

Epigenetic Regulation, Somatic Homologous Recombination, and Abscisic Acid Signaling Are Influenced by DNA Polymerase ϵ Mutation in *Arabidopsis* ^{W|OA}

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Based on abscisic acid (ABA) inhibition of seed germination and seedling growth assays, we isolated an ABA overly sensitive mutant (*abo4-1*) caused by a mutation in the *Arabidopsis thaliana* *POL2a/TILTED1(TIL1)* gene encoding a catalytic subunit of DNA polymerase ϵ . The dominant, ABA-insensitive *abi1-1* or *abi2-1* mutations suppressed the ABA hypersensitivity of the *abo4-1* mutant. The *abo4/til1* mutation reactivated the expression of the silenced *Athila* retrotransposon transcriptional silent information (*TSI*) and the silenced *35S-NPTII* in the *ros1* mutant and increased the frequency of somatic homologous recombination (HR) \sim 60-fold. ABA upregulated the expression of *TSI* and increased HR in both the wild type and *abo4-1*. *MEIOTIC RECOMBINATION11* and *GAMMA RESPONSE1*, both of which are required for HR and double-strand DNA break repair, are expressed at higher levels in *abo4-1* and are enhanced by ABA, while *KU70* was suppressed by ABA. *abo4-1* mutant plants are sensitive to UV-B and methyl methanesulfonate and show constitutive expression of the G2/M-specific cyclin *CycB1;1* in meristems. The *abo4-1* plants were early flowering with lower expression of *FLOWER LOCUS C* and higher expression of *FLOWER LOCUS T* and changed histone modifications in the two loci. Our results suggest that *ABO4/POL2a/TIL1* is involved in maintaining epigenetic states, HR, and ABA signaling in *Arabidopsis*.

INTRODUCTION

Plants are sessile and unable to escape acute environmental stresses, but they have developed sophisticated responses to cope with and survive stress conditions. Plants are able to quickly counteract environmental stresses through actions, such as up- or downregulating gene expression and accumulating adaptive metabolic compounds. Abiotic stresses, such as drought, high salinity, and low temperature, induce the accumulation of the phytohormone abscisic acid (ABA) (Finkelstein et al., 2002; Koornneef et al., 2002). ABA modulates embryogenesis, maturation, and dormancy during seed development and plays vital roles in a wide range of biological processes throughout plant growth, development, and stress responses (Finkelstein et al., 2002; Koornneef et al., 2002). Through forward and reverse genetic studies, many genes involved in ABA responses have been isolated and characterized (Finkelstein et al., 2002;

Koornneef et al., 2002; Xiong et al., 2002; Shinozaki and Yamaguchi-Shinozaki, 2007). Increased ABA in plant cells inhibits DNA replication and cell division, which results in retarded plant growth (Finkelstein et al., 2002; Swiatek et al., 2002; Chandrasekharan et al., 2003). However, the molecular mechanism for ABA inhibition of plant growth is largely unknown. One hint is that ABA induces expression of the cyclin-dependent kinase inhibitor *KRP1/ICK1* (Wang et al., 1998). Cyclin-dependent kinases regulate the progression of the cell cycle in eukaryotes. Studies thus far have focused on ABA-mediated rapid physiological responses that are important for plant stress resistance in the short term. Whether ABA or an ABA-regulated signal can affect epigenetic states and genome stability, which might be important for the long-term adaptation of plants to environmental stresses and possibly contribute to plant evolution, is not known.

During the cell cycle, both DNA methylation patterns and specific chromatin structures must be duplicated with high fidelity throughout DNA replication. The DNA replication process involves DNA biosynthesis, transient disruption of parental nucleosomes, and nucleosome reassembly (Groth et al., 2007), and many different proteins participate in this complex process. During S-phase, the leading and lagging strand are replicated by different mechanisms in a continuous and discontinuous fashion, respectively. In the lagging strand synthesis, DNA polymerase α uses RNA primers initiated by the primase to synthesize a short

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DNA and form an RNA–DNA hybrid. Replication factor C binds to the RNA–DNA junction and recruits the homotrimeric PCNA (a ring-shaped replication factor) to encircle the DNA. Then, most possibly, the DNA polymerase δ replaces the DNA polymerase α , together with PCNA, to copy the DNA template (Garg and Burgers, 2005; Moldovan et al., 2007). Pol ϵ is a leading strand DNA polymerase during chromosomal DNA replication in yeast (Pursell et al., 2007). In yeast, DNA polymerase ϵ is a four-subunit complex (consisting of the catalytic subunit, POL2p, and three regulatory subunits, DPB2p, DBP3p, and DBP4p), and this structure is conserved in *Homo sapiens* (Pospiech and Syvaaja, 2003). In *Arabidopsis thaliana*, DNA polymerase ϵ subunits consist of DPB2 (encoded by At5g22110, corresponding to yeast DPB2p), DPB4 (encoded by At1g09030, corresponding to yeast DPB4p), and DBP3a (encoded by At5g50490, corresponding to yeast DBP3p) for the regulatory subunits, and POL2a and POL2b for the catalytic subunits (Jenik et al., 2005; Ronceret et al., 2005). A null mutation in either DPB2 or POL2a is lethal (Ronceret et al., 2005). Knockout mutants with T-DNA insertions in *POL2b* exhibit no morphological phenotypes (Ronceret et al., 2005). Studies of Pol ϵ in yeast and humans implicated it in DNA repair and recombination (Pospiech and Syvaaja, 2003). Pol ϵ , together with RPA (replication protein A), replication factor C, PCNA, and DNA ligase I, was found to refill the repaired DNA patch through a reconstituted protein system in mammals and yeast (Aboussekhra et al., 1995; Shivji et al., 1995). Genetic studies in yeast indicated the importance of both Pol ϵ and δ in filling the gaps of repair DNA (Pospiech and Syvaaja, 2003). Moreover, DNA polymerase ϵ binds double-stranded DNA and promotes epigenetic silencing at telomeres in budding yeast, but this activity is not associated with its polymerase activity (Tsubota et al., 2006). Establishment of transcriptional gene silencing (TGS) and histone deposition in yeast seems to be independent of DNA replication (Kirchmaier and Rine, 2001; Li et al., 2001; Green et al., 2005). In *Arabidopsis*, lesions in *POL2a/TIL1* lead to longer cell cycles and developmental defects (Jenik et al., 2005; Ronceret et al., 2005). Other biological roles for *POL2a* in planta have not yet been characterized because of the lack of a suitable mutant. In this study, we characterized an ABA overly sensitive mutant (*abo4-1*), a novel weak allele of *POL2a*. We found that the mutation in *ABO4/POL2a/TIL1* releases TGS in some genomic regions and greatly increases the frequency of somatic homologous recombination (HR); both effects were reinforced by ABA treatment. Our studies on the *abo4* mutant reveal previously unknown functions of *POL2a* in epigenetic regulation, HR, and ABA signaling in plants.

RESULTS

Phenotypic Characterization of an ABA Overly Sensitive Mutant, *abo4-1*

An ABA overly sensitive mutant, *abo4-1*, was isolated from an ethyl methanesulfonate–mutagenized population of *Arabidopsis* (Columbia [Col] *gl1* background) using a root bending assay on Murashige and Skoog (MS) medium containing 30 μ M ABA. The *abo4-1* mutant was backcrossed with the wild type four times before phenotypic analysis.

As shown in Figure 1A, different concentrations of ABA greatly inhibited root growth and made *abo4-1* cotyledons become yellow, but not wild-type. We also tested the seed germination sensitivity of *abo4-1* to ABA. In the absence of ABA, both *abo4-1* and the wild type showed similar seed germination rates (Figure 1B, MS). In the presence of different concentrations of ABA, both germination and postgermination growth of *abo4-1* were greatly inhibited compared with the wild type (Figure 1B, 0.3 μ M ABA). Figure 1C shows the statistical analysis of seedling greening rate (percentage of greening seedlings over all germinated seedlings) after germination on day 7 at different concentrations of ABA. Supplementation of 0.3 μ M ABA had a striking effect on *abo4-1* seedling greening, whereas even 0.4 μ M ABA did not significantly influence the greening rate of the wild type (Figure 1C).

We crossed two dominant ABA-insensitive mutants, *abi1-1* and *abi2-1*, to *abo4-1*. ABI1 and ABI2, two protein phosphatases 2C, are key negative regulators in ABA signal transduction pathways (Leube et al., 1998; Rodriguez et al., 1998; Gosti et al., 1999; Merlot et al., 2001). The germination and postgermination growth of *abo4-1 abi1-1* and *abo4-1 abi2-1* double mutants were compared in the presence of 1 μ M ABA. As shown in Figure 1D, the two double mutants showed similar ABA insensitivity as *abi1-1* and *abi2-1*. However, the *abi1-1* or *abi2-1* mutations did not affect the growth phenotypes of *abo4-1* under normal growth conditions. This result suggests that the enhanced ABA response in the *abo4-1* mutant is mediated by ABA signal transduction pathway(s).

abo4-1 Is a Weak Allele of *POL2a*

We used map-based cloning to identify the *ABO4* gene. *ABO4* (*At1g08260*) encodes a catalytic subunit of DNA polymerase ϵ (*POL2a*) (Jenik et al., 2005; Ronceret et al., 2005). This locus had been previously identified as *TILTED1* (*TIL1*) (Jenik et al., 2005). *POL2a* has 47 introns and 48 exons. A point mutation that changes Gly₅₃₄ to Arg (G to A in position 4171 counting from the first putative ATG, in the 13th exon) was found in *abo4-1* (Figure 1E). A Salk T-DNA insertion line (SALK_096341, *abo4-2*), in which the T-DNA was inserted at position 3972 (in the 12th exon), counting from the first putative ATG of the genomic coding sequence, was obtained. In yeast, the N-terminal catalytic domain of DNA polymerase ϵ is not essential for survival but is required for the DNA damage checkpoint control (Feng and D'Urso, 2001; Ohya et al., 2002). Because null mutations in *Arabidopsis* *POL2a* are embryonic lethal (Jenik et al., 2005; Ronceret et al., 2005), both *abo4-1* and *abo4-2* should be weak alleles of *POL2a*. *abo4-2* showed similar ABA sensitivity of seedling growth as *abo4-1* (Figure 1F). Because the homozygous *abo4-2* mutants were sterile in both male and female, we selected heterozygous T-DNA mutant lines and crossed them with *abo4-1*. The F1 seedlings showed *abo4-1* and wild-type growth phenotypes at a ratio of \sim 1:1 (62:64) when grown on MS medium and also showed *abo4-1* and wild-type ABA-sensitive phenotypes at a ratio of \sim 1:1 (57:58) when grown on MS medium containing 30 μ M ABA in the root bending assay. Seven sensitive seedlings were randomly picked up and checked by PCR, all were heterozygous plants of *abo4-1* and *abo4-2*, which is consistent with the above genetic analysis. These results

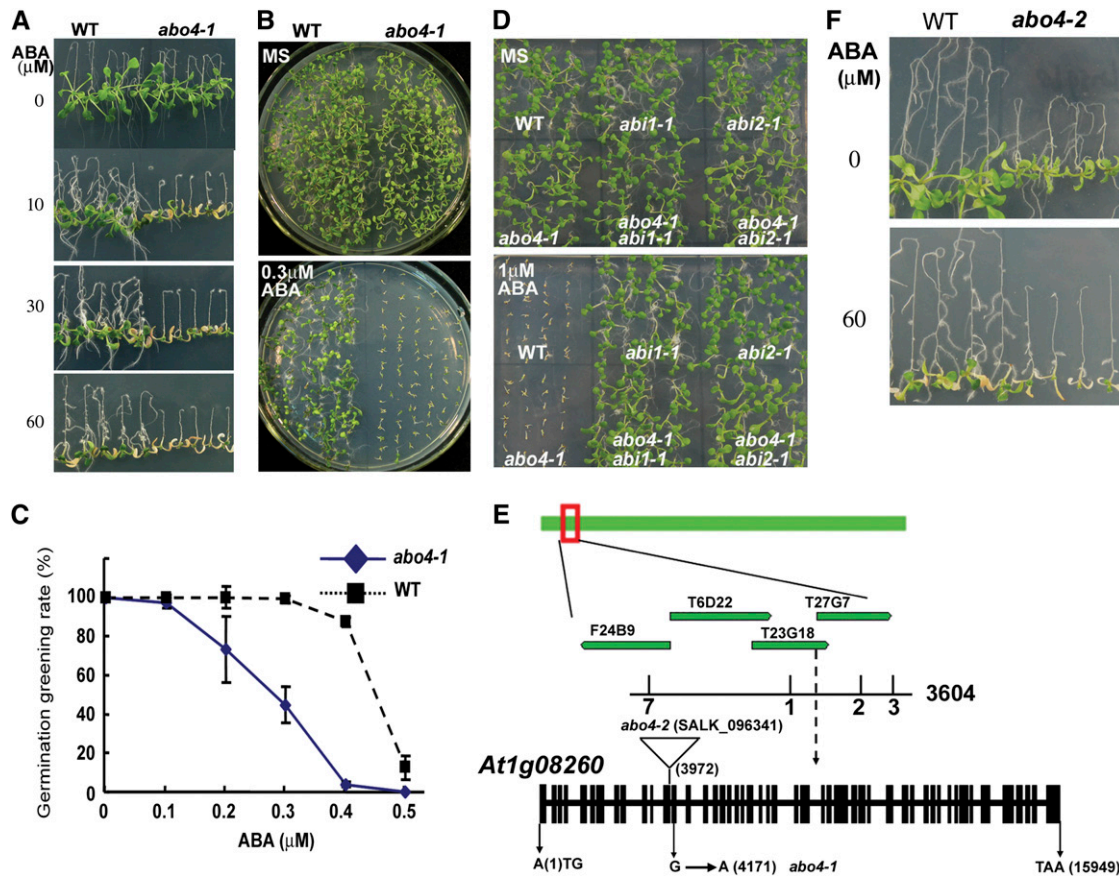


Figure 1. ABA Sensitivity of the *abo4-1* Mutant.

(A) ABA sensitivity of *abo4-1* seedlings grown on MS media containing different concentrations of ABA. Five-day-old seedlings grown on MS medium were transferred to MS medium containing different concentrations of ABA, respectively, and cultured for another 7 d before taking pictures.

(B) The seed germination sensitivity of *abo4-1* to ABA. Wild-type and *abo4-1* seeds were directly sowed on MS medium or MS medium containing 0.3 μM ABA, respectively. After being kept at 4°C for 3 d, the plates were removed to a growth chamber and cultured for 7 d before taking pictures.

(C) The statistic data of seed germination of wild-type and *abo4-1* on MS medium containing different concentrations of ABA. The experimental conditions were the same as in **(B)**. Values are means ± SE, $n = 3$ independent experiments. At each time, >100 seeds were counted.

(D) The dominant mutations of the negative regulators *ABI1* and *ABI2* block the ABA sensitivity of *abo4-1*. The seeds of wild-type, *abo4-1*, *abi1-1*, *abi2-1*, *abo4-1 abi1-1*, and *abo4-1 abi2-1* double mutants were germinated on MS medium containing no ABA or 1 μM ABA. The pictures were taken after seedlings were grown for 7 d in a growth chamber.

(E) Mapping-based cloning of *ABO4*. The *ABO4* locus was mapped between two BAC clones F24B9 (recombinants, 7 of 1802) and T27G7 (recombinants, 3 of 1802). Further mapping delimited the *ABO4* locus to a region within BAC T23G18. A point mutation (G4171A, in the 13th exon) was found in *AT1G08260/POL2a/TIL1*. A T-DNA insertion (*abo4-2*, SALK_096341) line was obtained with a T-DNA inserted at position 3972 (in the 12th exon).

(F) Comparison of ABA sensitivity of wild-type and *abo4-2* seedlings. The seedlings were first grown on MS medium for 5 d. *abo4-2* seedlings were picked up according to its grown phenotype and were, together with the wild type, transferred to MS medium containing different concentrations of ABA. Here, we only show the seedlings grown on MS medium containing 60 μM ABA for 7 d. The similar growth phenotypes were observed when *abo4-2* seedlings were grown on other concentrations of ABA (10, 20, 30, and 50 μM, respectively).

confirmed that *ABO4* is *At1g08260/POL2a/TIL1* (please also see the segregation analysis of F1 seedlings on methyl methanesulfonate [MMS] medium in Figure 2).

***abo4-1* Mutants Were Hypersensitive to MMS and UV-B Light**

In addition to bulk DNA synthesis, one of the important functions of DNA polymerase ϵ is nucleotide and base excision repair

(Dianov et al., 2003; Pospiech and Syvaaja, 2003). To evaluate if *ABO4/POL2a/TIL1* is involved in DNA repair, we examined the sensitivity of *abo4-1* seedlings to the DNA-damaging reagent, MMS, at different concentrations and to UV-B light. Both MMS and UV-B light produce double-strand DNA breaks (DSBs) in cells. The postgermination growth of *abo4-1* was completely inhibited by 125 ppm MMS, while the growth of wild-type plants was less affected (Figure 2A). Five-day-old seedlings grown on nutrient medium were transferred to nutrient medium

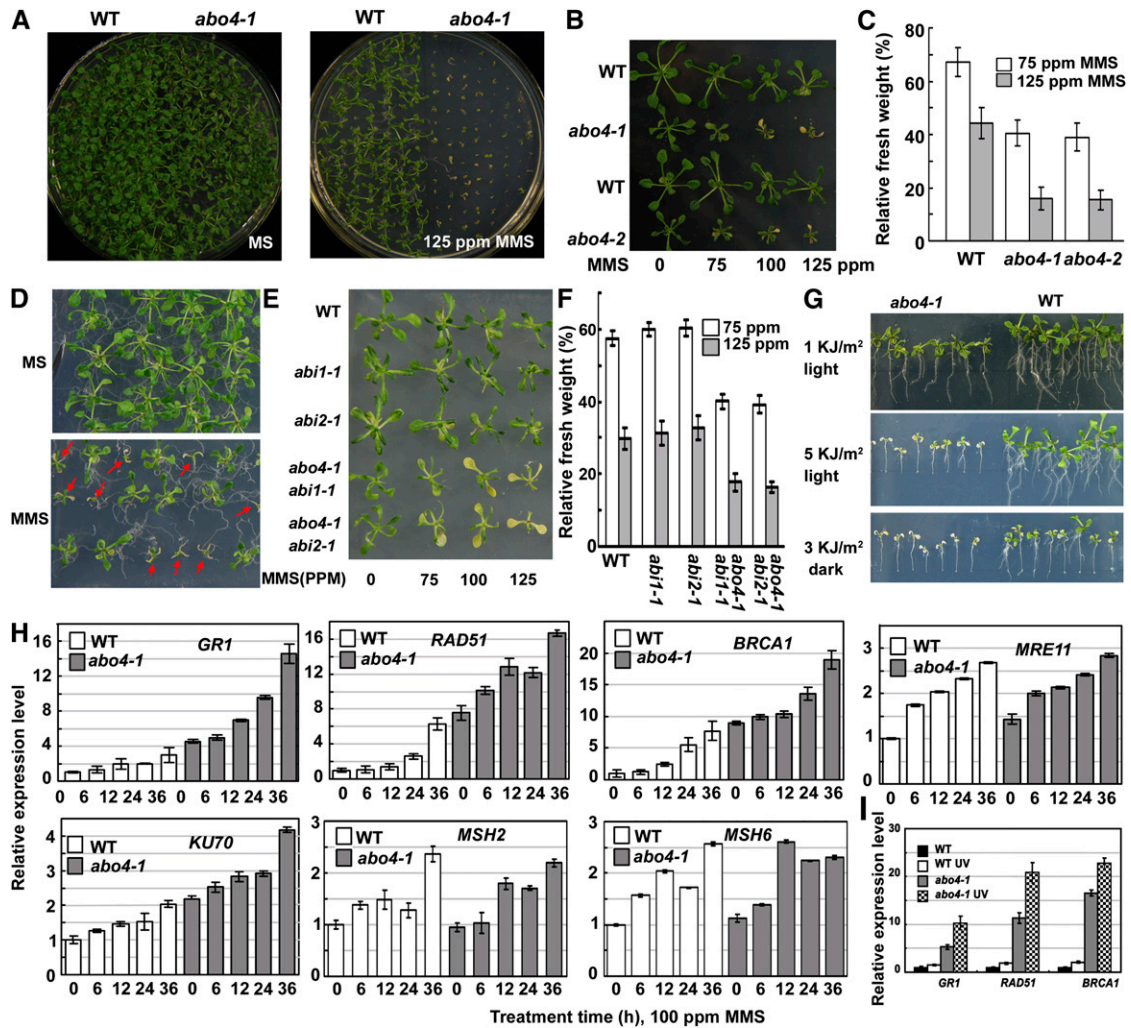


Figure 2. *abo4* Mutants Are Sensitive to DNA Damage.

(A) Comparison of the wild type and *abo4-1* seed germination on MS medium containing no (left) or 125 ppm (right) MMS. Seedlings were grown for 10 d on MS medium containing no MMS or 125 ppm MMS.

(B) Seedling growth sensitivity of wild-type *gl1*, *abo4-1* (*gl1*), the wild type, and *abo4-2* in MS liquid medium supplemented with different concentrations of MMS. Five-day-old seedlings were removed to the MS liquid medium containing different concentrations of MMS and cultured for 10 more days before taking pictures.

(C) Relative fresh weight of wild-type, *abo4-1*, and *abo4-2* seedlings grown on 75 and 125 ppm MMS. The fresh weight of wild-type, *abo4-1*, and *abo4-2* seedlings grown on MS agar medium for 10 d was used to compare with the fresh weight of seedlings grown on 75 and 125 ppm MMS, respectively. For each data point, 30 seedlings were collected and weighed. Data are the mean ± SD of three independent experiments.

(D) The segregation of F1 seedlings from heterozygous *abo4-2* mutant lines crossed with *abo4-1* grown on MS medium containing 100 ppm MMS (MMS sensitive to tolerant seedlings: 58/58). We randomly picked up 20 sensitive seedlings and checked by PCR, and all were heterozygous plants of *abo4-1* and *abo4-2*. Arrows point to the segregated mutants.

(E) *abi1* and *abi2* mutations did not improve the DNA damage sensitivity caused by MMS in *abo4-1*. The similar experiments were done as in **(B)** for *abo4-1 abi1-1* and *abo4-1 abi2-1* double mutant seedlings.

(F) Relative fresh weight of wild-type, *abi1-1*, *abi2-1*, *abo4-1 abi1-1*, and *abo4-1 abi2-1* seedlings grown on 75 and 125 ppm MMS. The fresh weight of wild type, *abi1-1*, *abi2-1*, *abo4-1 abi1-1*, and *abo4-1 abi2-1* seedlings grown on MS agar medium for 10 d was used to compare with the fresh weight of seedlings grown on 75 and 125 ppm MMS, respectively. For each data point, 30 seedlings were collected and weighed. Data are the mean ± SD of three independent experiments.

(G) *abo4-1* seedlings were more sensitive to UV-B light than the wild type. Five-day-old seedlings of the wild type and *abo4-1* in the same plate were treated with different amounts of UV-B light and then grown in light (for 1 and 5 kJ/m²) or first in dark for 2 d and then in light (for 3 kJ/m²). The pictures were taken after seedlings were grown in light for another 5 d.

(H) Comparison of the gene expression in the wild type and *abo4-1* under normal and MMS (100 ppm) treatment using real-time qRT-PCR. *GR1*, *RAD51*, *BRCA1*, *MRE11*, *KU70*, *MSH2*, and *MSH6* were selected for comparison. The transcripts of the wild type in normal condition were used as a

supplemented with different concentrations of MMS and incubated for 10 more days. As shown in Figure 2B, at 75, 100, and 125 ppm MMS, the growth of shoots and roots in both *abo4-1* and *abo4-2* was more severely affected compared with the wild type. Growth measurement of fresh weight is shown in Figure 2C, indicating that the growth of *abo4-1* and *abo4-2* was more greatly inhibited by MMS than that of the wild type. To further confirm the effects of *abo4-2* and *abo4-1* on MMS sensitivity, the F1 seedlings from heterozygous T-DNA mutant lines crossed with *abo4-1* were grown on MS medium containing 100 ppm MMS. As shown in Figure 2D, the ratio of MMS sensitive to tolerant seedlings was 1:1 (58:58). Twenty sensitive seedlings were randomly picked up and checked by PCR, and all were heterozygous for *abo4-1* and *abo4-2*. These data indicate that the *abo4* mutation cosegregated with MMS sensitivity. However, *abi1-1 abo4-1* and *abi2-1 abo4-1* double mutants showed similar MMS-sensitive growth phenotypes as the *abo4-1* single mutant (Figures 2E and 2F), suggesting that the pathways leading to the sensitivity of *abo4-1* to ABA and MMS are different. When treated with various dosages of UV-B light, the growth of *abo4-1* seedlings was more profoundly affected than the wild type (Figure 2G). These results suggest that POL2a has functions in DNA repair.

DSB-Inducible Genes Were Constitutively Expressed in *abo4-1* Mutants

In some DNA damage-sensitive mutants, such as *brusby1* (*bru1*) (Takeda et al., 2004), *tebichi* (*teb*) (Inagaki et al., 2006), the *nucleosome assembly protein1-1* (*nrp1-1*) *nrp2-1* (Zhu et al., 2006) and *fasciata1* (*fas1*) *fas2* double mutants (Endo et al., 2006; Kirik et al., 2006; Ramirez-Parra and Gutierrez, 2007), the expression of some DSB-inducible genes is enhanced. DNA damage caused by ionizing radiation, defects of nucleotide and base excision repair, or failure of mismatch repairing, can lead to DSBs which are repaired through the nonhomologous end joining (NHEJ) pathway (mainly in animals and plants) or through the HR pathway (mainly in yeast). We selected several marker genes functioning in DSB to check their expression. MEIOTIC RECOMBINATION11 (*MRE11*), one member of the MRN complex (composed of *Mre11*, *Rad50*, and *Nbs1*), plays a central role in early DSB repair (Puizina et al., 2004; Farah et al., 2005). *KU70* functions in repairing DNA damage through NHEJ pathway (Tamura et al., 2002), while *MSH2* and *MSH6* proteins play critical roles in DNA mismatch, repairing and limiting HR (Emmanuel et al., 2006; Li et al., 2006; Lafleur et al., 2007). *RAD51* is a homolog of the bacterial *RecA* recombinase in HR repair (Doutriaux et al., 1998). *Arabidopsis* BREAST CANCER SUSCEPTIBILITY1 (*BRCA1*) functions in concert with *Rad51* and other genes to control double-strand break repair and HR (Scully et al., 2004; Reidt et al., 2006). *GAMMA RESPONSE1* (*GR1*) is

also suggested to play some roles in HR (Lafarge and Montane, 2003). We tested the transcript levels of these genes under normal and genotoxic stress conditions by real-time quantitative RT-PCR (qRT-PCR). Without stress, the transcripts of *GR1*, *RAD51*, *BRCA1*, *MRE11*, and *KU70* were found to be higher in *abo4-1* mutants compared with the wild type (Figure 2H). After MMS treatment, the transcripts of the five genes were upregulated in both *abo4-1* and wild-type plants than under normal conditions at different time points (Figure 2H). It seems that *abo4* mutation did not influence the expression of *MSH2* and *MSH6*, but MMS treatment increased the transcripts of these two genes (Figure 2H). UV-B treatment also induced higher expression of *GR1*, *RAD51*, and *BRCA1* in both the wild type and mutant (Figure 2I).

abo4-1 Affected Cell Cycle Regulation

A previous study suggested important roles for POL2a in cell cycle regulation during embryo development (Jenik et al., 2005). Because *abo4* affects meristem development, we investigated the role of ABO4/POL2a in the cell cycle in meristem cells. A transgenic line carrying the *CYCB1;1* promoter fused with the β -glucuronidase (*GUS*) gene was crossed with *abo4-1*. In *Arabidopsis*, the *CYCB1;1* promoter is expressed upon entry into the G2-phase, and *GUS* staining marks cells in G2- and early M-phase (Doerner et al., 1996). As illustrated in Figure 3A (top), much more *GUS* activity was detected in root tips and shoot meristems of *abo4-1* and *abo4-2* than the wild type, suggesting that G2/M progression in *abo4-1* and *abo4-2* was delayed. MMS treatment strongly induced *GUS* expression (Figure 3A, middle), suggesting a crucial role of DNA DSB repair in G2/M progression. However, ABA treatment did not apparently affect the expression of *CYCB1;1* (Figure 3A, bottom), which is consistent with previous results showing that ABA only affects cell cycle progression in the G1 stage (Swiatek et al., 2002). We also tested the expression of *CYCB1;1*, *CYCA2;1*, *CDC2A*, and *HISTONE H4b* (S-specific *Histone4*) by qRT-PCR and found the transcripts of these genes were all more highly expressed in *abo4-1* than wild-type plants (Figure 3B). The higher expression of *H4b* in *abo4-1* suggests a prolonged S-phase, which is consistent with previous observations (Jenik et al., 2005).

abo4 Mutation Greatly Elevated the Frequency of HR

Because *abo4-1* is sensitive to genotoxic stress and DSBs are repaired in part by HR, we examined whether or not the *abo4* mutation influences the level of HR using a *GUS* reporter recombination substrate. Two transgenic lines, 1415 and 1406, with T-DNAs containing two inactive fragments of the *GUS* gene sharing an overlapping sequence in direct or inverted orientation

Figure 2. (continued).

standard for normalization.

(I) Comparison of the transcripts of *GR1*, *RAD51*, and *BRCA1* by qRT-PCR between the wild type and *abo4-1* under normal and UV-B (3 kJ/m², in dark for 6 h) treatment.

In both (H) and (I), three independent biological replications were performed for each experiment. The level of 18S rRNA was used as an internal control. Error bars indicate \pm SD.

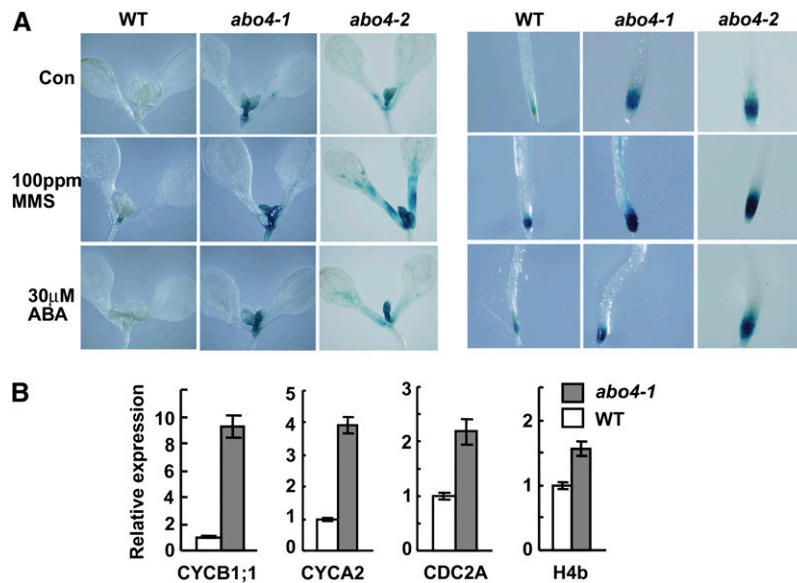


Figure 3. The *abo4* Mutation Increases Cell Cycle-Related Gene Expression.

(A) The representative examples of GUS staining. *CYCB1;1* promoter-GUS was expressed higher in shoot and root meristems of *abo4-1* and *abo4-2* than the wild type, and its expression was highly induced by MMS treatment but not affected by ABA.

(B) The relative expression levels of *CYCB1;1*, *CYCA2*, *CDC2A*, and *H4b* in the wild type and *abo4-1* analyzed by qRT-PCR. The expression of each gene in the wild type was used as a standard. The representative results were from one of three independent experiments. Error bars indicate \pm SD.

(Lucht et al., 2002) (Figure 4A) were selected for these experiments. If an intrachromosomal recombination event occurs between the overlapping regions, a functional GUS gene will be reconstituted and expressed, which can be detected as a blue sector after GUS staining. The *abo4-1* mutant was crossed with two recombination substrate lines 1415 and 1406, and we selected *abo4-1* mutants or wild-type plants carrying the homozygous recombination substrate transgene from the F3 population and used them for GUS staining analysis. More than 100 seedlings were analyzed for each assay. The number of GUS staining spots was various among different lines. On average, we detected 2.9 spots per wild-type plant and 140.4 spots per *abo4-1* plant in reporter line 1415, and one spot per wild-type plant and 59.3 spots per *abo4-1* plant in reporter line 1406 with our conditions (Figures 4B to F). We obtained similar high GUS recombination rates for *abo4-2* mutant in both 1415 and 1406 lines (Figure 4B, right). These results indicate that the *abo4* mutations greatly increase HR.

ABA Treatment Tended to Increase HR in Both the Wild Type and *abo4-1* Mutant

The influence of environment stresses, including pathogens, UV-B, UV-C, and x-rays, on somatic HR has been studied in plants (Schuermann et al., 2005; Boyko et al., 2006). However, the impact of key stress hormone ABA on HR has not yet been studied. Because of the sensitivity of *abo4-1* to ABA, we examined HR after ABA treatment. Here, we used a relatively low concentration of ABA, as a slightly higher concentration of ABA greatly inhibited *abo4-1* growth. Five-day-old seedlings were transferred to nutrient medium containing 5 μ M ABA and grown for 7 d. Then, the seedlings were moved to nutrient medium and grown for 2 d.

Because different plants have big variations in HR rate even if they are the same offspring, we used 100 to 120 seedlings and counted the GUS spots for each one and then obtained the average statistical number from these plants. Two independent experiments showed a similar HR increase in both the wild type and mutant. Figures 4C and 4E show the results of one typical experiment. The average number of blue sectors increased from 2.9 without ABA treatment to 7.1 with ABA treatment of the wild type, from 140.4 without ABA treatment to 159.4 with ABA treatment of *abo4-1* in line 1415, from 1 without ABA treatment to 1.5 with ABA treatment of the wild type, and from 59.4 without ABA treatment to 85.5 with ABA treatment of *abo4-1* in line 1406 (counting 100 to 120 seedlings for each line for each time). These results suggest that ABA treatment tended to increase HR frequency of both the wild type and the *abo4-1* mutant.

ABA Treatment Increases DNA Breaks in *abo4-1*

We tested whether the growth inhibition supersensitivity of *abo4-1* to ABA is at least partially due to a lesion in DNA repair. DNA breaks were analyzed using the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay after ABA treatment. The TUNEL assay sensitively detects increased 3'-OH break ends (Gao and Showalter, 1999). Nuclei in leaves were simultaneously detected by 4',6-diamidino-2-phenylindole staining. Under normal growth conditions, we did not detect any apparent TUNEL staining in both wild type and *abo4-1* nuclei (Figure 5A, top). Here, we used 30 μ M ABA to treat the seedlings, as this or higher concentrations of ABA apparently retarded the growth of mutant seedlings but does not greatly inhibit the growth of wild-type seedlings (the Col wild-type seedlings can tolerance

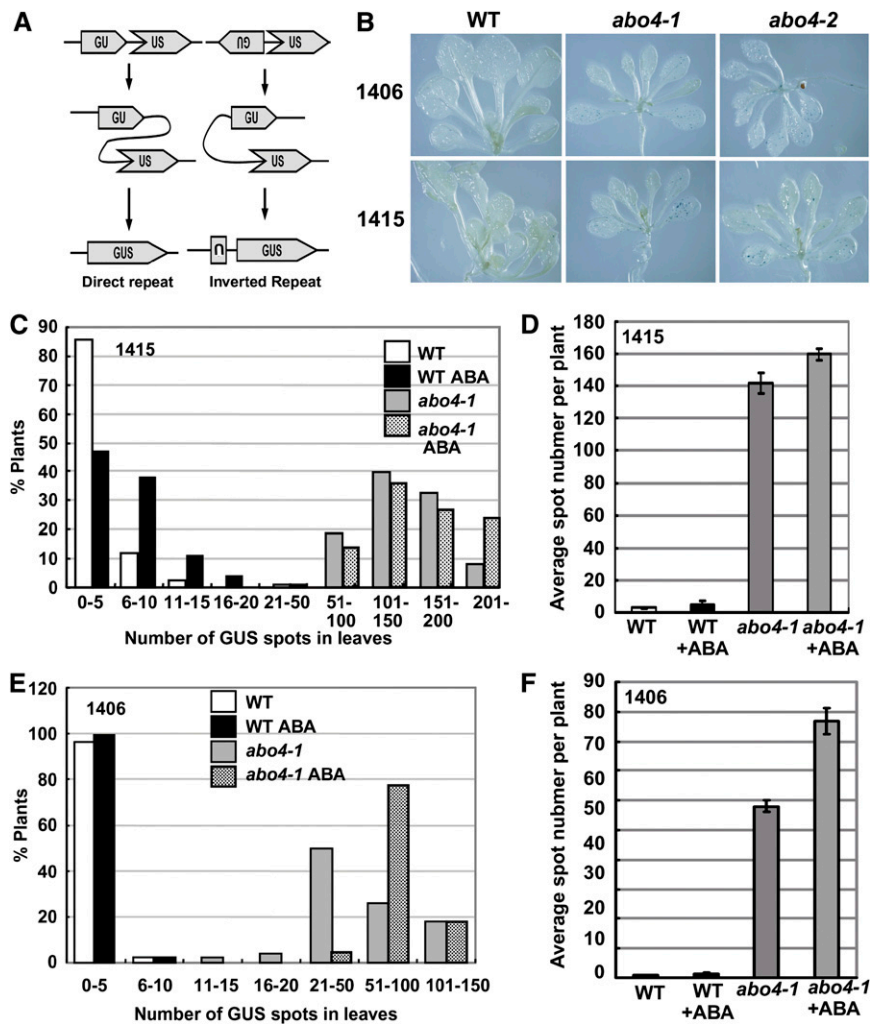


Figure 4. The Effects of *abo4* Mutation and ABA on HR.

(A) The diagram of GUS reporter constructs in direct (1415, left) and inverted repeat (1406, right) used in this study (adapted from Lucht et al., 2002). (B) Typical patterns of GUS staining in *abo4-1*, *abo4-2*, and the wild type carrying GUS reporter lines 1406 and 1415, respectively, under normal growth conditions.

(C) and (E) Quantitative analysis of HR in ~100 plants of *abo4-1* and the wild type carrying reporter line 1415 (C) and 1406 (E), respectively, under normal and ABA treatment conditions. The number of the GUS staining sectors was counted for each plant. The number of plants assayed was 115 for each assay. The proportions of plants showed frequency distribution of a given number of blue GUS spots in 1415 and 1406 populations.

(D) and (F) The average GUS spot number per seedling in ~100 *abo4-1* and wild-type seedlings under ABA and normal conditions. The number of plants assayed was 105 for each assay. Two independent experiments were performed. Error bars indicate \pm SD. The *abo4-1* showed a 48-fold increase in the number of blue sectors over the reporter line 1415 and 61-fold increase over the reporter line 1406 than the wild type under normal conditions.

as high as 150 μ M ABA). Treatment with 30 μ M ABA for 72 h greatly increased TUNEL staining in leaves of *abo4-1* but did not have any measurable influence on TUNEL staining in the wild type (Figure 5A, bottom), suggesting that ABA treatment increases DNA breaks in the *abo4-1* mutant.

ABA Increases the Expression of Some DSB-Inducible Genes

Because ABA treatment led to increased DSBs in *abo4-1* mutants, we analyzed the expression of *GR1*, *RAD51*, *BRCA1*,

KU70, *MRE11*, and *MSH2* after ABA treatment. ABA treatment increased the expression of *GR1* and *MRE11* but repressed the expression of *KU70*, *BRCA1*, and *RAD51*, especially at the later time points in both *abo4-1* and the wild type (Figure 5B). However, we did not observe any clear expression changes of *MSH2* (Figure 5B) and *MSH6* (data not shown) by ABA, suggesting the ABA might not impose influence on the expression of the two genes. These results indicate that the molecular mechanisms regulating the expression of these genes by ABA and MMS are different.

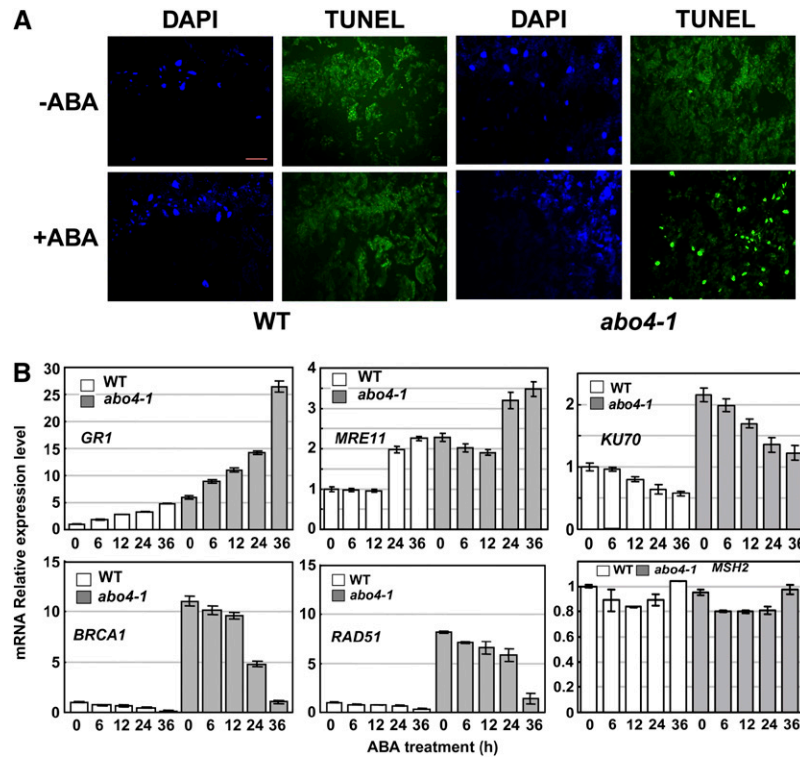


Figure 5. ABA Treatment Increases DNA Breaks and Upregulates *GR1* and *MRE11* Expression.

(A) The TUNEL assay under normal or ABA-treated conditions. Under the normal growth condition, TUNEL staining was negative in both the wild type and *abo4-1* mutant (–ABA). Under ABA (30 μ M for 3 d) treatment, only *abo4-1* showed many TUNEL (bright green fluorescent signal) nuclei, but the wild type still showed negative TUNEL nuclei. The 4',6-diamidino-2-phenylindole (DAPI) staining (blue color) was used to indicate the nuclei.

(B) Comparison of *GR1*, *MRE11*, *KU70*, *RAD51*, *BRCA1*, and *NSH2* expression by qRT-PCR under ABA treatment. The expression of *GR1* and *MRE11* was increased, and *KU70*, *RAD51*, and *BRCA1* decreased by ABA treatment (30 μ M) especially in the later time points in both *abo4-1* and the wild type. The expression of *MSH2* was not influenced by ABA. The transcripts of each gene under normal condition were used as a comparison standard. The representative data were from one of three independent experiments showing similar results. Error bars indicate \pm SD.

***abo4* Mutation Releases Transcriptional Gene Silencing of *35S-NPTII* in the *ros1* Mutant and *TSI* without Changing DNA Methylation**

Mutation in the *REPRESSOR OF SILENCING1* (*ROS1*) gene encoding a DNA demethylation enzyme led to the TGS of the originally activated *35S-NPTII*, *RD29A-LUC* gene, and endogenous *RD29A* gene (Gong et al., 2002). Screening for *ros1*_{RD29A-LUC} suppressors identified several genes that release the TGS of *NPTII* when mutated (Xia et al., 2006; Wang et al., 2007). To see whether the *abo4*/*POL2a* mutation is able to release the silenced *35S-NPT II* in *ros1*_{RD29A-LUC}, we crossed *abo4-1* with *ros1-1*_{RD29A-LUC} and made *abo4-1 ros1-1*_{RD29A-LUC} double mutant carrying *35S-NPTII* and *RD29A-LUC* transgenes. As shown in Figure 6A (middle), *abo4-1 ros1-1*_{RD29A-LUC} seedlings were resistant to kanamycin, suggesting that the *abo4* mutation reactivated the originally silenced *35S-NPTII* in *ros1-1*_{RD29A-LUC} mutant. RNA gel blot analysis confirmed the reexpression of *NPTII* gene in the *abo4-1 ros1-1*_{RD29A-LUC} double mutant (Figure 6B). However, as in the *ror1-1 ros1-1*_{RD29A-LUC} or *tousled ros1*_{RD29A-LUC} mutant (Xia et al., 2006; Wang et al., 2007), the *abo4-1* mutation did not release the TGS of *RD29A-LUC*

and endogenous *RD29A* in *ros1-1*_{RD29A-LUC} as analyzed by luminescence imaging assay (Figure 6A, left), RNA gel blot analysis of endogenous *RD29A* expression under ABA treatment (Figure 6B, *RD29A*), and *LUC* induction under NaCl treatment (Figure 6C). Under the same ABA and NaCl treatments, the expression of endogenous *RD29A* and *RD29A-LUC* in wild-type_{RD29A-LUC} was induced. As a positive control, a stress-inducible marker gene, *COR47* (Gong et al., 2002), was also induced in both wild-type_{RD29A-LUC} and *abo4-1*. These results suggest the diversification of the TGS pathways at different loci (Gong et al., 2002; Xia et al., 2006; Wang et al., 2007).

Several *Arabidopsis* mutants defective in DNA/chromatin assembly and replication showed instability of epigenetic states and release of TGS at the pericentromeric *Athila* retrotransposons, which are referred to as transcriptional silent information (*TSI*) (Steimer et al., 2000; Takeda et al., 2004; Elmayan et al., 2005; Kapoor et al., 2005; Inagaki et al., 2006; Wang and Liu, 2006; Xia et al., 2006; Zhu et al., 2006; Ramirez-Parra and Gutierrez, 2007; Wang et al., 2007). RNA gel blot analysis indicated that the *abo4-1* and *abo4-2* mutations released the TGS of *TSIs*, although the effect was much less than that of the *ddm1* mutation (Figure 6D).

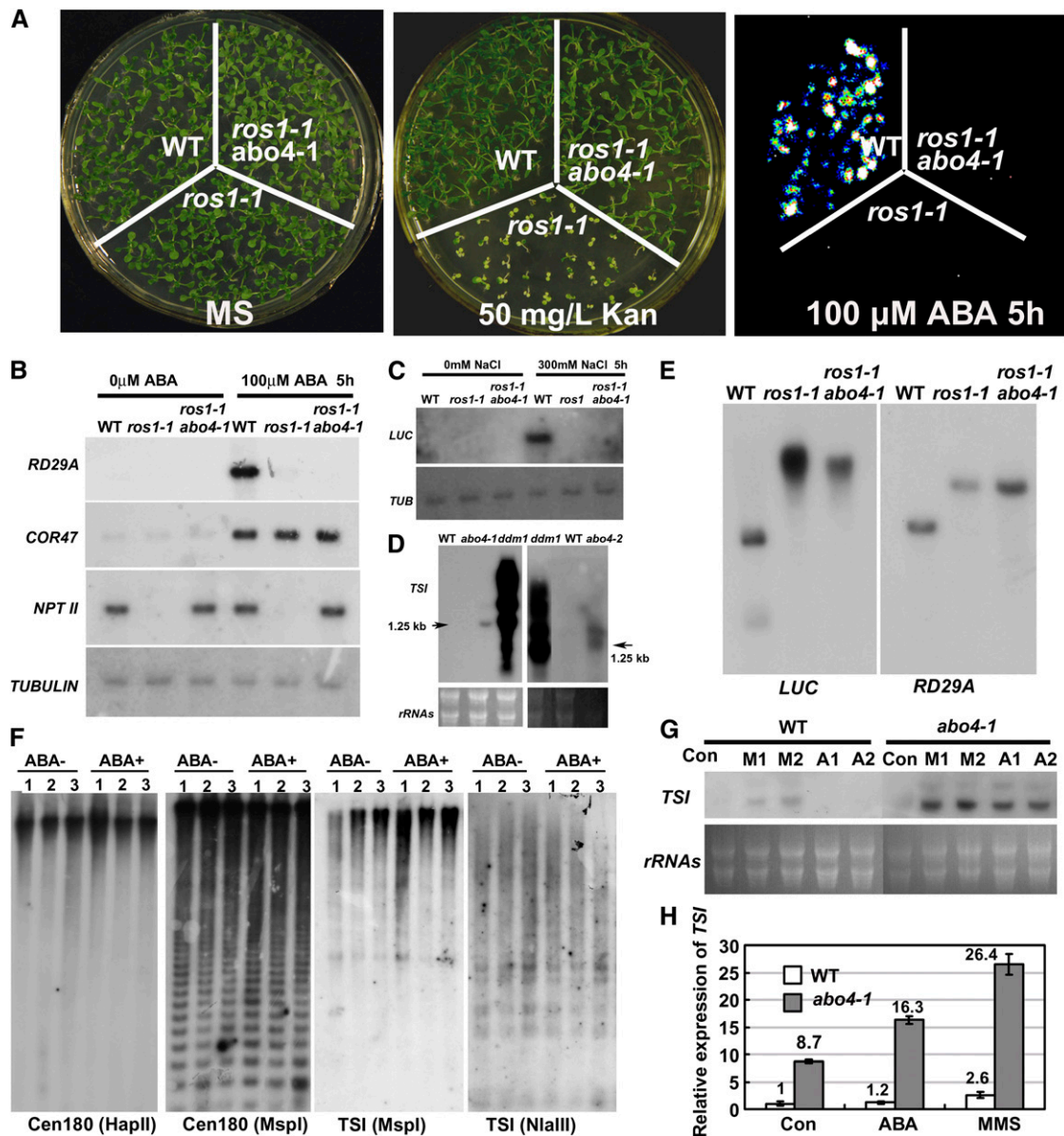


Figure 6. Releasing of TGS of *35S-NPTII* in *ros1* and *TSI* by *abo4* Mutation Independent on DNA Methylation.

(A) *abo4* mutation reactivated the silenced *35S-NPTII* but did not affect the silenced *RD29A-LUC* in *ros1-1* mutant. The seeds of wild-type_{*RD29A-LUC*}, *ros1-1*_{*RD29A-LUC*}, and *abo4-1 ros1-1*_{*RD29A-LUC*} carrying *RD29A-LUC* transgene were germinated and grown on MS medium (left plate) or MS medium supplemented with 50 mg/L kanamycin (middle plate) for 2 weeks. Both wild-type_{*RD29A-LUC*} and *abo4-1 ros1-1*_{*RD29A-LUC*} seedlings showed the kanamycin-resistant phenotype from expression of the *NPTII* gene, while *ros1-1*_{*RD29A-LUC*} seedlings were sensitive to kanamycin due to transgene silencing. Two-week-old seedlings were treated with 100 μ M ABA for 5 h to induce the expression of *RD29A-LUC*. *LUC* expression was only detected in wild-type_{*RD29A-LUC*} but not in *ros1-1*_{*RD29A-LUC*} and *ros1-1 abo4-1*_{*RD29A-LUC*} double mutants (right plate), indicating that *abo4* mutation did not reactivate the silenced *RD29A-LUC* in *ros1-1*.

(B) RNA gel blot analysis of endogenous *RD29A* and *35S-NPTII* expression under ABA treatment. Total RNAs extracted from different seedlings treated or not treated by 100 μ M ABA for 5 h were used for hybridization. A stress-inducible marker gene, *COR47*, was used as positive control to indicate that ABA treatment equally induced its expression in wild-type_{*RD29A-LUC*}, *ros1-1*_{*RD29A-LUC*}, and *ros1-1 abo4-1*_{*RD29A-LUC*}. *RD29A* expression was only detected in the wild type by ABA treatment, while *NPTII* transcripts were detected in both wild-type_{*RD29A-LUC*} and *ros1-1 abo4-1*_{*RD29A-LUC*}. The endogenous *RD29A* and the transgene copy of *35S-NPTII* were silenced in *ros1-1*_{*RD29A-LUC*}. *TUBULIN* was used as a loading control.

(C) *abo4* mutation did not release the TGS of *RD29A-LUC*. RNA gel blot analysis of *RD29A-LUC* expression in wild-type_{*RD29A-LUC*}, *ros1-1*_{*RD29A-LUC*}, and *ros1-1 abo4-1*_{*RD29A-LUC*} under 300 mM NaCl for 5 h. *LUC* transcripts were only detected in the wild type and not in *ros1-1* and *ros1-1 abo4-1*.

(D) *TSI* expression was enhanced in the *abo4-1* and *abo4-2* mutants. RNA gel blot analysis of *TSI* expression in *ddm1* was used as a control for comparison.

Because DNA methylation is the hallmark of heterochromatin and closely related to TGS, we measured the DNA methylation status in the *TSI*, centromeric DNA, and *RD29A* promoter regions by DNA gel blot analysis using DNA methylation-sensitive enzymes, as these regions are hypermethylated (Gong et al., 2002). DNA gel blot analysis indicated that *abo4-1* did not affect DNA methylation in both foreign and endogenous *RD29A* promoters (Figure 6E) as well as in *TSI* and centromeric DNA regions (Figure 6F, ABA⁻). The DNA methylation status of *TSI* and centromeric DNA was not affected by ABA treatment (Figure 6F, ABA⁺). Like *ror1/rpa2a* and *ts1* mutations (Xia et al., 2006; Wang et al., 2007), *abo4* mutation influences the epigenetic instability likely independent on DNA methylation.

Due to the sensitivity of *abo4-1* to ABA, we examined *TSI* expression under ABA treatment. Interestingly, *TSI* transcripts were upregulated by ABA only in *abo4-1* but were not detected in the wild type under our RNA gel blot conditions (Figure 6G). qRT-PCR analysis indicated that *TSI* expression was induced two times in *abo4-1* by ABA, but only 1.2 times in the wild type (Figure 6H). However, MMS treatment induced *TSI* transcripts in both the wild type and *abo4-1* but at a little higher level in *abo4-1* (2.6 times in wild type and three times in *abo4-1*) (Figures 6G and 6H).

***abo4* Mutants Show Early Flowering and Reduced Expression of *FLOWERING LOCUS C* and Increased Expression of *FLOWERING LOCUS T* with Changing Histone H3 Modifications**

abo4-2 plants grown on MS medium were a little smaller than *abo4-1* plants, and the two mutants were both noticeably smaller than the wild type when grown on agar plates or soil (Figures 7A and 7B). The F1 seedlings obtained from the crossing of *abo4-1* with heterozygous *abo4-2* showed *abo4-1* and wild-type growth phenotype at a ratio of ~1:1 (54:56) when grown in soil (Figure 7C). Under long-day conditions (16 h light/8 h dark), wild-type plants flowered at $\sim 24 \pm 1$ d after germination with a leaf number of $\sim 11 \pm 1$, while *abo4-1* plants flowered at $\sim 17 \pm 1$ d after germination and had a leaf number of 8 ± 1 (counting 50 plants each time, three times, Figures 7B and 7D). The F1 heterozygous *abo4-1* and *abo4-2* plants flowered earlier than *abo4-1* or *abo4-2* mutants (Figure 7B). *abo4-1* produced fewer seeds than the wild type (on an average 27 seeds per silique in *abo4-1* and 40 seeds per silique in the wild type, counting 50

siliques, Figure 7E). Occasionally, some plants were found to have fasciated stems and abnormal flowers (Figure 7F, wild-type stem; Figure 7G, *abo4-1* stem; Figure 7H, *abo4-2* stem; Figure 7I, wild-type flower; Figure 7J, *abo4-1* flower), suggesting stochastic incidents might have happened during development. The root meristem of *abo4-1* is much shorter than that of the wild type (Figure 7K, wild type; Figure 7L, *abo4-1*). The shoot apical meristem (SAM) in the wild type exhibits three classical zones: the central zone in the top, the peripheral zone in the middle, and the ribzone in the bottom (Xia et al., 2006). However, it is hard to clearly differentiate these three zones in the SAM of *abo4-1* (Figure 7M, wild type; Figure 7N, *abo4-1*).

The floral repressor *FLOWERING LOCUS C* (*FLC*) is a central player that quantitatively represses the floral pathway integrators *FLOWERING LOCUS T* (*FT*), *SOC1*, and *LFY* for delaying flowering (Boss et al., 2004; Amasino, 2005; Dennis and Peacock, 2007). We used qRT-PCR to check the expression of *FLC*, *FT*, and *AP1*, a downstream target of *FT*. Two-week-old seedlings grown on MS plates under long-day conditions (16 h light/8 h dark) were used to extract total RNAs. As shown in Figure 7O, the expression of *FLC* was lower in *abo4-1* and *abo4-2* compared to that in the wild type. However, the expression of *FT* and *AP1* was much higher in *abo4-1* and *abo4-2* than that in the wild type. Because the expression of *FLC* and *FT* is mediated by histone modifications (Amasino, 2005), and ABO4 suggests to be involved in epigenetic inheritance, we performed a chromatin immunoprecipitation (ChIP) analysis using histone H3K4me2 and H3K27me3 antibodies to test whether the *abo4* mutation affects epigenetic marks on *FLC* and *FT*. The dimethylation of Lys-4 of histone H3 (H3K4me2) is a hallmark of active genes in euchromatin, while the trimethylation of Lys-27 of histone H3 (H3K27me3) functions in the epigenetic silencing of *FLC* (Finnegan and Dennis, 2007; Greb et al., 2007). We found a clear increase of H3K27me3 in the first intron regions of *FLC* (*FLC*-P3 and *FLC*-P5, two fragments amplified by PCR; please see Cao et al., 2008), but only a little decrease in the promoter region of *FT* in *abo4-1* compared to the wild type (Figure 7P, top). However, a noticeable enrichment of H3K4me2 was found in the promoter of *FT* but no change (*FLC*-P5) or a little decrease (*FLC*-P3) of H3K4me2 in the first intron regions of *FLC* (Figure 7P, bottom). These results suggest that *abo4* mutation influences the histone H3 modifications around *FLC* gene body and *FT* promoter region.

Figure 6. (continued).

(E) *abo4* mutation did not affect the DNA hypermethylation in the transgene and endogenous *RD29A* promoter in *ros1-1_{RD29A-LUC}*. Genomic DNAs extracted from wild-type_{RD29A-LUC}, *ros1-1_{RD29A-LUC}*, and *ros1-1_{RD29A-LUC} abo4-1_{RD29A-LUC}* were digested with DNA methylation-sensitive enzymes *MluI* (A^mCG^mCGT) and *BstUI* (m^mCG^mCG) as described (Gong et al., 2002) and hybridized with ³²P-labeled *LUC* (for transgene *RD29A* promoter) or *RD29A* (for endogenous *RD29A* promoter).

(F) Methylation status of centromeric DNA (Cen180) or *TSI*s was neither influenced by *abo4* mutations nor by ABA treatment. Genomic DNAs were extracted from (1) the wild type; (2) *abo4-1*; (3) *abo4-2*; ABA⁻, without ABA treatment; ABA⁺, seedlings were treated with 10 μ M ABA for 7 d and digested by DNA methylation-sensitive restrictive enzymes *HpaII* (m^mC^mCGG methylation), *MspI* (m^mCCGG methylation), and *NlaIII* (m^mCATG methylation). The membranes were hybridized with ³²P-labeled Cen 180 bp or *TSI* fragment, respectively.

(G) Both ABA and MMS treatment increased the *TSI* transcripts more in *abo4-1* than the wild type. Seven-day-old seedlings were treated with MMS (M1, 50 ppm; M2, 100 ppm) for 7 d or treated with ABA (A1, 5 μ M; A2, 10 μ M) for 7 d. RNA gel blot analysis was performed using *TSI* as probe. RNAs were used as loading control.

(H) qRT-PCR analysis of *TSI* expression in the wild type and *abo4-1* treated by 5 μ M ABA or 50 ppm MMS for 7 d. Untreated wild type was used as a standard control. Three biological replications were performed for each experiment. Error bars indicate \pm SD.

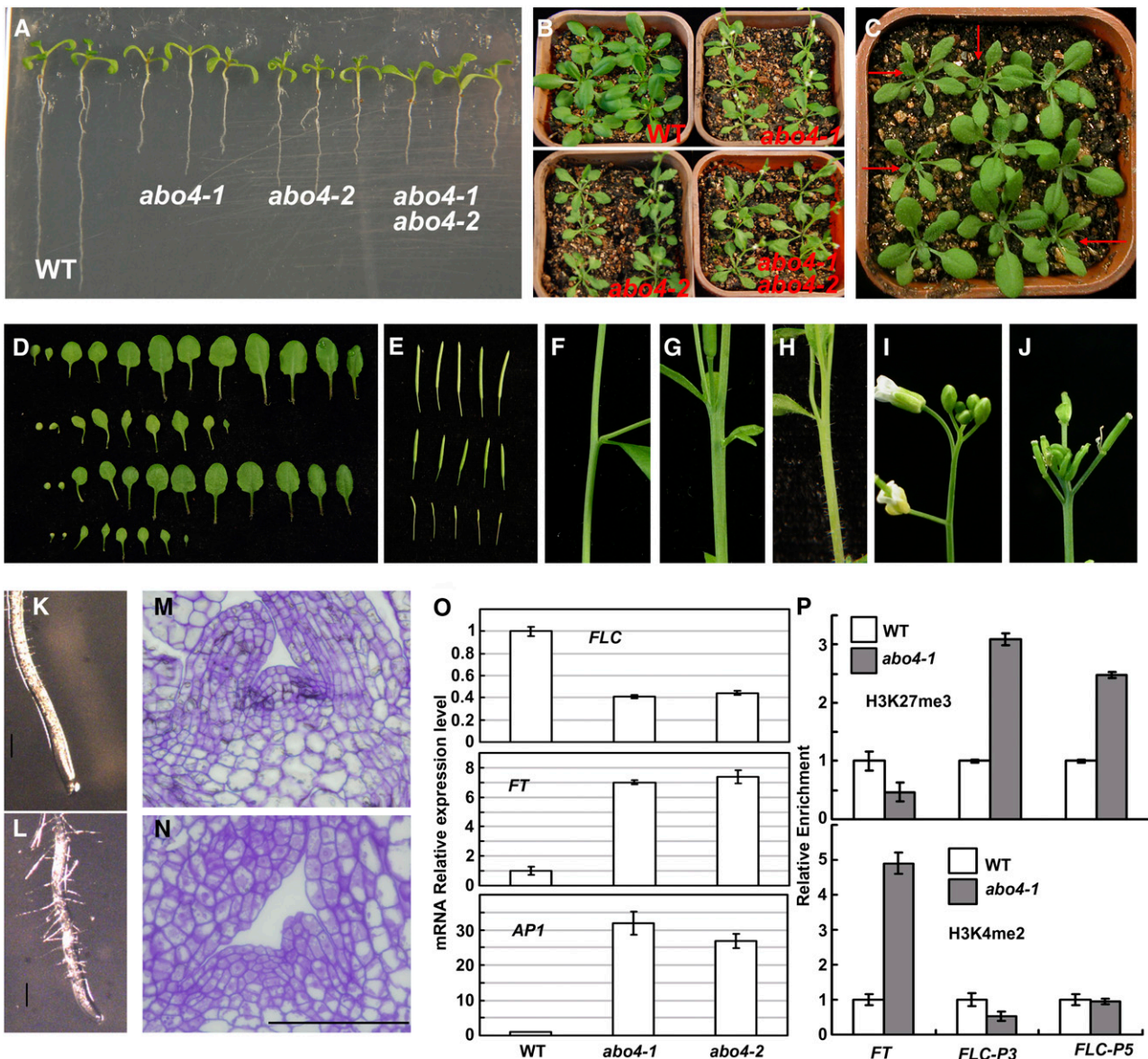


Figure 7. Growth phenotypes of *abo4* mutants.

(A) Seedling comparison of wild-type, *abo4-1*, *abo4-2*, and *abo4-1 abo4-2* heterozygous plants on MS agar plates. Please notice that *abo4-2* shows a smaller phenotype, and *abo4-1 abo4-2* heterozygous plants show *abo4-1* phenotype.

(B) Comparison of wild-type, *abo4-1*, *abo4-2*, and *abo4-1 abo4-2* heterozygous plants grown in soil.

(C) The F1 seedlings of heterozygous *abo4-2* crossed with *abo4-1*. The ratio of *abo4-1* to wild-type phenotype is ~1:1 (54:56). *abo4-2/abo4-1* mutants showing *abo4-1* phenotypes are indicated with arrows.

(D) Comparison of the rosette leaves of wild-type (*gl1*), *abo4-1(gl1)*, wild-type (Col), and *abo4-2* (Col) before flowering. *abo4-1* is in Col *gl1* background, and *abo4-2* is in Col background. Because there is no growth difference in Col *gl1* and Col except for Col *gl1* with no trichomes, we only used Col *gl1* as control in later analysis.

(E) Comparison of siliques in wild-type, *abo4-1*, and *abo4-2*. Please notice that no seeds were set in *abo4-2* siliques, and fewer seeds were set in *abo4-1* than the wild type.

(F) to (H) Comparison of stems of the wild type **(F)**, *abo4-1* **(G)**, and *abo4-2* **(H)**. Here, we only show *abo4-1* and *abo4-2* stems with fasciation phenotype. However, most of the *abo4-1* and *abo4-2* stems grew normally.

(I) and (J) Comparison of flowers of the wild type **(I)** and *abo4-1* **(J)**.

(K) and (L) Comparison of roots of the wild type **(K)** and *abo4-1* **(L)**. The root elongation zone is shorter in *abo4-1* than the wild type. Bars = 5 mm.

(M) and (N) Comparison of SAMs of the wild type **(M)** and *abo4-1* **(N)**. Three classical zones were clearly observed in the SAM of the wild type but not in the SAM of *abo4-1*. Bar = 100 μ m.

DISCUSSION

Plant hormones act as important regulators of cell division by controlling the expression of cell cycle-related genes (Dewitte and Murray, 2003). Previous studies suggested that ABA inhibits cell division through an increase in the expression of the cyclin-dependent kinase inhibitor *KRP1/ICK1* (Wang et al., 1998, 2000). However, inhibition of DNA synthesis by ABA treatment is considered to occur earlier than inhibition of either RNA or protein synthesis (Stewart and Smith, 1972), suggesting that ABA or ABA signaling might directly target existing DNA synthesis or cell cycle proteins to arrest cell division. Here, we showed that a mutation in *ABO4/POL2a/TIL1* leads to *Arabidopsis* plants that are overly sensitive to ABA. However, the ABA sensitivity of *abo4-1* was blocked by two of the ABA-insensitive mutations, *abi1* and *abi2*, suggesting that ABA itself is not directly targeting the POL2a protein. We also found that the growth of *fas1-101* (a mutation in chromatin assembly factor CAF-1) and *ror1-3* (a T-DNA insertion mutation in replication protein A2) (Xia et al., 2006) was also sensitive to ABA, but a polymerase α mutant (isolated as a *ros1-1* suppressor) (see Supplemental Figure 1 online) did not show ABA-sensitive phenotypes. ABA did not change the expression level of *ICK* between *abo4-1* and the wild type (data not shown). It is conceivable that ABA signal is transduced to downstream target(s), which then affect the DNA synthesis and cell cycles through POL2a and other proteins.

abo4-1 mutants are supersensitive to DNA-damaging agents MMS and UV-B light, suggesting that in *Arabidopsis*, POL2a plays a crucial role in DNA repair. We found that *abo4-1* constitutively expresses higher levels than the wild type of *GR1*, *BRCA1*, *RAD51*, *MER11*, and *KU70*, all of which are induced in response to the accumulation of DNA damage by MMS. These DNA damage-induced genes are thought to function in DNA repair, cell cycle checkpoints, or HR (Lafarge and Montane, 2003; Puizina et al., 2004; Inagaki et al., 2006). Interestingly, ABA treatment also induced more DNA breaks in *abo4-1* than in the wild type and increased the expression of *GR1* and *MRE11*, but suppressed the transcript levels of *RAD51*, *BRCA1*, and *KU70*. These data suggest that the plant responses to DNA damage caused by ABA are different from those caused by MMS because MMS highly induced the expression of all these genes.

In the *abo4-1* and *abo4-2* mutant, cell cycle progression is arrested at the G2/M-phase with a hallmark of high *CYCB1;1* expression. Recent studies indicated that defective cell cycle progression was also observed in mutants, such as *teb*, *bru1/tsk/mgo3*, *fas1*, *fas2*, *nrp1/nrp2*, *tsl*, *ror1/rap2a*, and *tso2-1 rnr2a-1*, all of which are defective in DNA repair and replication (Ehsan et al., 2004; Endo et al., 2006; Inagaki et al., 2006; Wang and Liu, 2006; Zhu et al., 2006). The *Arabidopsis* POL2a mutation leads to

the lengthening of the S-phase of the cell cycle with a high expression of *H4b* in *Arabidopsis* (this study; Jenik et al., 2005). It is possible that a POL2a lesion impairs DNA and chromatin replication, leading to more stalled replication forks during S-phase, which activates the S-phase checkpoint response. DNA synthesis and chromatin assembly must be precisely duplicated before cells enter into the mitotic stage. Retardation in the G2/M-phase with the accumulation of *CYCB1;1-GUS* expression could be important for cells to repair S-phase accumulated DNA damage. However, MMS, but not ABA treatment, increased the accumulation of *CYCB1;1-GUS*, suggesting that the DNA damages caused by MMS delay the cell cycle in G2/M-phase, which was not influenced by ABA. These results imply that the delayed cell cycle by ABA treatment did not happen in the G2/M but might be in the G1/S, which is supported by the previous results that ABA negatively influences the cell cycle progression at the G1/S transition but not in the G2/M in tobacco BY-2 cells or during maize (*Zea mays*) germination (Swiatek et al., 2002; Sanchez Mde et al., 2005). However, how ABA inhibits G1/S transition remains an enigma. Because ABA did not influence the cell cycle in G2/M in the *abo4* mutants, this means that ABA does not directly restrain the ABO4/POL2a activity, but instead, ABA might target an unidentified protein(s) that might mainly function in the G1/S and manipulate the activities of ABO4/POL2a and other proteins, such as FAS1 and ROR1/RPA2A. However, this protein would not affect the polymerase α , as its mutation did not show an ABA phenotype. These results suggest that POL2a, FAS1, and ROR1/RPA2A are in a different pathway from polymerase α in the cell cycle.

To our surprise, we observed a higher number of HR events in *abo4-1* than the wild type, suggesting that POL2a is involved in maintaining low HRs in the wild-type plants. This phenomenon contrasts with lower MAT switching with the yeast POL2 mutation (Holmes and Haber, 1999). In eukaryotes, DSBs are repaired by both NHEJ and the HR pathway. In the HR pathway, the undamaged sister chromatid is used as a DNA template to accurately repair the lesion strand in the late S-G2 phase. NHEJ mainly reconnects the broken DNA ends with no sequence similarity especially during the G1-phase when sister chromatids are not available (Kobayashi et al., 2008; Shrivastav et al., 2008). Yeast preferentially uses HR in DNA repair and possesses a high HR frequency, whereas plants and mammals prefer NHEJ repair. A model was proposed in which stalled replication forks were broken and repaired by HR before replication resumes when DNA replication encounters DNA damages (Rothstein et al., 2000). Studies in *Arabidopsis* indicate that greatly increased HR frequency was observed in *fas1*, *fas2* (defective in genes encoding chromatin assembly factor 1 subunits p150 and p60, respectively) (Endo et al., 2006; Kirik et al., 2006), or *cen2* (*centrin2*,

Figure 7. (continued).

(O) qRT-PCR analysis of *FLC*, *FT*, and *AP1* expression. In each case, RNAs from the wild type were used as a standard control for normalization for each RNA. The representative data were from one of three independent experiments showing similar results. The level of 18S rRNA was used as an internal control. Error bars indicate \pm SD.

(P) ChIP analysis of histone H3K27me3 and H3K4me2 modifications in the first intron of *FLC* (the same primers are used as in Cao et al., 2008) and in the *FT* promoter region by qRT-PCR. Three independent immunoprecipitations were performed for each experiment. The immunoprecipitated DNA was quantified by real-time qRT-PCR. We used *ACTIN* as internal controls for the H3K4me2 level and *TA3* for the H3K27me3 level. Error bars indicate \pm SD.

defective in a calcium binding EF-hand protein) (Molinier et al., 2004); while mutation in BRU1 (a novel protein involved in DNA repair and replication) (Takeda et al., 2004), RAD50 (Gherbi et al., 2001), RAD17, or RAD9 (Heitzeberg et al., 2004) only slightly increases HR, whereas *teb* (a homolog of human DNA polymerase θ) (Inagaki et al., 2006), *rad51c*, or *rad51d* (a bacterial RecA recombinase homolog) (Durrant et al., 2007), *mim* (hypersensitive to MMS, irradiation, and MMC, a structural maintenance of chromosome protein) (Mengiste et al., 1999; Hanin et al., 2000), and *ino80* (an ortholog of the SWI/SNF ATPase family) (Fritsch et al., 2004) mutations apparently decreased HR. We also observed that mutations in ROR1/RPA2A elevated the HR to a similar level as in *abo4* mutants (see Supplemental Figure 2 online). It is possible that the POL2a works together with ROR1/RPA2A and FAS1 to regulate HR as these three proteins also coparticipate the DNA synthesis and DNA nucleotide excision repair (Gaillard et al., 1996; Lindahl et al., 1997; Pospiech and Syvaaja, 2003). It seems that defects of these protein activities lead to lengthening of S-phase, which accumulates DSBs and in turn activates cell cycle checkpoints and induces HR. We found that ABA treatment increased the HR frequency in both *abo4-1* and wild-type plants. Interestingly, ABA downregulates the DNA end binding protein KU70, a key protein in NHEJ, which is consistent with a recent study on *KU70* gene expression (Liu et al., 2008). It is possible that ABA-induced DNA breaks may partly result from the lower expression of *KU70* because reduction of *KU70* might lead to lower efficiently rejoining repair of DNA breaks in NHEJ. As NHEJ and HR pathways compete with each other during the cell cycle, the reduced NHEJ might increase the DNA breaks and activate the expression of *MRE11* and *GR1*. GR1 has some homology with Ct IP, a mammalian protein phosphorylated by ATM (ataxia-telangiectasia mutated). MRE11 plays a crucial role in preventing the accumulation of DSBs resulting from stalled replication forks during DNA replication (Pichierrri and Franchitto, 2004). Ct IP physically and functionally interacts with the MRE11 complex, and both are required for efficient HR (Sartori et al., 2007).

However, the high HR frequency observed in *abo4-1* could have a more complex cause. A previous study indicates that MMS or other DNA breakage reagents are only able to increase HR severalfold (Lindhout et al., 2006) but not as high as in *abo4-1* mutants. Recent studies of *fas1* and *fas2* suggest that the delayed chromatin assembly at S-phase would be another important factor for increasing HR in *Arabidopsis* (Endo et al., 2006; Kirik et al., 2006). However, mutations in *ddm1*, a chromatin remodeling SWI2/SNF2 greatly affecting the DNA methylation and chromatin structures in the whole genome in *Arabidopsis* (Gendrel et al., 2002), do not apparently affect HR (Shaked et al., 2006), and mutations in another SWI/SNF ATPase *INO80* specifically reduce the HR (Fritsch et al., 2004). Considering that high HR mutants, including *cen2*, *fas1*, *fas2*, *abo4*, and *ror1*, are all involved in the NER pathway (Molinier et al., 2004; Endo et al., 2006; Kirik et al., 2006; this study), it is possible that another unidentified mechanism that might depend on the NER pathway, but be unique in plants, could regulate the HR. In any case, POL2a should be the core player because it directly synthesizes DNA, and defects of POL2a may lead to insufficient repair of damaged DNA and in turn increase HR.

The release of TGS in *abo4-1* indicates that POL2a plays an essential role in maintaining epigenetic states in *Arabidopsis*. In budding yeast, a genetic screen for rDNA silencing defects identified many genes, including Pol ϵ (Ehrenhofer-Murray et al., 1999; Smith et al., 1999). It was also found that the mouse Pol ϵ B subunit interacts with SAP18, a protein associated with the transcriptional corepressor Sin3, which possesses histone deacetylase activity and functions in TGS (Wada et al., 2002). These results suggest the functional conservation of Pol ϵ in maintaining epigenetic states in different organisms. We found that *abo4* mutation activated two originally silenced loci, including *TSI* and *35S-NPTII* in the *ros1-1_{RD29A-LUC}* mutant. However, the epigenetic regulation by ABO4/POL2a seems independent of DNA methylation as checked in several hypermethylation loci by DNA gel blot analysis. Previous studies on *fas1*, *fas2*, *ror1*, *bru1*, *tousled*, and *mom1* (Takeda et al., 2004; Elmayan et al., 2005; Kapoor et al., 2005; Vaillant et al., 2006; Xia et al., 2006; Wang et al., 2007) indicate that the TGS regulated by these genes are independent of DNA methylation, which is different from the TGS pathway mediated by RNA-direct DNA methylation and heterochromatin formation (Matzke et al., 2007). However, MOM1 is a chromatin remodeling factor not participating in DNA repair pathway (Shaked et al., 2006) and should be in the different pathway from *FAS1*, *FAS2*, *ROR1/RPA2*, *BRU1*, *TOUSLED*, and *ABO4/POL2a* in mediating TGS. These results suggest that several different pathways mediate the TGS in plants. Studies on ROR1/RPA2A and TOUSLED indicate that the released TGS of *35S-NPTII* in *ror1* and *tousled* mutants might be due to a change in histone modifications (Xia et al., 2006; Wang et al., 2007). Here, we found that *abo4* mutations influenced flowering time through manipulating the histone modifications in *FLC* and *FT* loci. In the *icu2-1* study, the authors find that ICU2 DNA polymerase α interacts with TFL2/LHP1 to regulate chromatin-mediated gene expression (Barrero et al., 2007). Together, these studies indicate that DNA replication machinery is involved in chromatin-mediated gene expression in plants.

Our findings that ABA increases HR and activates the expression of retrotransposons are biologically significant because somatic recombination and transposon activation may be important mechanisms for restructuring the genome as an adaptive response to environmental challenge (Grandbastien, 1998). Changes to the genome of somatic cells can be transmitted to offspring and may produce new resistance traits during the evolutionary process (Molinier et al., 2006). Because ABA accumulation is induced by environmental stresses, we suggest that ABA might affect genome stability through the DNA replisome and allow plants to effectively adapt to environmental stresses over the course of evolution. However, further studies are needed to dissect the molecular mechanisms of this process.

METHODS

Plant Materials and Growth Conditions

We used *Arabidopsis thaliana* Col accession with a *gl1* mutation resulting in no trichomes on the plants. The F2 ethyl methanesulfonate-mutagenized seeds were used for screening ABA-sensitive mutants. Root growth was analyzed by the root bending assay as described previously (Wu et al., 1996). Briefly, 5-d-old seedlings with 1- to 1.5-cm-long roots were

transferred from the vertical agar plates, one by one, onto a second agar medium that contained 20 to 30 μM ABA. The seedlings were arranged in rows, and the plates were oriented vertically with the roots pointing upward. After growth for another 5 to 7 d, the putative mutants that showed inhibited root growth were picked up. The root growth phenotypes of putative mutants were rechecked in the next generation, genetic analysis was performed by backcrossing with the wild type, and the ABA-sensitive phenotype of *abo4-1* was determined. All seedlings were cultured on agar-solidified MS medium supplemented with 3% (w/v) sucrose at 21°C under 24 h of light. Plant growth conditions in soil are the same as described previously (Xia et al., 2006).

Mapping-Based Cloning of ABO4

abo4-1 was crossed with an *Arabidopsis* Landsberg accession, and the F2 population (1802 samples) was used for mapping-based cloning by analyzing recombination events using simple sequence length polymorphism markers. The *abo4* mutation was first mapped to chromosome 1 between F12K11 and F14J9. Then, markers F24B9, T23G18, and T27G7 were used to narrow down the *abo4-1* mutation to the BAC clone T23G18. Sequencing the candidate gene *At1g08260* identified a G-to-A point mutation. A Salk T-DNA insertion line SALK_096341 (*abo4-2*) was requested from The Arabidopsis Information Resource. We isolated genomic DNA from the progeny of SALK lines and performed PCR analysis using primer pair LP 5'-ACCTTCTCCTTGATTTCGTCC-3' and RP 5'-CTATGGCTCTTTATGGGTTGC-3', which covered the T-DNA insertion region, to find the homozygous lines. Because *abo4-2* is sterile in both male and female, we used the heterozygous T-DNA plants (confirmed by PCR using primer pair RP 5'-CTATGGCTCTTTATGGGTTGC-3' and TF 5'-ATTTTGGCGATTTCGGAAC-3') to cross with *abo4-1* (confirmed by Derived Cleaved Amplified Polymorphic Sequences using primer pair 5'-GGTCATGTAGAGTGCCTTGAAGGTG-3' and 5'-GAATCATGCACACAAGAATGGGAAGC-3'; cut with *HphI*) to make heterozygous mutants that contained both the *abo4-1* and *abo4-2* mutations. The heterozygous plants of *abo4-1* and *abo4-2* showed *abo4-1* phenotypes.

Recombination Assays

The recombination reporter lines 1406 (direct repeat line) and 1415 (inverted repeat line) (Col accession) (Gherbi et al., 2001) were crossed with *abo4-1* and homozygous plants for both GUS reporter and *abo4-1* or for GUS reporter only (control plants), and samples were selected from F3 plants and used for GUS activity analysis. The GUS spot numbers, each of which indicates a recombination event, were then determined under a dissecting microscope. For ABA treatment, 5-d-old seedlings grown on MS medium were transferred to MS medium containing 5 μM ABA and grown for another 7 d. Then the seedlings were used for the GUS activity assay. GUS staining was done as described previously (Gong et al., 2002). Approximately 100 individual seedlings were analyzed for each sample.

DNA Damage Sensitivity Assays

For DNA damage assays, seeds were directly planted on MS medium containing different concentrations of MMS and grown for 2 weeks after germination, or 2-d-old seedlings were transferred to liquid MS medium supplemented with different concentrations of MMS and grown for another 10 d before taking pictures. For UV-B treatment, 5-d-old seedlings were exposed to different levels of UV-B light (UV 256 nm in a Stratalinker) and then were kept in the dark for 2 d before being removed to light for growth for 3 kJ/m^2 or directly grown under light (for 1 kJ/m^2 or 5 kJ/m^2). After 5 d in light, the pictures were taken. Each experiment was repeated at least three times independently. The representative results from one experiment are shown. The TUNEL assay was conducted as described previously (Wang and Liu, 2006) using the In Situ Cell Death Detection Kit-Fluorescein (Roche

Diagnostics). Five-day-old seedlings grown on MS medium were transferred to MS medium containing 30 μM ABA for 3 d before performing TUNEL staining. Images were captured with an Olympus BX51 microscope equipped with fluorescein isothiocyanate and UV light filters and a COOL SNAP HQ photometrics CCD camera.

RNA Gel Blot Analysis

Shoots of 2-week-old seedlings were dipped into solution containing 30 μM ABA for different times. For *TSI* expression analysis, 7-d-old seedlings were treated with MMS (50 and 100 ppm) for 7 d or treated with ABA (5 and 10 μM) for 7 d. For gene expression analysis in *abo4-1 ros1-1*, 2-week-old seedlings were treated with 100 μM ABA or 300 mM NaCl for 5 h. Total RNAs were extracted and hybridized with different probes as described previously (Xia et al., 2006). Twenty micrograms of total RNA from each sample was separated on 1.2% (w/v) agarose formaldehyde gels. *Tubulin* was used as a loading control. Probes were amplified with primer pairs provided in Supplemental Table 1 online.

DNA Gel Blot Analysis

DNA methylation analysis in *RD29A* promoter, *TSI*, and centromere DNA region was performed as described previously (Gong et al., 2002).

CYCB1;1::GUS Assay

The *Arabidopsis* Col plants carrying CYCB1;1::GUS were crossed with *abo4-1*. The homozygous plants for both CYCB1;1::GUS and *abo4-1* or only for CYCB1;1::GUS were selected from F3 and used for GUS staining (Colon-Carmona et al., 1999; Endo et al., 2006). For ABA or MMS treatment, 5-d-old seedlings were transferred to MS medium containing 30 μM ABA or 100 ppm MMS and grown for another 4 d before GUS staining.

Real-Time qRT-PCR

Approximately 2 μg of total RNA, extracted from seedlings under different conditions and digested with RNase-free DNase I, was used for RT-PCR, employing oligo(dT) primers with M-MLV (Invitrogen) in a 50- μL reaction. One microliter of the RT reaction was used as template to determine transcript levels of the tested genes on a PTC-200 DNA Engine Cycler (MJ Research) with Chromo4 Detector in 25- μL reactions. The levels of 18S rRNA were used as a reference, and the values given are expressed as a ratio of the values in the wild type. Three biological replications were performed for each experiment. The values shown represent averages of triplicate assays for each RT sample. PCR conditions were as follows: 5 min at 95°C (one cycle), 30 s at 95°C, 30 s at 58 to 61°C, and 60 s at 72°C (30 cycles). The primers and detail annealing temperatures used for real-time PCR are provided in Supplemental Table 1 online.

ChIP

ChIP was performed as described previously (Xia et al., 2006) using 2-week-old seedlings grown on MS plates under long-day conditions (16 h light/8 h dark). Anti-H3K4me2 and anti-H3K27me3 antibodies were purchased from Upstate Biotechnology. We used *ACT11N* as internal controls for the H3K4me2 level and *TA3* for the H3K27me3 level. PCR conditions are same as above using 58°C as annealing temperature. The primers used for ChIP are provided in Supplemental Table 1 online.

Accession Number

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession number NM_001123775 (ABO4/POL2A/TIL1/At1g08260).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Comparison of ABA-Sensitive Phenotypes in *ror1-3*, *fas1-101*, and *pol α* Mutants.

Supplemental Figure 2. Homologous Recombination Patterns in *ror1-3* (1415) and *ror1-3* (1406).

Supplemental Table 1. Primers Used in This Study.

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