Cell, Vol. 111, 803–814, December 13, 2002, Copyright ©2002 by Cell Press

ROS1, a Repressor of Transcriptional Gene Silencing in *Arabidopsis*, Encodes a DNA Glycosylase/Lyase

Zhizhong Gong,[4] Teresa Morales-Ruiz,[2] Rafael R. Ariza,[2] Teresa Roldán-Arjona,[2] Lisa David,[1] and Jian-Kang Zhu[1,3]
[1]Department of Plant Sciences
University of Arizona
Tucson, Arizona 85721
[2]Departamento de Genética
Universidad de Córdoba
14071 Córdoba
Spain

Summary

Mutations in the *Arabidopsis ROS1* locus cause transcriptional silencing of a transgene and a homologous endogenous gene. In the *ros1* mutants, the promoter of the silenced loci is hypermethylated, which may be triggered by small RNAs produced from the transgene repeats. The transcriptional silencing in *ros1* mutants can be released by the *ddm1* mutation or the application of the DNA methylation inhibitor 5-aza-2′-deoxycytidine. *ROS1* encodes an endonuclease III domain nuclear protein with bifunctional DNA glycosylase/lyase activity against methylated but not unmethylated DNA. The *ros1* mutant shows enhanced sensitivity to genotoxic agents methyl methanesulfonate and hydrogen peroxide. We suggest that ROS1 is a DNA repair protein that represses homology-dependent transcriptional gene silencing by demethylating the target promoter DNA.

Introduction

Epigenetic control of gene expression plays vital roles in development as well as in cellular responses to viruses, transposons, and transgenes in eukaryotes (Habu et al., 2001; Vaucheret and Fagard, 2001; Moazed, 2001; Richards and Elgin, 2002). The silencing of transgenes and endogenous genes in plants can occur at either the transcriptional (transcriptional gene silencing, TGS) or posttranscriptional (posttranscriptional gene silencing, PTGS) levels. Genetic analysis indicated that PTGS in diverse organisms is triggered by double-stranded RNAs (DsRNAs) (Zamore, 2002; Matzke et al., 2001). DsRNAs are cleaved into small sense and antisense RNAs (21–25 nt) by a double-stranded RNA specific ribonuclease III, Dicer (Bernstein et al., 2001). These small RNAs (smRNAs) are proposed to interact with other proteins to form an RNA-induced silencing complex (RISC) and target homologous mRNAs for degradation (Zamore, 2002).

TGS of transgenes is often associated with a high copy number of the transgenes, or insertion of the transgenes in certain genomic regions (Vaucheret and Fagard, 2001). Some transgenes driven by endogenous promoters can cause the methylation and transcriptional silencing of the corresponding endogenous genes in trans, which may be mediated by RNAs (Mette et al., 2000). Evidence for RNA-directed DNA methylation (RdDM) came from a study on an RNA viroid in tobacco (Wassenegger et al., 1994). It was shown that viroid cDNAs integrated into the host genome became methylated only when viroid RNA-RNA replication had taken place. In other studies, cytoplasmically replicated virus RNAs were found to specifically induce the methylation of its homologous DNA integrated in the host plant genome, suggesting that the signals for triggering nuclear DNA methylation come from the cytoplasm (Jones et al., 1998). DsRNAs can either initiate TGS by triggering the hypermethylation of homologous promoter DNA (Mette et al., 2000) or cause PTGS by targeting the transcribed region of genes (Dalmay et al., 2000). Recent work shows that smRNAs originated from DsRNAs may provide the signal that triggers RdDM (Mette et al., 2001).

Several genes that maintain TGS in plants have been cloned recently. DDM1, a SWI2/SNF2-like protein, regulates both DNA methylation and TGS (Jeddeloh et al., 1998, 1999; Vongs et al., 1993). MOM1 is another putative chromatin remodeling protein that participates in TGS, but *mom1* mutations release the TGS of transgenes without reducing methylation (Amedeo et al., 2000). In addition, histone H3 methyltransferase and DNA methyltransferase have been shown to function in TGS in *Arabidopsis* (Jackson et al., 2002; Lindroth et al., 2001; Bartee et al., 2001).

In contrast to the substantial progress toward understanding how silent (trans)genes are maintained in silenced states, little is known about how active (trans)genes are maintained in active states or kept from being silenced. In this study, we isolated an *Arabidopsis* mutation, *ros1*, which causes transcriptional gene silencing of an active transgene and an endogenous gene with a homologous promoter. The *ros1* mutation triggers hypermethylation in the promoter of the silenced loci, but does not alter the methylation levels in rDNA, centromeric DNA, or transposon DNA regions. The transgene repeats produce similar amounts of smRNAs in the wild-type and *ros1* mutant plants. Removing the transgene repeats from *ros1* mutant plants regains the expression of the homologous endogenous gene, suggesting that smRNAs produced by the transgene may act as a trigger for DNA hypermethylation and TGS in *ros1* mutants. The silenced state of *ros1* mutants can be completely released by the application of the DNA methylation inhibitor 5-aza-2′-deoxycytidine and partially released by the *ddm1* mutation. Some *ros1* mutants show developmental abnormalities in later generations. The *ROS1* gene was cloned and predicted to encode a nuclear protein of 1393 amino acids containing an endonuclease III domain. The *ros1* mutant shows enhanced sensitivity to hydrogen peroxide or the DNA alkylating agent methyl methanesulfonate, suggesting that ROS1 also functions in DNA repair. Our results suggest that ROS1 is a critical repressor of smRNA triggered DNA hypermethylation

[3]Correspondence: jkzhu@ag.arizona.edu
[4]Present address: College of Biological Sciences, China Agricultural University, Beijing 100094, China.

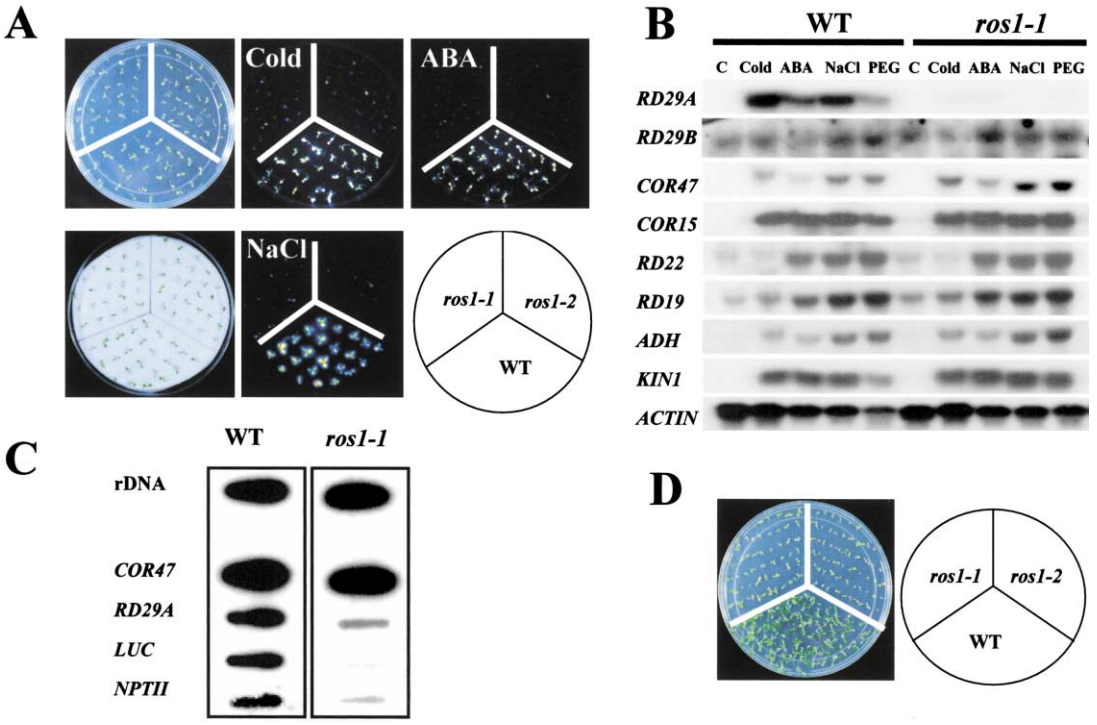

Figure 1. Transcriptional Gene Silencing in *ros1* Mutant Plants

(A) Expression of the *RD29A-LUC* transgene in *ros1* mutants. Wild-type, *ros1-1*, and *ros1-2* seedlings grown on MS agar plates for one week were treated with cold (4°C) for 24 hr, 100 μM ABA for 3 hr, or transferred to a filter paper soaked with 300 mM NaCl for 5 hr. Luminescence images were taken after each treatment. WT: wild-type.

(B) The endogenous *RD29A* gene is silenced in *ros1-1* mutant plants. Steady-state transcript levels of *RD29A* and other genes in wild-type and *ros1-1* mutant plants were determined by RNA blot analysis. Plants were either untreated (C) or treated with cold (4°C) for 24 hr, 100 μM ABA for 3 hr, 300 mM NaCl for 5 hr, or 30% PEG for 5 hr. The *ACTIN* gene was used as a loading control. WT: wild-type.

(C) Nuclear run-on assays of *RD29A-LUC*, *NPTII* (driven by the CaMV 35S promoter), and *RD29A* genes. *COR47* and rDNA were used as controls.

(D) Kanamycin sensitivity of *ros1* mutants. The seeds of wild-type (WT), and *ros1-1* and *ros1-2* mutants were planted on MS medium containing 35 mg/l kanamycin, and were cultured for two weeks.

and transcriptional gene silencing. Recombinant ROS1 protein is able to incise methylated but not unmethylated DNA in vitro, suggesting that the anti-silencing activity of ROS1 may be achieved by demethylation of the promoter DNA.

Results

Identification of *ros1* Mutations that Cause the Silencing of a Transgene and a Homologous Endogenous Gene

We developed a system to screen for *Arabidopsis thaliana* mutants with deregulated expression of the *RD29A-LUC* transgene, which consists of the firefly luciferase reporter under control of the ABA, drought, salt, and cold stress-responsive *RD29A* promoter (Ishitani et al., 1997). Expression of the *RD29A-LUC* transgene in our transgenic *Arabidopsis* line has been very stable for many generations over the last seven years. The *RD29A-LUC* plants (referred to as wild-type) were mutagenized with ethyl methanesulfonate (EMS) and mutants with abnormal bioluminescence in response to cold, osmotic stress, or ABA treatment were screened from the M2 population (Ishitani et al., 1997). One group of mutants failed to emit significant bioluminescence after treatment with low temperature, ABA, or osmotic stress. Two allelic mutants from this group were selected for detailed characterization. Figure 1 shows the luminescence images of the wild-type and mutant seedlings before treatment and after being treated with cold, ABA, or NaCl. Compared with the wild-type, all of the mutant seedlings emitted virtually no luminescence (Figure 1A). This non-luminescent phenotype is stable from the young seedling stage to late in development (data not shown). Subsequent studies led us to believe that the mutations caused the silencing of the *RD29A-LUC* transgene and the endogenous *RD29A* gene. The wild-type gene defined by the mutations was therefore named as *ROS1* for *R*epressor *O*f *S*ilencing *1*. The *ros1* mutants were each backcrossed with the wild-type plants. F1 plants resulting from the crosses between *ros1* mutant and wild-type plants showed a wild-type luminescence phenotype, and selfed F2 progenies segregated approximately 3:1 for wild-type:mutant, indicating that the *ros1* mutations are recessive, and in a single nuclear gene (data not shown). Crosses between the two mutants revealed that they are allelic (thus referred to as *ros1-1 and ros1-2*) (data not shown).

To determine whether the expression of the endoge-

nous *RD29A* and other stress-responsive genes is affected by the *ros1* mutation, total RNA from *ros1-1* mutant and wild-type plants treated with cold, NaCl, PEG, or ABA was analyzed by Northern hybridization. Figure 1B shows that *RD29A* expression under all treatments was almost completely blocked by the *ros1* mutation. In contrast, expression of the control gene *ACTIN* or other stress responsive genes representing various stress gene regulation pathways was not affected at all. Identical results were obtained with the *ros1-2* mutant (data not shown). These data suggest that the block of expression of the *RD29A-LUC* transgene and the endogenous *RD29A* gene in *ros1* mutants is because of gene silencing and not a defect in stress signaling. This notion is further supported by our later finding that *ros1* mutations also block the expression of the *NPTII* gene (Figures 1C and 1D), which is unrelated in sequence to *RD29A-LUC* or *RD29A*.

The Gene Silencing Caused by *ros1* Occurs at the Transcriptional Level

In order to differentiate PTGS from TGS, nuclear run-on assays were carried out (Dorweiler et al., 2000). Figure 1C shows that the pre-mRNA transcript levels of both the *RD29A* gene and the *LUC* gene are much lower in *ros1-1* than in wild-type plants. In comparison, there was no difference between *ros1-1* and wild-type plants in the pre-mRNA transcript level for the *COR47* gene that has the same stress-responsive cis-elements as in *RD29A*. These results indicate that the gene silencing in *ros1* mutants occurs at the transcriptional level.

Typically, TGS is related to a chromosomal region, and not to a specific promoter (Rine, 1999). We hypothesized that other genes that are adjacent to the *LUC* transgene or the endogenous *RD29A* gene may also be silenced. To determine whether the *NPTII* gene (linked to the *LUC* gene in the inserted T-DNA) is silenced, we planted *ros1* mutant and wild-type seeds on Murashige-Skoog (MS) nutrient medium supplemented with 35 mg/l kanamycin. As shown in Figure 1D, wild-type plants were resistant to kanamycin whereas the mutant plants were very sensitive and did not grow at all. Nuclear run-on assays show that *NPTII* gene transcription in *ros1* mutants is much lower than that in wild-type plants (Figure 1C). These results indicate that the entire T-DNA region including both the *LUC* and *NPTII* genes is silenced. However, the *RD29B* gene, which is adjacent to the endogenous *RD29A*, is not silenced (Figure 1B).

The *ros1* Mutation Leads to DNA Hypermethylation Specifically in the Promoter of the Silenced Loci

In *Arabidopsis*, the release of TGS by *ddm1* mutations is correlated with reduced DNA methylation (Jeddeloh et al., 1998). However, mutations in *MOM1* release the silencing of hypermethylated genes without noticeable changes in DNA methylation (Amedeo et al., 2000). To determine whether there is any methylation change in *ros1* mutants, we sequenced the upper strand of a 188 bp region of the *RD29A* promoter after bisulfite treatment. Compared with that of the wild-type, the promoter DNA in *ros1* mutant plants is substantially more heavily methylated (Figure 2A). Four out of seven CG sites in this region are completely methylated. Noticeably, no cytosine residue is methylated in the −85 to −150 region in the wild-type, but these are heavily methylated in the *ros1-1* mutants. To determine whether both the *RD29A-LUC* transgene and the *RD29A* endogenous gene are hypermethylated in the mutant, we carried out Southern analysis using two methylation sensitive restriction enzymes, BstUI (CGCG) and MluI (ACGCGT), and the *RD29A* coding sequence and the luciferase gene as probes. The CGCG/ACGCGT site is localized in the *RD29A* promoter region (Figure 2A). As shown in Figure 2B, the ACGCGT site was completely digested by MluI in the wild-type but not digested in *ros1-1* mutant when the luciferase gene was used as probe, and the CGCG site was completely digested by BstUI in the wild-type but not in *ros1-1* when the *RD29A* coding sequence was used as probe. These results show that the *ros1-1* mutation causes DNA hypermethylation in the promoter region of both the *RD29A-LUC* transgene and the *RD29A* endogenous gene. We did not find any methylation changes in the coding regions of *RD29A* or *LUC* as revealed by digestion with methylation sensitive enzymes *Hpa*II (CG methylation) and *Msp*I (CNG methylation) (data not shown).

We also checked the DNA methylation status in rDNA, centromeric DNA, and two retrotransposons (Jackson et al., 2002). As shown in Figure 2C, no differences were detected in rDNA, centromeric DNA, or the retrotransposons between *ros1* mutant and wild-type plants. Because the rDNA, centromeric DNA, and retrotransposon regions are already hypermethylated in the wild-type genome, any methylation-enhancing effect of *ros1* on these regions may be difficult to detect using methylation sensitive restriction enzymes. The *ddm1* mutation causes global DNA hypomethylation in the *Arabidopsis* genome. If the *ros1* mutations could cause global DNA hypermethylation, it should be easier to detect this in the *ros1/ddm1* double mutant. As shown in Figure 2B, digestion with methylation sensitive restriction enzymes did not reveal any difference between *ddm1* and the *ros1/ddm1* double mutant in the methylation of rDNA, centromeric DNA, and or the retrotransposons. These results suggest that *ros1* mutation causes DNA hypermethylation in specific DNA regions but does not cause global DNA hypermethylation.

DNA Methylation Inhibitor and the *ddm1* Mutation Release Transcriptional Gene Silencing in *ros1* Mutant Plants

The cytosine methylation inhibitor 5-aza-2′-deoxycytidine (5Aza-dC) has often been used to study the effect of DNA methylation (Chen and Pikaard, 1997). As shown in Figure 3A, there is no difference between the *RD29A-LUC* expression of *ros1-1* and wild-type seedlings after the 5Aza-dC treatment. When three-week-old seedlings were treated with 5Aza-dC, newly grown roots in *ros1-1* were found to have a strong luminescence response similar to that in wild-type plants (data not shown). We tested whether the recovery of *RD29A-LUC* expression in *ros1* by 5Aza-dC could be maintained after the inhibitor is removed. One week after 5Aza-dC was removed, *RD29A-LUC* expression in the inhibitor-treated *ros1-1*

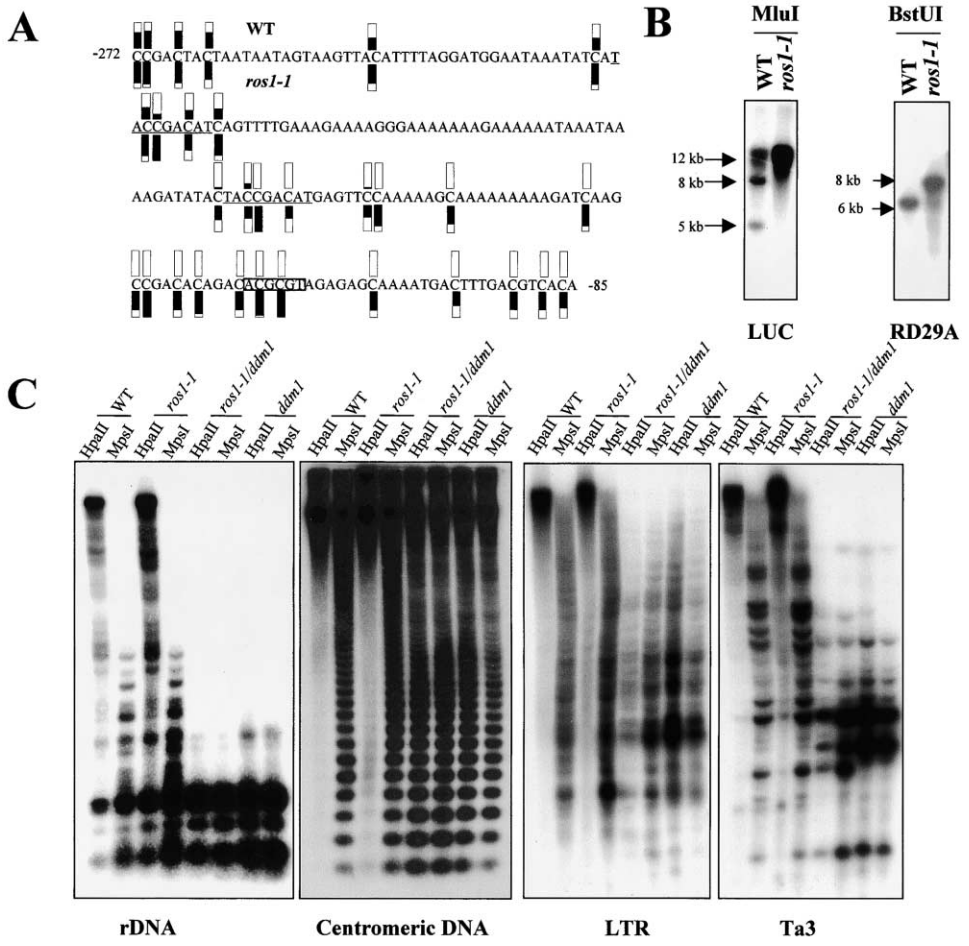

Figure 2. DNA Methylation Status in *ros1* Mutant Plants

(A) Genomic sequencing of the upper strand of *RD29A* promoter core region (−272–85) after bisulfite treatment in *ros1-1* (below sequence) and wild-type (above sequence) plants. The percentage of cytosine methylation is indicated by the extent of black bars and no methylation is indicated by white bars. Two binding sites of the CBF/DREB transcriptional activators are underlined.

(B) Methylation status of promoter regions in *RD29A-LUC* transgene and *RD29A* endogenous gene was analyzed by using methylation sensitive enzymes MluI (A^mCG^mCGT) and BstUI ($^mCG^mCG$). The ACGCGT/CGCG site is boxed in (A).

(C) Methylation status of rDNA, centromeric DNA, and retrotransposons in wild-type, *ros1*, *ddm1*, and *ros1*/*ddm1*double-mutant plants. DNA from wild-type (WT), *ros1-1*, *ddm1*, and *ros1*/*ddm1*double-mutant plants was digested with methylation sensitive enzymes *Hpa*II (CG methylation) or *Msp*I (CNG methylation), and hybridized with an rDNA, a 180 bp centromeric repeat, an Athila long terminal repeat (LTR), or a Ta3 probe (Jackson et al., 2002), respectively.

seedlings returned to untreated *ros1* level (data not shown). These results show that the release of gene silencing by 5Aza-dC in *ros1* mutant plants cannot be maintained in the absence of the methylation inhibitor.

Because the *ddm1* mutation can reduce DNA methylation in the whole genome, we also tested the effect of *ddm1* on the TGS in *ros1*. As shown in Figure 3B, the luminescence response of the *ros1*/ *ros1::ddm1/ddm1* double mutant was much higher than that of *ros1*/ *ros1::DDM1/DDM1* plants, but was still lower than that of *ROS1*/*ROS1:: DDM1/DDM1* plants. The result suggests a partial release of gene silencing in *ros1* by *ddm1*. Taken together, our data suggest that the *ros1* mutation causes TGS by failing to prevent DNA hypermethylation.

Small RNAs May Act as a Trigger for TGS in *ros1*

Recent studies suggest that small RNAs arising from promoters in transgene repeats could trigger the hypermethylation and silencing of homologous gene promoters (Mette et al., 2001). In *ros1* mutants, both the transgene and the homologous endogenous gene were silenced. We hypothesized that smRNAs may be produced from the *RD29A-LUC* transgene repeats, and the smRNAs subsequently causes the hypermethylation of the *RD29A* promoter in *ros1* mutants. The T-DNA in our *RD29A-LUC* plants is arranged in a complex repeat configuration (data not shown). We tested whether our wild-type and *ros1* mutant plants produce small RNAs from the promoter of the *RD29A-LUC* transgene repeats. As shown in Figure 4A, both the *ros1-1* mutant and wild-type plants produced ~23 bp smRNAs that hybridize with the *RD29A* promoter. The amount of smRNAs is similar in *ros1-1* and wild-type plants. This result shows that smRNAs are produced from the transgene repeats and the *ros1* mutation does not affect the accumulation of these smRNAs.

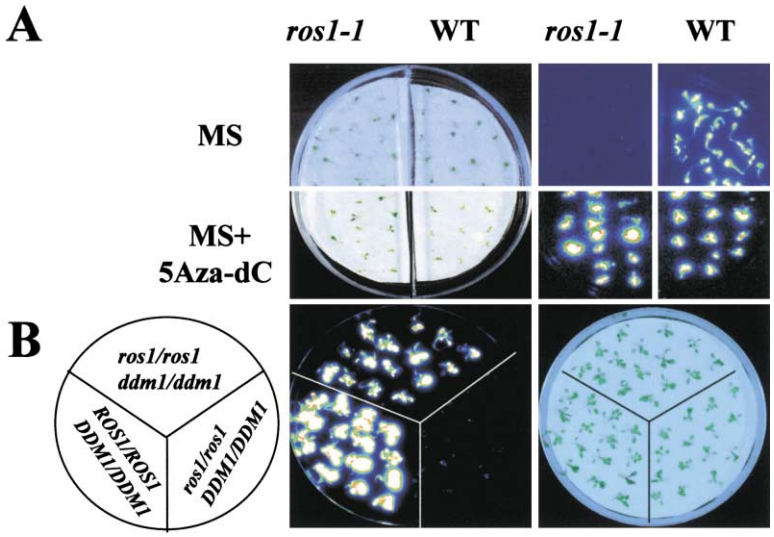

Figure 3. Effect of 5-aza-2′-deoxycytidine (5Aza-dC) and *ddm1* Mutation on *RD29A-LUC* Expression in *ros1-1* Mutant Plants

(A) Seedlings grown in MS medium for three days were transferred to an MS medium containing 50 μM 5Aza-dC. After one week, luminescence images were taken following a treatment with 100 μM ABA for 3 hr.

(B) *RD29A-LUC* expression from the *ros1* mutant, *ros1/ddm1* double-mutant, and the wild-type plants. The luminescence images were taken after a treatment with 300 mM NaCl for 5 hr.

We crossed the *ros1-1* mutant with wild-type plants without the *RD29A-LUC* transgene. From the segregating F2 population, *ros1* mutant plants without the *RD29A-LUC* transgene were selected. As shown in Figure 4B, expression of the endogenous *RD29A* gene was recovered to the wild-type level in these transgene-minus *ros1-1* mutant plants. These transgene-minus *ros1* mutant plants did not produce any smRNAs (Figure 4C), and showed a wild-type level of DNA methylation in the endogenous *RD29A* gene promoter (Figure 4D). The results suggest that smRNAs produced from the transgene repeats may be involved in the promoter DNA hypermethylation and TGS of the endogenous *RD29A* gene.

Epigenetic Effects of the *ros1* Mutation on Plant Development

In *ddm1/som* mutants, plant developmental abnormalities accumulated after inbreeding for more than three generations (Kakutani et al., 1996). In contrast, *mom1* mutants did not show developmental abnormalities even after many generations (Amedeo et al., 2000). In

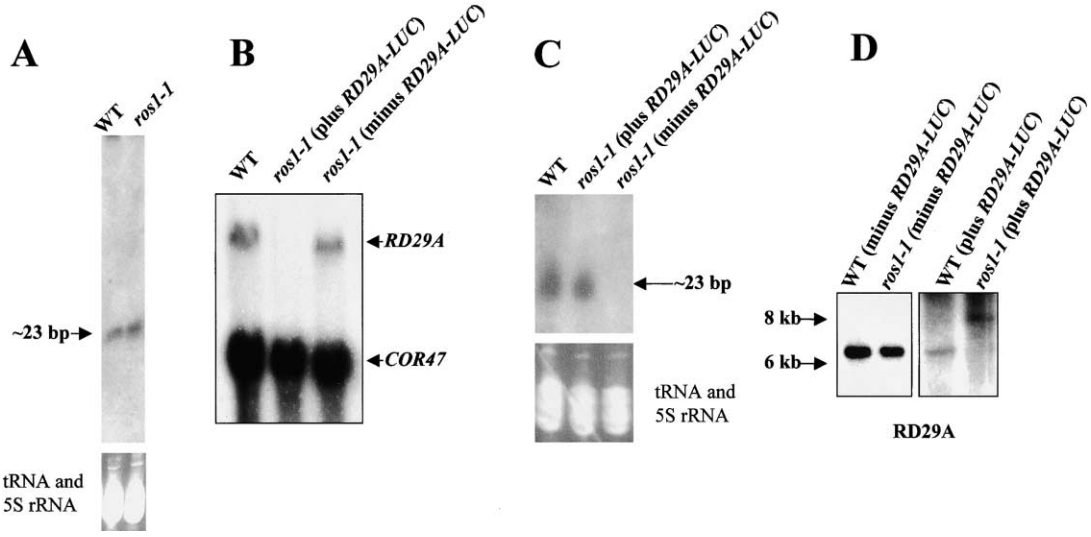

Figure 4. The Silencing Effect of *ros1* Mutation Is Dependent on smRNAs

(A) Detection of small RNAs in *ros1-1* mutant and wild-type plants. Total RNAs were extracted from two-week-old plants. The enriched low molecular weight RNAs were fractioned, blotted, and hybridized with ^{32}P-labeled *RD29A* promoter probe.

(B) Expression of endogenous *RD29A* gene is recovered in the *ros1-1* mutant plants when the *RD29A-LUC* transgene is removed (minus *RD29A-LUC*). Total RNAs extracted from two-week-old plants treated with 100 μM ABA were blotted and hybridized with ^{32}P-labeled *RD29A* and *COR47* gene probes.

(C) Small RNAs were not detected in *ros1-1* mutant plants without the *RD29A-LUC* transgene.

(D) DNA methylation in the endogenous *RD29A* promoter is reduced greatly after the *RD29A-LUC* transgene was removed from the *ros1-1* mutant. DNA from minus transgene *ros1-1* mutant and minus transgene wild-type plants were digested with BstUI and hybridized with ^{32}P-labeled *RD29A* cDNA.

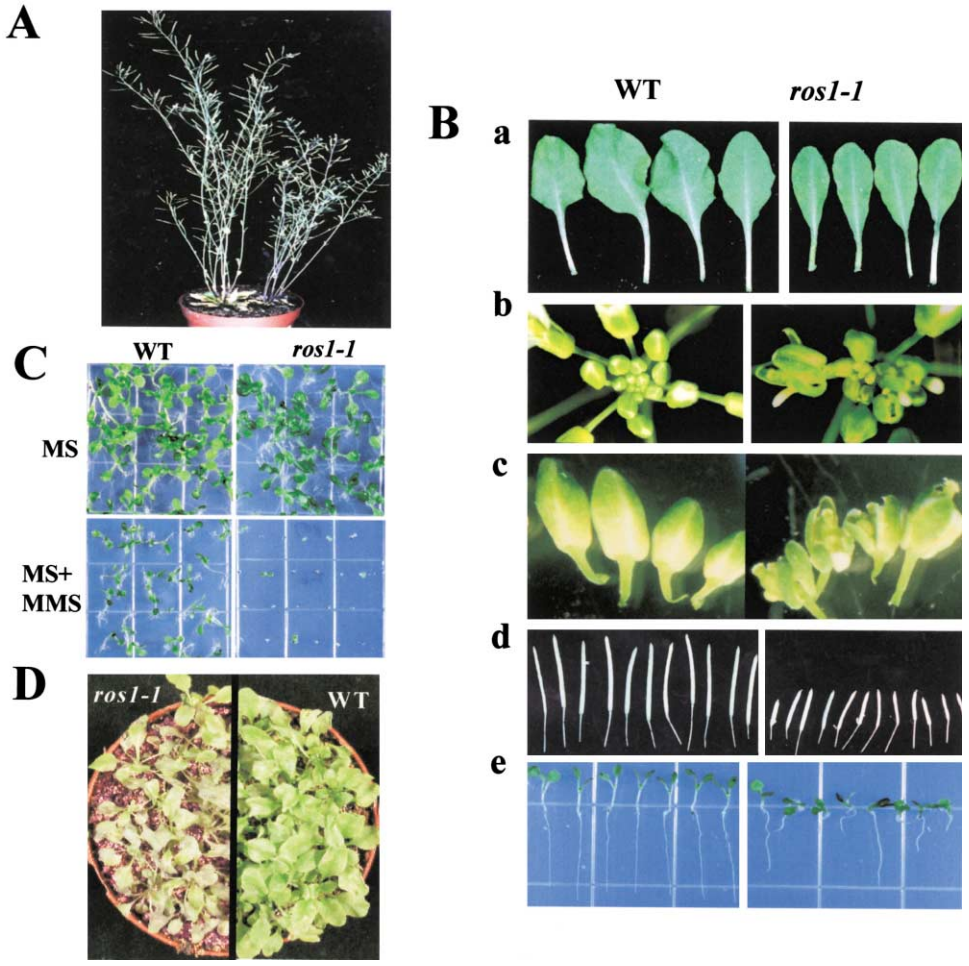

Figure 5. Aberrant Developmental Phenotypes of Some *ros1-1* Plants that Were Selfed for Four Generations

(A) The aberrant plant (right) is reduced in height compared to the wild-type (left).

(B) (a) The leaves of aberrant plants are narrower than those of wild-type plants. (b and c) Altered flower structure in aberrant *ros1* plants (right) as compared to flowers of wild-type plants (left). (d) The siliques of aberrant plants (right) are shorter and contain fewer seeds than those of wild-type plants (left). (e) Seedlings originated from aberrant *ros1* (right) and from wild-type (left) seeds.

(C) The germination of *ros1* mutant seeds is more sensitive to a DNA double-strand break agent methyl methanesulfonate (MMS). The seeds of wild-type (WT) and *ros1-1* mutant plants were plated on MS medium containing 50 ppm MMS and kept under normal growth conditions for 2 weeks. MS nutrient medium without MMS supplementation was used as control.

(D) *ros1-1* mutant plants are more sensitive to hydrogen peroxide. Three-week-old plants were sprayed with 1 mM H_2O_2 and the picture was taken one day later.

ros1-1 mutants, there were no apparent developmental phenotypes in the first three generations. However, from the fourth generation, some mutant plants showed aberrant phenotypes, which include flowering slightly earlier than wild-type plants, abnormal flowers, shorter siliques, and a reduction in height to about 2/3 of wild-type plants (Figures 5A and 5B). The aberrant *ros1-1* plants produced less than 5% of the amount of seeds produced by wild-type plants. However, the seeds from the aberrant *ros1-1* plants weigh approximately 150% as much as the wild-type seeds, suggesting that *ROS1* may affect imprinting (Adams et al., 2000). The progenies of the aberrant plants all appeared abnormal, with shorter hypocotyls and aberrant cotyledons, and the leaves were narrower than those of wild-type plants (Figure 5B). Later generations of these aberrant plants showed more severe aberrant phenotypes. The abnormalities appeared to occur early in development since the entire plants and not just some specific organs displayed the aberrant phenotypes. Some of the developmental abnormalities in *ros1*, such as decreased stature and narrower leaves, are similar to those in the *caf1* mutant (Jacobsen et al., 1999), while the reduced fertility phenotype has also been observed with the *ddm1* mutant (Kakutani et al., 1996).

Map-Based Cloning of the *ROS1* Gene

Initial mapping with selected markers from each of the five *Arabidopsis* chromosomes located *ROS1* to chromosome II. Fine mapping with Simple Sequence Length Polymorphism markers that we have developed delimited *ROS1* to a contig of four BAC clones, F2H17, F1O11, F13K3, and T1J8 (Figure 6A). Candidate open reading frames on these four BAC clones were amplified

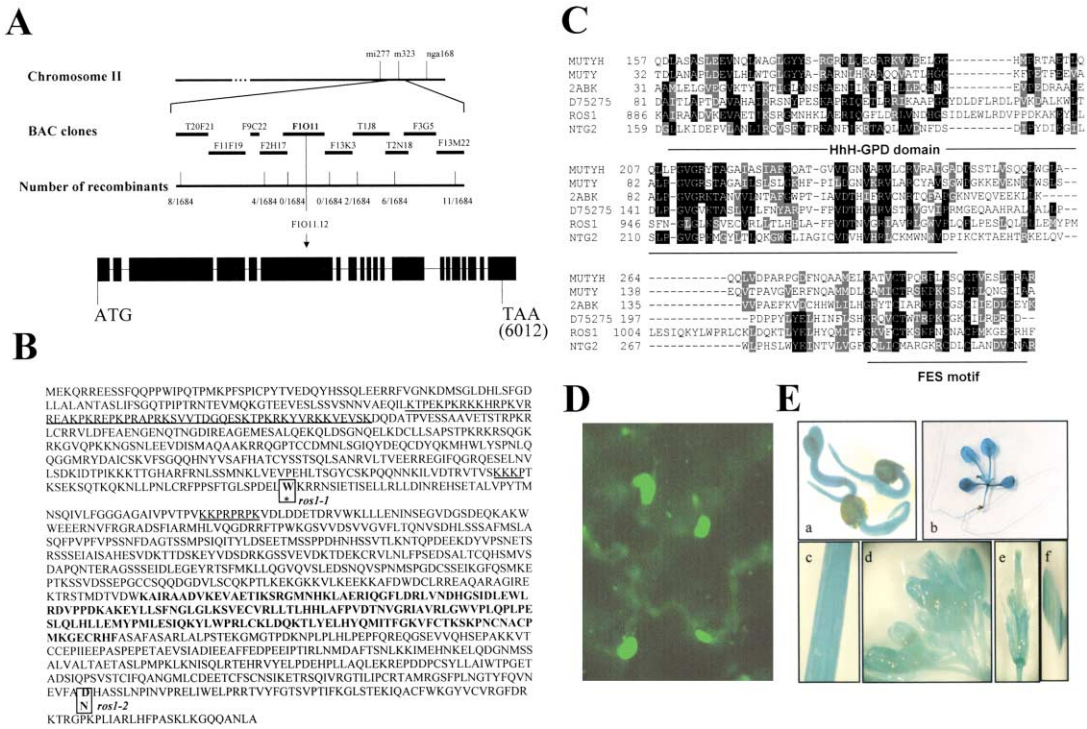

Figure 6. Positional Cloning, Sequence of the Predicted ROS1 Protein, ROS1-GFP Protein Localization, and *ROS1* Promoter::GUS Expression.

(A) Genetic and physical mapping and gene structure of *ROS1* (i.e., *F1O11.12*).

(B) Predicted amino acid sequence of *ROS1* and the positions of *ros1* mutations. Underlined are three putative nuclear localization signals. The region showing similarity to the HhH-GPD superfamily of proteins is highlighted in bold. The mutations in *ros1-1* and *ros1-2* are encircled.

(C) Sequence alignment of the HhH-GPD superfamily of proteins. The conserved HhH-GPD domain and distinct [4Fe-4S] cluster (FES motif) were underlined. The sequences used for the alignment are: MUTY, P17802 (*E. coli*); MUTYH, NP_036354 (human); 2ABK, 1311214 (*E. coli*); D75275 (*Deinococcus radiodurans*); and NTG2, Q08214 (yeast).

(D) ROS1-GFP protein is localized in the nucleus. The picture shows GFP signals in the nuclei of epidermis cells in a leaf of ROS1-GFP transgenic plants.

(E) *ROS1* promoter::GUS expression in various plant tissues. (a) Two-day seedlings. (b) Ten-day-old seedling. (c) Stem. (d and e) Flowers. (f) Silique.

from wild-type as well as *ros1-1* mutant plants and sequenced. The sequence analysis revealed a single nucleotide substitution in the hypothetical *F1O11.12* gene in the *ros1-1*mutant. This mutation (from TGG to TAG) is predicted to change Trp-469 to a premature stop codon (Figure 7B), resulting in an early truncation of the protein and thus may be considered as a null allele. The *F1O11.12* gene from *ros1-2* plants was sequenced and a single nucleotide substitution (from GAT to AAT) was found that would change Asp-1310 to Asn (Figure 7B). This mutation in an independent allele thus confirms that *F1O11.12* is the *ROS1* gene.

ROS1 Encodes a Nuclear Protein with an Endonuclease III Domain

A full-length *ROS1* cDNA was obtained by reverse transcriptase (RT)-PCR. Comparison between the cDNA and genomic DNA sequences revealed that the *ROS1* gene consists of 20 exons and 19 introns (Figure 6A). *ROS1* is predicted to encode a protein of 1393 amino acids with an estimated molecular mass of 156.5 kDa (Figure 6B). ROS1 contains an endonuclease III domain with significant similarities to base excision DNA repair proteins in the HhH-GPD superfamily (Figures 6B and 6C). This family contains a diverse range of structurally related DNA repair proteins including endonuclease III (DNA glycosylase/AP lyase) and MutY (A/G specific adenine glycosylase) proteins (Krokan et al., 1997; Scharer and Jiricny, 2001).

The fact that ROS1 contains a domain highly conserved in the HhH family of DNA glycosylases strongly suggests that one ROS1 function may be repairing damaged DNA in *Arabidopsis*. In order to determine whether ROS1 may function in DNA repair in planta, we tested the response of *ros1* mutants to the genotoxic agent methyl methanesulfonate (MMS), which causes base damages to DNA. The seeds of *ros1-1* and wild-type plants were planted on MS nutrient medium or MS nutrient medium containing 50 ppm MMS. As shown in Figure 5C, the germination of *ros1-1* but not wild-type seeds was decreased by 50 ppm MMS. The *ros1-1* mutant plants were also more sensitive to the oxidizing agent hydrogen peroxide (Figure 5D). The hydrogen peroxide-treated leaves in *ros1-1* but not in the wild-type withered. The results show that *ros1* mutant plants are more sensitive to genotoxic chemicals, and thus suggest a role of ROS1 in DNA repair.

We hypothesized that since the wild-type *ROS1* gene

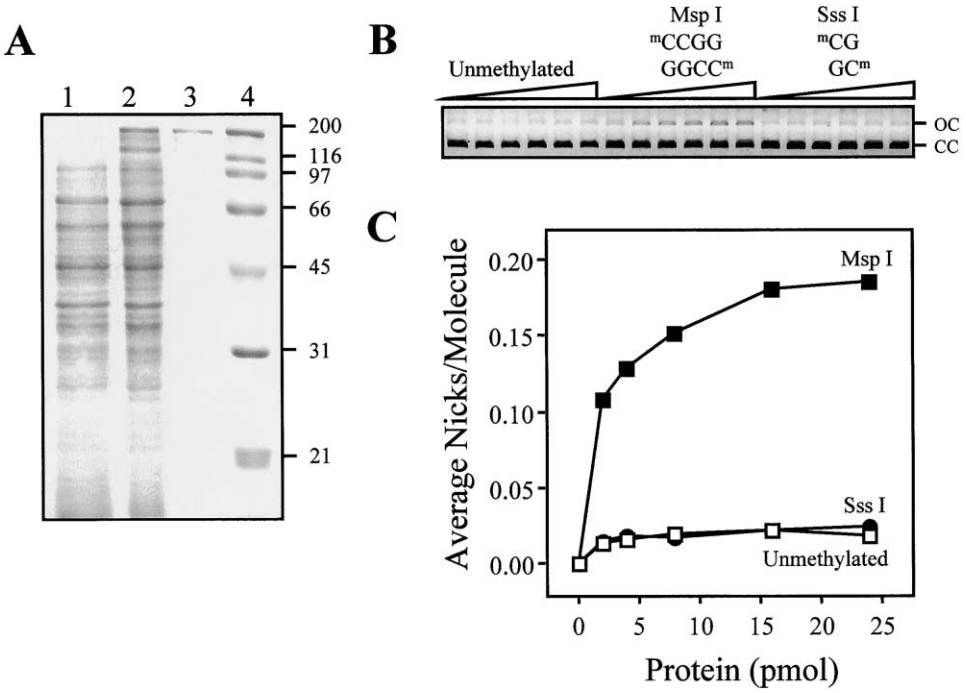

Figure 7. DNA Incision Activity of MBP-ROS1 Protein on Methylated DNA

(A) Purification of recombinant MBP-ROS1 protein. Fractions from various steps were separated on a 10% SDS-polyacrylamide gel stained with Coomassie Blue. Lane 1, uninduced cells; lane 2, induced cells; lane 3, MBP-ROS1 purified through amylose affinity column; lane 4, molecular mass markers with sizes indicated in kDa.

(B) DNA nicking activity. Purified closed-circular (CC) plasmid DNA was incubated with increasing amounts of MBP-ROS1, and the reaction mixtures resolved by electrophoresis. An inverted image of the gel is shown. Control reactions with nonmethylated plasmid were carried out in parallel.

(C) Quantification of the DNA nicking activity. The average number of nicks per plasmid molecule was estimated from the fraction of open-circular form (OC).

suppresses smRNA-triggered TGS and has a role in DNA repair, the ROS1 protein may be localized in the nucleus. The ROS1 protein has four predicted nuclear localization signal sequences (http://psort.nibb.ac.jp) (Figure 6B). To determine the localization of ROS1 protein, we fused ROS1 in-frame to the N terminus of the green fluorescent protein (GFP). The ROS1-GFP fusion protein was expressed in *Arabidopsis* plants under the cauliflower mosaic virus (CaMV) 35S promoter. Green fluorescence imaging of the transgenic plant leaves under a confocal microscope showed that the ROS1-GFP fusion protein is clearly localized in nuclei (Figure 6D).

The gene silencing phenotypes of *ros1* mutants were observed throughout the plant life cycle, which suggests that *ROS1* functions constitutively in all developmental stages. In order to determine the tissue and developmental expression pattern of the *ROS1* gene, *ROS1* promoter was fused with the β-glucuronidase reporter gene (GUS), and the resulting construct was introduced into wild-type *Arabidopsis* plants. GUS expression was observed in all plant tissues examined including both vegetative and reproductive organs (Figure 6E).

Nicking Activity of ROS1 Protein on Methylated DNA

We fused a ROS1 cDNA in-frame to the maltose binding protein (MBP) gene and expressed the fusion protein in *E. coli*. Cells containing the expression plasmid synthesized the MBP-ROS1 fusion protein (167.9 kDa) to a high level upon IPTG induction. The fusion protein was affinity purified by binding to an amylose column (Figure 7A).

Since the results described above suggest that ROS1 might prevent hypermethylation at promoter sequences by demethylation, we decided to test if the enzyme has any activity against DNA containing 5-methylcytosine. The amino acid sequence of ROS1 endonuclease III domain shows a characteristic invariant lysine (lys-953) residue in the HhH motif. This suggests that it is a bifunctional DNA glycosylase/lyase, able both to hydrolize the N-glycosyl bond linking bases to DNA and to cleave the phosphodiester backbone at the site where a base has been removed (Krokan et al., 1997). Therefore, its enzymatic activity may be analyzed by investigating its capacity to generate strand breaks in different DNA substrates. We prepared plasmid DNA methylated in vitro with either SssI methylase, which methylates cytosine residues within the sequence 5′-CG-3′, or MspI methylase, which methylates the external cytosine residues at 5′-CCGG-3′ sequences. As shown in Figures 7B and 7C, recombinant MBP-ROS1 did not have any strand-breaking activity on unmethylated or SssI-methylated plasmid, but was able to incise MspI-methylated DNA. The nicking activity was dependent on the protein concentration, and after 1 hr incubation, 16 pmol protein induced an average of 0.18 strand breaks per plasmid molecule. This activity is in the same range as that of previously characterized endonuclease III homologs on

different damaged substrates (Roldan-Arjona et al., 2000). It is important to note that pBluescript contains 388 CpG sites recognized by SssI methylase and only 13 targets for MspI methylase. Thus, MBP-ROS1 incises DNA containing 5-methylcytosine, but its catalytic activity in vitro is highly sequence-specific.

Discussion

We have shown that recessive mutations in the *ROS1* gene cause transcriptional silencing of two originally active loci, a T-DNA region (very close to marker AthGA-Pab in chromosome III, data not shown) and the endogenous *RD29A* gene (at the bottom of chromosome V). The simple recessive nature of the silencing phenotype suggests that the TGS is not sustained as soon as the *ros1* mutation is removed or rendered heterozygous. DNA bisulfite sequencing of the *RD29A* promoter region indicates that the *ros1* mutation leads to DNA hypermethylation in the affected loci. Interestingly, cytosine residues are more heavily methylated in a defined transcriptional region in *ros1* (Figure 2A), which contains the binding sites of the CBF/DREB transcriptional activators (Ishitani et al., 1997). This and other observations suggest that ROS1 negatively regulates DNA methylation only in some specific DNA regions and not in genomic DNA in general.

Our data show that the *RD29A-LUC* transgene repeats result in the generation of small RNAs from the *RD29A* promoter (Figure 5A). The WS ecotype of *Arabidopsis* has four *PAI* genes at three sites: an inverted repeat at one locus and singlet genes at two unlinked loci (Luff et al., 1999). The *PAI* inverted repeat induces methylation and silencing of the unlinked homologous genes, and this is likely also mediated through smRNAs (Luff et al., 1999). However, the methylation of the unlinked loci is maintained even after the *PAI* inverted repeat is removed, whereas the unlinked endogenous *RD29A* promoter methylation is dependent on the presence of the *RD29A-LUC* transgene. In this regard, the smRNA-dependent promoter DNA methylation and transcriptional silencing described here is more similar to that reported by Mette et al. (2000). Mette et al. (2000) reported that smRNAs generated by a compound *NOSpro* transgene cause silencing of an unlinked *NOSpro* locus, and the silencing is dependent on the transgene repeats. However, the silencing of the *NOSpro* genes occurs in the wild-type background. This is in contrast to the silencing described here, which only occurs in the homozygous *ros1* mutant background. The reason for the different sensitivities between *RD29A* and *NOSpro* to smRNA-induced silencing is unclear at present. It is possible that different genes differ in their sensitivity toward smRNA-induced DNA methylation and silencing. Genes that are resistant to smRNA-induced silencing and are only silenced in the *ros1* mutant background would be targets of ROS1. It is also possible that the difference seen in the two systems has to do with the level of smRNAs, and that ROS1 may also control the silencing of the *NOSpro* transgene. Therefore, in the *ros1* mutant background the frequency of *NOSpro* silencing might be increased. Future experiments will be able to test these possibilities.

The fact that the silencing of *RD29A-LUC* in *ros1* mutant plants can be released partially by the *ddm1* mutation and completely by DNA methylation inhibitor are consistent with a role of ROS1 in preventing DNA hypermethylation. There are two possible mechanisms underlying this function of ROS1. One is that ROS1 may prevent smRNAs from causing DNA methylation. Another possibility is that ROS1 may inhibit the hypermethylation of specific DNA sequences targeted by small RNAs through participation in the demethylation of the DNA. Our data support this latter hypothesis. *ROS1* encodes a protein with motifs conserved in bifunctional DNA glycosylases/AP lyases. DNA glycosylases initiate the base excision DNA repair pathway, which in most organisms removes common base modifications (oxidation, deamination, and alkylation) caused by endogenous agents (Lindahl and Wood, 1999). Usually, they are relatively small monomeric proteins that hydrolytically cleave the glycosylic bond between the target base and deoxyribose, releasing the free damaged base and leaving an apurinic/apyrimidinic (AP) site that must be further processed. According to their catalytic activity, DNA glycosylases are classified into two broad groups: monofunctional DNA glycosylases, which catalyze only hydrolysis of the glycosylic bond, and DNA glycosylases/lyases, bifunctional enzymes with an associated AP lyase activity that cleaves the DNA backbone at the site where a base has been removed (McCullough et al., 1999). Structural studies have revealed that all DNA glycosylases fall into two main structural families. The best characterized is the HhH-GPD family, which includes EndoIII, AlkA, MutY, and hOGG1 (Scharer and Jiricny, 2001). A lysine residue located at the HhH domain is conserved in all the bifunctional enzymes of this family (Krokan et al., 1997) and is also present in ROS1 (Lys-953).

The ability of recombinant MBP-ROS1 protein to induce strand breaks in DNA containing 5-methylcytosine suggests that ROS1 may be directly involved in DNA demethylation through a base excision repair mechanism. A role for DNA glycosylases in genome demethylation during cell differentiation in vertebrates has been previously suggested (Jost et al., 1995). Although the observed strand breaks might reflect excision of mispaired thymine residues arisen by spontaneous 5-methylcytosine deamination, the absence of nicking activity on a heavily methylated plasmid at CpG sequences seems to rule out this possibility. The significance of this strong sequence preference for the in vivo activity of the protein remains to be determined and will require a complete characterization of the substrate specificity of the enzyme. It should be pointed out that the *RD29A* promoter hypermethylation pattern observed in *ros1-1* mutant plants also includes CpG sequences. The sequence specificity of ROS1 in vivo may be affected by its potential interaction with smRNAs and other proteins.

The genome of *Arabidopsis* encodes several other proteins belonging to the HhH family of DNA glycosylases, all of them with similar DNA repair activities to homologs found in bacteria, fungi, or animals (Garcia-Ortiz et al., 2001; Roldan-Arjona et al., 2000). However, there are several characteristics that make ROS1 an atypical DNA glycosylase. It is much bigger (1393 amino acids) than typical DNA glycosylases, which are in the

200–400 amino acids range. The similarity to DNA glycosylases is limited to the endonuclease III domain, and the only recognizable feature in the rest of the sequence is a region rich in basic residues, which displays a weak similarity to H1 histones. A database search revealed three other large *Arabidopsis* proteins that are similar to ROS1 in the endonuclease III domain and also with an N-terminal basic region (data not shown). One of them is DME (Choi et al., 2002). *DME* is required for endosperm gene imprinting and its ectopic expression induces *MEA* expression and nicks the *MEA* promoter. Interestingly, DME may function by a mechanism other than demethylation of the *MEA* promoter since no 5-methylcytosine residues were found in the promoter (Choi et al., 2002).

ROS1 in *Arabidopsis* may function as a regulator of smRNAs-triggered epigenetic control of gene expression and development. The accumulated abnormal phenotypes in the later generations of *ros1* mutants indicate that some genes important in development must be affected by the loss of *ROS1* function. The *Arabidopsis ddm1* and *ddm2* mutations also lead to developmental abnormalities in later generations. However, the accumulated developmental phenotypes in *ddm1* and *ddm2* are associated with DNA hypomethylation (Ronemus et al., 1996; Kakutani et al., 1996), whereas the aberrant phenotypes in *ros1* mutant plants may be associated with DNA hypermethylation specifically in some genes.

The fact that *ros1* mutants were hypersensitive to DNA base damage reagents indicates one of the in vivo functions of ROS1 is to repair damaged DNA. The repair of DNA damage is an important step during chromatin assembly and requires both the recognition of altered DNA structures and the recruitment of repair proteins to the damage sites (Lindahl and Wood, 1999; Hu et al., 2001). After repair, the chromatin structure of repaired DNA must be reassembled in order to faithfully restore preexisting structures, especially in transcribed regions. Recent studies have revealed a mechanistic connection between gene silencing or chromatin remodeling factors and DNA repair proteins. For example, the mammalian TIP60 histone acetylase complex (Ikura et al., 2000) and the *Drosophila* RCAF complex (Tyler et al., 1999) are involved in chromatin remodeling as well as in DNA repair. Our results suggest that a DNA repair factor can serve as a repressor of smRNA-triggered DNA hypermethylation and TGS.

Experimental Procedures

Plant Growth, Mutant Isolation, RNA and DNA Blot Analysis, DNA Methylation Assays

Arabidopsis thaliana (ecotype C24) expressing the chimeric *RD29A-LUC* reporter gene (referred to as wild-type in this study) was mutagenized with ethyl methanesulfonate. Mutant screening, plant growth, and RNA analysis were as described (Ishitani et al., 1997). Nuclei were isolated from two-week-old seedlings treated with 100 μM ABA for 3 hr. Nuclear run-on assays were carried out as described (Dorweiler et al., 2000). DNA methylation was determined by Southern blot analysis using methylation sensitive restriction enzymes or by sequencing the genomic DNA after bisulfite treatment using the CpGenome DNA modification kit (Intergen). A total of 15 clones were sequenced for each genotype. Mutants were backcrossed to the wild-type for four times to eliminate other mutations from the genetic background. Seedlings were grown on Murashige and Skoog (MS) nutrient medium with 0.6% agar under constant white fluorescent light at 22 ± 2°C. For 5-aza-2′-deoxycytidine treatment, seedlings grown for three days or three weeks were transferred to MS liquid medium containing 50 μM 5Aza-dC (Sigma). Seedlings were subjected to luciferase imaging after being treated with 100 μM ABA for 3 hr. Detection of small RNAs was as described (Mette et al., 2000). Briefly, total RNAs were isolated from two-week-old plants grown in soil and small RNAs were enriched by precipitation with 5% PEG (MW 8000), 0.5 M NaCl. The enriched small RNAs were separated on a 15% polyacrylamide-7 M urea gel in 1 × TBE buffer. The 21-mer oligo-DNA was loaded as markers. RNAs were transferred to membrane and the filter was hybridized with ^{32}P-labeled *RD29A* promoter probe (~650 bp) at 33°C in perfectHyb plus hybridization buffer (Sigma). The filter was washed two times with 2XSSC, 0.1% SDS at 50°C for 15 min.

Positional Cloning

For genetic mapping, the homozygous *ros1-1* mutant in the C24 background with *RD29A-LUC* transgene was crossed to the wild-type of the Columbia ecotype without the *RD29A-LUC* transgene. The F2 population was screened for *ros1* mutants based on luminescence imaging and PCR genotyping for the presence of *RD29A-LUC*. Simple sequence-length polymorphism (SSLP) markers were developed and used for mapping. Using SSLP markers, *ros1* was first mapped to chromosome 2 between the markers mi227 and nga168. Markers T20F21-1, F2H17-1, F1O11-1, F13M22-1, T2N18-1, T1J8-1, and F13K3-1 were then used to narrow down the *ros1* mutation to within the following four BAC clones: F2H17, F1O11, F13K3, and T1J8. To identify the *ros1* mutation, candidate genes from wild-type and *ros1* mutant plants were sequenced.

Localization of ROS1-GFP Fusion Protein and Analysis of *ROS1* Promoter-GUS Expression

Arabidopsis poly(A) RNAs from seedlings were reverse transcribed with a 21-mer oligo(dT) primer and were used as templates for PCR amplification of *ROS1* cDNA by using the following primers: ROS1GFP-F: 5′-CCGCTCGAGTCAGAAATGGAGAAACAGAGGAGA GAAG ROS1GFP-R: 5′-GGAATTCAGGCGAGGTTAGCTTGTTGTCC CTTC. The resulting PCR fragment was cloned and sequenced, and inserted into the binary vector pEZTNL (kindly provided by Dr. David W. Ehrhardt) downstream from the CaMV 35S promoter. *Agrobacterium* strain LBA4404 containing this *ROS1-GFP* translational fusion was introduced into *Arabidopsis*.

The promoter region (−25 to −1565 from first ATG) of the *ROS1* gene was PCR-amplified and inserted into the pCAMBIA1380 binary vector. This *ROS1* promoter-*GUS* construct was introduced into *Agrobacterium* strain GV3101 and transformed into wild-type *Arabidopsis*, and 27 independent lines of hygromycin-resistant transgenic plants were obtained and analyzed.

ROS1 In Vitro Activity Assay

A cDNA clone encoding the C-terminal 1099 residues of ROS1 protein was subcloned into the pMal-c2X vector (New England Biolabs) to obtain a *malE-ROS1* in-frame fusion. Expression of the MBP-ROS1 fusion protein in *E. coli* strain BL21(DE3) cells was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside. The fusion protein was purified by affinity chromatography of the crude bacterial lysate on an amylose column (New England Biolabs).

Plasmid pBluescript KS+ (Stratagene) was purified from *E. coli* BL21 (DE3), a *dcm* strain, using a Maxi-plasmid purification kit (Qiagen). Twenty μg of plasmids were methylated in vitro in a 300 μl reaction containing 20 U of MspI or SssI methylases (New England Biolabs) under the conditions recommended by the manufacturer. After methylation, DNA was purified using a Mini-plasmid purification kit (Eppendorf). Nonmethylated plasmid was subjected in parallel to the same procedure and used as a control in assays. The methylation status was confirmed by digestion with MspI and HpaII endonucleases.

For the nicking assay, a reaction mixture (20 μl) containing 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 purified closed-circular plasmid DNA, was incubated at 37°C for 1 hr with increasing concentrations of purified MBP-ROS1 protein. Reactions were stopped by adding 1

μl of stop solution (0.4 M EDTA, 1% SDS), heated at 70°C for 5 min, and the mixtures loaded onto a 1% agarose gel. Gel images were captured to a DC120 Zoom Digital Camera (Kodak) and analyzed with Kodak DS 1D Image Analysis Software, version 2.0.2. 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 over closed-circular DNA was taken into account in all quantifications (Wood et al., 1995)

Acknowledgments

We thank Xuying Chen, Liming Xiong, Manabu Ishitani, Yan Lin, Chun-Hai Dong, Yan Guo, A. Díaz-Bermúdez, Becky Stevenson, and Xuhui Hong for assistance; Dr. Eric Richards for providing *ddm1* and *ddm2* seeds, and rDNA and centromeric DNA probes; Dr. Steve Jacobsen for providing *cmt3* seeds and helpful suggestions; and Drs. Rich Jorgensen and Vicki Chandler for helpful advice and critical reading of the manuscript. This work was supported by NSF grants IBN-9808398 and DBI-9813360 to J.-K.Z. and MCYT grant BMC2001-1797 to T.R.-A.

Received: June 24, 2002
Revised: October 22, 2002

References

Adams, S., Vinkenoog, R., Spielman, M., Dickinson, H.G., and Scott, R.J. (2000). Parent-of-origin effects on seed development in *Arabidopsis thaliana* require DNA methylation. Development *127*, 2493–2502.

Amedeo, P., Habu, Y., Afsar, K., Scheid, O.M., and Paszkowski, J. (2000). Disruption of the plant gene *MOM* releases transcriptional silencing of methylated genes. Nature *405*, 203–206.

Bartee, L., Malagnac, F., and Bender, J. (2001). *Arabidopsis cmt3* chromomethylase mutations block non-CG methylation and silencing of an endogenous gene. Genes Dev. *15*, 1753–1758.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363–366.

Chen, Z.J., and Pikaard, C.S. (1997). Epigenetic silencing of RNA polymerase I transcription: a role for DNA methylation and histone modification in nucleolar dominance. Genes Dev. *11*, 2124–2136.

Choi, Y., Gehring, M., Johnson, L., Hannon, M., Harada, J.J., Goldberg, R.B., Jacobsen, S.E., and Fischer, R.L. (2002). DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in *Arabidopsis*. Cell *110*, 33–42.

Dalmay, T., Hamilton, A., Rudd, S., Angell, S., and Baulcombe, D.C. (2000). An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell *101*, 543–553.

Dorweiler, J.E., Carey, C.C., Kubo, K.M., Hollick, J.B., Kermicle, J.L., and Chandler, V.L. (2000). *mediator of paramutation1* is required for establishment and maintenance of paramutation at multiple maize loci. Plant Cell *12*, 2101–2118.

Garcia-Ortiz, M.V., Ariza, R.R., and Roldan-Arjona, T. (2001). An OGG1 orthologue encoding a functional 8-oxoguanine DNA glycosylase/lyase in *Arabidopsis thaliana*. Plant Mol. Biol. *47*, 795–804.

Habu, Y., Kakutani, T., and Paszkowski, J. (2001). Epigenetic developmental mechanisms in plants: molecules and targets of plant epigenetic regulation. Curr. Opin. Genet. Dev. *11*, 215–220.

Hu, F., Alcasabas, A.A., and Elledge, S.J. (2001). Asf1 links Rad53 to control of chromatin assembly. Genes Dev. *15*, 1061–1066.

Ikura, T., Ogryzko, V.V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J., and Nakatani, Y. (2000). Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell *102*, 463–473.

Ishitani, M., Xiong, L., Stevenson, B., and Zhu, J.K. (1997). Genetic analysis of osmotic and cold stress signal transduction in *Arabidopsis*: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. Plant Cell *9*, 1935–1949.

Jackson, J.P., Lindroth, A.M., Cao, X., and Jacobsen, S.E. (2002). Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature *416*, 556–560.

Jacobsen, S.E., Running, M.P., and Meyerowitz, E.M. (1999). Disruption of an RNA helicase/RNAse III gene in *Arabidopsis* causes unregulated cell division in floral meristems. Development *126*, 5231–5243.

Jeddeloh, J.A., Bender, J., and Richards, E.J. (1998). The DNA methylation locus *DDM1* is required for maintenance of gene silencing in *Arabidopsis*. Genes Dev. *12*, 1714–1725.

Jeddeloh, J.A., Stokes, T.L., and Richards, E.J. (1999). Maintenance of genomic methylation requires a SWI2/SNF2-like protein. Nat. Genet. *22*, 94–97.

Jones, L., Thomas, C.L., and Maule, A.J. (1998). De novo methylation and co-suppression induced by a cytoplasmically replicating plant RNA virus. EMBO J. *17*, 6385–6393.

Jost, J.P., Siegmann, M., Sun, L., and Leung, R. (1995). Mechanisms of DNA demethylation in chicken embryos. Purification and properties of a 5-methylcytosine-DNA glycosylase. J. Biol. Chem. *270*, 9734–9739.

Kakutani, T., Jeddeloh, J.A., Flowers, S.K., Munakata, K., and Richards, E.J. (1996). Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. Proc. Natl. Acad. Sci. USA *93*, 12406–12411.

Krokan, H.E., Standal, R., and Slupphaug, G. (1997). DNA glycosylases in the base excision repair of DNA. Biochem. J. *325*, 1–16.

Lindahl, T., and Wood, R.D. (1999). Quality control by DNA repair. Science *286*, 1897–1905.

Lindroth, A.M., Cao, X., Jackson, J.P., Zilberman, D., McCallum, C.M., Henikoff, S., and Jacobsen, S.E. (2001). Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. Science *292*, 2077–2080.

Luff, B., Pawlowski, L., and Bender, J. (1999). An inverted repeat triggers cytosine methylation of identical sequences in *Arabidopsis*. Mol. Cell *3*, 505–511.

Matzke, M., Matzke, A.J.M., and Kooter, J.M. (2001). RNA: Guiding gene silencing. Science *293*, 1080–1083.

McCullough, A.K., Dodson, M.L., and Lloyd, R.S. (1999). Initiation of base excision repair: glycosylase mechanisms and structures. Annu. Rev. Biochem. *68*, 255–285.

Mette, M.F., Aufsatz, W., van der Winden, J., Matzke, M.A., and Matzke, A.J. (2000). Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J. *19*, 5194–5201.

Mette, M.F., Matzke, A.J., and Matzke, M.A. (2001). Resistance of RNA-mediated TGS to HC-Pro, a viral suppressor of PTGS, suggests alternative pathways for dsRNA processing. Curr. Biol. *11*, 1119–1123.

Moazed, D. (2001). Common themes in mechanisms of gene silencing. Mol. Cell *8*, 489–498.

Richards, E.J., and Elgin, S.C. (2002). Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. Cell *108*, 489–500.

Rine, J. (1999). On the mechanism of silencing in *Escherichia coli*. Proc. Natl. Acad. Sci. USA *96*, 8309–8311.

Roldan-Arjona, T., Garcia-Ortiz, M.V., Ruiz-Rubio, M., and Ariza, R.R. (2000). cDNA cloning, expression and functional characterization of an *Arabidopsis thaliana* homologue of the *Escherichia coli* DNA repair enzyme endonuclease III. Plant Mol. Biol. *44*, 43–52.

Ronemus, M.J., Galbiati, M., Ticknor, C., Chen, J., and Dellaporta, S.L. (1996). Demethylation-induced developmental pleiotropy in *Arabidopsis*. Science *273*, 654–657.

Scharer, O.D., and Jiricny, J. (2001). Recent progress in the biology, chemistry and structural biology of DNA glycosylases. Bioessays *23*, 270–281.

Tyler, J.K., Adams, C.R., Chen, S.R., Kobayashi, R., Kamakaka, R.T., and Kadonaga, J.T. (1999). The RCAF complex mediates chromatin assembly during DNA replication and repair. Nature *402*, 555–560.

Vaucheret, H., and Fagard, M. (2001). Transcriptional gene silencing in plants: targets, inducers and regulators. Trends Genet. *17*, 29–35.

Vongs, A., Kakutani, T., Martienssen, R.A., and Richards, E.J. (1993). *Arabidopsis thaliana* DNA methylation mutants. Science *260*, 1926–1928.

Wassenegger, M., Heimes, S., Riedel, L., and Sanger, H.L. (1994). RNA-directed de novo methylation of genomic sequences in plants. Cell *76*, 567–576.

Wood, R.D., Biggerstaff, M., and Shivji, M.K.K. (1995). Detection and measurement of nucleotide excision repair synthesis by mammalian cell extracts in vitro. Methods *7*, 163–175.

Zamore, P. (2002). Ancient pathways programmed by small RNAs. Science *296*, 1265–1269.

Accession Numbers

The GenBank accession number for the ROS1 sequence reported in this paper is AAD24633.