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A SNF2-like protein facilitates dynamic control of DNA methylation

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DRD1 is a SNF2-like protein previously identified in a screen for mutants defective in RNA-directed DNA methylation of a seed promoter in *Arabidopsis*. Although the initial study established a role for DRD1 in RNA-directed DNA methylation, it did not address whether DRD1 is needed for *de novo* or maintenance methylation, or whether it is required for methylation of other target sequences. We show here that DRD1 is essential for RNA-directed *de novo* methylation and acts on different target promoters. In addition, an unanticipated role for DRD1 in erasure of CG methylation was shown when investigating maintenance methylation after segregating away the silencing trigger. DRD1 is unique among known SNF2-like proteins in facilitating not only *de novo* methylation of target sequences in response to RNA signals, but also loss of methylation when the silencing inducer is withdrawn. The opposing roles of DRD1 could contribute to the dynamic regulation of DNA methylation.
Keywords: demethylation; *de novo* methylation; maintenance methylation; RNA-directed DNA methylation; SNF2-like protein

INTRODUCTION

RNA-directed DNA methylation has been documented in plants (Mathieu & Bender, 2004) and in human cells (Kawasaki & Taira, 2004; Morris *et al*, 2004). Genetic analyses in *Arabidopsis* have identified the DNA methyltransferases that catalyse *de novo* cytosine (C) methylation in response to RNA signals (Cao *et al*, 2003; Aufsatz *et al*, 2004) and maintain symmetrical C(N)G methylation (N is A, T or C) in cooperation with histone-modifying enzymes (Jones *et al*, 2001; Aufsatz *et al*, 2002a; Jackson *et al*, 2002; Malagnac *et al*, 2002). A plant-specific SNF2-like putative

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chromatin-remodelling protein, DRD1, was identified in a screen for mutants impaired in RNA-directed DNA methylation and silencing of the seed-specific α' promoter (Kanno *et al*, 2004). From the initial genetic screen, it was unclear whether *drd1* mutants are deficient in *de novo* methylation or in maintenance methylation. In the case of RNA-directed DNA methylation, *de novo* methylation leads to the modification of Cs in all sequence contexts within the region of RNA–DNA sequence identity, whereas maintenance methylation refers to perpetuation of symmetrical C(N)G methylation during DNA replication in the absence of the RNA trigger (Matzke *et al*, 2004). To further understand the role of DRD1 in RNA-directed DNA methylation, we examined separately the effects of a *drd1* mutation on RNA-directed *de novo* methylation and on maintenance methylation. We report here that DRD1 influences both of these processes in a manner that facilitates the dynamic regulation of DNA methylation.

RESULTS AND DISCUSSION

Transgenic systems to study RNA-mediated silencing and methylation of the seed-specific α' promoter (Kanno *et al*, 2004) and the constitutive nopaline synthase (NOS) promoter (Aufsatz *et al*, 2002b) have been described previously. These systems rely on a double-stranded RNA—encoded at a silencer *H* complex (hygromycin resistance)—that is homologous to the respective target promoter sequence. The double-stranded RNAs are processed to short RNAs, 21–24 nucleotides in length, which are thought to trigger *de novo* methylation and silencing of the homologous target promoter at an unlinked target *K* complex (kanamycin resistance; supplementary Fig 1 online). In the absence of the silencer, the respective target promoter is active and unmethylated (Fig 1A: left, α' promoter; right, NOS promoter), whereas it is repressed and methylated in the presence of the silencer (Fig 1B: left, α' promoter; right, NOS promoter).

In *drd1* mutants, non-CG methylation of the target α' promoter is greatly reduced, as evidenced by complete digestion with methylation-sensitive restriction enzymes abbreviated F, Sc, Ps, Pa, E and Ba (Fig 1C, left: data are for third-generation *drd1-6* plants). By contrast, substantial CG methylation is retained,
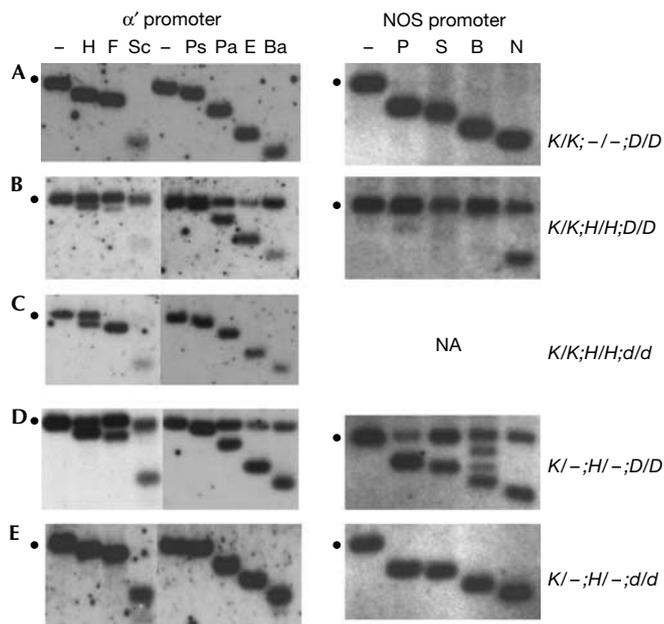
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Fig 1 | Analysis of *de novo* methylation. Methylation of the target α′ promoter was analysed using restriction enzymes diagnostic for non-CG methylation (F, Sc, Ps, Pa, E, Ba) or CG methylation (H). Methylation of the nopaline synthase (NOS) promoter was analysed using restriction enzymes diagnostic for non-CG methylation (N), CG methylation (P, B) or both (S). The respective methylation-sensitive enzymes were added after a standard digest with non-methylation-sensitive restriction enzymes ('−' lanes). The position of the methylated fragment is denoted by the dots to the left of each blot. Shifts to the smaller fragment(s) indicate no methylation at the site(s) tested. Maps of the promoters showing positions of restriction enzyme sites and the probes used for hybridization are depicted in supplementary Fig 1 online. Genotypes of the plants analysed are shown to the right; dashes indicate a hemizygous transgene locus. Bold letters to the left represent boxed genotypes shown in the breeding schemes in Fig 2. Abbreviations: *K*, target complex; *H*, silencer complex; *d*, *drd1-6* mutant; *D*, wild type; NA, not applicable. Abbreviations of enzymes and their recognition sequences (sensitivity to C methylation indicated by the superscript 'm'): α′ *promoter*: B, *Bst*UI (ᵐCGᵐCG); Ba, *Bam*HI (GGATᵐCᵐC); E, *Eco*T22I (ATGᵐCAT); F, *Fnu*4HI (GᵐCᵐNGᵐC, if N is C); H, *Hpy*CH4IV (AᵐCGT); *NOS promoter*: N, *Nhe*I (GCTAGᵐC); P, *Psp*1406I (AAᵐCGTT); Pa, *Pag*I (TᵐCATGA); Ps, *Pst*I (ᵐCTGᵐCAG); S, *Sac*II (ᵐCᵐCGᵐCGG); Sc, *Scr*FI (CᵐCᵐNGG (if N is C).

as indicated by the persistent double band generated by the restriction enzyme abbreviated H (Fig 1C, left). Similar findings for *drd1-1* led to the suggestion that DRD1 is important primarily for non-CG methylation (Kanno *et al*, 2004).

To analyse whether DRD1 is needed for RNA-directed *de novo* methylation of target sequences, crosses were made to generate F1 plants in which a naive target *K* complex was combined with the silencer *H* complex in either wild-type (*D/D*; Fig 2D) or homozygous *drd1-6* (*d/d*) plants (Fig 2E). Methylation of the target promoter was then examined in the resulting F1 progeny.

In wild-type F1 plants, the target α′ promoter showed increased methylation in CGs and in non-CGs after introducing the silencer

H complex (Fig 1D, left). The level of methylation observed in wild-type F1 plants approximated that seen in plants in which the target complex and silencer complex had been together in the same genome for several generations (Fig 1B, left). The similar methylation patterns indicate that the maximum attainable level of RNA-directed methylation of the target α′ promoter is essentially reached in the first generation containing both transgene complexes. By contrast, the target α′ promoter did not acquire detectable methylation after being combined with the silencer *H* complex in homozygous *drd1-6* plants (Fig 1E, left), which showed a hybridization pattern identical to that of the unmethylated target α′ promoter (Fig 1A, left). The lack of methylation was not because of inadequate production of RNA signals, as indicated by the continued presence of α′ promoter short RNAs in *drd1-6* plants (Fig 3A).

The target NOS promoter also becomes methylated *de novo* at CGs and non-CGs in wild-type plants after introducing the respective silencer *H* construct (Fig 1D, right; Aufsatz *et al*, 2004), but it failed to acquire measurable methylation in the *drd1-6* mutant (Fig 1E, right). Bisulphite sequencing confirmed that no detectable methylation was induced at the target NOS promoter in *drd1-6* mutants, whereas methylation was observed at Cs in all sequence contexts within the region of RNA–DNA sequence identity in wild-type plants (supplementary Fig 2 online). NOS promoter short RNAs were detected in the F1 plants (Fig 3B), indicating that RNA signals were available but unable to induce methylation in the *drd1-6* mutants. In contrast to the α′ promoter, the level of NOS promoter methylation was less in F1 progeny than in plants in which the target and silencer had been together for several generations (compare Fig 1D, right, with Fig 1B, right). Despite this difference, the results show that DRD1 is indispensable for RNA-directed *de novo* methylation of two distinct promoters.

The efficiency of maintenance methylation was examined in wild-type and *drd1-6* plants after crossing out the respective silencer *H* complexes to remove the source of the RNA signals. In wild-type F2 progeny descended from DRD1 wild-type parents (Fig 2F), the target α′ promoter lost both CG and non-CG methylation after segregating away the silencer complex (Fig 4F, left), resulting in a hybridization pattern that is indistinguishable from the unmethylated α′ promoter (Fig 1A, left). An identical pattern of methylation (Fig 4G, left) was observed in wild-type F2 progeny descended from the *drd1-6* mutant (Fig 2G). Bisulphite sequencing confirmed the loss of non-CG methylation and showed only some residual methylation at two CG dinucleotides (supplementary Fig 3A online). Thus, in the α′ promoter silencing system, almost all methylation is lost in wild-type progeny when the source of the RNA signal is withdrawn. Unexpectedly, however, in *drd1-6* progeny lacking the silencer *H* complex (Fig 2H,I), substantial CG methylation was detected, even though non-CG methylation was lost. Retention of CG methylation is again exemplified by the persistent double band generated by the restriction enzyme abbreviated H. This double band was observed in *drd1-6* progeny that are homozygous (Fig 4H, left) and hemizygous (Fig 4I, left) for the target *K* complex, indicating no dependence on dosage of the target promoter. Bisulphite sequencing confirmed the enhanced maintenance of CG methylation in the *drd1-6* mutant (supplementary Fig 3B online) relative to wild-type plants (supplementary Fig 3A online).
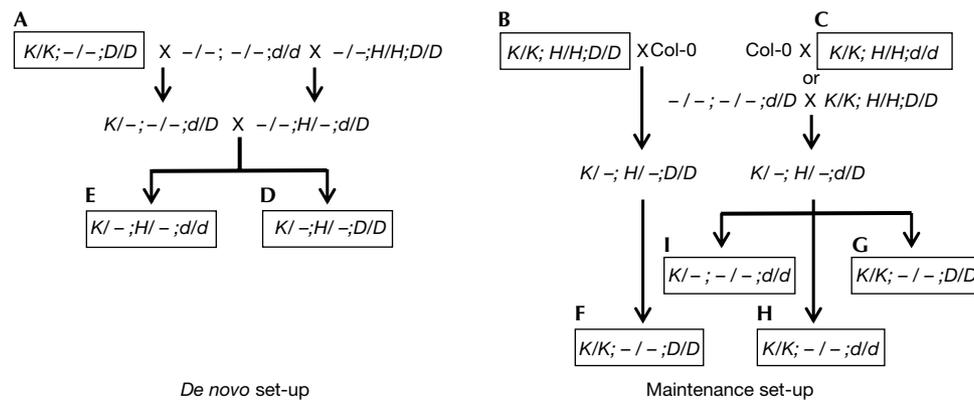
Fig 2 | Breeding schemes. *De novo* methylation analysis: wild-type plants that were homozygous for either the target *K* complex (*K/K*;−/−;*D/D*) or the silencer *H* complex (−/−;*H/H*;*D/D*) were crossed with a transgene-free, homozygous *drd1-6* mutant (−/−;−/−;*d/d*), producing respective heterozygous *drd1-6* progeny (*K/−*;−/−;*d/D* and −/−;*H/−*;*d/D*). Heterozygous *drd1-6* plants containing either the target *K* complex or silencer *H* complex were crossed and F1 progeny that were either *drd1-6* mutant (*K/−*;*H/−*;*d/d*) or wild type (*K/−*;*H/−*;*D/D*) were identified by genotyping. Maintenance methylation analysis: α′ *promoter system*: segregating the target *K* complex from the silencer *H* complex in wild-type (*K/K*;*H/H*;*D/D*) or homozygous *drd1-6* plants (*K/K*;*H/H*;*d/d*) required an initial backcross to untransformed wild-type plants (Col-0). This produced progeny that were hemizygous for the *K* and *H* complexes in either wild-type (*K/−*;*H/−*;*D/D*) or heterozygous *drd1-6* (*K/−*;*H/−*;*d/D*) backgrounds. *The nopaline synthase (NOS) promoter system*: the *K/K*;*H/H*;*D/D* plants were likewise crossed to Col-0 to produce wild-type progeny (*K/−*;*H/−*;*D/D*). To generate the *K/−*;*H/−*;*d/D* genotype, wild-type plants that were homozygous for the respective target *K* complex and silencer *H* complex (*K/K*;*H/H*;*D/D*) were crossed with a transgene-free, heterozygous *drd1-6* mutant (−/−;−/−;*d/D*). For both silencing systems, wild-type plants (*K/−*;*H/−*;*D/D*) were selfed and the resulting F2 progeny genotyped to identify those containing *K* and lacking *H* (*K/K*;−/−;*D/D*). The heterozygous *drd1-6* plants (*K/−*;*H/−*;*d/D*) were also selfed and the resulting F2 progeny were genotyped to identify those containing *K* and lacking *H* in wild-type (*K/K*;−/−;*D/D*) and homozygous *drd1-6* mutant (*K/(K)*;−/−;*d/d*) backgrounds. The boxed and lettered genotypes correspond to the lettered blots in Figs 1,4.

A comparable result was obtained when analysing maintenance methylation in the NOS promoter system. In contrast to the α′ promoter, the target NOS promoter retains significant methylation in the absence of the silencer *H* complex in wild-type plants: although non-CG methylation seems to be lost (exemplified by complete digestion with the enzyme abbreviated N (*Nhe*I)), considerable CG methylation remains, as shown by only partial digestion with restriction enzymes abbreviated P (*Psp*1406I) and B (*Bst*UI) (Fig 4F, right). Similarly to the α′ promoter, however, the NOS promoter shows increased CG methylation in *drd1-6* progeny. This is evidenced by poorer digestion with enzymes P and B in *drd1-6* mutants (Fig 4H,I, right) than in wild-type plants (Fig 4F,G, right). By contrast, cleavage by enzyme N, which is diagnostic of non-CG methylation, is equivalent in both *drd1-6* mutants and wild-type plants (Fig 4F–I, right). Collectively, these findings show a previously unsuspected role for DRD1 in the complete erasure of CG methylation after segregating away the silencer *H* complex that encodes the RNA trigger.

The results of the experiments reported here, which have examined separately *de novo* and maintenance methylation, necessitate a revision of the original proposal that DRD1 is primarily important for non-CG methylation (Kanno *et al*, 2004). DRD1 is required for RNA-directed *de novo* methylation of Cs in all sequence contexts, including methylation of CG dinucleotides (Fig 5). However, DRD1 is also necessary for efficient loss of methylation, particularly CG methylation, once the source of the RNA signal is removed. The latter requirement is more evident for the target α′ promoter, which loses almost all CG methylation in wild-type plants after segregating away the silencer complex but shows elevated CG methylation in the absence of the silencer in
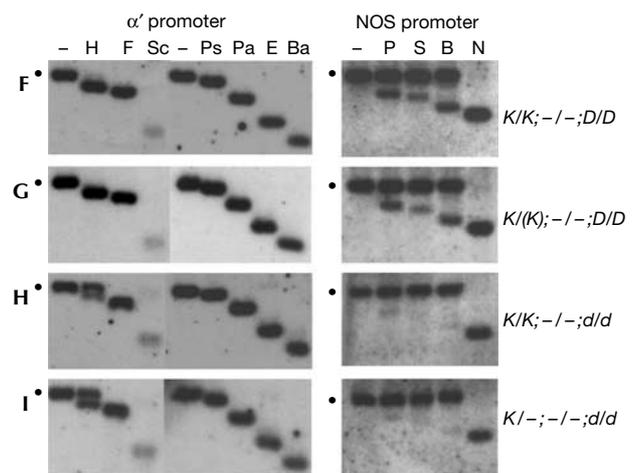
the *drd1-6* mutant. Therefore, the methylation pattern observed in third-generation *drd1* plants can be explained as an additive defect caused by inadequate erasure of CG methylation coupled to failed *de novo* methylation, which affects predominantly non-CG nucleotide groups (Fig 5). Whether the remaining CG methylation in *drd1* mutants is due to more robust maintenance or to a deficiency in either passive loss or active removal of CG methylation is not known. Passive loss occurs when there is a failure to maintain methylation during successive rounds of DNA replication. By contrast, active demethylation requires a demethylase activity and can take place in nondividing cells (Santos *et al*, 2002). In animal systems, DNA glycosylases, which are normally involved in base excision repair, have been reported to be involved in active demethylation of CG dinucleotides (Kress *et al*, 2001). In *Arabidopsis*, two large proteins that contain DNA glycosylase domains, DEMETER (Xiao *et al*, 2003; Kinoshita *et al*, 2004) and REPRESSOR OF SILENCING 1 (Gong *et al*, 2002), have been implicated in the removal of CG methylation and the re-activation of silenced genes. Additional work is required to clarify how DRD1 contributes to the loss of CG methylation.

Although DRD1 seems to be a common requirement for RNA-directed DNA methylation, the two target promoters tested here differ in the rate of their response to the introduction or withdrawal of RNA signals in wild-type plants. The target α′ promoter is very responsive, acquiring a full level of methylation in the first generation that combines the target and silencer complexes and losing most methylation in the first generation after crossing out the silencer complex. By contrast, the target NOS promoter does not attain the full level of RNA-directed methylation in the F1 generation and retains substantial CG methylation
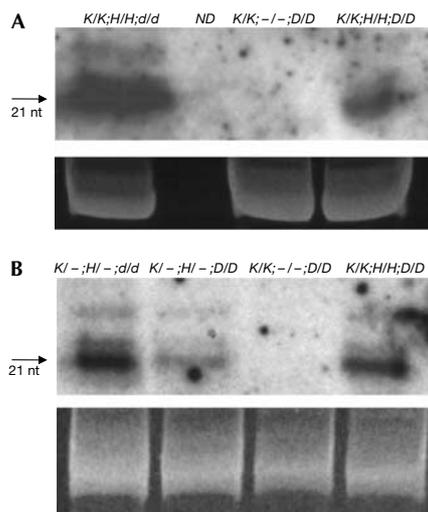
Fig 3 | Short RNA analysis. (**A**) Northern blot analysis of α′ promoter short RNAs in wild-type plants containing only the target complex (*K/K;−/−;D/D*) or both the target and silencer complexes (*K/K;H/H;D/D*), or in the *drd1-6* mutant (*K/K;H/H;d/d*). (**B**) Northern blot analysis of nopaline synthase (NOS) promoter short RNAs in F1 progeny from the '*de novo*' cross (Fig 2) that were homozygous for the *drd1-6* mutation (*K/−;H/−;d/d*) or wild type (*K/−;H/−;D/D*), and in wild-type plants that were either homozygous for the target *K* complex only (*K/K;−/−;D/D*) or double homozygous for the target *K* complex and silencer *H* complex (*K/K;H/H;D/D*). In wild-type plants, NOS promoter short RNAs are approximately twice as abundant in homozygous *H/H* as in hemizygous *H/−* plants. The quantities of short RNAs are increased in *drd1-6* mutants, perhaps because they are not used and turned over efficiently in these plants. Ethidium bromide staining of the principal RNA species in the samples is shown as a loading control. ND indicates that α′ promoter short RNAs were not tested in the genotype *K/−;H/−;D/D*.

Fig 4 | Maintenance methylation analysis. Methylation of the target promoter at the respective *K* complex (α′ promoter (left) and nopaline synthase (NOS) promoter (right)) after segregating away the respective silencer *H* complex was studied using methylation-sensitive restriction enzymes as described in the legend to Fig 1. The genotypes of the plants analysed are shown to the right. The bold letters to the left represent the boxed genotypes shown in the breeding scheme in Fig 2. '*K/(K)*' denotes both homozygous *K/K* and hemizygous *K/−* plants. The plants shown in (**G,H,I**) are siblings.

after removing the source of the trigger RNA. The reasons for these differences in response time are not known. In both systems, the respective short RNAs seem to be present in comparable amounts. Variations in the sequence composition, structure or chromatin condensation state of the two target complexes might alter their sensitivity to RNA signals (Matzke *et al*, 2004). For example, there are several copies of the NOS promoter at the corresponding target *K* complex (Aufsatz *et al*, 2002b), whereas only a single copy of the α′ promoter is present at the corresponding target *K* complex (Kanno *et al*, 2004). The higher density of CG dinucleotides in the NOS promoter (supplementary Fig 2 online) may contribute to more efficient maintenance of CG methylation at this promoter. By contrast, the α′ promoter, which is comparatively deficient in CG dinucleotides (supplementary Fig 3 online), is not repressed by CG methylation but instead seems to be silenced by non-CG methylation (Kanno *et al*, 2004). As shown here, this non-CG methylation must be established *de novo* in each generation by a DRD1-dependent, RNA-directed pathway. The fact that the α′ promoter is silenced by non-CG methylation probably allowed us to identify the *drd1* mutant in the corresponding genetic screen. By contrast, proteins required for CG methylation, the DNA methyltransferase MET1 (Aufsatz *et al*, 2002a) and histone deacetylase 6 (Aufsatz *et al*, 2002b), were

identified in a genetic screen carried out on the NOS promoter silencing system.

Several other SNF2-like proteins, including DDM1 (Jeddeloh *et al*, 1999), Lsh (Dennis *et al*, 2001) and ATRX (Gibbons *et al*, 2000), can regulate DNA methylation. However, DRD1 is the only one of these factors that has been implicated both in RNA-directed *de novo* methylation of cytosines in all sequence contexts and in erasure of CG methylation. The *drd1* alleles identified in the original genetic screen contain mutations in functionally implicated regions of the SWI2/SNF2 ATPase domain (Kanno *et al*, 2004), suggesting that DRD1 functions as a chromatin-remodelling protein to disrupt histone–DNA contacts and/or displace nucleosomes. One possibility is that DRD1 is specialized to allow RNA signals to access homologous target DNA in a chromatin context. Depending on the availability of RNA signals and various DNA-modifying enzymes in different cell types, DRD1 activity could facilitate RNA-guided *de novo* methylation catalysed by DNA methyltransferases or demethylation of CG dinucleotides catalysed by DNA glycosylases. In this model, DRD1 is a pivotal factor in the dynamic regulation of promoter activity, which might contribute to developmental plasticity, epigenetic reprogramming or adaptation of plants to the environment. Future research will have to focus on determining the full scope of DRD1 activity by using genomics approaches to identify endogenous targets in the genome.

Speculation
In view of the proposed role of DRD1 in opening chromatin to RNA signals to create a substrate for *de novo* methylation, one can ask whether loss of CG methylation, which also requires DRD1, occurs at DNA sequences targeted by short RNAs (Fig 5, short
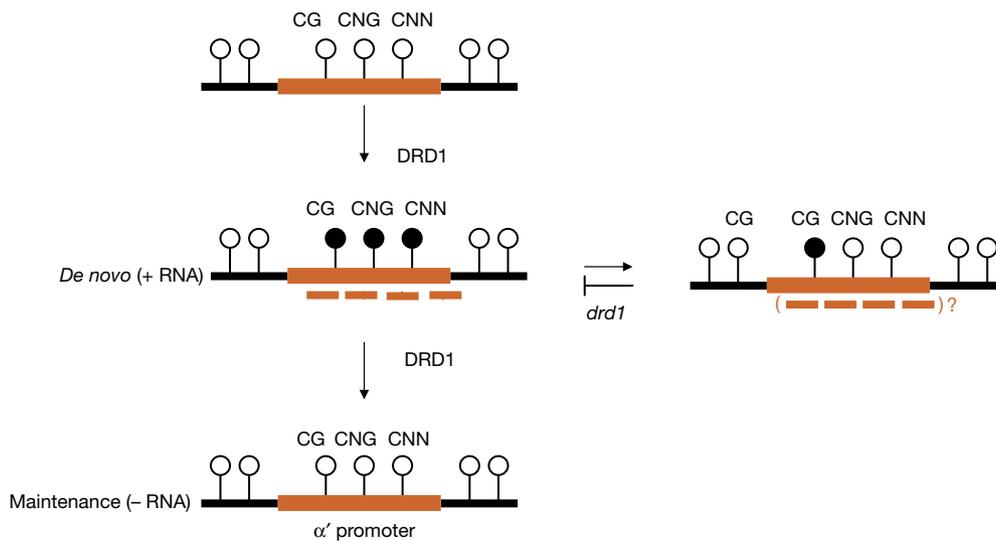
Fig 5 | Dual role of DRD1 in the dynamic regulation of RNA-directed DNA methylation. DRD1 is required for RNA-directed *de novo* methylation of Cs in all sequence contexts for the target promoters tested here. DRD1 is also needed for the complete erasure of methylation after segregation of the source of the RNA signal, as indicated by enhanced retention of CG methylation in *drd1* mutants. This is particularly clear for the target α' promoter, which normally loses most methylation after withdrawing the silencing trigger. Methylation is indicated by filled circles, the target promoter by the red line and short RNAs by red dashed lines.

RNAs in parentheses). Although the silencing complex that encodes the trigger RNA was segregated away in plants used to assess maintenance methylation, we cannot exclude the possibility that parental RNA signals are carried over into cells of the next generation, providing a short time period early in development when RNA-guided demethylation could occur. For example, DEMETER acts only in the central cell of the female gametophyte to remove CG methylation (Xiao *et al*, 2003; Kinoshita *et al*, 2004). Thus, whether RNA-directed *de novo* methylation or RNA-guided demethylation occurs would be governed by the differential cell-type-specific availability of either DNA methyltransferases or DNA glycosylases, respectively.

METHODS

Plant material and genotyping. All experiments were performed on *drd1-6* mutant (Kanno *et al*, 2004) or wild-type *Arabidopsis thaliana* (ecotype Columbia-0). The genotypes of plants used for molecular analyses were determined by PCR-based screens, as described in the supplementary information online.

DNA methylation analysis. DNA was isolated from genotyped plants and analysed for cytosine methylation, using methods described previously (Aufsatz *et al*, 2002a,b, 2004; Kanno *et al*, 2004). Southern data results were reproduced for at least two plants of each genotype. For the *drd1-6* mutant shown in Fig 1C (left), DNA was isolated from plants of the M3 generation (genotype *K/K;H/H;d/d*).

Short RNA analysis. NOS promoter and α' promoter short RNAs were isolated and analysed by northern blotting, as described previously (Aufsatz *et al*, 2002a,b, 2004; Kanno *et al*, 2004).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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