Role of the *Arabidopsis* DNA glycosylase/lyase ROS1 in active DNA demethylation

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DNA methylation is a stable epigenetic mark for transcriptional gene silencing in diverse organisms including plants and many animals. In contrast to the well characterized mechanism of DNA methylation by methyltransferases, the mechanisms and function of active DNA demethylation have been controversial. Genetic evidence suggested that the DNA glycosylase domain-containing protein ROS1 of *Arabidopsis* is a putative DNA demethylase, because loss-of-function *ros1* mutations cause DNA hypermethylation and enhance transcriptional gene silencing. We report here the biochemical characterization of ROS1 and the effect of its overexpression on the DNA methylation of target genes. Our data suggest that the DNA glycosylase activity of ROS1 removes 5-methylcytosine from the DNA backbone and then its lyase activity cleaves the DNA backbone at the site of 5-methylcytosine removal by successive β- and δ-elimination reactions. Overexpression of ROS1 in transgenic plants led to a reduced level of cytosine methylation and increased expression of a target gene. These results demonstrate that ROS1 is a 5-methylcytosine DNA glycosylase/lyase important for active DNA demethylation in *Arabidopsis*.

DNA methylation | epigenetics | transcriptional gene silencing

DNA cytosine methylation is important for many epigenetic processes including X chromosome inactivation, genomic imprinting, epigenetic changes during carcinogenesis, and silencing of transposons, of specific genes during development and of certain transgenes (1–5). The enzymes responsible for *de novo* as well as maintenance methylation at the 5′ position of cytosines have been well characterized, and mutations in these enzymes can release transcriptional gene silencing and cause various developmental phenotypes (2, 3, 6, 7). In contrast, the mechanism of DNA demethylation is less understood. DNA demethylation can be passive or active. Passive demethylation occurs automatically for newly synthesized DNA during replication if the new DNA is not acted upon by DNA methyltransferases. The biochemical mechanism of active DNA demethylation has been controversial (8).

The chemistry of demethylating 5-methylcytosine DNA is challenging because it requires the disruption of carbon–carbon bonds. Earlier work has shown that 5-methylcytosine was replaced by labeled cytosine during the demethylation reaction in erythroleukemia cells, indicating a replacement of the entire nucleotide or base alone (9). One potential mechanism to achieve this is through the action of 5-methylcytosine DNA glycosylase, which removes the methylcytosine from DNA leaving the deoxyribose intact (10). Local DNA repair then removes the abasic nucleotide and adds back an unmethylated cytosine nucleotide (11). Using chicken embryo nuclear extracts that can promote demethylation (12), a putative demethylase was purified; this was found to be a G/T mismatch repair DNA glycosylase (13). MBD4, a human homolog of the chicken enzyme, also has 5-methylcytosine DNA glycosylase activity (14). At least *in vitro*, these G/T mismatch repair DNA glycosylases appear to be inefficient demethylases because their 5-methylcytosine DNA glycosylase activity is very low compared to their strong G/T mismatch repair activities (12, 14, 15). A 5-methylcytosine DNA glycosylase/DNA demethylase activity was also identified and partially purified from normal and cancerous humans cells (16, 17), but the enzyme responsible for this demethylase activity has not yet been cloned.

Demethylation through the removal of the entire 5-methylcytosine nucleotide was also suggested (18). However, the putative demethylase involved in this nucleotide excision repair has not been cloned. In addition, the methyl CpG-binding protein MBD2 was reported to be a DNA demethylase that hydrolyzes 5-methylcytosine to cytosine and methanol (19). This claim was contested and could not be reproduced by other laboratories (20, 21).

There has been a critical need for combined genetic and biochemical analysis to firmly establish the mechanism and *in vivo* function of DNA demethylases. Strong genetic evidence in *Arabidopsis* supports that certain DNA glycosylases function in active DNA demethylation (22, 23). Mutations in the DNA glycosylase domain-containing protein ROS1 in *Arabidopsis* cause DNA hypermethylation and transcriptional gene silencing (TGS) of the *RD29A-LUC* (firefly luciferase driven by the *RD29A* promoter) transgene and the endogenous *RD29A* gene (22). A maltose-binding protein (MBP) fusion with the C-terminal 1099 residues of ROS1 that includes the DNA glycosylase domain is capable of incising plasmid DNA methylated with MspI methylase, suggesting that ROS1 might be a DNA demethylase (22). In addition, another DNA glycosylase protein in *Arabidopsis*, Demeter, is known to be required for endosperm maternal allele-specific hypomethylation and expression of the imprinted *MEA* gene (23, 24).

In this report, we show that ROS1 has a 5-methylcytosine DNA glycosylase activity against several DNA substrates. Overexpression of ROS1 reduces DNA methylation at both the transgene and endogenous *RD29A* promoters. Together, our results support a role for ROS1 as a 5-methylcytosine DNA glycosylase in erasing DNA methylation and preventing transcriptional gene silencing.

Results

Recombinant ROS1 Protein Has a Relatively High 5-Methylcytosine Glycosylase Activity and Low G/T Mismatch Repair DNA Glycosylase Activity. A MBP fusion with a full length ORF of ROS1 was expressed and purified (Fig. 1*A*) and found to have incision activity against plasmid DNA methylated with either MspI or SssI methylase, but no activity against unmethylated plasmid (Fig. 1 *B* and *C*). To rule out the possibility that the observed activity might have come from a contaminating protein in the preparation, glutamic acid-1303, a residue conserved in the

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Abbreviations: MBP, maltose-binding protein; mROS1, mutated ROS1; wtROS1, wild-type ROS1; 8-oxoG, 8-oxo-7,8-dihydroguanine; TAP, tandem affinity purification.

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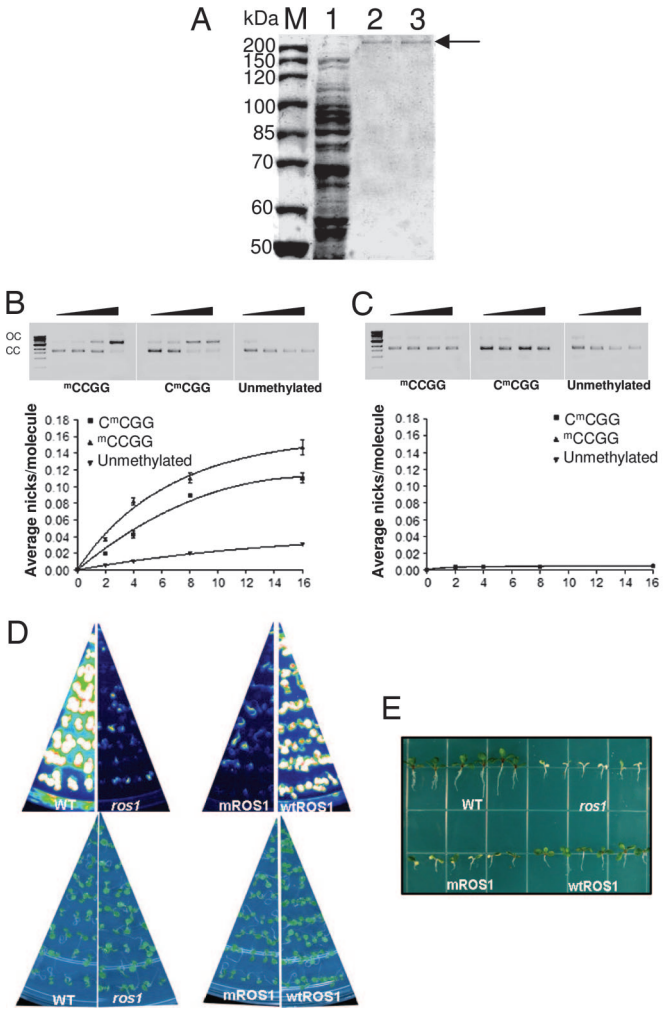

Fig. 1. Wild-type ROS1 (wtROS1) but not mutated ROS1 (mROS1) is functional in plants and have nicking activity on methylated plasmid DNA. (*A*) Purification of recombinant MBP-ROS1 and MBP-mROS1 fusion proteins. Shown is an SDS/PAGE gel stained with Coomassie blue. M, molecular mass markers; lane 1, Lysate of *E. coli* cells after induction of MBP-ROS1; lane 2, purified MBP-ROS1; and lane 3, purified MBP-mROS1. (*B* and *C*) DNA nicking activity. Purified closed circular (CC) plasmid DNA was incubated with increasing amounts of ROS1 (*B*) and mROS1 (*C*), and the reaction mixture resolved on an agarose gel. Control reaction with unmethylated plasmids was carried out in parallel. The plots represent quantification of DNA nicking activity. The average number of nicks per plasmid was estimated from the fraction of open circular form (OC). (*D*) Complementation of *ros1* mutant by ectopic expression of wtROS1 and mROS1. Seedlings grown on MS medium for 10 days were treated with at 4°C for 48 h before the luminescence images were taken. (*E*) Kanamycin sensitivity of *ros1* seedlings transformed with wtROS1 or mROS1. Seeds of wild type, *ros1*, and *ros1* transformed with wtROS1 and mROS1 were germinated on MS medium supplemented with kanamycin (35 μg/ml).

ROS1 subfamily of DNA glycosylases (Fig. 6, which is published as supporting information on the PNAS web site), was changed to lysine and the mutated ROS1 (mROS1) was fused with MBP (Fig. 1*A*). mROS1 had no incision activity against any of the DNA substrates (Fig. 1 *B* and *C*). When ectopically expressed in the *ros1* mutant, wild-type ROS1 (wtROS1) but not mROS1 rescued the silencing phenotype of *RD29A-LUC* and *35S-NPTII* transgenes (Fig. 1 *D* and *E*). These results confirm the DNA incision activity observed previously with truncated ROS1 and show that Glu-1303 is critical for the incision activity and *in vivo* function of ROS1. Unlike the N-terminally truncated ROS1 protein, which did not have activity against SssI-methylated plasmid (22), full-length ROS1 had such an activity. However, the specificity constant (k_{cat}/K_M) for DNA methylated with SssI was substantially lower than that with MspI methylase, indicating a greater preference of ROS1 for excision of external 5-methylcytosines (Table 1, which is published as supporting information on the PNAS web site).

To better define ROS1 activity, we performed DNA incision assays on oligonucleotides containing 5-methylcytosine in different sequence contexts. The oligonucleotides were labeled at the 5′ end with digoxigenin so that incision products could be detected by chemiluminescence. The end-labeled oligonucleotides annealed to unlabeled complementary strands were used to produce DNA substrates that are either hemimethylated or fully methylated at CpXpG (^{m}CCG) or CpG (C^{m}CG) contexts (Fig. 2*A*). Incubation of ROS1 with an oligonucleotide containing a fully methylated CpXpG site (methylation on both DNA strands at 16-nt position from 5′ end of labeled strand) and a hemimethylated CpXpG site (methylation on only one strand at 29-nt position from 5′ end of labeled strand) resulted in the generation of a cleavage product corresponding to only the fully methylated site (Fig. 2*B Left*). The result indicates that ROS1 is active on fully methylated but not hemimethylated DNA *in vitro*. Interestingly, the cleavage product migrated slightly faster than the β elimination product (expected size, 16 nt) (Fig. 2*B*). This finding suggests that ROS1 cleaves its substrate DNA by successive β- and δ-elimination reactions at the abasic site, after it removes the methylated cytosine base. A weak cleavage was observed on a substrate containing a fully methylated CpG site, and again no cleavage was observed on hemimethylated CpG (Fig. 2*B Right*). Incubation of the control protein mROS1 with the methylated CpXpG substrate failed to generate cleavage product (Fig. 2*C*). These results show that ROS1 can act on fully methylated DNA *in vitro*, probably by a β,δ-elimination mechanism.

Repair reactions catalyzed by all bifunctional DNA glycosylase/lyases proceed through a transient imino intermediate (Schiff base) that can be reduced by borohydride to form an irreversibly cross-linked complex between the enzyme and DNA substrate at the C1′ carbon of the deoxyribose ring (25–27). We carried out the demethylation reactions in the presence of $NaBH_4$ and analyzed the proteins by SDS/PAGE. In the presence of borohydride, ROS1 became labeled with digoxigenin as a result of irreversible cross-linking with the DNA substrate (Fig. 2*D*). No protein labeling was observed either in the absence of borohydride or when mROS1 was used. The observation suggests that the reaction catalyzed by ROS1 proceeds through a bifunctional DNA glycosylase/lyase mechanism.

The *RD29A* promoter is hypermethylated in *ros1* mutant but not in the wild type (22). We tested ROS1 activity on a 600-bp fragment of *RD29A* promoter, which has 20 sites that can be methylated with SssI and only one site that can be methylated with MspI methylase. The positions of the incisions were identified by the size of fragments on polyacrylamide gels (Fig. 2*E*). We found two bands corresponding to MspI methylated promoter after nicking by ROS1 and multiple bands corresponding to nicked products from SssI methylated promoter. No cleaved band could be detected with ROS1 on unmethylated *RD29A* promoter, or with mROS1 on any of the promoter substrates (Fig. 2*E*). These results show that ROS1 is very active in demethylating the *RD29A* promoter.

To rule out the possibility that the observed nicking activity of ROS1 might be due to the excision of mispaired thymine residues that arose by spontaneous conversion of 5-methylcytosine, we tested whether ROS1 has significant G:T mismatch repair activity. The chicken MBD4 protein has both 5-methylcytosine glycosylase and G:T mismatch repair activities (13) and was used here as a control. The DNA 5-methylcytosine glycosylase activity of ROS1 is 10–15 times higher than its G:T mismatch repair activity (Fig. 3 *A* and *B*). In contrast, the G:T mismatch repair

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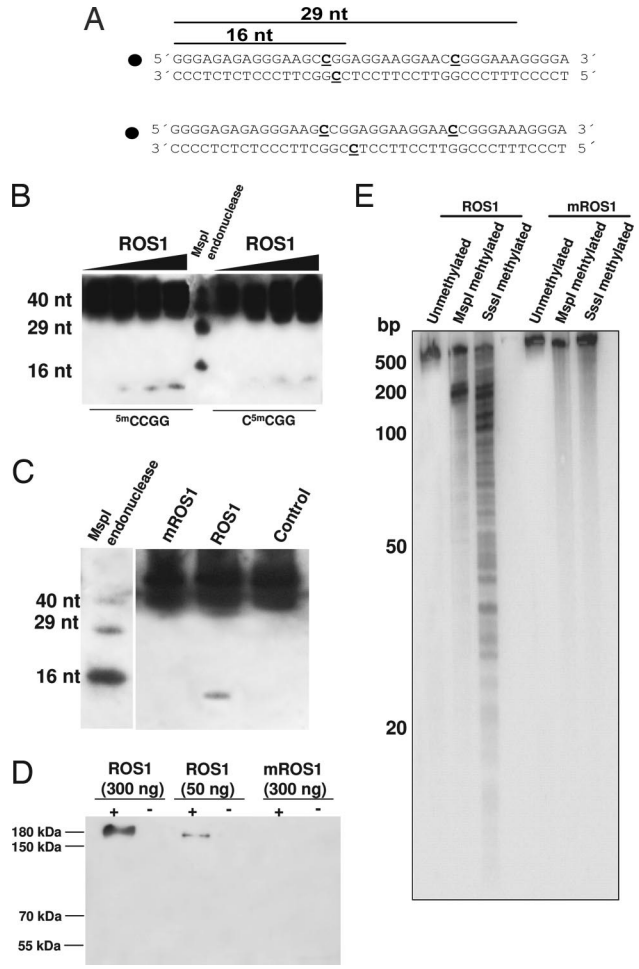

Fig. 2. 5-methylcytosine DNA glycosylase activity of ROS1 on oligonucleotide and promoter DNA substrates. (*A*) Sequences of methylated double stranded DNA oligonucleotides. The dot denotes digoxigenin labeling. Methylated cytosine is underlined. (*B*) Methyl-DNA cleavage activity of ROS1 using the substrates shown in *A*. (*C*) Comparison of wild-type ROS1 and mROS1 activity using the second substrate (^{5m}CCGG) shown in *A*. Control, no enzyme. (*D*) $NaBH_4$ trapping of the Schiff base reaction intermediate. Double-stranded oligonucleotide containing ^{5m}CCGG was incubated with purified ROS1 or mROS1 in the absence (−) or presence (+) of $NaBH_4$. Cross-linked enzyme-susbtrate complex was visualized by chemiluminescence detection after SDS/PAGE and transfer to nylon membrane. (*E*) A 600-bp fragment of the *RD29A* promoter methylated with either MspI or SssI methylase was used as a substrate in the demethylation reaction. The reaction product was resolved on a denaturing polyacrylamide gel, transferred to a nylon membrane, and hybridized with a labeled *RD29A* promoter cDNA probe. An unmethylated fragment of *RD29A* promoter was used as negative control.

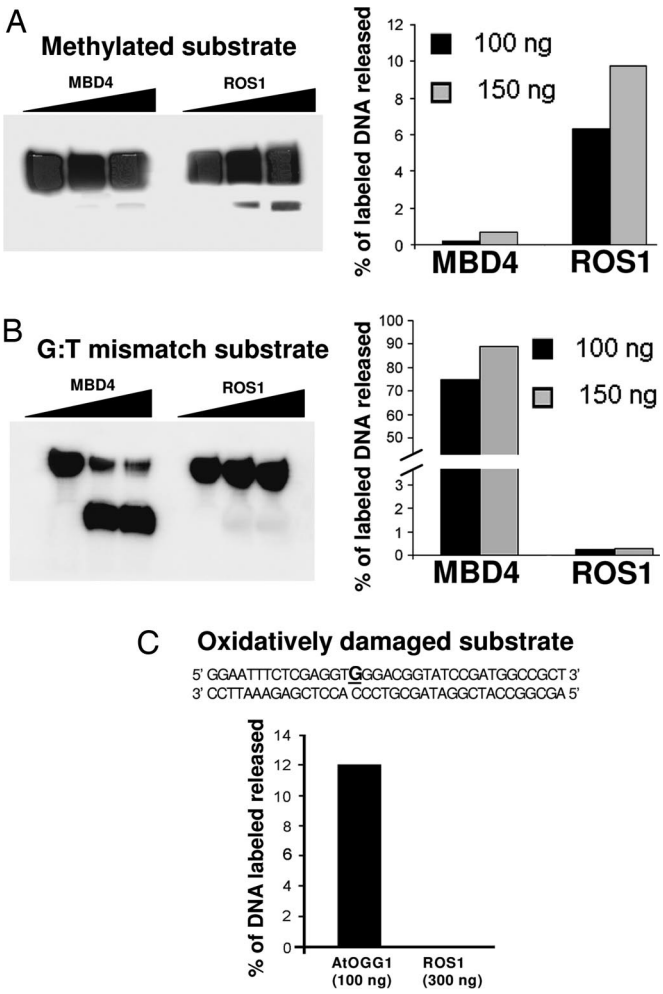

Fig. 3. ROS1 has low activity against mismatched DNA and no activity against damaged DNA. Radiolabeled double-stranded oligonucleotide containing ^{5m}CCGG (*A*) or G/T mismatch (*B*) were incubated with purified ROS1 or MBD4 recombinant protein. The reaction products were separated on 17% denaturing polyacrylamide gels. The released product as a percentage of total labeled DNA is shown in the graph on the right. (*C*) Radiolabeled double-stranded oligonucleotide containing 8-oxoG was incubated with ROS1 or AtOGG1 recombinant protein as described in *Materials and Methods*. The graph shows the released product as a percentage of total labeled DNA.

activity of MBD4 was 50–60 times higher than its DNA 5-methylcytosine glycosylase activity (Fig. 3 *A* and *B*). We also tested whether recombinant ROS1 protein has incision activity on DNA substrates containing 8-oxo-7,8-dihydroguanine (8-OxoG), which is typical of oxidatively damaged DNA (25, 28). Incubation of a 36-bp double-stranded substrate containing an 8-oxoG:C pair at a defined position with ROS1 recombinant protein did not generate any cleavage product, although we could detect an expected cleavage product when this substrate was incubated with the control enzyme AtOGG1 (25, 29) (Fig. 3*C*). The results suggest that ROS1 has a preference for 5-methylcytosine over G/T mismatch or damaged DNA substrates, and its main function is to demethylate DNA rather than to repair DNA mismatches or damaged bases.

5-Methylcytosine DNA Glycosylase Activity of ROS1 Protein from Plant Extracts. To determine the activity of native ROS1 protein from plants, ROS1 as well as mROS1 were ectopically expressed as an N-terminal tandem affinity purification (TAP)-tagged protein in *ros1* mutant plants. NTAPi-ROS1, but not NTAPi-mROS1, could partially rescue the *ros1* mutant defect in *RD29A-LUC* expression (Fig. 4*A*). The TAP tag allowed for a partial purification of ROS1 and mROS1 protein complexes from the transgenic plants (Fig. 4*B*). The addition of NTAPi-ROS1 to the demethylation reaction mixture produced two products, P1 and P2 (Fig. 4*C*). The cleavage product P2 indicates β and δ elimination by ROS1, whereas the cleavage product P1 is consistent with β elimination. The NTAPi-mROS1 protein preparation only yielded a weak band corresponding to P1 (Fig. 4*D*). The results support a β,δ-elimination mechanism of ROS1 in plants and the requirement of Glu-1303 for this activity. The product P1 may be due to the presence of other DNA glycosylase(s) in the partially purified ROS1 and mROS1 complexes.

Overexpression of ROS1 Causes DNA Hypomethylation and Increases *RD29A* Promoter Activity. Previous work showed that, in the wild-type background, the *RD29A* promoter has low levels of

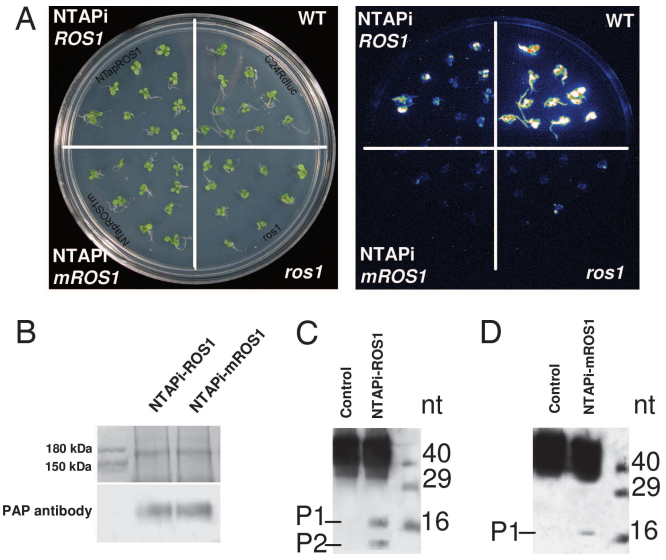

Fig. 4. 5-methylcytosine DNA glycosylase activity of ROS1 purified from plants. (*A*) Complementation of *ros1* mutant by ectopic expression of NTAPi-ROS1 and NTAPi-mROS1. Seedlings were treated with cold (4°C for 48 h) then the luminescence image was taken. (*B*) SDS/PAGE and Western blot analysis of NTAPi-ROS1 and NTAPi-mROS1 proteins using antibodies against the TAP tag. (*C* and *D*) Double-stranded oligonucleotide containing ^{5m}CCGG was incubated with NTAPi-ROS1 (*C*) or NTAPi-mROS1 (*D*). The reactions were carried out with NTAPi-ROS1/mROS1 protein immobilized on IgG-Sepharose beads. The putative β and $\beta\delta$ elimination products are indicated by P1 and P2, respectively.

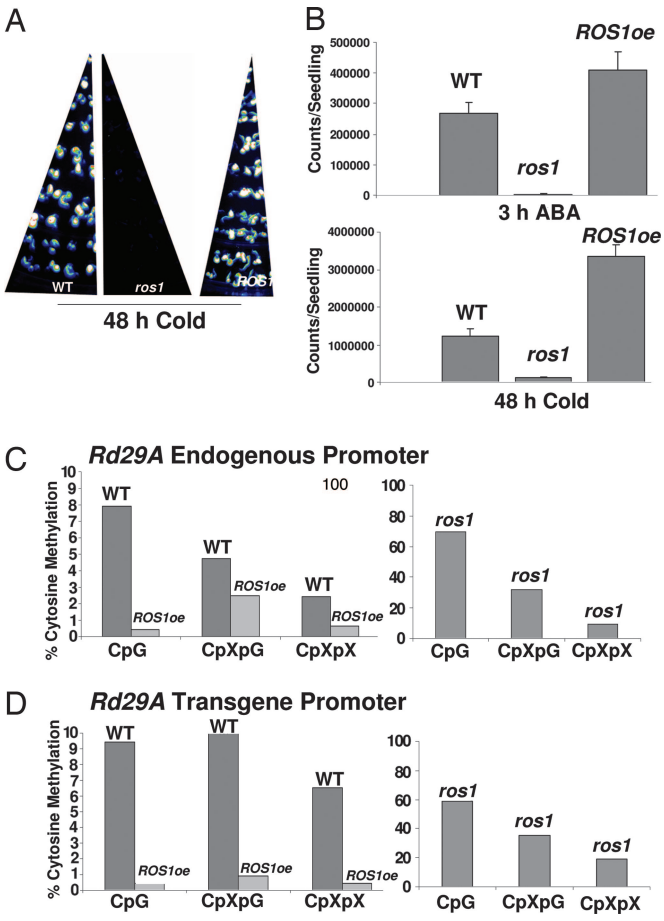

Fig. 5. Luminescence and DNA methylation phenotype of ROS1 overexpression plants. (*A* and *B*) Luminescence was analyzed after 100 μM ABA treatment for 3 h or cold treatment (4°C) for 48 h. (*C* and *D*) DNA methylation analysis of the endogenous (*C*) and transgene (*D*) *RD29A* promoter by bisulfite sequencing. Twenty clones were sequenced for each genotype.

DNA methylation but the methylation levels in the *ros1* mutant increase dramatically at all cytosine positions. We hypothesized that, in the wild type, a low level of *RD29A* promoter siRNAs triggered DNA methylation at the *RD29A* promoter, but the active demethylation activity of ROS1 prevented hypermethylation. Consistent with this hypothesis, in *dcl3* or *rdr2* mutants, *RD29A* promoter siRNAs are not produced, and even the low level of DNA methylation at the *RD29A* promoter that is present in the wild type is completely abolished (Z. Gao and J.-K.Z., unpublished result). To determine whether ROS1 activity is still limiting in the wild type and whether increased expression of ROS1 would reduce the DNA methylation to even lower levels, we tested the effect of overexpression of ROS1 on DNA methylation and activity of the *RD29A* promoter. We overexpressed ROS1 in the wild-type background under the control of the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter. Several transgenic lines were found to have increased *RD29A-LUC* transgene expression compared to the wild type, and one representative line is shown in Fig. 5 *A* and *B*.

Methylation levels at the endogenous and transgene *RD29A* promoters were determined by bisulfite sequencing. A low level of DNA methylation was found in the wild-type plants: 7.9% CpG methylation, 4.7% CpXpG methylation, and 2.5% CpXpX methylation at the endogenous *RD29A* promoter, and 9.4% CpG methylation, 10% CpXpG methylation, and 6.5% CpXpX methylation at the transgene *RD29A* promoter (Fig. 5 *C* and *D Left* and Fig. 7, which is published as supporting information on the PNAS web site). As reported previously, *ros1* mutant plants had very high levels of DNA methylation at both the endogenous and transgene *RD29A* promoters (Fig. 5 *C* and *D Right*). In the *ROS1* overexpression line, there was little or no methylation at both the endogenous and transgene *RD29A* promoters (Fig. 5 *C* and *D Left* and Fig. 7). The result suggests that ROS1 overexpression led to more demethylation, and consequently less 5-methylcytosine and increased expression of the *RD29A-LUC* transgene.

Discussion

Our genetic and biochemical data here support a base excision repair mechanism for DNA demethylation. Using methylated plasmid DNA, DNA oligonucleotides with well defined methylation patterns, or the methylated plant *RD29A* promoter DNA as substrates, we showed that ROS1 has a clear 5-methylcytosine glycosylase activity. Glu-1303, a residue conserved in the ROS1 subfamily of DNA glycosylases, is essential for this activity and for ROS1 function in plants. The 5-methylcytosine glycosylase activity of ROS1 is particularly high on methylated *RD29A* promoter, compared to the short DNA oligonucleotide substrates. *In vitro*, ROS1 has a preference for removing ^{5m}C in CpXpG sequences, and for fully methylated over hemimethylated sequences. A 5-methylcytosine DNA glycosylase partially purified from HeLa cells exhibited similar preferences (16, 17). However, it is unclear whether ROS1 may have these preferences *in vivo* because analysis of *ros1* mutant (22), and ROS1 overexpression (Fig. 5) plants suggested that ROS1 functions in erasing cytosine methylation in all sequence contexts. In addition, an independent study using a different oligonucleotide substrate found an opposite substrate specificity for ROS1 *in vitro*, i.e., a preference for removing ^{5m}C in CpG sequence context (30). It is possible that the activity of ROS1 *in vitro* is determined not only by the CpG or non-CpG context but also by other neighboring sequences. Alternatively, the different assay conditions used in the two independent studies may have caused the

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discrepancy in substrate preferences. In addition, the 5-methylcytosine DNA glycosylase activity of ROS1 *in vivo* could be influenced by other proteins that the enzyme may associate with.

Our work suggests that ROS1 possesses several enzymatic activities as part of a DNA demethylation mechanism. Its glycosylase activity removes ^{5m}C bases, and its AP (apurinic/apyrimidinic) lyase activity then nicks the DNA backbone at the abasic site. Our results suggest that this lyase activity removes the deoxyribose from the nicked abasic site in DNA, generating β,δ-elimination products. The final result of ROS1 action is thus a single nucleoside gap, which can then be filled by a polymerase and ligase after removal of a 3′ phosphate (8, 31). Interestingly, Demeter seems to generate a mixture of β- and β,δ-elimination products *in vitro* (23, 30). It is unclear whether this difference reflects intrinsic dissimilarities between ROS1 and Demeter, or is caused by different assay conditions used.

The chicken MBD4 protein has been suggested to be a DNA demethylase because it has a 5-methylcytosine glycosylase activity (13). However, this activity is low compared to its high G:T mismatch repair activity (13). In sharp contrast, we found that ROS1 has a very low G:T mismatch repair activity and its 5-methylcytosine glycosylase activity is 10–15 times higher (Fig. 3 *A* and *B*). Additionally, ROS1 is not active against damaged DNA (Fig. 3*C*). The results suggest that ROS1 is capable of specifically recognizing 5-methylcytosine in DNA.

Overexpression of ROS1 led to reduced DNA methylation at the *RD29A* promoter and increased expression of the *RD29A* promoter-driven luciferase gene. Together, the *ros1* mutant and ROS1 overexpression results indicate that DNA demethylases can be used to manipulate DNA methylation levels of certain target genes. In this regard, it will be of great interest to determine how ROS1 is targeted to specific genes. ROS1 is a large protein in which the glycosylase domain is only a small part. The rest of the protein may be important in targeting the glycosylase to specific loci through interaction with chromatin-associated partner proteins and/or with siRNAs that may be able to target both methyltransferases (3, 32) and demethylase complexes to specific chromatic regions.

Materials and Methods

Expression and Purification of ROS1. ROS1 and mROS1 (glutamic acid-1303 mutated to lysine) cDNAs containing the entire ORF were subcloned into the pMal-c2X vector. Expression of the MBP-ROS1 and MBP-mROS1 fusion proteins in *Escherichia coli* strain BL21(DE3) was induced by the addition of 0.25 mM isopropyl-1-thio-β-D-galactopyranoside at 27°C overnight. The fusion proteins were purified by affinity chromatography by using an amylose column.

In Vitro* Activity Assays.** ***Plasmid nicking assay. Plasmid pBluescript KS was purified from *E. coli* BL21 (DE3), a *dcm* strain, using a Maxi-plasmid purification kit (Qiagen). Twenty micrograms of plasmids were methylated *in vitro* in a 300-μl reaction containing 20 units MspI or SssI methylase. Nonmethylated plasmid was processed in parallel by using the same procedure but without methylase and was used as a control in the nicking assays. The methylation status was confirmed by digestion with MspI and HpaII restriction endonucleases. For the nicking assay, a reaction mixture (20 μl) containing the nicking buffer (40 mM Hepes-KOH, pH 8.0/0.1 M KCl/0.5 mM EDTA/0.5 mM DTT/0.2 mg/ml BSA) and 400 ng of purified, closed-circular plasmid DNA was incubated at 37°C for 1 h with purified MBP-ROS1 or MBP-mROS1 protein. Reactions were stopped by adding 8 μl of stop solution (0.4 M EDTA/1% SDS), heated at 70°C for 5 min, and the mixtures were loaded onto a 1% agarose gel. The average number of nicks per plasmid molecule was estimated from the fraction of remaining covalently closed-circular DNA by the Poisson distribution. The greater fluorescence of nicked circular DNA than closed-circular DNA was taken into account in all quantifications (33).

Oligonucleotide DNA duplex cleavage assay. Polyacrylamide gel-purified 40-mer oligonucleotides (Oligo 1, 5′-GGGAGAGAGGGAAGC^{5m}CGGAGGAAGGAAC^{5m}CGGGAAAGGGGA-3′; Oligo 2, 3′-CCCTCTCTCCCTTCGG^{5m}CCTTCCTTGGCCCTTTCCCCT-5′; Oligo 3, 5′-GGGAGAGAGGGAAG^{5m}CCGGAGGAAGGAA^{5m}CCGGGAAAGGGGA-3′; Oligo 4, 3′-CCCTCTCTCCCTTCGGC^{5m}CTTCCTTGGCCCTTTCCCCT-5′) containing external or internal ^{5m}C and labeled at the 5′ end with digoxigenin (Oligos 1 and 3) were purchased from Oligo ETC. To prepare double-stranded DNA substrates, 25 pmol of these oligos (Oligo 1 and 2, Substrate I; and Oligo 3 and 4, Substrate II) were annealed in 10 mM Tris·HCl (pH 8.0) and 20 mM NaCl. A polyacrylamide gel-purified 36-mer oligonucleotide (5′-GGAATTTCTCGAGGTGGGACGGTATCCGATGGCCGCT3′) (25 pmol) containing a single 8-oxoG at position 16 (underlined) and labeled at 5′ with digoxigenin was annealed in 10 mM Tris·HCl (pH 8.0) and 20 mM NaCl with a complementary one (50 pmol) containing C at the position opposite the single lesion. Annealing was performed by heating at 95°C for 10 min followed by slow cooling to room temperature.

Double-stranded oligonucleotides (300 fmol) were incubated at 37°C for 1 h in a reaction mixture containing nicking buffer and different amounts of ROS1 or mROS1 protein in a total volume of 10 μl. Reactions were stopped by adding 4 μl of formamide containing 1 mg/ml bromophenol blue and 10 mM EDTA, and the products were separated by 17% denaturing polyacrylamide gel containing 8 M urea. After electrophoresis, DNA was transferred to a positively charged nylon membrane by electroblotting. Digoxigenin-labeled molecules were visualized by chemiluminescent detection that involves reaction with anti-digoxigenin antibody conjugated to alkaline phosphatase and then addition of the chemiluminescence substrate CSPD (Roche Applied Science).

Cross-linking reaction with $NaBH_4$. Cross-linking reactions between ROS1 or mROS1 enzyme and substrates were performed by incubating the protein at 37°C for 1 h in the presence of 300 fmol of digoxigenin-labeled external cytosine methylated double-stranded DNA substrate in a reaction mixture (10 μl) containing nicking buffer and 50 nM $NaBH_4$. After incubation, the samples were mixed with SDS/PAGE loading buffer and denatured at 100°C for 5 min, and electrophoresis was carried on 7.5% SDS–polyacrylamide gels. Proteins were transferred onto a nylon membrane by electroblotting, and the digoxigenin-labeled DNA-enzyme complex was visualized by chemiluminescent detection (Roche).

Activity on mismatched DNA. The following oligonucleotides were used: T oligo, 5′-GACTGGCTGCTCC*T*GGGCGAAGTGCCC-3′; G oligo, 5′-GGGCACTTCCGCCCGGGAGCAGCCAGTC-3′; Oligo 3, 5′-GGGAGAGAGGGAAG^{5m}CCGGAGGAAGGAA^{5m}CCGGGAAAGGGGA-3′; Oligo 4, 3′-CCCTCTCTCCCTTCGGC^{5m}CTTCCTTGGCCCTTTCCCCT-5′.

The T oligo and Oligo 4 were labeled at 5′ end by using T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. The reaction was terminated by heating at 65°C for 20 min. The labeled T oligo and Oligo 4 were annealed with G oligo and Oligo 3, respectively, in 10 mM Tris·HCl (pH 8.0) and 20 mM NaCl. The mixture was heated at 95°C for 5 min and then slowly cooled to room temperature over a period of 2–3 h. The unincorporated [γ-^{32}P]ATP was removed from labeled duplex by passage through a G-25 microcolumn (Amersham Pharmacia).

Labeled duplex (20 nM) was equilibrated in the nicking buffer, and the reaction was initiated by adding purified ROS1 or MBD4 recombinant protein followed by incubation at 37°C for 1 h. Reactions were stopped by adding 4 μl of formamide containing 1 mg/ml bromophenol blue and 10 mM EDTA. The products were separated on 17% denaturing polyacrylamide gels contain-

ing 8 M urea. The gels were exposed to a PhosphorImager screen (Amersham Pharmacia), and the reaction products were quantified by using ImageQuant 5.2 software.

Activity on promoter DNA. A 600-bp fragment corresponding to the *RD29A* promoter was cloned into pBSK vector. The pBSK–*RD29A* promoter construct was methylated with MspI or SssI methylase. The *RD29A* promoter was released from the construct by digestion with BamHI and used as substrate in cleavage assays by ROS1.

The *RD29A* promoter fragment (300 nmol) was incubated at 37°C for overnight in a reaction mixture containing the nicking buffer and different amounts of ROS1 and mROS1 protein in a total volume of 10 μl. Reactions were stopped by adding 4 μl of formamide with 1 mg/ml bromophenol blue and 10 mM EDTA, and the products were separated by 7% denaturing polyacrylamide gels containing 8 M urea. After electrophoresis, DNA was transferred to a positively charged nylon membrane (Nylon PLUS, Amersham Pharmacia) by electroblotting. Purified *RD29A* promoter cDNA probe was labeled in the presence of [α-^{32}P]dCTP using Ready-To-Go DNA labeling kit (Amersham Pharmacia). Hybridization was carried out overnight at 42°C, and washing was done according to standard protocols (34). The image was visualized by using a PhosphorImager as described above.

Plant Growth and Luciferase Imaging. The wild-type (C24 containing the *RD29A-LUC* transgene), *ros1* mutant (22), and transgenic lines generated in this paper were grown in a controlled room with 16 h light and 8 h darkness at 22°C. Seedlings to be used for luciferase (LUC) imaging or to be planted on MS supplemented with kanamycin (35 μg/μl) were obtained after seeds were surface-sterilized and stratified for 3–4 days at 4°C and sowed on MS agar medium. Image acquisition and processing was performed as described (35).

Transgenic Plant Analysis. ROS1 and mROS1 cDNAs were cloned by digestion with EcoRI and XhoI and inserted into pEntry3C vector (Invitrogen). These clones were used for cloning ROS1 and mROS1 in pMDC32 for overexpression (36) and NTAPi destination binary vector (37) for generating NTAPi fusion. The recombination reactions were carried out as per LR reaction mix II Gateway technology manual (Invitrogen). The binary constructs were transferred to *Agrobacterium tumefaciens* GV3101 by electroporation for floral dip transformation. The ROS1 and mROS1 cDNA overexpression constructs and the NTAPi-ROS1 and NTAPi-mROS1 constructs were transformed into both wild-type and *ros1* mutant background. Transgenic lines were selected on MS medium supplemented with hygromycin (for ROS1 and mROS1 overexpression) or glufosinate ammonium (for NTAPi-ROS1 and NTAPi-mROS1 constructs). Transgenic lines were imaged after treatment with ABA for 3 h or cold for 48 h. They were also evaluated on MS medium supplemented with kanamycin.

DNA Methylation Analysis. DNA methylation analysis was carried out by using EZ DNA methylation kit (Zymo Research). Genomic DNA (2.0 μg) was digested with EcoRI, EcoRV, and HindIII, purified, and treated with sodium bisulfite for 16 h. The treated DNA was cleaned up following the manufacturer's instructions and used for subsequent PCR. To distinguish between the endogenous and transgene *RD29A* promoter, we used a common forward primer in the *RD29A* promoter and a different reverse primer. Primer information is available upon request. After PCR, the products were cloned in pGEMT (Promega), and individual clones were sequenced by using M13F primer. The sequences were then aligned with ClustalW (http://searchlauncher.bcm.tmc.edu/multialign/multialign.html) and compared with the DNA sequence from untreated samples.

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