

Arabidopsis *RAD51C* Gene Is Important for Homologous Recombination in Meiosis and Mitosis¹

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Rad51 is a homolog of the bacterial RecA recombinase, and a key factor in homologous recombination in eukaryotes. Rad51 paralogs have been identified from yeast to vertebrates. Rad51 paralogs are thought to play an important role in the assembly or stabilization of Rad51 that promotes homologous pairing and strand exchange reactions. We previously characterized two *RAD51* paralogous genes in Arabidopsis (*Arabidopsis thaliana*) named *AtRAD51C* and *AtXRCC3*, which are homologs of human *RAD51C* and *XRCC3*, respectively, and described the interaction of their products in a yeast two-hybrid system. Recent studies showed the involvement of *AtXrcc3* in DNA repair and functional role in meiosis. To determine the role of *RAD51C* in meiotic and mitotic recombination in higher plants, we characterized a T-DNA insertion mutant of *AtRAD51C*. Although the *atrad51C* mutant grew normally during vegetative developmental stage, the mutant produced aborted siliques, and their anthers did not contain mature pollen grains. Crossing of the mutant with wild-type plants showed defective male and female gametogeneses as evidenced by lack of seed production. Furthermore, meiosis was severely disturbed in the mutant. The *atrad51C* mutant also showed increased sensitivity to γ -irradiation and cisplatin, which are known to induce double-strand DNA breaks. The efficiency of homologous recombination in somatic cells in the mutant was markedly reduced relative to that in wild-type plants.

Chromosomal double-strand DNA breaks (DSBs) are produced by ionizing radiation (IR), oxygen free radicals, DNA cross-linking reagents, and DNA replication failure. There are two major repair pathways for DSBs: nonhomologous end joining (NHEJ) and homologous recombination (HR; Haber, 2000). In yeast, genetic analysis identified a set of genes, called the *RAD52* epistasis group genes, which is directly involved in HR (Baumann and West, 1998). This group contains the *Mre11*, *Xrs2*, *Rad50*, *Rad51*, *Rad52*, *Rad54*, *Rad55*, *Rad57*, and *Rad59* proteins (Paques and Haber, 1999). The eukaryotic Rad51 is structurally and functionally a homolog of the

Escherichia coli recombination protein RecA and is involved in both meiotic and mitotic recombination (Shinohara et al., 1992). The yeast genome contains more proteins with homology to RecA and Rad51. Two members of the Rad51 paralogs (*Rad55* and *Rad57*) have been identified (Kans and Mortimer, 1991; Lovett, 1994). In contrast to yeast, five Rad51 paralogs (*Rad51B*, *Rad51C*, *Rad51D*, *Xrcc2*, and *Xrcc3*) have been identified in mammals. These proteins share 20% to 30% sequence identity with Rad51 and with each other (Sonoda et al., 2001).

Previous studies reported several mutants of Rad51 paralogous genes. *RAD51C*, *XRCC2*, and *XRCC3* mutants in Chinese hamster cell lines (*CL-V4B*, *irs1*, and *irs1SF*, respectively) showed extreme sensitivity to cross-linking reagents. The frequencies of spontaneous chromosomal aberrations in these mutants were higher than those in wild-type cell line (Cui et al., 1999; Godthelp et al., 2002). The five knockout mutants of Rad51 paralogs in chicken B-lymphocyte DT40 cell lines were viable, but showed spontaneous chromosomal aberrations, hypersensitivity to DNA-damaging agents, and low levels of HR (Takata et al., 2001). *RAD51B*, *RAD51D*, and *XRCC2* knockout mutant mice died during embryonic development (Shu et al., 1999; Deans et al., 2000; Pittman and Schimenti, 2000). The *RAD51C* and *XRCC3* mutants in *Drosophila* (*Spn-D* and *Spn-B*, respectively) were partially sterile,

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defective for meiotic recombination, but not hypersensitive to DSB-inducing agents (Ghabrial et al., 1998; Abdu et al., 2003).

Physical interactions between the five Rad51 paralogs have also been demonstrated using yeast two-hybrid experiments and coimmunoprecipitation (Schild et al., 2000; Masson et al., 2001). Rad51 paralogs form two complexes, Rad51B-Rad51C-Rad51D-Xrcc2 (BCDX2) and Rad51C-Xrcc3 (CX3), both containing Rad51C as a component (Miller et al., 2004). Thus, Rad51C is the only Rad51 paralog that is a common component of distinct Rad51 paralog complexes. These complexes are thought to mediate DNA strand exchange events of Rad51 during the process of HR (Sung et al., 2003; Miller et al., 2004). Recently, Liu et al. (2004) demonstrated that Rad51C was required for resolution of Holliday junctions (HJs). The above studies suggest the involvement of Rad51C in not only the early stages but also the late stages of HR.

In plants, we previously cloned and characterized two *RAD51* paralogous genes from Arabidopsis (*Arabidopsis*

thaliana) named *AtRAD51C* and *AtXRCC3*, which are homologs of human *RAD51C* and *XRCC3*, respectively (Osakabe et al., 2002). Both *AtRAD51C* and *AtXRCC3* transcripts were detected in a variety of tissues, with the highest level of expression in flower buds, and were induced by γ -irradiation. Our previous results suggested that *AtRad51C* and *AtXrcc3* act together and are involved in DNA repair and meiotic recombination. Recently, *AtXRCC3* and *AtRAD51* knockout mutants have been identified and characterized in Arabidopsis (Bleuyard and White, 2004; Li et al., 2004). *atrad51* and *atxrcc3* mutants developed normally in vegetative stage but showed male and female sterility, due to chromosome fragmentation after pachytene in both types of gametogenesis. These results suggested that *AtRad51* and *AtXrcc3* play important roles in both male and female meiosis.

In this study, we characterized an *atrad51C* mutant of Arabidopsis. Based on the findings, we discuss the possible role of *AtRad51C* protein in mitotic and meiotic recombination in plant cells.

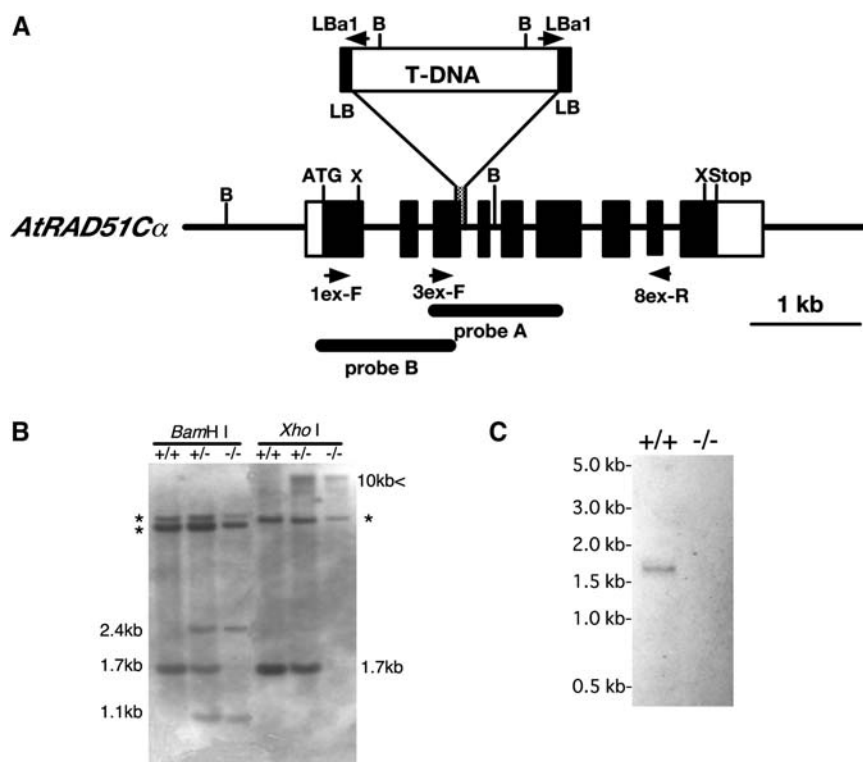


Figure 1. Molecular analysis of *atrad51C* T-DNA insertion. **A**, Genomic organization of the *AtRAD51C* locus, and the position of the T-DNA insertion at the *AtRAD51C* locus. Black boxes represent the exons, and white boxes represent 5' and 3' untranslated regions. Thick lines indicate probe regions for Southern- and northern-blotting analyses (probe A for Southern blotting and probe B for northern blotting). Arrows indicate PCR primer positions to determine the junction sequences of the T-DNA insertion. **B**, Southern-blotting analysis of the T-DNA insertion in the *AtRAD51C* locus. In these experiments, 5 μ g of genomic DNA from *AtRAD51C*^{+/+} (wild type), *AtRAD51C*^{+/-} (heterozygous), and *atrad51C*^{-/-} (homozygous) plants were digested with *Bam*HI or *Xho*I. The digested DNAs were blotted and probed with the DIG-labeled probe A shown in section A. Asterisks show the nonspecifically hybridized bands because these bands were observed in all lanes regardless of genotypes (*+/+*, *+/-*, *-/-*). **C**, Northern-blotting analysis of *AtRAD51C* expression in flower buds of *AtRAD51C*^{+/+} and *atrad51C*^{-/-} plants. In these experiments, 10 μ g of total RNAs from flower buds of wild-type and mutant plants were blotted and probed with DIG-labeled probe B shown in section A. RNA *M_r* markers (0.5–5.0 kb) are shown on the left.

RESULTS

Identification of an *AtRAD51C* T-DNA Insertion Mutant in Arabidopsis

To investigate the function of *AtRAD51C* gene in Arabidopsis, we searched for loss-of-function mutants. A single line, SALK_021960, was found in Salk Institute T-DNA insertion collections (Alonso et al., 2003), and the corresponding allele was named *atr51c*. The *AtRAD51C*/T-DNA junctions were amplified by PCR and sequenced to determine the position of the T-DNA insertion. Figure 1A provides detailed characterization of the T-DNA insertion at the *AtRAD51C* gene. According to the direct sequencing analysis of PCR products, the T-DNA insertion was surrounded by two left borders in opposite orientations. The insertion caused an 11-bp deletion of the end of the third exon and a 41-bp deletion of the beginning of the third intron in the *AtRAD51C* locus. We also analyzed the feature of T-DNA insertion at *AtRAD51C* locus by Southern-blot analysis. Genomic DNA isolated from *atr51c* and wild-type plants were digested with either *Bam*HI or *Xho*I and hybridized with digoxigenin (DIG)-labeled probe A spanning the T-DNA insertion site (Fig. 1, A and B). The results indicated that the length of the insertion was more than 10 kb and composed of several adjacent T-DNAs.

Next, we analyzed the transcription level of *AtRAD51C* gene in the *atr51c* mutant by northern-blotting analysis. In this experiment, total RNA from flower buds of wild-type and *atr51c* mutant were tested because relatively higher levels of *AtRAD51C* transcripts were detected in flower buds (Osakabe et al., 2002). As shown in Figure 1C, a 1.6-kb mRNA was detected in wild-type flower buds but not in *atr51c* (Fig. 1C). Thus, we concluded that the *atr51c* mutant could not express a functional Rad51C protein.

AtRad51C Is Not Crucial for Vegetative Development

The *atr51c* mutant plant did not show any abnormal phenotypes during the vegetative developmental stage under the standard growth conditions described in "Materials and Methods" (Fig. 2A). Homozygous *atr51c*^{-/-} plants were indistinguishable from heterozygous *AtRAD51C*^{+/-} and wild-type *AtRAD51C*^{+/+} plants until the plants started to produce siliques. The numbers of rosette and cauline leaves, size, and the growth rate of *atr51c*^{-/-} plants were also similar to those of *AtRAD51C*^{+/-} and *AtRAD51C*^{+/+} plants (data not shown). Thus, the loss of *AtRad51C* function did not affect vegetative growth. Cytological analysis also showed that mitosis of petals from the *atr51c*^{-/-} plant was normal as that of the *AtRAD51C*^{+/+} plant (Fig. 3).

The *atr51c* Mutant Plant Is Sterile

Next, we determined the sterility phenotypes in the progeny of self-fertilized heterozygous *AtRAD51C*^{+/-}

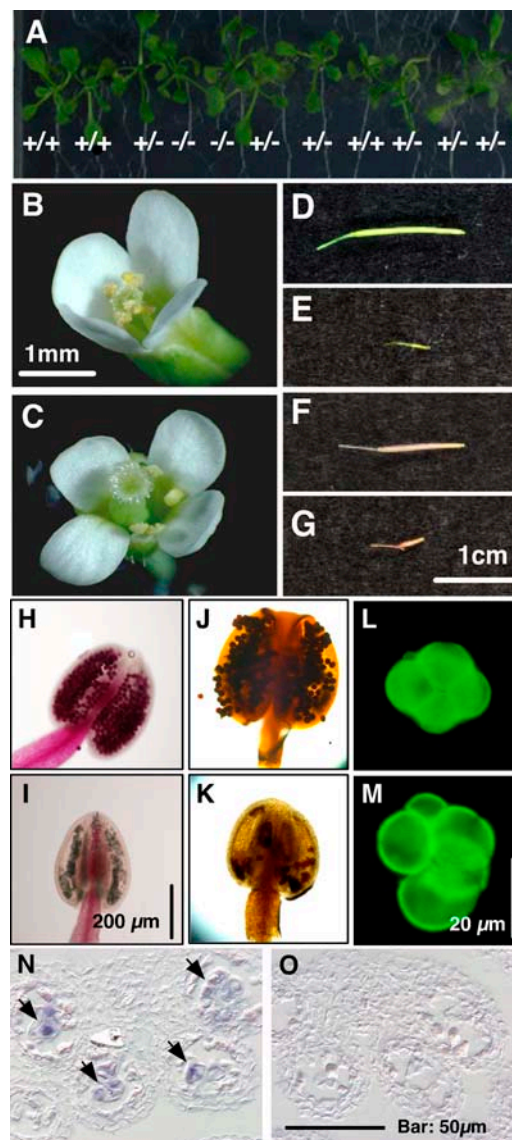


Figure 2. *atr51c* mutant plants are sterile. A, Fifteen-day-old *AtRAD51C*^{+/+}, *AtRAD51C*^{+/-}, and *atr51c*^{-/-} plants on MS agar medium. +/+, +/-, and -/- indicate *AtRAD51C*^{+/+}, *AtRAD51C*^{+/-}, and *atr51c*^{-/-} plants, respectively. B and C, Open flowers of wild-type (B) and *atr51c* mutant plants (C). D and E, Siliques of wild-type (D) and *atr51c* mutant plants (E). F and G, Siliques resulting from cross-fertilization between wild-type female and wild-type male (F; as a control), and *atr51c* mutant and wild-type male (G). H to K, Anthers of wild-type (H and J) and *atr51c* mutant plants (I and K). Anthers from 2-mm buds were stained with Alexander's solution (1969; H and I) or stained with I₂-KI (J and K). L and M, Tetrad of wild-type (L) and *atr51c* mutant plants (M) stained with aniline blue. N, *AtRAD51C* expression was detected in only PMCs by in situ hybridization. Black arrows indicate signals. O, No hybridization signal was detected when a sense probe was used.

plants. A total of 188 plants were tested, and 151 (80.3%) were found to be fertile and 37 (19.7%) were sterile, corresponding to a 3:1 segregation expected for a single Mendelian locus ($\chi^2=2.45$, $0.1 < P < 0.25$ for 3:1 segregation ratio). Using a PCR assay, we identified

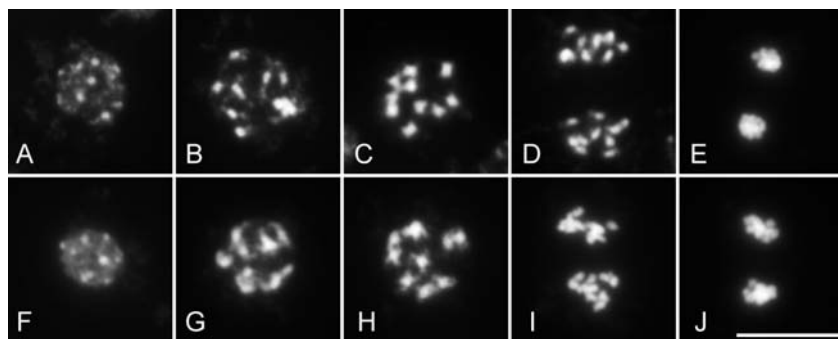


Figure 3. Mitosis in the petal of wild-type plants (A–E) and *atrada51C*^{-/-} mutant plants (F–J). A and F, Interphase; B and G, prophase; C and H, metaphase; D and I, anaphase; E and J, telophase. Calibration bar = 10 μ m.

the genotype of the *atrada51C* allele in these plants. Thirty-seven of the 188 plants (19.7%) were homozygous for the T-DNA insertion in the *AtRAD51C* gene (*atrada51C*^{-/-}), while 103 of 188 (54.8%) were heterozygous (*AtRAD51C*^{+/-}) and 48 of 188 (25.5%) were wild type (*AtRAD51C*^{+/+}). This analysis of individual plants confirmed that sterile plants are homozygous for the *atrada51C* allele.

Heterozygous *AtRAD51C*^{+/-} plants and homozygous *atrada51C*^{-/-} mutant plants showed normal flower development similar to the wild-type plants (Fig. 2, B and C). In addition, the fertility of heterozygous plant was similar to that of wild-type plants. However, *atrada51C* plants produced contractional siliques (Fig. 2E), and almost all these siliques were devoid of seeds. Thus, AtRad51C is required for fertility, but it is not crucial for vegetative development.

atrada51C Mutant Plants Are Defective in Male and Female Gamete Development

To determine the cause(s) of the sterility phenotype in *atrada51C* plants, we first examined the development of male gametes. To assess pollen grain viability, anthers were dissected from the wild-type and mutant flower buds and stained with Alexander's solution (Alexander, 1969; Fig. 2, H and I) or the iodine potassium iodide solution (I₂-KI; Fig. 2, J and K). In Alexander staining,

the viable pollen stained red to deep red, while the aborted pollen stained green. On the other hand, the mature pollen, which accumulates starch, stained deep brown by I₂-KI. None of the 50 examined *atrada51C* anthers contained any normal pollen grains (Fig. 2, I and K). Although wild-type plants produced normal tetrad including four spores in meiosis (Fig. 2L), the mutant produced abnormal tetrad-like structures, called polyads, including multiple spores ranging in number from five to seven (Fig. 2M). These findings suggest developmental arrest of the male gamete. In this regard, anthers of heterozygous plants were indistinguishable from those of wild-type plants. Thus, male gametogenesis is severely disturbed in the *atrada51C* mutant plants. We also determined the expression of *AtRAD51C* in meiosis by in situ hybridization. Transverse sections of anthers showed strong hybridization signals in only pollen mother cells (PMCs; Fig. 2N); no signals were detected in mature pollen grains (data not shown). This result was consistent with the important function of AtRad51C in meiosis.

Next, to investigate female gametogenesis in *atrada51C* mutants, *atrada51C* plants as female parents were cross-pollinated with pollens from wild-type plants. The cross-fertilization resulted in plants with contractional siliques, similar to self-fertilization of *atrada51C* mutant plants (Fig. 2G). Furthermore, an average of 0.035 seeds per silique ($n = 114$) was

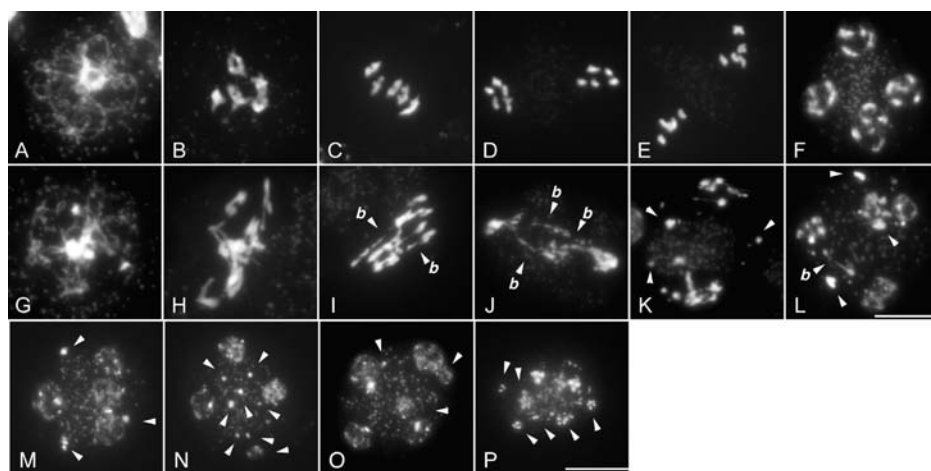


Figure 4. Meiosis is severely disrupted in *atrada51C* mutant plants. A to F, Wild type; G to P, *atrada51C*. A and G, Pachytene stage; B and H, diakinesis; C and I, metaphase I; D and J, anaphase I; E and K, metaphase II; F and L, anaphase II; M to P, telophase II. Arrows with b in sections I, J, and L indicate bridges. Arrows indicate abnormal chromosomal fragments. Calibration bar = 10 μ m.

observed. In contrast, when wild-type plants as female parents were cross-pollinated with wild-type pollens, the cross-fertilization resulted in plants produced 22.5 seeds per silique ($n = 27$). These results suggest that female gametogenesis is also disturbed in the *atrad51C* mutant plants.

Meiosis Is Severely Disrupted in *atrad51C* Plants

For further characterization of the *atrad51C* sterility, we examined meiotic progression in *atrad51C* and wild-type PMCs by fluorescence microscopy after 4',6'-diamidino-2-phenylindole staining of chromosomes. We observed the progression of normal meiosis in wild-type PMCs as described previously by Ross et al. (1996). Briefly, normal meiosis proceeded by completing synapsis of homologous chromosomes at pachytene (Fig. 4A) and further condensation and separation at diplotene to diakinesis (Fig. 4B), leading to the formation of five condensed bivalents at metaphase I (Fig. 4C). Homologous chromosomes separated from each other and migrated to the opposite poles of the cell at anaphase I (Fig. 4D). Individual chromosomes condensed again and aligned on the metaphase II plate (metaphase II; Fig. 4E). In anaphase II, sister chromatids separated and migrated to the opposite cell poles (Fig. 4F), resulting in decondensation of four groups of five chromatids, with partitioning of the cytoplasm, and finally the formation of a tetrad of haploid nuclei. In *atrad51C* PMCs, chromosome pairing seemed to occur as in wild-type PMCs during the pachytene stage (Fig. 4, A and G). In diakinesis, chromosomes were further condensed, but the normal bivalent formation was impaired (Fig. 4H). Chromosome fragments and bridges between bivalents were observed in the metaphase I stage (Fig. 4I). These two anomalies were more evident in anaphase I (Fig. 4J). In metaphase II, the majority of the visible chromosome fragments were aligned on the spindle, but some fragments were dispersed throughout the cytoplasm (Fig. 4K). We also found bridges in anaphase II, suggesting that fused chromosomes or chromatid fragments were still present at this stage (Fig. 4L). The result of meiosis in *atrad51C* PMCs was the aforementioned polyads (Fig. 2M), containing variable number of cells with variable DNA contents (Fig. 4, N–P).

atrad51C Plants Are Hypersensitive to a Cross-Linking Reagent, Cisplatin

We performed the sensitivity test to a cross-linking reagent, cisplatin, in *atrad51C* and wild-type plants. Cross-linking reagents produce intra- and interstrand DNA cross-links. Both intra- and interstrand cross-links induce DSBs during DNA synthesis, and these DNA lesions are thought to be repaired predominantly by HR as one of the major repair pathways during S phase. Especially, interstrand DNA cross-links are exclusively repaired by HR (De Silva et al., 2002;

Sasaki et al., 2004). Therefore, this test aims to provide evidence for the role of AtRad51C in HR processes. Cisplatin sensitivity was scored based on the number of true leaves produced on cisplatin-containing Murashige and Skoog (MS) agar medium. In cisplatin-free medium, both the wild-type and mutant plants produced six true leaves at 15 d after germination. Wild-type plants also produced six true leaves on 15 μM cisplatin medium and four to five true leaves on 30 μM cisplatin medium at 15 d after germination on 15 μM cisplatin medium (Fig. 5A). In mutant plants, the average numbers of true leaves were reduced to 50% and 60% on 15 μM and 30 μM cisplatin medium, respectively, compared to those in wild-type plants. In addition, the mutant plants produced two true leaves on 30 μM and 50 μM cisplatin medium, but these true leaves were not fully opened (Fig. 5B, center of 30 μM and 50 μM sections). After 11 d treatment with cisplatin, the plants were transferred onto cisplatin-free MS agar medium and allowed further growing. Wild-type plants first treated with 30 μM and 50 μM cisplatin produced additional true leaves, although wild-type plants treated with 50 μM cisplatin produced abnormal leaves with a slender leaf shape (Fig. 5C, top of the 50 μM section). On the other hand, the mutant plants that were first treated with cisplatin did not produce any additional leaves when allowed to grow in cisplatin-free medium (Fig. 5C, center of each section), and these plants later died (data not shown). We also tested the sensitivity of *atrad51B* null mutant plants (Osakabe et al., 2005) to cisplatin by the sensitivity test described in this article. When 3-d-old seedlings of *atrad51B* were treated with 30 μM cisplatin, *atrad51B* plants produced three to four true leaves (Fig. 5, A and B, bottom of the 30 μM sections). In contrast to the case of *atrad51C*, true leaves of *atrad51B* were expanded, and additional true leaves were also produced after transfer of the plants to cisplatin-free medium, although *atrad51B* plants produced abnormal leaves with a slender leaf shape (Fig. 5C, bottom of 30 μM section). These results indicated that *atrad51C* was more sensitive to cisplatin than *atrad51B*. Next, we tested the sensitivity to cisplatin by root growth assay on MS agar medium containing 15 or 30 μM cisplatin. Unexpectedly, the root growth of *atrad51C*^{-/-} plants was not different from that of *AtRAD51C*^{+/+} and *AtRAD51C*^{+/-} plants by 3-d cisplatin treatment (Fig. 5D). We also found that the root growth *atrad51B* was not different from that of wild type (data not shown).

We also tested the sensitivity to cisplatin in cultured cells derived from wild type and *atrad51C*. Wild-type and mutant calli were transferred to callus-inducing medium (CIM) containing 0 to 30 μM of cisplatin, and the growth of these calli was scored visually after 3 weeks. As shown in Figure 6A, 15 μM cisplatin severely affected the growth of *atrad51C* cells, while wild-type cells grew normally similar to wild-type cells grown on cisplatin-free CIM. Thirty millimolar cisplatin reduced the growth of both mutant and wild-type cells. These results suggested that *AtRAD51C*

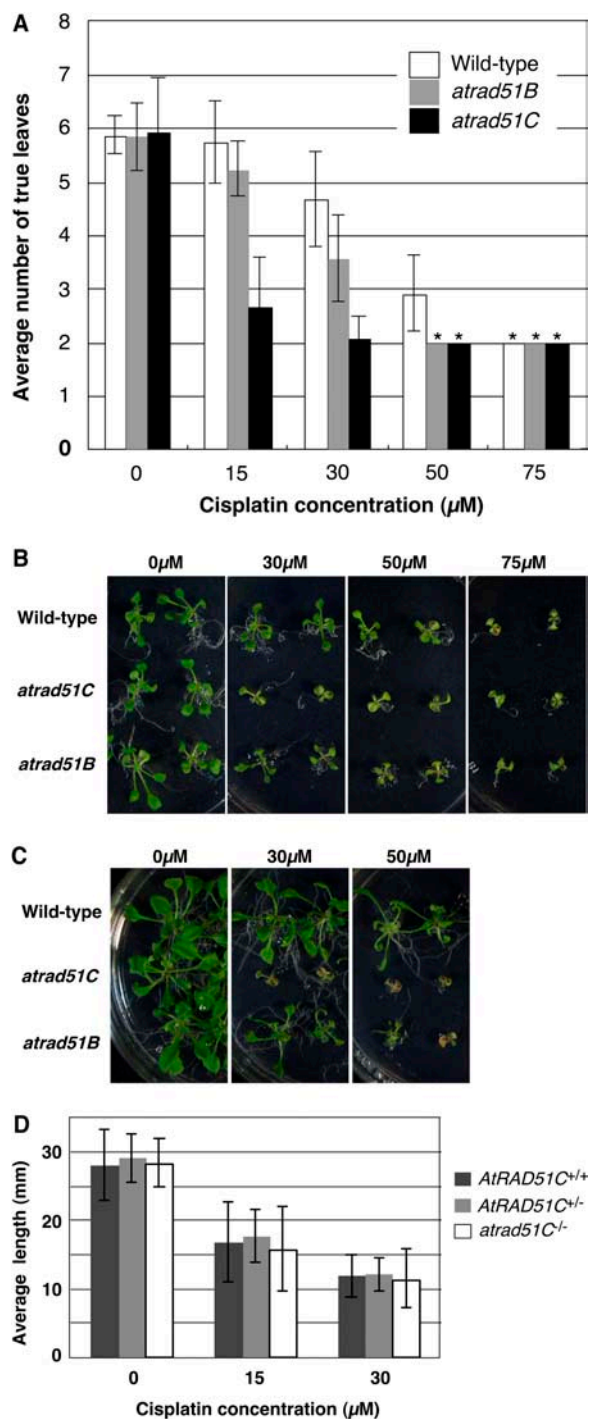


Figure 5. Cisplatin sensitivity of wild-type, *atrad51C*, and *atrad51B* plants. **A**, Production of true leaves in wild-type, *atrad51C*, and *atrad51B* plants at 12 d after treatment with 15 to 75 μM cisplatin. Asterisks indicate true leaf production with small and slender leaves. These leaves were not fully opened. Data represent mean \pm SD of 50 plants. **B**, Fifteen-day-old wild-type, *atrad51C*, and *atrad51B* plants treated or untreated with cisplatin. **C**, Three-week-old wild-type, *atrad51C*, and *atrad51B* plants treated or untreated with cisplatin. Fifteen-day-old plants untreated and treated with 30 μM and 50 μM cisplatin were transferred on MS agar medium without cisplatin, and cultured for additional 6 to 7 d. Top of each section, wild-type plants; center of each section, *atrad51C* plants; bottom of each section,

functions as a factor of DSB repair in the somatic cells of Arabidopsis.

To confirm that the hypersensitive phenotype of the *atrad51C* mutant to cisplatin is indeed due to the loss of function of *AtRAD51C* locus, a 4.2-kb *Xba*I-*Sal*I genomic *AtRAD51C* DNA fragment containing 1.3-kp 5'-upstream, 2.0-kb coding, and 0.9-kb 3'-downstream regions was cloned into the binary vector pZH1 (M. Kuroda and S. Toki, unpublished data) and used for transformation with root segments from wild type and the *atrad51C* mutant plants. Transgenic calli derived from wild-type and the mutant plants were selected on CIM containing hygromycin and further used for the sensitivity test to cisplatin. As shown in Figure 6B, complementation of *atrad51C* with the wild-type *AtRAD51C* resulted in wild-type sensitivity to cisplatin, demonstrating that the hypersensitivity of *atrad51C* to cisplatin was indeed because of the mutated *AtRAD51C* gene.

atrad51C Plants Are Hypersensitive to γ -Irradiation

We performed the sensitivity test to γ -irradiation in order to confirm the involvement of AtRad51C in DSB repair induced by another genotoxic stresses other than cisplatin. We irradiated 3-d-old seedlings of wild type and *atrad51C* to γ -irradiation. A dose of 100 Gy (Gy) strongly suppressed development of true leaves (Fig. 7, bottom of the center section). Mutant plants produced one or two thin and small true leaves at 12 d postirradiation and did not produce additional true leaves after two more weeks. These sensitive plants turned yellow and died (data not shown). In contrast, wild-type plants irradiated with 100 Gy showed only minimal growth defects (Fig. 7, top of the center section). However, 200 Gy strongly suppressed the development of true leaves in both wild-type and mutant plants (Fig. 7, right section). Considered together with the result of the hypersensitivity of *atrad51C* to cisplatin, we thought that AtRad51C is involved in efficient repair of the DSBs.

The Defect of Rad51C Reduced Frequencies of HR in Somatic Cells

For further characterization of AtRad51 function in HR, the *atrad51C* plants were monitored for somatic HR using an in planta recombination assay with a reporter gene consisting of two overlapping fragments of the β -glucuronidase (GUS) gene separated by a hygromycin-resistant marker (Swoboda et al., 1994, Gherbi et al., 2001). The GUS sequences have an

atrad51B plants. **D**, Comparison of the root growth of *AtRAD51C*^{+/+}, *AtRAD51C*^{+/-}, and *atrad51C*^{-/-} plants after 3-d cisplatin treatment. Six-day-old seedlings were transferred to cisplatin-containing MS agar medium and incubated for another 3 d. Root length of each plant was measured using the public domain NIH Image program (developed at the United States National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image>). Data represent mean \pm SD of 30 to 40 measurements.

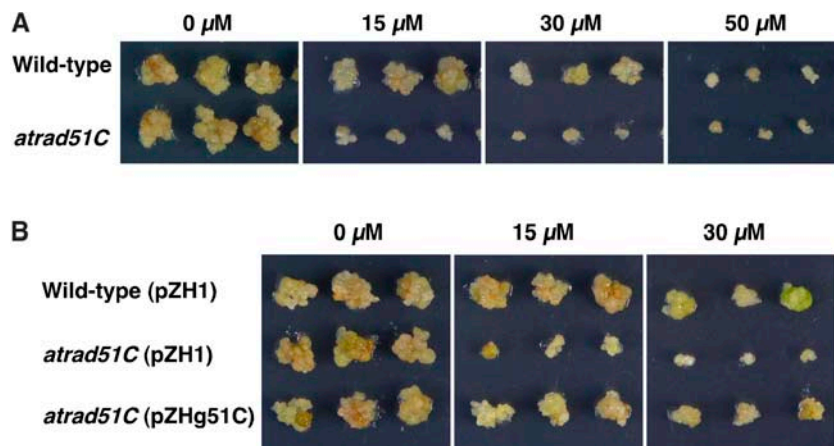


Figure 6. A, Cisplatin sensitivity of wild-type and *atrad51C*-cultured cells. Calli were obtained from excised root segments of wild-type and *atrad51C* plants. These calli were maintained on CIM and subcultured every 3 weeks. Seven-day-old calli were transferred and grown on CIM containing 0 to 50 μM cisplatin. The sensitivity to cisplatin was scored visually 3 weeks later. B, Complementation of *atrad51C*. Calli from wild-type roots transformed with pZH1 (wild type [pZH1]), calli from the *atrad51C* roots transformed with pZH1 (*atrad51C* [pZH1]), and calli from the *atrad51C* roots transformed with pZHg51C (*atrad51C* [pZHg51C]) were maintained on CIM containing hygromycin. Sensitivity to cisplatin of transgenic callus was scored as mentioned above. The expression of *AtRAD51C* mRNA in calli from the *atrad51C* roots transformed with pZHg51C was similar to that in wild-type callus based on reverse transcription-PCR analysis (data not shown).

overlap of 618 bp, and recombination event between the two overlapping sequences can produce a functional GUS gene resulting in blue-colored GUS spots in cells, which are detected by histochemical staining with 5-bromo-4-chloro-3-indoryl glucuronide. We used the two GUS recombination reporter lines, 1406 (direct-repeat recombination reporter line) and 1415 (inverted-repeat recombination reporter line), for in planta recombination assay (Gherbi et al., 2001). The GUS recombination reporter line 1406 or 1415 was crossed with the heterozygous *AtRAD51C*^{+/-} plant. The frequency of somatic HR (HRF) was monitored in F3 progeny plants homozygous for the GUS recombination substrate and homozygous for the *atrad51C* mutation allele or the wild-type *AtRAD51C* allele. The results are summarized in Table I and are presented as frequency distribution histogram in Figure 8. Spontaneous HRF of the *atrad51C*^{-/-} plants was reduced 2-fold compared to that of the wild-type plants (Table I; Fig. 8, A and B). Somatic HRF in wild-type plants was enhanced with approximately 4- to 5-fold induction after the treatment with 5 μM cisplatin (Table I; Fig. 8, C and D, and images, top). We also measured somatic HRF for bleomycin-treated plants. Bleomycin is a γ-ray mimetic agent and is known to induce DSBs

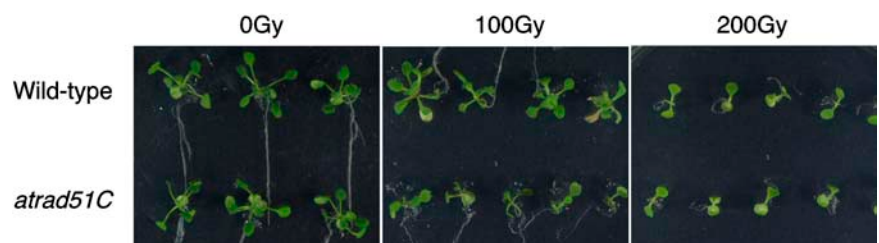
(Favaudon, 1982). After bleomycin treatment, somatic HRF in wild-type plants was also enhanced with 4- to 6-fold induction (Table I; Fig. 8, E and F). In contrast, somatic HRF in the *atrad51C* mutant plants increased by only 2- to 3-fold after cisplatin or bleomycin treatment (Table I; Fig. 8, C–F, and images, bottom). Thus, *atrad51C* showed hyporecombination phenotype for somatic chromosomal recombination.

DISCUSSION

We reported previously the isolation and characterization of Arabidopsis *RAD51* paralogous genes *AtRAD51C* and *AtXRCC3* (Osakabe et al., 2002). Transcription of *AtRAD51C* and *AtXRCC3* was detected in various tissues, with the highest level of expression in flower buds, and was induced by γ-irradiation. In this report, we characterized the *atrad51C* mutant caused by T-DNA insertion in the Arabidopsis *RAD51C* gene.

Phenotypic analysis of the *atrad51C* plant indicated that *AtRad51C* does not play a crucial role in vegetative development under normal growth conditions, but it is essential for male and female meiosis (Figs. 2 and 4). The lack of *AtRad51C* resulted in severely

Figure 7. Sensitivity of wild-type and *atrad51C*^{-/-} plants to γ-irradiation. In these experiments, 3-d-old seedlings of wild-type and mutant plants were either left untreated (as 0 Gy) or irradiated with a dose of 100 Gy or 200 Gy. The photographs were taken at 12 d after irradiation. Top of each section, wild-type plants; bottom of each section, *atrad51C* plants.



disrupted meiosis with chromosome fragmentations and appearance of bridges between chromosomes during meiosis I, and these anomalies were also present during meiosis II (Fig. 4), similar to the case of the *atxrc3* mutant (Bleuyard and White, 2004; Bleuyard et al., 2005; Li et al., 2005; this study). It is noted that other Rad51 family proteins, including AtXrc3, cannot fully substitute the function of AtRad51C in meiosis because the sterility of *atrad51C* is quite severe. The *atdmc1* mutant was deficient for meiotic homologous chromosome synapsis, arrested by the 10 univalents observed at metaphase I (Coureau et al., 1999). The *atrad51* mutant also failed to synapse homologous chromosomes and formed synaptonemal complexes (Li et al., 2004). Chromosome fragmentations were also observed at early stage of meiosis I in *atrad51* (Li et al., 2004). In contrast to AtDmc1 and AtRad51, AtRad51C and AtXrc3 are not required for synapsis but essential for postsynaptic events in meiosis (Bleuyard and White, 2004; this study). Thus, the functions of Arabidopsis Dmc1, Rad51, and Rad51 paralogs in meiosis are different, but AtRad51C acts in concert with AtXrc3 to ensure the correct progression of meiosis. Recently, Bleuyard et al. (2004) reported that the *atspo11-1* mutation suppressed the fragmentation phenotype that arose from meiosis I of the *atxrc3* mutant, but chromosome fragmentations are still observed in anaphase II of the *atxrc3/spo11-1* double mutant. In contrast to the case of *atxrc3/spo11-1*, no chromosome fragmentation is observed in anaphase II of the *atrad51/atspo11-1* double mutant (Li et al., 2004). Grelon et al. (2001) reported the presence of bridges in anaphase I of the *spo11-1* mutant but no bridges in anaphase II. These observations suggest that AtXrc3, and presumably also AtRad51C, functions in a post-AtRad51 role in the repair Spo11-induced DSBs or DSBs in homologous chromosomes and sister chromatids damaged spontaneously or by a minor meiotic endonuclease other than Spo11. Re-

cent studies of Rad51 paralogs in mammals suggest the involvement of Rad51 paralogs in modification of HJ (Braybrooke et al., 2003; Yokoyama et al., 2003; Liu et al., 2004) and that Rad51C and Xrc3 contribute to HJ resolution (Liu et al., 2004). These findings support the hypothesis that the defect of meiosis in *atrad51C* with chromosome fragmentations and bridges is due to the absence of HJ resolution activity as thought in the case of *atxrc3* (Bleuyard and White, 2004). Furthermore, the fragmentations in anaphase II of *atrad51C* mutants seem to arise from unresolved sister chromatid.

On the other hand, earlier studies suggested that the Rad51 paralogs also act in early steps of the HR process (Bishop et al., 1998; Godthelp et al., 2002). Recently, Forget et al. (2004) showed the localization of Xrc3 at sites of DNA breaks within 10 min after exposure to γ -irradiation and that such localization is independent of Rad51. In plants, meiosis is initiated by the formation of DSB, which is introduced by Spo11, in a manner similar to that in yeast (Grelon et al., 2001; Li et al., 2004). Further studies should be performed to determine the localization of AtRad51C and AtXrc3 following DSB formation by Spo11 in meiosis to understand the function of AtRad51C and AtXrc3 in HR.

Based on our previous findings of the interaction between AtRad51C and AtXrc3, and AtRad51B and AtRad51C (Osakabe et al., 2002, 2005), and of the inducibility of *AtRAD51B*, *AtRAD51C*, and *AtXRCC3* genes in response to genotoxic stress inducing DSBs (Osakabe et al., 2002, 2005), we hypothesized the involvement of AtRad51C in DSB repair and the increased sensitivity of *atrad51C* to genotoxic stresses compared to wild-type plants, although the *atrad51C* mutants plant did not show any abnormal growth in vegetative development under the standard growth condition used in our experiment. As seen in Figure 5, *atrad51C* showed hypersensitivity to the cross-linking

Table 1. Somatic HR in *atrad51C* and wild-type plants

Data are numbers of plants tested (*n*), total blue-stained recombination spots (*N*), and the mean number of spots per plant (*M*) of chromosomal recombination assays of wild-type and *atrad51C* plants under three different treatments.

	Wild-Type			<i>atrad51C</i>			Fold Reduction
	<i>n</i>	<i>N</i>	<i>M</i> (Enhancement)	<i>n</i>	<i>N</i>	<i>M</i> (Enhancement)	
No genotoxic stresses							
DR ^a	50	241	4.82	50	117	2.34	0.49
IR ^b	50	357	7.14	50	176	3.92	0.55
Cisplatin treatment							
DR	50	1285	26.3 (5.46)	50	368	7.36 (3.15)	0.28
IR	50	1562	31.2 (4.38)	50	396	7.92 (2.02)	0.25
Bleomycin treatment							
DR	50	1521	30.4 (6.31)	50	378	7.56 (3.23)	0.25
IR	50	1671	33.4 (4.68)	50	371	7.42 (1.89)	0.22

^aResults with the direct-repeat reporter line 1406.

^bResults with the inverted-repeat reporter line 1415.

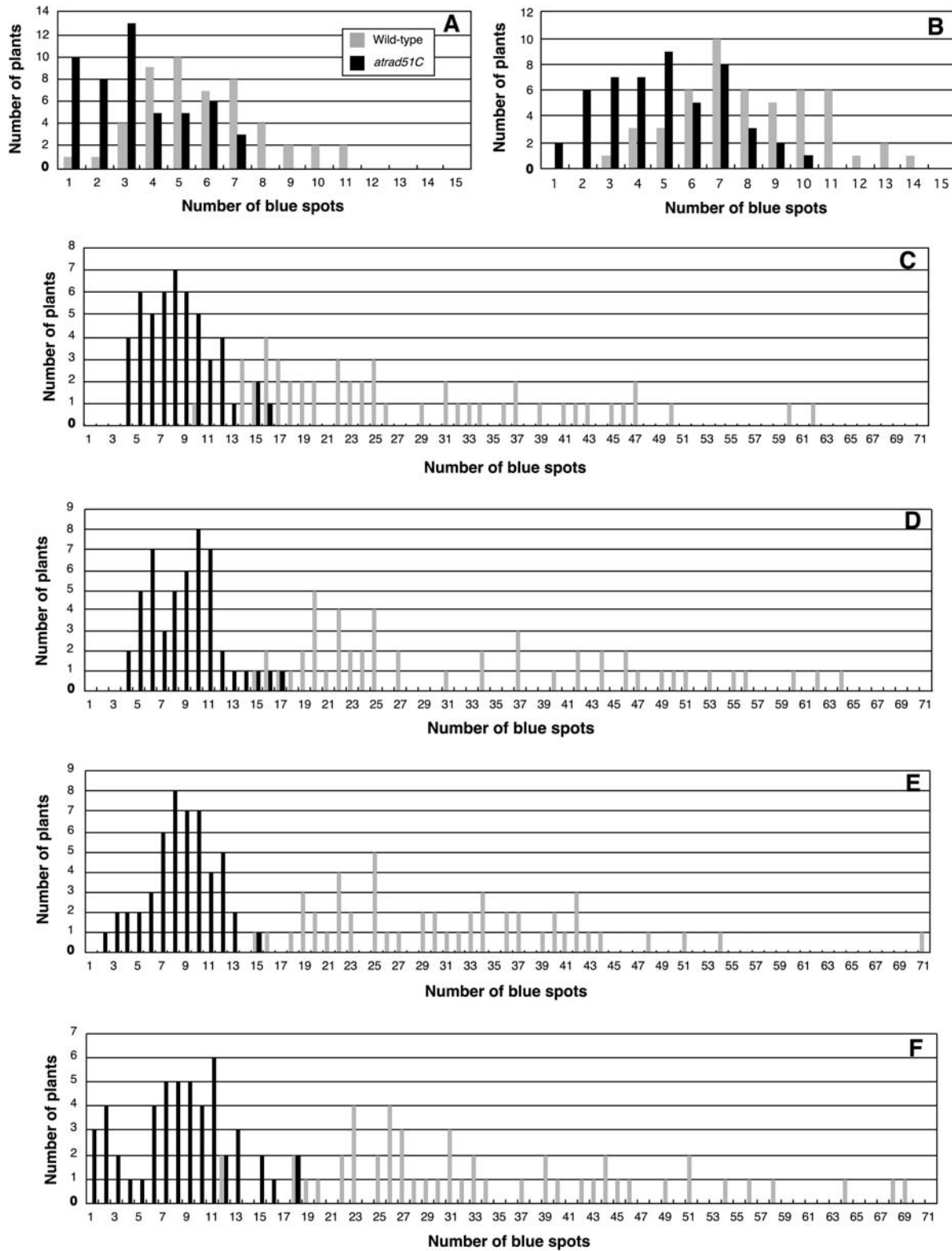


Figure 8. Frequencies of intrachromosomal HR in *atrads51C* and wild-type plants. A to F, Frequency distribution histogram shows the proportions of plants with a given number of blue GUS spots in the direct repeat (A, without genotoxic stresses; C, with cisplatin treatment; E, with bleomycin treatment) and inverted repeat (B, without genotoxic stresses; D, with cisplatin treatment; F, with bleomycin treatment) populations. *atrads51C* mutant and wild-type plants are shown as black and white bars, respectively. Images, Visualization by histochemical staining of recombination events in the direct-repeat line 1406. Top left, Wild-type plant without genotoxic stress; top right, wild-type plant with cisplatin treatment; bottom left, *atrads51C* plant without genotoxic stress; bottom right D, *atrads51C* plant with cisplatin treatment.

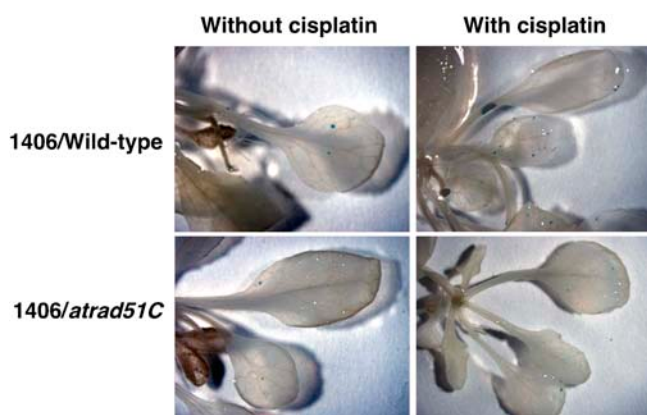


Figure 8. (Continued.)

reagent as reported in chicken DT40 and Chinese hamster CL-V4B cells with impaired Rad51C function (Takata et al., 2001; Godthelp et al., 2002; Drexler et al., 2004; Sasaki et al., 2004). Cisplatin forms DNA cross-links, which are intrastrand cross-links and interstrand cross-links (Zamble and Lippard, 1995). Intra- and interstrand DNA cross-links disturb DNA synthesis and lead to DSBs during DNA replication (Dronkert and Kanaar, 2001; De Silva et al., 2002). In this regard, HR seems to repair this type of DNA damage efficiently during S to G2 phase. Indeed, hypersensitivity to cross-linking reagents is a consistent feature of HR repair in deficient mutants described in vertebrate cell lines (Liu et al., 1998; Takata et al., 2000; Sasaki et al., 2004). Furthermore, a recent study reported that the Arabidopsis mutant with impaired Xrcc3, which is the interaction partner of AtRad51C (Osakabe et al., 2002), also shows hypersensitivity to the cross-linking reagent (Bleuyard et al., 2004). Unexpectedly, the root growth of *atrad51C* and *atrad51B* was not affected by cisplatin treatment in contrast to the production of true leaves (Fig. 2B). According to studies on yeast and mammals, damaged DNA by cross-linking reagents such as cisplatin and mitomycin C (MMC) is repaired through three potential repair pathways: nucleotide excision repair (NER), postreplication repair/translesion DNA synthesis, and HR repair (Dronkert and Kanaar, 2001). Other than mutants of HR factor, two mutants and antisense-suppressed plants show higher sensitivity to the cross-linking reagents: antisense *AtRAD1* (NER factor; Gallego et al., 2000) and *atrev3* (translesion DNA synthesis factor; Sakamoto et al., 2002). MMC inhibits both the production of true leaves and growth of roots of antisense *AtRAD1* plants (Gallego et al., 2000). MMC also inhibits the root growth of the *rev3* mutant plant, although the effect of MMC on the production of true leaves has not been determined (Sakamoto et al., 2002). These results suggest that the contribution of HR repair to the cross-linked DNA repair might be tissue specific, although the cooperation of several DNA repair pathways must be operational for the efficient repair of cross-linked DNAs in plant cells.

As shown in Figure 7, *atrad51C* seedlings also showed hypersensitivity to γ -irradiation, which is a direct DSB inducer (Dizdaroglu and Bergtold, 1986; Thacker, 1999). In contrast to our result, Bleuyard et al. (2005) reported that *atrad51C* does not show any increased sensitivity to γ -irradiation compared to wild-type plants. However, they used imbibed seeds in their sensitivity tests to γ -irradiation. When Arabidopsis embryo becomes fully mature, the cells stop division, and it is thought that the cells of Arabidopsis embryo are completely arrested in the G1 phase for long-term quiescence (Laufs et al., 1998; Preuss and Britt, 2003). In vertebrates and higher plants, NHEJ is thought to be the predominant repair pathway, especially in mature organisms and during the G1 and early S phases of the cell cycle. On the other hand, HR is important during the late S and G2 phases of the cell cycle when the sister chromatid is available as a template and during early development (Essers et al., 2000; Richardson and Jasin, 2000). Thus, NHEJ repair of the DSBs in seeds was predominant during germination after γ -ray irradiation, and the role of AtRad51C in HR is thought to be masked by NHEJ activity in the range of lower dose of γ -irradiation.

It is interesting to note that *atrad51C* mutant plants were more sensitive to cisplatin than *atrad51B*, when we tested the sensitivity to cisplatin by the method used in this study (Fig. 5, B and C). Previous studies of mammals suggested that Rad51 paralogs form two complexes, BCDX2 and CX3, both containing Rad51C (Miller et al., 2004). Moreover, Lio et al. (2004) reported that inhibition of Rad51C reduced the protein levels of Xrcc3 and Rad51B in four other Rad51 paralogs, although the reduction of Rad51B was less and more transient. Thus, it is thought that Rad51C is a central component of Rad51 paralog complexes and that it may regulate the amount of Rad51 paralog complexes. We also showed the interaction between AtRad51C and AtXrcc3, and AtRad51B and AtRad51C (Osakabe et al., 2002, 2005). Several mechanisms could explain the higher sensitivity of *atrad51C* than *atrad51B*. First, Arabidopsis might possess two different types of Rad51 paralog complexes similar to vertebrates, and, if CX3 and BCDX2 complexes participate in DSB repair in a different manner, the sensitivity to cisplatin of the *atrad51C* mutant should be more severe than that of other Rad51 paralog mutants. The other possibility is that AtRad51C possesses multiple functions in HR processes, such as the assembling step of Rad51-nucleoprotein filament (Sung et al., 2003; Miller et al., 2004) and the resolution step of HJs (Liu et al., 2004). Our results clearly showed a higher sensitivity of *atrad51C* compared to *atrad51B*, but the hypothesis of different functions for Arabidopsis Rad51 paralogs and Rad51 paralog complexes remains to be confirmed.

In addition to the hypersensitivity to genotoxic stresses, Rad51C deficiency causes impaired sister chromatid cohesion and lack of induction of sister chromatid exchange after genotoxic treatment, as well as increased levels of chromosome aberrations (God-

thelp et al., 2002). Previous studies using chicken DT40 cells also indicated that the deficiency of Rad51C resulted in low levels of both spontaneous and DNA cross-linking reagents-induced sister chromatid exchange (Takata et al., 2001; Sasaki et al., 2004). Rad51C deficiency also results in reduced frequencies of somatic recombination in Rad51C knockout DT40 and CL-V4B cells (Takata et al., 2001; Drexler et al., 2004). To determine the direct involvement of AtRad51C in HR, we measured the frequencies of somatic recombination in the *atrad51C* mutant with GUS recombination reporter system developed by the group of B. Hohn (Swoboda et al., 1994; Gherbi et al., 2001). Using this system, we confirmed that AtRad51C required the efficient recombination in somatic cells (Table I; Fig. 8). We used both direct- and inverted-repeat recombination reporter lines (1406 and 1415) for the evaluation of somatic HRF in the *atrad51C* mutant. In the case of the direct repeat, the production of a functional recombinant GUS gene occurs by gene conversion/crossover or single-strand annealing mechanisms; on the other hand, only a gene conversion/crossover produced a functional recombinant GUS gene for the inverted repeat (Gherbi et al., 2001; Dubest et al., 2004). Somatic HRF in *atrad51C* reduced in both direct- and inverted-repeat reporter lines at similar rates under or without genotoxic stresses (Table I). This finding suggested that AtRad51C participates in the processes of single-strand annealing as well as that of gene conversion. Dubest et al. (2004) reported that the deficiency of AtErcc1 shows hyporecombination phenotype as similar to *atrad51C*. In this regard, Ercc1 (Rad10) is a key component of NER as well as recombination as part of a structure-specific endonuclease (Davies et al., 1995; Niedernhofer et al., 2001; Motycka et al., 2004). As seen in the case of *atrad51C*, the somatic HRF is reduced in *atercc1* compared to wild type, but some stimulation is observed after bleomycin treatment. This indicates that AtRad51C and AtErcc1 play a cooperative role in somatic recombination and also in DSB repair, especially in repair of DNA cross-links as discussed above.

In conclusion, AtRad51C is essential for meiosis and is required for efficient repair of damaged DNA induced by cisplatin and γ -irradiation in somatic cells. In addition, we provided direct evidence for the involvement of AtRad51C in HR in somatic cells of higher plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was used in this study. The *atrad51C* T-DNA insertion line (Salk_021960) was found by searching the T-DNA express database (<http://signal.salk.edu>) established by the Salk Institute Genomic Analysis Laboratory (Alonso et al., 2003). Plants were grown on a soil mixture of equal parts of vermiculite and commercial soil (Sakata Super Mix; Sakata Seed) in a growth chamber at 22°C under 16/8-h photoperiod at 65 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with cool-white fluorescent lamps. Sterile

plants were incubated on MS medium (Murashige and Skoog, 1962) solidified with 0.25% gelrite (Wako; MS gelrite plate) in a growth chamber at 22°C under 16/8-h photoperiod at 65 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with cool-white fluorescent lamps.

Isolation of DNA and RNA

Genomic DNA was isolated from 2- to 4-week-old sterile plants with the DNeasy Plant Maxi kit (Qiagen). Total RNA was isolated from flower buds of 4-week-old plants using the RNeasy Plant Mini kit (Qiagen) according to the instructions provided by the supplier.

DNA Sequencing

Sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The reaction products were analyzed with an automatic DNA sequencer (ABI PRISM 3100-Avant Genetic Analyzer; Applied Biosystems).

PCR Genotyping Assay

Because the kanamycin-resistance gene present in *atrad51C* T-DNA insertion is silenced, the plant genotypes of SALK_021960 for the *atrad51C* mutation were identified by PCR. The wild-type *AtRAD51C* locus was identified by PCR with primers 3ex-F (5'-TTGCCCGGTATT ACTACATCTTGCTCTGA-3') and 8ex-R (5'-ATGCGATTGCTCGGTGTAAGTGCAGAC-3'). The mutant locus was identified by PCR with LBa-1 (5'-GCGTGGACCGCTTGCTGCAACT-3') and 8ex-R. The left and right sides of the T-DNA border sequences were amplified with primers 1ex-F (5'-ATGCGATTGCTCGGTGTAAGTGCAGAC-3') and LB-a1, and primers 8ex-R1 and LB-a1, respectively (Fig. 1A). PCR products were determined by direct sequencing.

Hybridization

Southern- and northern-blotting analyses were performed according to the standard protocols. DNA probes were prepared by PCR DIG probe synthesis kit (Roche) according to the manufacturer's instruction. For southern-blotting analysis, 3ex-F and 6ex-R (5'-ATGCGATTGCTCGGTGTAAGTGCAGAC-3') were used as primers. For northern-blotting analysis, 1ex-F and 3ex-R (5'-GCAAAGACTCCTCCTCGTGAAGC-3') were used as primers. Hybridization and washing were performed under high-stringency conditions with 0.1 \times SSC.

Cytological Analysis

Viability of pollen grains was examined by staining with Alexander's solution (Alexander, 1969) or the I₂-KI (Jefferies, 1977). Tetrads were dissected and stained with aniline blue according to Smith and McCully (1978). Mitotic chromosome preparations were obtained from petals of young floral buds, and meiotic chromosomes were prepared from anthers of young floral buds by enzyme maceration method (Murata and Motoyoshi, 1995; Ross et al., 1996; Armstrong et al., 2001; Nakayama et al., 2004). Whole inflorescences were fixed in freshly prepared fixatives (3:1 ethanol-acetic acid mixture) at 4°C. After rinsing in deionized water for 30 min twice, the floral buds were macerated with an enzyme mixture, which contained 0.8% Cellulase Onozuka RS (Yakult), 0.3% Macerozyme R200 (Yakult), 0.14% Cytohellicase (Sigma-Aldrich), 0.14% Pectolyase Y23 (Seishin Pharmaceutical), and 0.1 mM ethylenediaminetetraacetic acid at pH 4.2 at 37°C for 1 h. After dissection of petals or anthers, the chromosomes were spread by tapping with forceps and dried in air. The chromosomes were counter stained with 1.0 $\mu\text{g}/\text{mL}$ of 4',6'-diamidino-2-phenylindole in Vectashield (Vector Laboratories) and observed with a Zeiss Axioplan II using Cascade blue filter set. Fluorescent images were captured using a cooled CCD camera (PentaMax, Photometrics) and analyzed using IPLab (Scanalytics) and Adobe Photoshop. Meiotic stages of wild type were detected as described previously (Ross et al., 1996).

RNA in Situ Hybridization

Plant materials were fixed in 4% (w/v) paraformaldehyde in 100 mM sodium phosphate buffer, pH 7.4, overnight at 4°C, dehydrated through

a graded ethanol series and *t*-butanol series, and finally embedded in Paraplast Plus (Sherwood Medical). Microtome sections (6–10 μm thick) were placed onto silane-treated glass slides. The antisense and sense RNAs of *AtRAD51C* were labeled with DIG through in vitro transcription of linearized pBluescript KS(+) carrying a fragment of the entire coding sequence of the *AtRAD51C* cDNA. Hybridization and immunological detection were conducted according to the methods of Kouchi and Hata (1993).

Cisplatin Treatment and γ -Ray Irradiation

To study the effect of cross-linking reagents on Arabidopsis plants, we used cisplatin (cis-diamminedichloroplatinum [II]; Wako Pure Chemical Industries). For assay of cisplatin sensitivity based on production of true leaves, 3-d-old seedlings germinated on MS agar medium were transferred onto MS agar medium containing appropriate concentrations of cisplatin. Plants were scored at 12 d posttreatment of cisplatin for the formation of true leaves. To test the effects of cisplatin on root growth, 3-d-old seedlings were transferred onto MS agar medium containing appropriate concentrations of cisplatin and set vertically for 3 d. After 3-d cisplatin treatment, root length was measured. To test the effect of cisplatin on callus growth, calli were obtained from root segments of 2-week-old wild-type and *atrad51C* plants. Roots were cut into 3- to 5-mm segments and placed onto callus-inducing medium (3.3 g/L B5 basal minimal salts [Wako], 0.5 g/L MES, pH 5.7, 1 mL/L vitamin stock solution [0.5 mg/mL nicotinic acid, 0.5 mg/mL pyridoxine, and 0.5 mg/mL thiamine-HCl], 100 mg/L myo-inositol, 20 g/L Glc, 5 mg/L indoleacetic acid, 0.5 mg/L 2,4-dichlorophenoxyacetic acid, 0.06 mg/L kinetin, and 0.75% bactoagar). Wild-type and *atrad51C* calli were subcultured on CIM every 2 weeks. Five-day-old calli were transferred onto CIM containing appropriate concentration of cisplatin. Sensitivity to cisplatin was scored visually at 2 weeks posttreatment of cisplatin for callus growth. For assay of sensitivity to γ -ray irradiation, 3-d-old seedlings germinated on MS agar medium were irradiated using a ^{60}Co source at a dose rate of 14 Gy/min. Plants were further cultured on MS agar medium and scored at 12 d postirradiation for formation of true leaves. After scoring the sensitivity, genomic DNAs were isolated from the tested plants to determine their genotypes.

Complementation Test

For complementation of *atrad51C*, 4.2-kb *Xba*I-*Sal*I genomic *AtRAD51C* DNA fragment containing 1.3-kb 5'-upstream, 2.0-kb coding, and 0.9-kb 3'-downstream regions was subcloned from bacterial artificial chromosome DNA F4L23 (GenBank accession no. AC002387) into binary vector pZH1 (M. Kuroda and S. Toki, unpublished data) to yield pZHg51C. pZH1 and pZHg51C vectors carry the hygromycin-resistant gene as a selectable marker. The binary vectors were introduced in *Agrobacterium tumefaciens* strain EHA105. Wild-type and *atrad51C* plants were transformed by the root transformation method described by Nam et al. (1997). Transgenic calli were selected and maintained on CIM containing 20 $\mu\text{g/L}$ hygromycin (Wako). These calli were later analyzed for *AtRAD51C* mRNA expression and the sensitivity to cisplatin.

In Planta Recombination Assay

The GUS recombination substrate lines 1406 (direct-repeat recombination substrate line) and 1415 (inverted-repeat recombination substrate line) were crossed with the heterozygous *AtRAD51C*^{+/-} plant. F3 seeds from plants homozygous for the GUS recombination reporter and heterozygous for the mutant *atrad51C-1* allele were germinated on MS medium. The aerial part from each of 3-week-old plants was incubated at 37°C for 48 h in sterile staining buffer (100 mM sodium phosphate buffer [pH 7.0], 0.4 mg/mL 5-bromo-4-chloro-3-indolyl glucuronide, 0.25 mM potassium ferricyanide, 0.25 mM potassium ferrocyanide, and 0.1% Triton X-100). Genomic DNA was isolated from the remaining root of each plant for genotyping. The number of blue spots, resulting from a recombination event, on each plant was determined visually under a dissecting microscope. For the DNA damage-induced recombination assay, 2-week-old plants were transferred onto MS agar medium containing 5 μM cisplatin or 0.05 $\mu\text{g/L}$ bleomycin for 7 d, and used for GUS histochemical staining.

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