ZIP4 in homologous chromosome synapsis and crossover formation in rice meiosis

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Summary

In budding yeast, the ZMM complex is closely associated with class I crossovers and synaptonemal complex (SC) formation. However, the relationship between the ZMM genes remains unclear in most higher eukaryotes. Here, we identify the rice *ZIP4* homolog, a member of the ZMM gene group, and explore its relationship with two other characterized ZMM genes, *MER3* and *ZEP1*. Our results show that in the rice *zip4* mutant, the chiasma frequency is greatly reduced, although synapsis proceeds with only mild defects. Immunocytological analyses of wild-type rice reveal that ZIP4 presents as punctuate foci and colocalizes with MER3 in prophase I meiocytes. Additionally, ZIP4 is essential for the loading of MER3 onto chromosomes, but not vice versa. Double-mutant analyses show that *zip4 mer3* displays a greater decrease in the mean number of chiasmata than either of the *zip4* or *mer3* single mutants, suggesting that ZIP4 and MER3 work cooperatively to promote CO formation but their individual contributions are not completely identical in rice. Although *zep1* alone gives an increased chiasma number, both *zip4 zep1* and *mer3 zep1* show a much lower chiasma number than the *zip4* or *mer3* single mutants. These results imply that the normal functions of ZIP4 and MER3 are required for the regulation of COs by ZEP1.

Key words: Rice, Meiosis, Synapsis, Crossover, ZIP4, MER3, ZEP1

Introduction

Meiosis is a specialized set of cell divisions and plays a crucial role in ensuring the accuracy of the life cycle of sexually reproducing organisms. During meiosis, a single round of DNA replication is followed by two sequential cell divisions, creating cells that have half the number of chromosomes of the initial cell. A special concern is that homologous chromosomes must separate at meiosis I before sister chromatids separate at meiosis II. During meiotic prophase I, highly organized processes involving homologous chromosome recognition, alignment, recombination and synapsis promote faithful segregation of homologous chromosomes (Gerton and Hawley, 2005; Li and Ma, 2006). To date, a number of genes have been characterized in these complicated events that have allowed us to understand the important process of meiosis better.

Most of our current understanding of meiotic homologous recombination (HR) originates from studies in fungi such as the yeast *Saccharomyces cerevisiae*. In meiosis, homologous recombination ultimately yields non-crossover (NCO) and crossover (CO) products, ensuring the genetic diversity and correct segregation of homologs. HR is initiated by programmed DNA double-strand breaks (DSBs) generated by the SPO11 protein (Bergerat et al., 1997; Keeney et al., 1997). These DNA ends are resected by the MRX complex to yield 3′ single-stranded DNA (ssDNA) overhangs (Borde, 2007; Cao et al., 1990; Connelly and Leach, 2002; Ivanov et al., 1992). With the help of recombinases such as RAD51 and DMC1, and various accessory proteins, one of the free DSB ends invades its homologous intact double-stranded DNA and forms a stable single-end invasion (SEI) intermediate (Bishop et al., 1992; Hunter and Kleckner, 2001; Sung and Robberson, 1995). In the double-strand break repair (DSBR) model, the SEI intermediate proceeds into a double Holliday junction (dHJ); this in turn can be resolved into either CO or NCO products (Hunter and Kleckner, 2001; Szostak et al., 1983). However, recent studies in *S. cerevisiae* suggest that dHJs give rise primarily or exclusively to COs, whereas NCOs seem to be derived from a synthesis-dependent strand annealing (SDSA) pathway in which no dHJs are formed (Allers and Lichten, 2001; Bishop and Zickler, 2004; Börner et al., 2004). To date, a diverse collection of proteins (ZIP1, ZIP2, ZIP3, ZIP4, MER3, MSH4, MSH5 and SPO16) involved in the dHJ-promoted CO pathway have been identified. Referred to as ZMM proteins, these proteins seem to promote SEI stability and dHJ formation, and thus affect CO generation (Börner et al., 2004; Lynn et al., 2007; Shinohara et al., 2008).

There are two genetically separate pathways for CO formation. One of these is dependent on those ZMM proteins and sensitive to interference (when the presence of one CO inhibits additional COs nearby), whereas the other one is insensitive. In *S. cerevisiae*, *zmm* mutants show dramatic reduction of CO frequency and loss of interference, indicating that this ZMM-dependent CO pathway is interference sensitive (Börner et al., 2004; Chen et al., 2008; Shinohara et al., 2008; Tsubouchi et al., 2006). Most of the remaining COs rely on MUS81 and MMS4 (EME1) proteins and are insensitive to interference (Argueso et al., 2004; de los Santos et al., 2003; Hollingsworth and Brill, 2004). Lines of evidence to support the two-pathway hypothesis

exist for meiotic crossovers in *Arabidopsis* and in humans (Copenhaver et al., 2002; Housworth and Stahl, 2003), and probably in rice (Wang et al., 2009). However, not all organisms use both types of COs. Exceptions include *Schizosaccharomyces pombe* and *Caenorhabditis elegans* (Osman et al., 2003; Zalevsky et al., 1999).

In *S. cerevisiae*, ZMM proteins work coordinately in the same crossover pathway (Börner et al., 2004), but their biochemical functions are diverse. MER3 is a DNA helicase that unwinds duplex DNA in the 3′ to 5′ direction, extends the DNA joint made by RAD51 and facilitates the formation of the dHJ during meiotic HR (Mazina et al., 2004; Nakagawa et al., 2001; Nakagawa and Kolodner, 2002). MSH4 and MSH5 are homologues of bacterial MutS proteins and probably form a heterodimeric complex to stabilize and preserve dHJs (Snowden et al., 2004). ZIP1 is a central element component of synaptonemal complex (SC) (Sym et al., 1993), and ZIP3 is a SUMO E3 ligase (Cheng et al., 2006). ZIP2 and ZIP4 are a WD40-like repeat protein and a tetra-tricopeptide repeat (TPR) protein, respectively (Perry et al., 2005). The TPR motif is a protein–protein interaction module found in a number of functionally different proteins that facilitates specific interactions with a partner protein(s). ZIP4 might modify protein interactions with ZIP2 and ZIP3 in the same CO pathway (Tsubouchi et al., 2006).

In addition to the reduced COs, other important features in *S. cerevisiae zmm* mutants are synaptonemal complex assembly defects. Homologues are held together during the meiotic prophase I by a proteinaceous structure known as the SC (Page and Hawley, 2004). It is not known how mature SCs are formed; however, several known ZMM proteins make up a so-called synapsis initiation complex and play important roles in SC formation in *S. cerevisiae* (Fung et al., 2004). ZIP3 recruits both ZIP2 and ZIP4 to chromosomes, and further induces ZIP1 polymerization (Agarwal and Roeder, 2000; Chua and Roeder, 1998; Tsubouchi et al., 2006). MSH4 might also be involved in the formation of normal SCs (Novak et al., 2001). However, the functions of these proteins for SC assembly are divergent in other organisms. In *Arabidopsis*, no apparent defects in chromosome synapsis are observed in *msh5* and *zip4* mutants (Chelysheva et al., 2007; Higgins et al., 2008; Lu et al., 2008). In the *msh4* mutant, chromosome synapsis might be incomplete but the defects are not severe (Higgins et al., 2004). Similar incomplete synapsis is also found in severe *mer3* (*rck*) alleles (Chen et al., 2005). In mice, SCs fail to assemble normally without *MSH4* or *MSH5* (de Vries et al., 1999; Edelmann et al., 1999; Kneitz et al., 2000). In the mouse *Zip4h* mutant, synapsis that initiates goes to completion, but synapsis initiation appears to be decreased (Adelman and Petrini, 2008).

As an important food crop in the world, rice (*Oryza sativa*) is also becoming a model monocot for molecular biological studies. Many genetic and genomic studies, including those related to meiosis, have been carried out in rice. Two ZMM components, MER3 and ZEP1, have been described in rice recently. In the rice *mer3* mutant, the number of bivalents and chiasmata is significantly reduced. ZEP1 is a transverse filament (TF) protein and constitutes the central element of the SC in rice. However, in the *zep1* mutant, 12 bivalents are present at metaphase I and segregate normally at anaphase I (Wang et al., 2010). Given the totally different phenotypes of *zep1* and *mer3* in rice, ZEP1 and MER3 might not collaborate with each other as closely as ZIP1 and MER3 do in budding yeast. Here, we identified the homolog of *ZIP4* in rice and investigated its role during CO formation and synapsis. Using both genetic and cytological analysis, we further investigated the roles of ZIP4, MER3 and ZEP1 during synapsis and CO formation in rice.

Results

Identification of a sterile mutant in rice

We obtained a sterile rice mutant that arose in tissue culture from the *japonica* rice variety Nipponbare. The mutant plants (60 in total examined) showed normal vegetative growth and plant morphology, but were almost sterile (supplementary material Fig. S1A,B). We only obtained five fertilized seeds from 30 evaluated panicles with 3721 spikelets, whereas the seed setting in wild-type was 85.35% among 30 evaluated panicles.

The viability of male and female gametes was examined in this mutant. After staining mutant pollens with 1% I_2–KI solution, we found that nearly all of them were shrunken and could not be stained (supplementary material Fig. S1C,D). Even when using a mutant plant as the maternal recipient pollinated with wild-type pollen, no seed was set. Consequently, both male and female mutant gametes were grossly impaired. The self-fertilized heterozygous plants produced progenies (fertile, 99; sterile, 30) with a segregation ratio of ~3:1 ($\chi^2=0.21$; $P>0.05$), consistent with the conclusion that a single nuclear recessive allele controlled the sterile phenotype of the mutant.

Positional cloning and molecular characterization of the rice *ZIP4* gene

We performed positional cloning to investigate the molecular basis of the defects in the mutant plant. From the F2 and F3 population generated by crossing the heterozygous plant with Zhefu802, an *indica* rice variety, 499 sterile plants were used for genetic analysis. The locus was first mapped on the long arm of chromosome 1, which was further narrowed to a 102 kb region. Based on the annotations of the rice genome database (RiceGAAS http://ricegaas.dna.affrc.go.jp/), we found a candidate gene (*Os01g66690*) annotated as the putative *ZIP4* (*SPO22*). The corresponding 953 residue protein was similar to the *Arabidopsis* ZIP4 (SPO22) protein (378/949, 39% identity; 562/949, 59% positive; supplementary material Fig. S2). We sequenced the *Os01g66690* gene of the mutant. A single base pair deletion was detected at position 348 of the fourth exon, resulting in a premature stop codon at the 630th amino acid residue. Here, we designated *Os01g66690* as *ZIP4* in rice, and the mutant as *zip4*. To verify whether the *ZIP4* corresponded to the mutant locus, a complementation test was conducted. Transformation of a plasmid that contained the entire *ZIP4* gene succeeded in rescuing the sterile phenotype of the mutant plants. These results confirm that the nucleotide deletion in *ZIP4* is indeed responsible for the sterile phenotype of the mutant plant.

The full-length cDNA of the rice *ZIP4* gene was obtained by performing 3′ rapid amplification of cDNA ends (3′ RACE) and 5′ RACE. The 3′ non-coding region of the *ZIP4* transcript was 118 nucleotides in length and the 5′ non-coding region was 132 nucleotides long. The *ZIP4* gene had five exons and four introns, and its cDNA was 3112 bp in length with an ORF of 2862 nucleotides encoding a protein of 953 amino acids (supplementary material Fig. S3). A Pfam search indicated that ZIP4 was a tetratricopeptide repeat (TPR)-containing protein. Tetratricopeptide repeat domains, which mediate protein–protein

interactions, are found in numerous and diverse proteins. Thus, ZIP4 might be involved in protein–protein interactions.

The rice *zip4* mutant is defective in meiosis

To clarify the cause of sterility in the rice *zip4* mutant, meiotic chromosomes in pollen mother cells (PMCs) of both wild-type and *zip4* lines were investigated. The meiotic stages were defined mainly by degree of chromatin condensation combined with the canonical definitions. In the wild type, at leptotene, meiotic chromosomes became visible as single threads, evenly dispersed throughout the nucleus (supplementary material Fig. S4A). At zygotene, the chromosome threads aggregated into a characteristic group. Pairing and synapsis occurred between homologous chromosomes (supplementary material Fig. S4B). At pachytene, synapsis is completed, and fully synapsed chromosome pairs were visible (supplementary material Fig. S4C). At diakinesis, chromosomes condensed into 12 bivalents (supplementary material Fig. S4D). At metaphase I, all of the 12 extremely condensed bivalents aligned on the equatorial plate (supplementary material Fig. S4E). At anaphase–telophase-I, the homologous chromosomes separated and migrated to the opposite poles of the cell (supplementary material Fig. S4F). During the second meiotic division, the sister chromatids segregated and the tetrads formed in a mitosis-like process (supplementary material Fig. S4G–I).

In the rice *zip4* mutant, meiotic chromosome behavior was almost the same as that in the wild type from leptotene to pachytene (Fig. 1A–C). However, abnormalities appeared at diakinesis. Both bivalents and univalents co-existed in mutant PMCs (Fig. 1D). We found no diakinesis cell with 12 bivalents. At metaphase I, this defect became even more obvious (Fig. 1E–I). Chromatin condensation was abnormal and displayed various phenotypes. First, compact bivalents were found in some mutant metaphase I cells (Fig. 1E,F). They seemed to be normal. Second, bivalents with irregular edges were also detected. They displayed various twisted shapes and probably did not condense as fully as usual (Fig. 1G, arrowheads). Third, some one-chiasma bivalents were stretched longer than those in the wild type (Fig. 1G,H, arrows). Furthermore, we even found rather long chromosomes that were broken at the equatorial plate. These might have been chromosome bridges (Fig. 1I, arrow). During the subsequent anaphase I, the univalents segregated randomly, resulting in an unequal distribution of chromosomes (Fig. 1J). The second meiotic division subsequently occurred and resulted in the formation of tetrads with nuclei of different sizes, indicating that they contained aneuploid numbers of chromosomes (Fig. 1K,L).

To ask whether the meiotic chromosome defect was caused by non-homologous recombination in *zip4*, we performed fluorescence in situ hybridization (FISH) analysis using two different probes to monitor the homologous chromosome status. One is 5S rDNA located on chromosome 11, and the other is a BAC clone, OSJNBa0012J05, on the long arm of chromosome 8. When any 5S rDNA signal existed on a bivalent, there were always two signals on the same bivalent (supplementary material Fig. S5A, arrowheads). We found that 33 bivalents displayed this phenotype in 174 *zip4* metaphase I cells. In the remainder, the two separated 5S rDNA signals were on two different univalents (supplementary material Fig. S5B, arrowheads). The same situation was observed on chromosome 8 when using the BAC clone a0012J05 as the cytological marker (supplementary material Fig. S5, arrows). Only 18 bivalents had two BAC signals in 174 mutant cells; the rest were univalents for chromosome 8. In addition, none of these probe-labeled bivalents ($n=51$) presented as chromosome bridges. These results cannot exclude the possibility that non-homologous recombination forms chromosome bridges, but clearly the majority of the few bivalents that do occur result from normal homologous crossing-over in rice *zip4* PMCs.

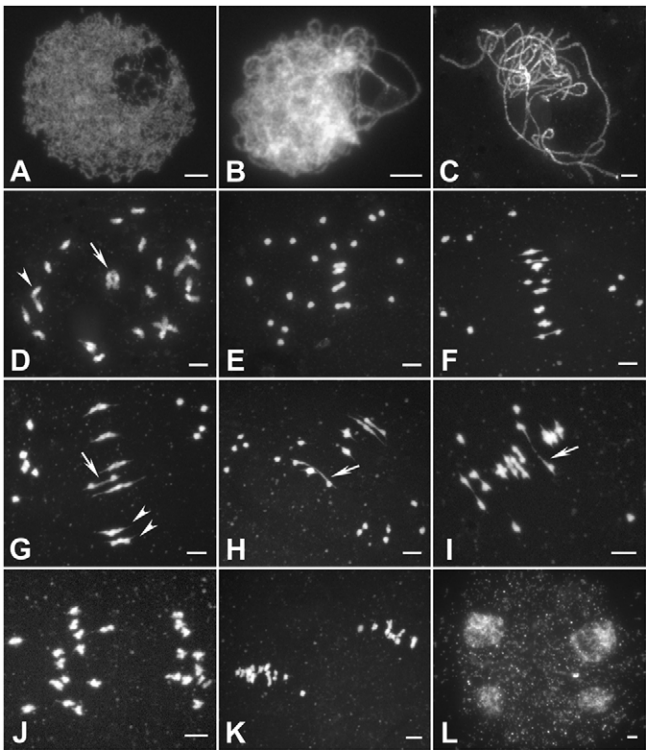

Fig. 1. Meiosis in rice *zip4* pollen mother cells (PMCs). (**A**) Leptotene. (**B**) Zygotene. (**C**) Pachytene. (**D**) Diakinesis. Cells have variable numbers of bivalents. The arrow indicates a bivalent with two chiasmata and the arrowhead indicates a bivalent with one chiasma. There are seven chiasmata in this diakinesis cell. (**E–I**) Metaphase I. Arrows indicate very drawn out bivalents (G,H) and a chromosome bridge with two broken places (I). Arrowheads indicate twisted bivalents (G). (**J**) Anaphase I. (**K**) Metaphase II. (**L**) Tetrad. This tetrad has aneuploid numbers of chromosomes based on relative sizes of nuclei. Scale bars: 5 μm.

These results show that ZIP4 participates in rice meiosis. Loss of *ZIP4* function profoundly disturbs the normal progress of meiosis, leading to the sterile phenotype of the mutant plants.

Reduced chiasma frequency in the rice *zip4* mutant

To quantify the chiasma number in each PMC in the wild type and *zip4* mutant, we investigated both the number and the shape of bivalents at diakinesis using the criteria previously described (Sanchez Moran et al., 2001). Bivalents with two chiasmata appear ring-shaped, whereas bivalents with one chiasma appear rod-shaped. For example, in supplementary material Fig. S4D, the diakinesis cell contained one bivalent with three chiasmata, six bivalents with two chiasmata and five bivalents with only one chiasma. So the chiasma number for this cell was 20. In the wild type, the chiasma frequency was 20.59 per cell (Table 1), and the chiasma distribution deviated from a Poisson distribution between different PMCs ($\chi_{[22]}^2=89.24$, $P<0.01$; supplementary material Fig. S6A) and also between different chromosomes ($\chi_{[3]}^2=925.77$,

Table 1. Chiasma frequency in different genotypes

Genotype	Chiasmas per cell
Wild type	20.59±1.62 (n=76)
mer3	5.59±2.07 (n=64)
zip4	6.05±1.97 (n=164)
zip4 mer3	3.13±1.70 (n=116)
zip4 zep1	3.79±1.91 (n=81)
mer3 zep1	3.85±1.84 (n=75)
zip4 mer3 zep1	2.60±1.62 (n=77)

$P<0.01$; supplementary material Fig. S6B). By contrast, the rice *zip4* mutant formed an average of 6.05 chiasmata (Table 1) corresponding to 5.20 bivalents per PMC (n=164). The chiasma number per cell was quite variable, ranging from 1 to 13. For example, in Fig. 1D, this mutant PMC had only seven chiasmata (two bivalents with two chiasmata, three bivalents with one chiasma and 14 univalents). Statistical analysis showed that the distribution of the remaining chiasmata per PMC in *zip4* was consistent with a predicted Poisson distribution ($\chi_{[13]}^2$=19.26, $P>0.1$; supplementary material Fig. S6C). In addition, the distribution of chiasmata per chromosome did not deviate significantly from the predicted Poisson distribution ($\chi_{[3]}^2$=4.85, $P>0.1$; supplementary material Fig. S6D). These data show that chiasma frequency is dramatically reduced and the majority of the residual chiasmata in *zip4* distribute randomly.

Residual chiasma frequency in the *zip4 mer3* double mutant is reduced significantly compared with that in the *zip4* or *mer3* single mutants

To gain a deeper understanding of the relationship between ZIP4 and MER3 in rice, we made the *zip4 mer3* double mutant and monitored the whole meiosis process in their PMCs. In *zip4 mer3*, chromosome behavior was very similar to that of either *zip4* (supplementary material Fig. S7G–I) or *mer3* (supplementary material Fig. S7J–L). The most obvious aberrations were observed from diakinesis to anaphase I. At diakinesis and metaphase I, univalents co-existed with bivalents in mutant PMCs (supplementary material Fig. S7M–O). There was a lot of nondisjunction at anaphase I, presumably resulting from random segregation of univalent homologues. Because most single mutations cause reduced chiasma number, considering chiasma reduction in all these single and double mutants, we counted the few chiasmata that were formed in the rice *zip4 mer3* double mutant (3.13±1.70, n=116; Table 1) and compared this with the corresponding data for the *zip4* (6.05±1.97, n=164; Table 1) and *mer3* single mutants (5.59±2.07, n=64; Table 1). We found a significant decrease in the mean number of chiasmata per PMC between *zip4* and *zip4 mer3* ($t_{[278]}$=12.93, $P<0.01$), and between *mer3* and *zip4 mer3* ($t_{[178]}$=8.59, $P<0.01$). Additionally, the few chiasmata that occur are distributed randomly among cells ($\chi_{[8]}^2$=2.07, $P>0.1$; supplementary material Fig. S8A) and chromosomes ($\chi_{[3]}^2$=7.01, $P>0.05$; supplementary material Fig. S8B). This suggests that the two genes might have different functions in forming COs during rice meiosis.

The phenotypes of *zip4 zep1*, *mer3 zep1* and *zip4 mer3 zep1* mutants are similar to that of *zip4* and *mer3* single mutants

To determine whether the *zep1* mutation affects the defects observed in *zip4* or *mer3*, we made the *zip4 zep1* and *mer3 zep1* double mutants as well as the *zip4 mer3 zep1* triple mutant and compared meiotic chromosome behavior with that in *zip4*, *mer3* and *zep1* single mutants. In *zep1*, the 12 bivalents were tightly connected side by side in diakinesis PMCs (supplementary material Fig. S7D, arrows), and no univalents were observed in metaphase I PMCs (supplementary material Fig. S7E,F). In either *zip4* or *mer3*, diakinesis and metaphase I PMCs showed a variable number of bivalents (supplementary material Fig. S7G–L). Furthermore, bivalents and univalents co-existed in diakinesis and metaphase I PMCs in *zip4 zep1* (supplementary material Fig. S7P–R), *mer3 zep1* (supplementary material Fig. S7S–U) and *zip4 mer3 zep1* (supplementary material Fig. S7V–X). By comparing the phenotypes between *zep1*, *zip4* and *zip4 zep1*, we found that the kinds of aberrant morphology and behavior of meiotic chromosomes in *zip4 zep1* were very similar to those in *zip4* rather than in *zep1*. And the aberrations of meiotic chromosomes in *mer3 zep1* were similar to those in *mer3* rather than in *zep1*. Chiasma number was reduced in all these single, double and triple mutants, except *zep1*. Thus, we quantified the chiasma frequency in *mer3 zep1* (3.85±1.84, n=75; Table 1), *zip4 zep1* (3.79±1.91, n=81; Table 1) and *zip4 mer3 zep1* (2.60±1.62, n=77; Table 1) and found that *zip4 zep1* had fewer chiasmata than the *zip4* single mutant ($t_{[243]}$=8.54, $P<0.01$) and *mer3 zep1* had fewer than the *mer3* single mutant ($t_{[137]}$=5.24, $P<0.01$). According to these results, we propose that both *ZIP4* and *MER3* function upstream of *ZEP1* during rice meiosis and *ZEP1* also participates in forming crossovers.

zip4, *mer3* and *zip4 mer3* mutants show different ZEP1 distribution patterns

To investigate the effect of the rice *zip4* mutation on synapsis, dual immunolocalization was performed using antibodies against REC8 and ZEP1. REC8 is a component of the cohesion complex and is required for axial element formation and homolog pairing. It can be used as a marker to follow early meiotic events during prophase I in rice (Shao et al., 2011). ZEP1 forms the central element of the synaptonemal complex and its distribution indicates the extent of synapsis in rice (Wang et al., 2010).

During zygotene in wild-type meiocytes, ZEP1 initially localized onto the chromosomes as punctate foci and quickly extended into linear signals (Fig. 2A). In *zip4* meiocytes, long linear ZEP1 signals were found in most cells (Fig. 2B), implying that SC polymerization was not grossly affected. At pachytene, ZEP1 signals in wild-type meiocytes were located along the entire chromosomes (Fig. 2F). We did observe ZEP1 signals of normal length in *zip4* (Fig. 2G), but only in relatively long spikelets, implying a delay in completing synapsis. We therefore analyzed the frequency of different meiotic stages in the developing spikelets from wild-type and mutant plants grown in the same paddy fields (supplementary material Fig. S9). In the wild type, most microsporocytes (81.25%, n=128) in 2.8–3.0 mm spikelets showed typical leptotene. In longer spikelets, meiosis was further advanced: pachytene cells mainly (55.38%, n=195) existed in 3.8–3.9 mm spikelets. In *zip4*, we found that some cells had full synapsis of all chromosomes (9.43–28.40%) but only in relatively longer spikelets (4.0–4.4 mm). In short, the meiotic cell cycle is probably delayed at the zygotene–pachytene–diplotene transition in *zip4*.

We also carefully investigated the distribution of ZEP1 signals in *mer3*. In early zygotene, ZEP1 appeared as foci and short linear signals, similar to that observed in the wild type. From late

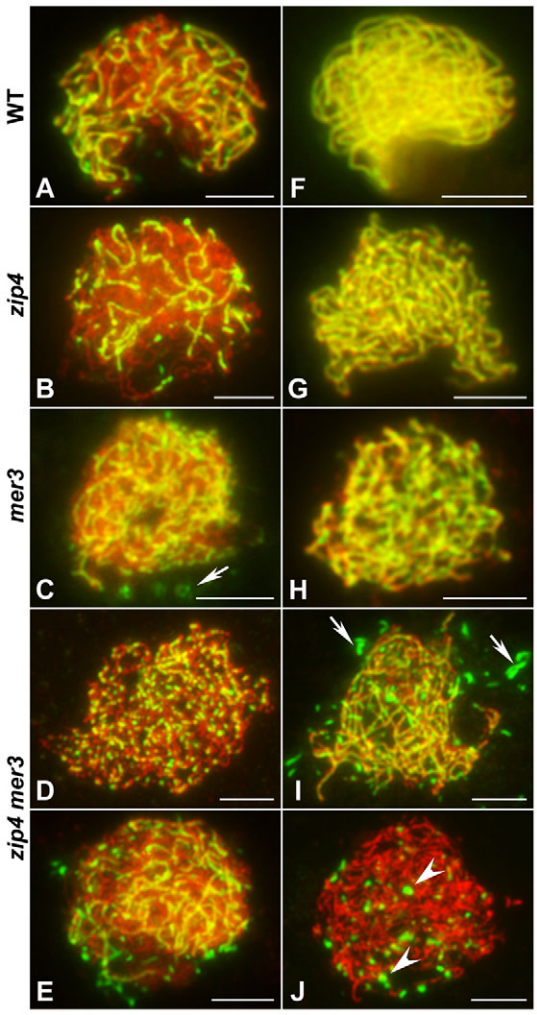

Fig. 2. Dual immunolocalization of REC8 and ZEP1 in wild-type, *zip4*, *mer3* and *zip4 mer3* PMCs. Figures show the merged images (yellow) of REC8 (red) and ZEP1 (green). (**A–E**,**J**) Zygotene. In wild-type zygotene PMCs, long linear ZEP1 signals could be observed (A). *zip4* and *mer3* show similar patterns of ZEP1 localization (B,C). The arrow indicates the released ZEP1 linear signals in *mer3* (C). 44.90% (n=245) of PMCs display numbers of ZEP1 foci (D), whereas the remaining 55.10% of PMCs contain long linear ZEP1 signals (E). Arrowheads indicate the bright ZEP1 aggregates in *zip4 mer3* zygotene PMCs (J). (**F–I**) Pachytene. Full-length ZEP1 signals could be found in rice *zip4* (G) and *mer3* (H) PMCs. Arrows indicate the released ZEP1 signals in *zip4 mer3* (I). Scale bars: 5 μm.

zygotene to pachytene, continuous ZEP1 signals were observed (Fig. 2C,H), suggesting that SC extension was not severely disrupted. However, a few ZEP1 linear signals were found to be released from chromosomes (Fig. 2C, arrow), showing that the maintenance of SCs might be slightly affected.

In *zip4 mer3*, at early zygotene, most ZEP1 proteins localized on chromosomes as foci, and short lines were rarely detected. From middle zygotene to pachytene, about 44.90% (n=245) of meiocytes still displayed numbers of ZEP1 foci (Fig. 2D). The remaining 55.10% of meiocytes contained long linear ZEP1 signals (Fig. 2E); however, 91.11% of these cells also had released ZEP1 linear signals (Fig. 2I, arrows). In addition to ZEP1 foci and linear signals, bright ZEP1 aggregates were also found in 18.51% cells (Fig. 2J, arrowheads). Taken together, synapsis seems to be severely affected in the rice *zip4 mer3* double mutant.

ZIP4 proteins present as punctuate foci and completely colocalize with MER3

To define the spatial and temporal distribution of ZIP4 accurately during rice meiosis, dual immunolocalization was carried out on wild-type microsporocytes using polyclonal antibodies against REC8 and ZIP4, raised in rabbit and mouse, respectively. In the wild type (n=250), ZIP4 foci appeared slightly later than REC8 signals at early leptotene. Almost all ZIP4 foci colocalized with REC8 and were located at one end of the REC8 signals (Fig. 3A). The average number of ZIP4 foci was about 110±28 (n=12; range, 72 to 153) at this stage. The number of ZIP4 foci increased rapidly (Fig. 3B) and reached its peak at late leptotene to early zygotene (mean, 301±45; n=30; range, 224 to 376; Fig. 3C). At late zygotene to early pachytene, most ZIP4 foci still persisted on chromosomes (Fig. 3D). With the progression of meiosis, however, the number of ZIP4 foci decreased rapidly, and only a few residual foci could be seen by late pachytene (focus number range, 0 to 86; n=15; Fig. 3E). The ZIP4 focus disappeared at diplotene and could not be detected thereafter (Fig. 3F).

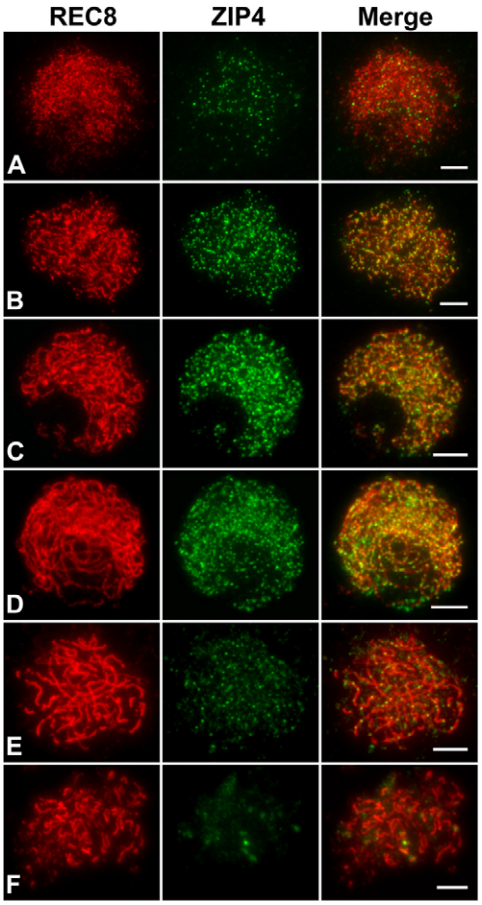

Fig. 3. Distribution of ZIP4 in wild-type PMCs. ZIP4 (green) presents as punctuate foci on chromosomes (REC8 labeled, red). (**A**) Early leptotene. (**B**) Middle leptotene. (**C**) Late leptotene to early zygotene. The number of ZIP4 foci increases rapidly. (**D**) Late zygotene to early pachytene. (**E**) Late pachytene. A few ZIP4 foci remain on the chromosomes. (**F**) Diplotene. No ZIP4 foci are detected. Scale bars: 5 μm.

Immunolocalization studies were also performed on rice *zip4* PMCs. During meiosis I, no ZIP4 signals could be detected, even in late leptotene to early zygotene during which ZIP4 foci were normally most prominent (supplementary material Fig. S10A).

A previous study has shown that MER3 proteins appear as punctuate foci and show a distribution pattern similar to ZIP4 proteins in early prophase I (Wang et al., 2009). We therefore performed dual immunostaining experiments using antibodies against both MER3 and ZIP4 to compare their localization patterns. Interestingly, MER3 foci and ZIP4 foci almost completely colocalized in all of the wild-type nuclei observed (n=100; supplementary material Fig. S11A). All the analysis shows that ZIP4 proteins mainly exist in early prophase I and the distribution of ZIP4 and MER3 is very similar.

ZIP4 is required for normal loading of MER3, but not vice versa

Considering the close cytological similarity between ZIP4 and MER3, we performed dual immunolocalization experiments to investigate the mutual dependences in the loading of ZIP4 and MER3. In most *zip4* meiocytes (n=453), no obvious MER3 signals were detected, from early leptotene to pachytene (Fig. 4B). Some MER3 signals were found in a small number of meiocytes (n=20), but those MER3 signals were not aggregated into foci as they were in the wild type (Fig. 4C). This suggests that the proper localization of MER3 onto chromosomes relies on the presence of ZIP4. Of course, other possibilities cannot be ruled out. Western blotting was performed to test the MER3 levels in both wild-type and rice *zip4* mutant panicles. Unfortunately, we failed to obtain clear signal even in the wild type, probably because of the low level and spatiotemporal limitations of the proteins. However, ZIP4 signals still presented as normal foci on meiotic chromosomes in *mer3* meiocytes, as in the wild type (supplementary material S10B). This suggests that the proper localization of MER3 onto chromosomes or the stable association of MER3 with chromosomes is likely to rely on the presence of ZIP4.

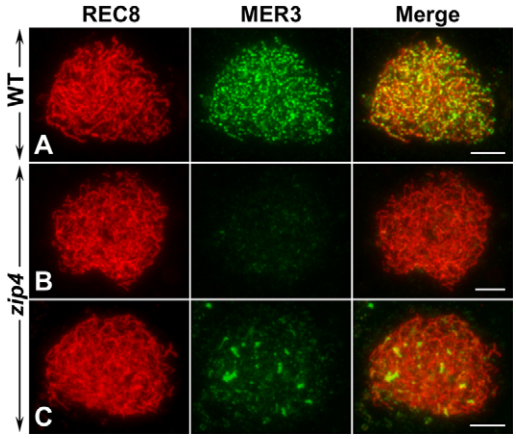

Fig. 4. Dual immunolocalization of REC8 and MER3 in wild-type and rice *zip4* PMCs. (**A**) Wild type. Late leptotene to early zygotene. MER3 (green) presents as punctuate foci on chromosomes (REC8 labeled, red). (**B**,**C**) *zip4*. Late leptotene to early zygotene. No obvious MER3 signals are observed in most rice *zip4* PMCs (B). Some MER3 aggregates are detected in a few *zip4* PMCs (C). Scale bars: 5 μm.

ZIP4 foci localize on ZEP1 signals during early zygotene

To investigate whether there was any cytological relationship between ZIP4 and synapsis, especially in the leptotene–zygotene transition stage, we carried out immunolocalization with anti-ZIP4 and anti-ZEP1 antibodies in wild-type PMCs. In the wild type, ZEP1 appeared as very faint signals when the ZIP4 foci are first detectable at early leptotene. These ZEP1 signals presented randomly in the nucleoplasm but did not colocalize with ZIP4 foci (Fig. 5A). Very quickly, the ZEP1 signals became brighter, and some gathered into several thick aggregates. These aggregates also did not show obvious colocalization with ZIP4 at middle leptotene (Fig. 5B). At early zygotene, the ZEP1 aggregates stretched into short linear signals, and showed high colocalization with ZIP4 foci. 94.59% of ZEP1 short stretches (n=533) had at least one ZIP4 focus. Interestingly, most ZIP4 foci located at one end of ZEP1 linear signals at early zygotene (91.78%; n=426), suggesting that ZEP1 might assemble from the sites where ZIP4 is located (Fig. 5C,D). Subsequently, during late zygotene, almost all ZIP4 foci localized onto the ZEP1 signals (Fig. 5E). However, because the ZEP1 signals were long segments and the number of ZIP4 foci was very high, very few of the ZIP4 signals were at the end of a ZEP1 segment, or rather, the vast majority are internal. Therefore, although it is probable that synapsis initiates from ZIP4-enriched sites in rice, not all ZIP4 foci can be associated with synaptic initiation.

In addition, we also examined ZIP4 signals in *zep1* PMCs. ZIP4 proteins presented as quite distinct and bright foci on meiotic chromosomes (supplementary material Fig. S10C), suggesting that ZIP4 loading was independent of ZEP1. ZIP4

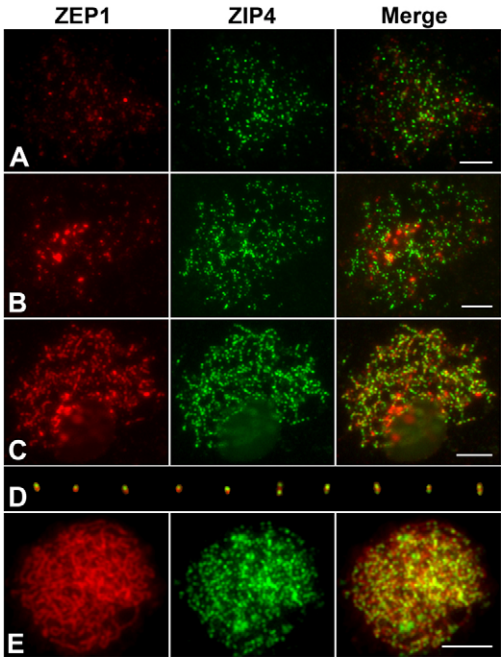

Fig. 5. Dual immunolocalization of ZEP1 and ZIP4 in wild-type PMCs. (**A**) Early leptotene. (**B**) Middle leptotene. ZEP1 (red) does not show colocalization with ZIP4 (green) when they first appear. (**C**) Early zygotene. 94.59% of ZEP1 short stretches (n=533) have at least one ZIP4 focus. (**D**) Enlarged images of ZEP1 stretches selected from C, showing end location of ZIP4. Most ZEP1 stretches (91.78%, n=426) have one end ZIP4 signal, whereas the rest have two end signals. (**E**) Late zygotene to early pachytene. All ZIP4 foci localize on the ZEP1 fragments. Scale bars: 5 μm.

distribution was not obviously affected, even in *mer3 zep1* (supplementary material Fig. S10D), and ZIP4 foci still colocalized with MER3 in *zep1* mutants (supplementary material Fig. S11B). Therefore, the connection between ZIP4 and MER3 was not disrupted by the mutation of *ZEP1*.

Discussion

ZIP4 is involved in meiotic crossover formation in rice

In the present study, we demonstrate that loss of ZIP4 protein results in a reduction in COs, visualized cytologically as chiasmata. The chiasma frequency is reduced from 20.59 per cell in the wild type to 6.05 in the rice *zip4* mutant, indicating that ZIP4 is essential for the formation of about 70% of COs in rice. The results are similar to that observed in *zip4* mutants from other organisms (Adelman and Petrini, 2008; Chelysheva et al., 2007; Tsubouchi et al., 2006). We conclude that ZIP4 has an evolutionally conserved function in crossover formation among eukaryotes. Recent studies have revealed that there are two classes of COs in yeast, and parallel results are also obtained in *Arabidopsis* (Chelysheva et al., 2007; Copenhaver et al., 2002; Higgins et al., 2008; Mercier et al., 2005). Similar observations in the *mer3* mutant support the hypothesis that two classes of COs might also coexist in rice (Wang et al., 2009). Here, statistical analysis of the data in the rice *zip4* mutant also suggests that the remaining chiasmata tend to distribute randomly among PMCs and chromosomes. However, we should consider the limitation of the Poisson test. When the total number of COs is low, a Poisson distribution would be expected regardless whether the interference is present or not. Even if a mutant has functional interference, the observed distribution of chiasmata would still show a Poisson distribution. Consequently, our conclusion that *zip4* lacks interference is weak. In addition to CO reduction, other kinds of chromosome aberrations were detected in *zip4* mutant PMCs. First, meiotic chromosomes were distorted and some metaphase I bivalents were dramatically drawn out. We propose that metaphase I is greatly prolonged in *zip4*. Tension depends on bi-orientation of homologous chromosomes; when it is not achieved at all kinetochores, the spindle checkpoint is activated and anaphase is delayed. Metaphase I tension would therefore keep pulling on the bivalent kinetochores for a long time and might cause these defects. Moreover, chromosome bridges existed in a few mutant PMCs. Although pairing occurs between homologous chromosomes in most cases, some non-homologous pairing cannot be excluded by our FISH experiments.

The contributions of ZIP4 and MER3 in crossover formation are not completely equivalent

In budding yeast, in all single and double *zmm* mutants, COs occur at ~15% of wild type, indicating similar contributions of each ZMM component in forming class I COs (Börner et al., 2004). Similar observations have been made in *Arabidopsis*, where the number of residual chiasmata in *Atmsh5-1* is not significantly different from that observed in *Atmsh4* or *Atmsh4 Atmsh5* (Higgins et al., 2008). The mean number of chiasmata per PMC between *Atzip4* and *Atzip4 Atmsh4* also do not exhibit a significant difference (Chelysheva et al., 2007), suggesting that these genes function in the same pathway for CO formation. In rice, based on the random distribution of the remaining chiasmata in *zip4* and *mer3* single mutants, we presume that both ZIP4 and MER3 are required in the interference-sensitive CO pathway. Although the statistical analysis in our study indicates a severe reduction in the class I COs in general, it did not provide evidence that the interference-sensitive CO pathway is completely disrupted in either of the *zip4* or *mer3* single mutant, so the results of the epistasis test would be misleading if both are hypomorphs. Nevertheless, because the mean number of chiasmata in *zip4 mer3* is prominently reduced compared with either the *zip4* or *mer3* single mutant, we prefer the hypothesis that ZIP4 and MER3 work cooperatively to promote CO formation, and the contribution of these two genes are not equivalent in forming class I COs in rice; the loss of function of either one partially disrupts the formation of COs. The other possibility is that, although no ZIP4 signal could be detected in *zip4*, we cannot guarantee that the mutation completely destroys its biological function. If so, then the defects (CO reduction) would be more severe in the double mutant than either single mutant even if *ZIP4* and *MER3* function in the same CO formation pathway.

ZEP1 might have a double role in meiotic crossover formation in rice

In the *S. cerevisiae zip1* mutant, homologous chromosomes are aligned but not synapsed during pachytene. In addition, the *zip1* null mutation shows relatively minor defects in both chromosome recombination and segregation (Sym et al., 1993; Sym and Roeder, 1994). Similarly, in the rice *zep1* mutant, 12 bivalents were observed in each nucleus at diakinesis and metaphase I, and COs are increased to a certain degree, supporting the hypothesis that SCs might be involved in inhibiting excessive CO formation (Sym and Roeder, 1994; Wang et al., 2010). In this study, all of the *zip4 zep1*, *mer3 zep1* and *zip4 mer3 zep1* double and triple mutants show decreased numbers of bivalents and chiasmata. The phenotypes are much more similar to those in *zip4* and *mer3* than to *zep1* (supplementary material Fig. S7). These results suggest that normal function of both ZIP4 and MER3 are required for the downregulation of COs by the SC during pachytene.

In addition, statistical analysis shows that the mean numbers of chiasmata in the double mutants *zip4 zep1* and *mer3 zep1* are even less than that in the *zip4* and *mer3* single mutants, respectively. This implies that ZEP1 (or nascent SC) also promotes CO formation at an early stage of meiosis in rice. This implication is consistent with the view from budding yeast that ZIP1 probably plays a role in recombination early during synapsis, when homolog axes are first closely juxtaposed at the sites of recombination (Lynn et al., 2007). Taken together, ZEP1 protein (or the SC) might have a dual function: one to facilitate CO determination at early zygotene and another to inhibit excess COs at pachytene.

MER3 loading depends on ZIP4 but not vice versa

In budding yeast, ZIP4 acts with ZIP2 and ZIP3 to mediate synapsis by promoting ubiquitylation (Perry et al., 2005). ZIP4 also forms a complex with SPO16 (Shinohara et al., 2008). Localization of ZIP4 to meiotic chromosomes is dependent on SPO16, ZIP1 and ZIP3. However, MER3 is a DNA helicase that catalyzes the unwinding of HJs (Nakagawa and Kolodner, 2002) and might have close relationships with other HJ-related proteins. Although the association of ZIP4 and MER3 with other recombination proteins has been investigated, to date there are no detailed analyses of the relationship between ZIP4 and MER3. Our present study reveals a correlation between ZIP4 and MER3

in rice. First, we show that during rice meiosis, ZIP4 foci colocalize with MER3 in wild-type PMCs. Second, colocalization between ZIP4 and MER3 is independent of normal ZEP1 function. Third, MER3 does not normally form foci in *zip4*, suggesting that ZIP4 is important for proper loading of MER3 onto chromosomes or the stability of its association with chromosomes. Fourth, the normal loading of ZIP4 in *mer3* implies that ZIP4 loading is independent of MER3. Fifth, no direct interactions between ZIP4 and MER3 proteins are detected in the yeast two-hybrid protein system (supplementary material Fig. S12). Thus, it is likely that ZIP4 and MER3 do not interact with each other directly. Some other intermediate proteins might participate in bridging ZIP4 and MER3 during meiosis.

ZEP1 polymerizations are grossly affected in the *zip4 mer3* double mutant

Studies of ZMM deficiency in *S. cerevisiae* have revealed that the polymerization of ZIP1 is impaired, indicating a defect in synapsis, which supports the idea that synapsis proceeds from, and depends on, ZMM proteins in budding yeast (Börner et al., 2004; Tsubouchi et al., 2006). However, the contribution of ZMM to synapsis varies between species. In *Arabidopsis*, SCs assemble normally with only mild defects in the *Atzip4* mutant (Chelysheva et al., 2007). Synapsis is also not prevented in *Arabidopsis mer3* (*rck*), *msh4* and *msh5* mutants (Chen et al., 2005; Higgins et al., 2004; Higgins et al., 2008). These results imply that ZMM proteins are not essential for SC polymerization in *Arabidopsis*. In the present study, analyses of the rice *zip4* mutant revealed that the SC protein ZEP1 assembles normally with only mild defects, indicating that ZIP4 might not be required for ZEP1 assembly. Similarly, in the *mer3* mutant, normal ZEP1 signals were observed, suggesting that ZEP1 assembly does not depend on MER3 either. However, analysis of the ZEP1 distribution pattern in the *zip4 mer3* double mutant suggests that SC extension is defective from early zygotene onward, but with delayed, partial and/or unstable SC installation still occurring in some PMCs. This pattern is different from that in either the *zip4* or *mer3* single mutants, but it is very similar to that in *zmm* mutants in budding yeast.

Previous studies suggest that the only requirement for SC formation might be close juxtaposition of two axes (Kleckner, 2006). In organisms where SC formation depends on DSBs, recombination might bring homolog axes locally into sufficiently close proximity for transverse filament proteins to nucleate and extend along the homolog axes (Börner et al., 2004; Lynn et al., 2007; Zickler and Kleckner, 1999). ZMM proteins might thus mainly promote recombination reactions, but not participate in synapsis per se (Lynn et al., 2007). The different ZEP1 distribution patterns between double and single mutants lead us to propose that, in the absence of both ZIP4 and MER3, homolog axes might not close enough for ZEP1 nucleation and extension because of the delay in and/or lack of recombination intermediate processing. By contrast, in the *zip4* or *mer3* single mutants, although the processing might be slightly affected, the recombination association can still bring homolog axes into sufficiently close physical proximity for ZEP1 assembly.

Synapsis might initiate from ZIP4-enriched recombination sites in rice

In budding yeast, SC assembly depends on ZMM proteins (Börner et al., 2004; Fung et al., 2004). In addition, during early zygotene, ZIP1 signals often colocalize with other ZMM components, suggesting that synapsis proceeds from ZMM sites in budding yeast (Agarwal and Roeder, 2000; Chua and Roeder, 1998; Shinohara et al., 2008; Tsubouchi et al., 2006). Recently, ZIP1 foci in leptotene were also reported in some studies (Börner et al., 2004; Shinohara et al., 2008). Here, we demonstrate that, during rice meiosis, ZEP1 aggregates, which might correspond to polycomplexes (PCs) before SC formation, do not colocalize with ZIP4 foci when they first appear at leptotene, implying that ZIP4 is present on chromosomes before ZEP1 stably localizes. This observation is consistent with the result that ZIP4 localizes normally onto chromosomes in the *zep1* mutant. In addition, both observations support the idea obtained from budding yeast that the subset of sites that will engage the ZMM complex is chosen before, and independently of, the SC (Bishop and Zickler, 2004). When synapsis occurs during early zygotene, ZIP4 foci colocalize well with ZEP1 signals. The result indicates that synapsis might mainly initiate from ZIP4-indicated recombination sites, although SC assembly does not rely on ZIP4 per se.

Our studies also revealed that these ZIP4 foci are often found at ends of partially elongated ZEP1 linear signals at early zygotene. Similar localizations have also been found in budding yeast and *Sordaria*, implying that synapsis frequently occurs unidirectionally from synaptic initiation sites. The implication contrasts with the long-standing assumption that SC polymerizes in both directions (Shinohara et al., 2008; Tsubouchi et al., 2008; Zickler et al., 1992). Hence, the fact that ZIP4 foci frequently locate at ends of nascent ZEP1 stretches suggests that synapsis might also proceeds outward in a single direction from the sites where ZIP4 locates in rice.

Based on the localization pattern of ZEP1 and ZIP4, we propose a hypothetical SC assembly process in rice. In leptotene, because homologs are not close enough for ZEP1 nucleation and extension, abundant ZEP1 proteins are assembled to form disordered aggregates (PCs). At this stage, those aggregates do not show obvious colocalization with ZIP4. Once homologs are closely aligned at zygotene, ZEP1 aggregates disperse and then bind to homolog axes at ZIP4-bound synaptic sites, which might be the closest association sites between homolog axes at this stage. Then, ZEP1 proteins polymerize and zipper from those sites to form ordered SCs along the aligned homologous chromosomes.

What is the relationship between ZMM proteins, CO I and synapsis in rice?

In budding yeast, ZMM foci (synapsis initiation sites) correspond to final class I COs (Agarwal and Roeder, 2000; Chua and Roeder, 1998; Fung et al., 2004). However, such correspondence is not universal among higher eukaryotes. The number of ZMM foci in both *Arabidopsis* and mouse significantly exceeds the number of COs (Chelysheva et al., 2007; de Vries et al., 1999; Higgins et al., 2004). One frequently cited explanation for this excess is that plants require abundant additional early recombinational interactions to ensure efficient homologous pairing of their relatively large chromosomes (Higgins et al., 2004; Zickler and Kleckner, 1999). In rice, from late leptotene to early pachytene, the ZMM focus number is consistently much larger than the number of final class I COs (Wang et al., 2009). During early pachytene, the ZMM focus number is still much higher than the final COs, requiring that these excess ZMM foci must be even further regulated after the formation of full-length

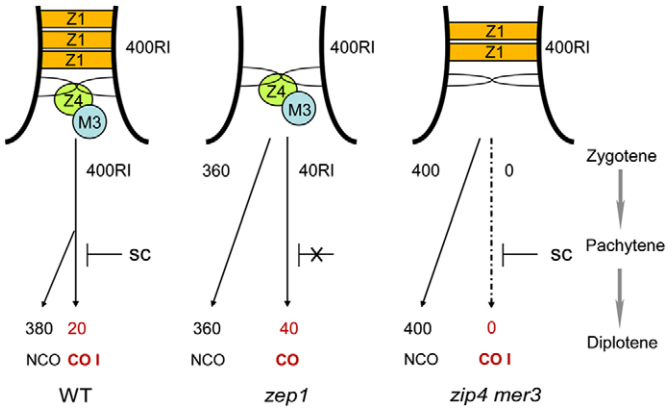

Fig. 6. Model of class I CO formation in rice. A schematic representation of the correlation among ZMM proteins, class I COs and synapsis in rice. During rice homologous recombination, ZIP4 and MER3 bind to a subset of recombination intermediates (RIs), which are finally processed into either class I COs or NCOs. At pachytene, the number of class I COs is kept within a range by regulation through the SC. In *zip4 mer3*, all stable RIs are disrupted and no class I COs are formed. In *zep1*, the stable RI number is decreased, but all of them are processed into COs because of the lack of putative suppression by SC regulation. The value presented here is not an exact number; it is only used to demonstrate the model more plainly. M3, MER3 protein; Z1, ZEP1 protein; Z4, ZIP4 protein; RI, recombination intermediate.

SC. DSBs that do not become COs must be repaired to generate NCOs instead (Bishop and Zickler, 2004; Martini et al., 2006). Therefore, it is reasonable to propose that those abundant ZMM foci result in NCOs in rice, with some probability of leaving a simple gene conversion footprint of that event.

In this study, we carefully investigated the relationship between ZIP4, MER3 and ZEP1 in rice through both genetic and cytological analysis. Based on all conclusions described above, a model is postulated to illustrate the correlation between ZMM proteins, class I COs and synapsis in rice (Fig. 6). At early stages in prophase I, recombination machinery promotes the recognization and alignment between homologs. Non-ZEP1 ZMM proteins bind to a subset of recombination intermediates, which are finally processed to either class I COs or NCOs. During zygotene, ZEP1 proteins bind to homologs at those sites and polymerize outward to form SCs along the whole length of homologs. Those nascent SCs might bring homologs into a closer juxtaposition and might be required for further progression of recombination intermediates. Loss of function of ZIP4 and MER3 might disrupt progression of almost all the intermediates, whereas mutation of ZEP1 only disrupts maturation of most but not all of the intermediates. Stable recombination intermediates might be destined to become COs. However, because of the regulation of the SC, most intermediates are resolved into NCOs in the wild type. In the rice *zep1* mutant, although the number of recombination intermediates is reduced, all those intermediates are resolved into COs as a result of the lack of regulation by the SC.

Materials and Methods

Plant materials and growth conditions

The rice (*Oryza sativa*) sterile mutant *zip4* was isolated from a *japonica* cultivar, Nipponbare. The F2 and F3 mapping population was generated from a cross between heterozygous plant (*zip4*/+ genotype) and Zhefu802, a polymorphic *indica* cultivar. Plants exhibiting sterility were selected for gene mapping. Although homozygotes of both *zip4* and *mer3* single mutants are almost sterile, their heterozygotes (*zip4*/+ and *mer3*/+) are fertile with normal seed set. To obtain the *zip4 mer3* double mutant, a *zip4*/+ single heterozygote was crossed to a *mer3*/+ single heterozygote. Then we used PCR screening to find out which F1 progeny were the desired *zip4*/+ *mer3*/+ double heterozygotes. These double heterozygous plants were self-pollinated and produced F2 progeny. Finally, we screened the F2 progeny and obtained the *zip4 mer3* double homozygous plant reported on here. The *zip4 zep1* and *mer3 zep1* double mutants were obtained by the same way. The *zip4 mer3 zep1* triple mutant resulted from crossing a *zip4*/+ *mer3*/+ double heterozygote and a *zep1*/+ single heterozygote. All plants were grown in the paddy fields either in Beijing (China) or in Sanya (Hainan Province, China) during the natural rice growing season.

Positional cloning

For fine mapping of *zip4*, STS markers were developed based on sequence differences between the *indica* variety 9311 and the *japonica* variety Nipponbare according to the data published on http://www.ncbi.nlm.nih.gov. The primer sequences are listed in supplementary material Table S1.

Complementation test

An 8.38 kb genomic DNA fragment containing the entire *ZIP4* coding region was inserted into the binary vector pCAMBIA1300 to generate the transformation plasmid for complementation test. The plasmid was transformed into *Agrobacterium tumefaciens* EHA105 by electroporation, and then into *zip4*/+ rice embryonic calli.

Cloning full-length *ZIP4* cDNA

Total RNA was extracted from rice panicles (~4–6 cm) using the TRIzol Reagent (Invitrogen). After treatment with DNase I (Invitrogen), 2 μg total RNA was reverse transcribed to synthesize cDNA using oligo (dT) primer and Superscript III (Invitrogen). 3′ rapid amplification of cDNA ends and 5′ rapid amplification of cDNA ends was performed according to the protocol of the kit (3′-Full RACE Core Set and 5′-Full RACE Core Set, Takara). For 3′ RACE, three rounds of PCRs were performed using the same adaptor primer (P-ada) and a set of *ZIP4* gene specific primers (HP-1F, HP-2F, HP-3F). For 5′ RACE, the RNA was reverse transcribed with 5′ (P) labeled primer (HP-4Rp); the first and second PCRs were performed using two sets of *ZIP4*-specific primers (HP-5, HP-6). The resulting 3′ RACE-PCR and 5′ RACE-PCR products were cloned and sequenced. All primer pairs are listed in supplementary material Table S1.

Y2H assay

The transactivation assay and Y2H assay were carried out using a Matchmaker LexA Two-Hybrid system (Clontech). The ORFs of *ZIP4* was amplified with primer pair Z4-Y2H from wild-type rice panicle cDNA and cloned into the vector pLexA to construct pLexA-ZIP4. This construct was used to transform the recipient strain EGY48 containing p8op-*lacZ*. Transformants were selected on selective medium plates at 30℃ for 4 to 6 days.

For the Y2H assay, the ORFs of *MER3* and *ZEP1* were amplified with gene-specific primer pairs (M3-Y2H, Z1-Y2H) and cloned into the vector pB42AD. Both of these constructs were transformed into EGY48 containing p8op-*lacZ* and pLexA-ZIP4, respectively. Cotransformants were selected on selective culture medium (SD/–Ura/–Trp/–His) at 30℃ for 4 to 6 days. The activation ability was assayed on Gal/Raf (–His/–Leu/–Trp/–Ura)/X-gal plate. All primer pairs are listed in supplementary material Table S1.

Antibody production

The anti-REC8, anti-PAIR2 and anti-MER3 polyclonal antibodies have been described (Wang et al., 2009). The anti-ZEP1 polyclonal antibody was described (Wang et al., 2010). To generate the antibody against ZIP4, an 84 bp fragment of *ZIP4* cDNA (amino acids 1–28) was amplified from rice panicle cDNA with primers HP-Ab; sequences are listed in supplementary material Table S1. This fragment was inserted into the expression vector pGEX-6P-1 (Amersham). The GST fusion ZIP4 peptide was expressed in BL21 (DE3) and purified with Glutathione Sepharose 4B (GE Healthcare). The anti-ZIP4 polyclonal antibody was obtained by immunizing a mouse with the fusion peptide. Specificity of the anti-ZIP4 antibody was checked in immunolocalization experiments.

Meiotic chromosome preparation

Young panicles of both wild type and *zip4* were harvested and fixed in Carnoy's solution (ethanol: glacial acetic, 3:1). Microsporocytes undergoing meiosis were squashed in an acetocarmine solution. Slides with chromosomes were frozen in liquid nitrogen. After removing the coverslips, the slides were dehydrated through an ethanol series (70%, 90% and 100%). Chromosomes were counterstained with 4′,6-diamidino-phenylindole (DAPI) in an antifade solution (Vector Laboratories, Burlingame, CA). Chromosome images were captured under the ZEISS A2 fluorescence microscope with a micro-CCD camera.

Fluorescence in situ hybridization

FISH analysis was conducted as described previously (Zhang et al., 2005). Two repetitive DNA elements were used as the FISH probes: pTa794 clone containing the coding sequences for the 5S ribosomal RNA genes from wheat (Cuadrado and Jouve, 1994) was used to monitor the short arm of chromosome 11. The other is a BAC clone, OSJNBa0012J05, on the long arm of chromosome 8 (Tang et al., 2007). Chromosomes were counterstained with DAPI in Vectashield anti-fading solution (Vector Laboratories). Original images were captured under the ZEISS A2 fluorescence microscope with a micro-CCD camera.

Immunofluorescence

Fresh young panicles were fixed in 4% (w/v) paraformaldehyde for 30 minutes at room temperature. Anthers in the proper stage were squashed using a dissecting needle in PBS solution and covered with a coverslip. After freezing in liquid nitrogen and removing the coverslip, the slide was dehydrated through an ethanol series (70%, 90% and 100%) before immunostaining. Slides were then incubated in a humid chamber at 37℃ for 4 hours with different antibody combinations diluted 1:500 in TNB buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.5% blocking reagent). After three rounds of washing in PBS, Texas-Red-conjugated goat anti-rabbit antibody and fluorescein isothiocyanate-conjugated sheep anti-mouse antibody (1:1000) were added to the slides. The chromosomes were counterstained with DAPI in an antifade solution (Vector Laboratories). All images were captured under the Carl Zeiss Axio Imager A2 microscope with a micro-CCD camera. To determine whether there was any staining signal in mutant PMCs, the background was adjusted to a suitable level of brightness. All fluorescence images were edited with Photoshop CS2 software.

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