Regulatory link between DNA methylation and active demethylation in Arabidopsis

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De novo DNA methylation through the RNA-directed DNA methylation (RdDM) pathway and active DNA demethylation play important roles in controlling genome-wide DNA methylation patterns in plants. Little is known about how cells manage the balance between DNA methylation and active demethylation activities. Here, we report the identification of a unique RdDM target sequence, where DNA methylation is required for maintaining proper active DNA demethylation of the Arabidopsis genome. In a genetic screen for cellular antisilencing factors, we isolated several REPRESSOR OF SILENCING 1 (ros1) mutant alleles, as well as many RdDM mutants, which showed drastically reduced ROS1 gene expression and, consequently, transcriptional silencing of two reporter genes. A helitron transposon element (TE) in the ROS1 gene promoter negatively controls ROS1 expression, whereas DNA methylation of an RdDM target sequence between ROS1 5′ UTR and the promoter TE region antagonizes this helitron TE in regulating ROS1 expression. This RdDM target sequence is also targeted by ROS1, and defective DNA demethylation in loss-of-function ros1 mutant alleles causes DNA hypermethylation of this sequence and consequently causes increased ROS1 expression. Our results suggest that this sequence in the ROS1 promoter region serves as a DNA methylation monitoring sequence (MEMS) that senses DNA methylation and active DNA demethylation activities. Therefore, the ROS1 promoter functions like a thermostat (i.e., methylstat) to sense DNA methylation levels and regulates DNA methylation by controlling ROS1 expression.

DNA demethylase | ROS1 | methylstat | sensor | rheostat

DNA methylation is a conserved epigenetic mark important for development and stress responses in plants and many animals (1–4). Genome-wide DNA methylation patterns are dynamically regulated by establishment, maintenance, and removal activities (4, 5). In plants, de novo DNA methylation is controlled by the RNA-directed DNA methylation (RdDM) pathway, in which complementary pairing between long non-coding RNAs and siRNAs mediates cytosine methylation in a sequence-specific manner (2, 4, 6, 7). Best characterized in Arabidopsis, RdDM involves a complex array of regulators and mainly targets heterochromatic regions that are enriched with transposon elements (TEs) and other DNA repeat sequences (8, 9). Once established, Arabidopsis DNA methylation is maintained via different mechanisms depending on the cytosine contexts [i.e., CG, CHG, CHH (H represents A, T, or C)]. CG and CHG methylation is maintained by MET1 and CMT3, respectively (2), whereas CHH methylation within pericentromeric long TEs can be catalyzed by CMT2 and CHH methylation at other loci is established de novo by DRM2 during every cell cycle (2, 10). In contrast to DNA methyltransferases that establish and/or maintain cytosine methylation, plant 5-methylcytosine DNA glycosylases initiate a base excision repair pathway that erases DNA methylation, thereby generating, together with the establishment and maintenance activities, a dynamic landscape of DNA methylation (11, 12).

The Arabidopsis REPRESSOR OF SILENCING 1 (ROS1) is a major DNA demethylase that prunes DNA methylation for dynamic transcriptional regulation (12). ROS1 counteracts the RdDM pathway to prevent DNA hypermethylation (5, 12–14). Interestingly, ROS1 gene expression is suppressed in mutants defective in RdDM (14–17) or MET1 (16), suggesting that DNA methylation and active demethylation activities are coordinated. However, the mechanism underlying such coordination remains elusive. Here, we report the identification of a regulatory element at the ROS1 promoter that monitors DNA methylation levels and accordingly modulates active DNA demethylation of the Arabidopsis genome by controlling ROS1 expression. In a genetic screen for Arabidopsis mutants that are defective in transcriptional antisilencing of transgenic reporter genes, we isolated several ros1 mutant alleles and many RdDM mutants with drastically repressed ROS1 gene expression. We found that a helitron TE in the ROS1 gene promoter negatively regulates ROS1 expression, and that such negative regulation is antagonized by RdDM activities. RdDM of a sequence between ROS1 5′ UTR and the promoter TE positively correlates with ROS1 gene expression. In addition, defective active DNA demethylation resulted in hypermethylation of this sequence, concomitant with enhanced ROS1 gene expression. ChiP analysis of ROS1 occupancy at this region confirmed the existence of coregulation between DNA methylation and active demethylation. These results provide important insights into how cells sense the balance between DNA methylation and demethylation, and fine-tune active DNA demethylation accordingly.

Significance

DNA methylation is critical for transposon silencing and gene regulation. DNA methylation levels are determined by the combined activities of DNA methyltransferases and demethylases. This study found a 39-bp DNA methylation monitoring sequence (MEMS) in the promoter of the DNA demethylase REPRESSOR OF SILENCING 1 (ROS1) gene of Arabidopsis plants. DNA methylation of MEMS is responsive to both RNA-directed DNA methylation and ROS1-dependent active demethylation. Thus, MEMS can sense DNA methylation and demethylation activities, and it regulates genomic DNA methylation by adjusting ROS1 expression levels. Our results suggest that the ROS1 promoter, with the MEMS and an adjacent helitron transposon element, functions as a “methylstat” that senses and regulates genomic DNA methylation levels.


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The authors declare no conflict of interest.

Data deposition: The high-throughput sequencing data generated in this study have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE53031, GSE64499, and GSE58790). An additional dataset used in this study is GEO accession no. GSE44209.

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Results and Discussion

Mutations in ROS1 and Its Regulator Increased DNA Methylation 1 Cause the Silencing of Cauliflower Mosaic Virus 35S Promoter::Sucrose Transporter 2 and 2 × Cauliflower Mosaic Virus 35S Promoter::Hygromycin Phosphotransferase II Transgenes. We previously reported an efficient transgene-based genetic screen for cellular antisilencing factors in Arabidopsis (18, 19). In this system, a sucrose transporter 2 (SUC2) gene was driven by the constitutive cauliflower mosaic virus 35S promoter (35S) and hygromycin phosphotransferase II (HPTII) gene was driven by the 2 × 35S promoter in Arabidopsis (Fig. 1A). When grown on sucrose-containing nutrient media, the transgenic plants (thereafter referred as WT) absorb too much sucrose from the medium, resulting in root growth inhibition (20). In this genetic screen, we obtained seven new alleles of ROS1 (Fig. 1B and Fig. S1). Quantitative RT-PCR analysis showed that the transcript levels of SUC2 and HPTII were drastically reduced in ros1 mutant plants (Fig. 1C and D). Bisulfite sequencing showed that the 35S promoter had higher levels of DNA methylation in ros1 than in the WT, especially in region A, although the promoter was already methylated in the WT (Fig. 1E). We also recovered eight new alleles of increased DNA methylation 1 (IDM1) (Fig. 1B and Fig. S1), a gene necessary for ROS1 function in active DNA demethylation, by creating a favorable chromatin environment (21). The results show that active DNA demethylation is required for preventing transcriptional silencing of the reporter genes. The large number of new ros1 and idm1 mutant alleles provides valuable information regarding structure–function relationships for these two important epigenetic regulators.

RdDM Mutations Cause the Silencing of 35S::SUC2 and 2 × 35S::HPTII Transgenes. Our genetic screen also recovered many RdDM mutants, including six new alleles of nrpd1-12, 2 nrpe1-17, 2 nrpd2-5, dcl3-1, dfl1-1, ago4-1, dmr2-2, ddr1-1, 4 kifs1, 2 idn1-1, and 3 idn2 (Fig. 2A and Fig. S3A). In the RdDM pathway mutants that were tested, the SUC2 and HPTII transgenes were silenced (Fig. 2B and C and Fig. S3A). Bisulfite sequencing showed that the 35S promoter is hypermethylated in the nrpd1-12 mutant compared with WT (Fig. 1E). Although the RdDM pathway is known to be required for epigenetic silencing of some transgenes and numerous endogenous loci, including many transposon repeats (22–25), our forward genetic screen here identified the RdDM as an antisilencing pathway. The notion of the RdDM pathway also having an antisilencing role is consistent with a previous report (17).

RdDM Maintains ROS1 Expression to Prevent the Silencing of 35S::SUC2 and 2 × 35S::HPTII Transgenes. Because the ros1 and RdDM mutants were recovered from the same genetic screen, we hypothesized that RdDM may prevent the silencing of the 35S::SUC2 and 2 × 35S::HPTII transgenes by maintaining ROS1 expression. We examined ROS1 gene expression, and found that ROS1 was dramatically repressed in all of the tested RdDM mutants (Fig. 2D and Fig. S3B), which is consistent with previous reports (14, 15, 17). To test whether the SUC2 and HPTII silencing in the RdDM mutants is caused by down-regulation of ROS1, we tried to restore ROS1 mRNA levels in nrpe1-17 plants by transgenic expression of ROS1 with a 2-kb native promoter. The attempt failed, as revealed by the long root phenotype of the ROS1 promoter (PROS1::ROS1/nrpe1-17 plants on sucrose-containing medium (Fig. 3A)). Quantitative RT-PCR results showed that the RNA levels of SUC2, HPTII, and ROS1 in these transgenic plants remained low (Fig. 3B). As a control, the PROS1::ROS1 transgene could rescue the root phenotype of ros1-12 mutant plants (Fig. S4). These results show that like the endogenous ROS1, the ROS1 transgene requires RdDM activity for expression. ROS1 gene promoter contains a helitron TE, which is located 155 bp upstream of the 5′ UTR of ROS1. Deletion of this helitron TE from the ROS1 promoter enabled transgenic expression of PROS1-ΔTE::ROS1 in RdDM mutants, and consequently suppressed the silencing of SUC2 and HPTII (Fig. 3A and B). The results confirmed that the antisilencing role of RdDM is indeed because it is required for ROS1 expression. The results also suggest that the helitron TE negatively regulates ROS1 gene expression, and that RdDM-dependent methylation is required for ROS1 expression only when this TE is present in ROS1 promoter. We hypothesized that an unknown RdDM target sequence at the ROS1 locus functions to antagonize the TE to ensure ROS1 expression.

RdDM Regulates the DNA Methylation Level of DNA Methylation Monitoring Sequence in the ROS1 Promoter. To investigate how RdDM activity may antagonize the helitron TE in regulating ROS1 gene expression, we examined DNA methylation patterns.
in the ROS1 promoter. We performed individual bisulfite sequencing to compare DNA methylation levels in the region between the 5′ UTR and the helitron TE in WT, npd1-12, and npd1-17. NRPD1 and NRPE1 encode the largest subunits of Pol IV and Pol V, respectively. Dysfunction of Pol IV and Pol V, both of which are major components of the canonical RdDM pathway (2, 6, 7, 26), substantially reduced DNA methylation levels at the examined region, especially in CHG and CHH contexts (Fig. 4A). Similar patterns were observed in npd1-3 and npd1-11 plants compared with their WT control Columbia-0 (Col-0), as revealed in whole-genome bisulfite sequencing (Fig. S5A). In the methylated ROS1 promoter region, Pol IV-dependent and Pol V-dependent 24-nt siRNAs were detected by small RNA sequencing (27) (Fig. S5B). Consistently, Pol V could bind to this region, as shown by published NRPE1 ChIP sequencing data (28) (Fig. SSC). These results demonstrated that RdDM directly controls DNA methylation in this particular sequence in the RdDM mutants is concomitant with ROS1 gene repression, we hypothesized that this sequence may serve as an indicator of general RdDM activities to allow a dynamic coordination between DNA methylation and active DNA demethylation through transcriptional regulation of ROS1. Accordingly, we referred to this sequence as the DNA methylation monitoring sequence (MEMS) in subsequent analyses.

DNA Methylation Level of MEMS Is also Regulated by Active DNA Demethylation. We found that DNA methylation at MEMS is also regulated by ROS1-dependent active demethylation, as shown by the increased DNA methylation levels when ROS1 is dysfunctional (Fig. 4A). In ros1-12 plants compared with WT plants, the levels of DNA methylation in CG, CHG, and CHH contexts were increased by 287%, 205%, and 196%, respectively (Fig. 4A). Similarly, DNA hypermethylation was observed in ros1-4 in comparison to its Col-0 WT control (Fig. S5A), further suggesting that MEMS is targeted by ROS1-dependent DNA demethylation. To confirm the regulation of MEMS by ROS1-dependent DNA demethylation, chromatin occupany of ROS1 at MEMS was examined by ChIP. We used ros1-4 plants complemented with transgenic ROS1 that was driven by its native promoter and was epitope-tagged with 3× FLAG. As shown in Fig. 4B, anti-FLAG antibody detected ROS1 signals at MEMS and its neighboring regions, consistent with the local DNA hypermethylation in ros1 mutants (Fig. 4A and Fig. S5A). Therefore, DNA methylation at MEMS is controlled by both RdDM and ROS1-dependent active demethylation.

We next asked whether DNA hypermethylation at MEMS might be accompanied by transcriptional elevation of ROS1. Five point-mutation alleles of ros1 mutants, including ros1-11 to ros1-15, were examined. Quantitative RT-PCR results showed that ROS1 gene expression was increased in ros1 mutants compared with their WT control Columbia-0 (Col-0), as revealed in whole-genome bisulfite sequencing (Fig. S5A). In the methylated ROS1 promoter region, Pol IV-dependent and Pol V-dependent 24-nt siRNAs were detected by small RNA sequencing (27) (Fig. S5B). Consistently, Pol V could bind to this region, as shown by published NRPE1 ChIP sequencing data (28) (Fig. S5C). These results demonstrated that RdDM directly controls DNA methylation in this particular sequence in the RdDM mutants is concomitant with ROS1 gene repression, we hypothesized that this sequence may serve as an indicator of general RdDM activities to allow a dynamic coordination between DNA methylation and active DNA demethylation through transcriptional regulation of ROS1. Accordingly, we referred to this sequence as the DNA methylation monitoring sequence (MEMS) in subsequent analyses.
expression was increased in four of the five ros1 mutant alleles, with the exception of ros1-12, which contains a C-to-T mutation, and thus a premature stop codon in the fourth exon (Fig. 4C and Fig. S1). RNA decay can be triggered by mutations that cause premature stop codons (29) and may account for a lack of increased RNA levels of ROS1 in ros1-12. The increased ROS1 expression in all other examined ros1 alleles indicates a positive correlation between MEMS DNA hypermethylation and ROS1 gene up-regulation, further supporting a role of MEMS in sensing DNA methylation and demethylation activities and regulating ROS1 expression.

DNA methylation in gene promoters is commonly considered as transcriptionally repressive. However, DNA methylation of MEMS has a positive role in regulating ROS1 gene expression. Unlike promoter methylation, gene body methylation is preferentially associated with transcribed genes (8, 30). DNA methylation in the ROS1 gene body is unaffected in npd1-3 and npe1-11 mutants (Fig. S6), indicating that RdDM regulation of ROS1 gene expression is independent of DNA methylation in the ROS1 gene body. Mutation of ROS1 causes DNA hypermethylation in nearly 5,000 genomic regions, many of which are not RdDM targets (21). Thus, ROS1 down-regulation by defective RdDM may cause DNA hypermethylation in these non-RdDM-targeted regions.

In the npd1 mutants, CG hypermethylation was observed at the 3’ end of the heltron TE that is adjacent to MEMS (Fig. S5A). It is unlikely that ROS1 gene repression in npd1 is a consequence of this hypermethylation, because a greater level of CG hypermethylation in the same region exists in ros1 mutants (Fig. S5A), which displayed increased ROS1 gene expression. This conclusion is also supported by the observation that ROS1 expression is suppressed in met1 mutant plants (16), in which CG methylation is abolished. Recently, it was shown that Pol V can be recruited by preexisting CG methylation at RdDM target loci (31). Therefore, dysfunction of MET1 may impair RdDM at MEMS, and thereby decrease methylation-dependent ROS1 gene expression. Indeed, DNA methylation at MEMS is lost in met1-3 mutant plants (32) (Fig. S7).

The DNA methylation at MEMS and its adjacent region in the ROS1 promoter tends to be variable (Figs. S5A and S7), although our results show that the methylation at MEMS decreases in npd1 and npe1 mutants and increases in ros1 mutants. The reason for this high variability is presently unclear but may be a reflection of the dynamic regulation of DNA methylation at the ROS1 promoter in plants. It is also unclear how the heltron TE negatively regulates ROS1 gene expression. One possibility is that the TE in ROS1 promoter causes a chromatin conformation that is unfavorable for transcription initiation. DNA methylation at the adjacent MEMS may help attract a transcriptional activator to overcome the transcriptional suppression by the TE.

Like a thermostat that senses temperature changes and maintains a stable temperature, a methylstat may exist in plant cells to monitor DNA methylation levels and accordingly adjust DNA methylation and/or active demethylation activities to maintain genomic DNA methylation patterns. Our results suggest that the ROS1 promoter functions as a methylstat that senses both DNA methylation and demethylation activities and accordingly fine-tunes ROS1 expression levels. In met1-3 plants, CG hypomethylation at 5S ribosomal DNA is followed by progressive increases in CHH methylation levels in successive generations as a result of RdDM and lack of ROS1 expression, resulting in re-establishment of transcriptional silencing (16). In humans, the genome-wide DNA methylation landscape is dynamically fine-tuned, as shown by a strong correlation between biological age and DNA methylation increases at some loci and decreases at other loci (33). It is possible that a similar methylstat may exist in mammals to regulate DNA methylation.

Materials and Methods

Plant Materials. All mutant and transgenic Arabidopsis plants used in this study were in the Col-0 genetic background. Seeds were surface-sterilized in 20% (vol/vol) bleach, rinsed with sterilized water, and sown on half-strength Murashige and Skoog (MS) medium plates with 1% (wt/vol) agar. The root phenotype was observed when 2% (wt/vol) sucrose was added to the medium. When it was necessary to avoid the side effects of sucrose accumulation and to monitor other phenotypes, sucrose was replaced with 1% glucose. After 2 d of stratification at 4 °C, plates were moved to a growth chamber at 22 °C with 160 μmol m−2 s−1 fluorescent light (16 h of light and 8 h of dark).

Screening for Ethyl Methanesulfonate-Mutagenized Mutant Population. M2 seeds from ethyl methanesulfonate-mutagenized plants were grown vertically on half-strength MS medium with 2% (wt/vol) sucrose for 1 wk. In this condition, root growth of WT plants was severely inhibited, whereas putative mutants with long roots were selected and transferred to soil. To map the mutated genes, mutants were crossed to Landsberg erecta ecotype WT plants. We selected a mapping population of 96 F2 seedlings that showed long roots when grown on half-strength MS medium with 2% (wt/vol) sucrose. In this medium, hygromycin (20 μg/mL) was added to exclude the seedlings that lack the 35S:SU2 transgene. After rough mapping with bulk segregant analysis, the mutant genome was sequenced to find the mutated genes.

Plasmid Construction and Mutant Complementation. For complementation, ROS1 genomic DNA with an ~2-kb promoter region was amplified from Col-0 genomic DNA and cloned into the pENTR/D-TOPO entry vector.
Bisulfite Sequencing of Individual Loci. Two hundred nanograms of genomic DNA was treated with the BisulfiFlash DNA Modification Kit (Epigentek) following the manufacturer’s protocols. Two microliters of bisulfite-treated DNA was used for each PCR assay (20-μL final volume) using ExTaq (Takara) with primers specific to the corresponding regions (Table S1). PCR products were cloned into the pGem-T easy vector (Promega) following the supplier’s instructions. For each region in each sample, at least 16 independent top-strand clones were sequenced and analyzed.

Real-Time RT-PCR. For real-time RT-PCR, total RNAs were extracted with the RNeasy Plant Kit (Qiagen). From 1 μg of total RNA, cDNAs were generated using poly (T) primer and M-MuLV Reverse Transcriptase (New England Biolabs) in a 20-μL reaction mixture. The resulting cDNAs were used as templates for real-time PCR with IQ SYBR green supermix (Bio-RAD). PCR was performed with a CFX96 real-time PCR detection system (Bio-RAD). TUB8 was used as an internal control. All of the primers used are listed in Table S1.

ChiP Assay. ChiP assays were performed as described by Wierzbicki et al. (28). The antibodies used were anti-FLAG (no. F1804; Sigma). ChiP products were diluted with 80 μL of TE buffer, and 2 μL was used for each quantitative PCR reaction. At least two biological replicates were performed.

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Fig. S1. Diagram of mutations identified in this study. The positions of mutations are counted from the first ATG in the genomic sequence. The changed amino acid is indicated in parentheses. The asterisk represents a stop codon.
Fig. S2. Diagram of mutations identified in this study. The positions of mutations are counted from the first ATG in the genomic sequence. The changed amino acid is indicated in parentheses. The asterisk represents a stop codon. AGO4, Argonaute 4; DTF1, DNA-binding transcription factor 1; KTF1, Kow domain-containing transcription factor 1.
Fig. S3. Dysfunctional RdDM caused transcriptional silencing of ROS1 and SUC2 transgenes. Expression of SUC2 (A) and ROS1 (B) in RdDM mutants is shown. Values are mean ± SD of three biological replicates, where the fold changes are normalized to transcript levels in WT. *dcl3*, dicer-like 3.

Fig. S4. Root phenotype of ros1-13 mutant was rescued by ROS1 gene driven by both its native promoter and the promoter with TE deletion.
Fig. S5. DNA methylation status and NRPE1 enrichment at ROS1 promoter. (A) DNA methylation status of ROS1 promoter in Col-0, nrpd1-3, nrpe1-11, and ros1-4 mutants. The result from whole-genome bisulfite sequencing is shown. Vertical bars on each track indicate DNA methylation levels. TSS, transcription start site. (B) Small RNA levels at ROS1 promoter in Col-0, nrpd1-3, and nrpe1-11 (1). (C) NRPE1 enrichment at ROS1 promoter. ChIP sequencing data (2) are from neomorph.salk.edu/pol_epigenomes/browser.html.


Fig. S6. DNA methylation status at ROS1 gene in Col-0, nrpd1-3, and nrpe1-11 mutants. The result from whole-genome bisulfite sequencing is shown. Vertical bars on each track indicate DNA methylation levels.
DNA methylation status at ROS1 promoter in Col-0 and met1-3 mutant. The result from whole-genome bisulfite sequencing (1) is shown. Vertical bars on each track indicate DNA methylation levels. TSS, transcription start site.


**Table S1. List of primers used in this study**

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<th>Primer name</th>
<th>Sequences (5′→3′)</th>
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<td>pEN-pROS1</td>
<td>CACCGCTTCACTTTGCTTGGCACA</td>
<td>Cloning ROS1 genomic DNA into pENTR vector</td>
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<tr>
<td>ROS1-r</td>
<td>GGCAGAGGTTAGTTCTGTTGTC</td>
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<td>CATATCAGTTGGAGCAATGGACGATGACGATGACGATA</td>
<td>Deletion of TE in ROS1 promoter</td>
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<td>ROS1prodelR</td>
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<td>Deletion of TE in ROS1 promoter</td>
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<td>ROS1proBiF</td>
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<td>Bisulfite sequencing of ROS1 promoter</td>
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<tr>
<td>ROS1proBiR</td>
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ACT2, Actin 2; f or F, forward; GUS, β-glucuronidase; qPCR, quantitative PCR; r or R, reverse; TUB8, Tublin 8.