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Mutations in a Conserved Replication Protein Suppress Transcriptional Gene Silencing in a DNA-Methylation-Independent Manner in *Arabidopsis*

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Summary

Mutations in the DNA glycosylase/lyase ROS1 cause transcriptional silencing of the linked *RD29A-LUC* and *35S-NPTII* transgenes in *Arabidopsis* [1]. We report here that mutations in the *Arabidopsis RPA2* locus release the silencing of *35S-NPTII* but not *RD29A-LUC* in the *ros1* mutant background. The *rpa2* mutation also leads to enhanced expression of some transposons. Neither DNA methylation nor siRNAs at any of the reactivated loci are blocked by *rpa2*. Histone H3 methylation at lysine 4 was increased and histone H3 methylation at lysine 9 was decreased at the *35S* promoter in the *ros1rpa2* mutant compared to the *ros1* background. *RPA2* encodes a nuclear protein similar to the second subunit of the replication protein A conserved from yeast to mammals. Ectopic expression of the *Arabidopsis RPA2* could complement the yeast *rfa2* (*rpa2*) mutant. These results suggest an essential role of RPA2 in the maintenance of transcriptional gene silencing at specific loci in a DNA-methylation-independent manner. In addition, we found that *rpa2* mutants are hypersensitive to the genotoxic agent methyl methanesulphonate, and the RPA2 protein interacts with ROS1 in vitro and in vivo, suggesting that RPA2 also functions together with ROS1 in DNA repair.

Results and Discussion

Suppression of *35S-NPTII* Transcriptional Silencing in *ros1* by the *rpa2* Mutation

Mutations in the bifunctional DNA glycosylase/lyase ROS1 cause transcriptional gene silencing (TGS) at the transgene and endogenous *RD29A* promoters [1]. A low level of *RD29A* promoter small interfering RNAs (siRNAs) are generated from the transgene repeat, which is presumably the trigger for cytosine methylation at both the transgene and endogenous *RD29A* promoters. In the wild-type background, the functional ROS1 presumably demethylates the DNA, thereby counteracting the siRNA-directed DNA methylation [1]. Apart from the silencing of endogenous *RD29A* locus and *RD29A-LUC* (firefly luciferase driven by the *RD29A* promoter) transgene, the linked kanamycin-resistance gene *35S-NPTII* (neomycin phosphotransferase II driven by the CaMV 35S promoter) in the transgene repeat is also transcriptionally silenced in *ros1* [1].

We generated ~20,000 lines of a T-DNA-mutagenized population of *ros1* plants. The population was screened for suppressors of *ros1* by plating the seeds on kanamycin-containing medium. One of the suppressor mutants that could grow on kanamycin (Figure 1A) was designated as *rpa2-1*. The kanamycin resistance (Figure 1A) combined with luciferase-imaging results (see Figure S1A in the Supplemental Data available with this article online) showed that the *rpa2* mutation suppresses the silencing of *35S-NPTII* but not the closely linked *RD29A-LUC* in *ros1*.

Northern-blot analysis showed that unlike *ros1* plants, *ros1rpa2* plants accumulated the *NPTII* transcript, albeit to a lower level than that in wild-type plants (Figure 1B). The *ros1rpa2* mutant did not accumulate *LUC* or endogenous *RD29A* transcripts (Figure 1B), consistent with its lack of luminescence. Nuclear run-on results showed that the suppression of *35S-NPTII* silencing occurs at the transcriptional level (Figure S1B).

RPA2 Encodes the Second Subunit of Replication Protein A

We determined the site of T-DNA insertion in *ros1rpa2-1* by using thermal asymmetric interlaced PCR. An insertion was found in the 9th exon of the gene At2g24490. Ectopic expression of At2g24490 cDNA in the *ros1rpa2-1* mutant restored kanamycin sensitivity (Figure 2A), thus confirming that At2g24490 is the *RPA2* gene. We obtained another T-DNA allele, designated as *rpa2-4*, from the SALK T-DNA collection (Figure 2B). Both mutant alleles of *rpa2* are early flowering, and expression of the wild-type *RPA2* cDNA in *ros1rpa2-1* also complemented the early-flowering phenotype of this mutant (data not shown).

RPA2 encodes a 279 amino acid protein similar to the second subunit of replication protein A (RPA). RPA is the major single-stranded DNA (ssDNA) binding protein in eukaryotes, and this heterotrimeric protein interacts with a variety of other proteins that ultimately govern how genetic information is copied or repaired [2]. The binding to ssDNA is mediated by a highly conserved domain (Figure S1C) known as the oligonucleotide/oligosaccharide binding fold (OB-fold) [2].

To confirm that the *Arabidopsis RPA2* encodes a functional RPA, we ectopically expressed it in a yeast

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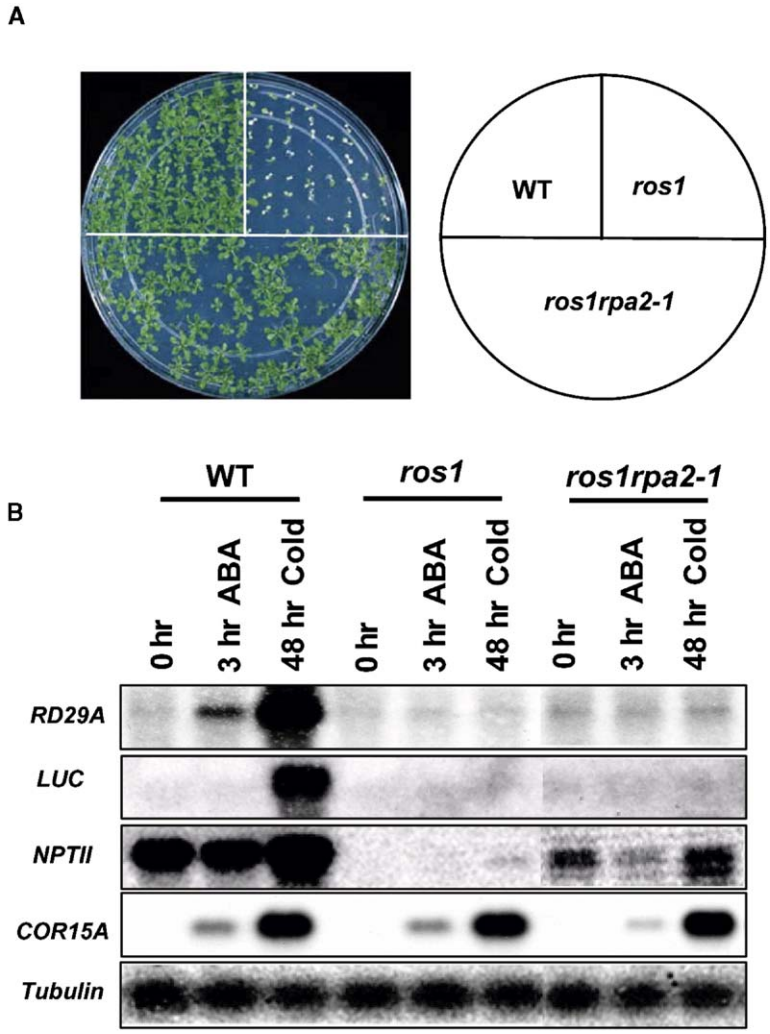

Figure 1. The *rpa2-1* Mutation Suppresses the Silencing of *35S-NPTII* But Not *RD29A-LUC* Transgene in *ros1*

(A) Kanamycin tolerance of *ros1rpa2-1* seedlings. WT (wild-type C24 containing *RD29A-LUC*), *ros1*, and *ros1rpa2-1* seeds were germinated on MS medium supplemented with 35 mg/L kanamycin. Seedlings were photographed 15 days after germination.

(B) Transcript levels of *RD29A*, luciferase, *NPTII*, and *COR15A* in WT, *ros1*, and *ros1rpa2-1* seedlings, as determined by northern analysis. *Tubulin* was used as a loading control. Note that *LUC* transcript was not visible in ABA-treated WT as a result of rapid turnover.

mutant (*rfa2-2*) defective in RPA2 [3]. The *rfa2-2* mutant has defects in DNA replication and exhibits increased sensitivity to hydroxyurea, particularly at 34°C [3]. As shown in Figure 2C, the mutant cells harboring the vector alone exhibited much higher sensitivity to hydroxyurea at 34°C, and this sensitivity was rescued in cells harboring the *Arabidopsis RPA2*. The result supports that the *Arabidopsis* gene encodes a functional RPA2.

To determine the subcellular localization of RPA2 protein, we fused green fluorescence protein (GFP) in frame to the N terminus of RPA2. The GFP-RPA2 fusion was expressed in transgenic *Arabidopsis* plants under the control of *CaMV 35S* promoter. Confocal imaging of the roots of the transgenic plants showed that GFP-RPA2 fusion protein is localized in the nucleus (Figure 3D). *RPA2* promoter-β-glucuronidase reporter and RT-PCR analysis showed that RPA2 is expressed ubiquitously in all plant tissues (Figures S1D and S1E).

The *rpa2* Mutation Does Not Affect DNA Methylation or siRNA Accumulation

Transcriptionally silenced genes show marks of heterochromatin, i.e., increased DNA methylation and/or histone H3 lysine 9 methylation [4, 5]. In *Arabidopsis*, mutations in various DNA methyltransferases [6–10], histone methyltransferases [11–13], histone deacetylases [14, 15], and other chromatin remodeling proteins such as DDM1 [4, 16], MOM1 [17], and DRD1 [18] can lead to the release of TGS [19–22].

Bisulfite sequencing showed that there is no difference in cytosine methylation at the *35S* promoter between the wild-type and *ros1*, or between *ros1* and *ros1rpa2-1* plants (Figure S2A). These results indicate that the transcriptional silencing of *35S-NPTII* in *ros1* is not due to increased methylation of the *35S* promoter, and the release of TGS in *ros1rpa2* cannot be correlated with loss of cytosine methylation.

Bisulfite sequencing also showed that compared to the wild-type, both *ros1* and *ros1rpa2-1* had higher levels of methylation at endogenous and the transgene *RD29A* promoters (Figures S2B and S2C). Southern analysis confirmed that hypermethylation of endogenous and transgene *RD29A* promoters in *ros1* was not changed by the *rpa2* mutation (Figure S2D). Southern analysis with methylation-sensitive isoschizomers MspI and HpaII did not detect a difference among the wild-

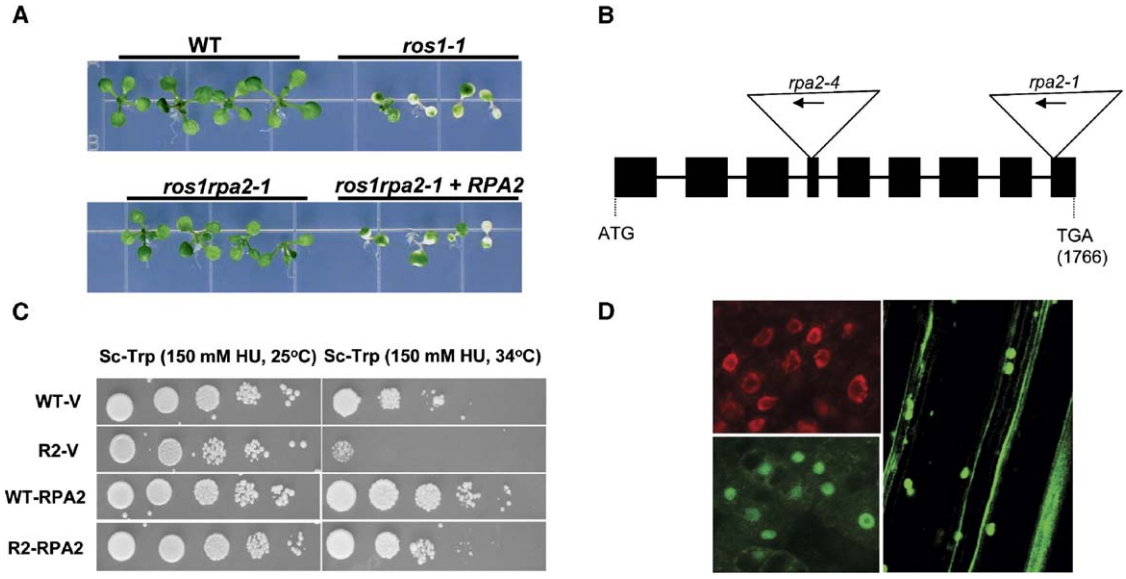

Figure 2. RPA2 Functional Complementation and Subcellular Localization

(A) Complementation of *ros1rpa2-1* mutant by ectopic expression of *RPA2*. The plants were grown on 35 mg/L kanamycin.

(B) Relative positions of T-DNA insertions in *rpa2-1* and *rpa2-4* alleles. Black blocks represent exons, and black lines represent introns.

(C) Complementation of the yeast *rfa2* (i.e., *rpa2*) mutant (R2) by ectopic expression of *Arabidopsis RPA2*. Wild-type and mutant cells transformed with the p414GPD vector (WT-V and R2-V, respectively) or with *RPA2* (WT-RPA2 and R2-RPA2) were incubated at 25°C or 34°C in the presence of hydroxyurea (HU).

(D) GFP-RPA2 fusion protein is localized in the nucleus. The upper-left panel shows the nuclear position after staining with DAPI. The lower-left and the -right panels show GFP-RPA2 protein localization in nuclei.

type, *ros1*, and *ros1rpa2-1* in the methylation patterns for *rDNA*, 180 bp centromeric DNA, and *5S rDNA* (Figure S3), indicating that the *rpa2* mutation does not affect global DNA methylation.

Evidence suggests that the initial trigger for the establishment of transcriptional gene silencing (TGS) is siRNAs [23–25]. We investigated the effect of *rpa2* mutation on the production of siRNAs. No siRNAs corre-

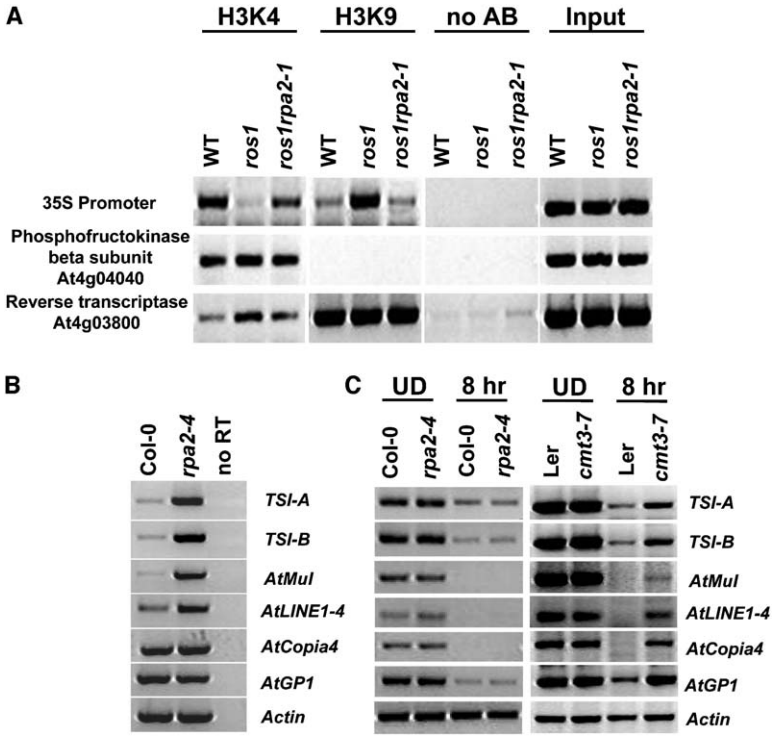

Figure 3. The *rpa2* Mutation Alters Histone-Methylation Patterns at the *35S* Promoter and Affects Transposon Expression in a DNA-Methylation-Independent Manner

(A) CHIP analysis of histone methylation at the *35S* promoter. Equal amount of chromatin was used for immunoprecipitation as shown by equal amplification in the Input lanes. At4g04040 and At4g03800 were used as controls for anti-H3K4 and anti-H3K9 antibodies, respectively. No AB denotes precipitation without antibody as a negative control.

(B) Expression analysis of the transposons in the wild-type (Col-0) and *rpa2-4* by RT-PCR. Specific amplification is indicated by absence of a band in no RT (without reverse transcriptase) lane. See Supplemental Experimental Procedures for information on the number of PCR cycles used for each transposon or *Actin*.

(C) DNA-methylation levels of transposons were not different between the wild-type (Col-0) and *rpa2-4*. McrBC PCR was carried out on untreated (UD) and McrBC-treated (8 hr) DNA. *cmt3-7* was used as a control. Ler indicates a wild-type background for *cmt3-7*.

sponding to the *35S* promoter were found in wild-type, *ros1*, or *ros1rpa2-1* plants (data not shown). siRNAs from the *RD29A-LUC* transgene promoter were still present in *ros1rpa2-1* (Figure S4A). In addition, siRNA levels corresponding to several endogenous loci were not affected in *ros1rpa2-1* (Figures S4B and S4C). These results suggest that TGS of *35S-NPTII* is not triggered by *35S* promoter siRNAs, and the *rpa2* mutation does not block siRNA production.

The Release of *35S-NPTII* Silencing in *rpa2* Involves Changes in Histone Methylation

Dimethylation of histone H3 at Lys4 (H3K4) is known to be associated with transcriptionally active genes, whereas dimethylation of histone H3 at Lys9 (H3K9) is associated with transcriptionally silent loci [4, 5, 26]. We carried out chromatin immunoprecipitation (ChIP) experiments with antibodies specific for H3 dimethyl K4 or H3 dimethyl K9. As shown in Figure 3A, in the wild-type plants, the *35S* promoter was associated with strong H3K4 methylation and weak H3K9 methylation. In contrast, *ros1* plants had decreased levels of H3K4 methylation but elevated levels of H3K9 methylation at the *35S* promoter (Figure 3A). Consistent with the release of TGS at the *35S* promoter, *ros1rpa2-1* had increased H3K4 methylation and reduced H3K9 methylation compared to *ros1* (Figure 3A). ChIP results from the active control gene At4g04040 and silent control gene At4g03800 [27] showed that the antibodies were specific for their association with chromatin (Figure 3A). These results suggest that TGS at the *35S* promoter in *ros1* plants correlates with heterochromatic histone-methylation patterns. In *ros1rpa2-1* plants, there was a loss of heterochromatic mark and a gain in euchromatic mark, resulting in reactivation of the *35S* promoter.

The *rpa2* Mutation Enhances the Expression of Some Transposons

Transposons constitute some of the major targets of TGS in plant genomes [4, 28–31]. The *rpa2-4* mutation led to increased expression of a DNA transposon, *AtMu1*, and retrotransposons *AtLINE1-4*, *TSI-A*, and *TSI-B*, although the extent of increase is difficult to assess with the RT-PCR method (Figure 3B). The expression of *AtCOPIA4* or *AtGP1* was not altered in *rpa2-4*.

As shown in Figure 3C, at all the transposons we found heavy DNA methylation, which is evident from reduced PCR amplification after McrBC digestion, but there was no difference between *rpa2-4* and Col-0, indicating that the increase in transposon expression in *rpa2* mutant plants was not accompanied by loss of DNA methylation. Control experiments showed that the assay could detect loss of CpXpG methylation in *cmt3-7* [7]. These results suggest that the *rpa2* mutation releases the TGS of a subset of transposons, and this occurs in a manner independent of DNA methylation.

rpa2 Mutant Plants Are Defective in DNA Damage Repair

RPA2 from humans, yeast, and *Drosophila* are known to have a central role in DNA damage repair [32–34]. We investigated whether RPA2 is important for DNA damage repair in *Arabidopsis* by testing the response of *rpa2* mutants to genotoxic treatments. Both *ros1rpa2-1* (Figure 4A) and *rpa2-4* (Figure S5A) were extremely sensitive to methyl methanesulfonate (MMS), whereas the wild-type plants were tolerant. As reported previously [1], *ros1* was also sensitive to MMS, although the sensitivity was less than that of *ros1rpa2-1* (Figure 4A). We also tested the sensitivity of *ros1rpa2-1* (Figure S5B) and *rpa2-4* to UV stress (Figure S5C) or bleomycin (data not shown), but no difference was observed between the different genotypes.

These results suggest that *Arabidopsis* RPA2 has an important role in certain DNA damage-repair processes in planta. This is consistent with the capacity of RPA2 to rescue the hydroxyurea sensitivity of the yeast *rfa2* mutant (Figure 2C). Our results also indicate that RPA2 does not function in the repair of DNA damage caused by UV or bleomycin. It is possible that a *RPA2*-like gene (At3g02920) may function in such DNA repair.

RPA2 Interacts Physically with ROS1

The human uracil DNA-glycosylase UNG, an enzyme in DNA base-excision repair, interacts with RPA2 and PCNA at the replication foci [35]. ROS1 is a bifunctional DNA glycosylase/lyase hypothesized to remove 5-methyl cytosine and other modified bases through a base-excision-repair mechanism [1]. Because both ROS1 and RPA2 have an in planta function in DNA repair, we investigated whether ROS1 may interact with RPA2. We carried out a yeast two-hybrid assay where ROS1 was used as a bait and RPA2 as prey. High β-galactosidase (β-gal) activity was observed when the RPA2 was cotransformed with ROS1 (Figure 4B), indicating a strong interaction.

The interaction between ROS1 and RPA2 was confirmed in vitro with protein pull-down assays. Whereas MBP-ROS1 could not pull-down ^{35}S-methionine-labeled UBP26 (negative control), it could pull-down ^{35}S-methionine-labeled RPA2 (Figure 4C). We also tested the in vivo interaction between RPA2 and ROS1 by using bimolecular fluorescence complementation [36]. For this, ROS1 was translationally fused to the C-terminal 86 amino acid portion of YFP (pUCSPYCE-ROS1), and RPA2 was translationally fused to the N-terminal 155 amino acid portion of YFP (pUCSPYNE-RPA2). As seen in Figure 4D, a yellow fluorescent signal was seen when pUCSPYCE-ROS1 and pUCSPYNE-RPA2 were cobombarded in *Arabidopsis* leaves, indicating that the two proteins interacted in vivo. No fluorescence was observed when the pUCSPYCE vector was cobombarded with pUCSPYNE-RPA2, or when pUCSPYCE-ROS1 was cobombarded with the pUCSPYNE vector (Figure S6). Through physical interactions with ROS1 and possibly other DNA glycosylases, RPA2 may target these DNA repair enzymes to the replication foci to initiate pre- and/or postreplicative base-excision repair.

Conclusions

The *rpa2* mutation releases TGS of the *35S-NPTII* transgene but not the endogenous *RD29A* or *RD29A-LUC* transgene. RPA2 is also required for the silencing of only a subset of transposons. Therefore, there are distinct mechanisms for the maintenance of TGS at the different loci. Although RPA2 has been studied extensively in yeasts and animals as a result of its critical

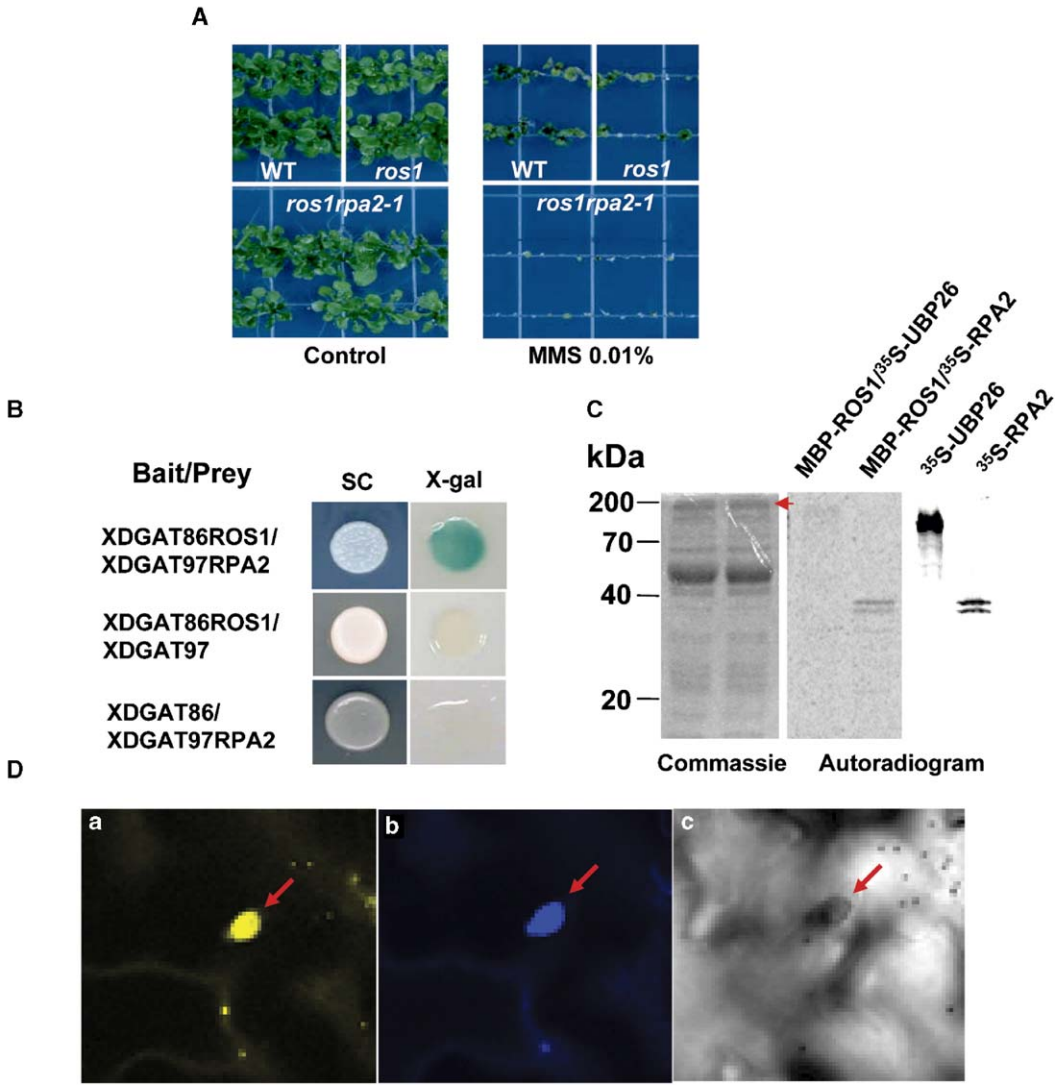

Figure 4. *rpa2* Mutants Are Hypersensitive to MMS, and RPA2 Interacts with ROS1 In Vitro and In Vivo

(A) *ros1rpa2-1* is sensitive to the DNA-alkylating agent methyl methane sulphonate (MMS). The mutant and wild-type seeds were sown on MS-media containing 0.01% MMS.

(B) Interaction between RPA2 and ROS1 in the yeast two-hybrid assay. Shown are yeast colonies (left) and corresponding β-Gal lift assay (right).

(C) Interaction between RPA2 and ROS1 in the pull-down assay. MBP-ROS1 fusion protein (shown by the red arrow) can pull-down ^{35}S-RPA2 but not ^{35}S-UBP26. The Commassie-stained gel (left panel) shows that equal amount of protein was used for pull-down. The middle panel shows the pull-down, whereas the right panel shows the labeled proteins used for pull-down.

(D) In vivo interaction between ROS1 and RPA2 as determined with bimolecular fluorescence complementation. (D_a) YFP signal in the nucleus indicating positive interaction between ROS1 and RPA2; (D_b) position of nucleus as shown by DAPI staining; (D_c) bright-field image of the cell. Arrow points to the nucleus.

role in DNA replication, recombination, and repair [32, 33], its function in heterochromatin formation and transcriptional silencing has not been explored. Our work and that of Elmayan et al. (in this issue of *Current Biology*, [37]) thus demonstrate a novel function of RPA2 and reveal a link between DNA replication, repair, and transcriptional silencing.

It is possible that heterochromatin formation at the *35S* promoter in the absence of a siRNA signal occurs as a result of spreading of heterohromatic marks from the nearby *RD29A* promoter. Because there is no difference in DNA cytosine methylation at the *35S* promoter between the wild-type and *ros1*, it appears that only the methylated H3K9 heterochromatin mark is spread and is sufficient for silencing the *35S* promoter. This spreading resembles the spreading in fission yeast, where heterochromatin can spread significantly outward from the initiating region that produces siRNAs to nearby regions [38].

RPA2 may be involved in the spreading of heterochromatin and/or in the maintenance of histone modification patterns during DNA replication. If there is a

blockage in the spreading of heterochromatic marks from the *RD29A* promoter to the *35S* promoter, then already established H3K9 methylation at *35S* would need to be maintained following cell division, in order to effectively repress the *NPTII* gene. During DNA replication, there is a redistribution of histones, resulting in a dilution of histone modifications in daughter chromatids [39]. To regain the level of histone modifications, the histones from the parent provide a molecular bookmark that enables the formation of new modifications in histones. At RPA2-independent loci, the maintenance and/or spreading of histone modification patterns during DNA replication may involve the RPA2-like protein, encoded by At3g02920. Alternatively, the problem of renewal of histone modifications following replication at the RPA2-independent loci may be circumvented because of some unidentified factors specific to these loci.

Supplemental Data

Supplemental Data include Experimental Procedures, six Supplemental Figures, and one Supplemental Table and are available with this article online at: http://www.current-biology.com/cgi/content/full/15/21/1912/DC1/.

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