibility of DRD1/Pol IVb-dependent silencing is the inefficient maintenance of CNN methylation during DNA replication in the absence of the inducing RNA. Thus, euchromatic promoters that are silenced by CNN methylation but not CG methylation, exemplified by the solo LTR of LTRCO family and the transgene α′ promoter, can be reactivated in dividing cells if production of the RNA trigger is discontinued. A second way to lose methylation, even from nonreplicating DNA, is through the activity of DNA glycosylase domain-containing proteins such as ROS1 and DEMETER, which are thought to promote active demethylation of cytosines through a base-repair-type mechanism (Kapoor *et al*, 2005). The intriguing finding that ROS1 is downregulated in *drd1* and pol IVb mutants, and hence presumably diminished in its capacity to mediate demethylation, might explain the failure to fully erase CG methylation from target promoters after removing the RNA trigger in these mutants (Kanno *et al*, 2005a). The even more dramatic downregulation of ROS1 in *nrpd1a* and *rdr2* mutants strengthens the proposed connection between ROS1 and the entire Pol IV pathway. Although the exact nature of this connection remains to be determined, we speculate that DRD1 and Pol IVb can cooperate with either DNA methyltransferases to catalyze cytosine methylation, or DNA glycosylases to actively remove methylated cytosines. This facilitates dynamic regulation of DNA methylation, which helps to maintain euchromatic promoters in a potentially reversible epigenetic state (Figure 6).

What are the functions of the DRD1/Pol IVb pathway in plants? An obvious one is to silence transposons that integrate into euchromatin and/or that are too small to be stably packaged into constitutive heterochromatin (Tran *et al*, 2005). In addition to subduing transposition, silencing of transposon promoters in euchromatin can prevent spurious transcription of intergenic regions or antisense transcription into neighboring plant genes. The DRD1/Pol IVb pathway might serve an additional beneficial function for plants by providing a genome-wide system of short RNA-regulated, transposon-derived promoters and/or enhancers that modulate gene expression under adverse growth conditions. The fact that certain stresses induce some short RNAs (Sunkar and Zhu, 2004) and reactivate retrotransposon LTRs to influence transcription of adjacent genes (Kashkush *et al*, 2003) suggests that these elements may function in reversible DRD1/Pol IVb-dependent silencing. There are more than 3000 annotated solo LTRs from *Copia*- and *Gypsy*-type elements in the *A. thaliana* genome (Peterson-Burch *et al*, 2004), many of which are located in euchromatic chromosome arms. Similarly to the solo LTRs described here, these uncharacterized solo LTRs could provide epigenetically controlled rheostats or switches for neighboring genes,

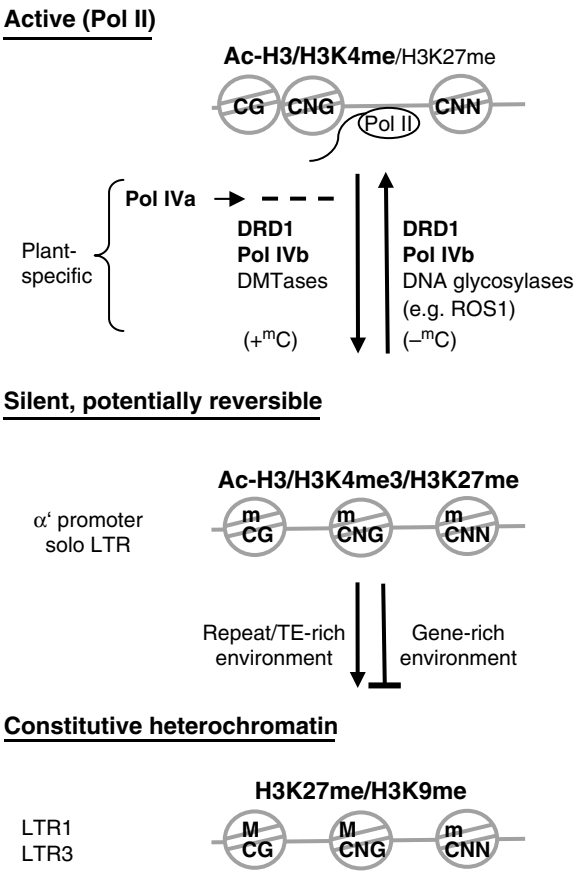

Figure 6 Model for reversible silencing of euchromatic genes by DRD1 and Pol IVb. Promoters of active genes transcribed by RNA polymerase II (Pol II) lack cytosine methylation and are enriched in euchromatic histone modifications (acetyl-H3 and H3K4me); some H3K27me may also be present. When short RNAs produced by Pol IVa are available, DRD1 and Pol IVb cooperate with DNA methyltransferases (DMTases) to induce *de novo* methylation ('m') of cytosines in all sequence contexts in the region of RNA–DNA complementarity. Methylated, silenced promoters retain euchromatic marks and can show increased H3K27me. For euchromatic promoters that are sensitive to CNN methylation, such as the transgene α′ promoter and solo LTR, silencing can be reversed in dividing cells if the RNA trigger is removed, resulting in loss of CNN methylation. Cytosine methylation can also potentially be removed in nondividing cells through active demethylation by DNA glycosylase-domain-containing proteins such as ROS1, which might also involve the DRD1/Pol IVb machinery (Kanno *et al*, 2005a). Sequences in repeat or transposon (TE)-rich genomic environments, such as LTR1 and LTR3 of the LTRCO family, can be subjected to additional epigenetic modifications, including H3K9me and persistent C(N)G methylation (capital 'M') to ensure stable silencing in constitutive heterochromatin. Sequences in gene-rich regions, such as the transgene α′ promoter and solo LTR, appear to be protected from the additional layer of epigenetic modifications, keeping them in a potentially reversible epigenetic state.

whose increased or decreased expression might have adaptive significance for the plant. If so, the elaboration of the DRD1/Pol IV pathway in the plant kingdom might reflect the need for rapid, reversible changes in gene expression that enable immobile plants to cope with environmental challenges.

Materials and methods

Plant material

Plants were grown on soil under long day conditions (16 h light/8 h dark at 24°C). All experiments were performed on *drd1-1* (Kanno *et al*, 2004), *nrpd2a-4*, and *nrpd1b-1* mutants (Kanno *et al*, 2005b) or wild-type *A. thaliana* (ecotype Columbia-0) plants. Seeds of other mutants were kindly provided by EJ Richards (*met1-1*), AJ Herr (*nrpd1a-4*), and JC Carrington (*rdr2-1*).

RNA isolation and RT–PCR

Total RNA was isolated from 3-week-old seedlings of the *drd1* mutant and the parental line as described previously (Mette *et al*, 1999). Polyadenylated RNA was prepared with the Oligotex mRNA midi kit (Qiagen, Hilden, Germany). RT–PCR was performed as described in a previous publication (Kanno *et al*, 2005b). The primer sets used for RT–PCR were LTR_625-F and LTR_625-F for *IG/LINE*; LTR1RTv-F and LTR1RTv-R for *LTRCO1;* LTR3RTv-F LTR3RTv-R for *LTRCO3;* IG1-F and IG1-R for *IG1*; 01420IGf and 01420IGr for *IG1**; and GAPA-F and GAPA-R for *GAPA* (Supplementary Table III).

SSH

SSH (Diatchenko *et al*, 1996) was performed with 2 μg of poly(A) RNA, using the PCR-Select cDNA subtraction kit (Clontech, Palo Alto, USA) according to the supplier's instructions. The mutant line was used as tester and the wild type as driver cDNA. The subtracted *drd1-1* library was cloned into vector pGEM-T (Promega, Mannheim, Germany). For the screening procedure, 576 individual bacterial cultures were grown according to the manual of the PCR-Select differential screening kit (Clontech, Palo Alto, USA). cDNA fragments were amplified by PCR, spotted on cDNA arrays and hybridized with forward and reverse subtracted cDNA according to supplier's instruction. 3′ RACE reactions were performed as described by the Marathon cDNA amplification kit (Clontech, Palo Alto, USA). Rapid amplification of 5′ cDNA ends was carried out using the FirstChoice RLM-RACE kit (Ambion, Huntingdon, UK) according to the manufacturer's instructions and products were cloned into pGEM-T (Promega, Mannheim, Germany). All PCR reactions used for cloning and RACE procedures were performed with a polymerase mix with proof-reading capacity (BD Advantage 2 PCR Enzyme System, Clontech, Palo Alto, USA). For primer information for 5′ and 3′ RACE of *L18* and *IG/LINE*, see Supplementary Table III.

cDNA-AFLP

In total, 500 ng of double-stranded cDNA prepared from polyadenylated RNA was used for AFLP analysis with restriction enzymes *Bst*YI and *Mse*I as described previously (Vos *et al*, 1995; Breyne *et al*, 2003) except that an additional selection (Breyne *et al*, 2003) for the reduction of the total number of cDNA fragments was excluded. For preamplifications, an *Mse*I primer without selective nucleotides was combined with a *Bst*YI primer mixture of equal amounts containing either a T or a C at the 3′ end. PCR conditions were as described (Vos *et al*, 1995). The amplification products were diluted 600-fold and 5 μl aliquots were used for selective amplifications using a ^{32}P-labeled *Bst*YI primer and *Taq* DNA polymerase (Fermentas, St Leon-Rot, Germany). A total of 128 primer combinations were used in a screen for differential transcripts with selective *Bst*-YN × *Mse*-NN primers (Breyne *et al*, 2003). The amplification products were separated on 5% polyacrylamide gels using the Sequigel system (Biorad, Vienna, Austria). Dried gels were exposed for 1–5 days to Kodak Biomax autoradiography films (Sigma Aldrich, Vienna, Austria). Robust and reproducible differential amplification products were further characterized as described by CWB Bachem (available online at www.dpw.wau.nl/pv/). Primers used for screening and re-amplification were as described previously (Breyne *et al*, 2003).

Real-time PCR

RNA extraction and cDNA synthesis were performed as described in a previous publication (Kanno *et al*, 2005b). The cDNA was diluted to 75 μl with DEPC-treated double distilled water, and 5 μl was used in a 30 μl PCR reaction. The mixture was set up with 15 μl of Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Lofer, Austria), 5 μl cDNA, and 2 μl of each primer (660 nm final). PCR was performed after a preincubation as suggested by the supplier (50°C for 2 min, 95°C for 2 min) by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 23 s, and extension at 72°C for 30 s. The comparative threshold cycle (C_t) method was used to determine relative RNA levels (User Bulletin no. 2, Applied Biosystems). *Ubi* (At5g25760) is used as the internal reference, and expression levels are relative to the *drd1* mutant plant. The primer sets used for real-time RT–PCR were LTR_625-F and LTR_625-R for *IG/LINE*, at5g27850-F and at5g27850-R for *L18*, 2g36490-F and 2g36490-R for *ROS1*, IG1-F and IG1-R for *IG1*, IG2-F and IG2-R for *IG2*, IG5-F and IG5-R for *IG5*, 1g21310-F and 1g21310-R for *At1g21310.1*, 1g76930-F and 1g76930-R for *At1g76930.2,* and UBI-F and UBI-R for *UBI* (Supplementary Table III).

Methylation analysis

Methylation of the solo LTR, LTR1, LTR3, and IG5, and PCR analysis were performed as described previously (Kanno *et al*, 2005b) (see Supplementary Figures 2A and 4C for positions of restriction sites investigated). For primer design, LTR flanking sequences were screened to identify unique sites suitable for LTR-specific PCR analysis. As a PCR control, an equal amount of unrestricted DNA was subjected to PCR. The primer sets used were LTR-F and LTR-R for solo LTR, LTR1-F and LTR1-R for LTR1, LTR3-F and LTR3-R for LTR3, and drd(5)ChiP2r and drd(5)ChiPf for IG5 (Supplementary Table III).

ChIP

ChIP was performed as described in http://www.epigenome-noe.net/researchtools/protocol.php?protid=13&PHPSESSID=4d57ea22991e633bf475ab562b612100. The chromatin (prepared from whole plants 3–4 weeks after germination, just before bolting) was immunoprecipitated with antibodies to: histone H3 (trimethyl K4) ChIP grade (ab8580; Abcam, Cambridge, UK), dimethyl K9 (prepared in the laboratory of T Jenuwein), monomethyl K27 (prepared in the laboratory of T Jenuwein), acetyl histone H3 (06-599; Upstate, Dundee, UK), and unmodified histone H3 (ab1791; Abcam, Cambridge, UK). The PCR was carried out in a total reaction volume of 25 μl for 3 min at 95°C followed by 30–34 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min with 6 min of final elongation step. The PCR products were separated by 1.5% agarose gel and stained with ethidium bromide. The results shown for H3K4me3, H3K9me2, H3K27me, and acetyl-H3 were reproduced in three independent experiments; results for anti-H3 (unmodified) were reproduced in two independent experiments. The primer sets used for PCR were as follows: H3F and H3R for At4g03770.2, B8F and B8R for At4g04040, TUB8f and TUB8r for *TUB8*, α′pro5′ and chip-gfp for Target (transgene α′ promoter), LTR-F and LTR-R for solo LTR, LTR1-F and LTR1-R for LTR1, LTR3-F2 and LTR3-R2 for LTR3, 01420IGf and 01420IGr for IG1, drd(2)ChiPr and drd(2)ChiPf for IG2, and drd(5)ChiP2r and drd(5)ChiPf for IG5 (Supplementary Table III).

Short RNA analysis

Short RNAs were isolated from *Arabidopsis* inflorescence tissue using the mirVana miRNA isolation kit (Ambion, Huntingdon, UK). Short RNAs (50 μg) were analyzed by Northern blot hybridization as described previously (Mette *et al*, 2000). For blotting, a Hybond N^+ membrane (Amersham, Vienna, Austria) was used. The probe for solo LTR siRNAs was a 376 bp riboprobe (Supplementary Figure 1), which was amplified from genomic DNA using primers LTR-F and LTR-R (Supplementary Table I) and cloned into pGEM-T (Promega, Mannheim, Germany). This plasmid was linearized with *Nde*I and ^{32}P-labelled transcripts were synthesized using T7 polymerase (Roche, Vienna, Austria). Hybridization was carried out overnight at 42°C as described previously (Mette *et al*, 2000). The blots were washed twice in 2 × SSC, 0.2% SDS at room temperature for 30 min. The blots were exposed overnight at −80°C.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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