The Plant Cell, Vol. 19: 3090–3099, October 2007, www.plantcell.org © 2007 American Society of Plant Biologists

The Catalytically Active Tyrosine Residues of Both SPO11-1 and SPO11-2 Are Required for Meiotic Double-Strand Break Induction in *Arabidopsis*

Frank Hartung,[a] Rebecca Wurz-Wildersinn,[a] Jörg Fuchs,[b] Ingo Schubert,[b] Stefanie Suer,[a] and Holger Puchta[a,1]

[a] Botanisches Institut II, Universität Karlsruhe, 76128 Karlsruhe, Germany
[b] Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung, 06466 Gatersleben, Germany

SPO11, a homolog of the subunit A of the archaebacterial topoisomerase VI, is essential for double-strand break (DSB)–induced initiation of meiotic recombination. In contrast with single homologs in animals and yeasts, three homologs are present in *Arabidopsis thaliana* and other higher plants. Whereas At SPO11-3 is involved in somatic endoreduplication, At SPO11-1 and, as recently shown, At SPO11-2 are essential for the initiation of meiotic recombination. Further defining the role of At SPO11-2, we were able to demonstrate that it is required for proper chromosome segregation, as its loss resulted in aneuploidy in the surviving progeny. The double mutant *spo11-1 spo11-2* does not differ phenotypically from the single mutants, indicating that both proteins are required for the same step. Contrary to the observations for the At *rad51-1* single mutant, the combination of *spo11-2* and *rad51-1* did not lead to chromosome fragmentation, indicating that SPO11-2, like SPO11-1, is required for DSB induction. As the meiotic phenotype of both single SPO11 mutants can be reversed by complementation using the full-length genes but not the same constructs mutated in their respective catalytically active Tyr, both proteins seem to participate directly in the DNA breakage reaction. The active involvement of two SPO11 homologs for DSB formation reveals a striking difference between plants and other eukaryotes in meiosis.

INTRODUCTION

Meiosis initiates the transition from the diploid to the haploid phase in the life cycle of all sexually reproducing eukaryotic organisms. After induction of double-strand breaks (DSBs), alignment of the maternal and paternal chromosomes, and recombination between homologs, two subsequent rounds of meiotic chromosome segregation yield haploid spores. Surprisingly, recent cytological, genetic, and molecular investigations revealed many species-specific variations in the meiotic process previously thought to be highly conserved (Caryl et al., 2003; Pawlowski and Cande, 2005; Hamant et al., 2006; Mezard et al., 2007). A central conserved step in meiosis is the induction by DSBs, allowing the invasion of allelic DNA strands (Keeney and Neale, 2006). For yeast, it could be demonstrated that the factor directly involved in DSB formation is the SPO11 protein (Keeney et al., 1997). SPO11 shows homology to the subunit A of the archaebacterial topoisomerase VI, which belongs to the class II topoisomerases (Bergerat et al., 1997; Gadelle et al., 2003). The *SPO11* gene is conserved in lower and higher eukaryotes. Initiation of homologous recombination by DSB formation in meiosis is not the only function of SPO11; homolog pairing and formation of the synaptonemal complex (SC) also is dependent on the presence of SPO11 in many but not all organisms (Celerin et al., 2000; Pawlowski and Cande, 2005; Hamant et al., 2006). In *Drosophila melanogaster* and *Caenorhabditis elegans*, meiotic recombination is eliminated if SPO11 is absent; however, normal SCs are formed between the homologs (Dernburg et al., 1998; McKim and Hayashi-Hagihara, 1998).

Previously, we were able to demonstrate that mono- and dicotyledonous plants harbor three distantly related homologs of SPO11: *SPO11-1*, *-2*, and *-3* (Hartung and Puchta, 2000, 2001). Two ethyl methanesulfonate–induced allelic *Arabidopsis thaliana SPO11-1* mutant lines in the Columbia (Col-0) background and a T-DNA insertion line in the ecotype Wassilewskija have been described to possess a severe meiotic phenotype, resulting in almost sterile plants that produce only a few seeds (Grelon et al., 2001). The formation of bivalents and chiasmata was severely reduced, and stable synapsis could not be observed (Grelon et al., 2001). In the *spo11-1* background, meiosis results in polyads of random DNA content instead of the typical tetrads. By contrast, the SPO11-3 protein is, as one subunit of the topoisomerase VI, involved in endoreduplication in somatic cells. T-DNA insertion lines of At *SPO11-3* were severely impaired in cellular endoreduplication required for plant cell enlargement during normal development (Hartung et al., 2002; Sugimoto-Shirasu et al., 2002, 2005; Sugimoto-Shirasu and Roberts, 2003).

Due to the lack of suitable mutants, the question of the biological function of SPO11-2 remained elusive for a long time. Recently, it has been reported that the meiotic phenotype of an At *spo11-2* T-DNA insertion line is virtually indistinguishable from At *spo11-1* (Stacey et al., 2006). However, no analysis in a homozygous double mutant background was performed, and the role of SPO11-2 was not defined further. In this article, we address these questions and provide evidence that both SPO11-1 and SPO11-2 proteins are directly involved in the induction of DSBs to properly initiate meiosis in *Arabidopsis*.

[1] Address correspondence to holger.puchta@bio.uni-karlsruhe.de.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Holger Puchta (holger.puchta@bio.nui-karlsruhe.de).
www.plantcell.org/cgi/doi/10.1105/tpc.107.054817

RESULTS

At SPO11-2 Is Required for Fertility

An insertion mutant for At *SPO11-2* (At1g63990) (GABI line 749C12) was obtained from the GABI collection (Rosso et al., 2003) and will be referred to as *spo11-2-3* to distinguish it from the already described lines 1 and 2 (Stacey et al., 2006). The T-DNA is located in the last intron of the coding region, thereby shortening the protein by 44 amino acids encoded by the last exon (Figure 1A). A small deletion of 12 bp occurred, and the T-DNA insertion is orientated in a tandem arrangement back to back with two left borders fused to the genomic sequence. No expression of the full-length mRNA could be detected in RT-PCR experiments using primers spanning the insertion site (Figure 1A, primers P1 and P2 or P3, and Figure 4C) in the mutant. Expression of the mRNA upstream of the T-DNA insertion was detectable by means of RT-PCR at a similarly low level as in the wild type using primer pairs P4 and P5 (Hartung and Puchta, 2000). By screening the progenies of heterozygous mutant plants, homozygous plantlets could be identified using PCR with primers P1 and P2. The homozygous plants displayed no visible differences during development until the onset of blossom. Anthers were shorter and aged earlier in the mutant in comparison to heterozygous or wild-type plants (Figure 1B). Siliques were much shorter, partially empty, and contained only a small number of seeds (Figure 1B). We obtained ~150 to 200 seeds (4 to 7 mg) per *spo11-2-3* plant compared with 2000 to 3000 from the wild-type plants (50 to 80 mg).

Flow Cytometric Analysis and Chromosome Counting Demonstrates That SPO11-2 Is Required for Proper Segregation

To further characterize whether the loss of SPO11-2 has direct consequences for the surviving progeny, we performed a flow cytometric analysis. We speculated that viable progeny might result from random segregation of chromosomes during meiotic divisions in pollen mother cells of *spo11-2-3*. Such random segregation should result mainly in aneuploidic offspring. Therefore, we measured the relative DNA content of viable progenies of *spo11-2-3* mutant seeds via flow cytometry in comparison to Col wild-type plantlets as an internal standard. The offspring of *spo11-2-3* showed severely impaired growth in most cases and had either a higher (17 of 20 plantlets) or the same (3 out of 20) but never a lower DNA content than Col-0 plantlets (Figure 2A). The amount of surplus DNA indicates the presence of extra chromosomes per

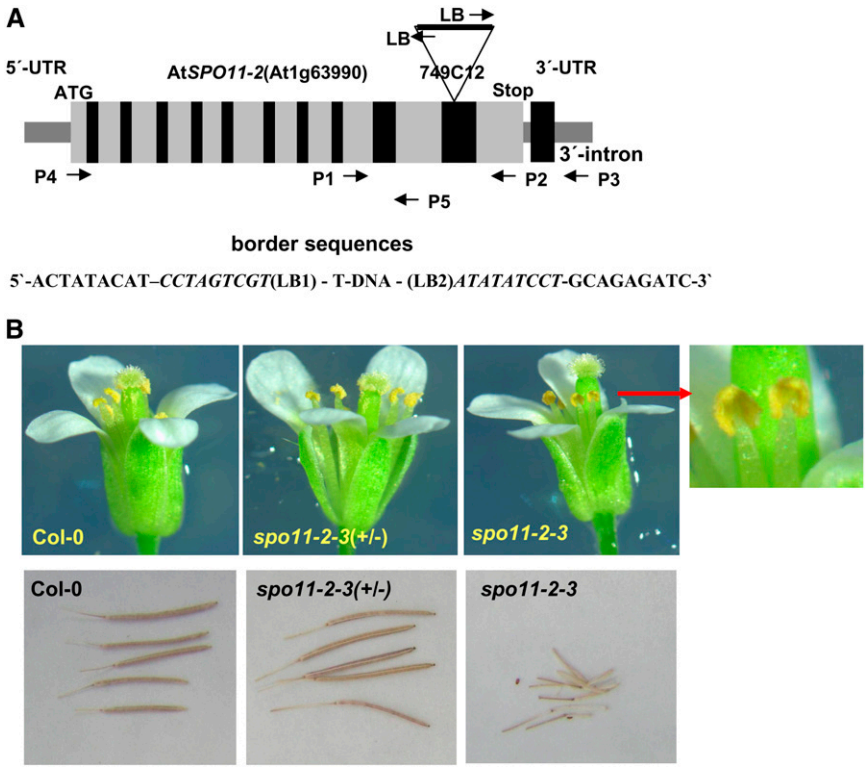

Figure 1. Molecular and Phenotypic Features of the At *spo11-2-3* Mutant.

(A) Schematic drawing of the At SPO11-2 gene, including both untranslated regions (UTRs); exons are shown in light gray and introns in black. The T-DNA insertion is depicted as a white triangle and the primers used as directional arrows. The border sequences contain nine bases of flanking genomic sequence on each side and nine bases of the respective left border (LB) sequence.

(B) Flowers and siliques of Col-0 and of the *spo11-2-3* mutant. The anthers of the homozygous *spo11-2-3* mutant appear shorter than in heterozygous state and in the wild type Col-0. The homozygous *spo11-2-3* mutant produces only few seeds in comparison to wild-type and heterozygous plants.

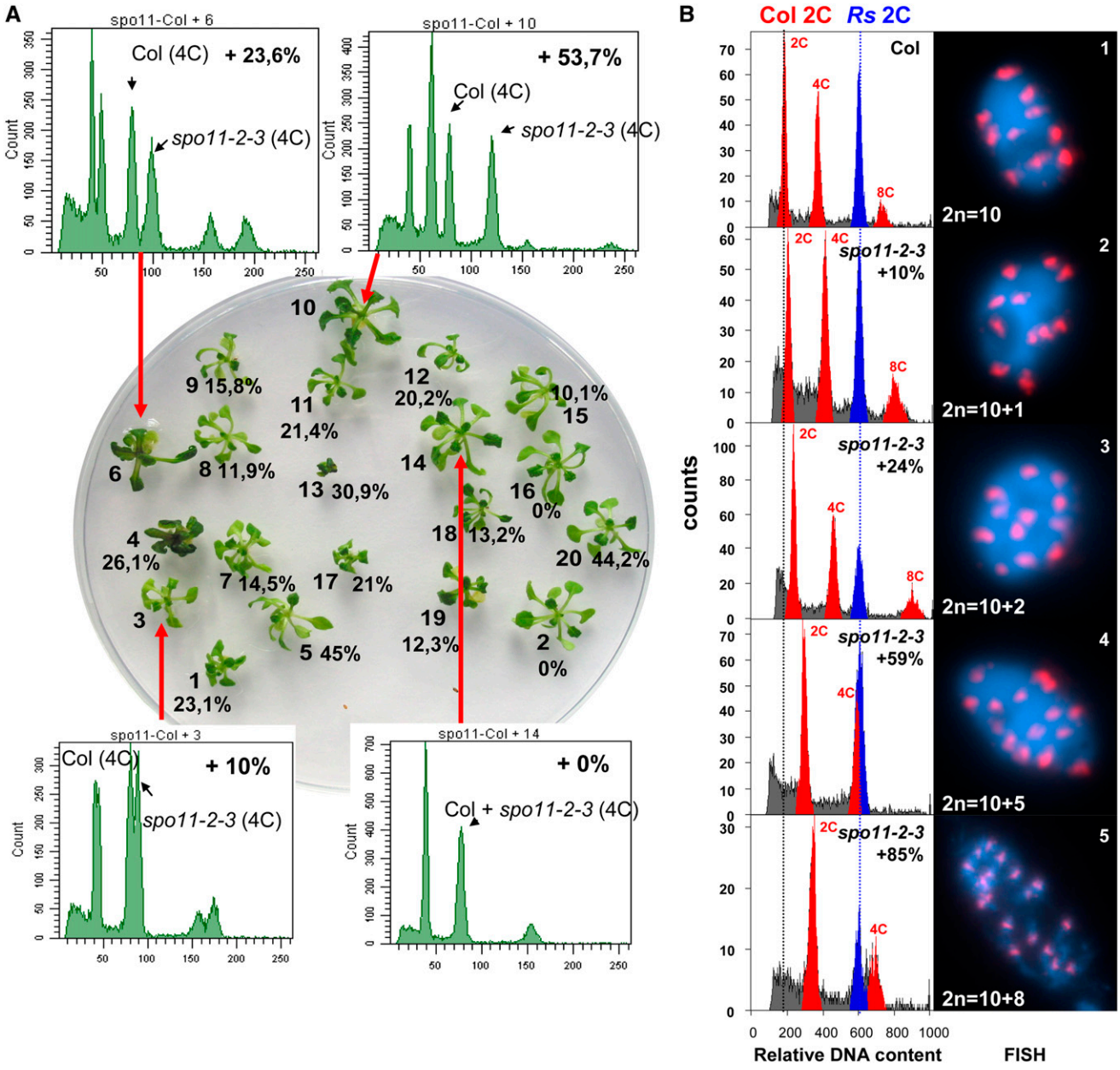

Figure 2. Flow Cytometric Analysis and Chromosome Counting of At *spo11-2* Progeny.

(A) The relative nuclear DNA content of 20 different plantlets derived from seeds of a *spo11-2* plant was analyzed via flow cytometry compared with Col wild-type plantlets as internal standard. Seventeen of them possessed a higher DNA amount than the wild type Col-0. Three plantlets displayed the same 2C and 4C DNA content as wild-type plants. The increase in DNA content of the individual mutant plantlets is given as a percentage.

(B) Left: Relative DNA content of Col wild type and four individual spo11-2 offspring plantlets with different amounts of surplus DNA (red peaks) measured by flow cytometry in comparison to *Raphanus sativus* (*Rs*; blue peak) as internal standard. Right: Corresponding flow-sorted 2C interphase (numbers 1 to 4) or (pro-) metaphase (number 5) nuclei after FISH with the centromeric 180-bp repeat.

haploid chromosome set according to the flow karyotype of *Arabidopsis* (Samoylova et al., 1996).

To confirm the flow cytometric data, we performed fluorescence in situ hybridization (FISH) with a centromeric 180-bp repeat on either flow-sorted 2C leaf nuclei or meristematic root nuclei of individual mutant plants with different amounts of surplus DNA. The maximal number of signals that occurs in the nuclei reflects the number of centromeres and therewith of chromosomes within individual mutants. For plantlets with surplus DNA of 10, 24, 59, and 85%, we counted one, two, five, and eight additional chromosomes (Figure 2B), proving the reliability of flow karyotyping.

These data clearly indicate that spores with more than the haploid chromosome set are frequently transferred. The resulting aneuploid plants were completely sterile. Thus, chromosome segregation is indeed severely disturbed in the *spo11-2-3* T-DNA insertion line. Comparable results were obtained investigating 33 plantlets derived from *spo11-1-3* mutant seeds (data not shown).

Double Mutant Analysis Indicates That *spo11-2* Is Disturbed in the Same Early Step in Meiosis as *spo11-1*

To further elucidate the defect in meiosis, we performed cytological studies using the T-DNA insertion mutant line At *spo11-1-3* (SALK_146172) from the SALK-collection (Alonso et al., 2003; Stacey et al., 2006). Furthermore, we crossed both the At *spo11-1-3* and At *spo11-2-3* mutant lines with each other and with the At *rad51-1* line (Li et al., 2004).

The first meiotic division of pollen mother cells of the *spo11-1* and *-2* mutants is impaired because the homologs neither pair nor form SCs due to the lack of initial DSB formation (Grelon et al., 2001; Stacey et al., 2006). The same phenotype was displayed by our *spo11-1-3* and *spo11-2-3* single mutants. Notably, the double homozygous mutant *spo11-1 spo11-2* showed neither a more pronounced nor a less severe phenotype in meiosis. The double mutant is rather indistinguishable from the single mutants (Figures 3, columns B to D).

The two mutants and the double mutant showed normal chromosome condensation but no bivalents in the diplotene stage (Figure 3, columns B to D, lines 2 to 4). As a consequence, no ordered arrangement of five bivalents can be found in metaphase I, and missegregation of the univalents occurred in anaphase I and II (Figure 3, columns B to D, lines 5 to 7). No chromosome fragments or brightly stained dots as in the *rad51-1* single mutant (Li et al., 2004) could be detected at this stage. Finally, the mutants show formation of polyads containing unequal amounts of DNA instead of the regular tetrads as found in the wild type (Figure 3, B8 to D8).

Crossing the single mutants of *spo11-1* and *-2* to *rad51-1* resulted in a clear *spo11* phenotype as described above (Figure 3, E1 to E8 for *spo11-2-3*). RAD51 is the key factor for homologous strand exchange and is necessary for proper meiotic recombination. The phenotype of the *rad51-1* mutant is characterized by chromosome breakage (Li et al., 2004). Our data demonstrate that SPO11-2 acts earlier than RAD51 and that the initiation of meiotic homologous recombination does not occur in the double mutants, thus suppressing the *rad51-1* phenotype. The same holds true for *spo11-1 rad51* double mutants (Li et al., 2004; data not shown). Taken together, the data clearly demonstrate that the defect in the *spo11-2* mutant occurs at the same stage during male meiosis as in the *spo11-1-3* mutant. Thus, both SPO11-1 and -2 seem to be involved in the induction of DSBs.

Seed Set of the *spo11-1 spo11-2* Double Mutant Indicates That Both Genes Are Epistatic

Since *spo11-1-3* produced ~0.5 to 1% and *spo11-2-3* ~10% seeds in comparison to the wild type, we expected that, if both genes act in independent, parallel pathways, even less seeds might be produced in the double mutant. Whereas we harvested 47, 36, 30, 24, and 12 seeds from five individual At *spo11-1-3* plants, two individual double mutants yielded 25 and 26 seeds, respectively. Thus, seed yield was not further reduced in the double mutant in comparison to *spo11-1-3* (29.8 ± 13.1). Moreover, plants double heterozygous for *spo11-1* and *-2* yielded a normal amounts of seeds. Since then, we propagated the double heterozygous plants for three more generations without detecting any failures in germination or seed production.

The Catalytically Active Tyr Residues of SPO11-1 and SPO11-2 Are Both Required for DSB Induction

We were able to demonstrate that both SPO11-1 and SPO11-2 are required for the initiation of DSBs in meiotic recombination. Evidence exists that in yeast, multimers of SPO11 might be formed just before the DSB formation (Maleki et al., 2007; Sasanuma et al., 2007). Therefore, theoretically, two putative roles for both proteins could be envisaged: one of them is the protein directly breaking the DNA and the other acts as accessory protein; alternatively, both proteins might be actively involved in breaking the DNA. Tyr-135 of yeast *SPO11* was described to be essential for DSB formation (Bergerat et al., 1997; Diaz et al., 2002). Furthermore, the active Tyr is necessary for the forming of a tight binding state of SPO11 and DNA prior to the DSB formation itself (Prieler et al., 2005). To analyze if the active Tyr is essential for DSB induction in both *Arabidopsis* proteins, we tried to complement the phenotype of *spo11-1-3* and *spo11-2-3* mutants with the corresponding wild-type genomic DNA as a control and with the same constructs having the catalytically active Tyr substituted by Phe. According to multiple alignments, the active Tyr residues of *SPO11-1* and of *SPO11-2* are Tyr-103 and Tyr-124, respectively (Hartung and Puchta, 2000). We cloned the complete At *SPO11-1* and *-2* genes, including 553 bp (=cSPO11-1) or 704 bp (=cSPO11-2) of the promoter region and 496 bp of the 3′-UTR for each into the binary vector pPZP221 (Hajdukiewicz et al., 1994). A single base exchange (adenine to thymine) was achieved by primer-directed mutagenesis (Figure 4A), in both cases replacing Try by Phe. The mutated constructs were also cloned into pPZP221 and sequenced in their full length.

Using the floral dip method, we transformed heterozygous *spo11-1-3* or *spo11-2-3* plants with the respective construct. Several T-DNA insertion lines were identified. We analyzed four independent *spo11-1-3* and 12 independent *spo11-2-3* lines, which were homozygous for the respective mutation and carried the wild-type construct. From these, three out of four (*spo11-1-3* + cSPO11-1) and all 12 lines (*spo11-2-3* + cSPO11-2) produced similar numbers of seeds as the wild-type plants, indicating that the defect can be fully complemented by the respective wild-type gene (Figure 4B). We analyzed the meiotic stages in the complemented plants and found no visible differences to meiosis in wild-type cells (data not shown).

Interestingly, none of the lines carrying the point-mutated construct showed complementation. We tested five lines for *spo11-1-3* + cpmSPO11-1 and seven lines for *spo11-2-3* + cpmSPO11-2 (cpm is complementation constructs point mutated), respectively, but did not find a single complementation event. Examples for siliques of complemented and noncomplemented

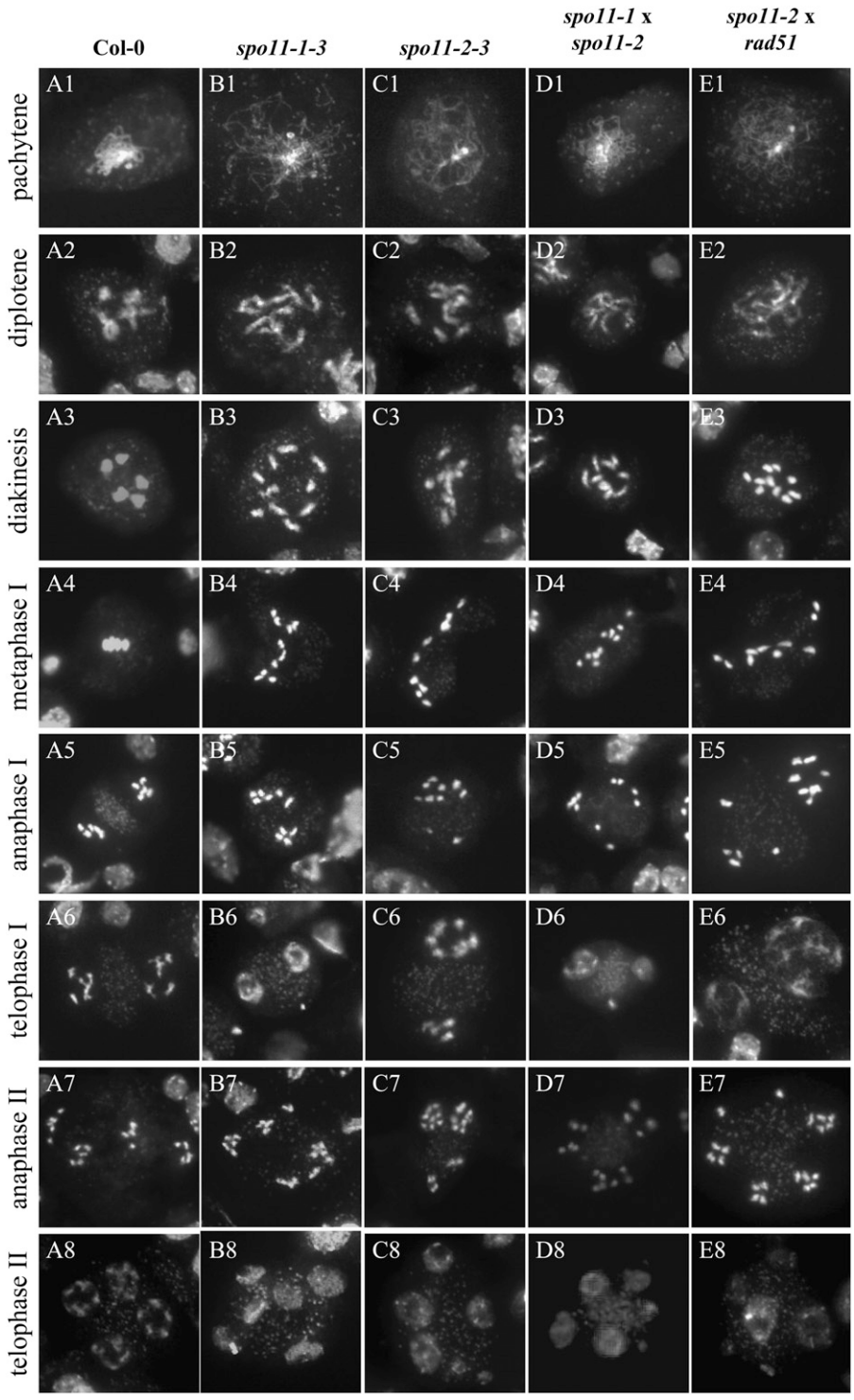

Figure 3. DAPI Staining of Different Meiotic Stages during Pollen Development.

Wild-type meiosis (**[A1]** to **[A8]**) is characterized by synapsis of homologous chromosomes and the formation of bivalents during pachytene stage of prophase **(A1)**. During diplotene **(A2)** and diakinesis **(A3)**, chromosome pairs strongly condense and detach from the nuclear envelope in preparation for the first meiotic division. During metaphase I, homologs congress at the metaphase plate **(A4)**. During anaphase I, homologous chromosomes separate **(A5)**. During telophase I, chromosomes decondense **(A6)**. During anaphase II, the chromatids separate **(A7)** and decondense in telophase II and end up in tetrads (i.e., haploid cells **[A8]**). Due to the lack of homologous pairing, *spo11-1-3* **(B)**, *spo11-2-3* **(C)**, and the double mutant **(D)** do not form bivalents during diplotene (**[B2]** to **[D2]**). Separation of homologs is disturbed as indicated by the absence of congressed bivalents at the metaphase plate (**[B4]** to **[D4]**). Therefore, missegregation of chromosomes occurs in anaphase I (**[B5]** to **[D5]**) and II (**[B7]** to **[D7]**), resulting in polyads (**[B8]** to **[D8]**) instead of tetrads formed in the wild type **(A8)**. The double mutant *spo11-2-3 rad51-1* (**[E1]** to **[E8]**) shows a phenotype virtually identical to *spo11-2-3* (**[C1]** to **[C8]**).

lines and the total numbers of lines investigated are shown in Figure 4B. One explanation of the noncomplementation phenotype of the mutants transformed with the point-mutated constructs could be lack of RNA expression. To test this, we performed RT-PCR analyses of different transformed lines. For these analyses, we used the same amount of total RNA from (1) Col-0 wild type, (2) the original mutant lines *spo11-1-3* and *spo11-2-3*, (3) lines homozygous for the original mutation but carrying the functional complementation construct, and (4) carrying the point mutated construct. As shown in Figure 4C, all lines express the respective SPO11 RNA except the original mutants *spo11-1-3* and *spo11-2-3*. Therefore, the lack of complementation in the lines transformed with the respective point-mutated construct is not caused by missing RNA expression. Thus, the active Tyr residues of both SPO11 proteins seem indeed to be indispensable for meiotic DSB induction in *Arabidopsis*.

DISCUSSION

For years the biological role of the *SPO11-2* gene in plants remained elusive. Our initial analysis revealed a very weak expression in *Arabidopsis*, mainly in the reproductive tissues (Hartung and Puchta, 2000). For a long time no insertion mutant was available in the public *Arabidopsis* insertion libraries. Only very recently was a report published describing that the loss of *SPO11-2* results in a defect early on in meiosis (Stacey et al., 2006). Independently, we were also able to obtain a line from the GABI collection. The insertion of the T-DNA in this mutant is located near the 3′ end of the gene. Since the mutant produced mRNA at a similar level as the wild type, a truncated protein missing the C-terminal 44 amino acids with residual activity might still be present in the cells. Thus, the mutant analyzed might not present a complete null phenotype. Nevertheless, we found severe phenotypic changes: only few seeds were produced, and most of them were aneuploid and led to infertile plants. The semisterile phenotype could be reverted by reintroducing the intact *SPO11-2* gene under its own promoter but not by an identical construct harboring a point mutation that alters the catalytically active Tyr into Phe.

The finding that, in contrast with all other nonplant organisms, two functional SPO11 homologs are required for proper meiosis in *Arabidopsis* clearly indicates that both proteins have a nonredundant function in the initiation of meiotic recombination. Cytological analysis could not discriminate between the phenotype of both mutants; however, this does not necessarily mean that both proteins are involved in exactly the same step during initiation of meiotic recombination. The fact that the *spo11-1-3 spo11-2-3* double mutant showed no further reduction in viable seed production in comparison to the *spo11-1-3* mutant suggests that both proteins are not involved in a parallel but in the same pathway. In accordance with this finding, both SPO11 mutants suppress the *rad51* phenotype. No traces of fragmented chromosomes (Li et al., 2004) could be observed during meiosis in both *spo11 rad51* double mutants. This demonstrates not only that SPO11-1 and -2 are required earlier on in meiosis than RAD51 but also that in the absence of one or the other SPO11 homolog, no DSB is formed.

All three *spo11-1* lines described by Grelon et al. (2001) produced a seed amount comparable to our *spo11-2-3* mutant. Stacey et al. (2006) described a *spo11-2-2* mutant harboring a T-DNA insertion in close proximity to the insertion site of the *spo11-2-3* line described here. *spo11-2-2* produced only 0.3% of seeds compared with nearly 10% in *spo11-2-3*. Taking a closer look at the integration site reveals that there is only a subtle difference of seven amino acids for which a truncated protein putatively expressed in *spo11-2-2* is longer than in *spo11-2-3*. This short stretch of amino acids is located outside of the functional domains conserved (Bergerat et al., 1997; Hartung and Puchta, 2000). Therefore, it is difficult to explain the phenotypic difference between the *SPO11-2* mutants by a different activity of the residual protein.

Stacey et al. (2006) observed that plants doubly heterozygous for the *SPO11-1* and *-2* T-DNA insertions were partially sterile. This phenomenon is known as nonallelic noncomplementation (Schulze-Lefert, 2004). We did not find a similar phenomenon with our mutants. By contrast, our heterozygous double mutants of *SPO11-1* and *-2* were fully fertile, showing no reduced fertility and enabling us to analyze its meiotic behavior. Moreover, the offspring of *spo11-1-3 spo11-2-3* heterozygotes could be propagated further for at least three generations without showing sterility or aneuploidy. For us, the most simple explanation for these conflicting results is the possibility that the nonallelic noncomplementation reported for *SPO11-1* and *-2* double heterozygous mutant plants (Stacey et al., 2006) is the result of a special effect like negative complementation in this particular mutant combination and not a general property of the two genes.

Are SPO11-1 and SPO11-2 Part of a Single DSB-Inducing Protein Complex?

In contrast with the wild-type genes, we were not able to complement the *spo11-1* or *spo11-2* mutants with the respective genes mutated in their catalytically active Tyr residues. We regard the possibility that the lack of complementation was due to a defect in expression as unlikely. First, we could demonstrate that the mRNA amount originating from mutated and wild-type transgenes in both mutants was similar (Figure 4C). Moreover, we noted that in the wild-type background, the mutated transgenes led to a certain reduction of the number of seeds (data not shown). This could be taken as a hint that the mutated proteins might even hinder DSB induction by negative complementation.

The simplest hypothesis to explain our findings is that a heterologous complex consisting of functional SPO11-1 and -2 proteins is required for DSB induction. In bakers' yeast, at least 11 other proteins are necessary for DSB induction during onset of meiosis, and most of these proteins are conserved in multicellular eukaryotes (Keeney, 2001; Caryl et al., 2003; Arora et al., 2004; Pawlowski and Cande, 2005; Hamant et al., 2006). Recently, the in vivo assembly of a SPO11 complex has been shown in *Saccharomyces cerevisiae* (Sasanuma et al., 2007). These authors used wild-type SPO11 constructs and also those with a point mutation at the catalytically active Tyr fused to a GAL4 or FLAG binding domain. Via chromatin immunoprecipitation, they found a number of complexes containing the different SPO11 fusion

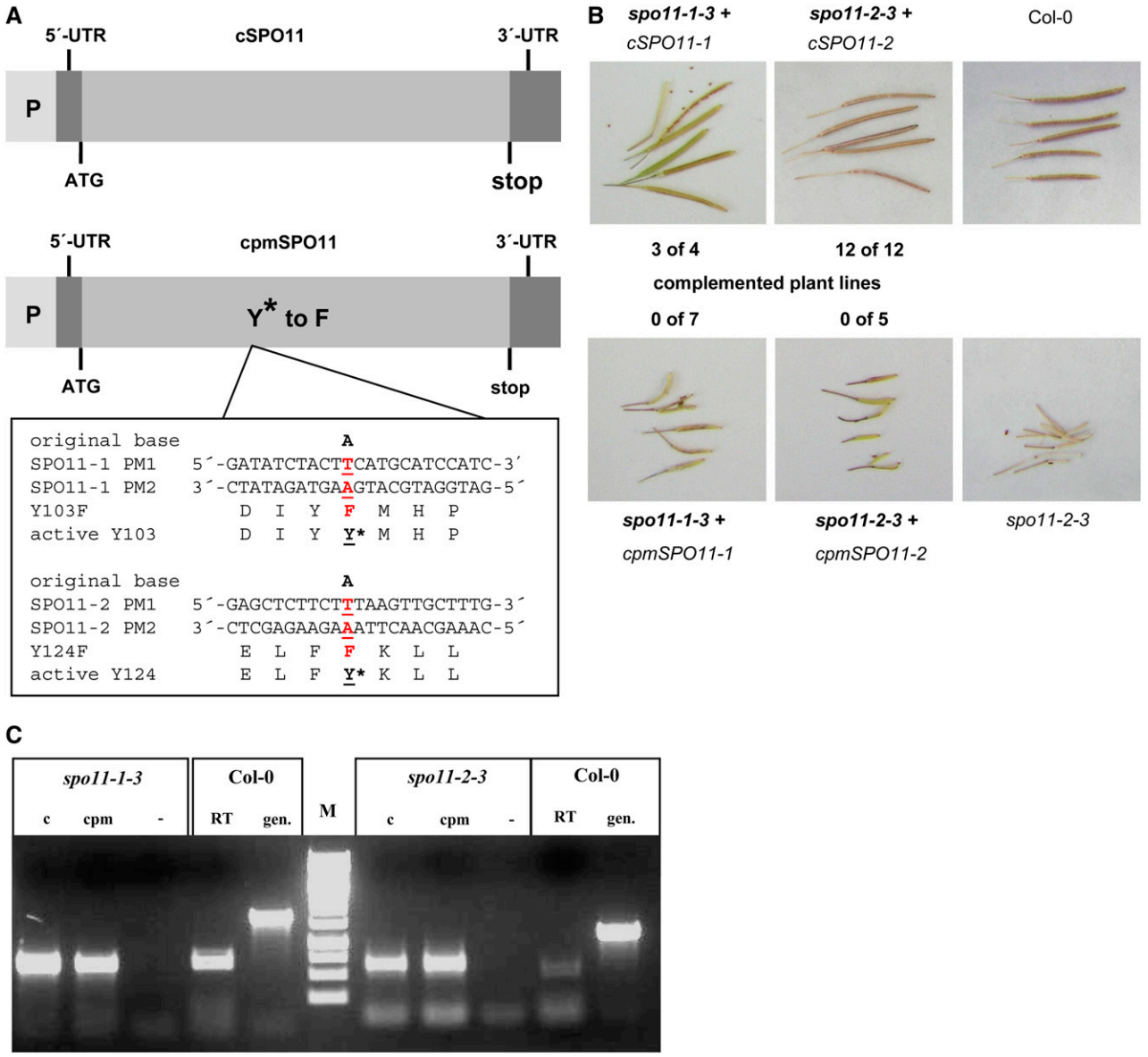

Figure 4. Complementation of *spo11-1* and *spo11-2* Mutants.

(A) Wild-type and altered constructs with the sequence mutated by primer-directed PCR mutagenesis of both constructs and the primers PM1 and 2 for both genes are shown. The mutations changed adenine into thymine, and the resulting amino acid is Phe instead of Tyr, as indicated by the asterisk.
(B) Siliques exemplary for plants transformed successfully with the constructs for complementation or with the point-mutated constructs are shown. All lines analyzed were confirmed to be homozygous for their respective T-DNA insertion in SPO11-1 or -2 by PCR. Numbers of successfully complemented mutants are given between the silique pictures. Whereas nearly all lines transformed with the intact construct showed complementation, none of the lines transformed with the point-mutated form could be rescued. c, functional complementation construct; cpm, complementation construct point mutated.
(C) RT-PCR analysis from *spo11-1-3* and *spo11-2-3*, transformed with the functional or the point-mutated complementation construct, are shown. All reverse transcription reactions were done using the same amount of total-RNA (5 μg) but different PCR cycle numbers for the detection of SPO11-2 cDNA (SPO11-1, 33 cycles; SPO11-2, 38 cycles). All transformed lines expressed the respective SPO11 RNA, whereas the original mutants did not. Lanes: c, functional complementation construct; cpm, complementation construct point mutated; −, the respective homozygous *spo11* mutant; RT, RT-PCR from wild-type RNA; gen., PCR of genomic wild-type DNA.

proteins. Furthermore, these interactions are only visible at meiosis and require the additional proteins REC102, 104, and 114. This might explain why we did not succeed, although we attempted to show an interaction between At SPO11-1 and -2 in vivo by split–yellow fluorescent protein analysis. Such a putative protein complex might only be present during the onset of meiosis in plant cells and might be regulated by posttranslational modification (e.g., phosphorylation). Alternatively, an interaction may require additional proteins specific for meiotic cells, such as REC102, 104, 114, or SKI8 in *S. cerevisiae* (Arora et al., 2004; Li et al., 2006; Maleki et al., 2007; Sasanuma et al., 2007). Besides the putative complex formation, another intriguing question is if SPO11-2 is epistatic with all the proteins that depend on SPO11-1–induced DSBs or if they are involved in SC formation, such as MER3, HOP1, MND1, and others (Couteau et al., 1999; Bleuyard et al., 2004; Li et al., 2005; Mercier et al., 2005; Pawlowski and Cande, 2005; Hamant et al., 2006; Kerzendorfer et al., 2006; Mezard et al., 2007). Furthermore, it is still unclear if indeed both proteins are transiently attached to the broken DNA ends or if only one of them is. It could be envisaged that both proteins act as a heterodimer and each DNA strand is broken by a different SPO11 homolog, a mechanism resembling that of yeast topoisomerase II (Liu and Wang, 1998, 1999). Alternatively, the concerted action of two catalytically active Tyr residues in a dimeric configuration might be essential to generate a DSB as proposed for SPO11 in *S. cerevisiae* (Sasanuma et al., 2007). Thus, although we were finally able to define an essential functional role for At SPO11-2 in the initiation of DSBs during meiotic recombination in plants, a series of new questions arises concerning the details of the DSB initiation process.

METHODS

Cytological Analysis of Male Meiosis

For comparison of the phenotype between mutants of SPO11-1 and -2, we used a previously described T-DNA insertion line of SPO11-1 from the SALK collection named *spo11-1-3*. This line was originally described by Stacey et al. (2006) and is different from the line *spo11-1-3* described by Pradillo et al. (2007). We refer to the SALK-line 146172 as *spo11-1-3* (Alonso et al., 2003).

The 4′,6-diamidino-2-phenylindole (DAPI) staining of pollen mother cells was performed as described (Maluszynska and Heslop-Harrison, 1991) and modified according to Bleuyard et al. (2004). Inflorescences were collected and incubated in 2 mM 8-hydroxyquinoline for 30 min at room temperature. After removal of the liquid, the plant material was destained two or three times for 30 min in a mixture of methanol and acetic acid in a 3:1 ratio. In this fixative, storage at −20°C is possible.

Fixed inflorescences were washed twice in 0.01 M citrate buffer (4 mL of 0.1 M citric acid, 6 mL of 0.1 M sodium citrate, and 90 mL of distilled water, pH 4.8) for 5 min and then treated with 2% cellulase (Sigma-Aldrich) and 0.2% pectinase (Sigma-Aldrich) in citrate buffer for 30 min in a moist chamber at 37°C. This digestion was followed by washing the inflorescences twice for 5 min in citrate buffer.

Buds that did not exceed a size of 0.5 mm were prepared in 15 μL of 45% acetic acid. After adding a cover slip, the pollen grains were gently squeezed out of the dissected anthers. The slides were frozen in liquid nitrogen for 30 s, and then the cover slips were removed with a razor blade. The air-dried preparations were stained with DAPI (1.5 μg/mL) in Vectashield mounting medium (Bleuyard et al., 2004). Meiotic stages of pollen mother cells were observed using an epifluorescence microscope with an appropriate DAPI filter.

Molecular Characterization of the Transformants

Transformation of *Arabidopsis thaliana* was performed as described (Clough and Bent, 1998). Due to the sterility of the *spo11* mutants, heterozygous plants had to be used for the transformation. For genotyping of *spo11-2-3*, DNA was extracted from a small leaf of transformants grown in soil. For PCR analysis, first primers (P1, 5′-TCCTGATCTGCCAATTCTTG-3′; P2, 5′-CCATGACAATAGAGAGCTTC-3′; Figure 1A) were used enabling an amplification of the sequence that is interrupted in the mutant. With this primer combination, DNA extracts of homozygous plants showed no signal, whereas heterozygous plants produced a PCR band indicative for the uninterrupted wild-type gene. As a positive control, in DNA extracts of homozygous and heterozygous plants, the presence of the insertion was proved using a left border–specific primer (GABI-LB, 5′-TTGGACGTGAATGTAGACAC-3′) combined with the forward primer P1, which binds upstream of the insertion site in the genomic sequence.

Flow Cytometric Estimation of the DNA Content and FISH

Nuclei of one to two fresh young leaves of individual At *spo11-2-3* mutant and Col wild-type plants were either isolated simultaneously or separately together with *Raphanus sativus* cv Voran (as internal standard), stained with propidium iodide as described (Galbraith et al., 1983), and analyzed on a FACSAria or FACStar^{PLUS} flow cytometer (BD Biosciences). Propidium iodide fluorescence was measured using a 610-nm band-pass filter. Usually, a total of 10,000 nuclei per sample were analyzed. The DNA content of the mutants was calculated based on the ratios of values of the 2C or 4C peak means between the wild type and mutant.

Flow sorting of nuclei, amplification and labeling of the 180-bp centromeric repeat of *Arabidopsis*, FISH, and microscopic evaluation were performed as described previously (Pecinka et al., 2004; Berr et al., 2006).

Complementation Experiments

To rescue the observed phenotypes and to investigate whether a functional protein is necessary for a successful complementation, we generated different constructs. For normal complementation, the constructs consisted of the entire gene (SPO11-1 or -2), including 553 or 704 bp of the promoter region, respectively, and 496 bp of the 3′-UTR each. The genomic region was amplified using linker primers and a high proofreading polymerase (Phusion-*Taq*; New England Biolabs). The fragments cloned into the vector pPZP221 (Hajdukiewicz et al., 1994) were sequenced and subsequently used for introducing point mutations. An adenine-to-thymine mutation was achieved by primer-directed mutagenesis (Figure 4). All constructs were transformed via *Agrobacterium tumefaciens* and the floral dip method (Clough and Bent, 1998) into the plant lines. After screening for successfully transformed plants using hygromycin selection, we tested the genotype of the transformed plants by PCR with primers from outside of the complementation constructs. Only plants homozygous for the respective T-DNA insertion in *SPO11-1* or *-2* were analyzed further.

RT-PCR Analyses

RNA from young *Arabidopsis* plantlets was isolated using the RNeasy plant mini kit from Qiagen according to the instructions of the manufacturer. Reverse transcription was performed using the RevertAid first-strand cDNA synthesis kit from Fermentas according to the instructions of the manufacturer. We modified the reverse transcription reaction using an oligo(dT) anchor primer, consisting of 17 Ts and two wobble bases instead

of the oligo(dT) primer (consisting of 17 Ts only) originally supplied. As input amount, we used 5 μg of total RNA for each RT reaction.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: At3g13170 (*SPO11-1*), At1g63990 (*SPO11-2*), and At5g20850 (*RAD51*).

ACKNOWLEDGMENTS

We thank Bernd Reiss for the kind gift of the At *rad51-1* mutant, Manfred Focke for thorough reading of the manuscript, and Sabine Zeiler and Andrea Kunze for technical assistance. This work was supported by Deutsche Forschungsgemeinshaft Grant Pu137/6.

Received August 6, 2007; revised October 10, 2007; accepted October 15, 2007; published October 26, 2007.

REFERENCES

Alonso, J.M., et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana.* Science **301:** 653–657.

Arora, C., Kee, K., Maleki, S., and Keeney, S. (2004). Antiviral protein Ski8 is a direct partner of Spo11 in meiotic DNA break formation, independent of its cytoplasmic role in RNA metabolism. Mol. Cell **13:** 549–559.

Bergerat, A., de Massy, B., Gadelle, D., Varoutas, P.C., Nicolas, A., and Forterre, P. (1997). An atypical topoisomerase II from Archaea with implications for meiotic recombination. Nature **386:** 414–417.

Berr, A., Pecinka, A., Meister, A., Kreth, G., Fuchs, J., Blattner, F.R., Lysak, M.A., and Schubert, I. (2006). Chromosome arrangement and nuclear architecture but not centromeric sequences are conserved between *Arabidopsis thaliana* and *Arabidopsis lyrata.* Plant J. **48:** 771–783.

Bleuyard, J.Y., Gallego, M.E., and White, C.I. (2004). The atspo11-1 mutation rescues atxrcc3 meiotic chromosome fragmentation. Plant Mol. Biol. **56:** 217–224.

Caryl, A.P., Jones, G.H., and Franklin, F.C. (2003). Dissecting plant meiosis using *Arabidopsis thaliana* mutants. J. Exp. Bot. **54:** 25–38.

Celerin, M., Merino, S.T., Stone, J.E., Menzie, A.M., and Zolan, M.E. (2000). Multiple roles of Spo11 in meiotic chromosome behavior. EMBO J. **19:** 2739–2750.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana.* Plant J. **16:** 735–743.

Couteau, F., Belzile, F., Horlow, C., Grandjean, O., Vezon, D., and Doutriaux, M.P. (1999). Random chromosome segregation without meiotic arrest in both male and female meiocytes of a dmc1 mutant of Arabidopsis. Plant Cell **11:** 1623–1634.

Dernburg, A.F., McDonald, K., Moulder, G., Barstead, R., Dresser, M., and Villeneuve, A.M. (1998). Meiotic recombination in *C. elegans* initiates by a conserved mechanism and is dispensable for homologous chromosome synapsis. Cell **94:** 387–398.

Diaz, R.L., Alcid, A.D., Berger, J.M., and Keeney, S. (2002). Identification of residues in yeast Spo11p critical for meiotic DNA double-strand break formation. Mol. Cell. Biol. **22:** 1106–1115.

Gadelle, D., Filee, J., Buhler, C., and Forterre, P. (2003). Phylogenomics of type II DNA topoisomerases. Bioessays **25:** 232–242.

Galbraith, D.W., Harkins, K.R., Maddox, J.M., Ayres, N.M., Sharma, D.P., and Firoozabady, E. (1983). Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science **220:** 1049–1051.

Grelon, M., Vezon, D., Gendrot, G., and Pelletier, G. (2001). AtSPO11-1 is necessary for efficient meiotic recombination in plants. EMBO J. **20:** 589–600.

Hajdukiewicz, P., Svab, Z., and Maliga, P. (1994). The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol. Biol. **25:** 989–994.

Hamant, O., Ma, H., and Cande, W.Z. (2006). Genetics of meiotic prophase I in plants. Annu. Rev. Plant Biol. **57:** 267–302.

Hartung, F., Angelis, K.J., Meister, A., Schubert, I., Melzer, M., and Puchta, H. (2002). An archaebacterial topoisomerase homolog not present in other eukaryotes is indispensable for cell proliferation of plants. Curr. Biol. **12:** 1787–1791.

Hartung, F., and Puchta, H. (2000). Molecular characterisation of two paralogous SPO11 homologues in *Arabidopsis thaliana.* Nucleic Acids Res. **28:** 1548–1554.

Hartung, F., and Puchta, H. (2001). Molecular characterization of homologues of both subunits A (SPO11) and B of the archaebacterial topoisomerase 6 in plants. Gene **271:** 81–86.

Keeney, S. (2001). Mechanism and control of meiotic recombination initiation. Curr. Top. Dev. Biol. **52:** 1–53.

Keeney, S., Giroux, C.N., and Kleckner, N. (1997). Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. Cell **88:** 375–384.

Keeney, S., and Neale, M.J. (2006). Initiation of meiotic recombination by formation of DNA double-strand breaks: Mechanism and regulation. Biochem. Soc. Trans. **34:** 523–525.

Kerzendorfer, C., Vignard, J., Pedrosa-Harand, A., Siwiec, T., Akimcheva, S., Jolivet, S., Sablowski, R., Armstrong, S., Schweizer, D., Mercier, R., and Schlogelhofer, P. (2006). The *Arabidopsis thaliana* MND1 homologue plays a key role in meiotic homologous pairing, synapsis and recombination. J. Cell Sci. **119:** 2486–2496.

Li, J., Hooker, G.W., and Roeder, G.S. (2006). *Saccharomyces cerevisiae* Mer2, Mei4 and Rec114 form a complex required for meiotic double-strand break formation. Genetics **173:** 1969–1981.

Li, W., Chen, C., Markmann-Mulisch, U., Timofejeva, L., Schmelzer, E., Ma, H., and Reiss, B. (2004). The Arabidopsis AtRAD51 gene is dispensable for vegetative development but required for meiosis. Proc. Natl. Acad. Sci. USA **101:** 10596–10601.

Li, W., Yang, X., Lin, Z., Timofejeva, L., Xiao, R., Makaroff, C.A., and Ma, H. (2005). The AtRAD51C gene is required for normal meiotic chromosome synapsis and double-stranded break repair in Arabidopsis. Plant Physiol. **138:** 965–976.

Liu, Q., and Wang, J.C. (1998). Identification of active site residues in the "GyrA" half of yeast DNA topoisomerase II. J. Biol. Chem. **273:** 20252–20260.

Liu, Q., and Wang, J.C. (1999). Similarity in the catalysis of DNA breakage and rejoining by type IA and IIA DNA topoisomerases. Proc. Natl. Acad. Sci. USA **96:** 881–886.

Maleki, S., Neale, M.J., Arora, C., Henderson, K.A., and Keeney, S. (2007). Interactions between Mei4, Rec114, and other proteins required for meiotic DNA double-strand break formation in *Saccharomyces cerevisiae.* Chromosoma **116:** 471–486.

Maluszynska, J., and Heslop-Harrison, J.S. (1991). Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana.* Plant J. **1:** 159–166.

McKim, K.S., and Hayashi-Hagihara, A. (1998). mei-W68 in *Drosophila melanogaster* encodes a Spo11 homolog: evidence that the mechanism for initiating meiotic recombination is conserved. Genes Dev. **12:** 2932–2942.

Mercier, R., Jolivet, S., Vezon, D., Huppe, E., Chelysheva, L., Giovanni, M., Nogue, F., Doutriaux, M.P., Horlow, C., Grelon, M., and Mezard, C. (2005). Two meiotic crossover classes cohabit in Arabidopsis: One is dependent on MER3, whereas the other one is not. Curr. Biol. **15:** 692–701.

Mezard, C., Vignard, J., Drouaud, J., and Mercier, R. (2007). The road to crossovers: Plants have their say. Trends Genet. **23:** 91–99.

Pawlowski, W.P., and Cande, W.Z. (2005). Coordinating the events of the meiotic prophase. Trends Cell Biol. **15:** 674–681.

Pecinka, A., Schubert, V., Meister, A., Kreth, G., Klatte, M., Lysak, M.A., Fuchs, J., and Schubert, I. (2004). Chromosome territory arrangement and homologous pairing in nuclei of *Arabidopsis thaliana* are predominantly random except for NOR-bearing chromosomes. Chromosoma **113:** 258–269.

Pradillo, M., Lopez, E., Romero, C., Sanchez-Moran, E., Cunado, N., and Santos, J.L. (2007). An analysis of univalent segregation in meiotic mutants of *Arabidopsis thaliana*: A possible role for synaptonemal complex. Genetics **175:** 505–511.

Prieler, S., Penkner, A., Borde, V., and Klein, F. (2005). The control of Spo11's interaction with meiotic recombination hotspots. Genes Dev. **19:** 255–269.

Rosso, M.G., Li, Y., Strizhov, N., Reiss, B., Dekker, K., and Weisshaar, B. (2003). An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol. Biol. **53:** 247–259.

Samoylova, T.I., Meister, A., and Misera, S. (1996). The flow karyotype of *Arabidopsis thaliana* interphase chromosomes. Plant J. **10:** 949–954.

Sasanuma, H., Murakami, H., Fukuda, T., Shibata, T., Nicolas, A., and Ohta, K. (2007). Meiotic association between Spo11 regulated by Rec102, Rec104 and Rec114. Nucleic Acids Res. **35:** 1119–1133.

Schulze-Lefert, P. (2004). Plant immunity: The origami of receptor activation. Curr. Biol. **14:** R22–R24.

Stacey, N.J., Kuromori, T., Azumi, Y., Roberts, G., Breuer, C., Wada, T., Maxwell, A., Roberts, K., and Sugimoto-Shirasu, K. (2006). Arabidopsis SPO11-2 functions with SPO11-1 in meiotic recombination. Plant J. **48:** 206–216.

Sugimoto-Shirasu, K., Roberts, G.R., Stacey, N.J., McCann, M.C., Maxwell, A., and Roberts, K. (2005). RHL1 is an essential component of the plant DNA topoisomerase VI complex and is required for ploidy-dependent cell growth. Proc. Natl. Acad. Sci. USA **102:** 18736–18741.

Sugimoto-Shirasu, K., and Roberts, K. (2003). "Big it up": Endoreduplication and cell-size control in plants. Curr. Opin. Plant Biol. **6:** 544–553.

Sugimoto-Shirasu, K., Stacey, N.J., Corsar, J., Roberts, K., and McCann, M.C. (2002). DNA topoisomerase VI is essential for endoreduplication in Arabidopsis. Curr. Biol. **12:** 1782–1786.