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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, synapsis

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 *DMC1*, may be generated by the duplication of an ancestral gene derived from the ancestor of eukaryote *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; Pawlowski *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 Gynheung An (Jeong *et al.*, 2006). 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 remeasured. 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 nthe 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 I_2–KI 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 *RAD51*C 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 bacterial artificial chromosome clone OSJNBa0004A24 (kindly provided by Professor Rod A. Wing of the University of Arizona) using restriction enzymes *Pst*I and *Sac*I (Supplementary Fig. S1 ay *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*, *DMC1a*, *DMC1b*, *RecA1*, *RecA2*, *RecA3*, and *RecA4* named by Lin *et al.* (2006), and *RAD51A1* and *RAD51A2* named by Rajanikant *et al.* (2008). At least six members of this family, *RAD51A2*, *RAD51C*, *RAD51D*, *DMC1b*, *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.

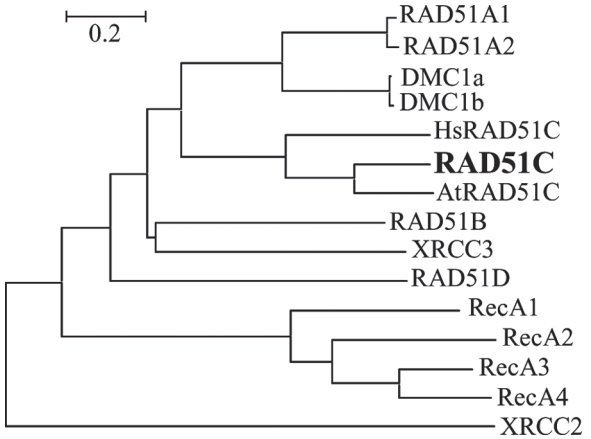

Fig. 1. Phylogenetic relationship of rice RecA/RAD51 family proteins and *Arabidopsis* (AtRAD51C; accession no. ACA14294) and human (HsRAD51C; AAC39604) RAD51C proteins. For rice *RecA/RAD51* family genes encoding multiple proteins, only the sequence of the largest protein is compared with that of other proteins.

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

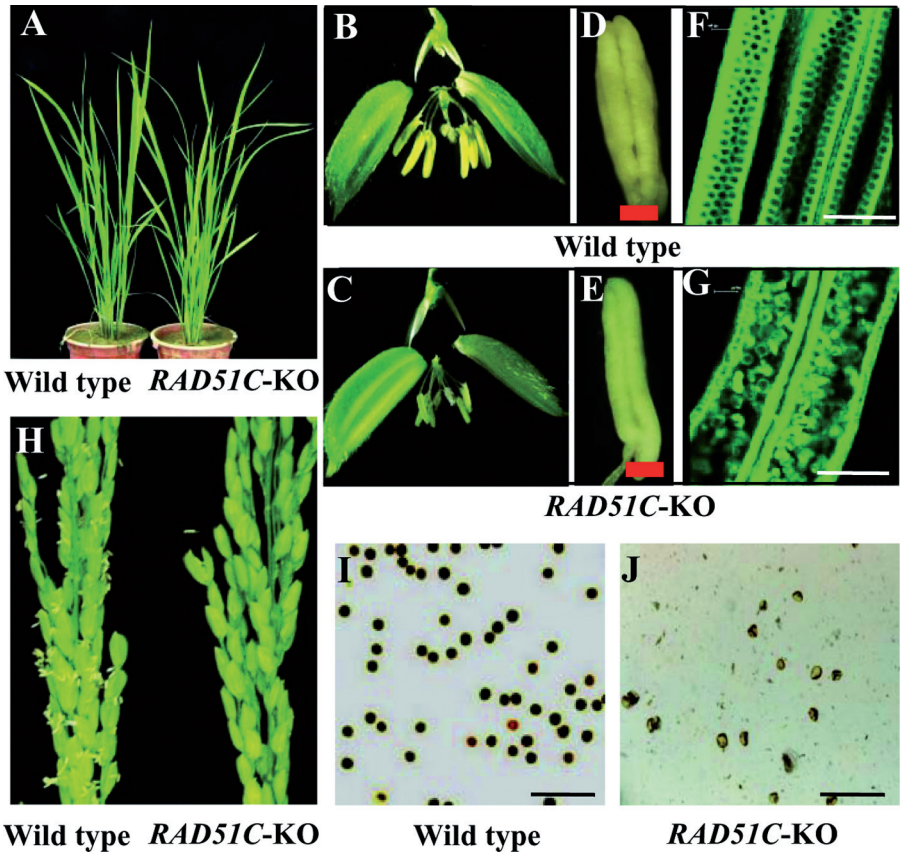

Fig. 2. Phenotypes of *RAD51C*-KO plants. Dongjin is the wild type (WT). Bar=150 μm. (A) Vegetative development stage. (B and C) Spikelets. (D and E) Anthers. (F and G) Photographs of eosin-B-stained anthers under confocal microscopy. (H) Panicles. (I and J) Iodium potassium iodide-stained pollen.

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 T_2 generation from three T_1 heterozygous T-DNA insertion (4D-50016) plants was analysed. A total of 265 T_2 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

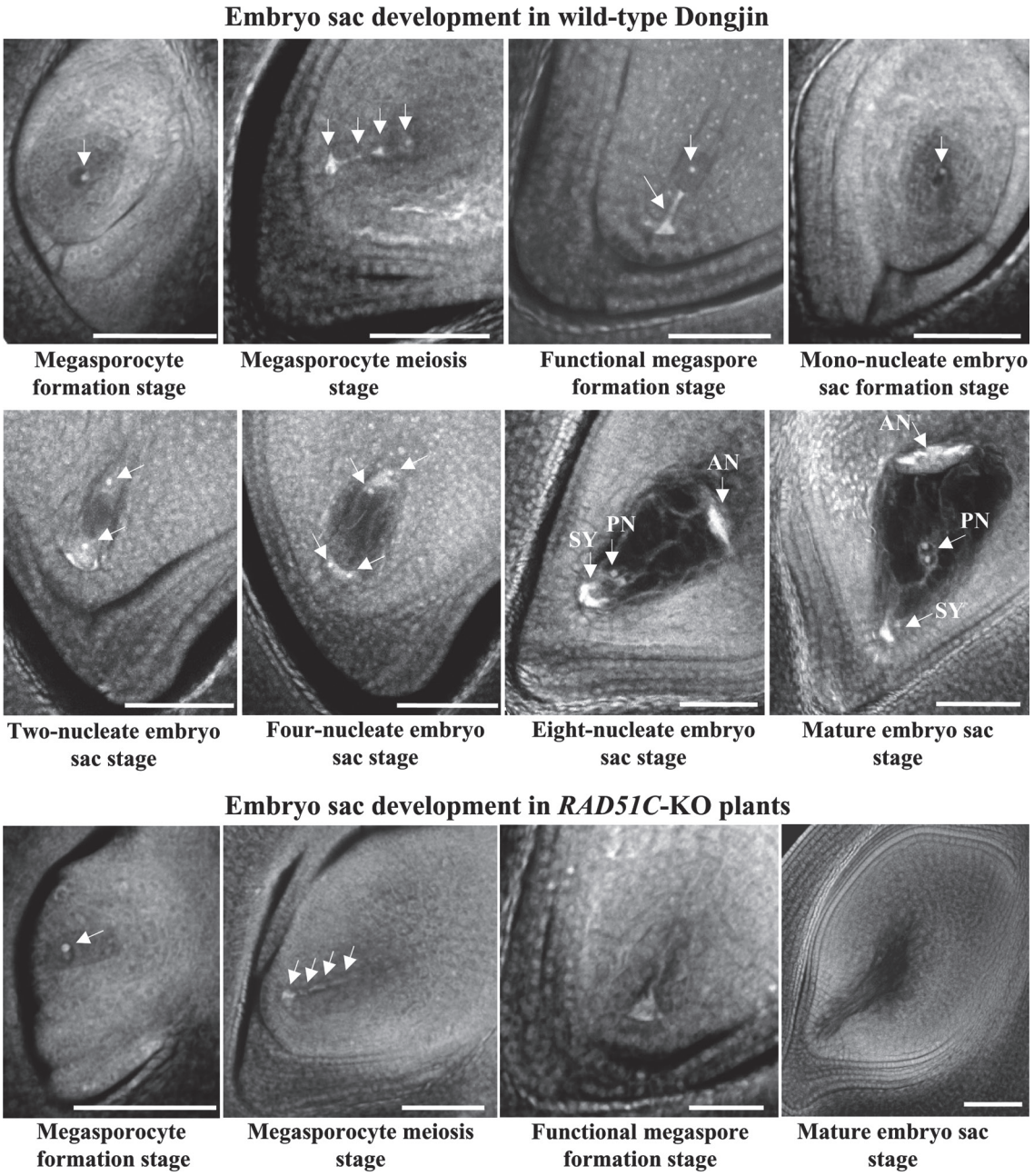

Fig. 3. Development of the embryo sac in *RAD51C*-KO and wild-type (Dongjin) plants. Arrows indicate the megasporocyte or megaspore. AN, antipodals; PN, polar nucleus; SY, synerids. Bar=50 μm.

Table 1. Reciprocal crosses between RAD51C-KO and wild-type (Dongjin) plants

Cross combination	No. of seeds/ no. of spikelets	Seed setting rate (%)
Dongjin (♀)×wild-type siblings segregated from the *RAD51C*-KO line (♂)	42/99	42
Wild-type siblings segregated from the *RAD51C*-KO line (♀)×Dongjin (♂)	57/94	60
Dongjin (♀)×*RAD51C*-KO line (♂)	0/115	0
	0/176	0
	0/217	0
RAD51C-KO line (♀)× Dongjin (♂)	0/83	0
	0/124	0
	0/86	0

fertile. 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 F_1 hybrid was backcrossed (BC) with Dongjin twice, selecting for the presence of the insertion, and the progeny were selfed to produce a BC_2F_2 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 BC_3F_2 population consisting of 23 plants was also developed from crossing a heterozygous 4D-50016 plant with japonica rice variety Zhonghua 11. Consistent with the results from the BC_2F_2 population, the sterile phenotype was only associated with *RAD51C*-KO plants (19, 22, 23, and 26) in the BC_3F_2 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 T_0 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 T_1 families from two fertile T_0 plants (RAD51C-KO-C52 and RAD51C-KO-C55) were further analysed individually for their fertility and the existence of the *RAD51C* transgene. All the T_1 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 megasporocyte 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 diplotene 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 $\mu l\ l^{-1}$ 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 $\mu l\ l^{-1}$ or 80 $\mu l\ l^{-1}$ MMS. MMC also affected the growth of both *RAD51C*-KO and control plants (Fig. 8B). However, the growth of the former was

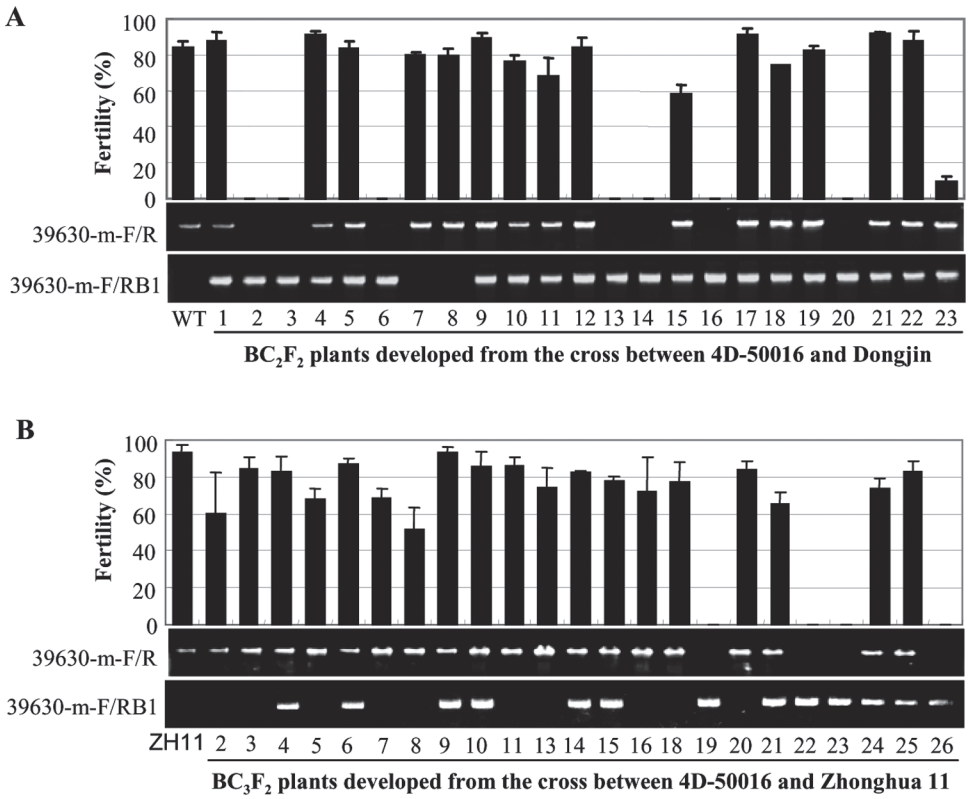

Fig. 4. Genotype and fertility of BC_2F_2 and BC_3F_2 populations segregated for T-DNA inserted in the *RAD51C* gene. 39630-m-F and 39630-m-R are *RAD51C*-specific PCR primers, and RB1 is a T-DNA-specific primer. Plants showing only the band amplified by 39630-m-F and RB1 were *RAD51C*-KO plants with a homozygous T-DNA insertion. Plants showing only the band amplified by 39630-m-F and 39630-m-R were negative plants without a T-DNA insertion. Plants showing both PCR amplification bands were heterozygous plants with heterozygous T-DNA insertion. The bar represents the mean (three panicles) ±SD. (A) Plant fertility of a BC_2F_2 population developed from the cross between the *RAD51C*-T-DNA insertion line (4D-50016) and wild-type (WT) Dongjin. (B) Plant fertility of a BC_3F_2 population developed from the cross between the *RAD51C*-T-DNA insertion line and rice variety Zhonghua 11 (ZH11).

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 megaspores 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

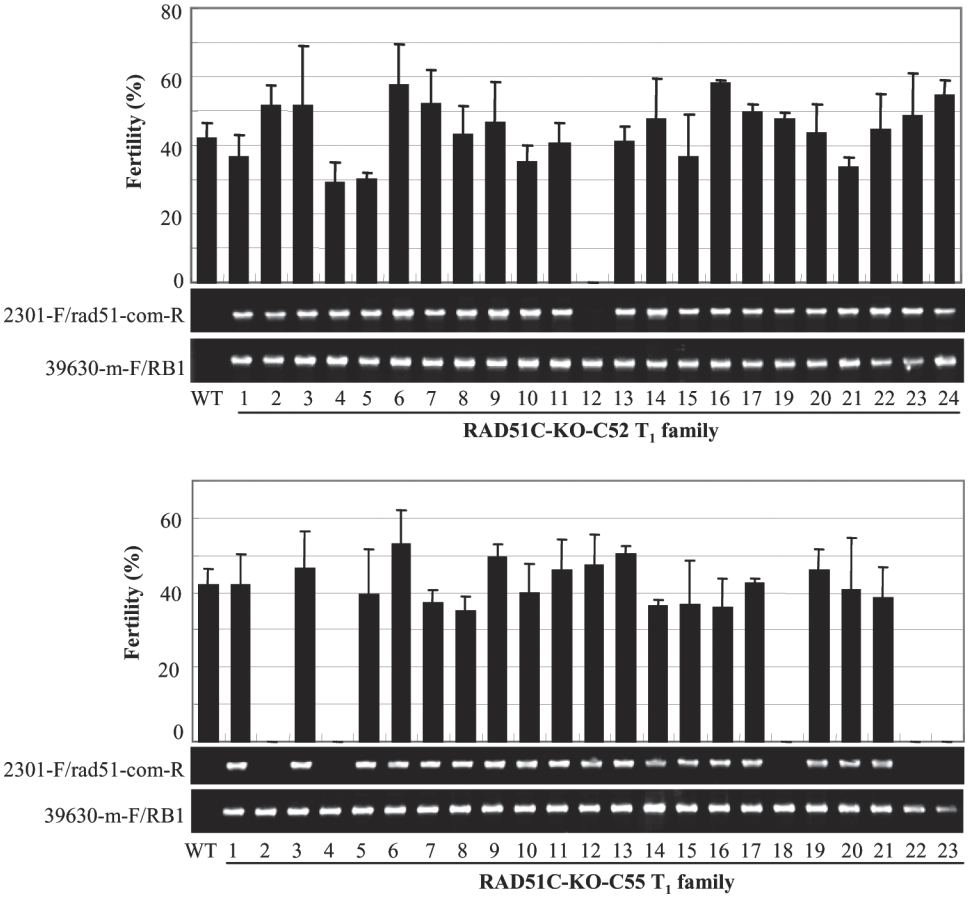

Fig. 5. The fertility of rice plants (RAD51C-KO-C) in two T_1 families was associated with the existence of the *RAD51C* transgene in a *RAD51C*-KO background. The bar represents the mean (three panicles) ±SD. The vector-specific primer 2301-F and *RAD51C* promoter-specific primer rad51-com-R were used to examine the existence of the *RAD51C* transgene. The *RAD51C*-specific primer 39630-m-F and T-DNA-specific primer RB1 were used to examine the existence of a T-DNA insertion in *RAD51C*. The *RAD51C*-KO plant, which was the recipient of the *RAD51C* transgene, had the genetic background of Dongjin (wild type, WT).

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 (Smeenk *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,

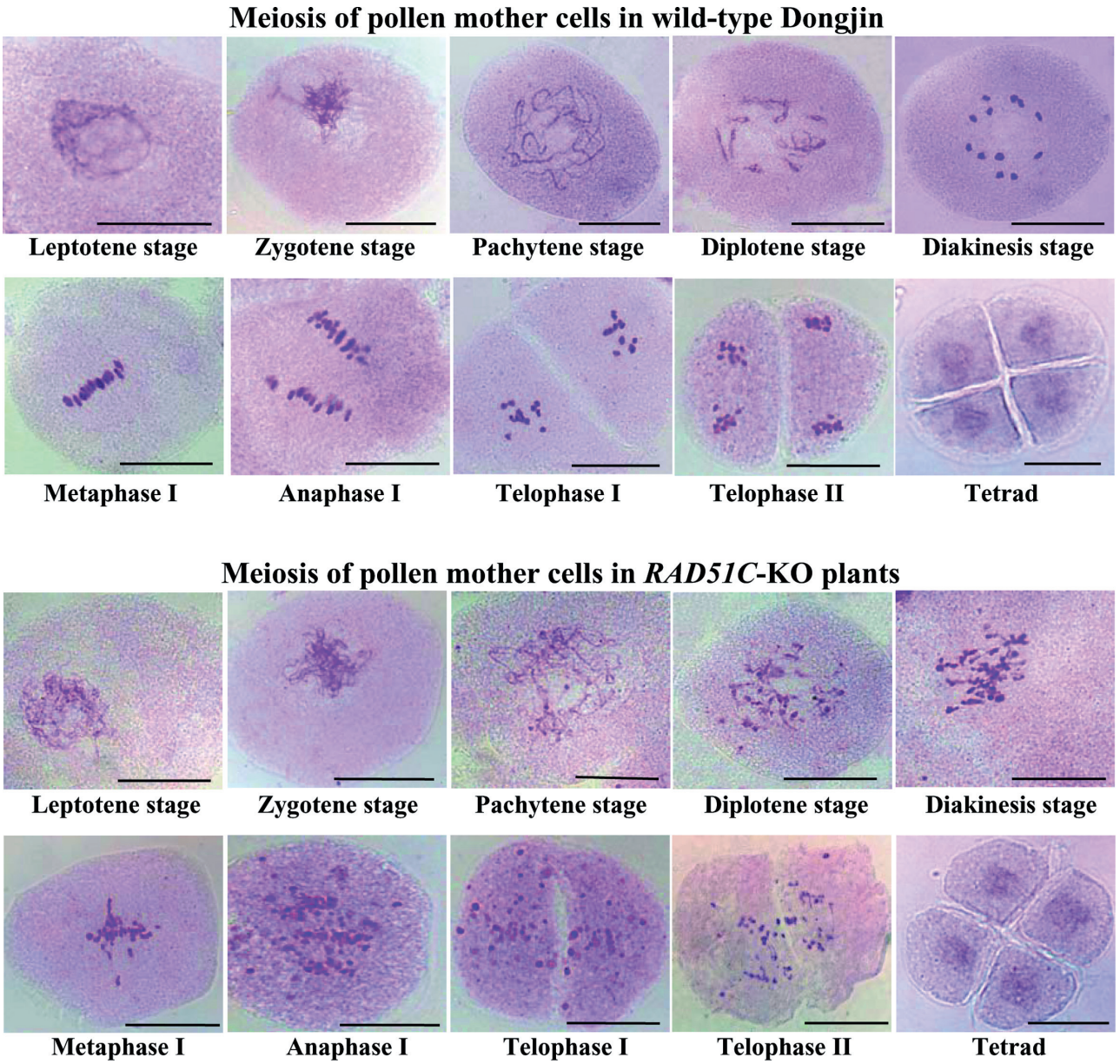

Fig. 6. Cytological analysis of meiotic processes of pollen mother cells in *RAD51C*-KO and wild-type plants. Bar=25 μm.

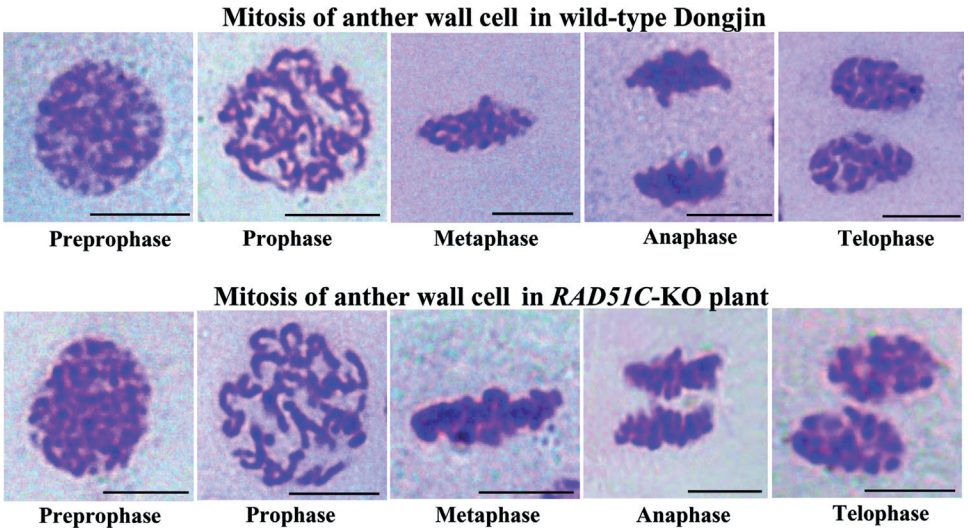

Fig. 7. Cytological analysis of mitotic processes of anther wall cells in *RAD51C*-KO and wild-type plants. Bar=5 μm.

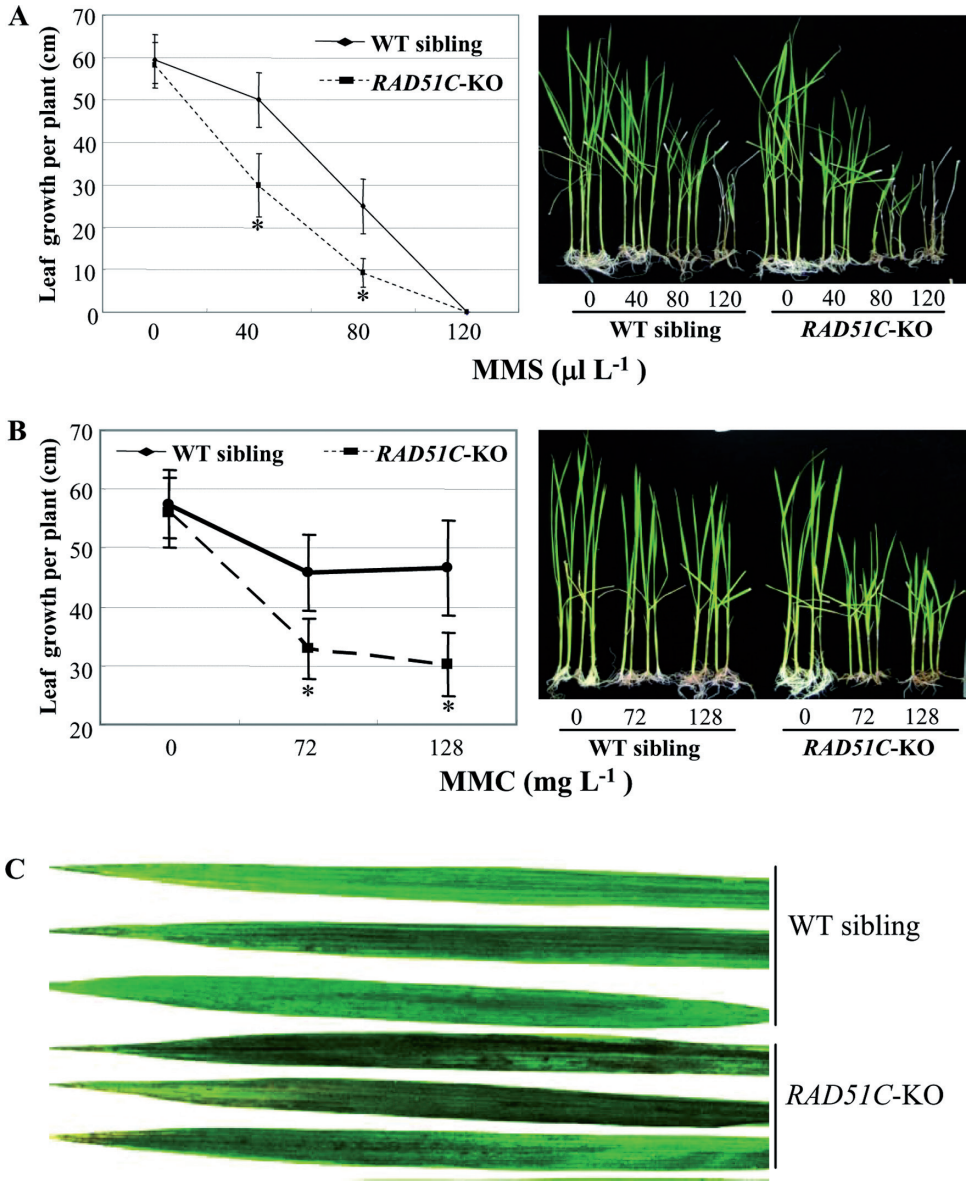

Fig. 8. *RAD51C*-KO plants were hypersensitive to MMS, MMC, and UV-C irradiation. Each data point represents the mean (averaged from eight plants) ±SD. A significant difference between *RAD51C*-KO plants and wild-type (WT) siblings segregated from the *RAD51C*-KO line was detected at $P < 0.0005$ (*). (A) Performance of rice plants after treatment with different concentrations of MMS. (B) Performance of rice plants after treatment with different concentrations of MMC. (C) Rice flag leaves after treatment with UV irradiation.

whereas imbibed *atrad51c* seeds are not sensitive to γ-irradiation compared with the wild type (Bleuyard *et al.*, 2005). The different responses of the *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 *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 *et al.*, 2005; Bleuyard *et al.*, 2005). However, *Drosophila RAD51C* does not appear to be involved in DSB repair in somatic cells, although it is specifically required during meiosis (Abdu *et al.*, 2003). The present results suggest that rice *RAD51C* also appears to be the functional homologue of *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 *et al.*, 2009). MMS methylates DNA on N^7-deoxyguanine and N^3-deoxyadenine (Vazquez *et al.*, 2008), while MMC causes interstrand DNA cross-linking (Lehoczky *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 *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 inter-strand 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 DSB repair (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 consistent 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 synapsis, 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. Rice *RecA/RAD51* gene family.

Table S3. Genetic complementation of the *RAD51C*-KO line.

Acknowledgements

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