

# The Central Element Protein ZEP1 of the Synaptonemal Complex Regulates the Number of Crossovers during Meiosis in Rice

Mo Wang,<sup>a</sup> Kejian Wang,<sup>a</sup> Ding Tang,<sup>a</sup> Cunxu Wei,<sup>b</sup> Ming Li,<sup>a</sup> Yi Shen,<sup>a</sup> Zhengchang Chi,<sup>b</sup> Minghong Gu,<sup>b</sup> and Zhukuan Cheng<sup>a,1</sup>

<sup>a</sup>State Key Laboratory of Plant Genomics and Center for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

<sup>b</sup>Key Laboratory of Crop Genetics and Physiology of Jiangsu Province/Key Laboratory of Plant Functional Genomics of Ministry of Education, Yangzhou University, Yangzhou 225009, China

**ZEP1, a transverse filament (TF) protein, is the rice (*Oryza sativa*) homolog of *Arabidopsis thaliana* ZYP1. In the *Tos17*-insertional *zep1* mutants, homologous chromosomes align along the entire length of the chromosome, but the synaptonemal complex is not assembled in early prophase I. Crossovers are well formed, and 12 bivalents could be detected from diakinesis to metaphase I, which leads to equal chromosomal segregation in anaphase I. Moreover, the number of crossovers has a tendency to be increased compared with that in the wild type. These phenomena are different from the TF mutants identified so far in other organisms. Chiasma terminalization of the bivalent, which occurs frequently in the wild type, seldom occurred in *zep1*. Transmission electron micrographs and immunodetection using an antibody against ZEP1 showed that ZEP1 is the central element of the synaptonemal complex. Although PAIR2 and MER3 were loaded normally in *zep1*, their dissociation was delayed severely compared with the wild type. In addition, ZEP1 is reloaded onto chromosomes in early microspores as the chromosome decondense, suggesting that ZEP1 might have other biological functions during this process.**

## INTRODUCTION

For all sexually propagating eukaryotes, meiosis is the crucial process of producing haploid gametes and consists of two rounds of chromosome division following a single round of DNA replication. The first division (meiosis I) is a process in which homologous chromosomes pair, synapse, recombine, and segregate; in the second division (meiosis II), which resembles mitosis, sister chromatids segregate toward opposite poles.


The synaptonemal complexes (SCs) are meiosis-specific proteinaceous structures that enhance the close attachment of homologous chromosomes in prophase I. SC assembly starts from early prophase I with the formation of the axial elements, which elongate along the chromosome arms and connect the sister chromatids (de Boer and Heyting, 2006). Transverse filament (TF) proteins are in the center of SCs and perpendicularly connect the lateral elements. In budding yeast, TFs first assemble at the sites of synapsis initiation complexes and then stretch along the homologs. TF proteins have been reported in


many organisms, including budding yeast, *Drosophila melanogaster*, *Caenorhabditis elegans*, mouse, and *Arabidopsis thaliana* (Meuwissen et al., 1992; Sym et al., 1993; Heyting, 1996; Page and Hawley, 2001; MacQueen et al., 2002; Colaiácovo et al., 2003; de Vries et al., 2005; Higgins et al., 2005). Interestingly, these TF proteins have poor homology at the amino acid level but exhibit significantly similar structures. They all have a coiled-coil domain in the central region with globular domains at both ends (Page and Hawley, 2004). The C termini of the TFs have S/TPXX motifs, which are reported to interact with DNA. When TF proteins are assembled, they form parallel homodimers with the N termini overlapping in the center of the SCs and the C termini connected to the lateral elements.


ZIP1 in budding yeast was the first TF protein identified (Sym et al., 1993; Storlazzi et al., 1996). The assembly of SCs in budding yeast is closely coordinated with the initiation and maturation of homologous recombination events. ZMM complexes, which are required to implement interference-sensitive (class I) crossovers (COs), contain seven collaborating members, including ZIP1, ZIP2, ZIP3, ZIP4, MSH4, MSH5, and MER3. These proteins colocalize and are always present at the sites where SC polymerization initiates, so the ZMM proteins are referred to as the synapsis initiation complex and are markers of class I COs (Fung et al., 2004; Tsubouchi et al., 2006; Lynn et al., 2007). In mutants of non-ZIP1 ZMM components, ZIP1 always localizes to chromosomes as dots at the early stage of prophase I; while at pachytene, it forms polycomplexes that are never associated with chromosomes rather than the string-like signals

<sup>1</sup> Address correspondence to zkcheng@genetics.ac.cn.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Zhukuan Cheng (zkcheng@genetics.ac.cn).

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along the entire chromosomes in the wild type (Agarwal and Roeder, 2000; Novak et al., 2001; Borner et al., 2004; Cheng et al., 2006). In the *zip1* mutant, the immunosignals of ZIP2, ZIP3, and RAD51/DMC1 resemble those in wild-type nuclei, while those of Msh4 and Msh5 in *zip1* become fainter (Chua and Roeder, 1998; Agarwal and Roeder, 2000; Shinohara et al., 2000; Novak et al., 2001; Shinohara et al., 2008).

*c(3)G* encodes the 744–amino acid TF protein in *Drosophila* females. Mutation of *c(3)G* results in the loss of all COs, indicating that all COs in *Drosophila* females depend on C(3)G (Page and Hawley, 2001; Bogdanov et al., 2002). In *C. elegans*, two components of the central element of SCs, SYP-1 and SYP-2, have been reported (MacQueen et al., 2002; Colaiácovo et al., 2003). Both SYP-1 and SYP-2 are coiled-coil proteins that may cooperate to span the width of synaptic axial elements. In both *syp-1* and *syp-2* mutants, 12 univalents are present in the diakinesis nuclei, reflecting a failure during chiasma formation. Therefore, TFs are essential for the formation of COs in *C. elegans*, just like that in *Drosophila* (MacQueen et al., 2002; Colaiácovo et al., 2003; Hillers, 2004). In the *syp-2* mutant, the Rad51 foci in early prophase I are similar to those of the wild type, but these foci fail to decrease at late pachytene and persist until early diakinesis. A similar phenomenon was also observed in *syp-1* (Colaiácovo et al., 2003). Sycp1 has been identified as a TF protein in mouse. Homologous chromosomes in *sycp1* spermatocytes can form normal axial elements but do not synapse, and >90% of COs disappear in *sycp1* (de Vries et al., 2005). In the *sycp1* mutant, the number and distribution of MSH4 foci are similar to those observed in the wild type, but they do not disappear at the appropriate time compared with the wild type. The same behavior was also observed for RAD51/DMC1 in *sycp1*.

Higgins et al. (2005) used an *in silico* method as described by Bogdanov et al. (2003) to overcome the lack of primary sequence homology and identified a TF protein in *Arabidopsis*. There are two redundant TF proteins in *Arabidopsis*, ZYP1a and ZYP1b, together referred to as ZYP1. In *Arabidopsis* plants treated with small interfering RNA for ZYP1, the number of foci of MLH1 (a marker of late recombination events destined to be COs) declines only slightly, which indicates that the formation of COs in *Arabidopsis* is less dependent on TF (Higgins et al., 2005; Jackson et al., 2006). Interestingly, nonhomologous chromosomes assembled to form bivalents and/or multivalents in the ZYP1 RNA interference (RNAi) plants, which may imply a new function of the TF proteins in *Arabidopsis*.

In both mitosis and meiosis, chromosomes must condense to facilitate chromosome segregation, and the condensin complex plays an important role in this process. The condensin complex (13S condensin) comprises two structural maintenance of chromosome (SMC) subunits (SMC2/CAP-E and SMC4/CAP-C), which form a heterodimer in a V-shape, as well as three non-SMC subunits (CAPD2, CAP-G, and CAP-H) (Hirano, 2002; Yu and Koshland, 2003; Nasmyth and Haering, 2005). The SMC superfamily proteins have ATP binding domains at both the N and C termini and two extended coiled-coil domains separated by a hinge in the middle. However, a condensin protein specific to meiosis has not been reported.

In this study, we identified the homolog of ZYP1 in rice (*Oryza sativa*), ZEP1, and characterized its function using both *Tos17*-

insertion mutants and RNAi plants. Our data suggest that ZEP1 is the central element of SCs in rice. In the mutants of *zep1*, the SCs do not assemble normally, but COs are always formed between homologous chromosomes, which led to equal chromosome segregation in meiosis I. In addition, the chromosomes tended to condense in early microspores separated from the tetrad in *zep1*, which impeded the initiation of the first pollen mitosis and finally led to defects in pollen development.

## RESULTS

### Identification of ZEP1

To identify a putative ZYP1 gene in rice, a BLAST search was performed using the *Arabidopsis* ZYP1a and ZYP1b amino acid sequences. The two homology searches produced the same candidate with significant similarity at locus Os04g0452500. By means of RT-PCR and rapid amplification of cDNA ends (RACE) PCR with gene-specific primers, we redefined the cDNA sequence, which differed remarkably from the predicted sequence, and named the gene ZEP1 for its functional homology with ZIP1 in *Saccharomyces cerevisiae* and ZYP1 in *Arabidopsis*. Nucleotide sequence analysis of the ZEP1 cDNA revealed that it is comprised of 3391 bp with an open reading frame of 2610 bp. ZEP1 has 21 exons and 20 introns, which differs from the predicted structure (see Supplemental Figure 1 online). The corresponding protein sequence of ZEP1 is most similar to ZYP1b (362/874 residues identical and 555/874 residues positive). It also shows high similarity to ZYP1a (357/880 residues identical and 567/880 residues positive; see Supplemental Figure 2 online). By means of RT-PCR, we revealed that ZEP1 is expressed mainly in young panicles and roots, and to a lesser extent in leaves and stems (see Supplemental Figure 3A online). Although the expression of ZEP1 is not meiosis specific, ZEP1 protein was present only in the panicle and could not be detected in vegetative organs (see Supplemental Figure 3B online).

The ZEP1 protein contains 869 amino acid residues, with a central coiled-coil region of 650 residues (amino acids 64 to 713; COILS-Prediction of Protein Coiled-Coil Regions available at [http://www.ch.embnet.org/software/COILS\\_form.html](http://www.ch.embnet.org/software/COILS_form.html)) (Bogdanov et al., 2003). The N-terminal globular domain of 63 residues (amino acids 1 to 63) is basic, which is different from the acidic N termini of the mouse and budding yeast TF proteins but consistent with the *Arabidopsis* protein. The C-terminal globular domain of 156 residues (amino acids 714 to 869) has a high pI (9.44) and putatively binds DNA (ProtParam tool available at <http://www.expasy.ch/tools/protparam.html>) (Bogdanov et al., 2003). Additionally, the C terminus of ZEP1 contains two S/TPXX motifs, SPET (amino acids 751 to 754) and SPIT (amino acids 767 to 770), which are possibly involved in DNA binding (Suzuki, 1989).

### Isolation of the *Tos17*-Insertion Mutant of ZEP1

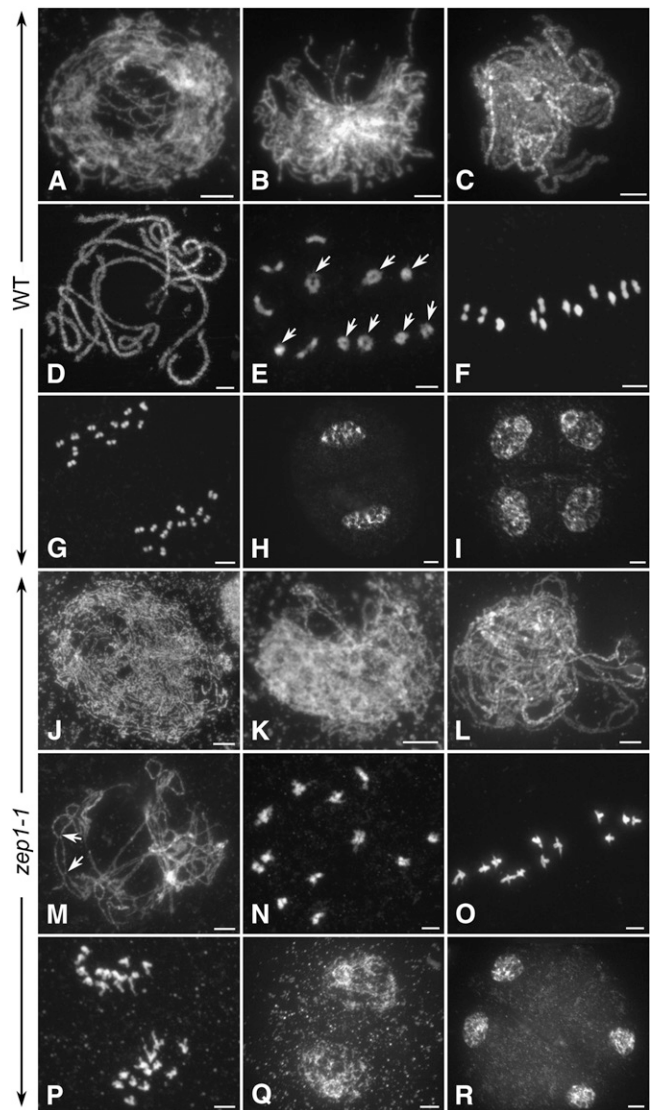
Four single *Tos17*-insertion mutant lines of the ZEP1 gene were identified by screening the public insertion line collections. Sequence analysis of their PCR products confirmed that *Tos17* was inserted in exon 8 of two independent lines and in exon 12

and intron 12 of the other two lines, which were referred to as *zep1-1*, *zep1-2*, *zep1-3*, and *zep1-4*, respectively (see Supplemental Figure 1 online). The four homozygous *zep1* mutants showed normal vegetative growth and development, but with decreased seed sets of 32.68, 34.55, 48.35, and 56.67%, respectively. Their pollen fertilities were 59.18, 57.63, 71.59, and 91.28%, respectively, when evaluated by 1% I<sub>2</sub>-KI solution staining (see Supplemental Table 1 online). We also checked both seed setting and pollen grain fertility evaluated with I<sub>2</sub>-KI staining of the *ZEP1/zep1* heterozygote plants. The seed setting and pollen fertility were normal (see Supplemental Figure 4 online), indicating that the *ZEP1* gene function is sporophytic. In the progeny of self-fertilized heterozygous plants of *zep1* mutants, plants with normal fertility and lower fertility segregated in a ratio of 3:1 (89:26 for *zep1-1*), implying that the lower fertility mutation in *zep1* is a monogenic recessive mutation. By conducting immunoblotting, we found that both *zep1-1* and *zep1-3* expressed truncated *ZEP1* proteins (see Supplemental Figure 3B online). Because of the differences in pollen fertilities and seed sets, we proposed that *zep1-1* and *zep1-2* are strong alleles, whereas *zep1-3* and *zep1-4* are weak alleles. Thus, we mainly focused on *zep1-1* to characterize the phenotype of *zep1* and study *ZEP1* function.

### The *zep1* Mutant Shows Abnormal Synaptic Behaviors and More COs during Prophase I

To determine whether the low fertility was a result of meiosis defects, the meiotic chromosomes of pollen mother cells at different stages from both the wild type and *zep1-1* were investigated. During early prophase I of the wild type, the homologous chromosomes began to pair (Figures 1A and 1B). The SCs were fully assembled at pachytene (Figures 1C and 1D) and fell apart at diplotene. At diakinesis, several ring-shaped bivalents could always be found in the same nucleus, indicating that the chiasmata of these bivalents had already been terminated (Figure 1E). All 12 of the extremely condensed bivalents lined up on the equatorial plate at metaphase I (Figure 1F). From anaphase I to telophase I homologous chromosomes segregated away from each other and migrated to the opposite poles (Figures 1G and 1H). Meiosis II is a relatively short process compared with meiosis I, and the sister chromatids of each chromosome segregate just like in mitosis. After meiosis II, four microspores were produced (Figure 1I).

In *zep1-1*, the chromosome behavior was almost the same as in the wild type from leptotene to zygotene (Figures 1J and 1K). At early pachytene, the two threads of the bivalents were always visible, although the homologous chromosomes could align perfectly along the entire chromosome length (Figure 1L). At late pachytene, all of the chromosomes were separated into single threads, while the two homologous chromosomes of each bivalent were still connected by several chiasmata, indicating that COs had occurred during the early stages (Figure 1M). At diakinesis, 12 bivalents could be detected in every nucleus (Figure 1N). We found that the two homologous chromosomes of the bivalents were tightly connected side-by-side and ring-shaped bivalents were seldom detected, implying that the chiasmata were not terminated efficiently at diakinesis in *zep1-1*. At



**Figure 1.** Meiotic Chromosomes Stained with 4',6-Diamidino-2-Phenylindole in Both Wild-Type and *zep1-1* Pollen Mother Cells.

- (A) to (I) The wild type.  
 (J) to (R) in *zep1-1*. Bars = 5  $\mu$ m.  
 (A) and (J) Leptotene.  
 (B) and (K) Zygotene.  
 (C) and (L) Early pachytene.  
 (D) and (M) Pachytene. In *zep1-1*, the COs are visible as indicated by arrows in (M).  
 (E) and (N) Diakinesis. The ring-shaped bivalents in the wild type are indicated by arrows in (E). In *zep1-1*, homologous chromosomes connect with each other tightly and ring-shaped bivalents are seldom observed (N).  
 (F) and (O) Metaphase I. In *zep1-1*, the two centromeres of each bivalent were dragged out by spindle fibers (O).  
 (G) and (P) Anaphase I.  
 (H) and (Q) Telophase I.  
 (I) and (R) Telophase II.

metaphase I, 12 bivalents in *zep1-1* were lined up on the equatorial plate (Figure 1O), but the two centromeres of each bivalent were dragged out far away in opposite directions by spindle fibers. At anaphase I, the homologous chromosomes segregated normally and moved toward the opposite poles (Figure 1P). During meiosis II, no significant defects could be detected in *zep1-1*, and tetrads were normally formed at the end of meiosis (Figures 1Q and 1R).

As indicated above, the two homologous chromosomes in *zep1-1* could align well at pachytene stage, and the COs were obvious and easily counted (Figures 2A and 2B). In the wild type, by contrast, homologs connected to each other closely at pachytene and the COs were not visible until diplotene (Figures 2C and 2D). We found that each chromosome pair in *zep1-1* formed several COs, implying that COs had a tendency to increase compared with the wild type, in which only  $\sim 1.73$  COs per pair are formed (Wang et al., 2009). Because pachytene

chromosomes were relatively longer and difficult to trace along their length, the total number of COs in an entire pollen mother cell of *zep1-1* was hard to estimate. Thus, we counted only the COs occurring between two cytological markers, OSJNBa0088116 and 5S rDNA, near the end and close to the centromere, respectively, of the short arm of chromosome 11 (Figures 2C to 2F). Using this measure, the average number of COs on the short arm of chromosome 11 was 3.47 ( $n = 17$ ) in *zep1-1* and 0.92 ( $n = 26$ ) in the wild type, consistent with COs being increased to a certain degree in *zep1-1*.

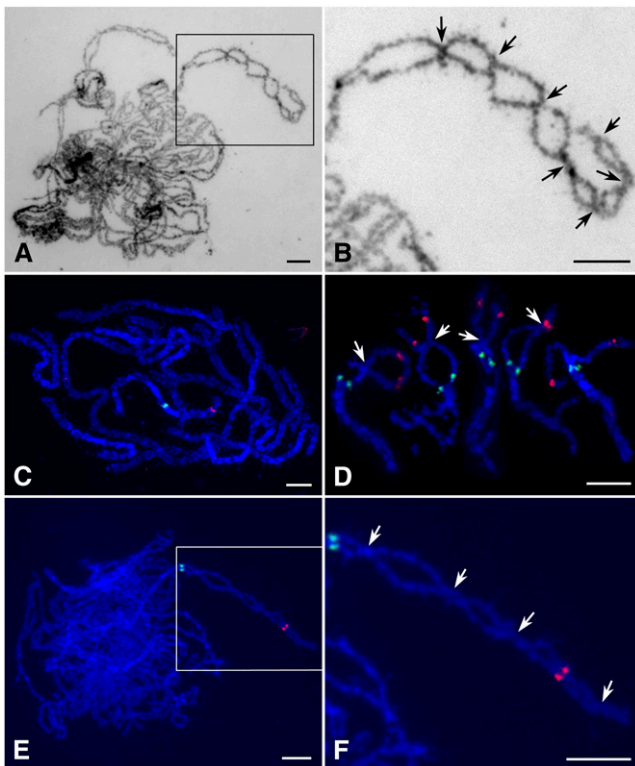
We performed interference to confirm that the abnormal chromosome behavior and low fertility were caused by the loss of *ZEP1* gene function in *zep1-1*. Among the 73 *ZEP1*<sup>RNAi</sup> transgenic plants, 56 showed decreased seed sets ranging from 3.33 to 68.45%. We selected the lowest fertility transgenic plant, named *ZEP1*<sup>RNAi-3</sup>, for further cytological analysis. The chromosome behavior of *ZEP1*<sup>RNAi-3</sup> also resembled that of *zep1-1* (see Supplemental Figures 5A to 5D online).

We also observed meiosis of the other three mutants, *zep1-2*, *zep1-3*, and *zep1-4*, and found that they exhibited the same meiotic characters as those of *zep1-1* (see Supplemental Figures 6A to 6C online). Even though some abnormal chromosome behavior was observed in *zep1*, chromosome segregation was still equal during meiosis. Therefore, the decreased pollen fertility of *zep1* does not appear to be caused by chromosome segregation defects.

### ZEP1 Is the TF Protein of SCs in Rice

To investigate the precise spatial and temporal distribution of ZEP1 as meiosis progresses, dual immunolocalization was performed using polyclonal antibodies against REC8 and ZEP1 (also known as RAD21-4) raised in mouse and rabbit, respectively. REC8 is a component of the cohesion complex and is required for sister chromatid cohesion, axial element formation, and homolog pairing (Molnar et al., 1995; Bhatt et al., 1999; Klein et al., 1999; Cai et al., 2003; Zhang et al., 2006). Thus, REC8 can be used as a marker to monitor early meiotic events during prophase I. REC8 was first present as punctuate foci at premeiotic interphase. At late leptotene and zygotene, these foci assembled to form linear signals and became thicker thereafter, corresponding to the condensing chromosomes (Figures 3A to 3C). At pachytene, REC8 was distributed over all the paired chromosomes (Figure 3D). After diakinesis, REC8 gradually disappeared (Figure 3G).

ZEP1 was first detected as punctuate foci at early leptotene (Figure 3B). From middle leptotene to zygotene, ZEP1 gradually elongated and formed short linear signals (Figure 3C). We found that all of the ZEP1 signals colocalized with the REC8 linear signals at this stage. Once the nucleus entered early pachytene, all ZEP1 signals aligned perfectly along the entire chromosome (Figure 3D). During this stage, the ZEP1 signals overlapped very well with the REC8 signals in length, whereas they were narrower than the REC8 signals in width and almost situated in the central region of the REC8 signals, showing that ZEP1 is the central element of SCs in rice (Figure 3E). The contiguous linear ZEP1 signals were maintained until late pachytene. At diplotene, ZEP1 fragments rapidly dissociated from the SCs and scattered as



**Figure 2.** Detection of COs in Both the Wild Type and *zep1-1*.

(A) *zep1-1* pachytene chromosomes stained with 4',6-diamidino-2-phenylindole (DAPI).

(B) Magnified view of the boxed region in (A); visible COs of one chromosome arm are indicated by arrows.

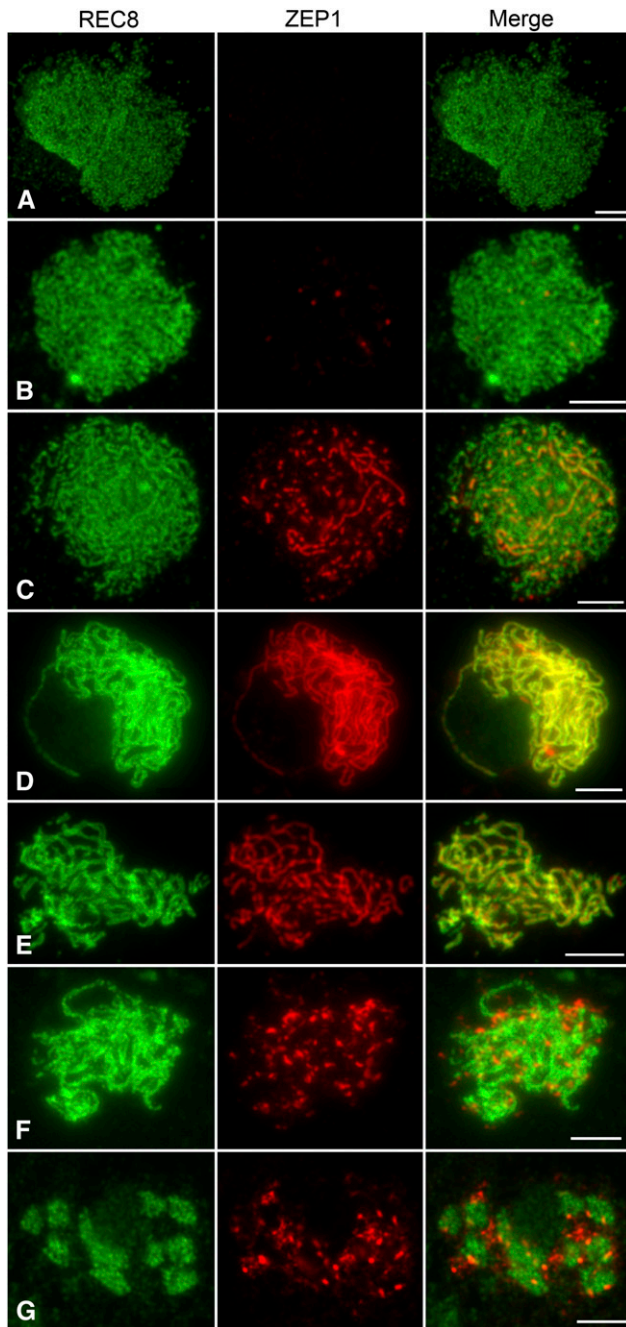
(C) Pachytene chromosome in the wild type probed with OSJNBa0088116 (red) and 5S rDNA (green) located near the end and close to the centromere, respectively, of the short arm of chromosome 11.

(D) Selected early diplotene chromosomes in the wild type probed with the same markers as in (C); arrows indicate COs.

(E) *zep1-1* pachytene chromosomes probed with the same two markers.

(F) Magnified view of the boxed region in (E); arrows indicate COs.

Bars = 5  $\mu$ m.



**Figure 3.** Dual Immunolocalization of REC8 and ZEP1 in the Wild-Type Prophase I.

- (A) Early leptotene.  
 (B) Late leptotene.  
 (C) Zygotene.  
 (D) Early pachytene. ZEP1 signals are narrower than the REC8 signals.  
 (E) Late pachytene. The double threads of REC8 signals of each bivalent align in parallel, while the single thread of ZEP1 situates in the center between REC8 signals, indicating that ZEP1 is the central element of SCs.  
 (F) Diplotene.  
 (G) Diakinesis.  
 Bars = 5  $\mu$ m.

aggregates in the nucleoplasm (Figure 3F). At diakinesis, all ZEP1 signals were completely separated from the chromosomes and gradually depleted from the nucleoplasm (Figure 3G).

Immunolocalization studies were also performed using antibodies against REC8 and ZEP1 on meiocytes of the *zep1-1* mutant. In the absence of ZEP1, the spatial and temporal distribution of REC8 was quite similar to that in the wild type. However, the two threads of REC8 on the homologous chromosomes at pachytene in *zep1-1* were separated from each other, while those in the wild type were very close and always appeared to be a single thread (Figures 5 and 6). This phenomenon indicates that chromosome pairing in *zep1-1* is not as intimate as that in the wild type. After diplotene, REC8 in *zep1-1* started to dissociate from the chromosomes, so REC8 can still be used as a good marker to indicate chromosome axes in the *zep1-1* mutant. In contrast with what was observed in the wild type, no ZEP1 signal was detected in *zep1-1* (see Supplemental Figure 7A online). We also conducted the same immunodetection for the other three *zep1* mutants and found besides some dot-like ZEP1 signals generated in *zep1-3*, very weak signals were detected in centromeric regions in *zep1-2* and *zep1-4* (see Supplemental Figure 7B online). However, no ZEP1 signal could be detected in *ZEP1<sup>RNAi-3</sup>* (see Supplemental Figure 7C online), just like in *zep1-1*.

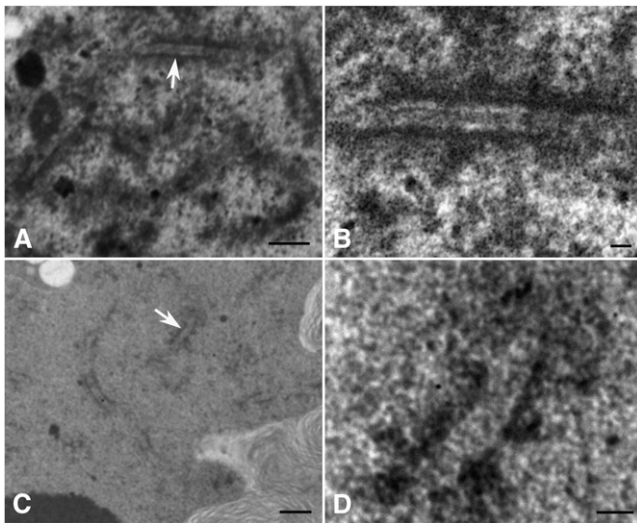
To elucidate further the deficient formation of SCs in *zep1*, we conducted transmission electron microscopy (TEM) analysis of *zep1-1* and wild-type pollen mother cells. In the wild type, axial elements coaligned and the TF proteins, which connected axial elements to each other, formed obvious central elements at the pachytene stage (Figures 4A and 4B). In *zep1-1*, axial elements could still align, but the central elements were absent, showing that the formation of SCs was abolished in the mutant (Figures 4C and 4D). Thus, both immunolocalization and TEM observations confirmed that ZEP1 is the TF protein in rice and constitutes the central element of SCs.

#### The Number of MER3 Foci Increased and the Depletions of Both PAIR2 and MER3 Were Delayed in *zep1-1*

Dual immunolocalization experiments were also conducted in both the wild type and *zep1-1* to investigate whether elimination of ZEP1 affected the distribution of meiotic proteins, including PAIR2 and MER3. PAIR2, the rice ortholog of HOP1 in *S. cerevisiae* and ASY1 in *Arabidopsis*, is first detected in premeiotic S/G2 cells and associates with axial elements through early prophase I. During synapsis, PAIR2 is removed from the chromosome and diffuses into the nucleoplasm (Nonomura et al., 2006). MER3 is a good marker to show most recombination events in rice (Wang et al., 2009). The distributions of MER3 and PAIR2 were investigated by immunodetection. In addition, the antibody against REC8 was also used to indicate the chromosome axes.

MER3 signals were first detected at early leptotene and began to decrease at zygotene in the wild type (Figures 5A to 5C). The stage at which MER3 appeared in *zep1-1* resembled that in the wild type. However, the MER3 foci at late leptotene in *zep1-1* (average 340,  $n = 5$ , range 299 to 370) were much more abundant than in the wild type (average 213,  $n = 5$ , range 182 to 241) (Figure





**Figure 4.** Transmission Electron Micrographs Showing SCs in the Wild Type and *zep1-1*.

(A) and (B) The wild type.

(C) and (D) *zep1-1*. Bars = 5  $\mu\text{m}$  in (A) and (C) and 1  $\mu\text{m}$  in (B) and (D). (A) and (C) Nucleus at pachytene stage with segments of SCs.

(B) and (D) Higher-magnification image of a stretch of SCs indicated by arrows in (A) and (C), respectively. In the wild type, the central elements of SCs are between the lateral elements and are less electron dense compared with lateral elements, whereas in *zep1-1*, no obvious central elements are visible.

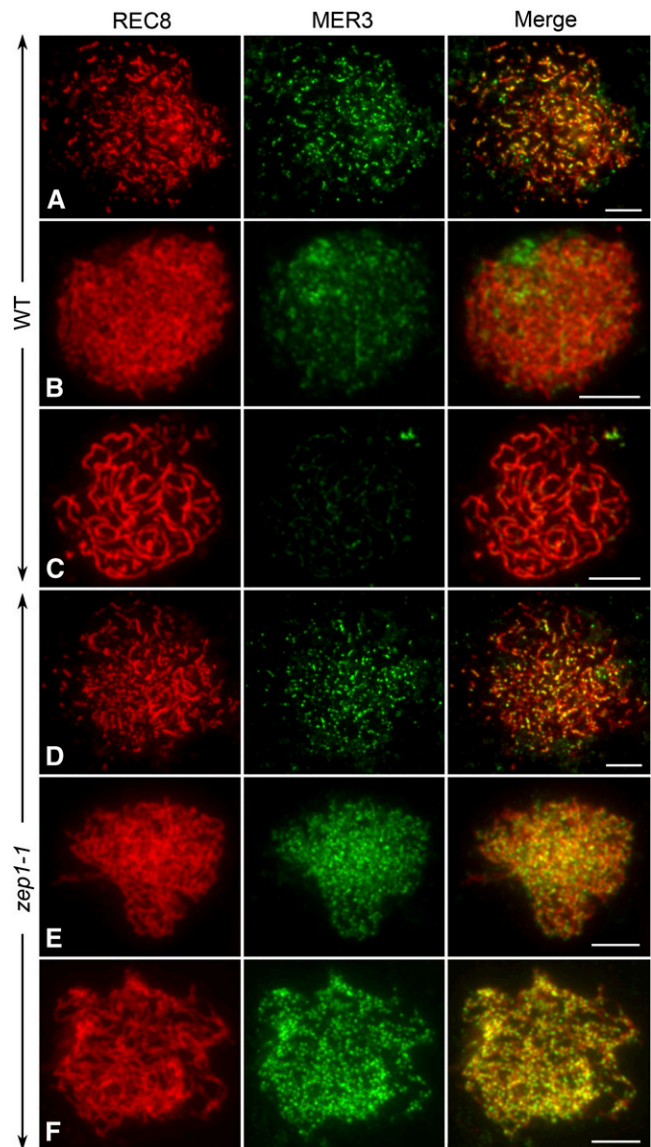
5D; see Supplemental Figure 8 online). When the homologous chromosomes paired, the number of MER3 foci decreased in both *zep1-1* and the wild type, but the rate of decrease was much slower in *zep1-1* (Figure 5E; see Supplemental Figure 8 online). At pachytene, when the MER3 foci in the wild type had almost disappeared (average 49,  $n = 5$ , range 38 to 67), there were still more foci in *zep1-1* (average 224,  $n = 5$ , range 169 to 278), with only a small decrease compared with the number at zygotene (average 246,  $n = 5$ , range 180 to 320) (Figure 5F; see Supplemental Figure 8 online). Some MER3 foci even persisted until diakinesis in *zep1-1*, which was never observed in the wild type.

Additionally, the process of PAIR2 depletion was much different between the wild type and *zep1-1*. In the wild type, PAIR2 was first detected in premeiotic interphase and was always associated with axial elements through leptotene (Figure 6A). When synapsis occurred during zygotene, PAIR2 started to be removed from the chromosomes and released into the nucleoplasm (Figure 6B). At pachytene, only a few PAIR2 fragments were left on the chromosomes (Figure 6C). As in the wild type, the emergence of PAIR2 in *zep1-1* was observed in premeiotic interphase. However, the signal of PAIR2 in *zep1-1* totally overlapped with REC8 from leptotene to diakinesis (Figures 6D to 6F). After diakinesis, both PAIR2 and REC8 gradually disappeared in *zep1-1*.

Taking these data together, although the loading of PAIR2 and MER3 occurred normally in *zep1-1*, depletion of both proteins was delayed severely compared with the wild type. In addition, the number of MER3 foci increased remarkably in absence of ZEP1.

### The Loading of ZEP1 Requires both Homologous Chromosome Pairing and Recombination

To study the roles of homologous pairing and recombination in the process of SC formation, we selected two null mutants, *pair2* and *mer3*, which represent defects in chromosome pairing and recombination (Nonomura et al., 2006; Wang et al., 2009),



**Figure 5.** Dual Immunolocalization of REC8 and MER3 in Both the Wild Type and *zep1-1*.

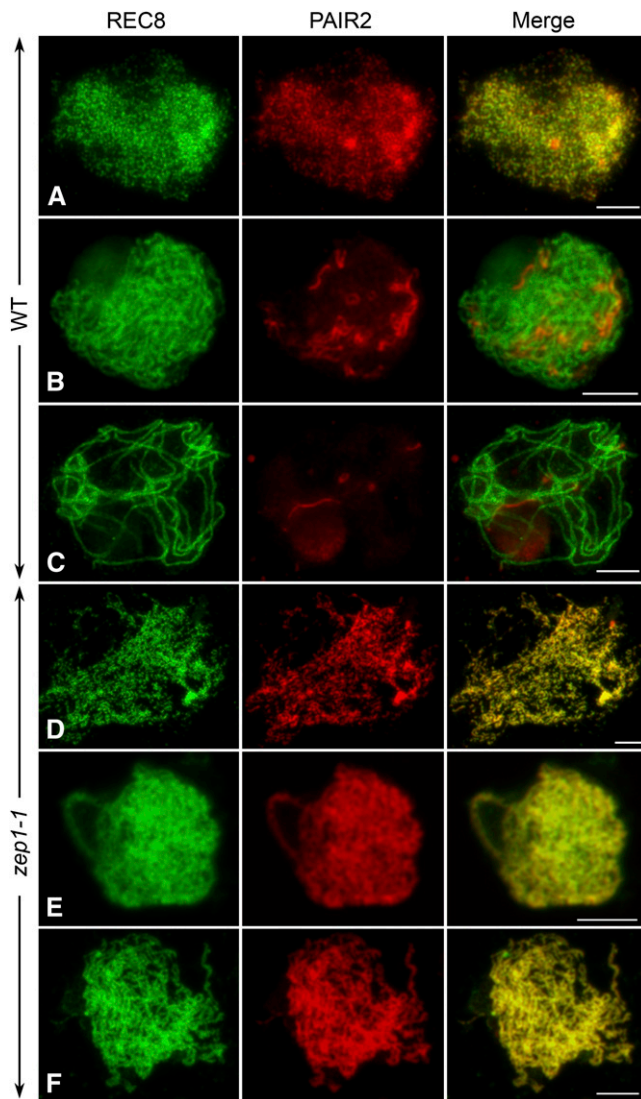
(A) to (C) The wild type.

(D) to (F) *zep1-1*. Bars = 5  $\mu\text{m}$ .

(A) and (D) Leptotene. The MER3 foci in *zep1-1* are more abundant than in the wild type.

(B) and (E) Zygotene.

(C) and (F) Pachytene. At pachytene, when MER3 foci in the wild type have almost disappeared, they are still abundant in *zep1-1*.



**Figure 6.** Dual Immunolocalization of REC8 and PAIR2 in Both the Wild Type and *zep1-1*.

(A) to (C) The wild type.  
(D) to (F) *zep1-1*. Bars = 5  $\mu$ m.  
(A) and (D) Early leptotene.  
(B) and (E) Zygotene.

(C) and (F) Pachytene. The PAIR2 signals in *zep1-1* still overlap with REC8 at pachytene, when PAIR2 in the wild type is completely disassociated from the chromosome.

respectively, to investigate the loading of ZEP1. In addition, REC8 was also used as a marker to monitor the meiotic chromosomes in prophase I. We found that in both mutants, REC8 could be normally loaded onto prophase I chromosomes, while the localization of ZEP1 was defective (see Supplemental Figures 7D and 7E online). These results indicated that SC assembly requires both chromosome pairing and recombination. In the *pair2* mutant, only a few ZEP1 foci could be detected in the prophase I nuclei (see Supplemental Figure 7D online). However,

in the *mer3* mutant, although the ZEP1 foci were abundant at the pachytene stage, the short linear signals were always distributed randomly in the nucleoplasm and were seldom loaded onto the chromosomes (see Supplemental Figure 7E online). Thus, normal loading of ZEP1 and further SC formation depend on both homologous pairing and recombination events.

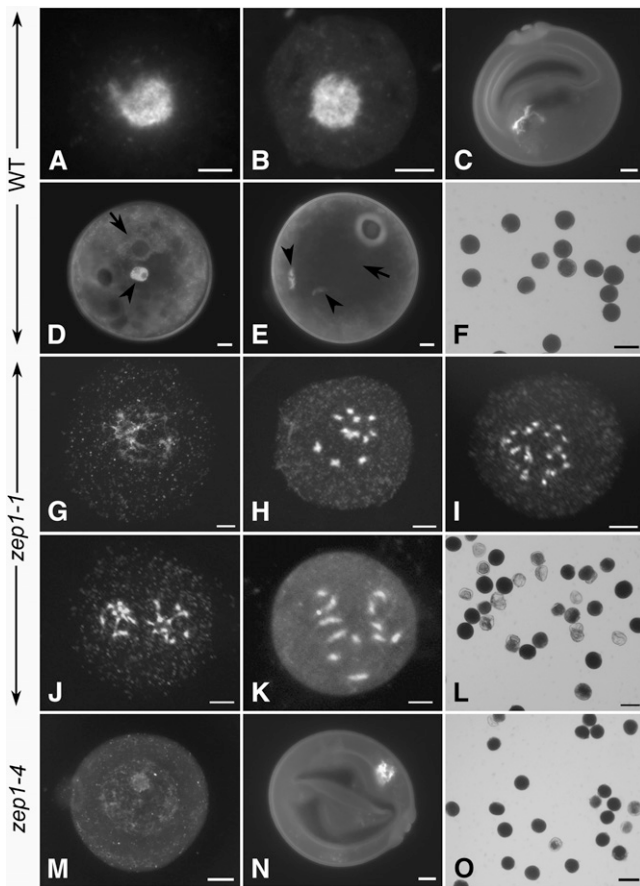
### Precocious Chromosome Condensation in Early Microspores Leads to Partial Abortion in *zep1*

As indicated above, chromosome segregation in *zep1-1* was equal throughout meiosis. In order to find the real cause of the decreased fertility in *zep1-1*, chromosome behavior after meiosis in both the wild type and *zep1-1* was further investigated. In the wild type, four early microspores were generated from a single pollen mother cell after meiosis (Figure 7A). As the early microspore developed, the pollen wall was gradually formed (Figure 7B). Meanwhile, the nucleus was moved to the peripheral region by the enlarging vacuole (Figure 7C), and each uninucleate microspore underwent an asymmetric mitotic division (pollen mitosis I) to produce two unequal daughter cells: the vegetative cell and the generative cell (Figure 7D). After nuclear migration, the generative nucleus completed the second mitotic division near the germinal pole, giving rise to the tricellular pollen (Figures 7E and 7F).

Interestingly, in contrast with the decondensed chromatin in the early microspores in the wild type, severely condensed chromosomes were observed in  $\sim 60\%$  of the early microspores in the *zep1-1* mutant (Figures 7G and 7H). We also observed that in a few early microspores, the chromosomes underwent equal segregation (Figures 7I and 7J). After pollen wall formation, the condensed chromosomes were still located in the center of the uninucleate pollen (Figure 7K). The abnormal chromosome condensation and nuclear location caused the microspore to be arrested at the uninucleate stage, finally leading to sterile pollen formation (Figure 7L). To test this hypothesis, we checked the microspore chromosome condensation status for the other three *zep1* mutants and found that chromosome condensation events could be detected in 54.35, 12.67, and 5.38% of the microspores, respectively, in *zep1-2*, *zep1-3*, and *zep1-4* (see Supplemental Table 1 online). In the weak allele *zep1-4*, the pollen formation was very similar to that in the wild type (Figures 7M to 7O). Nevertheless, the chromosome condensation of the uninucleate microspores in *ZEP1<sup>RNAi-3</sup>* was much more severe than that in *zep1-1* (see Supplemental Figures 5E and 5F online). It seems that the more chromosome condensation occurred in the microspores, the lower the fertility of the resulting pollen.

### ZEP1 Reloads onto Chromosomes in Early Microspores When Chromosomes Are Decondensed

The defect in maintaining chromosome decondensation in the absence of ZEP1 indicates that ZEP1 might be involved in chromosome decondensation. We reanalyzed the behavior of ZEP1 through the entire meiotic process in both the wild type and the *zep1* mutants. To our surprise, we found that ZEP1 could be reloaded onto chromosomes for two more rounds after diakinesis in the wild type. The first round was at prophase II when the



**Figure 7.** Microspore Development in the Wild Type, *zep1-1*, and *zep1-4*.

(A) to (F) The wild type.

(G) to (L) *zep1-1*.

(M) to (O) *zep1-4*. Bars = 50  $\mu$ m in (F), (L), and (O) and 5  $\mu$ m in the others.

(A) Early microspore just separated from tetrad.

(B) Early microspore with pollen wall formation.

(C) Uninucleate microspore with the nucleus migrating to the periphery of the cell.

(D) Bicellular pollen, with the arrowhead indicating the generative cell and the arrow indicating the vegetative nucleus.

(E) Tricellular pollen, with arrowheads indicating the generative cells and arrow indicating the vegetative nucleus.

(F) Mature pollen grains stained with 1% I<sub>2</sub>-KI solution.

(G) Early microspore in *zep1-1* with less condensing chromosomes.

(H) Microspore in *zep1-1* with 12 precocious condensed chromosomes in the central region.

(I) and (J) Two different microspores of *zep1-1* with the condensed chromosomes tended to conduct equal segregation. Before the nucleus division (I) and after the nucleus division (J).

(K) Uninucleate microspore in *zep1-1*.

(L) Mature pollen grains stained with I<sub>2</sub>-KI in *zep1-1*.

(M) Early microspore in *zep1-4* with decondensed chromosomes locating centrally within the cell, just like that in the wild type (B).

(N) Uninucleate microspore in *zep1-4* resembling that observed in the wild type (C).

(O) Mature pollens stained with I<sub>2</sub>-KI in *zep1-4*.

[See online article for color version of this figure.]

chromosomes decondensed into chromatins (Figure 8A), and the second round was from telophase II until the first mitosis of the microspore, during which time the chromosomes were also in a decondensed chromatin state (Figures 8D and 8E). However, the chromosomes were highly condensed between the two stages, such as in metaphase II (Figure 8B) or anaphase II (Figure 8C). We found that all of the ZEP1 signals were localized in the nucleoplasm as the chromosomes condensed from metaphase II to anaphase II. However, after the first pollen mitosis, ZEP1 totally disappeared and could not be detected thereafter (Figure 8F). In contrast with the wild type, no ZEP1 immunosignal was detected in the corresponding stages in *zep1-1* (Figures 8G to 8I) or *zep1-2*; however, those signals could constantly be detected in *zep1-3* and *zep1-4* (Figures 8J to 8L) at the same stages. We suspect that the truncated ZEP1 proteins in *zep1-3* and *zep1-4* might still perform their functions, so the chromosome decondensation status was maintained, just like that in the wild type.

As described before, the defects in chromosome pairing or recombination would lead to failure of SC assembly in *mer3* or *pair2*, respectively. However, in both the *mer3* and *pair2* mutants, chromosome decondensation was not obviously affected in the uninucleate microspores, though the chromosomes were unequally segregated in meiosis. Immunodetection was also performed to show whether ZEP1 localized normally at the corresponding stages in the mutants. We found that ZEP1 could still localize to chromosomes at prophase II, telophase II, and the early microspore stages in both *mer3* (Figures 8M to 8O) and *pair2* (Figures 8P to 8R), just like in the wild type. Thus, the results also support the idea that ZEP1 might be important for maintenance of chromosome decondensation during early microspore development.

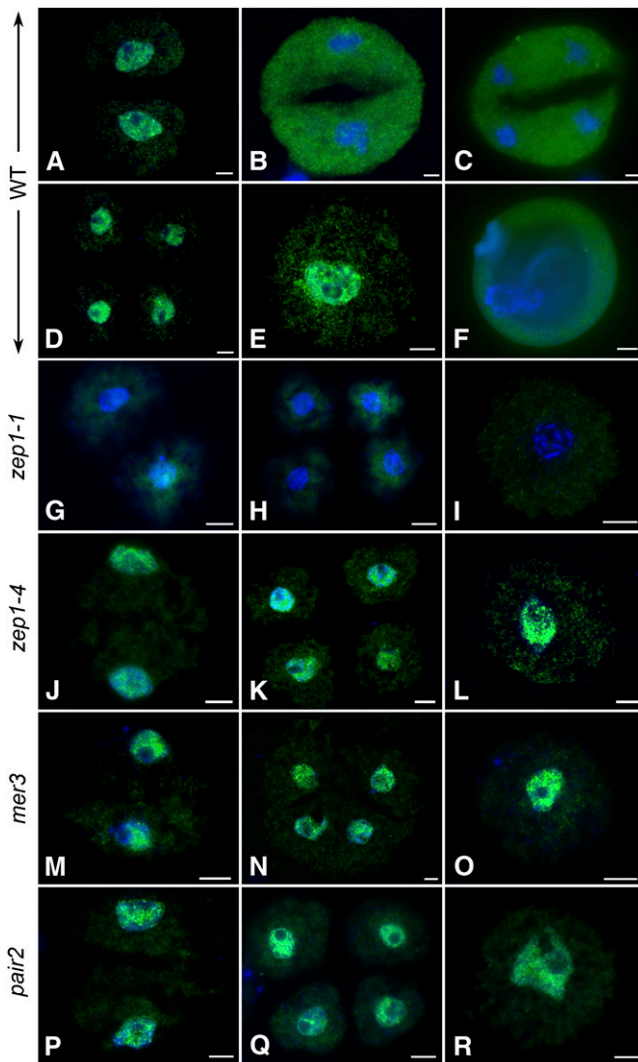
## DISCUSSION

### Synapsis Is Dispensable for CO Formation in Rice

The role of synapsis in CO formation has diverged among different organisms. In animals, such as *C. elegans*, mouse, and *Drosophila* females, SCs have been shown to be required for CO formation. In *C. elegans*, the inappropriate repair of double strand breaks in two synapsis-deficient mutants, *syp-1* and *syp-2*, induces apoptosis in most of the meiocytes. In the remaining meiocytes, only univalents can be observed at late prophase I (MacQueen et al., 2002; Colaiácovo et al., 2003). C(3)G is a TF protein that has been identified in *Drosophila* females. In the null mutant of *c(3)G*, double strand breaks seem to be inhibited and COs are accordingly unable to form (Page and Hawley, 2001). In fact, the initiation of recombination is not necessary for synapsis in *Drosophila* females and *C. elegans*, and they use specific pairing sites to achieve initial homolog recognition upon entering meiosis (McKim et al., 1993; McKee et al., 2000; de Boer and Heyting, 2006). In mouse, 90% of chiasmata in the wild type are dependent on SCYP1, and only univalents can be observed in *syp1* at metaphase I.

The situation is different in *S. cerevisiae* and *Arabidopsis*. In *S. cerevisiae*, the effect of the TF protein on CO formation varies according to the strain background, growth temperature,





**Figure 8.** The Localization of ZEP1 during Meiosis II and Microspore Development in the Wild Type, *zep1-1*, *zep1-4*, *mer3*, and *pair2*.

(A) to (F) The wild type.

(G) to (I) *zep1-1*.

(J) to (L) *zep1-4*.

(M) to (O) *mer3*.

(P) to (R) *pair2*. Bars = 5  $\mu$ m.

(A) Prophase II. ZEP1 (green signal) reloads on chromosomes after its previous disassociation at diakinesis.

(B) Metaphase II with condensed chromosome; ZEP1 disassociates from chromosome and moves into the nuclearplasm.

(C) Telophase II.

(D) Tetrad. As chromosomes decondense, ZEP1 is reloaded onto the chromosome again.

(E) Early microspore.

(F) Uninucleate pollen.

(G), (J), (M), and (P) Prophase II in *zep1-1*, *zep1-4*, *mer3*, and *pair2*, respectively.

(H), (K), (N), and (Q) Tetrad in *zep1-1*, *zep1-4*, *mer3*, and *pair2*, respectively.

(I), (L), (O), and (R) Early microspore in *zep1-1*, *zep1-4*, *mer3*, and *pair2*, respectively. No ZEP1 is detected from meiosis II to early microspore in

sporulation conditions, and so on. The *zip1* mutant from the strain BR arrests during prophase I, and COs are reduced severely during growth at 30 or 34°C (Sym et al., 1993; Borner et al., 2004). However, in the strain SK1, the role of the TF protein in CO formation varies with different growth temperatures. At 33°C, defects in converting double strand breaks to single-end invasions result in most of the *zip1* cells being arrested in meiosis and having severely altered sporulation. However, at 23°C, single-end invasions of the *zip1* mutant cells in the SK1 strain can reach the wild-type level after a substantial delay, and most of the *zip1* cells can go through meiosis II and produce spores (Borner et al., 2004). In *Arabidopsis* *ZYP1*<sup>RNAi</sup> plants, 70 to 80% of the wild-type MLH1 foci (the markers of chiasmata) can be assembled on the chromosomes, even though the multivalents are formed by nonhomologous chromosome association at metaphase I. Moreover, CO interference may still be maintained in *zyp1* (Higgins et al., 2005).

Although the SC is often considered to be a universal feature of meiosis, it is absent from several organisms, including *Schizosaccharomyces pombe*, *Aspergillus nidulans*, and *Drosophila* males (Rasmussen, 1973; Egel-Mitani et al., 1982; Molnar et al., 2003; Page and Hawley, 2004). In the absence of SCs, *Drosophila* males achieve homologous pairing and segregation in a special way rather than by meiotic recombination and chiasma formation (Page and Hawley, 2004). By contrast, in *S. pombe* and *A. nidulans*, large numbers of COs occur and homologs successfully disjoin without benefit of an SC (Kleckner, 2006). This lack of SCs in the two fungi makes it difficult to elucidate why the SC has evolved as a very common feature of the meiotic program (Kleckner, 2006). Here, the rice *zep1* mutant, which retains CO formation and successful homolog segregation, resembles the phenotype of *S. pombe* and *A. nidulans*.

While the sequence and biological functions of most meiosis-specific proteins are highly conserved among organisms, the lower level of sequence conservation among TF proteins may cause divergent functions in homologous recombination among different organisms.

### The Role of ZEP1 in Early Recombination Events

TF proteins have been reported to be required for programmed depletion of recombination-related proteins in many organisms. In mouse *sycp1*, 50 to 70% of the RAD51/DMC1, RPA, and MSH4 foci found in zygotene continued to persist into early diplotene, while most or all of these foci disappeared at the same stage in the wild type (de Vries et al., 2005). Additionally, the persistence of RAD51 foci into late prophase I was also observed in the mutants *syp-1* and *syp-2* in *C. elegans* (Colaiacovo et al., 2003). The delayed depletion of recombination-related proteins implies that meiotic recombination might be impeded at the step of single-end invasion.

In *S. cerevisiae*, MER3 can unwind duplex DNA from 3' to 5' as a helicase (Nakagawa and Ogawa, 1999; Nakagawa et al., 2001).

*zep1-1*, but the loading of ZEP1 in the corresponding stages in *zep1-4*, *mer3*, and *pair2* resembles that in the wild type. Chromosomes are stained with DAPI (blue).

MER3 is a component of the ZMM complex, which corresponds to the interference-sensitive CO (Lynn et al., 2007). The rice MER3 ortholog also is involved in interference-sensitive CO formation and may directly participate in double strand break repair (Wang et al., 2009). There are ~12-fold more MER3 foci at late leptotene compared with the prospective COs, suggesting that the abundant ZMM foci are probably required for full synapsis in plants. Our data showed that there were many more MER3 foci in *zep1* compared with those in the wild type at the same stage and that these foci persisted until diplotene, or even until diakinesis, while those in the wild type gradually disappeared as cells were entering into pachytene. As a result of later depletion of MER3, the recombination events may be accordingly delayed at the step in which MER3 is involved.

One model of CO interference has been proposed in which synapsis initiates at prospective CO sites and further prevents nearby COs. According to this model, COs are free to initiate in regions where synapsis does not occur and are excluded from synapsed regions (Egel, 1978; Sym and Roeder, 1994). Although the sites of synapsis initiation complexes display interference before SC formation in budding yeast, the prevention of CO initiation near synapsis initiation complexes by synapsis still quite likely exists (Fung et al., 2004). Additionally, our data show that CO is increased in the absence of ZEP1, providing the direct evidence that synapsis can inhibit the formation of excessive COs. This may occur through preventing the initiation of recombination events to some extent by limiting the number of MER3 foci in each nucleus. Thus, we infer that ZEP1 is quite important for regulating the process of meiotic recombination, although it does not participate directly in the progress of recombination events.

### Chiasma Terminalization Relies on Regular Assembly of ZEP1

We found that ZEP1 played an important role in chiasma terminalization. At diakinesis in *zep1*, the condensed homologs associated with each other intimately and ring-like bivalents were seldom detected. Additionally, the terminal chromatin of *zep1* bivalents was less condensed than that in the wild type. Therefore, we infer that this phenomenon is caused by defective chiasma terminalization in the absence of ZEP1. Chiasma terminalization was first described by Darlington (1929) and now is defined as shifting the position of chiasmata to or toward the ends of bivalents before metaphase I. The phenomenon of chiasma terminalization varies remarkably in different organisms, and the degree of terminalization may change from complete terminalization to the complete absence of terminalization (Egel, 1979; Baptista-Giacomelli et al., 2000). Chiasma terminalization might be affected by many factors, such as heterochromatic regions and structural rearrangement. In grasses, chiasma terminalization has been well characterized among different species, such as maize (*Zea mays*) and oat (*Avena sativa*; Maguire, 1981; Baptista-Giacomelli et al., 2000). Yet, the molecular mechanism of chiasma terminalization is still unknown. We suspect that the defects of chiasma terminalization in *zep1* might be related to two aspects: one is the increased number of COs, and the other is the later depletion of PAIR2 and other meiotic elements.

### ZEP1 Might Be Involved in Maintaining Chromosome Decondensation during the First Mitotic Division in Rice Pollen Genesis

During pollen development, which is specific to plants, a series of nuclear migration and cell division events are elaborately regulated (Twell et al., 1998; Zhang et al., 2005b). In this study, we found that precocious chromosome condensation prevented polar nuclear migration in absence of ZEP1 and therefore resulted in pollen abortion in *zep1*. There are many mutants defective in development from microspore to mature pollen in *Arabidopsis*, such as *scp*, *gem1*, *duo1*, *duo2*, etc. (Chen and McCormick, 1996; Park et al., 1998; Durbarry et al., 2005). By characterizing those mutants, several proteins specific to microspore development were identified. However, none of them have been shown to function during meiosis.

In *Arabidopsis*, REC8 can relocalize to the interphase II nucleus, but its function following meiosis I has not been reported (Cai et al., 2003). To our surprise, unlike anything reported in other organisms, ZEP1 can also reload onto chromosomes for two more rounds from prophase II to the early microspore stage as the chromosomes decondense in the wild type. In addition, we found that ZEP1 could reload normally onto chromosomes in dyads, tetrads, and in early microspores in mutants of both *pair2* and *mer3*. This shows that the function of ZEP1 after meiosis I is independent of both PAIR2 and MER3. To a certain degree, the mechanism of ZEP1 reloading onto the chromosome during meiosis may be similar to INCENP, a component of the chromosomal passenger complex, which regulates key mitotic and meiotic processes by changing its position during cell division (Parra et al., 2003; Resnick et al., 2006; Ruchaud et al., 2007). In this respect, ZEP1 may have a parallel function like chromosomal passenger complex in normalizing chromosome behavior, but the exact function of ZEP1 after meiosis is still obscure.

The condensin complex plays a crucial role in chromosome condensation. In the presence of ATP, the condensin complex can produce supercoils in the protein-free region of closed circular DNA (Bazett-Jones et al., 2002). ZEP1 protein contains a domain (amino acids 61 to 530, domain A) with limited similarity to the prokaryote SMC proteins (conserved domains search in NCBI; <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), which often form homodimers and are involved in condensing chromatin (Soppa et al., 2002). In this study, the *Tos17* elements of *zep1-1* and *zep1-2* were inserted into domain A, while those of *zep1-3* and *zep1-4* were inserted downstream of this domain. The aberrant chromosome condensation in early microspores was observed mainly in *zep1-1* and *zep1-2*, rather than in *zep1-3* or *zep1-4*, although all of them were defective in SC formation. Thus, we suspect that the entire ZEP1 protein is required for SC formation, while domain A of ZEP1 might be important for maintaining chromosome decondensation in early microspore development. However, how ZEP1 prevents previous chromosome condensation in early microspores is still enigmatic. Although it is probable that ZEP1 might be involved in maintaining chromosome decondensation in early microspore development, our observations do not rule out the possibility that the defects of pollen development in *zep1* may be the indirect consequences of

the abnormal meiosis or other undetected defects. These suggestions still need to be tested with more investigations.

## METHODS

### Plant Materials

The *japonica* rice (*Oryza sativa*) variety, Nipponbare, was used in the present study as the wild type. The seeds of *Tos17* insertion lines NG6573, NE7025, NF0601, and NG4542 named as *zep1-1*, *zep1-2*, *zep1-3*, and *zep1-4*, respectively, were kindly provided by the Rice Genome Resource Center of the National Institute of Agrobiological Sciences. *pair2* and *mer3* were isolated previously (Wang et al., 2009). All plant materials were grown in the paddy fields.

### Cloning the Full-Length ZEP1 cDNA

Total panicle RNA extraction and reverse transcription experiments were performed as previously described (Wang et al., 2009). The cDNA was subject to PCR using Phusion (NEB) with gene-specific primers F1 (5'-CCTAGGGTTTCGACGACAG-3') and R1 (5'-GAATCTTCTGAAGCAACTCT-3'), and F2 (5'-TCTGACCTAGAAGG GAGGGT-3') and R2 (5'-CAGCA CCTCAATGTGAGCT-3') designed to amplify the predicted coding regions of ZEP1 by two halves. The PCR products were cloned into pMD18-T vector (Takara) and sequenced. The 3' RACE and 5' RACE were performed according to the protocol of the kit (3'-Full RACE Core Set and 5' -Full RACE Core Set; Takara). For 3' RACE, the first and second PCRs were performed using 3'RACE-F1 (5'-CCAACACTCAGAAGTCATTG-3') and 3' adaptor (5'-CTGATCTAGAGGT ACCGGATCC-3'), and 3'RACE nested-F (5'-CTTAGGCATCTGACCATCC-3') and 3'RACE nested-R (5'-TTCAGTTGATGCTGTACGC-3'), respectively. For 5' RACE, the RNA was reverse transcribed using 5' phosphorylated primer 5'RACE-RT (5'-CAACTTGGTATGCGTCA-3'). The first and second PCRs were performed using 5'RACE-S1 (5'-TCCGATATTGGAGGGAGCA-3') and 5'RACE-R1 (5'-AGGTGGATCCAGCGAGAGA-3'), and 5'RACE-S2 (5'-CTGGTTAAGGAGCAGGCTT-3') and 5'RACE-R2 (5'-GATAAAC CAGCTTCTGC-3'), respectively. The products of 3'RACE-PCR and 5'RACE-PCR were cloned and sequenced.

### Real-Time PCR for Transcript Expression Assay

Total RNA was extracted from the stem, leaf, root, and panicle of Nipponbare. Real-time PCR analysis was performed using the Chromo4 real-time PCR instrument (CFD-3240; Bio-Rad) and SYBR Green I (Invitrogen). RT-PCR was performed using ZEP1RT-F (5'-CAGCAGGAT-AATGAGCATAA-3') and ZEP1RT-R (5'-GGTCTCAGGACTAACCAACT-3') for *ZEP1*, and UBQF (5'-CAAGATGATCTGCCGCAATGC-3') and UBQR (5'-TTTAACCAGTCCATGAACCCG-3') for *Ubiquitin*. The real-time PCR results were analyzed using Opticon Monitor analysis software 3.1 (Bio-Rad). The experiment had four replicates.

### Tos17 Insertion Site Mapping

Primer pairs, TosP (5'-ATTGTTAGGTTGCAAGTTAGTTAAGA-3') and 6573P (5'-TCTCCTTGCAGAGATTCCGT-3'), were used to amplify the *Tos17* inserted regions of *zep1-1* and *zep1-2*. Primer pairs, TosP and 0601P (5'-TTTGCAGGCA ATCAATGAAA-3'), as well as TosP and 4542P (5'-CCCTCAATTCATTCTCAGCC-3'), were used to amplify the *Tos17* inserted regions of *zep1-3* and *zep1-4*, respectively. The PCR products were cloned into pMD18-T vector (Takara) and sequenced.

### Computational and Database Analysis

The methods of drawing the gene structure schematic and aligning amino acid sequences were previously described (Wang et al., 2009).

### Construction of the ZEP1 RNAi Cassette and Rice Transformation

A 414-bp fragment of ZEP1 cDNA sequence was amplified with the following primers: RNAiF, 5'-ATGCTCGAGCACAAGCAAGAGTTGCTT-CAG-3' (adding an *XhoI* site) and RNAiR, 5'-GCTAGATCTTTCATATTCCTACGGATTTC-3' (adding a *BglII* site). The construction and transformation were performed as described (Wang et al., 2009).

### Antibody Production and Protein Gel Blot Analysis

The antibodies against REC8, PAIR2, and MER3 were produced as described (Wang et al., 2009). To generate the antibody against ZEP1, a 561-bp fragment of ZEP1 cDNA (amino acids 426 to 612) was amplified with the following primers: ZEP1AbF, 5'-GCGGATCCCCAAACTTC-TGGAGGATTCT-3' (adding a *BamHI* site) and ZEP1AbR, 5'-GCGT-CGACGCTCAGAATCTTGCCTGTTTTC-3' (adding a *SalI* site). The construction, fusion peptide expression, and purification were performed as described (Wang et al., 2009). Additionally, 20 residues (amino acids 97 to 116) of ZEP1 were synthesized to generate an antibody from mouse for immunoblotting.

For protein gel blot analysis, all protein samples (20  $\mu$ g each) were separated by 10% SDS-PAGE and then electrophoretically transferred onto a polyvinylidene difluoride membrane. Hybridization was performed using polyclonal primary antibody against ZEP1 (diluted 1:2000). Goat anti-mouse antibody labeled by horseradish peroxidase (DingGuo) was used as the secondary antibody (diluted 1:500). The signal appeared after incubating in Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore).

### Meiotic Chromosome Preparation and Fluorescence in Situ Hybridization

Young panicles of both the wild type and mutants 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 cover slips, the slides were dehydrated through an ethanol series (70, 90, and 100%). Chromosomes were counterstained with DAPI in an antifade solution (Vector). Fluorescence in situ hybridization (FISH) analysis was conducted as described (Zhang et al., 2005a). The pTa794 clone containing the coding sequences for the 5S rRNA genes of wheat (*Triticum aestivum*; Cuadrado and Jouve, 1994) was used as one FISH probe, and the rice BAC OSJNBa008816 was used as the other FISH probe to monitor the short arm of chromosome 11. Chromosome images were captured under the Olympus BX51 fluorescence microscope with a microCCD camera.

### TEM

For TEM of the meiocytes in the wild type and the *zep1* mutant, the fixation and infiltration procedures were performed as described previously (Owen and Makaroff, 1995). Ultrathin sections were examined with a JEOL JEM-1400 transmission electron microscope at 80 kV. At least 50 ultrathin sections 100 nm thick were analyzed for pachytene pollen mother cells of both the wild type and the mutant individually.

### Immunofluorescence

Fresh young panicles were fixed in 4% (w/v) paraformaldehyde for 30 min at room temperature. Anthers in the proper stage were squashed on a slide with PBS solution and covered with a cover slip. After soaking in liquid nitrogen and removing the cover slip, the slide was dehydrated through an ethanol series (70, 90, and 100%) prior to being used in

immunostaining. Slides were then incubated in a humid chamber at 37°C for 4 h in different antibody combinations mentioned in the Results (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).

#### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: ZEP1, GU479042; REC8, AY371049; MER3, FJ008126; PAIR2, AB109238; ZYP1a, At1g22260; and ZYP1b, At1g22275.

#### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Schematic Representation of the *ZEP1* Gene.

**Supplemental Figure 2.** Alignment of ZEP1 with ZYP1a and ZYP1b from *Arabidopsis thaliana*.

**Supplemental Figure 3.** Expression Analysis of *ZEP1*.

**Supplemental Figure 4.** The Matured Panicle and Pollen Grains Stained with 1% I<sub>2</sub>-KI Solution in the *ZEP1/zep1-1* Heterozygote Plant.

**Supplemental Figure 5.** DAPI Staining of Meiotic Chromosomes in *ZEP1<sup>RNAi-3</sup>* Pollen Mother Cells.

**Supplemental Figure 6.** Pachytene Chromosomes Stained with DAPI in *zep1-2*, *zep1-3*, and *zep1-4* Pollen Mother Cells.

**Supplemental Figure 7.** Dual Immunolocalization of REC8 and ZEP1 in Different Meiotic Mutants.

**Supplemental Figure 8.** Average Number of MER3 Foci at Successive Stages of Meiotic Prophase I in Both the Wild Type and *zep1-1*.

**Supplemental Table 1.** Fertility Investigation of the *zep1* Homozygous Mutants.

#### ACKNOWLEDGMENTS

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