**Arabidopsis PTD Is Required for Type I Crossover Formation and Affects Recombination Frequency in Two Different Chromosomal Regions**

Pingli Lu a,b,*, Asela J. Wijeratne b,1, Zhengjia Wang b,c, Gregory P. Copenhaver d,e, Hong Ma a,*

a Institute of Plant Biology, Institute of Genetics, School of Life Sciences, Fudan University, Shanghai 200433, China  
b Department of Biology and the Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, PA 16802, USA  
c School of Forestry and Biotechnology, Zhejiang A&F University, Linan 311300, China  
d Department of Biology and the Carolina Center for Genome Sciences, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA  
e Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine, Chapel Hill, NC 27599-3280, USA  

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**ABSTRACT**

In eukaryotes, crossovers together with sister chromatid cohesion maintain physical association between homologous chromosomes, ensuring accurate chromosome segregation during meiosis I and resulting in exchange of genetic information between homologues. The *Arabidopsis PTD* (Parting Dancers) gene affects the level of meiotic crossover formation, but its functional relationships with other core meiotic genes, such as *AtSPO11-1*, *AtRAD51*, and *AtMSH4*, are unclear; whether PTD has other functions in meiosis is also unknown. To further analyze PTD function and to test for epistatic relationships, we compared the meiotic chromosome behaviors of *Atspo11-1 ptd* and *Atrad51 ptd* double mutants with the relevant single mutants. The results suggest that PTD functions downstream of *AtSPO11-1* and *AtRAD51* in the meiotic recombination pathway. Furthermore, we found that meiotic defects in *rck ptd* and *Atmsh4 ptd* double mutants showed similar meiotic phenotypes to those of the relevant single mutants, providing genetic evidences for roles of PTD and RCK in the type I crossovers pathway. Moreover, we employed a pollen tetrad-based fluorescence method and found that the meiotic crossover frequencies in two genetic intervals were significantly reduced from 6.63% and 22.26% in wild-type to 1.14% and 6.36%, respectively, in the *ptd-2* mutant. These results revealed new aspects of PTD function in meiotic crossover formation.

**KEYWORDS:** PTD; Meiosis; MSH4; Crossover; Recombination frequency

**INTRODUCTION**

Meiosis is a specialized cell division in eukaryotic sexual life cycles, producing haploid gametes by halving the parental diploid genome. The key events in meiosis are homologous chromosome pairing, synapsis, recombination, and segregation (Ma, 2005). Meiotic recombination increases genetic diversity by exchanging genetic materials between homologues (Felsenstein, 1974; Zickler and Kleckner, 1999), consequently increasing genetic variation among individuals of a population. Another important role for meiotic recombination is to ensure accurate homologue segregation during meiosis I. Proper segregation relies on the formation of special structures called chiasmata, which provide physical association between homologues. These connections are required for bivalent formation, eventually leading to accurate homologue segregation during anaphase I.

Molecular and genetic studies, mainly in yeast, have led to the development of the DNA double-strand break repair
(DSBR) model of meiotic recombination (Szostak et al., 1983), as reviewed by Keeney (Keeney, 2001). This model is also supported by genetic studies in other fungi, animals and plants (Ma, 2005; Handel and Schimenti, 2010). According to the DSBR model, meiotic recombination is initiated by SPO11 which generates double-strand DNA breaks (DSBs) (Esposito and Esposito, 1969; Keeney, 2001; Lichten, 2001). Homologues of SPO11 have been identified in fungi, animals, plants and protists (Ma, 2005; Malik et al., 2007; Handel and Schimenti, 2010), suggesting that the initiation of meiotic recombination is evolutionarily conserved (Barlow and Hulten, 1998; Demburg et al., 1998; McKim et al., 1998; Keeney et al., 1999; Romanienko and Camerini-Otero, 1999; Celerin et al., 2000; Grelon et al., 2001). In Arabidopsis thaliana, two SPO11 homologues, AtSPO11-1 and AtSPO11-2, are both required for meiotic recombination, with mutants in either gene displaying similar defects (Grelon et al., 2001; Stacey et al., 2006). Following DSBs formation, each end of the break is resected to form a single-stranded DNA (ssDNA) overhang. Afterward, the DSB repair protein RAD51 and its meiosis-specific homologue DMC1 bind to the ssDNA and facilitate its invasion into the intact duplex DNA of a homologous chromosome, forming an intermediate called a displacement loop (D-loop). The functions of RAD51 and DMC1 homologues are also highly conserved in many different organisms including plants (Hamant et al., 2006; Deyhle et al., 2007).

After ssDNA invasion, there are at least two repair pathways for meiotic crossovers, interference-sensitive (type I) and interference-insensitive (type II) pathways (Zalevsky et al., 1999; Bishop and Zickler, 2004). The interference-sensitive pathway, which experiences inhibition of additional crossovers near existing ones, relies on proteins like the MSH4–MSH5 heterodimer and a DNA helicase called MER3 in yeast. On the other hand, the interference-insensitive pathway generates randomly positioned crossovers, and depends on the MUS81/MMS4/EME1 endonuclease activities (Holllsworth and Brill, 2004; Berchwitz et al., 2007). In Arabidopsis, the interference-sensitive pathway produces approximately 80% of meiotic crossovers (Copenhaver et al., 2000; Romanienko and Camerini-Otero, 2000). The roles of MSH4 and MER3 and their homologues during meiotic crossovers formation have been well studied in yeast (Nakagawa and Ogawa, 1999; Hoffmann and Borts, 2004; Mazina et al., 2004). The Arabidopsis homologue of MER3 has been identified and functionally characterized independently by two groups, and named as RCK (ROCK-N-ROLLERS) and AtMER3; although slightly weaker than the AtMsh4 mutant, the rck Atmer3 mutant phenotypes suggested that it plays a role in the type I crossover pathway (Chen et al., 2005; Mercier et al., 2005).

Another important Arabidopsis gene for meiotic crossovers formation is PTD (Parting Dancers), which was identified as a plant specific gene (Wijeratne et al., 2006). Light and transmission electron microscopy (TEM) analyses showed that the number of chiasmata is reduced in the ptd male meiocytes, statistically consistent with a role of PTD in the same crossover pathway as that requires AtMsh4 and RCK/MER3 (Wijeratne et al., 2006). However, the ptd mutant phenotypes seemed less severe than that of the Atmsh4 mutant and the previous results did not exclude possible additional functions of PTD, which might not be detectable in a single mutant. Such a possible hidden function could sometimes be revealed by double mutant analysis. Also, genetic evidence for interaction between PTD and other meiotic genes is not available. To further investigate PTD function and to test for genetic interactions with other genes, we generated double mutants between ptd and mutants of other meiotic recombination genes in Arabidopsis, including Atspo11-1 ptd-2, Atrad51 ptd-1, rek-4 ptd-1 and Atmsh4 ptd-2 double mutants by genetic crosses and examined their meiotic phenotypes in comparison to the relevant single mutants. Our genetic analyses indicated that PTD acts downstream of AtSPO11 and AtRAD51, and works in the MSH4–MSH5-dependent crossover pathway. Furthermore, although the previous study revealed that crossover formation was greatly reduced at the whole genome level in ptd mutants compared to wild-type, the cytological analyses that were used could not examine the meiotic crossover frequency in different genomic regions. Therefore, we examined the crossover frequency in two intervals on chromosome 1 and 3 in ptd-2 mutants by employing the pollen tetrad-based fluorescence method (Francis et al., 2006). Our data show that crossover frequencies are reduced in ptd-2 mutants in the two intervals tested.

RESULTS

PTD functions downstream of AtSPO11 during meiotic recombination

Genetically testing whether two genes are involved in the same biological pathway can be achieved by comparing the phenotypes of the two corresponding single mutants with that of the double mutant. If they function in the same pathway, the double mutant phenotype is expected to be similar to the single mutants (when they are similar), or to that of the more severe single mutant (when they are different). Conversely, if the two genes are in different pathways, the double mutant is expected to show a more severe phenotype than either of the two single mutants. Previously, two independent T-DNA insertion mutants for PTD were identified. One is ptd-1 (SALK_127447), in which the T-DNA was inserted in the first exon, 40 bp downstream of the beginning of the protein-coding region; the other is ptd-2 (SAIL_567_D09), carrying an insertion in the fifth exon (430 bp downstream of the start codon) of a total of nine exons (Wijeratne et al., 2006). If a truncated protein is produced in the ptd-2 mutant, it lacks the conserved C-terminal portion of the PTD protein. Both ptd-1 and ptd-2 displayed similar fertility defects, and RT-PCR results showed that transcripts containing the full length CDS (coding domain sequence) were not detectable in either mutants (Wijeratne et al., 2006). Therefore, both alleles were used for further studies here.

To test whether PTD and AtSPO11-1 are involved in the same pathway during meiotic recombination, we obtained
Atspo11-1-1 ptd-2 double mutant plants by crossing AtSPO11-1/Atspo11-1-1 with PTD/ptd-2, and identifying double and single mutant F2 progeny using allele-specific PCR. Meiotic chromosome behavior in the double mutant were compared with that of the single mutants and wild-type controls using 4',6-diamidino-2-phenylindole (DAPI)-stained chromosome spreads (Ross et al., 1996). At leptotene to early zygotene stages, Atspo11-1-1, ptd-2 and Atspo11-1-1 ptd-2 meiocytes did not show obvious abnormalities compared to wild type (Fig. 1A, F, U and A_P; B, G, V and B_D). At the pachytene stage, cells with thick thread-like chromosomes, which are characteristic of fully synapsed chromosome, were observed in both wild type and the ptd-2 mutant cells (Fig. 1C and W). However, in the Atspo11-1-1 single and the Atspo11-1-1 ptd-2 double mutant cells, we did not observe typical pachytene-like chromosomes (Fig. 1H and C_D). At the subsequent diplotene stage, the four genotypes looked similar (Fig. 1D, I, X and D_D). However, at the diakinesis stage the three mutants (Fig. 1J, Y and E_D) had a reduced number of bivalents compared to wild-type cells, which consistently displayed five bivalents (Fig. 1E).

To quantify the bivalent defect seen in the single and double mutants, we counted the residual bivalents and chiasmata in Atspo11-1-1 and Atspo11-1-1 ptd-2 mutant cells. As previously reported (Wijeratne et al., 2006), a sample of 76 ptd-2 mutant meiocytes had an average of 2.5 chiasma and 1.8 bivalents per cell (Table 1). Atspo11-1-1 single mutants had an average of 0.71 chiasma and 0.71 bivalents per cell (n = 51 meiocytes) (Table 1), consistent with previous reports (Grelon et al., 2001). In Atspo11-1-1 ptd-2 double mutant, we found an average of 0.64 chiasma and 0.62 bivalents per cell (n = 53) at the diakinesis stage (Table 1). The number of chiasma and bivalents were not significantly different between Atspo11-1-1 and Atspo11-1-1 ptd-2 (P = 0.64) (statistical analyses are shown only for chiasma numbers), but significantly different between those in ptd-2 and Atspo11-1-1 ptd-2 (P < 0.001). Because the double mutant was more similar to the Atspo11-1 single mutant, PTD likely functions in the same pathway for meiotic recombination as AtSPO11-1 and downstream of Atspo11-1.

PTD acts at the downstream of AtRAD51 during meiotic recombination

To test whether PTD and AtRAD51 are involved in the same recombination pathway, we generated Atrad51 ptd-1 double mutants by crossing AtRAD51/AtRAD51 with PTD/ptd-1 plants, and conducting PCR genotyping analysis to identify double and single mutants in the F2 progeny. Chromosome behavior was analyzed in meiocytes from Atrad51, ptd-1, AtRAD51 ptd-1 and wild-type controls. At the leptotene and zygotene stages, the chromosome behaviors of ptd-1, Atrad51 and AtRAD51/ptd-1 were not significantly different from wild-type cells (Fig. 1A, K, P and F_D; B, L, Q and G_D). However, pairing forks, which are indicative of early synapsis and are present in both wild type and ptd-1 mutant, were not observed in either Atrad51 or AtRAD51 ptd-1 meiocytes. At the pachytene stage, completely synapsed chromosomes were frequently observed in both wild type and the ptd-1 mutants (Fig. 1C and R). However, in both Atrad51 and AtRAD51 ptd-1, obvious pachytene stage cells were not observed (Fig. 1M and H_D), indicating that these plants are defective in meiotic chromosome synapsis. At the diplotene stage, ptd-1 chromosome behaviors were indistinguishable from wild-type cells (Fig. 1D and S). At the diakinesis stage, ptd-1 meiocytes produced fewer bivalents (average bivalents per cell 2.78; n = 76) (Fig. 1T), compared to the five observed in wild type controls (Fig. 1E). AtRAD51 and the AtRAD51 ptd-1 meiocytes showed signs of chromosome fragmentations at the diplotene and the diakinesis stages (Fig. 1N and I_D, O and J_D). This phenotype is noticeably different from ptd-1, which lacked chromosome fragmentation (Fig. 1S and T). The difference in synapsis and chromosome fragmentation phenotypes of AtRAD51 and AtRAD51 ptd-1 compared to ptd meiocytes indicate that PTD acts downstream of AtRAD51 during meioocytes recombination in Arabidopsis.

RCK/MER3, AtMSH4 and PTD likely function in the same meiotic CO pathway

In Arabidopsis, genetic studies indicated that AtMSH4 and RCK/MER3 are required for type I crossovers (Higgins et al., 2004; Chen et al., 2005; Mercier et al., 2005). The single mutant phenotypes of AtMSH4, RCK/MER3 and PTD are similar, with reduction of the number of bivalents at meiotic prophase I (Higgins et al., 2004; Chen et al., 2005; Mercier et al., 2005; Wijeratne et al., 2006), supporting the hypothesis that PTD acts in the same pathway as AtMSH4 and RCK. Previous studies also reported a greater reduction of chiasma number in the Atmsb4 mutant (Higgins et al., 2004) than the rck or ptd mutants (Chen et al., 2005; Wijeratne et al., 2006), but it is possible that slight variations in growth conditions and/or experimental protocol could have caused the observed phenotypic differences. To verify the difference between the single mutants and to test the hypothesis that PTD acts in type I pathway of CO formation, we re-examined the Atmsb4 phenotypes in our lab and generated rck-4 ptd-1 and Atmsb4 ptd-2 double mutant plants by crossing corresponding single heterozygous plants, and then identifying double mutant and single mutant F2 plants using allele-specific PCR. We observed no obvious differences in meiotic chromosome behaviors in any of the genotypes (including wild type controls) from leptotene to pachytene stages (Fig. 2A–C, F–H, K–M, P–R, U–W, A_P–C_D and F_D–H_D). In the rck-4, Atmsb4 and ptd single mutants, the earliest observed meiotic defects occurred at the diakinesis stage, when all three single mutants displayed fewer bivalents compared to the wild-type cells, which had five bivalents per cell (Fig. 2E, J, O, T, and Y). Similar to the single mutants, meiotic chromosome behaviors of the rck-4 ptd-1 and Atmsb4 ptd-2 double mutants were also indistinguishable from wild type until diakinesis (Fig. 2E, J, O, T, and Y). To further define the crossover phenotype of the ptd, Atmsb4 and rck single and double mutants, we counted the numbers of bivalents and chiasmata remaining in their meiocytes at the
Fig. 1. Comparison of chromosome behaviors at meiotic prophase I among wild type (WT), Atspo11-1-1, Atrad51, ptd-1, ptd-2, Atspo11-1-1 ptd-2 and Atrad51 ptd-1 mutants.

Images show DAPI-stained chromosomes representing five sub-stages of meiotic prophase I in wild-type (A–E) and similar stages of meiosis from Atspo11-1 (F–J), Atrad51 (K–O), ptd-1 (P–T), ptd-2 (U–Y), Atspo11-1 ptd-2 (Ad–Ed) and Atrad51 ptd-1 (Fd–Jd).

Table 1
The average number of bivalent and chiasmata per meiocyte in different genotype mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total number of cells</th>
<th>Average number of bivalents per cell/remaining percentage</th>
<th>Average number of chiasmata per cell/remaining percentage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>50</td>
<td>5.0(100%)</td>
<td>9.7(100%)</td>
<td>This study</td>
</tr>
<tr>
<td>ptd-1</td>
<td>76</td>
<td>2.7(54%)</td>
<td>3.2(32.6%)</td>
<td>Wijeratne et al., 2006</td>
</tr>
<tr>
<td>ptd-2</td>
<td>76</td>
<td>1.8(36%)</td>
<td>2.5(25.5%)</td>
<td>Wijeratne et al., 2006</td>
</tr>
<tr>
<td>Atspo11-1-1</td>
<td>51</td>
<td>0.71(14.2%)</td>
<td>0.71(7.2%)</td>
<td>This study</td>
</tr>
<tr>
<td>Atmsh4</td>
<td>61</td>
<td>1.48(29.6%)</td>
<td>1.72(17.5%)</td>
<td>This study</td>
</tr>
<tr>
<td>rck-4</td>
<td>62</td>
<td>2.26(45.2%)</td>
<td>3.06(31.2%)</td>
<td>This study</td>
</tr>
<tr>
<td>Atspo11-1-1 ptd-2</td>
<td>53</td>
<td>0.62(12.4%)</td>
<td>0.64(6.5%)</td>
<td>This study</td>
</tr>
<tr>
<td>Atmsh4 ptd-2</td>
<td>58</td>
<td>1.33(26.6%)</td>
<td>1.66(16.9%)</td>
<td>This study</td>
</tr>
<tr>
<td>rck-4 ptd-1</td>
<td>61</td>
<td>1.77(35.4%)</td>
<td>2.39(24.4%)</td>
<td>This study</td>
</tr>
</tbody>
</table>
The average number of chiasmata and bivalents in *Atmsh4* single mutants (1.72 chiasmata; 1.48 bivalents; *n* = 61; Table 1), which is consistent with previous observations (Higgins et al., 2004), were significantly lower than those in *ptd-2* (2.5 chiasmata; 1.8 bivalents; *n* = 76; Table 1) (*P* = 0.001). In *Atmsh4 ptd-2* double mutant, we found an average of 1.66 chiasmata and 1.33 bivalents per cell (*n* = 58) (Table 1). There was no statistically significant difference between *Atmsh4* and *Atmsh4 ptd-2* (*P* = 0.722), however there was between *ptd-2* and *Atmsh4 ptd-2* (*P* < 0.001). These results suggested that PTD and AtMSH4 are involved in the same meiotic crossover formation pathway, but PTD seems to be less crucial than AtMSH4.

Furthermore, the *rck-4 ptd-1* double mutant meiocytes formed an average of 2.39 chiasmata and 1.77 bivalents per cell (*n* = 61; Table 1), whereas the *rck-4* single mutant showed an average of 3.06 chiasmata and 2.26 bivalents (*n* = 62; Table 1). There was no statistically significant difference between *rck-4* and *ptd-1* (*P* = 0.645). The slightly more severe phenotype of *rck-4 ptd-1* double mutant than either *ptd-1* (*P* = 0.004) or *rck-4* (*P* = 0.004) single mutant was statistically significant, making it possible that PTD and MER3/RCK act partially in parallel; for example, PTD might be contribute weakly to double Holliday junction (dHJ) formation, rather than just in dHJ resolution. However, considering the type I pathway contributes to around 85% crossover formation during *Arabidopsis* meiosis (Berchowitz et al., 2007; Higgins et al., 2008), and the residual chiasmata in the *ptd* and *rck* single mutants show random distributions, both PTD and MER3/RCK are needed for the formation of most of type I
The existence of a MER3/RCK homologue in *Arabidopsis* (Chen et al., 2005) also makes it possible that RCK is partially redundant with its homologue and the single mutant is not completely blocked in the step that involves its function. Therefore, the more severe phenotype of the *rck-4 ptd-1* double mutant can be explained if both single mutants are not completely defective in the respective functions. Therefore, we propose that RCK and PTD are mainly involved in the same pathway for meiotic crossover formation, but also have some possible functional differences.

**Generation of *ptd-2* mutant plant with fluorescence markers for tetrad analysis**

To quantify the effect that loss of PTD function has on meiotic crossover frequency, we employed the Fluorescent...
Tagged Line (FTL) system, a fluorescence-based tetrad analysis assay in *Arabidopsis* (Francis et al., 2007). This system utilizes the *quartet1-2* (*qrt1-2*) mutant, which produces tetrads of four attached fertile pollen grains that result from a single meiosis (Preuss et al., 1994; Francis et al., 2006, 2007). In the *qrt1-2* background, pairs of transgenes encoding fluorescent proteins of different colors expressed under the control of the pollen-specific promoter *LAT52* can be used to monitor CO frequencies (Twell et al., 1990). Because each *Arabidopsis* flower can produce approximately 700 tetrads (Ma, 2006), the FTL system provides a way to analyze meiotic recombination frequency in a large number of meioses.

Here, we used two FTL lines, “Interval 1” (I1) and “Interval 3” (I3), which mark two intervals on chromosomes 1 and 3.

### Table 2
Comparison analyses of meiotic recombination frequency in two independent intervals between wild type and *ptd-2* plants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total tetrads</th>
<th>No recombination</th>
<th>One recombination</th>
<th>Double recombination</th>
<th>Recombination frequency</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interval 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>550</td>
<td>477</td>
<td>73</td>
<td>0</td>
<td>6.63%</td>
</tr>
<tr>
<td><em>ptd-2</em></td>
<td>613</td>
<td>599</td>
<td>14</td>
<td>0</td>
<td>1.14%</td>
</tr>
<tr>
<td>Interval 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>620</td>
<td>349</td>
<td>270</td>
<td>1</td>
<td>22.26%</td>
</tr>
<tr>
<td><em>ptd-2</em></td>
<td>700</td>
<td>611</td>
<td>89</td>
<td>0</td>
<td>6.36%</td>
</tr>
</tbody>
</table>

Fig. 4. The locations of CFP and DsRed fluorescence markers on the genome, and the representative recombination events shown by tetrads analysis between the two markers.
A: The insertion sites of fluorescence markers CFP (cyan circle) and DsRed (red circle) on chromosomes (gray bars). The genetic intervals I1 and I3 used in this study are delineated with brackets. B: Fluorescence markers patterns in pollen tetrads indicating different meiotic recombination events happened between the markers region.
We carried out a similar analysis using the I3 line, which carries CFP and DsRed2 markers on the long arm of the chromosome 3 (Fig. 4A). The estimated genetic distance between the markers is 19.1 cM, and a ~23% recombination frequency was previously observed between the two markers in the I3 line (Francis et al., 2007). We observed a 22.26 cM map distance in wild type (n = 620 tetrads; Table 2). In contrast, I3 in ptd-2 experienced a statistically significant 71% reduction in CO frequency and had a recombination frequency of 6.36% (P < 0.0001; n = 700 tetrads; Table 2), similar to the genome-wide reduction. The results from analyzing crossover frequencies in I1 and I3 indicate that PTD is critical for meiotic crossover recombination in Arabidopsis.

**DISCUSSION**

PTD is a part of a conserved machinery involved in crossover formation

Our cytological observations indicate that Atspo11-1 is epistatic to ptd. Atspo11-1 is required to produce the DSBs that initiate meiotic recombination, and is also important for proper synopsis (Grelen et al., 2001). In the ptd-2 mutant cells, synaptonemal complex formation was not severely affected (Wijeratne et al., 2006), and pachytene stage cells with complete synopsis were often observed. In the Atspo11-1 ptd-2 double mutant cells, we did not see any pachytene-like cells, indicating that the double mutant phenotype is similar to the Atspo11-1-1 single mutant. These results suggest that the PTD function is dependent on SPO11-1 initiated DSBs, and also indicates that PTD affects the meiotic recombination pathway after synopsis is complete.

Additionally, our data also show that PTD is downstream of AtrAD51 in the meiotic recombination pathway. RAD51 is required for repairing SPO11 induced DSBs, and has an evolutionarily conserved function in mediating strand invasion (Li et al., 2004; Deyhle et al., 2007). Our data suggests that PTD functions downstream of the strand invasion process. Previous studies demonstrated that ZMM proteins, including MSH4 and Mer3, facilitate the formation of stable single-end invasion (SEI) intermediates and double Holliday junctions (Börner et al., 2004), committing them to the interference-sensitive pathway, which generates approximately 80% of total crossovers in Arabidopsis (Berchowitz et al., 2007; Higgins et al., 2008). Our data indicated that the Atms81 ptd-2 double mutants were not significantly different from the Atms81 single mutants in chiasmata and bivalent frequencies, with a reduction of approximately 80% (Wijeratne et al., 2006). Additionally, a severe PTD RNA interference transgenic line driven by a meiosis specific AtDMCI promoter only give rise to an average number of 1.43 chiasmata per cell (P. Lu, unpublished data), close to that found in a class II mutant (such as mus81), but not that observed in a class I mutant (such as msh4), indicating that the double mutant phenotype is similar to the Atspo11-1-1 single mutant. These results suggest that the PTD function is dependent on SPO11-1 initiated DSBs, and also indicates that PTD affects the meiotic recombination pathway after synopsis is complete.

Meiotic crossover frequencies are significantly reduced in ptd-2 mutant

Pollen tetrads from mature flowers on the primary inflorescence were scored using fluorescence microscopy to generate a map distance based on the number of recombinant and non-recombinant meioses (Figs. 3B and 4B). Wild type control plants had a map distance of 6.63 cM for I1 (n = 550 tetrads; Table 2) which is consistent with previously published measurements (Francis et al., 2007). However, I1 in the ptd-2 mutant, had a map distance of 1.14 cM (n = 613 tetrads; Table 2). Therefore, the crossover frequency in I1 was reduced by 83% in ptd-2 compared to wild type, a statistically significant difference (P < 0.0001) and a decrease greater than the genome-wide reduction estimated from the number of chiasmata.

<table>
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</table>

Table 3

and 3, respectively, to estimate crossover frequency in ptd-2. Specifically, I1 carrying CFP (cyan fluorescent protein) and DsRed2 (red fluorescent protein 2) transgenes, linked in cis on chromosome 1 (Fig. 3A, red and blue bars) was crossed with PTD/ptd-2 (Fig. 3A, step 1). PTD/ptd-2 F1 plants were identified by PCR and plants with the CFP marker were identified by fluorescence microscopy and crossed with plants carrying the DsRed2 marker (Fig. 3A, step 2). PTD/ptd-2 plants carrying both CFP and DsRed2 markers were identified by examining pollen fluorescence and were allowed to self-fertilize; and ptd-2 plants with both fluorescence markers were identified in the F2 population (Fig. 3A, step 3). CO frequencies were measured by observing the segregation patterns of the fluorescent reporters in the pollen tetrads using fluorescence microscope (Fig. 3B). A similar strategy was used for the I3 line.
plants to facilitate meiotic recombination.


**Materials and Methods**

Plant materials and growth conditions

Plant growth conditions were as described previously (Wijeratne et al., 2006). The ptd-1 mutant carries a T-DNA insertion in the first exon 40 bp downstream of the start codon and the T-DNA insertion in ptd-2 is 430 bp downstream of the start codon in the fifth exon (Wijeratne et al., 2006). Both mutants lack transcripts with the complete coding regions (Wijeratne et al., 2006) and presumably could not produce the full-length protein. The wild-type plants used as control were the Columbia 0 ecotype. The AtSPO11/Atspo11-1, AtRAD51/AtRAD51-1, RCK/rck-4 and AtMSH4/AtMSH4 mutants were previously reported (Grelon et al., 2001; Higgins et al., 2004; Li et al., 2004; Chen et al., 2005).

**Generation of Atspo11-1-1 ptd-2, AtRAD51 ptd-1, rck-4 ptd-1 and AtMSH4 ptd-2 double mutants**

Genetic crosses were performed between the plants that were heterozygous for one of the meiotic mutations (e.g., PTD/ptd-1 and AtRAD51/AtRAD51 plants), resulting F1 seeds. Double-heterozygous F1 plants (e.g., AtRAD51/AtRAD51 PTD/ptd-1) and double homozygous F2 plants were identified using PCRs with gene-specific and T-DNA specific primers for each insertion. The following primers were used: (1) for AtSPO11 and Atspo11-1-1 alleles (oMC703 and oMC704 for wild type allele; oMC703 and oMC705 for T-DNA allele); (2) for AtRAD51 and AtRAD51 alleles (oMC1446 and oMC1447 for wild type allele; oMC1446 and oMC1286 for T-DNA allele); (3) for PTD and ptd-1 alleles (oMC1607 and oMC1608 for wild type allele; oMC1607 and oMC1863 for T-DNA allele); (4) for PTD and ptd-2 alleles (oMC1911 and oMC1912 for wild type allele; oMC1911 and oMC2009 for T-DNA allele); (5) for AtMSH4 and AtMSH4 alleles (oMC2232 and oMC2233 for wild type allele; oMC2232 and oMC1863 for T-DNA allele); (6) for RCK and rck-4 alleles (oMC1611 and oMC1612 for wild type allele; oMC1611 and oMC2009 for T-DNA allele). Primer sequences are shown in the Table 3. PCR was carried out under standard conditions using 10 pmol of each primer and 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 60 s.

**Analysis of cytological phenotypes and recombination frequency**

Meiotic chromosome behaviors of the wild-type, and single and double mutant cells were analyzed using chromosome spread approach as described previously (Wijeratne et al., 2006). To determine the recombination frequency, two different marker lines named II and I3 were used (Francis et al., 2007). II carries two fluorescence markers FTL992 (CFP) and FTL1313 (DsRed2) on chromosome 1; I3 carries FTL1500 (CFP) and FTL1371 (DsRed2) markers on chromosome 3 (Francis et al., 2007). They were crossed with PTD/ptd-2 plants to obtain plants that were homozygous for both ptd and qrt1-2 mutations and carry both markers (Fig. 3A). Pollen grains were collected in 1% PBS buffer. Wild type and recombinant pollen (as shown in Fig. 3B) were counted using a Nikon E1000 epi-fluorescence microscope (Melville, NY, USA). Recombination frequency was calculated using following formula: Recombination frequency = 100[(1/2T + 3 NPD)/n], T = tetratype,
NPD = nonparental ditgy, \( n \) = total number of tetrads (Perkins, 1949). The difference between map distances was compared by calculating a \( Z \)-score using the following equation:

\[
Z = \frac{|X1 - X2|}{\text{SQRT}(\text{Var}(X1 - X2))},
\]

where \( X1 \) and \( X2 \) are the two map distances (in Morgans) to be compared. More detailed information for the calculation can be found in the website: http://molbio.uoregon.edu/~fstahl/EquationsMapDistance.html.

\( Z \)-scores were converted to \( P \)-values using the \( P \)-value calculator function of the GraphPad software suite (http://www.graphpad.com/quickcalcs/PValue1.cfm). Differences in non-mean values of chiasma and bivalents were tested using Student’s \( t \)-tests conducted through website: http://udel.edu/~mcdonald/statstest.html for statistical analyses.

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