RESEARCH PAPER

The rice RAD51C gene is required for the meiosis of both female and male gametocytes and the DNA repair of somatic cells

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Abstract

The RecA/RAD51 family of rice (Oryza sativa) consists of at least 13 members. However, the functions of most of these members are unknown. Here the functional characterization of one member of this family, RAD51C, is reported. Knockout (KO) of RAD51C resulted in both female and male sterility in rice. Transferring RAD51C to the RAD51C-KO line restored fertility. Cytological analyses showed that the sterility of RAD51C-KO plants was associated with abnormal early meiotic processes in both megasporocytes and pollen mother cells (PMCs). PMCs had an absence of normal pachytene chromosomes and had abnormal chromosome fragments. The RAD51C-KO line showed no obvious difference from wild-type plants in mitosis in the anther wall cells, which was consistent with the observation that the RAD51C-KO line did not have obviously abnormal morphology during vegetative development. However, the RAD51C-KO line was sensitive to different DNA-damaging agents. These results suggest that RAD51C is essential for reproductive development by regulating meiosis as well as for DNA damage repair in somatic cells.

Key words: DNA damage repair, mitosis, Oryza sativa, reproductive development, synopsis

Introduction

During their lifetimes, all organisms suffer DNA damage, which includes double-strand breaks (DSBs), caused by intercellular events or environmental insults. DSBs can be generated spontaneously during DNA replication in dividing cells. Many endogenous and exogenous DNA-damaging agents, including ionizing radiation, DNA-methylating reagents, oxygen free radicals, and DNA cross-linking reagents, can also cause DSBs. DSB repair is important for genome stability, and eukaryotes are equipped with two major DSB repair pathways: homologous recombination (HR) and non-homologous end-joining (Li and Heyer, 2008; Weterings and Chen, 2008). The former pathway, which repairs DNA with homologous sequences, is a more accurate mechanism, while the latter pathway is an error-prone mechanism. Different organisms use one of the two major repair pathways predominantly to different extents (Bleuyard et al., 2006; Wyman and Kanaar, 2006; Agmon et al., 2009). HR is also important for cell division and generation of genetic diversity.

Many genes are involved in HR. Among these genes, Escherichia coli RecA and its eukaryotic homologue RAD51 have been extensively studied (San Filippo et al., 2008). Sequence and phylogenetic analyses suggest that the two eukaryotic RecA homologues, RAD51 and DMCI, may be generated by the duplication of an ancestral gene derived from the ancestor of eubacterial RecA/RAD51-like genes (Lin et al., 2006). RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3, frequently referred to paralogues of RAD51, are presumably generated from another ancestral gene derived from the ancestor of eukaryote RecA/RAD51-like genes, but they have relatively divergent functions (Lin et al., 2006; San Filippo et al., 2008). In addition, there are four RecA genes which are more similar to eubacterial RecA-like
genes than the eukaryotic RAD51-like genes in rice and Arabidopsis (Lin et al., 2006). Proteins encoded by RecA-like and RAD51-like genes share a highly conserved central RecA/RAD51 domain; they are suggested to be evolutionarily related and thus are classified into the RecA/RAD51 family (Lin et al., 2006). The RecA/RAD51 family of eukaryotes includes RAD51, RAD51B, RAD51C, RAD51D, DMC1, XRCC2, XRCC3, and RecA, but not every species has all these members (Lin et al., 2006). Genes of the RecA/RAD51 family play a vital role in HR, HR-dependent DNA repair, and/or other DNA repair processes in both somatic and meiotic cells (Couteau et al., 1999; Osakabe et al., 2002; Bleuyard and White, 2004; Bleuyard et al., 2005; Li et al., 2005; Hamant et al., 2006; Li and Ma, 2006).

Yeast is an excellent model organism for understanding meiosis. Cytological and molecular genetic studies support the idea that some members of the RecA/RAD51 family play an essential role in yeast meiosis, and it is similar in higher eukaryotes. The widely accepted model for recombination in meiosis is the DSB repair model (San Filippo et al., 2008). In yeast meiotic processes, homologous chromosome recombination is initiated by the generation of DSBs. DSBs are catalysed by the conserved topoisomerase-like enzyme SPO11, and the situation is similar in rice (Keeney, 2001; Yu et al., 2010). The RAD51 and RAD51 paralogues with the mediation of many other proteins bind to the single-stranded DNA generated from a DSB; then the nucleoprotein filament searches and attaches to the intact homologue to form a D-loop, which is triple-stranded DNA in which the two strands of a double-stranded DNA molecule are separated by a third strand of DNA (Li and Heyer, 2008). In mammals, some members of the RecA/RAD51 family are essential for the viability, and RAD51 or RAD51C deficiency can result in embryonic lethality (Sonoda et al., 1998; Kuznetsov et al., 2007). Recently, a viable mouse model with a hypomorphic allele of RAD51C was used to investigate the role of RAD51C in meiotic recombination; the results revealed that RAD51C is associated with resolution of the Holliday junction, which is a mobile junction between four strands of DNA, in mice (Kuznetsov et al., 2007).

In plants, the mutants of some RecA/RAD51 family members are sterile but do not show severe irregularities in vegetative growth (Couteau et al., 1999; Bleuyard and White, 2004; Li et al., 2004, 2005; Abe et al., 2005; Osakabe et al., 2005). The Arabidopsis RecA/RAD51 family consists of 11 members (Lin et al., 2006). Some mutants of Arabidopsis RecA/RAD51 family members (RAD51, RAD51C, XRCC3, and DMC1) cause abnormalities in meiosis, including chromosome fragmentation and defective homologue pairing and synapsis (Couteau et al., 1999; Osakabe et al., 2002; Bleuyard and White, 2004; Bleuyard et al., 2005; Li et al., 2005). Knockdown of rice DMC1 leads to abnormal bivalent formation and unequal chromosome segregation in meiosis (Deng and Wang, 2007). Maize (Zea mays) RAD51 is associated with chromosome synapsis and segregation in meiosis (Franklin et al., 2003; Pawlowska et al., 2003).

Some RecA/RAD51 family members are required for efficient HR and/or various types of DNA repair in somatic cells. Mammalian RAD51C, RAD51D, XRCC2, and XRCC3 play roles in DNA damage repair in somatic cells (Kurumizaka et al., 2001, 2002; Sigurdsson et al., 2001; Brenneman et al., 2002; French et al., 2002; Godthelp et al., 2002). RAD51C is needed for Holliday junction resolvase activity in human cells (Liu et al., 2004). In Arabidopsis, some RecA/RAD51 family members, including RAD51, RAD51B, RAD51C, RAD51D, XRCC3, and DMC1, are required for DNA repair, and mutants of these members are hypersensitive to different DNA-damaging agents (Couteau et al., 1999; Osakabe et al., 2002; Bleuyard and White, 2004; Bleuyard et al., 2005; Li et al., 2005; Osakabe et al., 2005; Durrant et al., 2007). Two RAD51 proteins showed ability to bind to single- and double-stranded DNA and strand annealing and strand exchange activities in vitro in physcomitrella moss (Physcomitrella patens; Ayora et al., 2002).

Although RecA/RAD51 family members in various species have been reported to be involved in meiosis and DNA damage repair in somatic cells, not all of them are involved in both biological activities, and the functions of these family members have differentiated during evolution (Lin et al., 2006). For example, disruption of Arabidopsis RAD51B, RAD51D, or XRCC2 does not influence meiosis (Bleuyard et al., 2005; Durrant et al., 2007), but yeast DMC1 only specifically functions in meiosis (Bishop et al., 1992). Mutants of Drosophila XRCC3 (spn-B) and RAD51C (spn-D) do not have defects in DNA repair in somatic cells (Abdu et al., 2003). Mammalian RAD51D is involved in DSBs and is specifically linked with telomere protection in both meiotic and somatic cells (Tarsounas et al., 2004). Rice RAD51 has also been reported to have ATPase activity that is stimulated by non-specific single-stranded DNA in vitro (Rajanikant et al., 2008). Thus it will be possible to understand the RecA/RAD51 family in a species only after elucidating the molecular functions of all members in this family.

The rice genome contains at least 13 RecA/RAD51 family genes based on publications (Lin et al., 2006; Rajanikant et al., 2008). However, only one of these, DMC1, has been functionally analysed in vivo (Deng and Wang, 2007). In this study, the rice RAD51C mutant was characterized. The results show that RAD51C is indispensable for meiosis in both female and male gametocytes, and it also plays a role in DNA damage repair in somatic cells. These results deepen our understanding of meiosis in rice.

Materials and methods

Rice materials and genotoxic treatment

Rice (Oryza sativa) RAD51C T-DNA insertion line 4D-50016, which had the genetic background of japonica (O. sativa ssp. japonica) variety Dongjin, was kindly provided by Professor Gyuneung An (Jeong et al., 2007). The genotype of this line was confirmed by PCR amplification using RAD51C-specific primers 39630-m-F and 39630-m-R, and the T-DNA primer RB1 (Supplementary Table S1 available at JXB online). Rice japonica varieties Dongjin and Zhonghua 11 were used in crosses with the homozygous 4D-50016 line.

The genotoxic treatments were performed according to reported procedures (Chang et al., 2009). In brief, surface-sterilized seeds of heterozygous 4D-50016 plants were germinated and grown on half-strength Murashige and Skoog medium supplemented with 0.3% phytagel for 12 d. After genotype assays, the leaf lengths of the RAD51C-knockout (KO) plants and wild-type siblings segregated from the 4D-50016 line were measured, and the plants were transferred to a standard rice culture solution supplemented with different concentrations of mitomycin C (MMC; Sangon, Shanghai, China) or methylmethane sulphonate (MMS; Sigma-Aldrich, St. Louis, MO, USA) for 20 d (Yoshida et al., 1976). After treatment, the lengths of leaves were re-measured. The
length difference of each leaf before and after treatment was noted and all the length differences of the leaves within a plant were summed as leaf growth. The averaged leaf growth from eight plants within a treatment was used as the index of treatment sensitivity (Chang et al., 2009). For analysing the sensitivity of rice plants to ultraviolet (UV)-C irradiation, rice plants at the tillering stage were exposed to UV light for 6 h at an irradiance of ~0.29 J m\(^{-2}\) s\(^{-1}\) (Chang et al., 2009).

**Database searches and phylogenetic analysis**

To identify the members of the rice RecA/RAD51 family, the sequences of Arabidopsis RecA/RAD51 family genes were used as queries to search different databases by BLAST analysis (Altschul et al., 1997). The databases searched were the Rice Genome Annotation Project (RGAP, http://rice.plantbiology.msu.edu/) and GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The positions of known motifs of the RecA/RAD51 proteins were determined by Motif Scanning (http://hits.isb-sib.ch). The Molecular Evolutionary Genetic Analysis program (version 3.1, Nei and Kumar, 2000) was used to generate phylogenetic trees by using the Neighbor-Joining method (bootstrap, 10 000 replicates).

**Phenotypic analyses**

To estimate the viability of pollen and anther of RAD51C-KO plants, mature flowers were dissected and stained using 1–K1 solution on a glass slide (Alexander, 1969). The stained pollen grains were observed using a light microscope under bright-field conditions. The anther and the developmental progression of embryo sacs were observed by WECLSM (whole-mount eosin B-staining confocal laser scanning microscope) under a Leica SP2 laser scanning confocal microscope (Zeng et al., 2007). Young panicles at the meiosis stage were fixed with Carnoy’s fixative to observe the meiotic chromosome behaviour (Chang et al., 2009).

**Gene expression analysis**

Total RNA from rice leaves and flowers was used for gene expression analysis. The RAD51C-specific primers rad51c-rt-f and rad51c-rt-r were used to detect the expression of RAD51C (Supplementary Table S1 at JXB online). Primers rad51c-2f and rad51c-2r were used to detect the different transcripts of RAD51C in different tissues (Supplementary Table S1). The expression level of the rice actin gene was used as reference for the mRNA level using gene-specific primers actin-F and actin-R (Supplementary Table S1). The assays were repeated at least twice. When similar results compared with the control were obtained in repeated experiments, only the result in one repetition is presented.

**Genetic complementation of the RAD51C-KO mutant**

An 8.9 kb fragment containing the RAD51C gene and its native promoter was obtained by digesting the Nipponbare artificial chromosome clone OSINBa0004A24 (kindly provided by Professor Rod A. Wing of the University of Arizona) using restriction enzymes PsiI and ScaI (Supplementary Fig. S1 at JXB online). The DNA fragment was cloned into a pCAMBIA2301 vector. The seeds of heterozygous 4D-50016 T-DNA insertion plants were used to induce the calli. After genotype determination, the homozygous 4D-50016 calli were selected for subculture. The pCAMBIA2301-RAD51C construct and the empty vector pCAMBIA2301 were separately transformed into the selected calli by Agrobacterium-mediated transformation as previously described (Lin and Zhang, 2005). Positive plants were confirmed by PCR amplification using T-DNA and the rice primer pair RB1 and 39630-m-R, and vector and rice primer pair 2301-f and rad51-com-r (Supplementary Table S1).

**Statistical analysis**

The significant differences between control and RAD51C-KO plants were analysed by the pair-wise \(t\)-test installed in the Microsoft Office Excel program.

**Results**

RAD51C is a single copy gene in the rice genome

Arabidopsis RAD51 family sequences were used to search different databases. This search identified 13 rice RecA/RAD51 family members (Supplementary Table S2 at JXB online), which included RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, DMClα, DMClβ, RecA1, RecA2, RecA3, and RecA4 named by Lin et al. (2006), and RAD51A1 and RAD51A2 named by Rajanikanth et al. (2008). At least six members of this family, RAD51A2, RAD51C, RAD51D, DMClβ, XRCC2, and RecA2, have 2–3 alternatively spliced transcripts based on the database searches (Supplementary Table S2). According to motif and domain prediction of the deduced protein sequences, all the members, except XRCC2, encode a RecA/RAD51 domain (Supplemental Fig. S2). The RecA/RAD51 domain has two conserved consensus motifs, Walker A (also known as the Walker loop or P-loop) and Walker B, which are crucial for nucleotide binding and ATP hydrolysis (Walker et al., 1982; Hanson and Whiteheart, 2005). The consensus sequences are GXXXXGKT/S (‘X’ indicating any amino acid) for Walker A and hhhhDE (‘h’ indicating a hydrophobic amino acid) for Walker B (Walker et al., 1982; Hanson and Whiteheart, 2005). All genes of this family except XRCC2 (which putatively encodes a protein harbouring only Walker A) encode both Walker A and Walker B motifs.

Rice RAD51C (LOC_Os01g39630) was chosen for further functional characterization. Comparative sequence analysis showed that RAD51C is a single copy gene. Phylogenetic analysis revealed that the protein encoded by RAD51C is more closely related to Arabidopsis (AtRAD51C) and human (HsRAD51C) RAD51C than to other rice Reca/RAD51 family proteins (Fig. 1). Rice RAD51C (accession no. BAG87648) has 61.8% and 39.1% sequence identity and 80.8% and 58.9% sequence similarity to AtRAD51C and HsRAD51C, respectively, and has only 5.9–32.0% identity and 10.6–48.9% similarity to other rice Reca/RAD51 family proteins.

![Fig. 1. Phylogenetic relationship of rice RecA/RAD51 family proteins and Arabidopsis (AtRAD51C; accession no. ACA14294) and human (HsRAD51C; AAC39604) RAD51C proteins.](https://academic.oup.com/jxb/article-abstract/63/14/5323/539940)
Knockout of RAD51C resulted in sterile rice

To study the function of rice RAD51C, a rice T-DNA insertion mutant line 4D-50016 was obtained; this line had a T-DNA inserted into the third exon of RAD51C (POSTECH; http://signal.salk.edu/cgi-bin/RiceGE), with the genetic background of japonica rice variety Dongjin (Supplementary Fig. S1A, B at JXB online). No RAD51C expression was detected in the homozygous 4D-50016 plants (Supplementary Fig. S1C); thus they are referred to as RAD51C-knockout (KO) plants in the following text. Two sequences of alternatively spliced transcripts of rice RAD51C, RAD51C-1 and RAD51C-2, from japonica rice variety Nipponbare were retrieved from GenBank (Supplementary Table S2). RAD51C-1 (GenBank accession no. AK060971) and RAD51C-2 (AK068701) putatively encode proteins consisting of 349 and 309 amino acids, respectively. Comparative analysis of the genomic and cDNA sequences of RAD51C (GenBank accession no. JN394076) in Dongjin identified two alternatively spliced transcripts in both leaf and panicle tissues. The first transcript was RAD51C-1, which had a sequence identical to the cDNA AK060971 from Nipponbare in the coding region (Supplementary Fig. S1A). The second transcript, named RAD51C-3, putatively encoded a protein consisting of 124 amino acids (Supplementary Figs S1A, S3). RAD51C-2 was not detected in the leaf and panicle tissues of Dongjin or in the indica (O. sativa ssp. indica) rice variety Minghui 63. RAD51C-1 and RAD51C-3 were co-expressed in various tissues of Dongjin, including stem, leaf, sheath, pistil, stamen, and spikelet at different developmental stages (including the stages covering meiosis when spikelets were 3–6 mm in size), and callus, with RAD51C-1 as the major transcript (Supplementary Fig. S3). Minghui 63 also expressed RAD51C-1 and RAD51C-3. The insertion of T-DNA into RAD51C blocked the expression of both RAD51C-1 and RAD51C-3 in the 4D-50016 line (Supplementary Fig. S1).

The RAD51C-KO plants did not show any obviously abnormal morphology during vegetative development (Fig. 2A); however, they failed to produce seeds. The general morphology of spikelets of RAD51C-KO plants did not differ from those of wild-type Dongjin except for the anthers (Fig. 2B, 2C). The anthers of wild-type plants were plumper than those of RAD51C-KO plants (Fig. 2D, 2E). The anthers of RAD51C-KO plants were filled with shrivelled pollen, whereas the anthers of wild-type plants were filled with orbicular pollen (Fig. 2F, 2G). Staining pollen with iodium potassium iodide showed that RAD51C-KO plants produced mostly aborted pollen (Fig. 2I, 2J). In addition, RAD51C-KO plants could not release pollen (Fig. 2H). These results suggest that RAD51C-KO plants appear to be male sterile due to abnormal male gametophyte development.

To ascertain whether RAD51C also influences female organ development, embryo sac formation in RAD51C-KO plants was examined. Compared with the processes of embryo sac development in wild-type Dongjin, RAD51C-KO plants were also able

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Fig. 2. Phenotypes of RAD51C-KO plants. Dongjin is the wild type (WT). Bar=150 μm. (A) Vegetative development stage. (B and C) Spikelets. (D and E) Anthers. (F and G) Photographs of eosin-B-stained anthers under confocal microscopy. (H) Panicles. (I and J) Iodium potassium iodide-stained pollen.
to form a megasporocyte in each embryo sac (Fig. 3). However, abnormalities were observed from the megasporocyte meiosis stage to the mature embryo sac stage in RAD51C-KO plants. The four megaspores formed via two meiotic divisions of the megasporocyte showed aberrant alignment in the tetrad of megaspores, and no functional megaspores were formed. This resulted in degenerated embryo sacs at the mature embryo sac stage of wild-type plants. These results suggest that RAD51C-KO plants may also be female sterile due to abnormal meiosis.

The inference that RAD51C-KO plants are both male and female sterile is supported by the analysis of reciprocal crosses between RAD51C-KO plants and wild-type Dongjin. No hybrid seed was obtained for 508 and 293 spikelets in three independent plants using RAD51C-KO plants as paternal and maternal recipients in crosses with Dongjin, respectively (Table 1). In contrast, reciprocal crosses between wild-type siblings segregated from the RAD51C-KO line and Dongjin showed that 42% and 60% of spikelets were fertile (Table 1). These results suggest that RAD51C-KO plants are completely female and male sterile.

To determine whether the sterile phenotype of RAD51C-KO plants was due to knockout of RAD51C, the T2 generation from three T1 heterozygous T-DNA insertion (4D-50016) plants was analysed. A total of 265 T2 plants were examined for their fertility. Among these plants, 66 plants with homozygous T-DNA insertion in RAD51C were sterile, whereas 141 plants with heterozygous T-DNA insertion in RAD51C and 58 wild-type siblings segregated from the 4D-50016 line were

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**Fig. 3.** Development of the embryo sac in RAD51C-KO and wild-type (Dongjin) plants. Arrows indicate the megasporocyte or megaspore. AN, antipodals; PN, polar nucleus; SY, synerids. Bar=50μm.
Table 1. Reciprocal crosses between RAD51C-KO and wild-type (Dongjin) plants

<table>
<thead>
<tr>
<th>Cross combination</th>
<th>No. of seeds/ no. of spikelets</th>
<th>Seed setting rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dongjin (♀)×wild-type siblings segregated from the RAD51C-KO line (♂)</td>
<td>42/90</td>
<td>42</td>
</tr>
<tr>
<td>Wild-type siblings segregated from the RAD51C-KO line (♀)×Dongjin (♂)</td>
<td>57/94</td>
<td>60</td>
</tr>
<tr>
<td>Dongjin (♀)×RAD51C-KO line (♂)</td>
<td>0/115</td>
<td>0</td>
</tr>
<tr>
<td>0/176</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0/217</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>RAD51C-KO line (♀)×Dongjin (♂)</td>
<td>0/83</td>
<td>0</td>
</tr>
<tr>
<td>0/124</td>
<td>0</td>
<td></td>
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<tr>
<td>0/86</td>
<td>0</td>
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</tr>
</tbody>
</table>

In order to exclude the possibility that any somatic change caused by tissue culture influenced the fertility of RAD51C-KO plants, a heterozygous 4D-50016 plant was crossed with wild-type Dongjin. An F₁ hybrid was backcrossed (BC) with Dongjin twice, selecting for the presence of the insertion, and the progeny were selfed to produce a BC₂F₂ population consisting of 23 plants. All RAD51C-KO plants (2, 3, 6, 13, 14, 16, and 20) were sterile, but the plants with a heterozygous T-DNA insertion (1, 4, 5, 9–12, 15, 17–19, and 21–23) or without the T-DNA insertion (7 and 8) were fertile (Fig. 4A). A BC₂F₂ population consisting of 23 plants was also developed from a crossing heterozygous 4D-50016 plant with japonica rice variety Zhonghua 11. Consistent with the results from the BC₂F₂ population, the sterile phenotype was only associated with RAD51C-KO plants (19, 22, 23, and 26) in the BC₂F₂ population (Fig. 4B). These results suggest that knockout of RAD51C is associated with sterility in rice.

The above inference was further confirmed by genetic complementation analysis of RAD51C-KO plants. RAD51C regulated by its native promoter and empty vector (control) were transformed into RAD51C-KO calli. Sixty independent positive plants transformed with RAD51C, named RAD51C-KO-C, and eight independent positive plants transformed with empty vector, named RAD51C-KO-V, were obtained. The fertility of 48 of the 60 T₀ RAD51C-KO-C plants was restored, but all eight RAD51C-KO-V control plants were still sterile (Supplementary Table S3 at JXB online). Plants in two T₁ families from two fertile T₀ plants (RAD51C-KO-C52 and RAD51C-KO-C55) were further analysed individually for their fertility and the existence of the RAD51C transgene. All the T₁ plants carrying the RAD51C transgene were fertile, whereas the negative siblings segregated from the RAD51C-KO-C plants were sterile (Fig. 5). All these results suggest that RAD51C is essential for rice fertility.

Knockout of RAD51C disrupted meiosis but not mitosis

The fact that RAD51C-KO plants had abnormal megasporeocyte meiosis (Fig. 3) led to examination of the processes of male gametophyte development. The first meiosis of pollen mother cells (PMCs) begins with chromosome condensation and formation of a thin thread-like structure at the leptotene stage (Ma, 2005). The PMCs in RAD51C-KO and wild-type (Dongjin) plants showed no obvious differences at the leptotene stage and subsequent zygotene stage (Fig. 6). However, abnormalities were observed from the pachytene stage in RAD51C-KO plants compared with wild-type plants. The PMCs of RAD51C-KO plants did not form a complete synaptonemal complex at the pachytene stage and exhibited visible abnormal chromosome fragments from the diplontene stage to telophase II, the end of the second meiosis. Because of these chromosome fragments, the numbers of bivalents in the PMCs of RAD51C-KO plants were difficult to distinguish at the diakinesis stage, at which stage 12 bivalents were clearly observed in the PMCs of wild-type plants. In the metaphase I stage, most of the chromosome fragments were aligned on the division plane of PMCs; however, some fragments were dispersed in the cytoplasm in RAD51C-KO plants. The chromosome fragments of PMCs of RAD51C-KO plants again became obvious at anaphase I and telophase I stages. However, despite these abnormalities in the first meiosis, the PMCs of RAD51C-KO plants could enter into the second meiosis to form tetrads. All these results suggested that RAD51C plays an important role in meiosis.

RAD51C was constitutively expressed in different rice tissues (Supplementary Fig. S3 at JXB online). To ascertain whether rice RAD51C also functioned in mitosis, the mitotic processes of anther wall cells in RAD51C-KO plants were examined. The mitotic processes, the pre-prophase, prophase, metaphase, anaphase, and telophase stages, showed no obvious difference from those in the cells of wild-type Dongjin (Fig. 7). In the prophase stage, the chromosomes are normal; no visible chromosome fragments, as seen in meiosis, were observed in the anther wall cells of RAD51C-KO plants. This cytological result is consistent with the observation that RAD51C-KO plants did not have obviously abnormal morphology during vegetative development (Fig. 2A). Thus, RAD51C may not be essential for somatic growth.

RAD51C-KO plants were sensitive to DNA-damaging agents

Previous studies have revealed that RAD51 paralogues from species other than rice are required for various types of DNA repair in somatic cells (Kurumizaka et al., 2001, 2002; Sigurdsson et al., 2001; Brenneman et al., 2002; French et al., 2002; Godthelp et al., 2002). To investigate whether RAD51C influenced the cellular response to DNA damage, the sensitivity to the alkylating agent MMS and the cross-linking agent MMC was analysed in RAD51C-KO plants and wild-type siblings (control) segregated from the RAD51C-KO line. An increased concentration of MMS influenced the growth of both RAD51C-KO and control plants; after treatment with 120 µl l⁻¹ MMS, all RAD51C-KO plants died, whereas some of the control plants survived (Fig. 8A). Furthermore, the RAD51C-KO plants grew significantly more slowly (P < 0.0001) than control plants in culture medium supplemented with 40 µl l⁻¹ or 80 µl l⁻¹ MMS. MMC also affected the growth of both RAD51C-KO and control plants (Fig. 8B). However, the growth of the former was...
significantly slower ($P < 0.0005$) than that of the latter after treatment with 72 mg l$^{-1}$ or 128 mg l$^{-1}$ MMC. The RAD51C-KO-C plants, which carried the transgene RAD51C in the genetic background of the RAD51C-KO line, were also examined for their responses to MMS and MMC. After MMS and MMC treatments, the RAD51C-KO-C plants showed a similar level of suppressed growth to the wild-type siblings segregated from the RAD51C-KO line (Supplementary Fig. S4 at JXB online).

UV irradiation induces various type of DNA damage (McCready and Marcello, 2003). The RAD51C-KO plants and control wild-type siblings were exposed to UV-C at the tillering stage. Several days after treatment, both types of plants developed necrotic lesions on their leaves (Fig. 8C). However, markedly more lesions were observed in RAD51C-KO plants compared with the control plants. All these results show that the somatic cells of RAD51C-KO plants are more sensitive to DNA-damaging agents than those of control plants, suggesting that RAD51C may play a vital role in DNA repair in somatic cells.

Discussion

Phylogenetic analysis has revealed that rice RAD51C is more closely related to Arabidopsis RAD51C than to other members of the Arabidopsis RecA/RAD51 family (Lin et al., 2006). The present results suggest that rice RAD51C appears to be the functional homologue of Arabidopsis RAD51C in both meiosis of reproductive cells and DNA damage repair of somatic cells. In addition, it has the highest sequence similarity to Arabidopsis RAD51C compared with other rice RecA/RAD51 family proteins.

Rice RAD51C is essential for meiosis

The completely sterile phenotype of RAD51C-KO plants indicates that RAD51C is indispensable for reproductive development. Comparative analysis of the development of PMCs in RAD51C-KO and wild-type plants suggests that RAD51C is required for at least the first meiotic processes of male gametocytes. RAD51C deficiency also resulted in abnormal meiospores that were formed by two meiotic divisions, indicating that RAD51C is also required for the meiosis of female gametocytes. These conclusions are supported by reciprocal crosses between RAD51C-KO and wild-type plants using RAD51C-KO plants as either paternal or maternal recipients, which were unable to produce hybrid seed.

Rice RAD51C is the sequence orthologue of Arabidopsis and mammalian RAD51C genes. Arabidopsis RAD51C is
involved in meiosis; its mutant rad51c-1 fails to form synapsis and leads to chromosome fragmentation that results in complete male and female sterility (Bleuyard et al., 2005; Li et al., 2005). Chromosome fragmentation was suppressed in a spo11-1/rad51c-1 double mutant, suggesting that the chromosome fragmentation of the rad51c-1 mutant is related to SPO11-1-generated DSBs (Li et al., 2005). The formation of DSBs initiates meiotic recombination (Grelon et al., 2001). Mouse RAD51C has two distinct functions in meiosis: RAD51-mediated recombination and homologous junction resolution (Kuznetsov et al., 2007). A deficiency of mouse RAD51C leads to early meiotic prophase I arrest in males and precocious separation of sister chromatids at meiotic metaphase II in females. The present results showed that the chromosome behaviour of male gametocytes in RAD51C-KO plants was obviously abnormal from the pachytene stage of the first meiosis. Chromosome fragmentation was observed, which suggests that knockout of RAD51C is leading to broken/unrepaired chromosome. Furthermore, the abnormal pachytene chromosomes suggest that the RAD51C-KO plant may be defective in synapsis and/or in homologue juxtaposition. Since meiotic recombination occurs between the leptotene and zygotene stages (Hamant et al., 2006), these results suggest that rice RAD51C may influence the early meiotic processes prior to the pachytene stage. All these results suggest that rice RAD51C appears to be the functional homologue of Arabidopsis and mammalian RAD51C in meiosis.

RAD51C is also important for somatic DNA repair in rice

In contrast to mammalian RAD51C (Sonoda et al., 1998; Kuznetsov et al., 2007), the present results suggest that rice RAD51C may not be essential for vegetative development under normal growth conditions. Arabidopsis RAD51B, RAD51C, and RAD51D are also not essential for vegetative growth (Li et al., 2005; Osakabe et al., 2005; Durrant et al., 2007). However, like some of its orthologues in other species, rice RAD51C appears to be involved in maintaining DNA stability in somatic cells following exposure to DNA-damaging agents. Human RAD51C plays an important role in DNA repair in somatic cells (Somyajit et al., 2010). Human RAD51C is involved in processing of branch migration and homologous junctions (Liu et al., 2004). Haploinsufficiency of hamster RAD51C causes increased sensitivity to DNA damage (Smeeenk et al., 2010). Arabidopsis RAD51C is also involved in DNA damage repair in somatic cells caused by cross-linking reagents (Abe et al., 2005; Bleuyard et al., 2005). Interestingly, atrad51c seedlings are hypersensitive to γ-irradiation (Abe et al., 2005), which is a direct DSB inducer.
Fig. 6. Cytological analysis of meiotic processes of pollen mother cells in RAD51C-KO and wild-type plants. Bar=25 μm.

Fig. 7. Cytological analysis of mitotic processes of anther wall cells in RAD51C-KO and wild-type plants. Bar=5 μm.
whereas imbibed \textit{atrad51c} seeds are not sensitive to γ-irradiation compared with the wild type (Bleuyard \textit{et al.}, 2005). The different responses of the \textit{Arabidopsis RAD51C} mutant to γ-irradiation at different developmental stages could be due to different DNA repair pathways being involved. Both HR and non-homologous end-joining contribute to DSB repair. HR-mediated repair is associated with DNA replication during synthesis and gap 2 phases of the cell cycle (Richardson \textit{et al.}, 2000). It is suggested that the cells of imbibed seeds are in the gap 1 phase; the role of AtRAD51C in DNA damage repair caused by γ-irradiation may not be notable in the imbibed seeds (Abe \textit{et al.}, 2005; Bleuyard \textit{et al.}, 2005). However, \textit{Drosophila RAD51C} does not appear to be involved in DSB repair in somatic cells, although it is specifically required during meiosis (Abdu \textit{et al.}, 2003). The present results suggest that rice \textit{RAD51C} also appears to be the functional homologue of \textit{Arabidopsis RAD51C} in somatic DNA repair caused by DNA-damaging agent.

The mechanisms of repairing DNA damage caused by the alkylating reagent MMS, the cross-linking reagent MMC, and UV irradiation are different (Chang \textit{et al.}, 2009). MMS methylates DNA on N7-deoxyguanine and N3-deoxyadenine (Vazquez \textit{et al.}, 2008), while MMC causes interstrand DNA cross-linking (Lehoczky \textit{et al.}, 2007). UV irradiation induces direct DNA damage, including strand breakage, thymine dimers, and other photoproducts, or indirect damage through reactive oxygen species (McCready and Marcello, 2003). In yeast, the damage caused by MMS can be repaired by base excision repair, HR, and DNA damage tolerance pathways, together with a functional synthesis-phase checkpoint (Vazquez \textit{et al.}, 2008). In addition, MMS can cause derived DSBs and this DSB repair
is reliant on the HR gene RAD51 (Ma et al., 2011). There are two main pathways to eliminate the damage of an interstrand cross-linking agent: nucleotide excision and HR repair (Lehoczky et al., 2007). Nucleotide excision repair, base excision repair, HR-dependent repair, and other DNA repair pathways can repair the damage caused by UV irradiation (Kimura et al., 2004). Furthermore, a recent study reported that UV irradiation is associated with HR (Yang et al., 2008).

Arabidopsis RAD51C can repair the DSBs caused by MMC and another DNA cross-linking reagent cisplatin (Abe et al., 2005; Bleuyard et al., 2005). Hypersensitivity to cross-linking agents is a constant character of HR in deficient mutants described in plant and vertebrate cell lines (Liu et al., 1998; Takata et al., 2000; Sasaki et al., 2004; Abe et al., 2005). As discussed above, the HR-dependent pathway is also involved in repair of DNA damage caused by other agents. The sensitivity of RAD51C-KO plants to MMS, MMC, and UV-C may be because the plants were defective in HR or in both HR and other DNA repair pathways. Since RAD51C-KO plants may have abnormal homologue juxtaposition and synopsis, which was associated with HR, during meiosis, further study is required to ascertain whether RAD51C contributes to DNA repair in somatic cells by HR.

Supplementary data

Supplementary data are available at JXB online.
Figure S1. The RAD51C gene and RAD51C-knockout mutant 4D-50016.
Figure S2. The schematic diagram of the domain structures of rice RecA/RAD51 family proteins.
Figure S3. Expression of transcripts RAD51C-1 and RAD51C-3 in rice variety Dongjin.
Figure S4. Plant responses to methylmethane sulphonate (MMS) and mitomycin C (MMC).
Table S1. PCR primers used for construction of vectors and gene structure and expression analyses.
Table S2. RecA/RAD51 gene family.
Table S3. Genetic complementation of the RAD51C-KO line.

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