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BRCA2 is a mediator of RAD51- and DMC1-facilitated homologous recombination in *Arabidopsis thaliana*

Katharina Seeliger, Stefanie Dukowic-Schulze, Rebecca Wurz-Wildersinn, Michael Pacher and Holger Puchta

Botanical Institute II, Karlsruhe Institute of Technology (KIT), Kaiserstraße 12, D-76131 Karlsruhe, Germany

Author for correspondence:
Holger Puchta
Tel: +49 72160848894
Email: holger.puchta@kit.edu

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Summary

- Mutations in the breast cancer susceptibility gene 2 (*BRCA2*) are correlated with hereditary breast cancer in humans. Studies have revealed that mammalian BRCA2 plays crucial roles in DNA repair. Therefore, we wished to define the role of the BRCA2 homologs in Arabidopsis in detail.
- As Arabidopsis contains two functional BRCA2 homologs, an At*brca2* double mutant was generated and analyzed with respect to hypersensitivity to genotoxic agents and recombination frequencies. Cytological studies addressing male and female meiosis were also conducted, and immunolocalization was performed in male meiotic prophase I.
- The At*brca2* double mutant showed hypersensitivity to the cross-linking agent mitomycin C and displayed a dramatic reduction in somatic homologous recombination frequency, especially after double-strand break induction. The loss of AtBRCA2 also led to severe defects in male meiosis and development of the female gametophyte and impeded proper localization of the synaptonemal complex protein AtZYP1 and the recombinases AtRAD51 and AtDMC1.
- The results demonstrate that AtBRCA2 is important for both somatic and meiotic homologous recombination. We further show that AtBRCA2 is required for proper meiotic synapsis and mediates the recruitment of AtRAD51 and AtDMC1. Our results suggest that BRCA2 controls single-strand invasion steps during homologous recombination in plants.

Introduction

In humans, mutations in the high-penetrance breast cancer susceptibility genes *BRCA1* and *BRCA2* are associated with up to an 80% probability of developing breast cancer (O'Donovan & Livingston, 2010). The cancer predisposition of carriers of Hs*BRCA1* and Hs*BRCA2* mutations is the result of defects in recombinational repair pathways. In addition to DNA repair, HsBRCA1 and HsBRCA2 are involved in other cellular processes, including cell cycle regulation and transcriptional control (for a review, see Yoshida & Miki, 2004; Boulton, 2006).

Surprisingly, BRCA1/2 homologs have been identified not only in animals but also in plants. In addition to a BRCA1 homolog (Lafarge & Montane, 2003; Reidt *et al.*, 2006; Block-Schmidt *et al.*, 2011), the *Arabidopsis* genome also contains two copies of the *BRCA2* gene, which share 96.8% sequence identity, except for the presence of a 450 bp insertion in an intron of At*BRCA2A* (Siaud *et al.*, 2004). The proteins encoded by the two At*BRCA2* genes are 1151 and 1155 amino acids long and are 94.5% identical to each other and 21% identical to the human protein. BRCA2 homologs have also been identified in several other plant species (for a review, see Trapp *et al.*, 2011).

Human BRCA2 interacts with the strand exchange protein RAD51, a central player in homologous recombination (HR) (Sharan *et al.*, 1997; Wong *et al.*, 1997; Chen *et al.*, 1998a,b; Katagiri *et al.*, 1998; Marmorstein *et al.*, 1998). The interaction between BRCA2 and RAD51 is mediated by BRCA2-specific BRC domains, which share high sequence homology across different species (Bork *et al.*, 1996; Bignell *et al.*, 1997; Wong *et al.*, 1997; Chen *et al.*, 1998b, 1999; Warren *et al.*, 2002; Galkin *et al.*, 2005; Thorslund & West, 2007). Although the sequence of the BRC repeat is well conserved, the number of BRC repeats is quite variable, ranging from 15 in *Trypanosoma brucei* to eight in humans and one in *Ustilago maydis* and *Caenorhabditis elegans* (Kojic *et al.*, 2002; Martin *et al.*, 2005; Hartley & McCulloch, 2008); there are four BRC repeats in the At-BRCA2 proteins. Interestingly, only the BRC2 motif (the BRC motifs are numbered in order of appearance in the Arabidopsis proteins) exhibits amino acid differences between the two Arabidopsis homologs (Siaud *et al.*, 2004).

As shown by Yang *et al.* (2002, 2005), human BRCA2 can bind to single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) and is thought to have a special affinity for ssDNA–dsDNA junctions, structures that are often caused by DNA damage. Recently, a number of groups have reported the successful expression and purification of full-length HsBRCA2 (Jensen *et al.*, 2010; Liu *et al.*, 2010; Thorslund *et al.*, 2010). *In vitro* experiments showed that HsBRCA2 mediates HsRAD51 filament formation and strand exchange after DNA damage by promoting the assembly of HsRAD51 on to HsRPA-coated

ssDNA. HsBRCA2 regulates RAD51-filament formation and DNA binding of RAD51 *in vitro*, which leads to an initiation of the homology search and strand exchange, and this regulation suggests that BRCA2 is also a mediator of RAD51-facilitated DNA repair *in vivo* (for a review, see O'Donovan & Livingston, 2010; Holloman, 2011; Maher *et al.*, 2011). The nuclear RAD51 foci that normally accumulate in response to DNA damage did not form in *brca2* cells (Sharan *et al.*, 1997; Yuan *et al.*, 1999), and RAD51 and BRCA2 colocalized in nuclear foci in somatic cells treated with ionizing radiation (Tarsounas *et al.*, 2004). It has also been shown that BRCA2 in mammals is highly expressed in S-phase and is recruited to stalled replication forks, suggesting that BRCA2 is important for recombinational repair associated with normal replication (Vaughn *et al.*, 1996; Scully, 2000; Scully & Livingston, 2000). Defects in HR resulting from the lack of BRCA2 have been documented for an array of different organisms (Moynahan *et al.*, 2001; Xia *et al.*, 2001; Cipak *et al.*, 2006; Lee & Baker, 2007).

Investigations into the role of BRCA2 in meiotic recombination in humans and other mammalian systems have been limited by the early embryonic lethality caused by the loss of functional BRCA2 (Suzuki *et al.*, 1997; Patel *et al.*, 1998; Yu *et al.*, 2000; Moynahan *et al.*, 2001; Tutt *et al.*, 2001). Mice or rats expressing truncated versions of BRCA2 or human BRCA2 were viable but sterile (Connor *et al.*, 1997; Sharan *et al.*, 2004; Cotroneo *et al.*, 2007). Recombination did not reach completion and synapsis failed in the BRCA2-deficient spermatocytes, in which meiosis did not progress beyond early prophase I (Connor *et al.*, 1997; Cotroneo *et al.*, 2007). These cells were also defective for the formation of foci of RAD51 and DMC1 (Sharan *et al.*, 2004). Homologs of BRCA2 in *U. maydis*, *C. elegans* and *Drosophila melanogaster* have been reported to be essential for meiosis as well (Kojic *et al.*, 2002; Martin *et al.*, 2005; Klovstad *et al.*, 2008; Ko *et al.*, 2008).

Several studies addressing the functions of the Arabidopsis BRCA2 proteins have been published (Siaud *et al.*, 2004; Dray *et al.*, 2006; Abe *et al.*, 2009; Wang *et al.*, 2010); however, a complete picture of the role of BRCA2 in DNA recombination in Arabidopsis has yet to be established. Partially contradicting results have been obtained with respect to mutant analysis, most probably because of differences in the cultivars and types of mutants used (Abe *et al.*, 2009; Wang *et al.*, 2010). With regard to somatic HR frequencies, previous studies analyzed only the single mutants (Wang *et al.*, 2010). It was shown that the AtBRCA2 proteins interact with AtRAD51 and its meiosis-specific paralog AtDMC1 (Siaud *et al.*, 2004; Dray *et al.*, 2006; Wang *et al.*, 2010). The interaction of AtBRCA2 with AtDMC1 suggests the involvement of AtBRCA2 in meiosis. Indeed, silencing At*BRCA2* by RNA interference disturbed meiotic division (Siaud *et al.*, 2004). Nevertheless, the At*BRCA2* knockdown in this line did not cause complete sterility, which indicates that AtBRCA2 might not be essential for meiosis. In another study, using lines of the Nossen background, it was reported that the AtBRCA2 double mutant was fertile (Abe *et al.*, 2009). In this study, we used At*brca2a/b* insertion mutants in the Columbia background. We show that AtBRCA2 plays an important role in DNA repair, especially in somatic HR. From our analysis of the double mutant, we were able to determine that AtBRCA2 is essential for male meiosis and that the absence of AtBRCA2 causes severe impairment of the development of the female gametophyte. We were also able to demonstrate that AtBRCA2 is crucial for the formation of AtRAD51 and AtDMC1 foci during male meiotic prophase I.

Materials and Methods

Plant material and growth conditions

For propagation, crossings and flower production for meiotic analysis, wild-type and mutant lines of *Arabidopsis thaliana* (L.) Heynh. (Columbia ecotype) were cultivated on soil and grown under a light : dark cycle of 16 : 8 h at 24°C. For sterile growth conditions, seeds were surface-sterilized and sown on agar plates with germination medium (GM: 4,9 g l^{-1} Murashige and Skoog medium plus vitamins and MES (2-(N-morpholino)ethanesulfonic acid, 10 g l^{-1} sucrose and 0.8% agar, adjusted to pH 5.7 with KOH) under otherwise similar growth conditions. The surface sterilization of seeds was performed by incubation in 6% sodium hypochlorite for 7 min followed by three washes with sterile water.

Seeds of T-DNA insertion mutants were obtained from the GABI (At*brca2a*; GABI_290C04, At4g00020) and SALK collections (At*brca2b*; SALK_037617, At5g01630) (Alonso *et al.*, 2003; Rosso *et al.*, 2003). Plants homozygous for T-DNA insertion were genotyped by PCR using primer pairs to identify the wild-type allele (*BRCA2A:* 5′- TGTATTGTCACTCTATTAGATAGACAGTGAGTA-3′/5′-TCGGTCCGCCCAGTGAGC-3′; *BRCA2B:* 5′-GCTCTGAATATCAGTAAACCTGC-3′/5′-AGGAAACCTCAAGTGGTGAT-3′) or the T-DNA insertion (*BRCA2A:* 5′-GTGATTGTCACTCTATTAGATAGACAGTGAGTA-3′/5′-TTGGACGTGAATGTAGACAC-3′; *BRCA2B:* 5′-GATTTAACCATGTGAACCAGTC-3′/5′-TCGGAACCACCATCAAACAG-3′). Both mutants were crossed to generate At*brca2* double mutants. Other mutants used were *rad51-1* (GK-134A01), kindly donated by Bernd Reiss (MPI, Köln, Germany), and *dmc1* (SAIL_170_F08), which was obtained from the Syngenta Arabidopsis Insertion Library (SAIL) collection via NASC (European Arabidopsis Stock Centre).

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from 2- to 3-wk-old plantlets using the RNeasy mini kit (Qiagen GmBH, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was conducted using the Revert Aid first-strand cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. Expression analysis was performed by semiquantitative PCR (β-tubulin: 5′-CCTGATAACTTCGTCTTTGG-3′/5′-GTGAACTCCATCTCGTCCAT-3′; a: 5′-CTTCAGTCCGTCTCGGATTC-3′; b: 5′-TGGAATTATTCACTGCCATATC-3′; c: 5′-GATATGGCAGTGAATAATTCCA-3′;

d: 5′-TCTGTATGTCCCATTAATATTC-3′; e: 5′-GTATTATGTGTGAGTACCAGA-3′; f: 5′-GGTGAGAAGAGTAGGCAGCTTCT-3′).

Sensitivity tests

Sensitivity tests were performed according to Bleuyard & White (2004) and modified as follows. Seeds of wild-type and mutant lines were surface-sterilized and sown on GM and GM containing 5 μg ml^{-1} mitomycin C (MMC). After 3 wk of growth, the numbers of real leaves were counted.

HR assay

To determine the rate of HR in At*brca2* mutant plants, *A. thaliana* single mutant plants (Columbia ecotype) were first crossed with wild-type plants carrying a β-glucuronidase (*uidA*) recombination substrate (termed GUS651 line, C24 ecotype) (Puchta *et al.*, 1995). Plants homozygous for both GUS651 and At*brca2a* or At*brca2b* were selected and crossed to obtain lines homozygous for GUS651 in an At*brca2a*$^{+/-}$ At*brca2b*$^{-/-}$ or segregated wild-type background. To determine HR frequencies in the double mutant, the seeds of At*brca2a*$^{+/-}$ At*brca2b* $^{-/-}$ and wild-type lines containing the recombination reporter were sterilized and sown on GM. After 1 wk, plants were transferred to solid GM with and without 5 μg ml^{-1} bleomycin. Three-wk-old seedlings, whose roots had been cut off for genotyping, were stained individually in 24-well plates. After 2 d at 37°C, the staining solution (46.5 ml of 100 mM Na_2HPO_4, pH 7; 2.5 ml of 1% X-GlcA (100 mg 5-bromo-4-chloro-3-indolyl-β-D-glucuronid dissolved in 10 ml of DMF (dimethylformamide)) was replaced with 70% ethanol to extract the chlorophyll, which facilitates the quantification of the blue sectors under a binocular microscope. With this approach, the double mutants could be identified, and HR frequencies could be determined for each plantlet. To calculate mean recombination frequencies, the HR assays were repeated three independent times with *c.* 160 plants for the segregating At*brca2a* $^{+/-}$ At*brca2b* $^{-/-}$ population and *c.* 40 plants for the control.

Analysis of embryo sac development and mature pollen grains

The preparation of embryo sacs and mature pollen grains is based on the description by Siddiqi *et al.* (2000). Inflorescences containing buds at different developmental stages were collected and fixed with FAA (3.7% formalin, 5% acetic acid, 50% ethanol) overnight at 4°C. The inflorescences were rinsed in acetone, starting with a 50% acetone solution, which was incrementally increased to 100% acetone in 10% steps. Flower buds were bleached in methyl benzoate for 2 h and then washed two times in low-viscosity paraffin oil (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). For the analysis of embryo sac development, ovules of appropriate stages were dissected on a slide, and the air-dried preparation was covered with a drop of low-viscosity paraffin oil. To analyze mature pollen grains, anthers of appropriate stages were isolated directly in a drop of low-viscosity paraffin oil on a slide. The analysis was performed using a microscope with differential interference contrast (DIC) optics (Zeiss Axio Imager.M1). Photographs were taken using AxioCam MRm, and images were processed with Zeiss AxioVision LE and Corel Draw X4.

Chromatin staining of pollen mother cells

Chromatin staining of pollen mother cells was performed according to Armstrong *et al.* (2009). Briefly, inflorescences were fixed in ethanol : acetic acid (3 : 1), and fixed flower buds of appropriate stages were isolated in fixative, washed in 0.01 M citrate buffer (pH 4.5) and digested in 0.3% cellulase (Sigma C1794) and 0.3% pectolyase (Sigma P5936) in a 0.01 M citrate buffer (pH 4.5) for 75 min at 37°C. Around four flower buds were squashed in water on a slide using a mounted needle. Approx. 7 μl of 60% acetic acid was added, and the suspension was stirred with a mounted needle. The slides were incubated for 30 s on a heat block set to 45°C. Fixation was accomplished by adding a ring of fixative around the suspension, and the preparation was dried with a hairdryer. VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, California, USA) was used for the staining of chromatin. Meiotic stages were defined using a fluorescence microscope (Zeiss Axio Imager.M1). Photographs were taken using AxioCam MRm, and images were processed with Zeiss AxioVision LE and Corel Draw X4.

Immunolocalization in pollen mother cells

Immunolocalization in pollen mother cells was performed according to Armstrong *et al.* (2009). Briefly, flower buds of appropriate stages were dissected, and isolated anthers were tapped out in 0.4% cytohelicase (Sigma C8274), 1.5% sucrose and 1% polylvinylpyrrolidone (Sigma MW 40000) directly onto a slide. For digestion, the slides were incubated at 37°C for 2 min in a humidified chamber. Chromosomes were spread by adding 0.1% Lipsol and stirring with a mounted needle for 1 min. Chromosomes were fixed with 4% formaldehyde. Air-dried preparations were washed twice in PBS containing 0.1% Triton X-100 and blocked with 1% BSA in PBS for 45 min at room temperature. Antibody staining was performed either overnight at 4°C or for 30 min at 37°C. Antibodies were diluted in 1% BSA in PBS containing 0.1% Triton X-100. Preparations were washed twice between incubations with different antibodies. The primary antibodies used in this study were anti-ASY1 (Armstrong *et al.*, 2002), anti-ZYP1 (Higgins *et al.*, 2005), anti-RAD51 (Mercier *et al.*, 2003) and anti-DMC1 (Sanchez-Moran *et al.*, 2007). All antibodies were a kind gift of F. Chris H. Franklin (University of Birmingham, UK). For simultaneous staining of proteins with primary antibodies from the same species, staining was performed consecutively. The first primary antibody applied to the samples was subsequently covered with labeled Fab fragments. Afterward, an additional fixation with 4% formaldehyde was performed for 12 min at room temperature. Then, incubations with the second primary antibody and appropriate secondary antibodies were

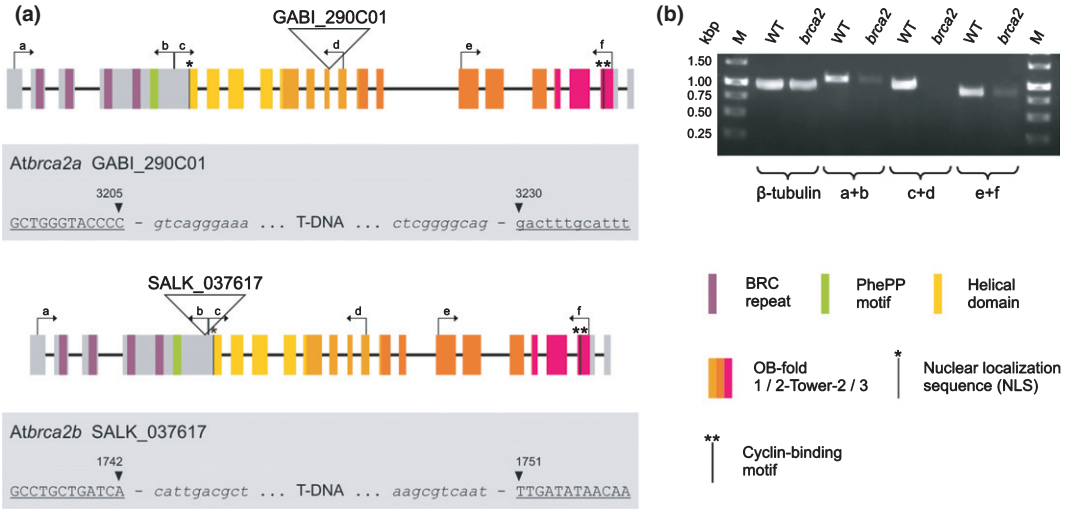

Fig. 1 Gene structure and T-DNA insertion mutant analysis of At*BRCA2A* and At*BRCA2B*. (a) Schematic drawing of the gene structure of At*BRCA2A* (At4g00020) and At*BRCA2B* (At5g01630) with the protein domains color-coded according to the key on the right (Siaud *et al.*, 2004; for a review see Trapp *et al.*, 2011). The T-DNA insertion sites for the mutants and detailed sequence information are indicated above and below the drawings. Primers used for expression analysis are marked with arrows. (b) Semiquantitative expression analysis of the At*brca2* double mutant shows that no full-length transcript of a *BRCA2* gene can be generated in the double mutant (kbp, kilobase pairs; M, marker; WT, wild-type; *brca2*, At*brca2* double mutant).

performed. Finally, VECTASHIELD mounting medium with DAPI was used for the staining of chromosomes. The analysis of the samples was performed as described earlier.

Results

Characterization of single insertion mutants

To identify At*BRCA2A/B* mutants (Siaud *et al.*, 2004), a database search of T-DNA insertion mutants on the SIGnAL webpage (Salk Institute Genomic Analysis Laboratory, La Jolla, California, USA) was conducted. Putative T-DNA mutants were identified, and one insertion line for each gene was selected for subsequent investigation. Exact insertion sites were determined for both mutants by sequencing PCR products containing the T-DNA border sequence–genomic sequence junction. The domain structure of AtBRCA2A/B and insertion sites for the mutants are shown in Fig. 1. The T-DNA insertion in At*BRCA2A* (GABI_290C01) is located within exon 10 in the middle of the gene, inside OB (oligonucleotide/oligosaccharide binding) fold 1. The T-DNA is inserted between nucleotides 4401 and 4426 and deletes 24 nucleotides: 16 from the 3′-end of exon 10 and eight from the downstream intron. The insertion in At*BRCA2B* (SALK_037617) is located in exon 4 near the nuclear localization sequence (NLS). This T-DNA insertion is located between nucleotides 3214 and 3223 and deletes eight nucleotides from exon 4. The mutant plants did not show any visible growth defects under standard conditions in comparison to wild-type plants.

The At*brca2* double mutant is sterile

An At*brca2* double mutant was generated by crossing T-DNA insertion mutants of At*BRCA2A* and At*BRCA2B*. Semiquantitative expression analysis showed that no full-length transcript of a BRCA2 gene can be generated in the double mutant (Fig. 1). The double mutant was viable throughout somatic growth whereas fructification resulted in small and empty siliques (Fig. 2). Additionally, anther filaments were clearly shortened compared with wild-type, while siliques from the single mutants were of normal length. Therefore, it appears that the role of the AtBRCA2 proteins in meiosis is redundant, and thus the lack of both proteins is needed to expose their meiotic function. To test

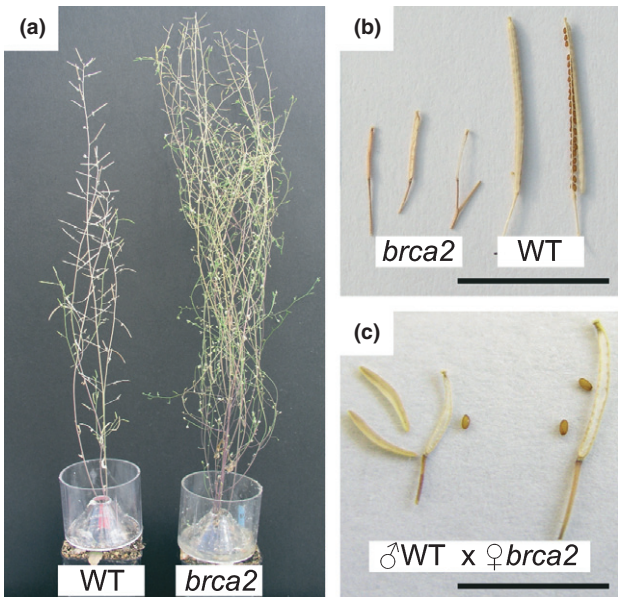

Fig. 2 Sterility of the At*brca2* double mutant. (a) Comparison of wild-type (WT) and At*brca2* double-mutant (*brca2*) plants in the fruit-ripening stage shows that the sterile At*brca2* double mutant was unable to produce normal siliques but has a very bushy habitus with many shoots. (b) Fructification in the At*brca2* double mutant results in small and empty siliques. (c) Back-crossing of At*brca2* double-mutant pistils with wild-type pollen leads to the production of very few seeds. Bars, 1 cm.

whether sterility could be attributed to either the male or female germ line, back-crosses to wild-type were performed using the At*brca2* double mutant as the mother plant and the wild-type as the father plant and vice versa. Interestingly, although wild-type pistils pollinated with mutant pollen were unable to produce seeds, the inverse combination did yield seeds, but only one or two per plant. Germinating these seeds did lead to the development of normal and healthy-looking plants (data not shown).

The At*brca2* double mutant is sensitive to MMC

Mutant plants with defects in DNA repair genes often show higher sensitivity to genotoxic agents than wild-type plants. To test At*brca2* single and double mutants for sensitivity to genotoxins, surface-sterilized seeds were sown on GM medium and medium containing 5 μg ml^{-1} of MMC. Because of the sterility of At*brca2* double mutants, this experiment was performed using seeds from a segregating line with the genotype At*brca2a*$^{+/-}$ At*brca2b*$^{-/-}$. On GM medium without MMC, wild-type plants, as well as the single and double mutants, produced seven to eight true leaves on average (Fig. 3). When treated with 5 μg ml^{-1} MMC, the wild-type and At*brca2* single mutants still had an average of seven to eight leaves, whereas approximately one-quarter of the plantlets derived from seeds of selfed At*brca2a*$^{+/-}$ At*brca2b*$^{-/-}$ plantlets had both decreased leaf numbers (down to three leaves) and decreased leaf size. The genotype of the small plants was determined by PCR. As suspected, genotypic characterization revealed that all of the small plants carried homozygous T-DNA insertions in both alleles of At*BRCA2A* and At*BRCA2B*. The FW of the plants was also determined (results not shown) and confirmed the results obtained visually. Despite being hypersensitive to MMC, the At*brca2* double mutant did not exhibit bleomycin sensitivity (results not shown). Thus, the double mutant seems to be specifically deficient in cross-link repair.

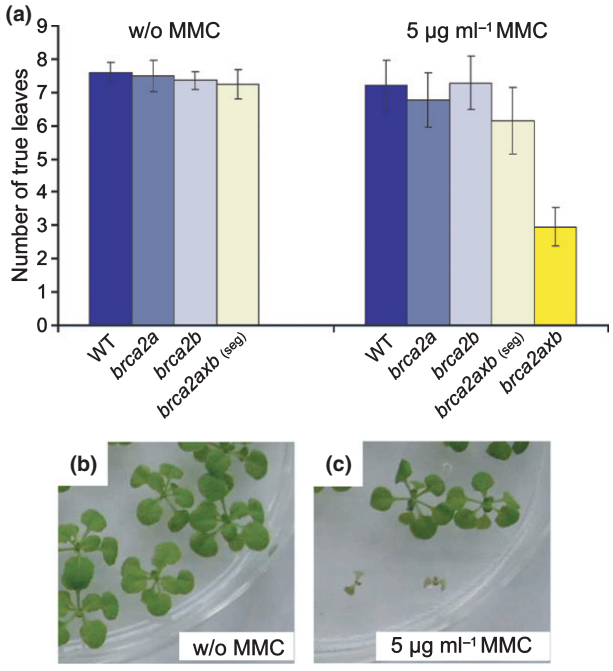

Fig. 3 Mitomycin C (MMC) sensitivity test of At*brca2* single and double mutants. (a) To test MMC sensitivity, plants were grown on agar plates prepared with or without (w/o) 5 μg ml^{-1} MMC. When untreated, the wild-type, single-mutant and segregating double-mutant plants display an average of seven to eight leaves per plantlet. When grown on agar containing 5 μg ml^{-1} MMC, the At*brca2* double-mutant plantlets display a significant reduction in leaf number, whereas the single mutants show no difference in leaf number compared with wild-type (*brca2axb* (seg) = seeds obtained by self-crossing of At*brca2a*$^{+/-}$ At*brca2b*$^{-/-}$). (b, c) Segregating At*brca2* double-mutant plantlets sown on agar without MMC (b) show no growth phenotype, which is in contrast to the growth differences observed when the At*brca2* double mutants were sown on agar containing MMC (c), in which case the double mutants were much smaller in size.

AtBRCA2 has an important role in somatic homologous recombination

We postulated that the sensitivity of At*brca2* double mutants to MMC is the result of defects in the repair of DNA double-strand breaks (DSBs) which arose as a consequence of DNA cross-links and which have to be repaired during DNA replication. A possible explanation for the insensitivity of the At*brca2* double mutant to bleomycin, which causes mainly single and DSBs, is that DSBs in somatic cells are mainly repaired by nonhomologous end joining (NHEJ) (reviewed in Puchta, 2005). Thus, it is possible that the repair of DSBs by NHEJ could mask potential defects in the efficiency of repair by HR. To quantify HR events, we used an *in planta* recombination assay with a reporter gene consisting of two overlapping and inverted β-glucuronidase (GUS) fragments, separated by a hygromycin resistance gene (Swoboda *et al.*, 1994; Puchta *et al.*, 1995; Schuermann *et al.*, 2005). The GUS repeats share an overlapping stretch of 566 bp, which allows intra- or interchromosomal recombination and restoration of a functional β-glucuronidase gene. With this system, HR events can be visualized as blue sectors. We used the GUS651 line (containing the recombination construct) to produce lines of the desired genotypes (see the Materials and Methods section). F3 progeny homozygous for the GUS substrate in segregated control plants or in an At*brca2a*$^{+/-}$ At*brca2b*$^{-/-}$ population were used to determine the frequency of HR. In three independent experiments, the recombination frequency in the At*brca2* double mutant was drastically lower compared with the wild-type control (Fig. 4, Table 1). Whereas genotoxic stress induced by bleomycin resulted in an average *c.* 10-fold increase in HR events in control plants, At*brca2* double mutants exhibited approximately the same number of HR events with and without bleomycin. At*brca2* double mutants showed only *c.* 4% of the wild-type HR events after treatment with bleomycin. Our results clearly demonstrate that AtBRCA2 has an almost essential role in HR in somatic cells.

AtBRCA2 is essential for male meiosis

Using the At*brca2* double mutant, we further characterized the meiotic phenotype. To examine mature pollen grains, flower buds from wild-type and At*brca2* mutant plants were fixed and cleared. Isolated anthers were analyzed using a microscope with DIC optics. Whereas wild-type anthers contained round-shaped

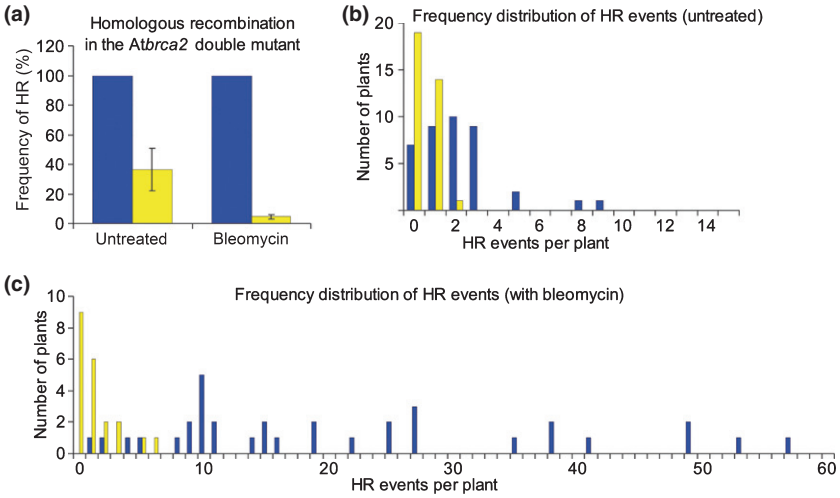

Fig. 4 Recombination frequencies in the At*brca2* double mutant. (a) The GUS651 reporter line was used to determine the frequency of recombination in At*brca2* double-mutant plants (*brca2*; yellow bars) in comparison to wild-type plants (WT; blue bars). The data in this panel represent the mean homologous recombination (HR) frequencies and standard deviations calculated from three independent experiments. When untreated, the double-mutant plants experienced significantly fewer HR events compared with the wild-type. This reduction was even more pronounced after treatment with bleomycin. (b, c) The distribution of HR frequencies of one representative experiment is shown for untreated (b) and bleomycin-treated (c) plants.

Table 1 Frequencies of somatic HR events in At*brca2* double mutants and segregated control plants

Control			At*brca2*			Ratio
n	*N*	m1	*n*	*N*	m2	m2/m1
Without genotoxic stress						
40	94	2.35	15	18	1.20	0.51
40	115	2.88	23	24	1.04	0.36
39	83	2.13	34	16	0.47	0.22
Mean		2.45 ± 0.39			0.90 ± 0.38	0.37
With bleomycin (5 μg ml^{-1})						
30	1130	37.67	16	18	1.13	0.03
38	204	5.37	41	10	0.24	0.04
34	721	21.21	21	27	1.29	0.06
Mean		21.42 ± 16.15			0.89 ± 0.56	0.04

Values represent the number of plants tested (*n*), the total number of blue-stained recombination sectors (*N*) and the mean number of sectors per plant per experiment (m1, control; m2, At*brca2* double mutant). The ratio calculated measures the frequency of recombination events in the mutant relative to the control.

pollen grains, anthers of the At*brca2* double mutant contained only empty and wizened pollen grains (Fig. 5). Details of the development of pollen mother cells (PMCs) were then investigated by fluorescence microscopy. Flower buds of different stages were isolated and treated as described in the Materials and Methods section. While early prophase stages progressed normally in At*brca2* double mutants, the formation of the synaptonemal complex seemed to be disturbed during the zygotene/early pachytene stage and chromosome pairing was not observed (Fig. 5). The first hints of fragmented chromosomes appeared at the onset of diakinesis as the chromosomes at this stage appeared visibly distorted. No bivalents were formed at the end of prophase I. In anaphase I, the chromosomes were entangled, and bridges and fragments of chromosomes were visible for the chromosomes that had separated. In the second meiotic division, these phenotypes were further exaggerated. In wild-type cells, tetrads were formed at the end of meiosis, whereas in At*brca2* double mutant cells, the chromosomes segregated abnormally, leading to polyads instead of tetrads. The meiotic phenotype of At*brca2* double-mutant PMCs is similar to the At*rad51* phenotype (Li *et al.*, 2004; Fig. 5) but different from the At*dmc1* phenotype (Couteau *et al.*, 1999). Severe fragmentation was observed in the At*brca2* double mutant and At*rad51* mutant but not in At*dmc1* mutants. All of these mutants are sterile and cannot form bivalents.

The development of the female gametophyte is severely affected in At*brca2* double mutants

Using the At*brca2* double mutant, we also analyzed the extent to which ovule development is affected. Flower buds were harvested, and pistils were dissected and opened lengthwise to isolate ovules. Ovules were cleared and observed by DIC microscopy. In wild-type plants, ovule megaspore mother cells undergo meiosis to yield four daughter cells, three of which later degenerate. The persisting cell is a precursor of the megaspore, which undergoes three nuclear divisions to produce the eight-nucleus embryo sac. The intermediate stages between the megaspore mother cell and the eight-nucleus embryo sac are accompanied by specific developmental stages of the integuments surrounding the embryo sac, which progressively develop according to changes in development. As shown in Fig. 6, At*brca2* double mutants are unable to pass through meiosis and do not produce a mature embryo sac. Mutant megaspore mother cells are still phenotypically identical to wild-type ovules; however, the following meiotic divisions are impeded. The primary megaspore mother cell persists throughout embryo sac development with no further divisions and degrades at a stage comparable to the wild-type mature female gametophyte. During this maturation, development of integuments and the embryo sac is similar to the wild-type with one exception: maturation of the wild-type embryo sac is associated with the appearance and enlargement of a vacuole, which the double mutant does not appear to develop. There were only a few cases in which a megaspore mother cell of At*brca2* double-mutant plants did proceed through meiosis to form a mature female gametophyte with an eight-nucleus embryo sac. This finding is consistent with the observation that seeds could be obtained at a low frequency by fertilizing double-mutant mother plants with wild-type pollen.

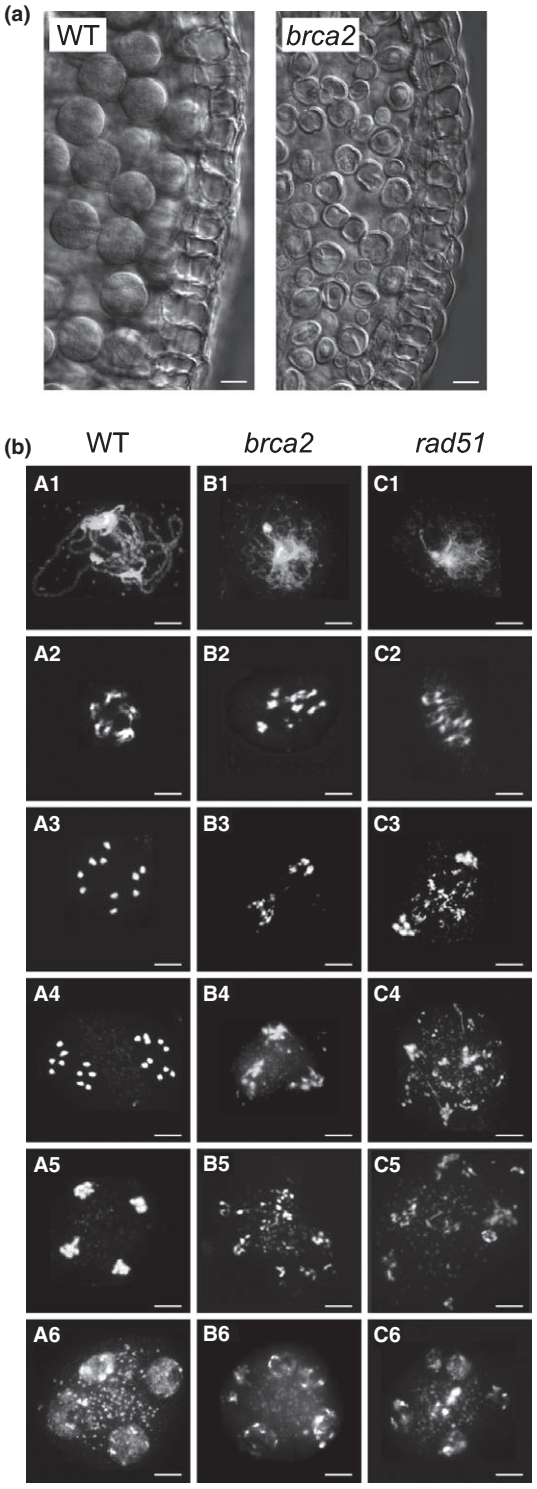

Fig. 5 Analysis of meiosis in At*brca2* double-mutant pollen mother cells (PMCs). (a) Isolated anthers of wild-type (WT) and At*brca2* double-mutant (*brca2*) flower buds were analyzed using a microscope with differential interference contrast optics. The pollen grains of wild-type anthers were round-shaped, whereas those from At*brca2* double-mutant anthers were empty and wizened. Bar, 10 μm. (b) PMCs of wild type (WT; A1–A6), At*brca2* double mutants (*brca2*; B1–B6) and *rad51* mutants (*rad51*; C1–C6) were isolated and chromatin was stained with 4′,6-diamidino-2-phenylindole (DAPI). At*brca2* double mutants and *rad51* mutants showed similar phenotypes: synapsis appeared disturbed during the zygotene/early pachytene stage (B1, C1), and bivalents did not form in diakinesis (B2, C2). The chromosomes looked entangled, and fragmentation and bridges were visible during anaphase I (B3, C3), a phenotype that grew worse in anaphase II (B4, C4). Segregation led to diffuse chromosome clusters in telophase II (B5, C5), and polyads were formed as the result of the meiotic divisions (B6, C6). Bars, 5 μm.

BRCA2 is essential for RAD51 and DMC1 focus formation during meiotic homologous recombination

To specify the role of AtBRCA2 in meiotic homologous recombination, immunolocalization studies were conducted to compare the localization of AtASY1, AtZYP1, AtRAD51 and AtDMC1 in wild-type and At*brca2* double-mutant PMCs (Fig. 7). At*rad51* and At*dmc1* were used as controls to confirm the specificity of the antibodies (Supporting Information, Fig. S1). AtASY1 is a HORMA domain containing protein and was shown to be essential for synapsis (Armstrong *et al.*, 2002) In wild-type cells, AtASY1 was localized at the chromosome axis, which is important for the early pairing events that lead to synapsis (Armstrong *et al.*, 2002; Sanchez-Moran *et al.*, 2007, 2008). In Arabidopsis there are two ZYP1 proteins – ZYP1a and ZYP1b – that are both recognized by the AtZYP1 antibody (Higgins *et al.*, 2005). AtZYP1 proteins are the central elements of the synaptonemal complex, and AtZYP1 can be used to identify chromosomes undergoing synapsis. AtRAD51 and AtDMC1 foci mark early recombination events during meiotic homologous recombination (Mercier *et al.*, 2003; Chelysheva *et al.*, 2007; Sanchez-Moran *et al.*, 2007; Vignard *et al.*, 2007). AtASY1 localization was not disturbed in the At*brca2* double mutant, demonstrating that the absence of BRCA2A/B does not interfere with normal chromosome axis formation. By contrast, the synaptonemal complex protein AtZYP1 was mislocalized in the mutant, an observation that is consistent with the absence of synapsis and bivalent formation as determined by staining with DAPI. Neither AtRAD51 nor AtDMC1 foci were observed in the At*brca2* double mutant. Thus, the localization of the recombinases AtRAD51 and AtDMC1 depends on AtBRCA2.

Discussion

BRCA2 is a prominent member of the homologous recombination machinery in mammals (reviewed in Liu & West, 2002; Yoshida & Miki, 2004; O'Donovan & Livingston, 2010; Holloman, 2011). Correlations between mutations in BRCA2 and breast and ovarian tumorigenesis are well established (reviewed in Stratton, 1996; Buchholz *et al.*, 1999; Ingvarsson, 1999; Modesti & Kanaar, 2001; Thompson & Schild, 2002; Shivji & Venkitaraman, 2004; Turner *et al.*, 2005; Mavaddat *et al.*, 2010). Homologs of BRCA2 can be found in seed plants but not in the moss *Physcomitrella patens* (Rensing *et al.*, 2008; Trapp *et al.*, 2011).

Male and female sterility of AtBRCA2 double mutant plants

Using *A. thaliana* as a model organism in an earlier study, an important function of AtBRCA2 in meiosis was demonstrated by

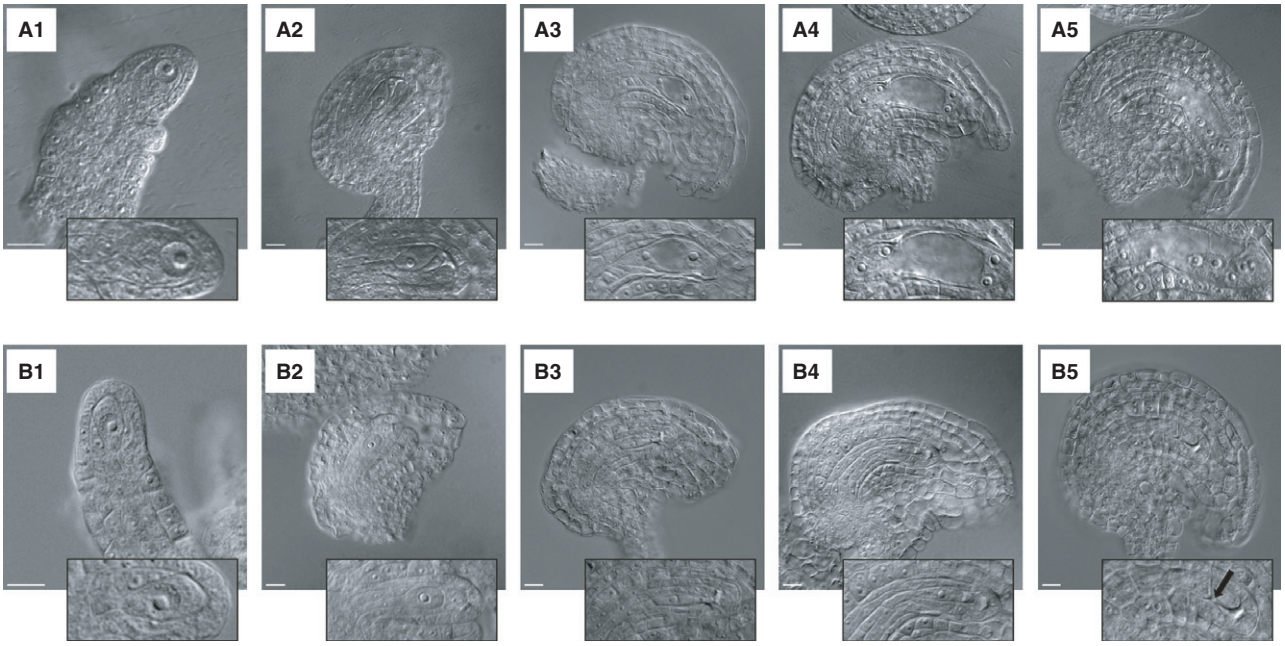

Fig. 6 Analysis of embryo sac development in At*brca2* double mutants. Ovules of wild-type (A1–A5) and At*brca2* double-mutant (B1–B5) plants were isolated and observed by differential interference contrast microscopy. In wild-type ovules, the megaspore mother cell (A1) went through meiosis and one of the four daughter cells persisted to become the megaspore (A2). After three nuclear mitotic divisions (A3, A4), the eight-nucleus embryo sac was produced (A5). In At*brca2* double mutants, the megaspore mother cells were still phenotypically identical to wild-type ovules (B1), but meiosis was impaired in most cases, and the primary megaspore mother cell persisted throughout embryo sac development with no further divisions (B2–B4) and degraded in a stage comparable to the wild-type mature female gametophyte (B5, see black arrow for degraded megaspore mother cell). Bar, 10 μm. The panels below the single stages show ×1.5 magnifications of the respective developing megaspore mother cell.

RNAi knockdown in a WS (Wassilewskija) background (Siaud *et al.*, 2004). The simultaneous knockdown of both AtBRCA2 genes resulted in partial sterility. However, with knockdown techniques, complete silencing of the target gene is difficult to achieve. Therefore, it is difficult to determine whether the targeted factor is essential for a specific pathway. Depending on the concentration of the remaining protein, phenotypes of different severity can arise: an At*RAD51* knockdown line generated by an RNAi construct was reported to be vital and fertile (Siaud *et al.*, 2004), whereas an At*rad51* T-DNA insertion line was completely sterile (Li *et al.*, 2004). Abe *et al.* (2009) reported that an At*brca2* double mutant in the Nossen background was fertile, whereas Wang *et al.* (2010) indicated that in their hands an At*brca2* double mutant in the Columbia background was sterile; it should be noted, however, that in this report the double mutant was not analyzed in detail. Here, we observed complete sterility by self-fertilizing the At*brca2* double mutant in the Columbia background. Therefore it seems that the type of mutant and perhaps the cultivar have great influence on meiotic impairment of mutants. In particular, the transposon mutants used by Abe *et al.* (2009) show a typical *brca2* somatic, but not meiotic phenotype. Interestingly, an RNAi-knockdown of BRCA2 in the Nossen background led to partially sterile plants, which supports the idea of the Ds (*Dissociation*) transposon mutants used in their study being hypomorphic mutants. To test whether sterility in the double mutant used in our study was the result of a complete failure of development of both germ lines, we performed back-crosses with the At*brca2* double mutant and wild-type plants. Interestingly, whereas pollen derived from the double mutant never resulted in progeny after fructification of wild-type plants, when the At*brca2* double mutant was the mother plant and was pollinated with wild-type pollen, approximately one-quarter of siliques contained one or two seeds. This result is in line with the observation that some female gametophytes developed embryo sacs and were able to overcome meiotic aberrations and produce seeds. Thus, loss of AtBRCA2 seems to have a stronger effect on male germ lines than on female germ lines. The same sterility phenotype was observed for Arabidopsis mutants lacking AtMND1, a protein that is required for homologous pairing in meiosis (Kerzendorfer *et al.*, 2006; Panoli *et al.*, 2006). This phenomenon seems not to be restricted to plants as a *brca2* null mutation in mice results in embryonic lethality as well. When BRCA2 expression in mice was reduced but not completely ablated, the mutants were infertile but able to develop to adulthood. Interestingly, the male *brca2* mice were completely sterile, while the female oocytes could pass through meiosis, undergo fertilization and develop into embryos, although those that did so experienced a high frequency of abnormalities (Sharan *et al.*, 2004).

The role of AtBRCA2 in somatic cells

We also examined the role of AtBRCA2 in DNA repair and recombination in somatic cells. Abe *et al.* (2009) showed that in the Nossen background both At*brca2* single mutants were hypersensitive to cisplatin and that the double mutant was more sensitive to γ-irradiation than the single mutants. By contrast, the

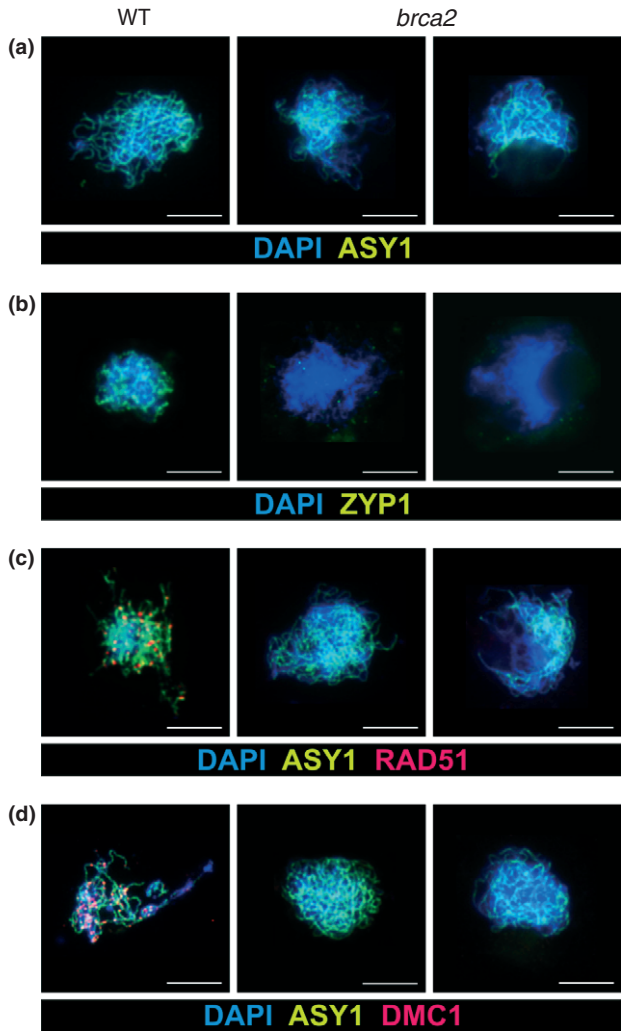

Fig. 7 Immunolocalization in At*brca2* double mutants. Pollen mother cells of wild-type (WT) and At*brca2* double mutants (*brca2*) were isolated and immunostained for ASY1 (a), ZYP1 (b), RAD51 (c) and DMC1 (d) in prophase I. Chromatin was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Whereas ASY1 localization in At*brca2* double-mutants pollen mother cells (PMCs) was normal, no proper ZYP1 localization could be detected. Furthermore, neither RAD51 foci nor DMC1 foci could be observed. Control staining in wild-type, At*rad51* and At*dmc1* PMCs confirmed the specificity of the antibodies (Fig. S1). Bars, 5 μm.

single mutants in the Columbia background displayed an allele-specific response to MMC and bleomycin, the At*brca2a* mutant showed enhanced sensitivity to high concentrations of MMC and bleomycin, whereas the At*brca2b* mutant did not (Wang *et al.*, 2010). Thus, it is possible that the cultivar and/or types of mutants also influence sensitivity to DNA-damaging agents. In our study, the At*brca2* double mutant in the Columbia background proved to be highly sensitive to low concentrations of MMC but was not sensitive to bleomycin. MMC causes mainly interstrand cross-links that have to be repaired during replication (Akkari *et al.*, 2000; Palom *et al.*, 2002). Thus, the sensitivity of At*brca2* mutants to MMC suggests the potential involvement of AtBRCA2 in repairing DNA cross-links, as has been demonstrated for mammalian BRCA2 (Yu *et al.*, 2000; Kraakman-van der Zwet *et al.*, 2002; Atanassov *et al.*, 2005; Ohashi *et al.*, 2005).

A study of HR frequencies without induction with bleomycin in At*brca2* mutants indicates that the HR frequency of the At*brca2a* mutant is *c.* 50% of that of the wild-type, whereas the HR frequency of the At*brca2b* mutant shows no difference from the wild-type (Wang *et al.*, 2010). In our study, the HR frequency of the At*brca2* double mutant was reduced to *c.* 30% of the wild-type, suggesting that the absence of both BRCA2A and B leads to a more drastic phenotype than the absence of BRCA2A alone. We were also able to show that the number of HR events in seedlings of the At*brca2* double mutant gets even lower in relation to the wild-type after treatment with bleomycin. These observations suggest that AtBRCA2 is an important factor in HR in somatic cells.

Wang *et al.* (2010) reported that AtBRCA2A has a specific role in systemic acquired resistance (SAR) as a transcriptional regulator, whereas AtBRCA2B seemed not to be involved in this process. Both AtBRCA2 homologs are required for DNA repair, but to a different extend: BRCA2B has the ability to only partially complement the loss of BRCA2A, whereas the loss of BRCA2B can be fully complemented by BRCA2A (Wang *et al.*, 2010). However, as revealed in our study, both Arabidopsis BRCA2 proteins have redundant functions in meiosis.

Also mutants of the RAD51 paralogs At*RAD51C* and At*XRCC3* are defective in meiotic recombination, leading to sterility of the respective mutants (Bleuyard & White, 2004; Abe *et al.*, 2005; Li *et al.*, 2005). Similar to the At*brca2* double mutant, the At*rad51c* mutant has somatic recombination defects as suggested by the mutant's reduced HR frequency (Abe *et al.*, 2005). Similarities in the sterility and MMC sensitivity of At*rad51*, At*xrcc3*, At*rad51c* and At*brca2* mutants are probably the result of the important contributions of these proteins to homologous recombination. These findings are in agreement with findings in other eukaryotes, such as mammals.

The role of BRCA2 in meiosis

To investigate the development of the female gametophyte, young ovules of different developmental stages were cleared and examined by DIC microscopy. In young ovules of wild-type plants, characteristic phases of embryo sac maturation appeared: a diploid megaspore mother cell passed through meiosis, and four haploid cells arose. Three of these cells degenerated, and the one that was left passed through three mitotic divisions, resulting in an eight-nucleus embryo sac corresponding to the mature female gametophyte. Each stage is correlated with a distinct progression of integument growth. This correlation allowed us to define the developmental stage of the embryo sacs in mutants with defects in meiosis. Embryo sacs from At*brca2* double mutants contained the megaspore mother cell but usually did not undergo proper meiotic division and, thus, did not progress to the eight-nucleus stage. Degenerated products of meiotic divisions appeared in mature ovules and additionally, in most cases, the vacuole, which arose in wild-type ovules during development in the middle of

the embryo sac, was not found. Sometimes, double-mutant megaspore mother cells passed through meiosis and developed mature gametophytes that resembled wild-type ovules. This observation explains the appearance of one or two seeds in approximately one-quarter of the siliques that originated by pollinating double-mutant plants with wild-type pollen. Here, we have shown for the first time that the development of the female gametophyte in Arabidopsis is severely affected by the absence of AtBRCA2.

By DAPI staining of PMCs, we were able to corroborate the data of Siaud *et al.* (2004), in which At*BRCA2* was knocked down by RNAi. PMCs of At*brca2* double mutants exhibited severe chromosomal aberrations during meiosis. In anaphase I, chromosomes arose as entangled structures that were connected by chromatin bridges, which led to chromosomal breaks and disparate separation of chromosomes. Defects in the separation of chromosomes resulted in polyads instead of tetrads after the second meiotic division. DAPI staining of the At*brca2* double mutants also suggested impaired bivalent formation as the mutants were found to have > five groups of chromosomes during diakinesis. Immunolocalization of AtASY1 and AtZYP1 in At*brca2* double mutants revealed correct assembly of the chromatin axis but profound defects in synapsis.

The role of BRCA2 in the recombination machinery of plants

Bivalent pairing and synapsis in prophase I is dependent on the formation and processing of DSBs (for a review, see Mercier & Grelon, 2008; Ronceret & Pawlowski, 2010; Osman *et al.*, 2011). Both RAD51 and its meiotic paralog DMC1 build filaments on ssDNA, which then invade homologous templates. The finding that AtDMC1 foci can be detected slightly earlier than AtRAD51 foci is consistent with the model that AtDMC1 nucleoprotein filaments promote interhomolog interaction in meiosis (Sanchez-Moran *et al.*, 2007; Vignard *et al.*, 2007). Both At*rad51* and At*dmc1* mutant PMCs have an asynaptic phenotype, but At*rad51* mutants additionally exhibit severe chromosome fragmentation (Couteau *et al.*, 1999; Li *et al.*, 2004). The difference between At*rad51* and At*dmc1* mutants could be explained by the fact that At*dmc1* mutants lose interhomolog interactions in favor of intersister interactions, whereas At*rad51* mutants lose homolog interactions entirely. The chromosomal aberrations that arise during meiosis in At*brca2* double mutants are similar to the chromosome structures observed in At*rad51* mutants but different from those in At*dmc1* mutants. To further analyze early recombination events in the At*brca2* double mutant, we conducted immunostaining of AtRAD51 and AtDMC1. In mammals, it was shown that RAD51 and DMC1 focus formation depends on BRCA2 (Sharan *et al.*, 2004), and BRCA2 was shown to be the mediator of strand invasion during HR (reviewed in Holloman, 2011; Maher *et al.*, 2011). For the first time in plants, we show that the loss of AtBRCA2 completely abolishes AtRAD51 and AtDMC1 focus formation during prophase I, indicating that AtBRCA2 is required for the correct localization of both recombinases. Loss of AtBRCA2 therefore mimics loss of AtRAD51, as in the At*rad51* At*dmc1* mutant in which the At*dmc1* mutant phenotype is suppressed by deleting At*RAD51* (Vignard *et al.*, 2007). Thus, we provided evidence that BRCA2 is a key mediator of RAD51- and DMC1-faciliated DNA repair in seed plants.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Control staining in wild-type, At*rad51* and At*dmc1* mutants.

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