DNA demethylation in Arabidopsis (Arabidopsis thaliana) is mediated by DNA glycosylases of the DEMETER (DME) family. Three DEMETER-LIKE (DML) proteins, REPRESSOR OF SILENCING1 (ROS1), DML2, and DML3, are expressed to protect genes from potentially deleterious methylation. In Arabidopsis, much of the DNA methylation is directed by RNA interference (RNAi) pathways and used to defend the genome from transposable elements and their remnants, repetitive sequences. Here, we investigated the relationship between DML demethylation and RNAi-mediated DNA methylation. We found that genic regions demethylated by DML enzymes are enriched for small interfering RNAs and generally contain sequence repeats, transposons, or both. The most common class of small interfering RNAs was 24 nucleotides long, suggesting a role for an RNAi pathway that depends on RNA-DEPENDENT RNA POLYMERASE2 (RDR2). We show that ROS1 removes methylation that has multiple, independent origins, including de novo methylation directed by RDR2-dependent and -independent RNAi pathways. Interestingly, in rdr2 and drm2 mutant plants, we found that genes demethylated by ROS1 accumulate CG hypermethylation, and we propose that this hypermethylation is due to the RDR2/DCL3/AGO4 pathway.

ROS1 is necessary for expression of an RD29A:: LUCIFERASE (RD29A::LUC) transgene and the endogenous RD29A gene (Gong et al., 2002). In RD29A::LUC plants, ROS1 removes RNA-directed DNA methylation (RdDM) from the promoters of RD29A::LUC and RD29A, as well as DNA methylation at other loci (Gong et al., 2002; Penterman et al., 2007; Zhu et al., 2007). RdDM is mediated by RNA interference (RNAi; Bender, 2004; Chan et al., 2005). aberrant transcripts produced from the RD29A::LUC reporter gene are processed into small interfering RNA (siRNA), which are likely loaded into ARGONAUTE4 (AGO4) and AGO6 effector complexes and used to direct methyltransferases to the promoters of RD29A::LUC and the endogenous RD29A gene (Gong et al., 2002; Zheng et al., 2007). It is not known if the antagonism between ROS1 and RdDM extends to endogenous loci demethylated by ROS1.

Current evidence suggests that the primary function of RdDM and RNAi is to defend the genome from transposable elements (Bender, 2004; Chan et al., 2005). At least two independent RNAi pathways can lead to RdDM. One pathway includes RNA-DEPENDENT RNA POLYMERASE6 (RDR6), SUPPRESSOR OF GENE SILENCING3/SILENCING DEFECTIVE2 (SDE2), SDE3, and AGO1, and the other RNAi pathway is composed of RNA polymerase IV (PolIV), RNA-DEPENDENT RNA POLYMERASE2 (RDR2), DICER-LIKE3 (DCL3), and AGO4 (Chan et al., 2005). Evidence suggests that the PolIV/RDR2/DCL3/AGO4 pathway catalyzes the majority of RdDM in Arabidopsis (Chan et al., 2005; Li et al., 2006; Lu et al., 2006; Pontes et al., 2006; Kasschau et al., 2007; Zhang et al., 2007). The PolIV/RDR2/DCL3/AGO4 pathway is required for de novo
methylation by the methyltransferase DOMAINS RE-
ARRANGED METHYLTRANSFERASE2 (DRM2) and for
the maintenance of non-CG methylation genome-
wide by DRM2 and CHROMOMETHYLTRANSFER-
ASE3 (Bender, 2004; Chan et al., 2005). The PolIV/
RDR2/DCL3/AGO4 pathway maintains non-CG
methylation by using a positive feedback loop (Pontes
et al., 2006; Zhang et al., 2007). At heterochromatic loci,
PolIV is hypothesized to generate single-stranded
RNA molecules (Herr et al., 2005; Kanno et al., 2005;
Pontes et al., 2006). RDR2 then synthesizes double-
stranded RNAs with which DCL3 endonuclease gen-
erates 24-nucleotide siRNAs, a hallmark of the PolIV/
RDR2/DCL3/AGO4 pathway (Xie et al., 2004; Herr
et al., 2005; Pontes et al., 2006; Kasschau et al., 2007).
The siRNAs then direct DRM2 to the loci from which they
originated, maintaining or establishing the level of
DNA methylation. PolIV then initiates the cycle
again to maintain non-CG methylation (Pontes et al.,
2006; Zhang et al., 2007), and METHYLTRANSFER-
ASE1 (MET1) maintains CG methylation, which may
not require additional cycles of the siRNA pathway
(Bender, 2004; Chan et al., 2005). Mutations that affect
any step of this pathway block de novo DNA methyl-
lation, significantly reduce genomic non-CG methylation,
and cause the derepression of several transposable
elements (Kanno et al., 2004, 2005; Xie et al., 2004;
Chan et al., 2005, 2006; Herr et al., 2005). The PolIV/RDR2/
DCL3/AGO4 pathway might have a role in establish-
ing methylation at endogenous loci demethylated by
ROS1 because ROS1 and AGO4 are antagonists in
transgenic RDR2::LUC plants (Zheng et al., 2007).

Mutations in RDR2, DEFECTIVE IN RNA-
DIRECTED DNA METHYLATION1 (DRD1), and genes
that encode PolIV reduce the level of ROS1 RNA
(Huettel et al., 2006, 2007). It is not known how
reduced ROS1 expression due to mutations in the
RDR2 and PolIV genes affects DNA demethylation
activity at endogenous ROS1 gene targets.

Here, we investigated the genetic relationship be-
tween DNA demethylation and DNA methylation in
Arabidopsis. We show that genomic regions demeth-
ylated by DML enzymes are enriched for siRNAs and
generally contain sequence repeats, transposons, or both.
The most common class of siRNA at DML target
genes was 24 nucleotides long, suggesting a role for
the PolIV/RDR2/DCL3/AGO4 pathway at these loci.
By using mutations in RDR2 and DRM2, we show that
ROS1 removes de novo methylation directed by the
PolIV/RDR2/DCL3/AGO4 pathway and an RDR2-
dependent RNAi pathway. Interestingly, in rdr2 and
drm2 mutant plants, we found that genes demeth-
ylated by ROS1 in wild-type plants accumulate meth-
ylation at CG sites, and we propose that this
hypermethylation is due to the ROS1 down-regulation
that occurs in these mutant backgrounds. Our obser-
vations support the hypothesis that DML enzymes
protect genes from the genome defense pathway by
demethylating nearby sequence repeats and transpos-
able elements.

RESULTS

Loci Demethylated by DML Enzymes Correspond to
Regions with Small RNAs, Repeats, and Transposons

DNA methylation is generally found at transposons,
repetitive sequences, and regions targeted by siRNAs.
We searched for these features at intergenic and genic
loci (n = 33 and n = 146, respectively) previously
identified as demethylated by DMLs (Penterman et al.,
2007) by using databases that contain sequenced
siRNAs and computationally identified repeats and
transposons (Gustafson et al., 2005; Lu et al., 2005;
Rajagopalan et al., 2006; Kasschau et al., 2007; Zhang
et al., 2007). Sequenced siRNAs were found at 60% (n =
88) of genes and 64% (n = 21) of intergenic loci that are
demethylated by DML enzymes (Fig. 1A). The gene set
demethylated by DMLs is significantly enriched for
siRNAs relative to the number of Arabidopsis genes
targeted by siRNAs (approximately 20%; Kasschau
et al., 2007). Transposons and various sequence repeats
were also common to genic (48%; n = 70) and intergenic
(55%; n = 18) loci demethylated by DML enzymes (Fig.
1A). When combined, 75% of genes (n = 110) and 76%
of intergenic loci (n = 25) demethylated by DMLs had
siRNAs, transposable elements and repeats, or both (Fig.
1A). These data show that loci demethylated by
DMLs are enriched for siRNAs and commonly overlap
with sequence repeats and transposable elements.

Multiple RNAi pathways function in Arabidopsis,
producing siRNAs that generally are 20 to 24 nucleo-
tides long. The size of siRNAs at a given locus can
provide clues as to which RNAi pathway targets that
locus. Data about the size of siRNAs were available for
105/109 loci targeted by DML demethylation and
siRNAs. At these loci, siRNAs ranged from 20 to 24
nucleotides in length, and multiple classes of siRNAs
were often observed at a single locus (Fig. 1B; data not
shown). The siRNA profile of DML-target loci was
skewed toward the longer siRNA classes (Fig. 1B).
Nearly 80% of all loci demethylated by DMLs were
targeted by a 24-nucleotide-long siRNA (Fig. 1B), which
is a hallmark of the PolIV/RDR2/DCL3/AGO4 path-
way (Xie et al., 2004; Herr et al., 2005; Kasschau et al.,
2007). These observations suggest that the PolIV/
RDR2/DCL3/AGO4 RNAi pathway targets DNA meth-
ylation to most loci demethylated by DML enzymes.

ROS1 Demethylation Antagonizes de Novo
Methylation Directed by the PolIV/RDR2/DCL3/AGO4
RNAi Pathway

The PolIV/RDR2/DCL3/AGO4 pathway protects
the genome from transposable elements and repetitive
dNA by establishing de novo methylation and main-
taining non-CG methylation (Chan et al., 2004, 2005,
2006). We used the rdr2-1 loss-of-function mutation to
test the role of this pathway in methylation of ROS1-
target loci because RDR2 is specific to and essential for
the PolIV/RDR2/DCL3/AGO4 pathway (Xie et al.,
2004; Lu et al., 2006; Pontes et al., 2006; Kasschau et al.,
An F1 plant heterozygous for ros1-3 and rdr2-1 was self-pollinated to generate a homozygous ros1-3; rdr2-1 F2 line, and the methylation levels of three ROS1-target genes (At1g26400, At1g34245, and At5g38550) in ros1-3; rdr2-1 F2 plants were measured and compared to wild-type and ros1-3 control plants. Hypermethylation of these loci was significantly less in ros1-3; rdr2-1 F2 plants relative to wild-type plants. For the At1g34245 locus (Fig. 2B), both CG and non-CG methylation levels were reduced in ros1-3; rdr2-1 F2 plants, although the levels of CG and CNG methylation were intermediate between the levels observed in wild-type and ros1-3 plants. Thus, full CNG and CNN hypermethylation of loci in ros1-3 mutants requires a PolIV/RDR2/DCL3/AGO4 pathway. These results are consistent with the high proportion of 24-nucleotide siRNAs at DML-target loci and indicate that ROS1 demethylation protects endogenous genes from methylation directed by the PolIV/RDR2/DCL3/AGO4 pathway.

The PolIV/RDR2/DCL3/AGO4 pathway both establishes and maintains non-CG methylation. In the experiments reported above, ROS1 and RDR2 activities were simultaneously lost when the ros1-3; rdr2-1 F2 double mutant plants were formed (ros1; rdr2 in Fig. 2, A–C). Thus, the reduction of non-CG methylation observed in the ros1-3; rdr2-1 genetic background might reflect the role the PolIV/RDR2/DCL3/AGO4 pathway has in both establishing and maintaining hypermethylation of ROS1-target loci. Alternatively, these results might reflect only the maintenance function of the PolIV/RDR2/DCL3/AGO4 pathway. To clarify the role of the PolIV/RDR2/DCL3/AGO4 pathway at these loci, we defined this pathway’s role in maintaining DNA hypermethylation in the ros1-3 mutant background. This was accomplished by allowing hypermethylation to be established and maintained by the PolIV/RDR2/DCL3/AGO4 pathway in a ros1-3; RDR2/rdr2-1 F2 mutant plant (see methylation data in Fig. 5, B–D), which was subsequently self-pollinated, and the effect of the loss of RDR2 activity upon maintaining hypermethylation was assessed by comparing the methylation levels of loci in ros1-3; rdr2-1 F3 progeny relative to ros1-3 plants (compare ros1; rdr2 in A and B to D and E, respectively), indicating that the PolIV/RDR2/DCL3/AGO4 pathway maintains CNG and CNN hypermethylation at these loci in ros1-3 plants.

We next determined the role of the PolIV/RDR2/DCL3/AGO4 pathway in establishing hypermethylation at ROS1-target loci by comparing the results obtained when ROS1 and RDR2 activity were simultaneously lost (ros1; rdr2 in Fig. 2, A–C) to those when the maintenance function of RDR2 was lost in ros1-3; rdr2-1 F3 progeny (compare ros1; rdr2 in A and B to D and E, respectively), indicating that the PolIV/RDR2/DCL3/AGO4 pathway maintains CNN hypermethylation at these loci in ros1-3 plants.

Figure 1. DML-target loci have features associated with DNA methylation. A, Percentage of DML-target genes and intergenic loci with siRNAs, transposons and repetitive elements, or both. B, Percentage of DML-target loci with 20-, 21-, 22-, 23-, and 24-nucleotide siRNAs.
plants. At At5g38550, it was not possible to distinguish the establishment and maintenance function of the PolIV/RDR2/DCL3/AGO4 pathway in hypermethylation of CNN sites because an equivalent reduction was observed in both plants (compare ros1; rdr2 in C and F). However, the establishment and maintenance function of the PolIV/RDR2/DCL3/AGO4 pathway in hypermethylation of CNG was distinguishable; the PolIV/RDR2/DCL3/AGO4 pathway was required for establishment, but not maintenance, of CNG hypermethylation (compare ros1; rdr2 in C and F), indicating that the PolIV/RDR2/DCL3/AGO4 pathway establishes de novo CNG methylation at At5g38550 in ros1 mutant plants. After establishment, a different pathway maintains CNG hypermethylation at At5g38550 in ros1-3 mutants. Collectively, these data show that DNA demethylation by ROS1 in wild-type plants removes methylation that in most, but not all, cases is established and maintained by the PolIV/RDR2/DCL3/AGO4 pathway.

ROS1 Removes Methylation That Has Multiple, Independent Origins

Mutations that disrupt the PolIV/RDR2/DCL3/AGO4 pathway do not affect the establishment or maintenance of CG hypermethylation at At1g26400, At1g34245, and At5g38550 in ros1 mutant plants (ros1; rdr2 in Fig. 2, A–C). The lack of a role by the PolIV/RDR2/DCL3/AGO4 pathway in CG hypermethylation might be a reflection of the fact that CG methylation is symmetric and is mediated by MET1 methyltransferase without input from an RNAi pathway (Chan et al., 2005). Alternatively, a redundant RNAi pathway, distinct from the PolIV/RDR2/DCL3/AGO4 pathway, could establish this CG hypermethylation. To test the latter idea, we investigated the role of the de novo methyltransferase DRM2 in establishing hypermethylation at At1g26400, At1g34245, and At5g38550 in the ros1-3 mutant background. If an RNAi pathway other than the PolIV/RDR2/DCL3/AGO4 pathway establishes hypermethylation in ros1 plants, then drm2 mutations should lower CG hypermethylation levels more than rdr2 mutations. This is because DRM2 is the only known de novo methyltransferase in Arabidopsis (Chan et al., 2005), so that both the PolIV/RDR2/DCL3/AGO4 pathway and the hypothetical redundant pathway would use DRM2 for de novo DNA methylation. A plant heterozygous for the ros1-3 and drm2-1 mutations was self-pollinated to generate a ros1-3; drm2-1 F2 line, and the methylation of these loci was measured in F3 plants (ros1; drm2 in Fig. 2, A–C). As expected, ros1-3; drm2-1 F2 line, and the methylation of these loci was measured in F3 plants (ros1; drm2 in Fig. 2, A–C). As expected, ros1-3; drm2-1 mutant plants displayed little or no CNG or CNN hypermethylation at At1g26400, At1g34245, and At5g38550. Thus, mutations in DRM2 prevented the establishment and/or maintenance of nearly all non-CG hypermethylation at loci in ros1-3; drm2-1 F3 plants (Fig. 2, A–C). By contrast, CG hypermethylation at At1g26400 and At5g38550 was not affected in ros1-3; drm2-1 F3 double mutants, suggesting
that DRM2 is not required for CG hypermethylation at these loci (ros1; drm2 in Fig. 2, A and C). CG sites at At1g34245 were also hypermethylated in ros1; drm2 plants relative to wild-type plants (ros1; drms in Fig. 2B). However, the CG methylation level at At1g34245 in ros1-3; drm2 was approximately 50% lower than in ros1-3 plants (ros1 in Fig. 2B), yet was still 3-fold higher than the wild type (WT in Fig. 2), indicating that DRM2 was required for a certain level of CG hypermethylation at At1g34245 in ros1 mutant plants. Taken together, the data indicate that ROS1 actively removes CG methylation that is mostly independent of DRM2.

The CNG and CNN methylation profiles at At1g26400 and At5g38550 in ros1-3; drm2 F3 plants were similar to the profiles observed in ros1-3; rdr2 F3 plants, indicating that DRM2 and RDR2 are required to establish the same patterns of CNG and CNN hypermethylation at these loci (Fig. 2, A and C). The CNG methylation level at At1g34245 in ros1-3; drm2 F3 plants was lower than the level measured in ros1-3; rdr2 F3 plants (Fig. 2B), suggesting that DRM2 establishes a certain level of CNG methylation at At1g34245 in ros1-3 plants in an RDR2-independent manner. De novo methylation by DRM2 requires RNAi (Chan et al., 2005). Therefore, these results suggest that ROS1 demethylation at At1g34245 removes de novo methylation that is directed by RDR2-dependent and -independent RNAi pathways.

Mutations in DRM2 and RDR2 Lead to CG Hypermethylation at ROS1-Target Loci

At1g26400, At1g34245, and At5g38550 all contain low levels of non-CG methylation in wild-type plants (Fig. 2, A–C; Penterman et al., 2007). Given the previous results showing non-CG DNA methylation by the PolIV/RDR2/DCL3/AGO4 pathway, single rdr2 and drm2 mutations in a wild-type ROS1 background would be expected to reduce non-CG methylation levels of these loci because ROS1 would remove methylation and no new non-CG methylation would be established by pathways using RDR2 and DRM2. The overall non-CG methylation level at At1g26400, At1g34245, and At5g38550 in rdr2 and drm2 single mutants was less or similar to the level observed in wild-type plants (Fig. 3, A–C).

As for CG methylation, the rdr2 and drm2 mutations should have no effect or reduce it if they are needed to re-establish CG methylation after ROS1 demethylation in wild-type plants. However, contrary to expectations, At1g26400, At1g34245, and At5g38550 were hypermethylated at CG sites in rdr2-1 and drm2-1 single mutants relative to wild-type plants (Fig. 3, A–C). The degree of hypermethylation was similar to that observed in ros1 mutant plants (Fig. 3, A–C).

Is the CG hypermethylation of ROS1-target loci general to mutants defective in RNAi and RdDM, or is it specific to the PolIV/RDR2/DCL3/AGO4 RNAi pathway? To address this question, At1g26400, At1g34245, and At5g38550 were bisulfite sequenced from plants bearing mutations in RDR6, which is required for

Figure 3. ROS1-target loci are hypermethylated in rdr2-1 and drm2-1 mutant plants. A to C, Percentage of methylation at At1g26400 (A), At1g34245 (B), and At5g38550 (C) in wild-type, ros1-3, rdr2-1, drm2-1, and rdr6-11 plants.
RdDM and transcriptional silencing of highly transcribed sense transgenes (Chan et al., 2005). No hypermethylation was observed at ROS1 target loci (Fig. 3, A–C). These observations suggest that ROS1-target loci are specifically affected by mutations in RDR2 and DRM2.

**ROS1 Is Down-Regulated in drm2 Mutant Plants**

rdr2-1 and drm2-1 mutations cause CG hypermethylation at ROS1 target loci (Fig. 3). The level of CG hypermethylation is similar to the level observed in ros1-3; rdr2-1 and ros1-3; drm2-1 double mutant plants (Fig. 2, A–C). Moreover, plants with mutations in RDR2 and PolIV genes have significantly reduced levels of ROS1 RNA (Huettel et al., 2006). Down-regulation of ROS1 might explain why ROS1 target loci become hypermethylated at CG sites in rdr2 and drm2 mutants. To test this idea, semiquantitative reverse transcription (RT)-PCR was used to compare ROS1 expression levels between rdr2, drm2, ROS1/ROS1; rdr2-1, and ROS1/ros1-3; DRM2/DRM2 plants. As previously shown, the expression of ROS1 was significantly down-regulated in rdr2-1 plants (Fig. 4; Huettel et al., 2006). In drm2 mutant plants, ROS1 expression was further reduced and could barely be detected by RT-PCR (Fig. 4). By contrast, ROS1 expression was not reduced in rdr6-T1 mutant plants (Fig. 4). These results show that proper ROS1 expression depends on DRM2 and RDR2, but not on RDR6. Thus, there is a positive correlation between the expression level of ROS1 and the CG methylation status of its target genes. In drm2 and rdr2 mutants, ROS1 expression is down-regulated and its target loci are hypermethylated at CG sites. In the wild type and rdr6-T1, there is a high level of ROS1 expression and ROS1-target loci are hypomethylated. ROS1-target loci might be hypermethylated at CG sites in drm2 and rdr2 plants because ROS1 expression is down-regulated.

**RDR2 Is Required for DNA Demethylation by ROS1**

To demonstrate that the down-regulation of ROS1 in rdr2-1 mutant plants affects ROS1 demethylation, we tested the ability of ROS1 to demethylate and restore CG hypermethylated loci to wild-type levels in siblings that differ in the presence and absence of RDR2 (Fig. 5A). We crossed ros1-3; RDR2/rdr2-1 plants to rdr2-1 single mutants to generate ros1/RDR2; rdr2/rdr2-1 and ros1/ROS1; rdr2-1 progeny (Fig. 5A). We compared the methylation levels of these progeny to the levels of the parents to see if reintroduction of ROS1 alleles in progeny could restore hypermethylated alleles to a more hypomethylated state. The level of CG methylation at At1g26400, At1g34245, and At5g38550 in heterozygous ros1/RDR2; rdr2/rdr2-1 progeny was much less than in both parents, indicating that these loci were demethylated by ROS1 (Fig. 5, B–D). However, in ros1/RDR2; rdr2-1 siblings, CG methylation levels at At1g26400, At1g34245, and At5g38550 were similar to or exceeded the methylation levels of the parents, indicating that the rdr2 mutation inhibited ROS1 demethylation (Fig. 5, B–D). These results show that the CG hypermethylation observed at ROS1 target loci in rdr2 and drm2 plants (Fig. 3, A–C) is because ROS1 demethylation requires a wild-type RDR2 allele.

**DISCUSSION**

In this study, we showed that the loci demethylated by DML enzymes contain features commonly targeted by the plant’s genome defense pathway. These features include transposable elements and their remnants, repetitive sequences, and siRNAs (Fig. 1A). A significant fraction of DML-target loci matched 24 nucleotide siRNAs (Fig. 1B), which suggested that DML demethylation removes methylation directed by the PolIV/RDR2/DCL3/AGO4 RNAi pathway (Xie et al., 2004; Kasschau et al., 2007). We demonstrated an antagonistic relationship between ROS1 and the PolIV/RDR2/DCL3/AGO4 pathway and uncovered evidence that suggests that ROS1 also removes DNA methylation directed by an RDR2-independent RNAi pathway (Fig. 2B). We found that DRM2 is also required for ROS1 expression and demonstrate that wild-type RDR2 and DRM2 alleles are required for DNA demethylation by ROS1 (Figs. 3, A–C, 4, and 5, B–D). Our data support the hypothesis that DNA demethylation by DML enzymes glucoseylates protects genes from the genome defense pathways and show one mechanism by which ROS1 demethylation is potentially regulated by DNA methylation pathways in Arabidopsis.

DML demethylation protects genes from potentially deleterious methylation (Penterman et al., 2007). At genes, DML enzymes primarily demethylate the 5′ and
ends, a pattern opposite to the overall distribution of wild-type DNA methylation (Zhang et al., 2006; Penterman et al., 2007; Vaughn et al., 2007; Zilberman et al., 2007). This led us to hypothesize that DML demethylation was a factor keeping gene ends methylation free. Our data show that nearby transposons and repeats are common characteristics of DML-demethylated genes (Fig. 1A). These features, which are inherent targets of the RNAi pathways and RdDM (Chan et al., 2005), are likely the reason methylation is directed to some DML-demethylated genes (Penterman et al., 2007). Thus, at some loci, DML demethylation protects genes from genome defense pathways that are targeting repeats and transposable elements.

Methylation within Arabidopsis genes is concentrated in the middle and distributed away from the 5’ and 3’ ends, suggesting that 5’ and 3’ methylation is detrimental to gene function (Zhang et al., 2006; Gehring and Henikoff, 2007; Vaughn et al., 2007; Zilberman et al., 2007). Our data and recent reports suggest that gene ends are kept free of methylation by two distinct mechanisms. The 5’ and 3’ ends of genes generally lack siRNAs, which indicates that genic ends are kept free of methylation by controlling where methylation is directed (Kasschau et al., 2007). However, our data indicate that when gene ends are targeted by siRNAs, DML demethylation can prevent the methylation of some gene ends (Figs. 1, A and B, and 2; Penterman et al., 2007). Thus, protecting gene ends from methylation involves two distinct layers of regulation: (1) siRNAs are generally not directed to the ends of genes (Kasschau et al., 2007); and (2) DML demethylases target gene ends that are targeted by siRNAs.

Our data also suggest that the methylation removed by ROS1 has multiple, distinct origins. In this study, we examined the role of the PolIV/RDR2/DCL3/AGO4 pathway and DRM2 in de novo methylation of loci in ros1 mutant plants. At At1g26400 and At5g38550 in ros1-3 plants, the PolIV/RDR2/DCL3/AGO4 pathway and the de novo methyltransferase DRM2 were required for non-CG hypermethylation, whereas CG hypermethylation did not require the PolIV/RDR2/DCL3/AGO4 pathway and DRM2 (Fig. 2, A and C). When and how CG hypermethylation is established at these loci is not clear. However, as CG methylation is efficiently maintained by MET1, establishment of CG methylation would only have to occur infrequently or in progenitor cells to be maintained indefinitely. We found that the PolIV/RDR2/DCL3/AGO4 pathway was only partially responsible for directing DRM2 de novo methylation at the At1g34245 locus, as ros1; rdr2 mutants had greater CG and CNG methylation than ros1; drm2 mutants (Fig. 2B). This

Figure 5. RDR2 is required for ROS1 DNA demethylation. A, Inheritance of hypermethylated loci by siblings that differ in the absence or presence of an RDR2 allele. B to D, Percentage of methylation at At1g26400, At1g34245, and At5g38550 in the parental genotypes (rdr2 and ros1-3; RDR2/rdr2-2) and their progeny (ROS1/ros1-3; RDR2/rdr2-1 and ROS1/ros1-3; rdr2-2). Eight to 10 clones from the parents were sequenced, and 17 to 20 clones from the progeny were sequenced.
observation implies that another distinct RNAi pathway directs DRM2 de novo methylation to Atg34245 in ros1-3 mutant plants. Thus, ROS1 demethylation might antagonize more than one RNAi pathway, which is consistent with its housekeeping function (Penterman et al., 2007).

Previously, ROS1 was shown to be transcriptionally down-regulated in plants with mutations in RDR2, DRD1, and the PolIV genes (Huettel et al., 2006). This effect on ROS1 expression appears to be specific to mutations that disrupt the PolIV/RDR2/DCL3/AGO4 RNAi pathway, because mutations in RDR6, which functions in another RNAi pathway, do not affect ROS1 expression (Fig. 4). ROS1 was also down-regulated in plants with mutations in the de novo methyltransferase DRM2 (Fig. 4). Because RDR2, DRD1, PolIV genes, and DRM2 encode enzymes that function at different, critical steps of the PolIV/RDR2/DCL3/AGO4 pathway (Li et al., 2006; Pontes et al., 2006), these observations suggest that it is the function of the pathway, rather than any one component, that is important for ROS1 expression. Our data also show that the PolIV/RDR2/DCL3/AGO4 pathway is necessary for ROS1 demethylation. In drm2 and rdr2 mutants, loci demethylated by ROS1 accumulate methylation at CG sites (Fig. 3, A–C), and hypermethylated alleles inherited from the parents are demethylated by ROS1 in progeny that have a wild-type RDR2 allele, whereas these alleles remain hypermethylated in homozygous rdr2 siblings (Fig. 4, A–D). Thus, down-regulation of ROS1 in rdr2 and drm2 mutants results in minimal ROS1 demethylation and a unique methylation pattern at its targets. Identifying the mechanisms of ROS1 down-regulation by mutations that disrupt the PolIV/RDR2/DCL3/AGO4 RNAi pathway is crucial to determining its functional significance in Arabidopsis.

MATERIALS AND METHODS

Transposon, Repeat, and siRNA Analysis

The data used to search for transposable elements, repeats, and siRNAs were described previously (Gustafson et al., 2005; Lu et al., 2005; Rajagopalan et al., 2006; Kasschau et al., 2007; Zhang et al., 2007) and can be accessed at the following databases: http://asrp.cgrb.oregonstate.edu, http://epigenomics.mcbdb.ucla.edu/smallRNAs, and http://mpss.udel.edu/at. The databases were queried using the coordinates for each DML target locus (Penterman et al., 2007). Size information for siRNAs was available for most loci except ones whose siRNAs were only reported by Lu et al. (2005). Annotation of transposable elements was based on RepliBase v10.0.1 (Jurka, 2000).

Plant Materials

The descriptions and genotyping protocols for ros1-3, dml2-1, and dml3-1 were previously described (Penterman et al., 2007). The rdr2-1 and rdr6-11 alleles were obtained from the Arabidopsis Biological Resource Center (Ohio State University). The drm2 allele was a kind gift from Steve Jacobsen (University of California, Los Angeles). The descriptions and genotyping protocols for rdr2-1, rdr6-11, and drm2 were previously described (Cao and Jacobsen, 2002; Peragine et al., 2004; Xie et al., 2004). The drm2 allele was introgressed into the Columbia-0 background four times prior to use in the experiments. All genetic experiments involving ros1-3 with rdr2-1 or dml1-1 were done in the ros1-3; dml2-1; dml3-1 background.

Bisulfite Sequencing

Methods and primer sequences used in bisulfite sequencing experiments were previously described (Penterman et al., 2007). Around eight to 10 clones were sequenced for all experiments unless noted otherwise.

RT-PCR Analysis of Genes Demethylated ROS1

Twenty-day-old plants were used to isolate total RNA as described (Choi et al., 2002). RT-PCR conditions and ACTIN11 primers were as previously described (Penterman et al., 2007). Primers for amplification of ROS1 transcripts were ROS1F (5′-TGGAAGGACCGTGGATTCT-3′) and ROS1R (5′-CCATCTTGCTGAGGACCAACA-3′).

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LITERATURE CITED
