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

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Edited by Steven Henikoff, Fred Hutchinson Cancer Research Center, Seattle, WA, and approved June 19, 2006 (received for review May 1, 2006)

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 α-elimination reactions. Overexpression of ROS1 in transgenic plants led to a reduced level of DNA 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 erythroblasts cells, indicating a replacement of the entire nucleotide or base lone (9). One potential mechanism to achieve this is through the action of 5-methylcytosine DNA glycosylase, which removes the methylcytosine from DNA leaving the deoxycytosine 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 demethylation 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. 1A) and found to have incision activity against plasmid DNA methylated with either MspI or SssI methylase, but no activity against unmethylated plasmid (Fig. 1B 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
rescued the silencing phenotype of the ro1 DNA substrates (Fig. 1Agius et al. (Fig. 1 to lysine and the mutated ROS1 (mROS1) was fused with MBP as supporting information on the PNAS web site), was changed ROS1 subfamily of DNA glycosylases (Fig. 6, which is published Wild-type ROS1 (wtROS1) but not mutated ROS1 (mROS1) is functional in plants and have nicking activity on methylated plasmid DNA. ( ) 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 rasT 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 rasT seedlings transformed with wtROS1 or mROS1. Seeds of wild type, rasT, and rasT 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. 1A). mROS1 had no incision activity against any of the DNA substrates (Fig. 1B and C). When ectopically expressed in the rasT mutant, wild-type ROS1 (wtROS1) but not mROS1 rescued the silencing phenotype of RD29A-LUC and 35S-NPTII transgenes (Fig. 1D 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 (kcat/KM) 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 (GGCC) and CpG (CCGG) contexts (Fig. 2A). 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. 2B 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. 2B). 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. 2B Right). Incubation of the control protein mROS1 with the methylated CpXpG substrate failed to generate cleavage product (Fig. 2C). 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 NaBH4 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. 2D). 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 incubations were identified by the size of fragments on polyacrylamide gels (2E). 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. 2E). 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. 3A and B). In contrast, the G:T mismatch repair
activity of MBD4 was 50–60 times higher than its DNA 5-methylcytosine glycosylase activity (Fig. 3A 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. 3C). 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. 4A). The TAP tag allowed for a partial purification of ROS1 and mROS1 protein complexes from the transgenic plants (Fig. 4B). The addition of NTAPi-ROS1 to the demethylation reaction mixture produced two products, P1 and P2 (Fig. 4C). The cleavage product P2 indicates G→T elimination by ROS1, whereas the cleavage product P1 is consistent with C→T elimination. The NTAPi-mROS1 protein preparation only yielded a weak band corresponding to P1 (Fig. 4D). The results support a G→T, C→T-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
DNA methylation but the methylation levels in the rosl 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 transgene expression compared to the wild type, but the methylation levels in the endogenous RD29A promoter (Fig. 5) plants suggested that ROS1 functions in erasing DNA methylation but the methylation levels in the rosl 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. 5A 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% CpxP methylation, and 2.5% CpxXP methylation at the endogenous RD29A promoter, and 9.4% CpG methylation, 10% CpxP methylation, and 6.5% CpxXP methylation at the transgene RD29A promoter (Fig. 5C and D Left and Fig. 7, which is published as supporting information on the PNAS web site). As reported previously, rosl mutant plants had very high levels of DNA methylation at both the endogenous and transgene RD29A promoters (Fig. 5C and D Right). In the ROS1 overexpression line, there was little or no methylation at both the endogenous and transgene RD29A promoters (Fig. 5C 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 glycosylase 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 CpG sequences. A 5-methylcytosine DNA glycosylase partially purified from HeLa cells exhibited similar preferences (16, 17). In vitro, ROS1 has a preference for removing 5mC in CpxP 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 rosl 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 5mC in CpxP sequence context (30). It is possible that the activity of ROS1 in vitro is determined not only by the CpxG or non-CpxG context but also by other neighboring sequences. Alternatively, the different assay conditions used in the two independent studies may have caused the
plasmids were methylated with Maxi-plasmid purification kit (Qiagen). Twenty micrograms of plasmid DNA was fraction of remaining covalently closed-circular DNA by the nicking assay, a reaction mixture (20 μl) containing nicking buffer and 50 nM NaBH₄. After incubation, the samples were mixed with SDS–PAGE loading buffer and denatured at 100°C for 5 min, and electrophoresis was carried out 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 Applied Science).

Cross-linking reaction with NaBH₄. 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, 50 nM NaBH₄, and 3 μg of 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 unitsMspI 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 accounted for in all quantifications (33).

Oligonucleotide DNA duplex cleavage assay. Polycrylamide gel-purified 40-mer oligonucleotides (Oligo 1, 5′-GGGAAGAGGGAAGGAC5mCCGGGAAAAGGGA-3′; Oligo 2, 5′-CCCTCTCCCTCTCTGCGGTT-CCCTCTCTCCCTCTCCCTCTCCCT-5′) containing external or internal 5mC 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 polycrylamide gel-purified 36-mer oligonucleotide (5′-GGGATTTCTCGAGGTGGGACGGTATC-CGATGCGGGCT3′) (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 chemiluminescent substrate CSPD (Roche Applied Science).

Activity on mismatched DNA. The following oligonucleotides were used: T oligo, 5′-GACTGCGCTCTCCCGGGCGAGGATGGCC-CCC-3′; G oligo, 5′-GGGCACCTTCCGCGGGAGGACGGCAGTC-3′; Oligo 3, 5′-GGGAAGAGGAAAGGAC5mCCGGGAAAAAGGGA-3′ Oligo 4, 5′-CCCTCTCCCTCTCTGCGGTTCCCTCTCTCCCTCTCCCTCTCCCTCTCCCTCTCCCT-5′. The T oligo and Oligo 4 were labeled at 5′ end by using T4 polynucleotide kinase in the presence of [γ-32P]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-32P]ATP was removed from labeled duplex by passage through a G-25 microcolumn (Amersham Pharmacia).

Labelled 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 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-32P]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 (5 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 m**ROS1** cDNAs were cloned by digestion with EcoRI and XhoI and inserted into pEntry3C vector (Invitrogen). These clones were used for cloning **ROS1** and m**ROS1** in pMDC32 for overexpression (36) and NTAPI destination binary vector (37) for generating NTAPI fusion. The recombinant 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 m**ROS1** cDNA overexpression constructs and the NTAPI-**ROS1** and NTAPI-m**ROS1** constructs were transformed into both wild-type and **ros1** mutant background. Transgenic lines were selected on MS medium supplemented with hygromycin (for **ROS1** and m**ROS1** overexpression) or glufoxamate ammonium (for NTAPI-**ROS1** and NTAPI-m**ROS1** 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.

We are grateful to Teresa Roldan-Arjona (Universidad de Córdoba, Córdoba, Spain) for providing us full-length **ROS1** cDNA, 8-oxoG oligo, and ArOGG1 clone and Xiaodong Cheng (Emory University, Atlanta, GA) for providing the MBD4 clone. This work was supported by National Institutes of Health Grant R01GM070795 (to J.-K.Z.).