MER3 is required for normal meiotic crossover formation, but not for presynaptic alignment in rice

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Summary

MER3, a ZMM protein, is required for the formation of crossovers in *Saccharomyces cerevisiae* and *Arabidopsis*. Here, *MER3*, the first identified ZMM gene in a monocot, is characterized by map-based cloning in rice (*Oryza sativa*). The null mutation of *MER3* results in complete sterility without any vegetative defects. Cytological analyses show that chiasma frequency is reduced dramatically in *mer3* mutants and the remaining chiasmata distribute randomly among different pollen mother cells, implying possible coexistence of two kinds of crossover in rice. Immunocytological analyses reveal that MER3 only exists as foci in prophase I meiocytes. In addition, MER3 does not colocalize with PAIR2 at the beginning of prophase I, but locates on one end of PAIR2 fragments at later stages, whereas MER3 foci merely locate on one end of REC8 fragments when signals start to be seen in early prophase I. The normal loading of PAIR2 and REC8 in *mer3* implies that their loading is independent of MER3. On the contrary, the absence of MER3 signal in *pair2* mutants indicates that PAIR2 is essential for the loading and further function of MER3.

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Key words: Rice, Meiosis, MER3, Crossover

Introduction

Meiosis is a specialized type of cell division that has an important role in the life cycle of all sexually reproductive organisms. During meiosis, one round of DNA replication is followed by two rounds of chromosome division, generating four haploid cells. Prophase I of meiosis has been the focus of research for several decades because of the occurrence of special chromosome interactions, including homology searching, pairing, recombination and synapsis, all of which are required for proper chromosome segregation in subsequent stages of meiosis (Li and Ma, 2006; Zickler and Kleckner, 1999). Any errors in these highly complex processes can result in nondisjunction, aneuploid formation or even failure of nuclear division (Higgins et al., 2004).

Based on genetic and molecular analyses of recombination in *Saccharomyces cerevisiae* as well as in other organisms, the double-strand break repair (DSBR) model has proposed (Sun et al., 1991; Szostak et al., 1983). According to this model, recombination is initiated by the formation of double-strand breaks generated by the conserved transesterase protein SPO11 (Keeney, 2001; Lichten, 2001). A protein complex which includes MRE11, RAD50 and XRS2 is required for the resection of the 5′ end of the break to yield a 3′ single-stranded tail (Symington, 2002). After loading two homologs of the bacterial RecA protein, RAD51 and DMC1, the single-stranded 3′ tail undergoes strand exchange with allelic sequences on the homolog to produce a structure called the displacement (D)-loop (Bishop, 1994; Hunter and Kleckner, 2001; Whitby, 2005).

The D-loop is further processed into either crossover (CO) or noncrossover (NCO) products. Recent studies indicate that soon after the formation of DSBs, the decision of becoming a CO or NCO is determined (Allers and Lichten, 2001; Bishop and Zickler, 2004; Borner et al., 2004). It is believed that the majority of DSBs are processed into NCOs via synthesis-dependent strand annealing (SDSA) (Allers and Lichten, 2001; Terasawa et al., 2007). However, the intermediates of SDSA have not yet been identified, probably because they are labile and/or short-lived (Shinohara et al., 2008). However, only a minority of DSBs are processed into COs. In budding yeast, most COs occur via single-end invasion (SEI) intermediates, which are formed by the interaction between the invading strand and the intact duplex. After capture of the second end of the intermediate, a four-way junction called a double Holliday junction (DHJ) is formed. Theoretically, different resolutions of DHJs will lead to either COs or NCOs, but in fact, all DHJs are resolved exclusively into COs (Allers and Lichten, 2001; Bishop and Zickler, 2004; Holliday, 1964; Hunter and Kleckner, 2001). In most organisms, some COs inhibit the occurrence of another CO nearby, which results in more evenly spaced crossovers than expected by a random distribution. This phenomenon is known as interference. In addition to interference-sensitive COs (class I), COs insensitive to interference (class II) were also found and studied recently (de los Santos et al., 2003; Hollingsworth and Brill, 2004).

Multiple ZMM proteins (ZIP1, ZIP2, ZIP3, ZIP4, MSH4, MSH5 and MER3) involved in the formation of class I COs have been identified in budding yeast. Single and double mutants of these genes have similar phenotypes, with significantly reduced COs and high frequency of univalent formation (Borner et al., 2004). In addition, extensive colocalization of these proteins, which is indicative of synapsis initiation complexes (SICs), has been reported in budding yeast (Fung et al., 2004; Tsubouchi et al., 2006). Both results indicate

that ZMM proteins work coordinately in the same recombination pathway (Borner et al., 2004). ZIP1 is an integral element of the synaptonemal complex (SC) and its linear signals from zygotene to pachytene are useful markers to discriminate assembled SCs. ZIP2, ZIP3 and ZIP4 are proposed to modify protein interactions (Agarwal and Roeder, 2000; Cheng et al., 2006; Perry et al., 2005; Tsubouchi et al., 2006). MSH4 and MSH5, two homologs of the bacterial MutS protein, appear to function as a heterodimer to stabilize strand invasion (Bocker et al., 1999; Snowden et al., 2004). MER3, a protein containing the DEXH box, is a DNA helicase that unwinds various duplex DNA in the 3′ to 5′ direction in an ATP-dependent manner (Nakagawa et al., 2001; Nakagawa and Kolodner, 2002a; Nakagawa and Kolodner, 2002b). Further study proposed that MER3 stabilizes nascent interactions via DNA heteroduplex extension to facilitate capture of the second DNA end that would lead to the formation of the DHJ (Mazina et al., 2004). However, MER3 is less understood than other ZMM proteins and the genetic requirements for its localization have not been determined, partially because of the lack of localization experiments (Shinohara et al., 2008).

Intriguingly, the frequency of class I and II COs vary in different organisms. Recently, the *Arabidopsis* homologs of *MSH4*, *MSH5*, *MER3* and *ZIP4* were investigated. All the mutants of these genes show a dramatic reduction of COs. In addition, the remaining COs are processed in a pathway that is not subject to interference, so it is proposed that the two CO pathways coexist in *Arabidopsis* (Chelysheva et al., 2007; Chen et al., 2005; Higgins et al., 2004; Higgins et al., 2008b; Lu et al., 2008; Mercier et al., 2005). However, not all organisms contain both CO classes. In *S. pombe*, only *MUS81*-dependent class II COs seem to occur, and these COs decrease significantly in *mms4 mus81* double mutants (Osman et al., 2003). By contrast, only class I COs seem to occur in *C. elegans* where interference is extremely robust. Only one CO happens in each bivalent, suggesting the occurrence of complete interference where one crossover completely suppresses the occurrence of another crossover in the same pair of chromosomes (Hillers and Villeneuve, 2003; Meneely et al., 2002). Until now, the situation in monocots remained unknown.

Rice is one of the most important food crops in the world, providing staple food for more than half of the world's population. Moreover, rice is becoming a model of molecular biological study in monocot plants, especially after the complete sequencing of the rice genome and the establishment of efficient *Agrobacterium*-mediated transformation systems. In spite of this, the molecular mechanism of meiosis is poorly understood in rice compared with *Arabidopsis* or maize (Jenkins et al., 2008). So far, only *PAIR1*, *PAIR2* and *MEL1* genes have been cloned using a *Tos17* insertion-tagging system (Nonomura et al., 2007; Nonomura et al., 2006; Nonomura et al., 2004). However, none of the genes that only affect COs have been characterized in rice until now.

Results

Characterization of the *mer3* mutant

We identified a spontaneous mutant showing complete sterility from the self-fertilization line of a *japonica* rice variety, Lunhui 422. This mutant showed a normal phenotype in the vegetative stage and could not be distinguished from Lunhui 422 based on its morphology (Fig. 1A,B). Following flowering, we found all the mature pollen grains of the mutant plant were empty and shrunken (Fig. 1C,D). The mutant could not set seeds when pollinated with mature pollen from wild-type plants, suggesting that megagametogenesis was also

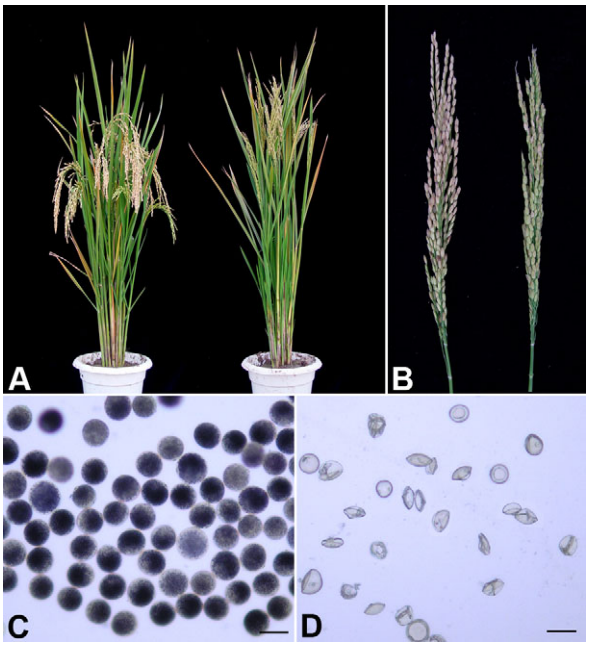

Fig. 1. Characterization of the *mer3* mutant phenotype. (A) Comparison of a wild-type plant (left) and a *mer3* mutant plant (right). (B) Comparison of a wild-type panicle (left) and a *mer3* panicle (right). (C) Fertile pollen grains in a wild-type plant. (D) Completely sterile pollen grains in a *mer3* plant. Scale bars: 50 μm.

affected in *mer3* mutants. We selected six individual plants with normal seed setting and harvested their seeds separately. In the next generation, two lines were all fertile plants, showing they were normal Lunhui 422. However, the other four lines all displayed sterile and fertile segregation, suggesting that they were generated from $mer3^{+/-}$ heterozygous plants. Among them, 159 plants were completely sterile whereas 521 plants were fertile, indicating the sterile phenotype of the mutant is controlled by a single recessive gene (X^2=0.95; P>0.05).

Isolation of the *MER3* gene

We isolated *MER3* using a map-based cloning approach. As the homozygous *mer3* is completely sterile, we constructed two populations by crossing heterozygous $mer3^{+/-}$ with Nanjing 11 and Balilla, individually. A total of 1048 F2 and F3 segregates showing the complete sterile phenotype were used for gene mapping. Linkage analysis mapped *MER3* on the long arm of chromosome 2, which was further delimited to a 61 kb region. Within this region, one candidate gene (*02g0617500*), annotated as an ATP-dependent helicase, showed high similarity with *MER3* from budding yeast and *MER3*/*RCK* from *Arabidopsis*. The mutants related to the two genes resulted in a reduced fertility in both budding yeast and *Arabidopsis* (Chen et al., 2005; Mercier et al., 2005; Nakagawa and Ogawa, 1999). Thus, this candidate gene in the *mer3* mutant was chosen to be amplified and sequenced. A 763 bp deletion, containing a 510 bp gene sequence and its 253 bp upstream region, was detected within this gene (Fig. 2). To verify that the sterile phenotype was caused by this deletion, the plasmid pCMER, containing the entire open reading frame (ORF), 2555 bp upstream sequence and 407 bp downstream sequence, was constructed and transformed into immature embryos from the heterozygous $mer3^{+/-}$ plants. Meanwhile, the plasmid pCMERC containing a partial ORF was

Fig. 2. Schematic representation of *MER3* gene and the location of *mer3* mutation. Exons are represented by black boxes and untranslated regions are shown in gray. The black bar indicates the 763 bp deletion in *mer3*.

constructed as a negative control. The genotypes of all the transgenic plants were investigated by PCR amplification using genome-specific primers. Only homozygous *mer3* plants were kept for further fertility evaluation. Twenty-three transgenic plants transformed with pCMER were identified, and all recovered their seed fertility, whereas 18 transgenic plants analyzed with pCMERC could not rescue their seed fertility. Therefore, we conclude the candidate gene, *MER3*, controls the sterile phenotype in rice.

To confirm the mutant phenotype was caused by the loss of function of the *MER3* gene, a RNA interference experiment was conducted to investigate whether the downregulation or silencing of *MER3* mimics the sterile phenotype and causes abnormal meiotic chromosome behavior. Transgenic plants expressing an inverted repeat of partial *MER3* were generated in Nipponbare. A total of 107/177 plants exhibited reduced fertility with a mean seed setting of 21.0%. Three completely sterile plants were selected for further analysis. RT-PCR analysis did not detect any residual *MER3* transcripts and immunostaining with an antibody against MER3 failed to find any MER3 protein. Both of these results indicate that the sterility was caused by the entire knockdown of MER3 in those plants.

The structure of *MER3* and its protein sequence

The full-length cDNA of the *MER3* gene was obtained by performing 5′- and 3′-RACE PCR with gene-specific primers. The *MER3* cDNA is comprised of 3962 bp with an ORF of 3615 bp (Fig. 2). *MER3* has 27 exons and 26 introns. The 1205 amino acid protein of MER3 shares significant identity with the *Arabidopsis* MER3/RCK protein (623/987 residues identical) (supplementary material Fig. S1). It also shows high similarity with *S. cerevisiae* MER3 protein (268/763 residues identical). A conserved domains search in NCBI revealed three conserved domains for the MER3 protein, namely, a DEXDc domain (residues 49-218), a HELICc domain (residues 271-432) and a SEC63 domain (residues 539-853).

To determine whether the transcripts of *MER3* in the mutant were altered or lost, further RT-PCR experiments were performed on young panicle tissue from the mutant. We found that the deletion brought about a new transcript caused by the fusion between a *mer3* fragment and its upstream gene transcript (NM_001053976) for *Os02g0617400*. Computational analysis showed that the truncated *MER3* sequence is embedded in the 3′ UTR of the fused transcript (Fig. 2). Theoretically, this fusion transcript will not change the ORF of NM_001053976, but only results in elimination of MER3 proteins.

Meiosis is abnormal in *mer3* mutant

To characterize whether the *mer3* sterility is a result of meiosis defects, the meiotic chromosomes in different stages of pollen mother cells (PMCs) from both wild-type and the *mer3* mutant were investigated (Figs 3 and 4). In the wild type, individual chromosomes became visible as thin threads at leptotene (Fig. 3A), synapsis of homologous chromosomes began at zygotene (Fig. 3B),

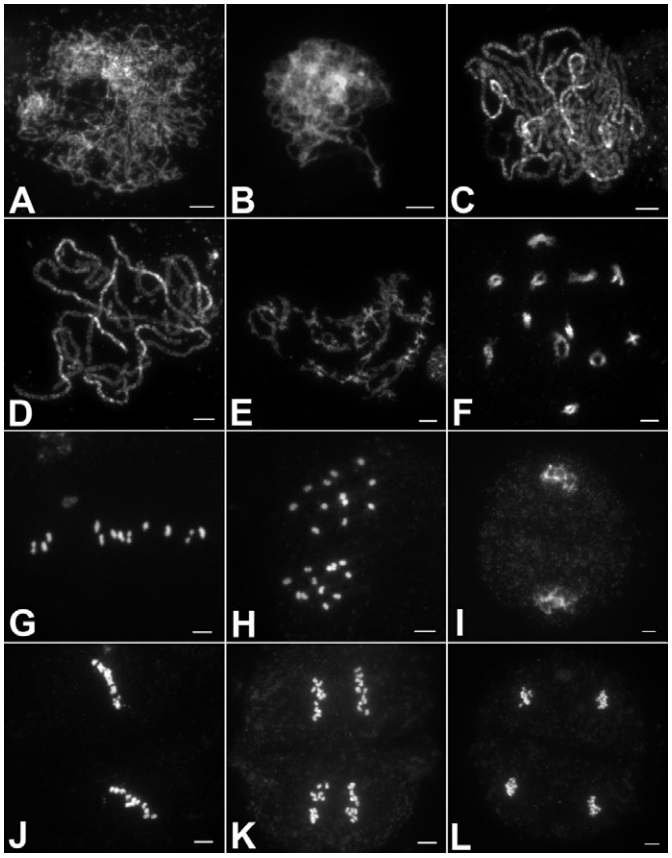

Fig. 3. Meiosis in wild-type Lunhui 422 rice. (A) Leptotene. (B) Zygotene. (C) Early pachytene. (D) Late pachytene. (E) Diplotene. (F) Diakinesis. (G) Metaphase I. (H) Anaphase I. (I) Prophase II. (J) Metaphase II. (K) Anaphase II. (L) Telophase II. Scale bars: 5 μm.

and pachytene was characterized by fully synapsed chromosomes along the SCs, indicating the final stage of recombination (Fig. 3C,D). SCs then fell apart and chiasmata, which correspond to crossovers formed in pachytene, were visible at diplotene (Fig. 3E). During diakinesis, the chromosomes condensed further and 12 bivalents were clearly observed (Fig. 3F). The bivalents aligned on the equatorial plate in metaphase I (Fig. 3G), and after that, homologous chromosomes separated and migrated in opposite directions at anaphase I (Fig. 3H). During the second meiotic division, the sister chromatids of each chromosome separated, as in mitosis, resulting in the formation of four sets of 12 chromatids (Fig. 3I-L).

In the *mer3* mutant, meiotic chromosome behavior resembled that of the wild type during leptotene and zygotene (Fig. 4A,B). We also found that the homologous chromosomes aligned normally in early pachytene (Fig. 4C). However, in mid-pachytene some regions of homologous chromosomes started to separate (Fig. 4D). This disassembly was even more evident following further condensation of chromosomes in late pachytene nuclei (Fig. 4E). Many homologous chromosomes separated from each other at diplotene (Fig. 4F). During diakinesis and metaphase I, the separation became more apparent, and in addition to normal bivalents, many univalents were formed (Fig. 4G-J). In metaphase I, univalents scattered throughout the entire nucleus whereas bivalents all lined up on the metaphase plate (Fig. 4I,J). In anaphase

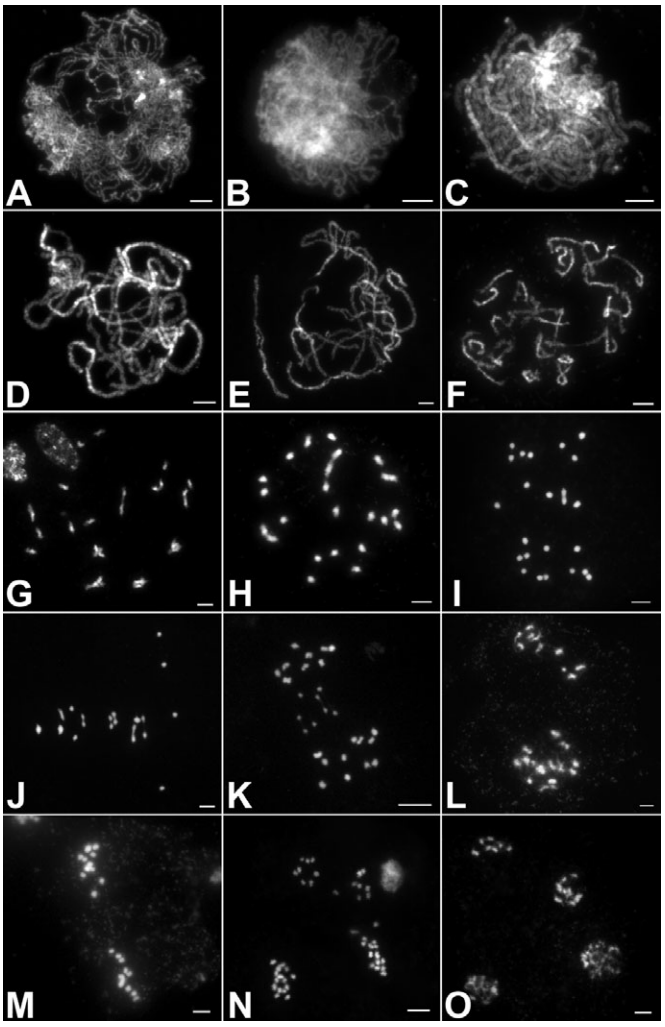

Fig. 4. Meiosis in the *mer3* mutant. (A) Leptotene. (B) Zygotene. (C) Early pachytene. (D) Middle pachytene. (E) Late pachytene. (F) Diplotene. (G,H) Diakinesis. (I) Metaphase I with one bivalent and 22 univalents. (J) Metaphase I with nine bivalent and six univalents. (K) Anaphase I. (L) Prophase II. (M) Metaphase II. (N) Anaphase II. (O) Telophase II. Scale bars: 5 μm.

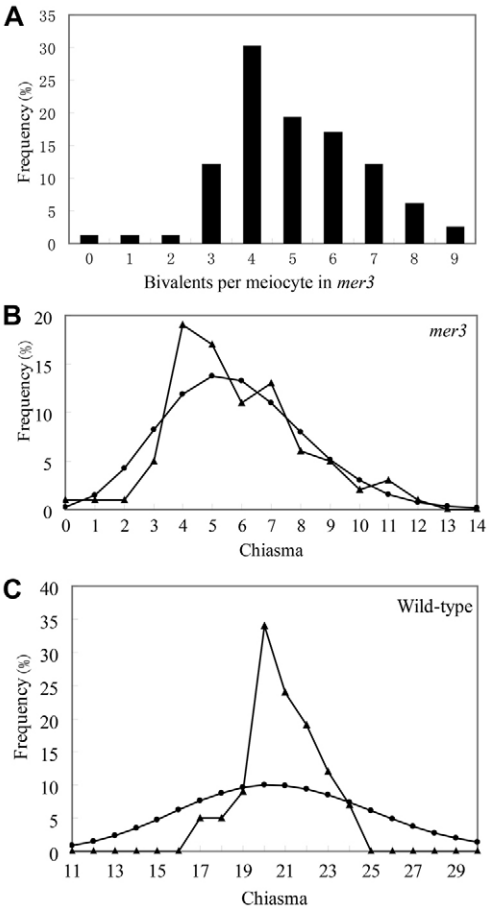

Fig. 5. Distribution of bivalents and chiasmata in both wild-type and *mer3* rice. (A) The frequency of bivalent number per meiocyte in *mer3*. (B) Chiasma distribution in *mer3*. (C) Chiasma distribution in the wild type. Triangles indicate observed distributions, whereas circles show predicted Poisson distribution.

I and telophase I, an uneven number of chromosomes could be seen in the two related daughter cells, as a result of random segregation of the univalents (Fig. 4K,L). After the second division, four spores with different numbers of chromosomes were detected (Fig. 4N,O). Cytological examination of meiotic chromosomes was also performed on the completely sterile RNAi plants, which revealed the same meiotic defects as those in *mer3* (data not shown).

The distribution of residual chiasmata in *mer3* is random

To investigate whether *MER3* mutation causes any CO variation, we quantified the chiasma frequency at metaphase I in both Lunhui 422 and *mer3* mutants as described (Sanchez Moran et al., 2001). The rod-shaped and ring-shaped bivalents were treated as having one and two chiasmata, respectively. In the wild-type Lunhui 422, the mean chiasma frequency from 115 PMCs was 20.8 per cell, compared with 5.8 per cell from 85 PMCs in *mer3* mutants. In addition, the mean bivalent frequency in *mer3* mutants reduced to 5.0 per meiocyte, in contrast to 12.0 in the wild type. In fact, no more than nine bivalents were observed in *mer3* mutants (Fig. 5A). Thus, the frequency of chiasma is reduced dramatically in *mer3*, leading to the subsequent reduction of bivalent frequency.

The number of bivalents was significantly reduced in *mer3* mutants because of the decrease of chiasma number. Therefore, the distribution of chiasma was counted and analyzed further in both *mer3* mutants (Fig. 5B) and the wild type (Fig. 5C). The frequency of chiasma number in *mer3* ranged from 0 to 12, and the distribution was very close to the predicted Poisson distribution ($\chi_{[11]}^2$=14.02; P>0.1), indicating the residual chiasmata distribute randomly among cells. However, the chiasma distribution among wild-type PMCs deviated significantly from a Poisson distribution ($\chi_{[23]}^2$=109.72; P<0.01). Thus, it is possible that two kinds of COs coexist in rice and MER3 might have an essential role in the formation of interference-sensitive COs.

MER3 does not colocalize with PAIR2 at early prophase I but locates on one end of PAIR2 fragments at later stages

The distribution and chronology of the MER3 protein were investigated by fluorescence immunolocalization on wild-type microsporocytes with the polyclonal antibody against MER3 fusion protein raised in mouse. The results revealed that MER3 was restricted to meiocytes in early prophase I of meiosis.

To determine MER3 distribution more accurately, a dual immunolocalization was conducted using antibodies against MER3 and PAIR2, raised in mouse and rabbit, respectively. PAIR2, which shows similarity to *S. cerevisiae* HOP1 and *Arabidopsis* ASY1, associates with unpaired chromosome axes at early meiosis I. PAIR2 can serve as a good marker to identify early meiotic events in plants (Nonomura et al., 2006). PAIR2 proteins were first visible during pre-meiotic S and G2 phases as punctuated foci in the nucleus (Fig. 6A). The foci then elongated and formed filamentous structures on the chromosomes (Fig. 6B,C,D). At early zygotene, the signals appeared as continuous lines indicative of the whole length of the chromosome axis (Fig. 6F). Then, with the synapses of homologous chromosomes, PAIR2 signals became thicker and started to be released from the chromosomes. During late zygotene or early pachytene, only a few residual signals could be observed on chromosomes (Fig. 6G). At the same time, many large foci were formed in the nucleoplasm and were maintained until the end of meiosis (supplementary material Fig. S2A).

MER3 showed up slightly later than PAIR2 and did not show obvious colocalization with PAIR2 foci when they began to appear (Fig. 6A,B). However, with the elongation of PAIR2 signals in early leptotene, more and more MER3 foci emerged and localized to the PAIR2 linear signals (Fig. 6C). Interestingly, we observed that most MER3 foci only located at one end of the PAIR2 short signals (Fig. 6E). The number of MER3 foci grew rapidly and reached its peak at late leptotene (average 267, n=8, range 225-303) (Fig. 6D). With the prolongation of PAIR2 signals, adjacent PAIR2 signals fused, but MER3 foci still persisted and were obviously on PAIR2-integrated linear signals. Markedly, some foci, which were only attached to one side of the linear PAIR2 signals, could also be detected in early zygotene (Fig. 6F). In late zygotene, MER3 and PAIR2 signals began to diminish almost simultaneously, but the elimination of PAIR2 signals was faster than that of MER3, so the residual signals of MER3 and PAIR2 did not show any relationship at late zygotene and pachytene (Fig. 6G). Both of the signals disappeared completely in late pachytene and could not be detected thereafter (Fig. 6H).

MER3 locates on one end of REC8 fragments in early stages

To further investigate MER3 localization in late zygotene and pachytene, dual immunolocalization was also conducted using antibodies against MER3 and REC8 (RAD21-4). REC8 is required for chromosome pairing, sister chromatid arm cohesion and normal homologous recombination (Bhatt et al., 1999; Cai et al., 2003; Chelysheva et al., 2005; Golubovskaya et al., 2006; Zhang et al., 2006). REC8, which shows high similarity with *Arabidopsis* DIF1/SYN1/REC8 and maize AFD1/REC8, was identified and analyzed by RNAi (Zhang et al., 2006). However, the distribution of REC8 in rice has not been described yet. Our data on meiocytes indicated that REC8 in rice acts similarly to that in *Arabidopsis*. REC8 was first visible in punctuated foci in premeiotic interphase nuclei (Fig. 7A). Those foci began to associate with condensing chromatin in early leptotene (Fig. 7B), and elongated to form linear signals in late leptotene (Fig. 7C). REC8 appeared as thin threads along the chromosomes in early zygotene and became thicker thereafter, corresponding to the condensing chromosomes (Fig. 7E). At pachytene, REC8 signals distributed over all paired chromosomes (Fig. 7F,G). In diplotene and diakinesis, the signals on chromosomes began to decrease (Fig. 7H). After metaphase I, most meiocytes were devoid of any signal, suggesting that all REC8 proteins had been totally degraded at this stage (Fig. 7I). As REC8 occurred early in premeiotic interphase nuclei and gradually disappeared before metaphase I, it can be used as a good marker to monitor prophase I, especially late zygotene and pachytene events in meiosis.

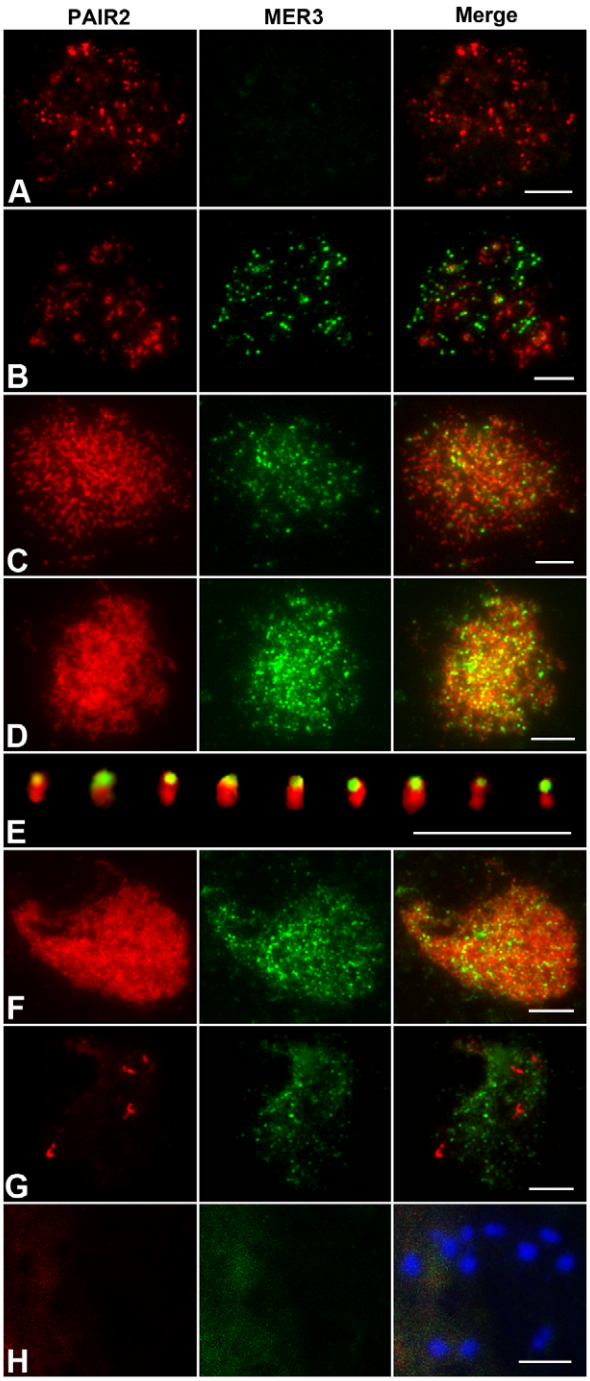

Fig. 6. Dual immunolocalization of PAIR2 and MER3 in wild-type rice. (A) Interphase. (B) Early leptotene. (C) Middle leptotene. (D) Late leptotene. (E) Enlarged images of PAIR2 fragments selected from C and D, showing one end location of MER3. (F) Early zygotene. (G) Late zygotene or early pachytene. (H) Diakinesis. Chromosomes are stained with DAPI (blue). Scale bars: 5 μm.

Dual immunostaining showed that MER3 appeared a little later than REC8. Almost all MER3 foci located at one end of REC8

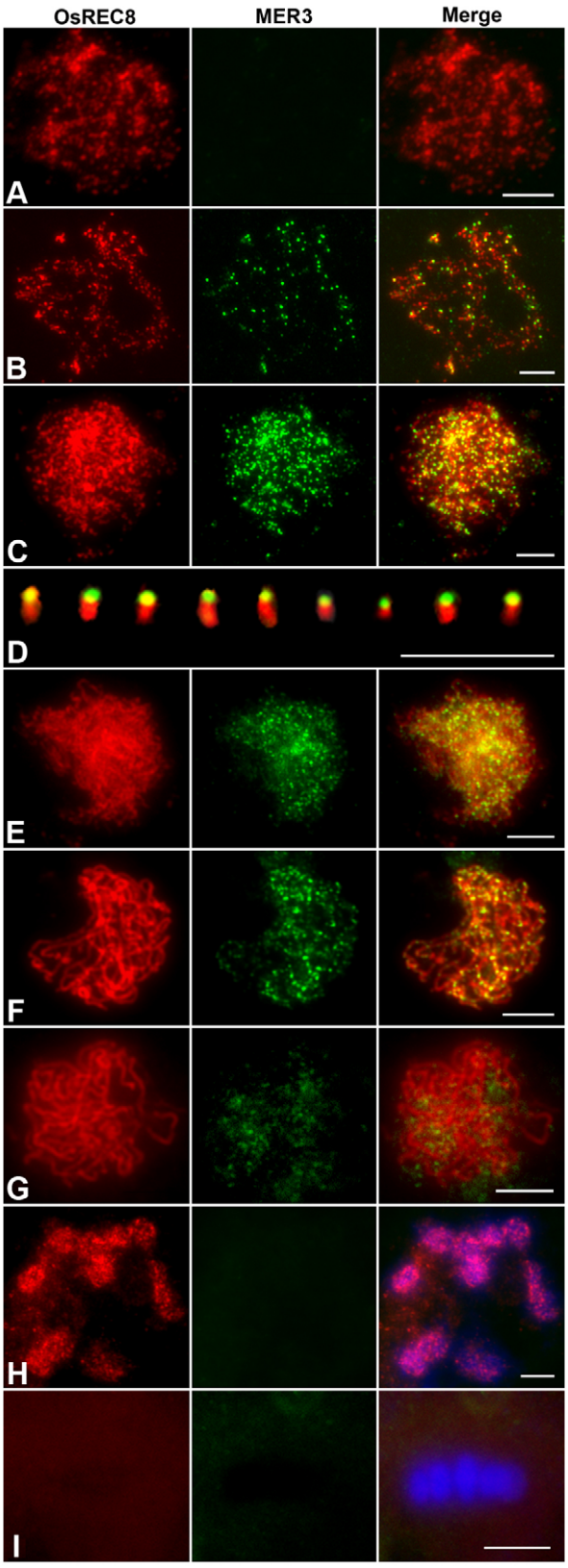

Fig. 7. Dual immunolocalization of REC8 and MER3 in wild-type rice. (A) Interphase. (B) Early leptotene. (C) Late leptotene. (D) Enlarged images of REC8 fragments selected from B and C, showing one end location of MER3. (E) Zygotene. (F) Early pachytene. (G) Middle pachytene. (H) Diakinesis. Chromosomes are stained with DAPI (blue). (I) Metaphase I. Chromosomes are stained with DAPI (blue). Scale bars: 5 μm.

linear signals as they became visible (Fig. 7B). During late leptotene and early zygotene, colocalization between MER3 and REC8 was the same as that observed between MER3 and PAIR2 (Fig. 7C,E). With the proceeding of homologous pairing in zygotene, the foci of MER3 decreased quickly and only about 134 foci (n=4, range 117-148) remained at early pachytene (Fig. 7F). In mid-pachytene, MER3 foci became diffused and gradually disappeared along the whole chromosomes. At the end of pachytene and its following stages, all bivalents were entirely free of MER3 signals (Fig. 7H,I).

MER3 is not required for loading of PAIR2 and REC8, whereas MER3 loading depends on PAIR2

As the deletion in *mer3* resulted in the alteration of *MER3* transcripts, it was important to find out whether the mutation also affected the synthesis of MER3 protein. As expected, no MER3 immunostaining signal was detected in the meiocytes of the *mer3* mutant (Fig. 8A,B,C,D), indicating that *mer3* is a null mutant. We further wanted to know whether elimination of MER3 also affected the distribution of PAIR2 and REC8. Therefore, immunostaining was also performed in *mer3* meiocytes using antibodies against PAIR2 and REC8.

The distribution of PAIR2 on chromosomes in *mer3* meiocytes did not exhibit any difference from that in wild-type meiocytes (Fig. 8A). Interestingly, the merged large foci, which usually occurred in the nucleoplasm in the wild type from pachytene through its following stages, did not appear throughout the entire meiosis in *mer3* meiocytes (supplementary material Fig. S2B). In *Arabidopsis*, large foci were also detected in the nucleoplasm and were thought to be PAIR2 aggregates before they degraded (Armstrong et al., 2002). Therefore, it is probable that PAIR2 in *mer3* is degraded immediately without any aggregate formation once it is released from the chromosomes.

REC8 in *mer3* behaved the same as that in wild-type meiocytes at most stages. However, in pachytene nuclei, the two paired threads of REC8 on the same pachytene chromosome in *mer3* were usually separate from each other (Fig. 8B), whereas those in the wild type were very close and always looked like a single thread. This observation is consistent with the fact that homologous chromosomes tend to disassemble in *mer3*. After diplotene, REC8 in *mer3* started to degrade. The degradation process of REC8 in *mer3* is similar to that observed in the wild-type meiocytes.

We also carried out immunodetection with different antibody combinations in a *pair2* mutant. REC8 was normally loaded on the prophase I chromosomes, but no PAIR2 signals could be detected in the same nuclei (Fig. 8C), indicating that the *pair2* mutant is a null mutant. In addition, no MER3 signals were observed in the *pair2* mutant (Fig. 8D), implying that the normal loading of MER3 depends on PAIR2.

Discussion

MER3 has an important role in the formation of COs in rice

In the present study, we identified the *MER3* gene in rice using a map-based cloning approach and analyzed meiotic chromosome behavior by cytological observations. In *mer3*-null mutants, the average number of residual chiasmata is 5.81 per cell, accounting for only ~28% of that in the wild type. Thus, MER3 has an important role in the formation of the majority of COs in rice.

In budding yeast, about 85% of COs are derived from the MER3-dependent pathway (Borner et al., 2004). In rice, about 72% of COs are attributed to the activity of MER3-dependent pathway; this frequency is very similar to that demonstrated in *Arabidopsis*, where

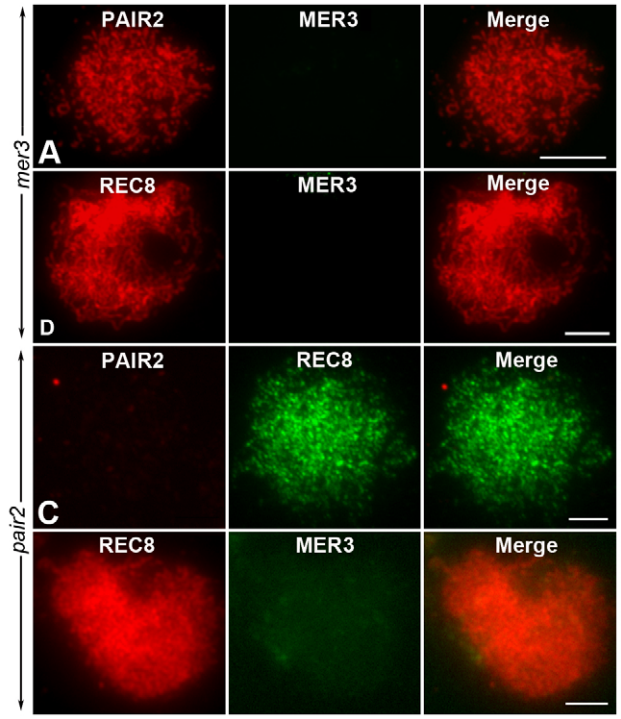

Fig. 8. Immunolocalization of PAIR2, REC8 and MER3 in *mer3* and *pair2*. (A) PAIR2 and MER3 in *mer3* at zygotene stage. (B) REC8 and MER3 in *mer3* at pachytene stage. (C) PAIR2 and REC8 in *pair2* at zygotene stage. (D) REC8 and MER3 in *pair2* at zygotene stage. Scale bars: 5 μm.

MER3 accounts for 68-77% of COs (Chen et al., 2005; Mercier et al., 2005). Therefore, it is reasonable to propose that MER3 maintains an evolutionally conserved function in the formation of COs among eukaryotes (Chen et al., 2005). Also, the contribution of the MER3-dependent pathway to meiotic COs in rice might be as much as that in *Arabidopsis*.

Our analyses of meiotic cells in *mer3* mutants show that the distribution of residual chiasmata is consistent with a Poisson distribution, reflecting the random distribution of the residual COs. By contrast, the distribution of COs in the wild type differs significantly from a Poisson distribution. By analogy with similar observations in budding yeast and *Arabidopsis*, those MER3-dependent COs might also be interference-sensitive COs. Therefore, this suggests that rice might also have two classes of COs and that MER3 has an essential role in the formation of interference-sensitive COs (class I).

MER3 might transfer onto PAIR2-indicating axial elements in early prophase I

PAIR2 is a protein that associates with axial elements (AEs) in rice, its distribution can well reflect the distribution of AEs in early stages (Nonomura et al., 2006). In addition, studies in budding yeast have confirmed that MER3 works downstream of the formation of DSBs. Dual immunostaining results clearly show that in the beginning, MER3 foci do not show any correlation with PAIR2 signals, but all MER3 foci locate on one end of PAIR2 filaments thereafter. These observations support the hypothesis that DSBs do not occur on AEs, but only transfer to one end of nascent AEs after DSBs formation on chromatin loops (Kleckner, 2006; van Heemst and Heyting, 2000). This implication is consistent with the view from *Arabidopsis* that ASY1 is required for normal meiotic recombination progression, but not for its initiation (Sanchez-Moran et al., 2007).

Presynaptic alignment might be determined before MER3 performs its function

Analyses of meiotic mutants in numerous organisms have led to the proposal that meiotic homolog juxtaposition involves three mechanistically distinct steps, namely recognition, presynaptic alignment and synapsis (Dawe, 1998; Tesse et al., 2003; Zickler and Kleckner, 1999). Studies in plants have demonstrated that RAD51 also participates in homology searching in addition to its function on meiotic recombination, implying that presynaptic alignment might be determined after the RAD51-mediated homology search (Franklin et al., 1999; Li et al., 2007; Li et al., 2004; Pawlowski et al., 2003). In all *mer3* mutants from different organisms, the number of chiasmata is dramatically reduced, but presynaptic alignment of homologous chromosomes proceeds normally, indicating that presynaptic alignment is independent of MER3 function and the subsequent class I recombination in rice. As class I recombination accounts for the majority of COs and mutation of class II genes has no obvious impact on presynaptic alignment (Berchowitz et al., 2007; de los Santos et al., 2003; Higgins et al., 2008a), it is likely that presynaptic alignment might be determined after single-end invasion facilitated by RAD51, but before DNA heteroduplex extension stimulated by MER3.

In budding yeast, the correlation between ZMM foci and crossovers has been studied, where the number of ZMM foci (60-70) is less than that of final crossovers (around 90). Mutations of *ZMM* genes reduce crossovers by only ~66%, implying that ZMM foci are corresponding to class I COs (Agarwal and Roeder, 2000; Chua and Roeder, 1998; Fung et al., 2004). However, such correspondence between ZMM foci and COs is not universal. The number of ZMM foci in both *Arabidopsis* and mouse significantly exceeds the number of COs (de Boer et al., 2006; Higgins et al., 2004). A similar pattern is found in rice: the number of MER3 foci (~267) in late leptotene is about 12-fold more than the prospective COs (~21). The abundance of ZMM foci was postulated to have a vital role in the homologous pairing of relatively long chromosomes (Higgins et al., 2004; Zickler and Kleckner, 1999). However, *mer3* mutants show normal homologous pairing, indicating that homologous pairing is independent of MER3 proteins. Studies in the fungus *Sordaria macrospora* suggest that SC formation needs more DSBs than does presynaptic alignment (Tesse et al., 2003). In addition, analyses in budding yeast and *Arabidopsis* reveal that synapsis is incomplete in *mer3* mutants (Borner et al., 2004; Chen et al., 2005). Considering rice has relatively long chromosomes but only a limited number of COs, we believe that those ZMM foci, which correspond to the final COs, might be too few to maintain the formed SCs on relatively long chromosomes. Thus abundant ZMM SICs are probably required for full synapsis in plants.

Materials and Methods

Materials

The spontaneous mutant, *mer3*, was found in a *japonica* rice variety, Lunhui 422. As *mer3* is completely sterile for both male and female gametes, six wild phenotype plants (theoretically, including both wild type and $mer3^{+/-}$ heterozygous plants), which existed in the population where mutants were found, were crossed with Nanjing11 (*indica*) and Balilla (*japonica*) individually to make mapping populations. All plants were planted in individual lines to monitor the segregation of F2 populations used for fine mapping. The *japonica* rice variety, Nipponbare, was used for rice transformation to generate RNAi plants. A *pair2* mutant identified from the *indica*

rice Zhongxian 3037 was also used in this study. All plant materials were grown in the paddy fields.

Molecular cloning of *MER3*

To fine map *MER3*, STS markers (P1-P8) were developed based on sequence differences between *indica* variety 9311 and *japonica* variety Nipponbare according to the data published in http://www.ncbi.nlm.nih.gov.

Molecular cloning of the full-length *MER3* cDNA

Total RNA was extracted from rice tissues using TRIpure reagent (BioTeke) as described by the supplier. 3 μg RNA was reverse-transcribed with Oligo-dT(18) primer using superscript II RNaseH reverse transcriptase (Invitrogen). Using LA-Taq polymerase (Takara), a 3897-bp fragment including the predicted coding region of the cDNA was amplified with primers RT1F and RT3R. 3′RACE was carried out using primers RACE18T, RACEF, RT4F and RT3F. 5′RACE was carried out as follows: cDNA was synthesized with 5′ phosphorylated primer 5RACE-RT. After treatment with RNaseH, the cDNA was ligated using T4 RNA ligase. PCR was performed with primers 5RACE-RS1 and 5RACE-RS2.

Construction of siRNA and rice transformation

A 222 bp fragment of *MER3* was amplified by PCR with the primer MERRNAi, this fragment was cloned into the pGEM-T vector (Promega) and sequentially cloned into the *Bam*HI/*Sal*I and *Bgl*II-*Xho*I sites of the pUCRNAi vector. Subsequently, the stem-loop fragment was cloned into the pCAMBIA2300-Actin vector. The resulting RNAi construct was transformed into *A. tumefaciens* strain and used for further rice transformation.

Complementation test

The complementary plasmid was constructed by cloning the 11495 bp *Sse*8387I genomic DNA fragment containing the entire *MER3* coding region into the pCAMBIA1300 vector. A control plasmid, containing 8900 bp of the truncated *MER3* gene was also constructed. Both of these plasmids were transformed into EHA105 and then into embryonic calli of *MER3*$^{+/-}$ plants. Primer JD3 verified the genotype of the transgenic plant.

Antibody production

To generate the antibody against PAIR2, a 333 bp fragment of *PAIR2* (amino acids 510-609) was amplified from AK064028 (RGRC-NIAS) with primer PAIRGST. After being cloned into the pGEM-T vector and sequenced, this fragment was ligated into the expression vector pGEX-4T-2 (Amersham) digested with *Bam*HI-*Xho*I. A 747-bp *REC8* cDNA fragment (amino acids 139-384) was amplified from rice panicle cDNA using primer REC8GST, the fragments were digested with *Eco*RI-*Xho*I and ligated into the expression vector pGEX-4T-2. A portion of the *MER3* cDNA corresponding to amino acids 987-1142 was amplified from rice panicle cDNA using primer MERHIS, the PCR products were then digested with *Bam*HI-*Xho*I and ligated into the expression vector pET-30a(+) (Novagen).

All of these expression vectors were transformed into BL21 (DE3) and were induced by addition of 0.3 mM IPTG to the culture medium. The PAIR2-fusion and REC8-fusion peptides, mainly expressed in the soluble fraction, were purified using the MagneGST Protein Purification System (Promega). The MER3-fusion peptides were accumulated in the insoluble fraction, the inclusion bodies were collected, washed, and dissolved in nickel-chelating binding buffer containing 8 M urea and purified using nickel-chelating Sepharose beads. Polyclonal antibodies of PAIR2 were raised in rabbits. Polyclonal antibodies of MER3 were raised in mouse, whereas polyclonal antibodies of REC8 were raised in both rabbits and mouse. No anti-MER3 signal was obtained in *mer3* and no anti-PAIR2 signal was found in *pair2*, indicating the specificity of their antibodies. The specificity of REC8 antibodies was checked by western blot.

Computational and database analysis

The gene structure schematic diagram was drawn using GSDS (http://gsds.cbi.pku.edu.cn/index.php). The alignment of amino acid sequences was performed with the Alignx module of Vector NTI Advance 9.0 (Invitrogen), and further modified using GeneDoc (http://www.nrbsc.org/gfx/genedoc/index.html). Primers were designed using the PrimerSelect program of Lasergene (DNASTAR, Madison, WI). All primers mentioned above are listed in supplementary material Table S1.

Meiotic chromosome preparation

Young panicles of both wild-type and *mer3* 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 Olympus BX61 fluorescence microscope with a microCCD 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 on a slide with PBS solution and covered with a coverslip. After soaking in liquid nitrogen and removing the coverslip, 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° for 4 hours in 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).

Accession number

The GenBank (http://www.ncbi.nlm.nih.gov/GeneBank) accession number for the *MER3* cDNA is FJ008126.

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