

# Antisilencing role of the RNA-directed DNA methylation pathway and a histone acetyltransferase in *Arabidopsis*

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**REPRESSOR OF SILENCING 1 (ROS1) is a DNA demethylation enzyme that was previously identified during a genetic screen for the silencing of both *RD29A-LUC* and *35S-NPTII* transgenes on a T-DNA construct. Here we performed a genetic screen to identify additional mutants in which the *35S-NPTII* transgene is silenced. We identified several alleles of *ros1* and of the following components of the RNA-directed DNA methylation (RdDM) pathway: *NRPD1* (the largest subunit of polymerase IV), *RDR2*, *NRPE1* (the largest subunit of polymerase V), *NRPD2*, *AGO4*, and *DMS3*. Our results show that the silencing of *35S-NPTII* in the RdDM pathway mutants is due to the reduced expression of *ROS1* in the mutants. We also identified a putative histone acetyltransferase (*ROS4*) from the genetic screen. The acetyltransferase contains a PHD-finger domain that binds to unmethylated histone H3K4. The mutation in *ROS4* led to reduction of H3K18 and H3K23 acetylation levels. We show that the silencing of *35S-NPTII* and some transposable element genes was released by the *ddm1* mutation but that this also required *ROS4*. Our study identifies a unique anti-silencing factor, and reveals that the RdDM pathway has an anti-silencing function due to its role in maintaining *ROS1* expression.**

transcriptional gene silencing | epigenetic | gene silencing | histone modification

**D**NA cytosine methylation is an important epigenetic modification in plants and animals. The molecular mechanism for RNA-directed DNA methylation (RdDM) has been well-established by both genetic and biochemical studies in *Arabidopsis* (1–4). Small RNAs (~24 bp) and long noncoding RNAs produced by a complex transcriptional machinery composed of polymerase (Pol) IV and Pol V, together with other proteins, target specific sequences for de novo DNA methylation (1–4). RdDM mainly occurs in transposons, retrotransposons, rDNA arrays, and centromeric repeat regions, and accounts for about 30% of the DNA methylation in the genome of *Arabidopsis* (5, 6).

DNA methylation can be removed passively during DNA replication or actively by the DNA glycosylase REPRESSOR OF SILENCING 1 (*ROS1*) and its homologs DEMETER (*DME*), DEMETER-LIKE 2 (*DML2*), and *DML3* (1, 2, 7, 8). *ROS1* was identified in a genetic screen in which the originally active *RD29A-LUC* was silenced in *ros1* mutants (9). *ROS1* is a key DNA demethylation component in vegetative tissues (9), and its transcript level is regulated by *MET1* and several RdDM components (10). Screening for second-site suppressors of *ros1* mutants by using silenced *RD29A-LUC* has enabled researchers to identify the main components of the RdDM pathway and has further indicated that RdDM and *ROS1* antagonistically regulate DNA methylation (11–14).

*ros1* mutations also cause the silencing of another transgene, *35S-NPTII*, which is in the same T-DNA construct as *RD29A-LUC* (9). *RD29A-LUC* has been used to identify *ROS1* and *ROS3* genes (9, 15). By screening for second-site suppressors of *ros1* using silenced *35S-NPTII*, we previously identified genes that are involved in DNA replication and maintenance of transcriptional silencing (7). Our results indicated that *RD29A-LUC* and *35S-NPTII* are

silenced and/or maintained by different mechanisms. In the current study, to find additional REPRESSOR OF SILENCING genes, we performed a forward genetic screen by using *35S-NPTII* as a selection marker. Unexpectedly, besides three alleles of *ros1* and a unique gene (*ROS4*), the genes we identified are mainly components in the RdDM pathway. We show that the mutations in RdDM components cause the silencing of *35S-NPTII* by down-regulating the expression of *ROS1*. Our results provide genetic evidence for an in vivo role of regulating *ROS1* expression by the RdDM pathway. The antisilencing factor *ROS4* encodes a putative histone acetyltransferase with a PHD-finger domain and a methyl-CpG-binding domain. *ROS4* preferentially binds to unmethylated histone H3K4, and the *ros4* mutation results in reduction of the overall H3K23 and H3K18 acetylation levels in plants. We also found that *ROS4* is required for the expression of some genes that are normally silenced by DNA methylation.

## Results

### Isolation of Mutants in Which the Expression of *35S-NPTII* is Silenced.

To identify genes that are required for the expression of *35S-NPTII*, we performed a forward genetic screen using an ethyl methanesulfonate (EMS)-mutagenized population of C24 transgenic plants carrying a T-DNA locus (used as the wild type). In the T-DNA locus, two transgenes, *RD29A-LUC* and *35S-NPTII*, are actively expressed for many generations without silencing (9). Seedlings carrying the T-DNA are able to grow on Murashige and Skoog (MS) medium containing more than 200 mg/L kanamycin (Kan). To identify mutants with reduced expression of *35S-NPTII*, we germinated M2 (germinated seeds of a mutagenized population) seeds on MS medium containing 50 mg/L Kan. After the seedlings had grown for 1 wk, Kan-sensitive plants were transferred to MS medium without Kan and allowed to recover. The putative mutants were confirmed on the Kan medium in the next generation. All of the identified mutants were determined to have lost Kan resistance when seeds were germinated and grown on MS medium supplemented with 50 mg/L Kan (Fig. 1A).

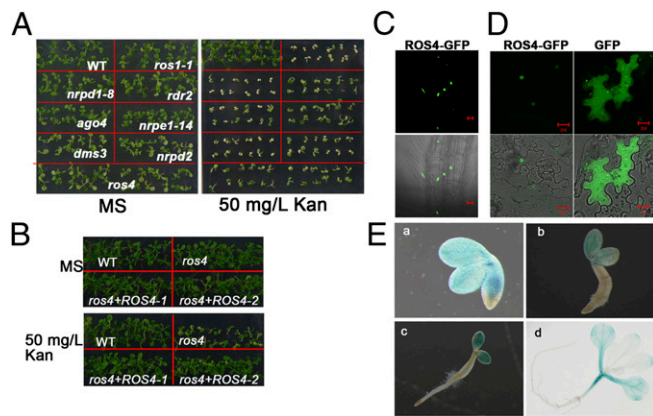
The mutants were crossed with Col-0, and the F2 (second generation from self-fertilized F1) plants that showed the Kan-sensitive phenotype and that carried the *NPTII* gene (as indicated by PCR) were used for map-based cloning. We identified 15 mutants that include three alleles of *ros1*, three alleles of *nripd1*, four alleles of *nrpe1*, two alleles of *nripd2*, and one allele each of *dms3*, *rdr2*, and *ago4* (Fig. S1). The *nripd1-8* (P438 mutated to L) and *nrpe1-14* (W1360 mutated to a stop codon) mutants were selected as the representative mutants in the RdDM pathway for further characterization. We also isolated a mutant, named *ros4*. *ros4* was created by a G-to-T mutation at

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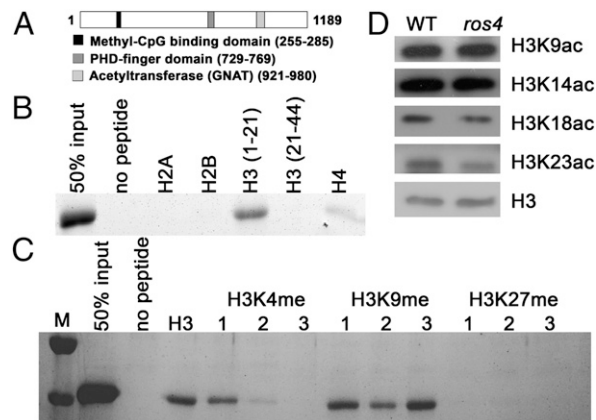
**Fig. 1.** Analysis of kanamycin sensitivity of *ros4* and other mutants and *ROS4* expression. (A) Kan sensitivity of *ros4* and RdDM pathway mutants. The seeds of *ros1-1*, *nprpd1-8*, *rdr2*, *ago4*, *nrpe1-14*, *dms3*, *nprpd2*, and *ros4* were germinated on MS medium or MS medium supplemented with 50 mg/L Kan for 7 d. (B) Complementation of the *ros4* mutant. A DNA fragment containing the *ROS4* promoter (−2499 to −1) driving *ROS4* full-length cDNA was transformed into the *ros4* mutant. Two independent lines were tested for Kan sensitivity on 50 mg/L Kan MS medium. (C) *ROS4*-GFP localization in a root of a transgenic line carrying *35S::ROS4-GFP*. (Upper) GFP fluorescence image. (Lower) GFP image combined with bright field. (Scale bars, 20  $\mu$ m.) (D) *ROS4*-GFP localization in a transient assay using tobacco leaves expressing *35S::ROS4-GFP* (Left). GFP was used as a control (Right). (Upper) GFP fluorescence image. (Lower) GFP image combined with bright field. (Scale bars, 20  $\mu$ m.) (E) The expression analysis of *ROS4* promoter::*GUS* in a transformed line at 12 h (a), 36 h (b), 48 h (c), and 14 d (d) after seed imbibition.

position 1351 of *AT3G14980*, which changes the amino acid E451 to a stop codon. The promoter (−2499 to −1) of *ROS4* driving *ROS4* full-length cDNA or *ROS4* full-length cDNA fused with *MYC* was able to complement the Kan-sensitive phenotype of *ros4* (Fig. 1B, showing only the *ROS4* promoter::*ROS4* cDNA).

***ROS4* Is a Nuclear Protein Expressed in Early Seedling Development.** For determination of subcellular localization, *ROS4* cDNA was amplified and fused with the N terminus of GFP and stably expressed in *Arabidopsis* or transiently expressed in tobacco epidermal cells under a *35S* promoter. The *35S::ROS4-GFP* transgene was able to complement the Kan-sensitive phenotype of *ros4*. Confocal microscopy showed that *ROS4*-GFP was localized in the nucleus when stably expressed in the root cells of transgenic *Arabidopsis* plants (Fig. 1C) or when transiently expressed in tobacco epidermal cells (Fig. 1D, Left). As controls, GFP alone was localized mainly in the cytoplasm (Fig. 1D, Right).

To determine the expression pattern of *ROS4*, we used transgenic plants carrying the *ROS4* promoter (−2499 to −1)::*GUS* ( $\beta$ -glucuronidase) fusion. *GUS* staining indicated that *ROS4* was expressed in cotyledons and hypocotyls during early seedling development, but the expression became less in true leaves and roots (Fig. 1E).

***ROS4* Encodes a Putative Histone Acetyltransferase with a PHD Domain That Preferentially Binds to Unmethylated Histone H3K4.** *ROS4* encodes a predicted polypeptide with 1,189 amino acids that has a high similarity to acyl-CoA *N*-acetyltransferases (GNAT family). There is a putative PHD-finger domain around residues 729–769, a putative methyl-CpG-binding domain around amino acids 255–285, and an acyl-CoA *N*-acetyltransferase domain around amino acids 921–980 (Fig. 2A). Previous studies have shown that PHD domain-containing proteins preferentially bind to H3K4me2/3 in *Arabidopsis* (16) and animals (17) or to H3K4me0 in animals (18, 19). Because many studies have shown that the PHD domain is responsible for histone binding (20), we expressed the PHD domain (amino acids 723–769) of *ROS4* in *Escherichia coli* and conducted a peptide pull-down assay with different combinations of histones. The PHD fragment bound

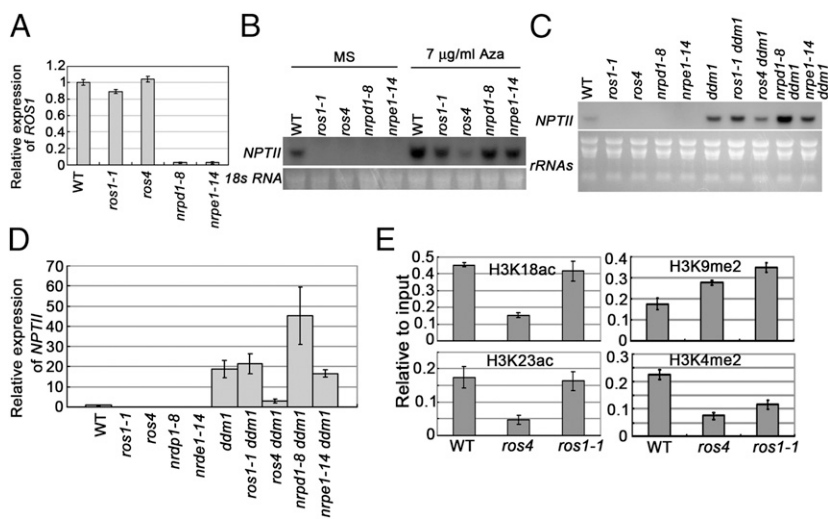


**Fig. 2.** PHD domain of *ROS4* binds to H3K4me0, and the mutation in *ROS4* leads to reduction of acetylation of H3K18 and H3K23. (A) Structural diagram of the *ROS4* protein. (B) In vitro assays of the binding of the recombinant PHD domain to unmodified histone peptides. H3 was divided into two fragments, H3 (1–21) and H3 (21–44). Note that the PHD domain also weakly binds to H4. (C) Effect of histone methylation on the binding of the PHD domain to the H3 tail. At least three independent experiments were done with similar results. (D) H3K9ac, H3K14ac, H3K18ac, and H3K23ac levels in the wild type and *ros4*. Total histone proteins were extracted from 5- to 7-d-old seedlings, and different antibodies were used for Western blotting; H3 was used as a loading control. M, size markers.

strongly to the H3 N-terminal tail (amino acids 1–21) without any modifications and bound very weakly to H4, but did not bind to H3 (amino acids 21–44), H2A, or H2B (Fig. 2B). The binding between the PHD domain of *ROS4* and H3 (amino acids 1–21) was substantially reduced by H3K4me2/3 and somewhat reduced by H3K4me1, but was not reduced substantially by H3K9 methylation. The fragment also did not bind to H3K27me1/2/3 (H3, amino acids 21–44) (Fig. 2C). These results indicate that, in contrast to the PHD fingers of AtING and Alfin-like proteins that were previously characterized in *Arabidopsis* (16) and bind to H3K4me2/3, the PHD finger of *ROS4* preferentially binds to unmethylated H3K4 (H3K4me0).

Because *ROS4* is a putative acetyltransferase that binds to H3K4me0, we explored whether H3 acetylation is affected by *ros4* mutation by performing Western blot analysis with different H3 acetylation antibodies. Because *ROS4* is mainly expressed early in seedling development, we extracted proteins from 5- to 7-d-old seedlings. Western blot analysis indicated that, relative to the wild type, H3K23ac was clearly reduced, H3K18ac was weakly reduced, and H3K9ac and H3K14ac were not changed in *ros4* (Fig. 2D).

**RdDM Pathway Mutations Lead to Silencing of *35S-NPTII* Due to a Lack of Expression of *ROS1*.** Previous studies showed that the expression of *ROS1* is regulated by the RdDM pathway (10). We confirmed that the expression of *ROS1* was greatly reduced by *nprpd1* and *nrpe1* mutations (Fig. 3A). In *ros4*, *ROS1* expression was not changed (Fig. 3A). Given that the RdDM pathway is known to silence the expression of target genes through siRNA-guided DNA methylation, mutations in RdDM pathway components should release rather than lead to transcriptional gene silencing (TGS) if *35S-NPTII* is directly regulated by the RdDM pathway. We suspected that the TGS of *35S-NPTII* in RdDM mutants might be due to down-regulation of *ROS1*. To test this hypothesis, we expressed *ROS1* cDNA under the constitutive *35S* promoter in *ros1*, *nprpd1*, *nrpe1*, and *ros4* mutants and found that the transgenic plants became resistant to Kan in *ros1*, *nprpd1*, and *nrpe1* (Fig. S2) but not in *ros4* (24 independent transgenic lines were tested). However, *ROS1* cDNA driven by its native promoter was able to complement the *ros1-1* mutant but was unable to recover the Kan-resistant phenotype in *nprpd1* (63 lines tested) or *nrpe1* (24 lines tested). These results indicate that *NRPD1* and *NRPE1*, but not *ROS4*, regulate the expression of *ROS1* and that



**Fig. 3.** ROS4 suppresses DNA methylation-mediated transcriptional gene silencing of *35S-NPTII*. (A) Quantitative RT-PCR analysis of *ROS1* expression in different mutants. Three independent experiments (each with three biological replicates) were done with similar results. (B) *NPTII* expression as indicated by Northern analysis in *ros4* and other mutants treated or not treated with 7  $\mu\text{g}/\text{mL}$  5-aza-cytidine. 18S RNA was used as the loading control. (C) The effect of the *ros4* mutation on *ddm1*-promoted *NPTII* expression as determined by comparing single and double mutants. (D) Quantitative RT-PCR assays of *NPTII* transcripts in different single and double mutants. Three independent experiments (each with three biological replicates) were done with similar results. (E) ChIP assay of H3K18ac, H3K23ac, H3K9me2, and H3K4me2 in the wild type, *ros4*, and *ros1-1*. Seven-day-old seedlings were used for ChIP assay with antibodies against H3K18ac, H3K23ac, H3K9me2, or H3K4me2. Three independent experiments (each with three biological replicates) were done with similar results. In A, D and E, error bars refer to standard error ( $n = 3$ ).

the silencing of *35S-NPTII* in *nrdp1* and *nrpe1* mutants is the result of the down-regulation of *ROS1*.

**ROS4 Is Required for the Expression of *35S-NPTII* Even When DNA Methylation Is Inhibited.** We next determined whether the TGS of *35S-NPTII* is regulated in a similar manner by *ROS1* and *ROS4*. When 7  $\mu\text{g}/\text{mL}$  of 5-aza-2'-deoxycytidine (Aza), a DNA methylation enzyme inhibitor, was combined with 50 mg/L Kan in the medium, Kan resistance was recovered in all four mutants (Fig. S3), indicating that the silencing of *35S-NPTII* is caused by DNA methylation. Northern blot analyses did not detect *NPTII* expression in any of the Kan-sensitive mutants, but detected *NPTII* expression in the wild type (Fig. 3B and C). Addition of 5-aza-cytidine increased the expression of *NPTII* in the wild type and in all mutants (Fig. 3B). Interestingly, expression of *NPTII* induced by 5-aza-cytidine treatment was much lower in *ros4* than in the wild type, *ros1*, *nrdp1*, or *nrpe1* (Fig. 3B). This result suggests that *ROS4* but not *ROS1* is required for the expression of *NPTII* that is released from silencing by 5-aza-cytidine. Furthermore, we used *ddm1*, a DNA hypomethylation mutant (in C24 ecotype, identified in another screen) (21), to compare the effects of mutations in *ROS1* or *ROS4* on *NPTII* expression by Northern blot and quantitative (q)RT-PCR analyses. *NPTII* transcripts were more abundant in *ddm1* than in the wild type, indicating that the TGS of *35S-NPTII* could be released by the mutation in *DDM1* (Fig. 3C and D). The expression of *NPTII* in *ddm1* was not affected by the *ros1* mutation but was greatly reduced by the *ros4* mutation, indicating that the *ros4* mutation inhibits *ddm1*-promoted expression of *NPTII*. We also found that the expression of *NPTII* in *ddm1* was enhanced by *nrdp1* but not by *nrpe1* (Fig. 3C and D), suggesting that *NRPD1* and *NRPE1* play different roles in regulating the DNA methylation and/or chromatin structure of the *35S* promoter.

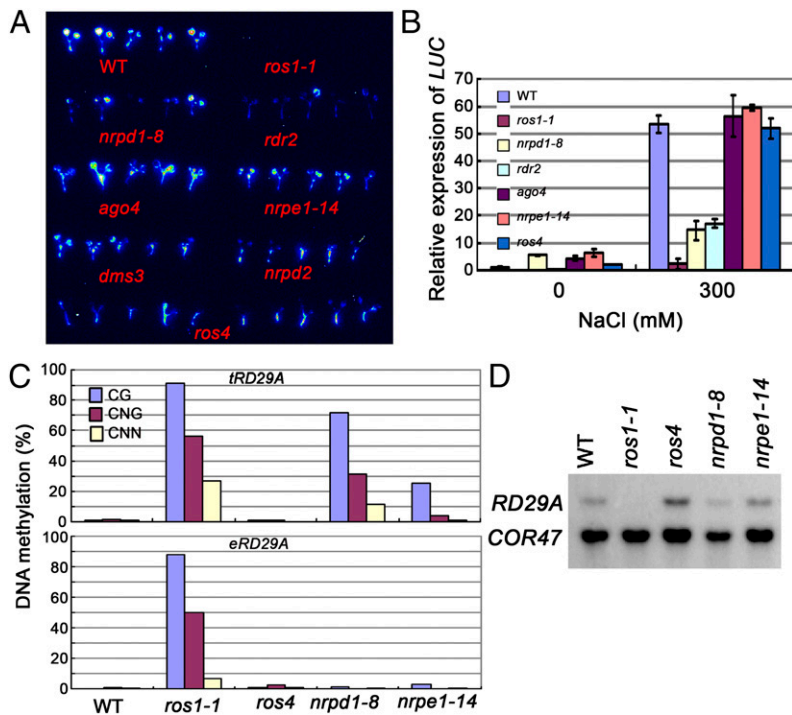
Next, we used the chromatin immunoprecipitation (ChIP) assay and different antibodies to analyze the histone H3 modifications in the *35S* promoter. We included *ros1-1* as a control. Previous studies indicated that H3K9me was increased and H3K4me was reduced in the *ros1* mutant (22, 23). As shown in Fig. 3E, both H3K18ac and H3K23ac levels were decreased in *ros4* relative to the wild type, but *ros1-1* did not show a clear change in the levels of H3K18ac and H3K23ac. Both *ros4* and *ros1-1* had a higher level of H3K9 dimethylation and a lower level of H3K4 dimethylation than the wild type. These results further support *ROS4* as important for histone H3K18 and H3K23 acetylation in vivo and suggest that *ROS1* may function either downstream of *ROS4* or independently of *ROS4* in the silencing of *35S-NPTII*. Our results also suggest that *ROS4* is critical for the expression of the *35S-NPTII* transgene that is normally silenced by DNA methylation, even when the DNA methylation is inhibited by 5-aza-cytidine treatment or by the *ddm1* mutation.

**Effect of *ROS4* and RdDM Factors on the Expression of *RD29A-LUC* or Endogenous *RD29A*.** Because *ROS1* is required for the expression of *RD29A-LUC* as well as *35S-NPTII*, we tested whether *RD29A-LUC* is silenced by *ros4* or the RdDM mutations. As shown previously (9) and confirmed here, *ros1-1* emitted undetectable luminescence (Fig. 4A). *nrdp1-8* and *rdr2* emitted less luminescence than *nrpe1-14*, *ago4*, *dms3*, and *nrdp2*, and the latter four emitted similar luminescence as the wild type. *ros4* mutation did not change the luminescence level. The expression of *RD29A-LUC* as indicated by luminescence was confirmed by qRT-PCR (Fig. 4B).

We measured the DNA methylation by bisulfite sequencing. In both the wild type and *ros4*, no DNA methylation could be detected in the *RD29A-LUC* transgene (*tRD29A*) promoter (Fig. 4C). However, the DNA methylation was increased to a higher level in *nrdp1-8* and *nrpe1-14* than in the wild type. DNA methylation was higher in *nrdp1-8* than in *nrpe1-14*, and was lower in both *nrdp1-8* and *nrpe1-14* than in *ros1-1*. These results indicate that *NRPD1* and *NRPE1* have different effects on *RD29A-LUC* and suggest that *RD29A-LUC* may also be regulated by an RdDM-independent pathway. The increased DNA methylation in *nrdp1-8* and *nrpe1-14* is probably due to down-regulation of *ROS1*, which leads to severe DNA hypermethylation, and part of this may be independent of the RdDM pathway and not suppressed by the *nrdp1* and *nrpe1* mutations.

DNA methylation of the endogenous *RD29A* (*eRD29A*) promoter was not detected in *nrdp1-8*, *nrpe1-14*, or *ros4* but was detected at a high level in *ros1-1* (Fig. 4C) (9). The expression of *RD29A* was consistently silenced in *ros1-1* but not in *ros4*, *nrdp1-8*, or *nrpe1-14* (Fig. 4D).

***ROS4* Is Important for *ddm1*-Promoted Expression of some Transposable Element Genes.** Because the *ros4* mutation led to reduction of the *ddm1*-promoted expression of *35S-NPTII*, we hypothesized that the expression of *ROS4*-targeted endogenous genes would be lower in the *ros4 ddm1* double mutant than in the *ddm1* single mutant. We compared the expression profiles among the wild type, *ddm1*, *ros4 ddm1*, and *ros4* by a microarray assay. We chose genes whose expression was reduced in *ros4*, was increased in *ddm1*, and was lower in *ddm1 ros4* than in *ddm1*. We selected six genes from our preliminary microarray data and checked their expression by qRT-PCR (Fig. 5A). Among them, the expression of three transposable element genes, *AT3G47330*, *AT4G02960*, and *AT4G28960*, was highly induced by the *ddm1* mutation, but the expression was attenuated when the *ddm1* mutation was combined with the *ros4* mutation. Because the expression of each is already silenced in the wild type, further reduced expression by *ros4* could not be detected. The expression of *AT5G03090*, *AT3G20810*, and *AT3G02550* was consistent with the microarray data, that is, these genes were expressed at higher levels in *ddm1* than in *ddm1 ros4* and at lower levels in *ros4* than in the wild type. Our results suggest

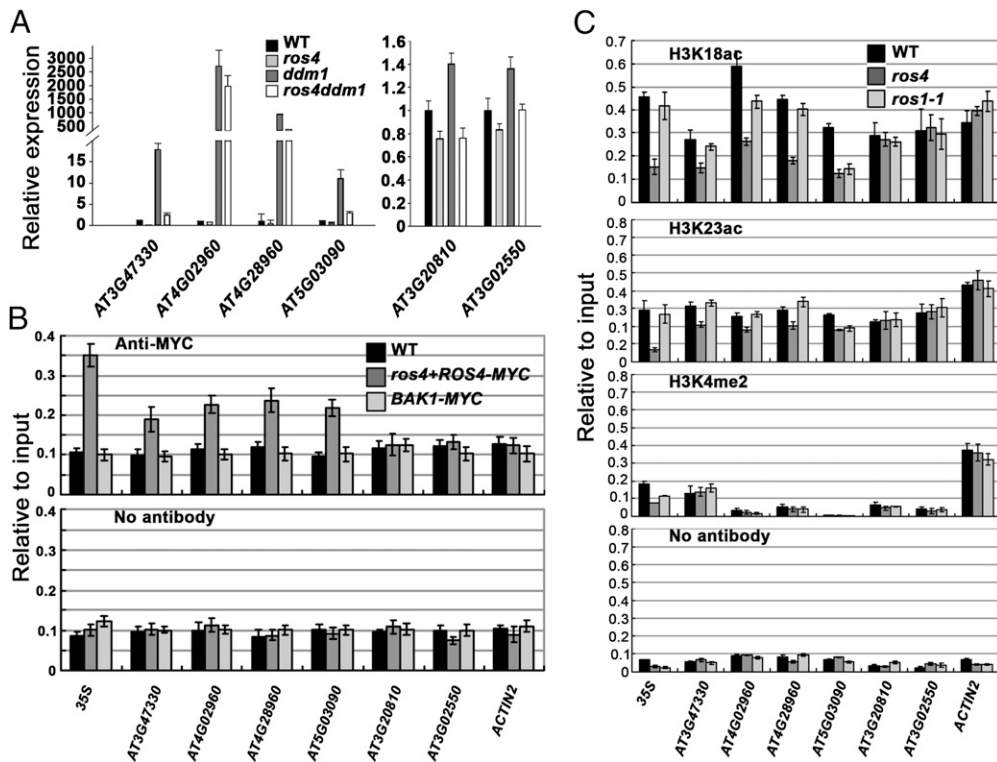


**Fig. 4.** ROS4 does not regulate the expression of *RD29A-LUC* or of the endogenous *RD29A* gene. (A) Luminescence images of different mutants. Ten-day-old seedlings were treated with 300 mM NaCl for 3.5 h before luminescence images were obtained. (B) Quantitative RT-PCR analyses of *LUC* transcripts in different mutants. Three independent experiments (each with three biological replicates) were done with similar results. Error bars refer to standard error ( $n = 3$ ). (C) DNA methylation analyses of the transgene *RD29A* (*tRD29A*) and the endogenous *RD29A* (*eRD29A*) promoter by bisulfite sequencing in the wild type, *ros1-1*, *ros4*, *nrpd1-8*, and *nrpe1-14*. (D) The expression of the endogenous *RD29A* gene in the wild type, *ros1-1*, *ros4*, *nrpd1-8*, and *nrpe1-14* under 300 mM NaCl treatment. The stress-inducible gene *COR47* was used as a control for effective stress treatments.

that ROS4 is critical for the expression of some endogenous genes that are normally silenced by DNA methylation, even when the DNA methylation is inhibited by the *ddm1* mutation.

**ROS4 Targets 35S-NPTII and Three Transposable Element Genes.** To determine whether ROS4 directly targets *35S-NPTII* and the other six genes, we used a ChIP assay. We transformed the *ros4* mutant with the *ROS4* promoter::*ROS4-Myc* and obtained transgenic

plants in which the Kan-sensitive phenotype of *ros4* was complemented. Because ROS4 can be detected in early seedling development, we used 5- to 7-d-old seedlings for our assays. ChIP assays using Myc antibody indicated that ROS4-Myc, but not BAK1-Myc (BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1, as an unrelated control protein), was enriched in the *35S* promoter, *AT3G47330*, *AT4G02960*, *AT4G28960*, and *AT5G03090*, but not in *AT3G20810*, *AT3G02550*, or *ACTIN2*



**Fig. 5.** ROS4 is important for the *ddm1*-promoted expression of endogenous genes. (A) The expression of six selected genes as determined by qRT-PCR in the wild type, *ros4*, *ddm1*, and *ros4 ddm1*. At least three independent experiments (each with three biological replicates) were done with similar results. (B) ROS4-Myc binds to the *35S* promoter and the promoters of four endogenous genes as indicated by ChIP assay. The ChIP assay was performed on 7-d-old seedlings of the *ros4* mutant complemented by the *ROS4* promoter::*ROS4-Myc*, the wild type, and a transgenic plant expressing BAK1-Myc. At least three independent experiments (each with three biological replicates) were done with similar results. (C) H3K18ac, H3K23ac, and H3K4me2 levels in the wild type, *ros4*, and *ros1-1*. The ChIP assay used 7-d-old seedlings and H3K18ac, H3K23ac, or H3K4me3 antibodies. No antibody was used as a negative control. In A–C, error bars refer to standard error ( $n = 3$ ).

(Fig. 5B), suggesting that the latter three genes are not targeted by ROS4. Although the expression of *AT3G20810* and *AT3G02550* was increased by *ddm1* and repressed when *ddm1* was combined with *ros4*, it is unlikely that ROS4 directly targets these two genes.

We then used antibodies for H3K18ac and H3K23ac in the ChIP assay, and found that H3K18ac and H3K23ac in the promoter regions of *AT3G47330*, *AT4G02960*, *AT4G28960*, and *AT5G03090* were apparently reduced in *ros4* relative to the wild type (Fig. 5C). H3K18ac and H3K23ac in the promoter regions of *AT3G20810*, *AT3G02550*, and *ACTIN2* were not changed by the *ros4* mutation. The mutation in *ROS1* only resulted in the reduction of H3K18ac and H3K23ac in the promoter of *AT5G03090*, but not in other promoter regions. Consistent with the results in Fig. 3E, H3K18ac and H3K23ac of the 35S promoter were greatly reduced in *ros4* relative to the wild type, but this was not the case in *ros1-1* (Fig. 5C). H3K4me2 levels were very low in the 35S promoter and the five selected genes; as a control, the *ACTIN2* promoter had a high level of H3K4me2. These results indicate that besides affecting the 35S promoter, ROS4 directly affects histone acetylation of some endogenous genes.

## Discussion

DNA methylation and histone modifications are crucial for regulating chromatin structure and gene transcription (3). DNA methylation tends to increase heterochromatin status and silence genes. Active DNA demethylation is an important mechanism for maintaining gene activity in both plants and animals (24). In this study, we characterized a PHD finger-containing protein that binds to H3K4me0 and suppresses gene silencing caused by DNA methylation. We showed that the expression of 35S-*NPTII* can be enhanced by 5-aza-cytidine treatment and by the *ddm1* mutation in the wild-type C24, indicating that the 35S promoter is already silenced in C24 wild-type plants by DNA methylation, although this silencing is not complete because there is still considerable expression of the transgene. Both ROS1 and ROS4 are needed to maintain the expression of 35S-*NPTII* in the wild type (Fig. S4). ROS1 and ROS4 may function in the same pathway. Because ROS4 does not control the *RD29A-LUC* transgene and ROS4-dependent H3K18 and H3K23 acetylation are not affected by ROS1, ROS4 may regulate the activity or targeting of ROS1 to a subset of loci subjected to active DNA demethylation. The histone acetylation activity of ROS4 may be important for creating a chromatin environment necessary for ROS1 to function. However, ROS4 may have additional functions in promoting the expression of some genes. This is indicated by our observation that when 5-aza-cytidine treatment or the *ddm1* mutation led to up-regulation of the expression of *NPTII* and some endogenous genes, the expression was reduced by the *ros4* mutation but not by the *ros1* mutation. It is also possible that ROS4 and ROS1 may prevent the silencing of 35S-*NPTII* and other genes via different mechanisms. During the preparation of this manuscript, Qian et al. (25) reported that ROS4/IDM1 recognizes methyl-CG, unmethylated H3K4, and unmethylated H3R2 marks on a subset of target loci of active DNA demethylation, and generates acetylated H3K18 and H3K23 marks, which are important for the DNA demethylation and attenuation of silencing of the recognized loci.

The T-DNA locus used for the genetic screening contains 35S-*NPTII* and *RD29A-LUC*. Previous studies indicated that the expression of these two genes is regulated by different epigenetic mechanisms (7). In this study, we found that both 35S-*NPTII* and *RD29A-LUC* are not regulated by one simple mechanism. We used Kan as a selection marker to identify the genes that positively regulate the expression of *NPTII*. Strikingly, most of the identified genes are components in the RdDM pathway. Our results revealed that the down-regulation of *ROS1* by mutations in these RdDM components was the cause of 35S-*NPTII* silencing (10).

It seems that NRPD1 and NRPE1 play similar roles in controlling 35S-*NPTII* due to their regulation of *ROS1*, because both mutations led to the silencing of 35S-*NPTII*. In the *ddm1* mutant background, however, *nrip1* but not *nripe1* appeared to be important in negatively regulating 35S-*NPTII*: *NPTII* transcripts were more abundant in the *nrip1-8 ddm1* double mutant than in the *ddm1* single mutant or the *nripe1-14 ddm1* double mutant. It is

possible that in addition to indirectly controlling 35S-*NPTII* via *ROS1*, Pol IV may also directly regulate this locus by generating silencing RNAs. The differing roles of Pol IV and Pol V are further suggested by our observation that *nrip1-8* and *nripe1-14* exhibited different levels of *RD29A-LUC* expression and different levels of transgene *RD29A* promoter DNA methylation. Given that Pol IV is required for siRNA biogenesis and Pol V transcripts are needed for the siRNAs to cause DNA methylation at RdDM target loci, it is possible that other transcripts from RNA polymerases such as Pol II might substitute for Pol V transcripts in the RdDM pathway (26).

Histone hyperacetylation is a major characteristic of actively transcribed genes, whereas histone hypoacetylation is a hallmark of transcriptionally inactive genes (27). In *Arabidopsis*, there are four histone acetyltransferase (HAT) families: GNAT (Gcn5-related N-acetyltransferase), MYST (MOZ, Ybt2, Sas2, and Tip60-like), p300/CBP, and TAF<sub>II</sub>250. *Arabidopsis* also has three histone deacetyltransferase (HDAC) families: RPD3/HDA1, HD2, and SIR2 (27). Previous studies have identified HDA6, a histone deacetylase that removes acetyl groups from the histone tail, as a key regulator that is not required for RNA-directed de novo methylation but is required for maintaining TGS by enhancing DNA methylation (28–30). HDA6 also regulates the TGS of both 35S-*NPTII* and *RD29A-LUC* (11). HDA6 is responsible for removing acetyl groups from H3K14, H4K5, and H4K12 (31). HD1, another plant-specific histone deacetylase, is required for H3K9 deacetylation (32). Histone H3 (H3K9, H3K14) and H4 (H4K5, H4K8, H4K12, H4K16) acetylations are catalyzed by different HATs (33). *ROS4* encodes a putative histone acetyltransferase that shows high similarity in the acetyltransferase domain to GNAT families (27). *ROS4* is a single-copy gene in *Arabidopsis*, and its orthologs are found in both dicot and monocot plants, but not in animals. The *ros4* mutation specifically causes a reduction in the acetylation of H3K18 and H3K23 but not of H3K9 and H3K14, suggesting that ROS4 might mainly acetylate H3K18 and H3K23 in vivo. We have also provided evidence that H3K18ac and H3K23ac are reduced by the *ros4* mutation in the 35S promoter and in the promoters of four endogenous target genes. Because H3K18 and H3K23 acetylation are only reduced but not completely absent in *ros4*, we suspect that other histone acetyltransferases might work redundantly with ROS4 in acetylating H3K18 and H3K23. ROS4 appears to preferentially associate with chromatin lacking methylated H3K4, suggesting that unmethylated H3K4 is a common epigenetic feature for ROS4 target loci.

## Materials and Methods

Seeds were sterilized and sown onto MS medium plates containing 2% (wt/vol) sucrose and 0.8% (wt/vol) agar. After 4 d at 4 °C, the plates were transferred to a growth chamber at 22 °C under long-day (23-h light/1-h dark) conditions. Seven-day-old seedlings were transferred to soil and kept in a growth room at 20 °C under 16-h light/8-h dark.

C24 carrying transgenes *RD29A-LUC* and 35S-*NPTII* (9) was used as the wild type. Except for the *ddm1* mutant, which was described before (21), all other mutants in this study were screened from the EMS-mutagenized population of the wild type. Wild-type seeds can germinate and grow on MS medium containing 50 mg/L kanamycin. M2 seeds were sown on MS medium containing 50 mg/L kanamycin. After seeds germinated and grew in a growth chamber for 1 wk, the kanamycin-sensitive seedlings were transferred to MS medium without kanamycin to recover. The putative mutants were confirmed on 50 mg/L kanamycin medium in the next generation and then backcrossed with the C24 wild type for genetics analysis. If F1 seedlings exhibited the C24 phenotypes and F2 progeny from self-fertilized F1 plants showed an approximate 3:1 segregation of kanamycin-resistant to -sensitive phenotypes, the mutants were selected and crossed with Col-0. The F2 plants that showed kanamycin sensitivity and also carried the *NPTII* gene (as indicated by PCR) were used for mapping. Map-based cloning was performed as described (23).

For the complementation assay, the region from -2499 to -1 of *ROS4* and the *ROS4* full-length cDNA-fused MYC tag were in-sequence-cloned into pCAMBIA1300 (relevant primers are listed in Table S1). Then, *Agrobacterium tumefaciens* strain GV3101 carrying the constructs was transformed into *ros4*. The vector containing 35S-*ROS1*, which was described before (9), was used to transform other mutants. Transgenic plants that were resistant to both 30 mg/L hygromycin and 50 mg/L kanamycin in the T2 (second generation of transgenic plants) generation were selected. Two independent lines were used for further study.

**RNA Analysis.** Real-time PCR was carried out to detect transcription levels of different genes. Total RNA was extracted from 5-d-old seedlings using TRIzol reagent (Invitrogen). After the RNA was incubated with RNase-free DNase I (New England Biolabs) at 37 °C to remove remaining DNA, 4 µg of RNA was reverse-transcribed to cDNA with M-MLV reverse transcriptase (Promega). The cDNAs were then diluted 10-fold, and 1 µL of the dilution was added to each 20-µL reaction system in SYBR Green Master Mix (TaKaRa). The PCR procedure was performed as described (34). Specific primers of each gene are listed in Table S1. EF1a was used as an internal control.

For Northern blotting, 15 µg of total RNA of each sample was used for detecting NPTII expression following the method previously described (23), except that 5-d-old seedlings were used. Total RNA used in *RD29A* and *COR47* expression assays was extracted after the seedlings were treated in 300 mM NaCl for 3.5 h. Primers are listed in Table S1.

**Bisulfite Sequencing.** The EZ Methylation-Gold Kit (Zymo Research) was used for DNA methylation analysis. A 500-ng quantity of DNA was added in each reaction system, and all operations followed the protocol supplied with the kit. Approximately 75 ng of bisulfite-treated DNA was used in the PCR, which was carried out with primers that were specific for each region (Table S1). PCR products were cloned using the pMD18-T vector (TaKaRa), and at least eight independent clones of each sample were sequenced.

**Histone Peptide-Binding Assay.** The following biotinylated histone peptides were purchased from Millipore and used in this binding assay: H2A (residues 12–406), H2B (12–407), H3 (1–21) (12–403), H3 (21–44) (12–404), H3K4me1 (12–563), H3K4me2 (12–460), H3K4me3 (12–564), H3K9me1 (12–569), H3K9me2 (12–430), H3K9me3 (12–568), H3K27me1 (12–567), H3K27me2 (12–566), H3K27me3 (12–565), and H4 (12–372). The PHD domain (residues 723–769) was cloned into the vector pGEX4T-2, expressed in *E. coli*, and purified by the glutathione Sepharose TM 4B column (GE Healthcare; 17-0756-01). Briefly, 0.5 µg of the peptides was incubated overnight at 4 °C with 5 µg of the GST-fused PHD-finger protein in a binding buffer [20 mM Hepes, pH 7.0, 250 mM NaCl, 0.1% (vol/vol) Triton X-100, 1 mM phenylmethylsulfonyl fluoride]. Streptavidin beads (Millipore) were then added and incubated for 1 h. After they were washed three times, the beads bound with proteins were analyzed by SDS/PAGE and stained with Coomassie blue.

**Chromatin Immunoprecipitation Assay.** ChIP was performed as described previously (35) using 5-d-old seedlings grown in a growth chamber at 22 °C under 23-h light/1-h dark conditions. The antibodies used in the ChIP assays were anti-H3K4me2 (Millipore; 17-677), anti-H3K9me2 (Millipore; 17-648), anti-H3K18ac (Millipore; 07-354), anti-H3K23ac (Millipore; 07-355), and anti-MYC (Sigma; F3165). Finally, ChIP products were dissolved in 50 µL of water, and 0.5 µL of the solution was used in each qPCR reaction. Specific primers are listed in Table S1.

**Histone Extraction and Western Blotting.** Seedlings were ground to powder in a mortar cooled with liquid nitrogen, and the powder was suspended in NIB buffer (250 mM sucrose, 60 mM KCl, 15 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 15 mM Pipes, pH 6.8, 0.8% Triton X-100). The suspension was centrifuged, and the pellet was resuspended in 0.4 M H<sub>2</sub>SO<sub>4</sub> and incubated for at least 1 h at 4 °C. The preparation was centrifuged again before acetone was added to precipitate the histone protein. The preparation was kept at –20 °C overnight before the proteins were dissolved in 4 M urea and analyzed by Western blotting. The antibodies used in the Western blotting were anti-H3K9ac (Millipore; 07-352), anti-H3K14ac (Millipore; 07-353), anti-H3K18ac (Millipore; 07-354), and anti-H3K23ac (Millipore; 07-355).

**Histochemical GUS Staining.** A DNA fragment containing the region from –2499 to –1 of *ROS4* was cloned into the pCambia1391 vector. The recombinant plasmid was introduced into *A. tumefaciens* strain GV3101, followed by transformation into C24 wild-type plants. Histochemical GUS staining was performed on transgenic T2 plants that were resistant to hygromycin as previously described (23).

**Localization of the ROS4-GFP Fusion Protein.** The full-length cDNA of *ROS4* without TAA (encoding a stop codon) was cloned into the modified vector pCambia1300 at the site just behind the 35S promoter. Then GFP was fused in-frame with *ROS4* at its C terminus. Transient expression in tobacco and GFP image acquisition were described previously (34).

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