AtSPO11-1 is necessary for efficient meiotic recombination in plants

Mathilde Grelon¹, Daniel Vezon, Ghislaine Gendrot and Georges Pelletier

Station de Génétique et d’Amélioration des Plantes, INRA de Versailles, Route de St-Cyr, 78026 Versailles Cedex, France

¹Corresponding author
e-mail: grelon@versailles.inra.fr

The Saccharomyces cerevisiae Spol1 protein catalyses DNA double-strand breaks (DSBs) that initiate meiotic recombination. The model plant Arabidopsis thaliana possesses at least three SPO11 homologues. T-DNA and ethyl-methane sulfonate mutagenesis allowed us to show that meiotic progression is altered in plants in which the AtSPO11-1 gene is disrupted. Both male and female meiocytes formed very few bivalents. Furthermore, no fully synapsed chromosomes were observed during prophase I. Later, in meiosis I, we observed that chromosomes segregated randomly, leading to the production of a large proportion of non-functional gametes. These meiotic aberrations were associated with a drastic reduction in meiotic recombination. Thus, our data show that initiation of meiotic recombination by SPO11-induced DSBs is a mechanism conserved in plants. Furthermore, unlike Drosophila and Caenorhabditis elegans, but like fungi, SPO11 is necessary for normal synopsis in plants.

Keywords: Arabidopsis thaliana/meiosis/mutants/recombination/SPO11

Introduction

In contrast to animals, plant organogenesis is a continuous process, and somatic recombination can therefore lead to transmittable genetic variations. Nevertheless, even in plants, meiosis remains the major source of genetic variability. Cross-overs and genetic conversion generate recombination between homologous chromosomes, reorganizing the allelic combinations among them. Cross-overs also establish physical links between homologous chromosomes (chiasmata) so that these remain linked together even when the synaptonemal complex (SC) disappears. This mechanism thus ensures proper segregation of chromosomes at anaphase I when chiasmata are released (for a review see Roeder, 1997), allowing random segregation of paternal and maternal chromosomes (inter-chromosomal recombination).

In budding yeast, meiotic recombination is initiated by double-strand breaks (DSBs) (Sun et al., 1989; Cao et al., 1990), which will undergo strand exchange interactions with an intact partner duplex. The formation of DSBs requires at least nine different genes (SPO11, RAD50, MRE11, XRS2, MER2, MEI4, REC102, REC104, REC114) (reviewed in Smith and Nicolas, 1998; Pâques and Haber, 1999). There are now several indications that DSBs in Saccharomyces cerevisiae are initiated by the Spo11 protein, which has been found linked to the 5' termini of the broken DNA molecules in rad50 yeast mutants (Keeney et al., 1997). Spo11 belongs to a novel family of type II topoisomerase (topoisomerase VI, subunit A), suggesting that Spo11 is the protein that catalyses the formation of DSBs through a 5'-phosphotyrosyl linkage (Bergerat et al., 1997). Sequence comparison of the members of the topoisomerase VI/SPO11 family, as well as structural analyses of one of its members (topoisomerase VI A of Methanococcus jannaschii; Nichols et al., 1999), permitted the definition of several conserved domains involved in DNA binding and cleavage (Bergerat et al., 1997; Aravind et al., 1998; Nichols et al., 1999). Evidence for the generation of DSBs during meiosis of other organisms has only been obtained for Schizosaccharomyces pombe (Cervantes et al., 2000; Zenvirith and Simchen, 2000), but SPO11 homologues have been identified in Drosophila melanogaster (mei-W68; McKim and Hayashi-Hagihara, 1998), Caenorhabditis elegans (Dernburg et al., 1998), S.pombe (REC12; Lin and Smith, 1994), Coprinus cinereus (Cummings et al., 1999; Celerin et al., 2000) and mammals (Keeney et al., 1999; Romanienko and Camerini-Otero, 1999; Shannon et al., 1999; Metzler-Guillemain and de Massy, 2000), suggesting that enzymatic generation of DSBs is conserved among all these organisms. As a matter of fact, meiotic defects (including absence of recombination) were found to be associated with SPO11 disruption not only in yeasts (Klapholz et al., 1985; Atcheson et al., 1987; Giroux et al., 1989; Lin and Smith, 1994), but also in Drosophila (McKim and Hayashi-Hagihara, 1998), C.elegans (Dernburg et al., 1998) and Coprinus (Celerin et al., 2000). Differences between species appeared when authors investigated the relationship between synopsis (homologous chromosome association through the SC) and SPO11 disruption. spo11 mutations in fungi co-ordinately affect recombination and SC formation (Giroux et al., 1989; Celerin et al., 2000), whereas SC formation in Drosophila and C.elegans was found to be independent of SPO11 (Dernburg et al., 1998; McKim et al., 1998). These results underline the importance of studying several independent models to understand the relationship between recombination and synopsis.

In addition to DSB formation and synopsis, additional functions in early chromosome pairing (close juxtaposition of homologous chromosomes; Loidl et al., 1994; Weiner and Kleckner, 1994) and S-phase replication rates (Cha et al., 2000) have been assigned to Spo11 in S.cerevisiae. The recent study of Cha et al. (2000) showed that early roles of Spo11 (chromosome pairing and replication control) can be uncoupled from later ones...
(DSB formation) in mutants where the tyrosine involved in DSB cleavage is replaced by a phenylalanine (spo11Y135F).

In plants, very few molecular data have been obtained on meiosis. Even less is known about meiotic recombination: the only functional data come from the analyses on the Arabidopsis dmc1 mutant that was shown to be deficient in bivalent formation during male and female meiosis (Couteau et al., 1999). The Lilium RecA homologues (LIM15 and RAD51) (Terasawa et al., 1995; Anderson et al., 1997) as well as the maize RAD51 protein (Franklin et al., 1999) have been studied by immunolocalization on meiotic chromosomes, implicating these proteins in several aspects of meiosis in plants, including homology searches between chromosomes, pairing and recombination. In addition, several synaptic mutants have been described in Arabidopsis (Ross et al., 1997) that could lead to the characterization of other genes potentially involved in recombination since most of these mutations are thought to reflect defects in recombination (Dawe, 1998). Until now, the only report of Arabidopsis mutants affected in meiotic recombination comes from Masson and Paszkowski (1997), who selected several lines according to their hypersensitivity to X-ray irradiation (Masson et al., 1997). Two of them were found to present defects in somatic and meiotic recombination (Masson and Paszkowski, 1997).

Sequencing of the Arabidopsis thaliana genome is nearly complete and allowed Hartung and Puchta (2000) to describe two Spo11 homologues in this model plant. A third Spo11-like coding sequence can be found in the DDBJ/EMBL/GenBank database (accession No. CAB86036). Until now, Spo11 genes have been found in every eukaryote tested, but only plants possess more than one homologue. The meaning of such sequence redundancy is not yet understood. The study presented here shows that mechanisms by which recombination initiates are conserved between yeast, animals and plants, and that disruption of the AtSpo11-1 gene is sufficient to drastically reduce meiotic recombination and pachytene-stage homologue juxtaposition.

Results

Isolation of spo11-1 mutants of A.thaliana

To identify mutants affected in meiosis, we screened the Versailles collection of T-DNA-transformed lines (Bechtold et al., 1993) as well as ethyl-methane sulfonate (EMS)-mutagenized lines for reduced fertility. Screening for sterility among mutagenized plants allowed two allelic mutations conferring a semi-sterile phenotype (Figure 1) to be isolated.

The semi-sterile phenotype segregated 3:1 as a single recessive mutation in both lines (see Materials and methods).

According to the results shown later, the T-DNA and EMS alleles were called spo11-1-1 and spo11-1-2, respectively.

Male and female fertilities are affected in spo11-1 mutants

Plants heterozygous for one of the spo11-1 mutations could not be distinguished from wild type. Homozygous spo11-1 mutant plants exhibited an overall morphology similar to wild type, except for seed production (Figure 1). Both mutant alleles produced very reduced amounts of seeds (on average 2 seeds per silique instead of 45 ± 5 for wild-type plants). Furthermore, 32% of these seeds were abnormal (dark, shrunken and non-viable).

Seeds counted on spo11-1 mutants come from self-fertilization of the plants. In order to examine the effects of spo11-1 mutation on male and female fertilities individually, a spo11-1-1 homozygous mutant plant was used as either the female or the male parent in crosses with a wild-type strain of the same line [ecotype Wassilewskija (Ws)]. When the mutant was used as the female parent, an average of 3.2 seeds per silique were recovered (counted from 51 siliques) and among them very few abnormal seeds were observed (0.2 per silique). When reciprocal crosses were performed (using the spo11-1-1 mutant as male parent), an average of 10.1 seeds per silique were obtained (counted from 46 siliques). Among these, 33% were abnormal (dark and shrunken). These data show that abnormal seeds observed in selfing progeny probably come from male gamete defects. Furthermore, these crosses indicate that both male and female fertilities are impaired by the spo11-1-1 mutation, explaining the low level of seed production observed in spo11-1 selfing progeny.

To investigate the basis of these gametic defects, we examined mature gametophytes in mutant plants (Figure 2). The life cycle of flowering plants is predominantly diploid (sporophytic). The production of gametes occurs within haploid gametophytes (male pollen grains and female embryo sacs) differentiated within the sporophyte after meiosis.
Mature pollen grains scored for viability by Alexander staining (Figure 2A and B) showed that spo11-1 mutants produced a large proportion of dead pollen in their anthers (shown by arrows for spo11-1-1 in Figure 2B) when compared with wild type (Figure 2A), where 100% of the mature pollen was stained pink. Furthermore, remaining ‘viable’ pollen grains in mutants (monitored by the colouration of the cytoplasm) were variable in size and shape. In vitro germination of spo11-1-1 pollen showed that ~11% of the pollen grains (among 2969 investigated) germinated, whereas 97% germination of wild-type pollen was observed (data not shown).

On the female side, embryo sacs (mature female gametophytes) develop within ovules. In every ovule, a single nucellar cell differentiates into the megaspore mother cell and undergoes meiosis to produce a tetrad of haploid megaspores. Only one of these survives, and after three mitotic divisions gives rise to an eight-nuclei coenocytic embryo sac, which will then cellularize into a seven-cell embryo sac (Figure 2C). As the embryo sac develops, surrounding ovule tissues (nucellus, integuments and funiculus) evolve to produce a fully curved (anatrophe) mature ovule (for a review see Schnitz, et al., 1995). Wild-type 2-mm pistils were cleared and observed by differential interference contrast microscopy (DIC). The mature embryo sac composed of seven mature cells could be observed (Figure 2C). In spo11-1-1 plants, however, only 3% (among 1358 observed) of the ovules showed such differentiated female gametophytes. In the remaining ovules, we observed fully developed ovules with no distinguishable embryo sac in 74% of the cases (Figure 2D). However, some intermediate stages of development were sometimes observed, as shown in Figure 2E and F, where apparently mature ovules (as defined by the anatrophe shape) contain embryo sacs with one, two or four nuclei, suggesting that meiosis occurred but differentiation of the embryo sac has stopped at different stages of development.

Data are shown for spo11-1-1, but were also observed for the spo11-1-2 allele.

Both male and female meiosis are altered in spo11-1 mutants

To further determine what stages of sporogenesis or gametogenesis are impaired in spo11-1 mutants, we observed the developing male and female gametophytes by DIC after clearing treatments.

Comparison of early steps of male sporogenesis did not reveal any difference between wild-type and mutant plants (Figure 3A and B): round pollen mother cells (PMCs) could be distinguished within the anther loculus. Later, when these cells had performed the two meiotic divisions, we could observe, in wild-type anthers, the characteristic tetrad of microspores encased in a callose wall (Figure 3C). In mutant plants (data are shown only for spo11-1-1), meiosis products encased in a callose wall were observed (Figure 3D), but instead of a regular tetrahedral structure, either asymmetric tetrads or ‘polyads’ containing more than four meiotic products could be distinguished.

Male meiosis was therefore investigated, staining chromosomes with 4’,6-diamidino-2-phenylindole (DAPI) (Figures 4, 5, 7 and 8). Wild-type Arabidopsis meiosis has already been described in detail by Ross et al. (1996) and Figure 4 summarizes its major stages. At metaphase I, the five bivalents of Arabidopsis are easily distinguishable (Figure 4A), each of them being composed of two homologous chromosomes linked together by chiasmata.
(see also Figure 7F). Anaphase I leads to the formation of dyads containing two pools of five chromosomes (Figure 4B). A partial decondensation of the chromosomes (Figure 4C) occurs before meiosis II, during which segregation of sister chromatids happens, producing four pools of five chromosomes (Figure 4D). Decondensation of chromosomes (Figure 4E) and cytokinesis then take place, producing tetrads of microspores encased in callose (Figures 4F and 3C).

In spo11-1 mutant PMCs, the 10 Arabidopsis chromosomes were condensed, but bivalents were rarely formed (Figure 5A). However, some chromosome bridges were observed (Figure 5B, arrow), showing that chromosomes can still sometimes form bivalents. Out of 84 spo11-1 cells in metaphase I or in the beginning of anaphase I, 36 contained chromosomes showing evidence of some crossover (39 chromosome bridges and 8 bivalents). These data suggest that ~7% of chiasmata are formed compared with wild type since an average of nine cross-overs per wild-type meiosis was reported by Copenhaver et al. (1998). Anaphase I proceeded, but because most homologous chromosomes were not linked together by chiasmata, segregation appeared to be random. Instead of the two pools of five chromosomes observed at metaphase II in wild-type cells (Figure 4B), variable partitioning of the 10 chromosomes was seen in mutant meiocytes (Figure 5C, D and E). Sister chromatids were then separated and cells with 20 chromosomes were observed (Figure 5F). Decondensation (Figure 5G) and cytokinesis occurred normally, producing the ‘polyads’ already described (Figure 3D) with variable number of cells (Figure 5H), reflecting the variation in numbers of pools of chromosomes obtained at the end of meiosis.

Female meiosis was also investigated after chromosome staining by propidium iodide and observation by confocal microscopy. Univalents were also observed at the end of prophase I (compare Figure 6C and A), leading to random segregation of the chromosomes at anaphase I (compare Figure 6B and D). Therefore, gametophyte development deficiencies described earlier appeared to be a consequence of aberrant meiosis in the mutants.

Chromosome behaviour during the earlier stage of meiosis was then analysed, comparing meiotic prophase I in wild type (Figure 7) and in the spo11-1-1 mutant (Figure 8). Figure 7A shows a leptotene nucleus with very thin chromosome threads. Later, synapsis progresses and can be identified in some nuclei where loops of paired and unpaired chromosomes alternate (Figure 7B, arrows). Synapsis is maximum at pachytene (Figure 7C and D), during which bivalents appear thick. Later, each member
of the bivalent tends to separate (Figure 7E), and chiasmata become visible at diakinesis (Figure 7F). Out of 90 cells in prophase I in wild type, we found six in leptotene (Figure 7A), 21 in diakinesis (Figure 7F) and 43 in pachytene (Figure 7C and D). The remaining cells (20) showed intermediate synapsis. The same analyses on the spo11-1-1 mutant did not allow the identification of any pachytene stage among 160 cells observed. Nevertheless, condensation progressed until diakinesis [eight cells were observed in leptotene (Figure 8A), 27 in diakinesis (Figure 8D) and 125 at intermediate stages of condensation like the one shown in Figure 8B and C], but fully synapsed chromosomes were never observed.

**Molecular characterization of spo11-1 mutations**

Plant DNA flanking the T-DNA insertion in the spo11-1-1 allele was cloned either by plasmid rescue or tail PCR. PCR mapping on the CIC YAC library showed that the two borders map on the same part of chromosome 3 on CIC clones 6H6 and 5G8. The Columbia allele of this gene was found in the database as part of a P1 clone sequenced on chromosome three of Arabidopsis (clone MJG9, DDBJ/EMBL/GenBank accession No. AP000375). The T-DNA insertion occurred between nucleotides 43553 and 43555. The EMS mutation (spo11-1-2) was mapped to chromosome 3, between morphological markers hy2 (12 cM)
and gll (30 cM), confirming the location of spo11-1 mutations on the top arm of chromosome 3 between 15 and 25 cM.

Southern analyses (not shown) showed that spo11-1-1 possessed two linked left borders (LB) and no right border (RB). Indeed, cloning and sequencing of the T-DNA flanking sequences showed that a recombined insertion had occurred (Figure 9). A first LB was truncated within the 24 bp repeat, which is observed frequently in T-DNA transformants (LB1; Figure 9). The RB region and the promotorless GUS coding sequence of the T-DNA were deleted, and replaced by a piece of the binary vector pGKB5 and a second LB, complementary to the first one (LB2; Figure 9).

We found that T-DNA insertion occurred in the first exon of AtSPO11-1 recently described by Hartung and Puchta (2000). T-DNA insertion in the spo11-1-1 allele disrupted the gene as early as the first exon (Figures 9 and 10), probably leading to a null mutant. The Ws sequence for AtSPO11-1 was obtained (DDBJ/EMBL/GenBank accession No. AF302928) and compared with the Columbia sequence, showing 96% identity at the nucleotide level. cDNA was obtained through RT–PCR (data not shown) and encodes a protein showing 98.6% identity with the Columbia sequence (Figure 10). Expression studies performed by RT–PCR showed that AtSPO11-1 is expressed at a low level and mainly, but not exclusively, in reproductive tissues (data not shown). Interestingly, alternate splicing as described previously by Hartung and

Fig. 6. Propidium iodide staining of developing ovules at meiosis, observed with a confocal laser scanning microscope. Metaphase I of wild type (A) or spo11-1-1 mutant (C). Metaphase II of wild type (B) or spo11-1-1 mutant (D). Scale bar = 10 μm.

Fig. 7. DAPI staining of wild-type PMCs during meiotic prophase. (A) Leptotene stage. (B) Partial homologous pairing (arrows). (C and D) Pachytene stages showing fully “synapsed” homologous chromosomes. Some bright pericentromeric regions are indicated by arrows. (E) Diplotene stage. (F) Diakinesis stage. Scale bar = 10 μm.
Puchta (2000) was constantly observed. It is not clear yet how this pattern of intron retention is developmentally regulated. Sequencing of the AtSPO11-1 gene in the EMS allele (spo11-1-2) revealed a C to T transition in the second exon of AtSPO11-1, transforming a glutamine codon CAA into a stop codon TAA (Figures 9 and 10), confirming that mutant phenotypes observed in both mutants are the result of a non-functional AtSPO11-1 gene.

Hartung and Puchta (2000) described a second Arabidopsis SPO11-like gene located on chromosome 1 (DDBJ/EMBL/GenBank accession No. AJ251990). In addition, a third sequence putatively encoding a protein with strong similarity to Spo11 was detected in the Arabidopsis genome (chromosome 5, DDBJ/EMBL/GenBank accession Nos AL162973, CAB86036). We checked that this third gene is transcribed (DDBJ/EMBL/GenBank accession number No. AF323679). Figure 10 shows an alignment of several SPO11/Topo VI A orthologues including the three Arabidopsis homologues. The level of amino acid identity between AtSPO11-1 and S.cerevisiae Spo11 is 30%. Conserved regions I–V, as defined by Bergerat et al. (1997), are indicated. Region I contains the active tyrosine site (indicated by an asterisk), which is invariant among all the proteins of this family and which has been shown to be necessary for DSB induction in S.cerevisiae (Bergerat et al., 1997).

**AtSPO11-1 disruption drastically decreases meiotic recombination**

The S.cerevisiae Spo11 protein is thought to catalyse DNA DSBs that initiate meiotic recombination. To examine recombination frequencies in a spo11-1 mutant background, lines heterozygous for the spo11-1-1 mutation (Ws ecotype) were crossed to Columbia (Col-0). Meiotic recombination rates were investigated in the progeny of F$_2$ plants either homozygous or wild type. Microsatellites showing polymorphisms between the two ecotypes used (Bell and Ecker, 1994) were chosen to measure recombination frequencies on two chromosome arms: the bottom of chromosome 1 between markers nga280 and nga111, and the top of chromosome 5 between markers nga106 and nga76. MAPMAKER (Lander et al., 1987) was used to convert data into genetic distance in centimorgans (Table 1). According to the recent RI map (http://nasc.nott.ac.uk/), markers nga280 and nga111 are separ-
**Fig. 10. Clustal_X alignment of the three Arabidopsis SPO11-like proteins (Columbia ecotype) (AtSPO11-1, DDBJ/EMBL/GenBank accession No. CAB81544; AtSPO11-2, accession No. CAB81545; AtSPO11-3, accession No. CAB86036) with S. cerevisiae (Sc; accession No. AAA5532) and mouse (accession No. AAD52563) SPO11 proteins as well as S. shibatae topoisomerase VI A protein (accession No. Q05208). Conserved motifs I–V, as defined by Bergerat *et al.* (1997), are indicated in black boxes and conserved amino acids are shadowed in grey. The location of the T-DNA insertion in the spo11-1 allele and the stop mutation of the spo11-2 allele are indicated by arrowheads. An asterisk shows the conserved tyrosine (in position 135 in *S. cerevisiae*) thought to be responsible for DNA cleavage (Bergerat *et al.*, 1997). Amino acids that differ between *Ws* and *Columbia* AtSPO11-1 are underlined. An isoleucine replaces the phenylalanine (position 5), a glutamic acid replaces the valine (position 26), a serine replaces the asparagine (position 39), a leucine replaces the phenylalanine (position 65), and there is an insertion of a valine in position 213 between the underlined threonine and glycine.**
bypass the requirement for Spo11 in *C. elegans* (Dernburg *et al.*, 1998) and *C. cinereus* (Celerin *et al.*, 2000), as in *S. cerevisiae* (Thorne and Byers, 1993). These data, taken together with the fact that *Arabidopsis* spo11-1 mutants are defective in recombination, suggest that initiation of recombination via Spo11-induced DSBs is conserved in plants, even if there is no biochemical proof so far that any Spo11 can make DSBs *in vitro*.

### Why are there three SPO11-like genes in *Arabidopsis?*

It is likely that events initiating meiotic recombination are conserved between plants and other eukaryotes, but the existence of at least three SPO11-like genes in the *Arabidopsis* genome remains to be explained. It has emerged from *Arabidopsis* sequencing project data that this genome shows an unexpectedly high level of genetic redundancy, since as many as 75% of gene products show significant similarity to another protein coded in the genome (Bancroft, 2000). Our study proves that AtSPO11-1 is a functional homologue of other eukaryote SPO11 genes. The severe phenotype of both spo11-1 mutants we isolated shows that the other two AtSPO11 genes are not functionally redundant with AtSPO11-1. Nevertheless, recombination is not null in spo11-1-1 mutant (see Table 1), and we obtained cytological evidence for the formation of some bivalents (Figure 5B). These results are different from what was obtained for yeast, *Drosophila* and *C. elegans* spo11 mutants, where no evidence for any meiotic recombination events was obtained (Klaholz *et al.*, 1985; Dernburg *et al.*, 1998; McKim *et al.*, 1998). Even if a T-DNA insertion can sometimes lead to a leaky mutation, we have good reason to believe that spo11-1-1 is null since the T-DNA insertion occurred within the first exon of the gene. Therefore, it is plausible that at least one of the remaining AtSPO11-like genes is responsible for the low level of recombination we measured in the spo11-1-1 mutant, restoring (very partially) AtSPO11-1 function. However, we cannot exclude the possibility that other protein(s) partially responsible for DSB formation exist in plants and not in other eukaryotes. Another possibility is that environmental factors might be able to induce DSBs in plants and not in other eukaryotes where reproductive cells are less exposed to external factors.

Concerning the functions of AtSPO11-2 and -3, we do not possess much data yet. The specificity of expression of these genes, as well as their disruption, will give more information on their role in plants. Several hypotheses can already be raised. First, they might be involved in recombination within organs other than meiocytes since most recombination events are known to be induced by DSBs (Piques and Haber, 1999). Another explanation might be that these other two sequences have topoisomerase functions independent of recombination. Spo11 function in *S. cerevisiae* was elucidated thanks to the isolation of a new type of topoisomerase II from *Sulfolobus shibatae* (topoisomerase VI; Bergerat *et al.*, 1997). In the Archaea, this topoisomerase is composed of two subunits: A and B. Subunit A is homologous to Spo11 and subunit B has ATP binding activity. Homologues of topoisomerase VI subunit A were found in every eukaryote studied and all have meiotic functions similar

### Table I. Effect of spo11-1 mutation on meiotic recombination

<table>
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<tr>
<th>Genotype</th>
<th>No.</th>
<th>Map distance(^a) nga11/280</th>
<th>Map distance(^a) nga76/106</th>
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<td>nd</td>
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<tr>
<td></td>
<td>27</td>
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<td>30.7 (45)</td>
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<td></td>
<td>36.8 (116)</td>
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</tr>
</tbody>
</table>

\(^a\)Map distance calculated in centimorgans in the progeny of sister plants either homozygous for the *spo11-1* mutation (plants 15, 27 and 39), heterozygotes (plant 48) or wild type (plants 6 and 10). Total numbers of plants tested for each marker are indicated in parentheses. nd, not done.

### Discussion

#### Initiation of meiotic recombination in plants via DSBs

In this paper, we show that disruption of *AtSPO11-1* induces perturbation of male and female meiosis, with the formation of very few bivalents at the end of prophase I. This absence of association between homologous chromosomes can be explained by an absence of crossing over, prerequisite to any chiasmata and therefore to bivalent stabilization. Another possibility could be that recombination proceeds normally, but premature release of the chiasmata leads to univalent formation, as has already been reported for the *dy* and *dxy* mutants of maize (Maguire, 1978; Maguire *et al.*, 1993). The drastic decrease of meiotic recombination observed in a *spo11-1* background indicates that the first hypothesis is the correct one.

There is now quite a lot of evidence that Spo11 is the protein involved in the induction of DSBs in yeast, necessary for cross-overs and therefore chiasma formation. Even if the formation of DSBs has only been demonstrated in yeasts, recent analyses showed that induction of DSBs by Spo11 is probably conserved in *C. elegans* (Dernburg *et al.*, 1998), *D. melanogaster* (McKim and Hayashi-Hagihara, 1998) and *C. cinereus* (Celerin *et al.*, 2000). In plants, it has been shown that artificially induced DSBs (via \(\gamma\)-irradiation) of *Lilium longiflorum* buds can increase the number of chiasma per bivalent (Lawrence, 1961). Irradiation can also partially

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to yeast Spo11. Until now, no B subunit homologue has been found, even in the fully sequenced *S. cerevisiae* and *C. elegans* genomes. However, a BLAST search revealed topoisomerase VI B subunit-like sequences in several plant genomes, including *Arabidopsis* (DDBJ/EMBL/GenBank accession Nos AB025629, BAB02486). The fact that plants are the only eukaryotes to possess a *topoVI* gene and more than one *topoVI* coding sequence might indicate that plants have conserved topoisomerase VI function in addition to a specialized meiotic SPO11-topoisomerase VI-related function.

**What is the link between synopsis and recombination in plants?**

The relationship and timing between different events in meiotic prophase, including chromosome synopsis and recombination, are very striking and subject to debate. Historically, it was proposed that chromosomes synapse before they recombine. In yeast, however, it is now obvious that DSBs occur during the early stages of meiotic prophase (Padmore et al., 1991), well before the SC assembles and triggers synopsis between homologues. In this organism, there is also some evidence that recombination and synopsis are tightly coupled events (reviewed in Zickler and Kleckner, 1999). The yeast *spo11Δ* and *spo11Y135F* mutants do not form any SC (Giroux et al., 1989; Cha et al., 2000), showing that SC formation in yeast is dependent upon Spo11 function and possibly upon DSB formation. The discovery that in *C. elegans* (Dernburg et al., 1998) and *Drosophila* (McKim et al., 1998) *spo11* mutant chromosomes can pair and synapse without any recombination is very intriguing, but these may be exceptions. Indeed, the *C. cinerea* *spo11* mutant was recently found to be unable to form mature SC (Celerin et al., 2000). The analyses we present in this paper show that, in plants also, synopsis is affected when SPO11 function is absent, since no pachytene stages were observed in the *Arabidopsis spo11-1* mutant. Therefore, it seems that compared with other eukaryotes, *Drosophila* and *C. elegans* have different control mechanisms for SC formation.

**Meiosis in spo11-1 mutants is abnormal but not prevented, leading to chromosomal missegregations and production of unbalanced gametes**

Male and female meiosis in *spo11-1* mutants is perturbed, with little formation of bivalents at the end of prophase I; nevertheless, chromosome segregation occurs. Male meiosis of *spo11-1* mutants produces aberrant 'tetrads' composed of more than four cells, having different sizes. In *Arabidopsis*, all the meiotic mutants described so far produce this type of polyad (He et al., 1996; Peirson et al., 1996; Ross et al., 1997; Bhatt et al., 1999; Couteau et al., 1999; Yang et al., 1999). Some of the genes affected have been cloned and their concern mutations that affect recombination steps (*dmc1*; Couteau et al., 1999), chromosome synopsis (*asy1*; Caryl et al., 2000), chromosome or chromatid segregation (*syn1*, Bai et al., 1999; *dfl1*, Bhatt et al., 1999; *ask1*, Yang et al., 1999) or late abnormalities of division (*ms5*/tkhm; Glover et al., 1998).

Gametogenesis in *spo11-1* mutants, like for many other plant meiotic mutants, is not abolished since differentiations of male and female gametophytes is observed. Microspore aneuploidy, followed by chromosome staining with DAPI, results in a large proportion of aborted or abnormal pollen grains (only 11% of *spo11-1* pollen grains germinate *in vitro*). Studies on chromosomal rearrangements and pollen grain irradiation have shown that although both male and female gametophytes are fairly tolerant to additions to the single set of chromosomes (trisomics, duplications), the gametophytes are particularly sensitive to deficiencies, therefore most unbalanced gametes are lethal. On the female side, we mainly observed mature ovules that failed to differentiate a functional embryo sac, and stopped at different stages of development (no nucleus, one, two or four nuclei embryo sacs), probably according to the level of aneuploidy of the meiosis products. Therefore, female fertility (3% of mature embryo sacs) fits with what is expected from random segregation of the 10 unpaired chromosomes of *Arabidopsis*, as was suggested by Couteau et al. (1999) for the *At-dmc1* mutant phenotype \( [(1/2)^3 = 0.03] \). We obtained approximately the same amount of seeds per silique in the progeny of self-pollinated homozygous mutant plants (2 seeds per silique) or in crosses using the mutant as female (3.2 seeds per silique). Therefore, the number of pollen grains able to fertilize is probably not a limiting parameter in *spo11-1* mutant fertility.

Among the few seeds produced in *spo11-1* mutant selfing progeny, 32% are abnormal. The analysis of reciprocal crosses using *spo11-1* mutants as male or female parent showed that the large proportion of aborted seeds observed in selfing progeny comes from a male defect and not a female one. These data are interesting since most of the data obtained on the consequences of unbalanced gametes on seed development come from studies on irradiated pollen used as pollinator on wild-type ovules (see e.g. Cave and Brown, 1954; Vizir et al., 1994). Therefore, *spo11-1* and other synaptic mutants represent new tools to study the impact of unbalanced macro- or microspores on gamete development, fertilization and seed development.

**Materials and methods**

**Plant material**

*Arabidopsis thaliana* (L.), ecotype Wassilewskija (Ws), was used to generate a collection of T-DNA insertional mutants by infiltration of adult plants (Bechtold et al., 1993). Three per cent of the T2 lines showed reduced fertility (scored on the basis of limited elongation of the siliques). Among them, *spo11-1* was isolated.

*spo11-1-2* was isolated from *Arabidopsis* ecotype Columbia (Col-O) seed stocks that were mutagenized by EMS, essentially as described by Chory et al. (1989). After individual screening of 300 M2 progeny, 17 lines affected in their fertility were found, among which *spo11-1-2* was characterized. This EMS line was backcrossed twice by the wild-type parent (Col-O ecotype).

**Genetic analyses**

The T-DNA mutant (in a Ws background) was initially found to segregate 63:1 for kanamycin resistance (one of the T-DNA markers), suggesting the presence of at least three unlinked inserts. After crossing to wild type, lines were obtained segregating 3:1 for kanamycin resistance and for the semi-sterile mutation. Linkage between the remaining T-DNA insert and the semi-sterile phenotype was checked by analysing the progeny of 100 plants resulting from self-pollination of a plant heterozygous for the remaining kanamycin insertion. Among these 100 plants, 25 exhibited the mutant phenotype and were resistant to kanamycin. For the remaining fertile plants, it was checked that each plant heterozygous for the
kanamycin resistance gene (segregating 3:1 in vitro for the kanamycin resistance) was also producing 25% semi-sterile plants in its selfed progeny, when every wild-type plant (100% of progeny sensitive to kanamycin) did not generate any semi-sterile plants after selfing. Three backcrosses with the Ws wild-type line were performed on several independent heterozygotes, checking the expected 1:1 segregation of the kanamycin resistance gene every generation.

In order to test for allelism, crosses using as female a plant heterozygous for the EMS mutation (25% of sterile plants in its progeny) and as male a plant heterozygous for the T-DNA mutation (progeny segregating 3:1 for the kanamycin resistance in vitro) yielded 13 sterile plants among 69 F1 examined, indicating that the two mutations are allelic.

Mapping of spo11-1. Oligonucleotides were deduced from plant genomic sequences flanking T-DNA insert. They were used for PCR screening of the CIC YAC library as described by Crescut et al. (1995) on three-dimensional YAC pools.

Mapping of spo11-1. Heterozygous plants were crossed with tester lines (NW, provided by NASC, Nottingham Arabidopsis Stock Center) in Landsberg erecta ecotype, carrying 2 or 3 different phenotypic markers per chromosome (Koomneel et al., 1983). The recombinant F2 plants were checked and the data analysed with MAPMAKER (version 3.0; SAS Institute, 1988).

Measurement of meiotic recombination in a spo11-1 background. Lines heterozygous for the spo11-1 mutation (Ws ecotype) were crossed to a Columbia strain (Col-0). F2 plants heterozygous for the spo11-1 mutation were selected according to their resistance to kanamycin. F2 plants were generated by selfing and characterized for several loci including AsSP011-1, and microsatellite markers showing polymorphisms between the two ecotypes Ws and Col-0 (Bell and Ecker, 1994). Levels of recombination were estimated choosing plants heterozygous for markers nga280 and nga111 or heterozygous for markers nga106 and nga76.

Isolation of plant T-DNA flanking sequences

One of the LB flanking genomic sequences (LB1) was obtained using thermally asymmetric interlaced PCR (TAIL PCR) according to Liu et al. (1995), using the arbitrary degenerate primer AD3 [5′-(AT)-GTGGNA(A/T)ANCANAGA-3′]. The three specific primers of the LB of the pGKB5 vector (Bouchez et al., 1993) are TAGS (5′-ATT-GCCCTTTCTTATCGACC-3′), TaIlb (5′-CGGGCTATTTGGAATAG-GACACTGG-3′) and LB-Bar1 (5′-CAACCTCTCAACTGGAAACG-GGCGCCA-3′). The other T-DNA border was isolated by performing kanamycin rescue experiments according to Bouchez et al. (1996). A 6 kbp PvuI fragment was cloned in pRES38; 3.1 kb of the cloned insert were found to be plant DNA.

Light and confocal microscopy

Observation of developing ovules and embryo sacs by DIC was performed as described by Motamayor et al. (1999).

Development of PMC was observed under DIC after clearing in Herr’s buffer (phenol:chloral hydrate: 85% lactic acid:xyylene:oil of clove; 1:1:1:0.5:1; www:ww) of fresh buds of different sizes.

Mature pollen grain viability was estimated as described by Alexander (1969).

DAPI staining of meiotic chromosomes was performed either according to the technique described by Maluszynska and Heslop-Harrison (1991) or according to that described by Ross et al. (1996).

For confocal laser scanning microscopy, anthers and pistils of different stages were observed as described by Motamayor et al. (1999).

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References


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