Arabidopsis RAD51C Gene Is Important for Homologous Recombination in Meiosis and Mitosis

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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 gametogoneses 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,
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.

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**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 μg of genomic DNA from *AtRAD51C*<sup>+/+</sup> (wild type), *AtRAD51C*<sup>+/−</sup> (heterozygous), and *atrad51C*<sup>−/−</sup> (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*<sup>+/+</sup>, and *atrad51C*<sup>−/−</sup> plants. In these experiments, 10 μg 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<sub>r</sub> 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 atrad51C. 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 atrad51C and wild-type plants were digested with either BamHI or Xhol 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 atrad51C mutant by northern-blotting analysis. In this experiment, total RNA from flower buds of wild-type and atrad51C 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 atrad51C (Fig. 1C). Thus, we concluded that the atrad51C mutant could not express a functional Rad51C protein.

AtRad51C Is Not Crucial for Vegetative Development

The atrad51C 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 atrad51C−/− 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 atrad51C−/− 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 atrad51C−/− plant was normal as that of the AtRAD51C+/+ plant (Fig. 3).

The atrad51C Mutant Plant Is Sterile

Next, we determined the sterility phenotypes in the progeny of self-fertilized heterozygous AtRAD51C+/− 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...
the genotype of the $atrad51C$ allele in these plants. Thirty-seven of the 188 plants (19.7%) were homozygous for the T-DNA insertion in the $AtRAD51C$ gene ($atrad51C^{2/2}$), while 103 of 188 (54.8%) were heterozygous ($AtRAD51C^{1/2}$) and 48 of 188 (25.5%) were wild type ($AtRAD51C^{1/1}$). This analysis of individual plants confirmed that sterile plants are homozygous for the $atrad51C$ allele.

Heterozygous $AtRAD51C^{1/2}$ plants and homozygous $atrad51C^{2/2}$ mutant plants showed normal flower development similar to the wild-type plants (Fig. 2, B and C). In addition, the fertility of heterozygous plants was similar to that of wild-type plants. However, $atrad51C$ 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.

$atrad51C$ Mutant Plants Are Defective in Male and Female Gamete Development

To determine the cause(s) of the sterility phenotype in $atrad51C$ 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 ($I_2$-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 $I_2$-KI. None of the 50 examined $atrad51C$ 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 $atrad51C$ 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 $atrad51C$ mutants, $atrad51C$ 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 $atrad51C$ mutant plants (Fig. 2G). Furthermore, an average of 0.035 seeds per silique ($n = 114$) was...
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 siliques (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 synaptic 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
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 XbaI-SalI genomic AtRAD51C DNA fragment containing 1.3-kb 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 Grey (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
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 \( \text{AtRAD51C}^{1/2} \) plant. The frequency of somatic HR (HRF) was monitored in F3 progeny plants homozygous for the GUS recombination substrate and homozygous for the \( \text{atrad51C} \) mutation allele or the wild-type \( \text{AtRAD51C} \) allele. The results are summarized in Table I and are presented as frequency distribution histogram in Figure 8. Spontaneous HRF of the \( \text{atrad51C}^{1/2} \) 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 \( \mu M \) cisplatin (Table I; Fig. 8, C and D, and images, top). We also measured somatic HRF for bleomycin-treated plants. Bleomycin is a \( \gamma \)-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 \( \text{atrad51C} \) mutant plants increased by only 2- to 3-fold after cisplatin or bleomycin treatment (Table I; Fig. 8, C–F, and images, bottom). Thus, \( \text{atrad51C} \) showed hyporecombination phenotype for somatic chromosomal recombination.

**DISCUSSION**

We reported previously the isolation and characterization of Arabidopsis \( \text{RAD51} \) paralogous genes \( \text{AtRAD51C} \) and \( \text{ATXRCC3} \) (Osakabe et al., 2002). Transcription of \( \text{AtRAD51C} \) and \( \text{ATXRCC3} \) was detected in various tissues, with the highest level of expression in flower buds, and was induced by \( \gamma \)-irradiation. In this report, we characterized the \( \text{atrad51C} \) mutant caused by T-DNA insertion in the Arabidopsis \( \text{RAD51C} \) gene.

Phenotypic analysis of the \( \text{atrad51C} \) 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...
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 \textit{atxrcc3} mutant (Bleuyard and White, 2004; Bleuyard et al., 2005; Li et al., 2005; this study). It is noted that other Rad51 family proteins, including AtXrcc3, cannot fully substitute the function of AtRad51C in meiosis because the sterility of \textit{atrad51C} is quite severe. The \textit{atdmc1} mutant was deficient for meiotic homologous chromosome synopsis, arrested by the 10 univalents observed at metaphase I (Couteau et al., 1999). The \textit{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 \textit{atrad51} (Li et al., 2004). In contrast to AtDmc1 and AtRad51, AtRad51C and AtXrcc3 are not required for synopsis 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 AtXrcc3 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 \textit{atxrcc3} mutant, but chromosome fragmentations are still observed in anaphase II of the \textit{atxrcc3/spo11-1} double mutant. In contrast to the case of \textit{atxrcc3/spo11-1}, no chromosome fragmentation is observed in anaphase II of the \textit{atrad51/spo11-1} double mutant (Li et al., 2004). Grelon et al. (2001) reported the presence of bridges in anaphase I of the \textit{spo11-1} mutant but no bridges in anaphase II. These observations suggest that AtXrcc3, 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. Recent 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 Xrcc3 contribute to HJ resolution (Liu et al., 2004). These findings support the hypothesis that the defect of meiosis in \textit{atrad51C} with chromosome fragmentations and bridges is due to the absence of HJ resolution activity as thought in the case of \textit{atxrcc3} (Bleuyard and White, 2004). Furthermore, the fragmentations in anaphase II of \textit{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 Xrcc3 at sites of DNA breaks within 10 min after exposure to \(\gamma\)-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 AtXrcc3 following DSB formation by Spo11 in meiosis to understand the function of AtRad51C and AtXrcc3 in HR.

Based on our previous findings of the interaction between AtRad51C and AtXrcc3, and AtRad51B and AtRad51C (Osakabe et al., 2002, 2005), and of the inducibility of \textit{AtRAD51B}, \textit{AtRAD51C}, and \textit{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 \textit{atrad51C} to genotoxic stresses compared to wild-type plants, although the \textit{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, \textit{atrad51C} showed hypersensitivity to the cross-linking

### Table 1. Somatic HR in \textit{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 \textit{atrad51C} plants under three different treatments.

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<td>(n)</td>
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<td>(M) (Enhancement)</td>
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<td>No genotoxic stresses</td>
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<td>DR(^a)</td>
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<td>241</td>
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<td>IR(^b)</td>
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<td>IR</td>
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<td>IR</td>
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\(^a\)Results with the direct-repeat reporter line 1406. \(^b\)Results with the inverted-repeat reporter line 1415.

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Figure 8. Frequencies of intrachromosomal HR in atrad51C 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. atrad51C 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, atrad51C plant without genotoxic stress; bottom right D, atrad51C plant with cisplatin treatment.
the cross-linking reagents: antisense-suppressed plants show higher 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 Brit, 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).

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 Brit, 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.

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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 recombinase 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 recombinated GUS gene occurs by gene conversion/crossover or single-strand annealing mechanisms; on the other hand, only a gene conversion/crossover produced a functional recombinated 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 AtErrc1 shows hyporecombination phenotype as similar to *atrad51C*. In this regard, Errc1 (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; Motyczka et al., 2004). As seen in the case of *atrad51C*, the somatic HRF is reduced in *aterrc1* compared to wild type, but some stimulation is observed after bleomycin treatment. This indicates that AtRad51C and AtErrc1 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 μmol m⁻² 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 μmol m⁻² 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 DNAeasy Plant Maxi kit (Qiagen). Total RNA was isolated from flower buds of 4-week-old plants using the RNaseasy 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 automated 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'-TTCGCGTGTAATTACATCCTGTGCTGA-3') and 8ex-R (5'-ATCTGCGATTGCGGTCGTTAATCGCAGAC-3'). The mutant locus was identified by PCR with LB-a1 (5'-AGCTGCGACGCTTGGCTGCACCAC-3') and LB-a1, and primers 3ex-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 8ex-R (5'-ATCTGCGATTGCGGTCGTTAATCGCAGAC-3') were used as primers. For northern-blotting analysis, 1ex-F and 3ex-R (5'-GCAAAGATCCTCCTCGTGAAGC-3') were used as probes. Hybridization and washing were performed under high-stringency conditions with 0.1X SSC.

**Cytological Analysis**

Viability of pollen grains was examined by staining with Alexander’s solution (Alexander, 1969) or the I-KI (Jefferys, 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 inferences 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% Cytohelicase (Sigma-Aldrich), 0.14% Pectolyase Y23 (Seishin Pharmaceutical), and 0.1 mM ethylendiaminetetraacetic 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 μg/mL of 4',6'-diamidino-2-phenylindole in Vectashield (Vector Laboratories) and observed with a Zeiss Axioskan 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 plBluescript 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-diaminedichloroplatinum [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 BS 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 concentrations 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 60Co 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 Xhel-Sall 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 (Gentbank accession no. AC023387) 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. 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 60Co 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.

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 homozigous for the GUS recombination reporter and heterozygous for the mutant atrad51C-T 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-indoly 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 μg cisplatin or 0.05 μg/L bleomycin for 7 d, and used for GUS histochemical staining.

Roles of AtRad51C in Meiotic and Mitotic Recombination

ACKNOWLEDGMENTS

We thank J.M. Lucht and B. Hohn for providing GUS recombination reporter lines. We thank the SALK Institute Genomic Analysis Laboratory and the Arabidopsis Biological Resource Center for providing the T-DNA insertion line. We also thank the Arabidopsis Biological Resource Center for providing the bacterial artificial chromosome DNA. We thank R. Aoto, E. Ozawa, A. Nagashii, Y. Nomura, and F. Suzuki for their technical help. Received May 9, 2005; revised July 26, 2005; accepted August 9, 2005; published September 16, 2005.

LITERATURE CITED


Grelon M, Vezon D, Gendrot G, Pelletier G (1994) Sequence of the RAD55 gene of


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