The AtRAD51C Gene Is Required for Normal Meiotic Chromosome Synapsis and Double-Stranded Break Repair in Arabidopsis

Wuxing Li, Xiaohui Yang, Zhenguo Lin, Ljudmilla Timofejeva, Rong Xiao, Christopher A. Makaroff, and Hong Ma*

Department of Biology and the Huck Institutes of the Life Sciences (W.L., Z.L., L.T., R.X., H.M.) and Intercollege Graduate Program in Plant Physiology (W.L., R.X., H.M.), the Pennsylvania State University, University Park, Pennsylvania 16802; Department of Chemistry and Biochemistry, Miami University, Oxford, Ohio 45056 (X.Y., C.A.M.); and Department of Gene Technology, Tallinn University of Technology, Tallinn 12618, Estonia (L.T.)

Meiotic prophase I is a complex process involving homologous chromosome (homolog) pairing, synapsis, and recombination. The budding yeast (Saccharomyces cerevisiae) RAD51 gene is known to be important for recombination and DNA repair in the mitotic cell cycle. In addition, RAD51 is required for meiosis and its Arabidopsis (Arabidopsis thaliana) ortholog is important for normal meiotic homolog pairing, synapsis, and repair of double-stranded breaks. In vertebrate cell cultures, the RAD51 paralog RAD51C is also important for mitotic homologous recombination and maintenance of genome integrity. However, the function of RAD51C in meiosis is not well understood. Here we describe the identification and analysis of a mutation in the Arabidopsis RAD51C ortholog, AtRAD51C. Although the atrad51c-1 mutant has normal vegetative and flower development and has no detectable abnormality in mitosis, it is completely male and female sterile. During early meiosis, homologous chromosomes in atrad51c-1 fail to undergo synapsis and become severely fragmented. In addition, analysis of the atrad51c-1 atspo11-1 double mutant showed that fragmentation was nearly completely suppressed by the atspo11-1 mutation, indicating that the fragmentation largely represents a defect in processing double-stranded breaks generated by AtSPO11-1. Fluorescence in situ hybridization experiments suggest that homolog juxtaposition might also be abnormal in atrad51c-1 meiocytes. These results demonstrate that AtRAD51C is essential for normal meiosis and is probably required for homologous synapsis.

Meiosis is essential for eukaryotic sexual reproduction, allowing the production of haploid gametes. In addition, meiotic recombination during the early stages of meiosis allows the exchange of genetic information, serving as an important source of genetic diversity. The success of meiosis depends on a complex and prolonged prophase I that involves homologous chromosome (homolog) pairing, synapsis, and recombination (Zickler and Kleckner, 1999; Page and Hawley, 2003; Schwarzacher, 2003). After pairing, the homologs continue to associate and this interaction has been referred to as homolog juxtaposition (Zickler and Kleckner, 1999). Recombination results in crossover events that correspond to cytologically observed chiasmata, which, in combination with sister chromatid cohesion, maintain the association between homologs in the form of bivalents, ensuring proper segregation of homologs at anaphase I. Synapsis, the formation of synaptonemal complexes (SCs) between closely associated chromosomes, has also been implicated to play important roles in meiotic prophase I, although its relationship with recombination differs among organisms.

Cytological and molecular genetic studies support the idea that homolog pairing, synapsis, and recombination are closely coupled events in normal meiosis. In particular, recombination and synapsis are often interdependent. In fact, a number of meiotic genes are required for both normal synapsis and recombination in yeast (Saccharomyces cerevisiae; Zickler and Kleckner, 1999). For example, in budding yeast, the SPO11 protein is required for both recombination and synapsis. SPO11 is a member of a family of type II topoisomerases and is required for the generation of double-stranded breaks (DSBs; Keeney et al., 1997) that initiate recombination. SPO11 homologs have been identified in both animals and plants, suggesting that the pathway for enzymatic generation of DSBs is conserved. In Arabidopsis (Arabidopsis thaliana), a SPO11 homolog, AtSPO11-1, is required for meiotic recombination,
suggesting that there is an AtSPO11-1-dependent mechanism for DSB formation in plants (Grelon et al., 2001). On the other hand, SPO11 homologs are required for recombination, but dispensable for synapsis in Caenorhabditis elegans and Drosophila melanogaster (Dernburg et al., 1998; McKim and Hayashi-Hagihara, 1998), indicating that requirements of specific genes for recombination and synapsis are not identical among organisms.

In the widely accepted DSB repair model for recombination (Szostak et al., 1983), DSBs facilitate the initiation of interaction between homologs via RAD51 and other proteins. RAD51, first discovered in yeast, is a homolog of the Escherichia coli RecA recombinase (Shinohara et al., 1992). Like RecA, RAD51 catalyzes DNA strand exchange in an ATP-dependent manner (Sung, 1994). In addition, a second yeast RecA homolog, DMC1, functions specifically in meiosis (Bishop et al., 1992). Biochemical studies indicate that RAD51/DMC1 binds to single-stranded DNA (ssDNA) that is formed following the generation of DSBs and then promotes strand invasion of the ssDNA into the intact homolog, producing a D-loop (Petukhova et al., 2000). Genetic studies in yeast indicate that a portion of DSBs leads to the formation of double Holliday junctions and crossover events, whereas other DSBs result in non-crossover events (Bishop and Zickler, 2004; Borner et al., 2004). Protein localization studies of RAD51 in maize (Zea mays) and Arabidopsis and molecular genetic analyses in Arabidopsis also support the idea that RAD51 is important for pairing, synapsis, and recombination (Franklin et al., 1999; Mercier et al., 2003; Pawlowski et al., 2003, 2004; Li et al., 2004).

Five RAD51 paralogs have been identified in mammals and birds, including RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3 (Shinohara et al., 1993; Tebbs et al., 1995; Albala et al., 1997; Dosanjh et al., 1998; Liu et al., 1998; Pittman et al., 1998; Schild et al., 2000). In cell cultures, mutations in these genes cause defects in DSB repair, increased chromosome instability, and broad-spectrum mutagen sensitivity (Takata et al., 2001) similar to the effects of rad51 mutations. These RAD51 paralogs have been found in two complexes, the RAD51C-XRCC3 complex and the RAD51B-RAD51C-RAD51D-XRCC2 complex, and RAD51 may interact with the RAD51C-XRCC3 complex (Masson et al., 2001; Liu et al., 2002). RAD51C is the only protein present in both complexes, suggesting an important role of RAD51C in recombination. The human RAD51C binds to ssDNA and double-stranded DNA and exhibits a DNA-stimulated ATPase activity (Lio et al., 2003), suggesting that it may have a similar biochemical function to that of RAD51. On the other hand, the fact that RAD51 and RAD51C are both required for normal DNA repair and recombination indicates that they do not have identical functions. This is further supported by in vitro analysis showing that RAD51C promotes branch migration and the resolution of double Holliday junctions in homologous recombination (Liu et al., 2004). The critical role of RAD51C in mitotic recombination suggests that it might be involved in meiotic recombination as well. In Drosophila, a putative RAD51C homolog, spnD, is required for normal fertility and oogenesis, but its role in meiosis has not been analyzed in detail (Abdu et al., 2003). Therefore, the function of RAD51C in animal meiosis is still not clear.

A putative RAD51C ortholog, AtRAD51C, is present in the Arabidopsis genome (Osakabe et al., 2002). We have identified an insertional line in the AtRAD51C gene, atrad51c-1, and report our analysis of this mutant here. An independent analysis of this insertional line and other mutants has been published recently (Bleuyard et al., 2005) and these two studies both indicate that the AtRAD51C gene is required for male meiosis. We provide additional detailed characterization of the atrad51c-1 mutant, including the result that this gene is also required for female meiosis, but it is not critical for mitosis under normal conditions. Fluorescence and transmission electron microscopy (TEM) results demonstrated that synapsis is abnormal and homolog juxtaposition might also be altered in this mutant, indicating that AtRAD51C may be required for both processes. Furthermore, atrad51c-1 mutant cells exhibit chromosome fragmentation in an AtSPO11-1-dependent manner, indicating that AtRAD51C is required for proper processing of AtSPO11-1-generated DSBs. The number of breaks in the atrad51c-1 cells is greater than the expected number of crossovers, suggesting that at least some of the unprocessed DSBs would have resulted in non-crossover events. Therefore, our results provide clues suggesting the involvement of non-crossover-generating DSBs in homolog juxtaposition. Here we also discuss a possible model for RAD51C function in early meiosis.

RESULTS

Identification of a T-DNA Insertion in the AtRAD51C Gene

Both Arabidopsis genomic and cDNA sequences reveal a putative homolog of the human RAD51C gene, At2g45280, which was named AtRAD51C (Osakabe et al., 2002). Sequence comparison indicates that the predicted AtRAD51C protein is more similar to the human RAD51C protein (37.7% identity and 59.8% similarity) than to other human and Arabidopsis RAD51 paralogs (21.3%–27.2% identities and 39.4%–48.8% similarities). Reverse transcription (RT)-PCR indicates that AtRAD51C is expressed widely, with the highest levels in floral buds (Fig. 1B). mRNA in situ hybridization detected strong AtRAD51C expression only in meiocytes (Fig. 1C), suggesting a function of AtRAD51C in meiosis.

To understand the in vivo function of AtRAD51C, we obtained a T-DNA insertional line (SALK_021960) that contained an insertion in the second intron of the AtRAD51C gene (Fig. 1A). The position of the insertion, named atrad51c-1, was confirmed by PCR

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Flowers from atrad51c-1 plants had normal morphology, including normal floral organ identity and number (Fig. 2, C and D). However, the atrad51c-1 plants were completely sterile, with small, empty seedpods (Fig. 2, G and H). Examination of the mutant flowers indicated that they were male sterile and mutant anthers failed to produce functional pollen grains (Fig. 2, D, F, and J). Some pollen grains were produced in the mutant, but they had abnormal sizes (Fig. 2F) and stained blue with Alexander’s solution, indicating that they were not viable (Fig. 2J). At the tetrad stage, atrad51c-1 anthers contained polyads with five to 10 microspores (Fig. 2L) instead of four in the wild type (Fig. 2K). When the stigmas of atrad51c-1 flowers were pollinated with wild-type pollen (16 flowers crossed), they failed to set seed, indicating that the atrad51c-1 mutant is also female sterile.

Because mutations in human RAD51C affect the DNA repair in somatic cells, and our RT-PCR results as well as microarray data (Obayashi et al., 2004; Zimmermann et al., 2004) indicate that AtRAD51C is also expressed in leaves and roots, we examined mitosis in root tips of wild-type and atrad51c-1 mutant plants to detect possible minor defects that may not be obvious at the plant or organ levels. Analysis in root tips of 4-d-old mutant plants indicated that mitosis in the mutant is very similar to that in wild-type plants, with no obvious differences being detected (Fig. 3; over 350 cells analyzed). Therefore, unlike the vertebrate RAD51C genes, AtRAD51C function appears to be dispensable for somatic growth in Arabidopsis under normal conditions.

**Male and Female Meiosis Is Disrupted in atrad51c-1 Plants**

Our morphological and genetic analyses suggest that atrad51c-1 may be defective in both male and female meiosis. To test this hypothesis, we compared both male and female meioses in wild-type and atrad51c-1 plants grown under standard conditions, using chromosome spreads stained with 4',6-diamidino-2-phenylindole (DAPI). Meiotic abnormalities were identified in both male and female meiocytes (Fig. 4). In wild-type plants, male meiosis I starts with the condensation of chromatin, forming distinct thin chromosome threads at leptotene (Fig. 4A). After further condensation and pairing of homologous chromosomes during zygote (Fig. 4B), juxtaposed homologous are observed at pachytene (Fig. 4C). Following further condensation (Fig. 4D and E), the chromosomes then aligned at the division plane (Fig. 4F). No obvious alterations were observed in atrad51c-1 meiocytes at leptotene (Fig. 4G); however, dramatic abnormalities were observed from zygote through metaphase I. In contrast to the wild type (Fig. 4B), close association of homologs was rarely detected in mutant meiocytes (Fig. 4H). In addition, typical pachytene images could not be found in atrad51c-1 meiocytes (Fig. 4I), suggesting that fully juxtaposed chromosomes may not be
present. Subsequent abnormalities were also observed during diplotene (Fig. 4J) and diakinesis (Fig. 4K). A striking feature of the atrad51c-1 mutant is the presence of numerous brightly stained spots. The abnormally large number of chromosomal entities was detected beginning at approximately diakinesis, suggesting that some DSBs were not repaired and subsequently were manifest as broken chromosomes. The chromosome fragments became more obvious at metaphase II, when they are highly condensed (atrad51c-1, an average of 28.7 ± 4.2 spots from 29 cells; for comparison, 31 atrad51c-1 mutant cells have an average of 32.4 ± 4.9 spots; only well-spread cells were counted, although >1,000 were observed with fragmentation for each mutant). To test the possibility that the observed chromosome fragmentation might be resulting in part due to the force of chromosome spreading, we also examined intact atrad51c meiocytes (n = 324) and found similar chromosome fragments (data not shown), whereas wild-type cells had the expected five bivalents at late diakinesis. Other abnormalities observed in atrad51c-1 meiosis II were likely the consequence of defects in meiosis I (Fig. 4, S–X).

Female meiosis in atrad51c-1 exhibited similar, but less severe, defects (Fig. 4EE–JJ) as those observed during male meiosis. In atrad51c-1 female meiocytes, association of homologs at zygotene was also altered (Fig. 4FF), and fully juxtaposed chromosomes as seen in normal pachytene could not be observed (Fig. 4GG).

Mutant female meiocytes also exhibited abnormal chromosome alignment and the presence of more than 10 chromosomal entities. However, fewer fragments were detected at metaphase I (Fig. 4II) and anaphase I (Fig. 4JJ) in female meiocytes than male meiocytes (13–15 chromosomal entities in 23 cells), suggesting that female meiosis may be less affected by the mutation.

**atrad51c-1 Is Defective in Homolog Juxtaposition and Synapsis**

The absence of normal pachytene chromosomes suggested that the atrad51c-1 mutant may be defective in synopsis and/or in homolog juxtaposition. To test this, we examined both wild-type and atrad51c-1 meiocytes using fluorescence in situ hybridization (FISH). From interphase to early zygotene, eight to 10 centromere signals were observed in wild-type and atrad51c-1 meiocytes (Fig. 5, A, B, E, and F). By late zygotene, 72% of the wild-type male meiocytes had five or fewer centromere signals (Fig. 5, C and D), whereas >100 meiocytes were observed with fragmentation for each mutant. To test the possibility that the observed chromosome fragmentation might be resulting in part due to the force of chromosome spreading, we also examined intact atrad51c meiocytes (n = 324) and found similar chromosome fragments (data not shown), whereas wild-type cells had the expected five bivalents at late diakinesis. Other abnormalities observed in atrad51c-1 meiosis II were likely the consequence of defects in meiosis I (Fig. 4, S–X).

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diakinesis stages, >100 cells for each stage; Fig. 5, G and H). This suggests that the atrad51c-1 mutation affects the juxtapositioning of the centromere regions of some chromosomes. Furthermore, most atrad51c-1 cells observed had one or two centromere signals that were larger than the rest and those in the wild type, whereas other centromere signals were often smaller and not as brightly stained as wild-type centromeres. These observations suggest that centromere regions in atrad51c-1 meiocytes may have abnormal structure.

Because paired centromeres could be nonhomologous, we next used a telomere-derived probe that labels, in addition to telomeres, an arm sequence adjacent to the centromere only on chromosome 1 (Armstrong et al., 2001), in order to further assess homolog interaction in atrad51c-1 meiocytes. Two strong signals corresponding to chromosome 1 were observed during interphase, leptotene, and early zygotene in both wild-type and atrad51c-1 meiocytes (Fig. 6). Beginning at late zygotene and extending through pachytene, diplotene, and diakinesis stages, one signal was observed in wild-type meiocytes (Fig. 6). In contrast, two sometimes adjacent signals were observed in the meiocytes of atrad51c-1 plants at the stages corresponding to the normal pachytene, diplotene, and diakinesis stages (Fig. 6). atrad51c-1 cells containing a single chromosome 1 signal were rarely (<20/300) observed. These results are consistent with our observations from DAPI staining and centromere FISH and confirm that, while there may be some association of homologs, the normal homolog juxtaposition is altered in the mutant. In addition to the chromosome 1 signal(s), similar numbers of telomere signals were observed in both wild type and atrad51c-1 (Fig. 6). However, the overall sizes of the telomere signals seemed to be smaller in atrad51c-1 meiocytes than those in wild-type cells (Fig. 6). In some instances, a telomere signal was not detected at some chromosome ends, possibly due to chromosome breakage.

To investigate possible atrad51c-1 defects in synapsis, SC formation was analyzed using TEM (Fig. 7). Wild-type meiotic chromosomes start to synapse at early zygotene (Fig. 7, A and B). Two lateral elements, aligned in parallel, become connected by transverse filaments to the central element forming the SC. Early recombination nodules associated with the SC structures can be observed in wild-type nuclei (Fig. 7B). In mutant meiocytes, at the stages corresponding to zygotene or early pachytene in wild-type meiocytes, SC structures can be seen as short stretches (less than 10% in total length of that in wild type), indicating that most chromosome regions remain unsynapsed (Fig. 7, D–F). Recombination nodules were not observed on the mutant SC central element among 73 cells that were examined with 25 to 30 sections for each cell. Furthermore, abnormal SCs consisting of more than two lateral elements (Fig. 7E) were sometimes observed (approximately one-third of the SCs in the

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**Figure 3.** Mitosis in root tips of 4-d-old wild-type (A–H) and atrad51c-1 (I–P) seedlings. Chromosomes (left) and microtubule structures (right) from the same cell were visualized with DAPI and antibodies against β-tubulin, respectively. Cells at different stages during the mitotic cell cycle were analyzed (preprophase, A, E, I, and M; metaphase, B, F, J, and N; anaphase, C, G, K, and O; and telophase, D, H, L, and P).
mutant), suggesting that synapsis might have occurred between nonhomologous chromosomes. However, no SC or SC-like structures were observed in mutant nuclei at late pachytene, suggesting that partially synapsed chromosomes did not proceed to become fully synapsed and that the partial SCs precociously disassembled during the stage corresponding to wild-type pachytene.

**AtSPO11-1 Is Important for Chromosome Fragmentation in atrad51c-1 Meiocytes**

It is known that, in yeast, SPO11-generated DSBs are required for normal recombination and synapsis and that the Arabidopsis AtSPO11-1 gene is required for pairing and recombination. The chromosome fragmentation observed in atrad51c-1 meiocytes suggests that they may represent a defect to process DSBs generated by the AtSPO11-1 protein. To test the relationship between AtSPO11-1-generated DSBs and chromosome fragmentation in atrad51c-1 plants, we generated the atspo11-1 atrad51c-1 double mutant and analyzed its meiosis (Fig. 8). The meiotic chromosome behavior in the double mutant was similar to that of the atrad51c-1 single mutant, including the presence of many univalents and occasional bivalents (Fig. 8, D and J). This was very different from that in the atrad51c-1 mutant (Fig. 4). In the vast majority of cells (>110), no more than 10 chromosomes were observed in male meiocytes of the double mutant from metaphase I through metaphase II, and 20 newly separated sister chromatids were observed at anaphase II (Fig. 4V), although, in a few cells (3 out of 110 examined), there was an extra DAPI-staining spot in the double mutant. Therefore, chromosome fragmentation was
nearly absent in the double mutant, indicating that AtRAD51C acts downstream of AtSPO11-1. We conclude that AtRAD51C likely plays an important role in processing AtSPO11-1-generated DSBs.

DISCUSSION

Our results clearly show that AtRAD51C is required to repair the meiotic DSBs generated by the AtSPO11-1 during meiosis I, consistent with the function of vertebrate RAD51C in mitosis. The atrad51c-1 mutant plants can undergo normal vegetative and flower development with apparently normal mitosis, although mutations in AtRAD51C and other RAD51 paralogs have recently been shown to cause increased sensitivity to the DNA cross-linking agent mitomycin C (Bleuyard et al., 2005), suggesting that AtRAD51C may have a conserved function in meiosis in both animals and plants. It is possible that vertebrate RAD51C also functions during meiosis; this will need to be tested using mutants, such as mouse knockouts or zebrafish mutants, if they are not embryonic lethal.

Conservation and Divergence of RAD51C and Other RAD51 Paralogs

Although RAD51C orthologs have not been identified in yeast and other fungal organisms, putative orthologs are found in plants and animals. In Drosophila, the spnD gene encodes a putative RAD51C homolog (Abdu et al., 2003), but the levels of amino acid sequence similarity between spnD and mammalian RAD51C are lower (approximately 30% identity and 50% similarity) than those between AtRAD51C and mammalian RAD51C proteins (approximately 40% identity and 60% similarity). Mutations in the spnD gene affect meiosis and oogenesis (Abdu et al., 2003), suggesting that RAD51C may have a conserved function in meiosis in both animals and plants. Therefore, analyses reported here and from others indicate that RAD51C is likely involved in multiple processes during the meiotic prophase I and mitotic cell cycle in animals and plants.
In contrast to mammalian and chicken RAD51C, AtRAD51C and spnD are not required for mitotic growth under normal conditions. A similar situation exists for RAD51 homologs. The mammalian and chicken RAD51 gene is required for the mitotic cell cycle and a mouse rad51 knockout is embryonic lethal (Lim and Hasty, 1996; Sonoda et al., 1999). In contrast, the Arabidopsis atrad51-1 mutant exhibits normal vegetative and flower development (Li et al., 2004). Similarly, Drosophila rad51/spnA and C. elegans rad51 mutants are also both viable, although they have elevated sensitivity to radiation (Rinaldo et al., 2002; Staeva-Vieira et al., 2003). Furthermore, the Arabidopsis atxrcc3 and Drosophila spnB (a putative XRCC3

**Figure 6.** FISH of a telomere probe in wild-type (A–D, I–L) and atrad51c-1 (E–H, M–P) male meiocytes. A FITC-labeled telomere probe (green) was hybridized to chromosome spreads. Chromosomes were counterstained with DAPI (red). Thick arrows denote the telomere-associated chromosome 1 signals at the centromere region. Stages included are interphase I (A and E); zygotene (B and F; inset at the top right corner of F shows the broken chromosome ends with no telomere signals in atrad51c-1); pachytene (C and G); diplontene (D and H; note that the telomere signals are detected at the center of the mutant cell, as indicated by a thin arrow), and telophase II (L and P).

**Figure 7.** TEM of male meiocyte nuclei in wild-type (A–C) and atrad51c-1 (D–F) plants. SCs (arrows) were observed at zygotene (A and B) and pachytene (C) stages in the wild type. In atrad51c-1, the majority of the axial elements (arrowheads) remained unpaired in nuclei corresponding to zygotene (D) or pachytene (F) stages; an occasional abnormal SC (thick arrows) was observed (D and E). Nu, Nucleolus; ERN: early recombination nodule. Bar in A equals 100 nm (same scale for C, D, and F); bar in B equals 500 nm (same scale for E).
homolog) mutants are viable (Abdu et al., 2003; Bleuyard and White, 2004). Therefore, RAD51 orthologs and paralogs seem to be less critical for the mitotic cell cycle than their counterparts in vertebrates. It is thought that RAD51 and its paralogs are required to maintain genome integrity during the cell cycle because even a small number of DSBs that can occur during DNA replication trigger checkpoint control and cell cycle arrest. The fact that RAD51 and some of its paralogs are not as critical in plants and Drosophila suggests that mitotic cell cycle regulation might be different in these organisms or that DSBs formed in mitosis could be repaired by alternative pathways (Li et al., 2004).

The Relationship between Pairing, Synapsis, and Recombination

Analyses in yeast, C. elegans, and Drosophila indicate that the relationship between synapsis and recombination is not conserved between yeast and invertebrate animals (Keeney et al., 1997; Dernburg et al., 1998; McKim and Hayashi-Hagihara, 1998; Peoples et al., 2002). Previously, phenotypic analysis of the atspo11-1 mutant suggests that a SPO11-dependent linkage between recombination and synapsis may exist in plants, but synapsis was not described in the atspo11-1 mutant (Grelon et al., 2001). Our results on synapsis in the atrad51-1 (Li et al., 2004) and atrad51c-1 (this study) mutants support a close relationship between recombination and synapsis. Our analysis strongly suggests that yeast and plants share highly conserved mechanisms for coupled recombination and synapsis. Furthermore, the Arabidopsis syn1 mutant defective in a Rec8 homolog also exhibits chromosome fragmentation. It is possible that the loading of AtrRAD51 is affected in the syn1 mutant, resulting in a failure to process SPO11-1-induced DSBs. Although the relationship between SYN1 and RAD51 in Arabidopsis is not clear, recent studies revealed that Rec8 may promote the assembly of RAD51 foci in rat and possibly in yeast (Eijpe et al., 2003; Zierhut et al., 2004). The relationship between pairing and recombination is much less clear than that for synapsis and recombination (Page and Hawley, 2003). It is known that the processing of SPO11-dependent DSBs has two possible outcomes—crossovers and non-crossovers—but the role of non-crossovers is not understood (Bishop and Zickler, 2004; Haber et al., 2004). RAD51 is localized in wild-type maize meiocytes initially to a very large number of foci, about 10 times the number of crossover sites, and then the number is reduced to be comparable to that of crossovers (Franklin et al., 1999). Recently, similar observations have been made from localization studies in mouse and Arabidopsis meiocytes (Moens et al., 2002; Mercier et al., 2003). Therefore, RAD51 seems to mark sites of DSBs for both crossovers and non-crossovers in early prophase I. Further analysis of RAD51 foci in pairing defective maize mutants provides strong evidence that RAD51 sites, including the non-crossover sites, are pairing sites (Pawlowski et al., 2003). The possible defect in homolog juxtaposition in the atrad51c-1 mutant suggests that it might be abnormal in pairing. It is possible that, in plants, RAD51C may also function in a complex with XRCC3, consistent with the observation that both atrad51c and atxrcc3 mutations exhibit similar abnormalities in meiosis (this study; Bleuyard and White, 2004; Bleuyard et al., 2004, 2005; see below). In addition, the number of chromosome fragments in atrad51c-1 cells exceeds those expected if only the subset of DSBs leading to crossovers is affected by these mutations, indicating that at least some of the non-crossover DSBs also are not repaired. Therefore, strand invasion following the generation of DSBs seems to be a critical mechanism for homology recognition and pairing. It is possible that processing of the DSB sites mediated by RAD51 and its paralogs ensure correct pairing between homologs.

A Model for ATRAD51, ATRAD51C, and ATRXRC3 Functions

Recent reports (Bleuyard and White, 2004; Li et al., 2004; Bleuyard et al., 2005) and this study showed that atrad51-1, atrad51c-1, and atxrcc3 mutants have related meiotic phenotypes, suggesting that they are involved in the same process. This is also supported by the observations that chromosome fragmentation in atrad51-1, atxrcc3, and atrad51c-1 mutants requires the function of AtSPO11-1 (Bleuyard et al., 2004; Li et al., 2004), although ATRXRC3 may have an additional function during meiosis II because atxrcc3 atspo11-1 exhibited severe chromosome fragmentation during anaphase II and telophase II (Bleuyard et al., 2004). At
the same time, the fact that each of these three mutants has very severe defects indicates that these genes have distinct functions, even though they code for homologous proteins.

It is known that RAD51 can bind to ssDNA and catalyze strand invasion by the ssDNA into a double-stranded DNA, forming a D-loop (Petukhova et al., 2000). In vitro studies indicate that mammalian XRCC3 and RAD51C can form a complex and RAD51C can complex with other RAD51 paralogs and at least some of these complexes can promote the loading of RAD51 onto DNA (Masson et al., 2001; Sigurdsson et al., 2001; Wiese et al., 2002; Lio et al., 2003). In addition, analysis of mutant cell lines revealed that the formation of RAD51 foci is reduced in both xrc3 and rad51c mutant cells and the formation of XRCC3 foci on chromosomes depends on RAD51C, but not on RAD51 (Lio et al., 2004; Yoshihara et al., 2004). Therefore, we propose that AtRAD51C and AtXRCC3 form a complex that facilitates the formation of AtRAD51 foci during meiosis. It is possible that RAD51 foci can still form in the absence of AtRAD51C or AtXRCC3, but in reduced numbers. If this is true, it would explain the remaining homolog interaction that was observed in the atrad51c-1 mutant. In addition, a smaller number of chromosome fragments was found in atrad51c-1 mutant meioocytes than in atrad51c-1 meioocytes, consistent with the idea that some DSBs might be processed in the atrad51c-1 mutant.

Mutant analysis has also been reported for the Arabidopsis DMC1 homolog, AtDMC1 (Couteau et al., 1999); the atdmc1 mutant exhibits abnormal chromosome segregation, but not chromosome fragmentation, suggesting that AtDMC1 is not required for the processing of meiotic DSBs. In addition, Drosophila does not possess a DMC1 ortholog, suggesting that its function may be provided by another RAD51 paralog. This is different from the situation in budding yeast in which both RAD51 and DMC1 are required for processing Spo11-generated meiotic DSBs (Shinohara et al., 1997). The phenotypic difference of atrad51-1, atrad51c-1, and atxrc3 mutants from that of the atdmc1 mutant suggests that the AtRAD51/AtRAD51C/AtXRCC3 genes may function upstream of AtDMC1. Recently, it was observed that a Chinese hamster mutant cell line defective in the RAD51C gene exhibits abnormal sister chromatid cohesion (Godthelp et al., 2002), suggesting a possible role for RAD51C in cohesion.

The model proposed above for Atrad51, AtRAD51C, and AtXRCC3 function may also explain why these proteins are dispensable for the mitotic cell cycle. It is likely that, during normal mitotic growth, only a small number of DSBs are generated, far fewer than the number of DSBs generated by Spo11. If so, the number of RAD51 foci needed during the mitotic cell cycle would be far fewer than during meiosis. Indeed, the RAD51 mRNA levels are higher in meiotic cells than other cells (Li et al., 2004). Therefore, whereas Atrad51C and AtXRCC3 may be important for the loading of RAD51 at numerous meiotic foci, they might not be needed when only a few RAD51 foci are needed during the mitotic cell cycle. This idea is supported by the observation that atrad51c and atxrc3 mutants are sensitive to DNA cross-linking agents (Bleuyard and White, 2004; Bleuyard et al., 2005), which presumably induce a large number of DNA lesions. The fact that atrad51-1 is also normal during mitotic growth suggests that some RAD51 paralog(s) might even be able to substitute for AtRAD51 when relatively little AtRAD51 activity is needed. The hypothesis that RAD51 is the primary player in the homology search and that RAD51C and XRCC3 play supporting roles is also supported by phenotypes of Drosophila mutants. The Drosophila spnA (rad51) mutant is hypersensitive to radiation, but spnB and spnD (xrc3 and rad51c) mutants are completely normal during mitotic development (Abdu et al., 2003; Staeva-Vieira et al., 2003), suggesting that SpnB and SpnD are less important than SpnA. It is possible that the molecular interactions between RAD51 and its paralogs RAD51C and XRCC3 are conserved between meiotic recombination in plants and mitotic DNA repair in Drosophila and mammals. Further experiments are needed to test the possible functional overlap between and distinct biochemical activities of Atrad51, AtRAD51C, and AtXRCC3, as well as other RAD51 paralogs.

MATERIALS AND METHODS

Plant Materials

The atrad51c-1 T-DNA insertional line was obtained from the SALK stock of Arabidopsis (Arabidopsis thaliana) T-DNA insertion lines. Both the wild-type and the atrad51c-1 mutant plants are of the Columbia ecotype. Unless otherwise indicated, the plants were grown under long-day conditions (16-h day/8-h night) at 22°C ± 2°C.

Phenotypic Analysis

Plants were photographed using a Sony (Tokyo) digital camera DSC-F707, and photographs of other fresh plant samples (flowers and siliques) were obtained using a Nikon (Tokyo) dissecting microscope with an Optronics (Goleta, CA) digital camera. Developing microspores from fresh floral buds and anthers prior to dehiscence were processed as reported for examining gametophyte development (Azumi et al., 2002). Chromosome spreads were prepared as previously described (Ross et al., 1996) and stained with DAPI (1 μg/ml). Digital images were obtained using a Nikon E400 fluorescence microscope with an Optronics camera. Female meiosis was analyzed essentially as described (Armstrong et al., 2001), using floral buds at stages 10 to 11 (Smyth et al., 1990) and photographed using a Nikon E800 fluorescence microscope with ImagePro software (Media Cybernetics, Silver Spring, MD) and a Hamamatsu CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). Mitosis images were obtained from root tip cells of 4-d-old seedlings (Li et al., 2004).

RT-PCR and RNA In Situ Hybridizations

RNA samples were extracted from young inflorescence tissues of wild-type and atrad51c-1 mutant plants. One microgram of total RNA from each sample was treated with RNase-free DNase I (Invitrogen, Carlsbad, CA) followed by inactivation of the DNase I. Reverse transcription was performed using Supertranscriptase II (Invitrogen) and synthesized cDNA was used as
the template for PCRs. The primers used for the PCRs were oMC 1594 (5′-ATGATTTACCTTGCGCCGCTA-3′) and oMC 193 (5′-CCTCCA-AGACCACCACTCACTCA-3′). As a control, the APTI cDNA in the same samples was amplified using primer pair oMC 571 (5′-TCCCGAGATCGTCTTGGACA-3′) and oMC 572 (5′-CCCTCCCTTGAACAGCTGTC-3′), based on a report that this gene is expressed constitutively in different Arabidopsis organs (Moffatt et al., 1994). For RNA in situ hybridization, sections of wild-type immature floral buds were hybridized with 7S-labeled Arabidopsis antisense and sense probes synthesized from a cDNA clone (amplified with primers oMC 1605 [5′-CAACAGATGGAGTAAGAACGTTCTGAC-3′]; and oMC 1995 [5′-CCGCGCCCCGACCTCGGACGATATTGCTGGCATTTGGCC-3′]), following previously described procedures (Drews et al., 1991; Flanagan et al., 1996). The similarities of A1RAD51C to other RAD51 paralogs are too low (44.4%–49.1%) for cross-hybridization.

Analysis of the atspo1-1 atrad51c-1 Double Mutant

Crosses were made between A1SPO1-1/atspo1-1 and A1RAD51C/atra RAD51C/ atspo1-1; A1SPO1-1/atspo1-1 were identified with gene-specific primers. The progeny of the double-heterozygous plants were genotyped with gene-specific primers and chromosome behavior in male meiosis of double-mutant plants was analyzed following DAPI staining. The primers used for genotyping the plants include oMC 1662 (5′-TGAATCTTCTTGGACGAT-TGGG-3′); oMC 1451 (5′-CAATCCGATTTGGGGCCTGTAACT-3′); oMC 645 (5′-GGGACGCTTGGCTCAACT-3′); oMC 703 (5′-AGTATCGG-GCGTAAAATCTCC-3′); oMC 704 (5′-TTTGGAGATCTTCTCCATGC-3′); and oMC 705 (5′-ACTCGGATCTTTGGGACA-3′).

FISH

Influorescences were fixed in acetic alcohol (ethanol/glacial acetic acid, 3:1) for 2 h at room temperature and stored at −20°C after replenishing the fixative. Staged anthers were spread onto slides and subjected to FISH using previously published procedures (Franz et al., 1996; Caryl et al., 2000) with modifications. The samples on slides were treated with a solution of 70% formamide in 2× SSC for 2 min at 80°C and dehydrated immediately through a graded ethanol series (5 min each in 70%, 90%, and 100%) at −20°C. The slides were dried at room temperature before the application of the probe. The pAL1 clone containing a pericentromeric 180-bp repeat (pAL1-CEN) was used to detect centromere sequences (Martinez-Zapater et al., 1986). Primary PCR amplification of pAL1-CEN was conducted using the M13 forward and reverse primers followed by random primer labeling in the presence of biotin-16-dUTP. The biotin-labeled probe was used in hybridization solution at 5 μg/mL and detected with 10 μg/mL fluorescein-conjugated streptavidin. Chromosome 1-associated telomere sequences were detected by hybridization with the 5′-end fluorescein isothiocyanate (FITC)-labeled oligonucleotide probe, FITC-CCCTAAATA, at 5 μg/mL. Slides are counterstained with DAPI, mounted, and viewed as above.

TEM

For TEM, the fixation and infiltration procedures were as described previously (Owen and Makaroff, 1995) with minor modifications (Li et al., 2004). To determine appropriate meiotic stages, thick cross-sections through the buds were cut, collected on glass slides, stained with 0.1% toluidine blue, and observed with a light microscope. Ultrathin sections were examined with a JEOL 1200 EXII transmission electron microscope (JEOL, Tokyo). At least 30 ultrathin sections were analyzed for each bud.

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Arabidopsis RAD51C Is Required for Meiosis

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