

PAIR3, an axis-associated protein, is essential for the recruitment of recombination elements onto meiotic chromosomes in rice

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ABSTRACT During meiosis, the paired homologous chromosomes are tightly held together by the synaptonemal complex (SC). This complex consists of two parallel axial/lateral elements (AEs/LEs) and one central element. Here, we observed that PAIR3 localized to the chromosome core during prophase I and associated with both unsynapsed AEs and synapsed LEs. Analyses of the severe *pair3* mutant demonstrated that PAIR3 was essential for bouquet formation, homologous pairing and normal recombination, and SC assembly. In addition, we showed that although PAIR3 was not required for the initial recruitment of PAIR2, it was required for the proper association of PAIR2 with chromosomes. Dual immunostaining revealed that PAIR3 highly colocalized with REC8. Moreover, studies using a *rec8* mutant indicated that PAIR3 localized to chromosomes in a REC8-dependent manner.

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INTRODUCTION

Meiosis is a highly conserved process in all sexually reproducing eukaryotic organisms. During meiosis, pairing and recombination between homologous chromosomes are essential for the production of genetically distinct haploid sperm and eggs. The close association between homologues is enhanced by a meiotic-specific ladderlike proteinaceous structure, the synaptonemal complex (SC). Ultrastructural analysis of the SC reveals a tripartite structure with two parallel lateral elements (LEs) and one central element (CE). SC assembly starts during the premeiotic S-phase and early meiotic prophase with the formation of a single proteinaceous axis, the axial element (AE), along the two sister chromatids of each chromosome (the LE is called AE before synapsis of the homologous chromosomes; de Boer and Heyting, 2006). From zygotene to pachytene, the AEs of homologues are connected to each other along their entire length by the transverse filaments (TFs), and the CEs are formed in the middle, during a process called synapsis. At

the diplotene stage, the SC disassembles and homologous chromosomes are separated, except at the chiasmata sites.

TF proteins have been characterized in several model organisms, including budding yeast (Zip1; Sym *et al.*, 1993), mammals (Sycp1; Meuwissen *et al.*, 1992), *Caenorhabditis* (Syp-1 and Syp-2; MacQueen *et al.*, 2002; Colaiacovo *et al.*, 2003), *Drosophila* (C(3)G; Page and Hawley, 2001), and *Arabidopsis* (ZYP1a and ZYP1b; Higgins *et al.*, 2005). Although these TF proteins exhibit similar structures, they show a high degree of sequence divergence (Page and Hawley, 2004). A recent study revealed that ZEP1, the TF protein in rice, showed significant similarity with ZYP1 (Wang *et al.*, 2010), suggesting that TF proteins are probably conserved in the plant kingdom.

AE assembly is one of the earliest known events of meiosis and is essential for the formation of cytologically characteristic leptotene chromosomes (Zickler and Kleckner, 1999; Wang *et al.*, 2009a). The AE was initially defined by electron microscopy as a structure that forms between sister chromatids, along their entire length, before synapsis. Genetic and biochemical studies have identified AE components and showed that AEs play important roles in chromosome condensation, pairing, recombination, TF assembly, and prohibiting double-strand breaks from entering into recombination pathways that involve sister chromatids (Zickler and Kleckner, 1999; Page and Hawley, 2004; Severson *et al.*, 2009).

Several AE components have been described in yeast, mammals, and *Caenorhabditis elegans*. The budding yeast Red1 and Hop1 are meiosis-specific proteins that localize to chromosomes. Mutants of *red1* fail to make any discernible AE or SC structures and

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Abbreviations used: AE, axial element; CE, central element; DAPI, 4',6-diamidino-phenylindole; LE, lateral element; PBS, phosphate-buffered saline; SC, synaptonemal complex; TF, transverse filament.

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exhibit normal chromosome compaction, while *hop1* mutants form long fragments of AEs but without any SCs and are defective in axial chromosome compaction (Rockmill and Roeder, 1990; Loidl *et al.*, 1994; Smith and Roeder, 1997; Zickler and Kleckner, 1998). Synaptonemal complex proteins (SYCP) SYCP2 and SYCP3 are two known mammalian AE proteins that contain predicted coiled coils near the C terminus. They localize to both unsynapsed AEs and synapsed LEs. In the absence of either SYCP3 or SYCP2, AE/LE structures are disrupted (Dobson *et al.*, 1994; Lammers *et al.*, 1994; Offenberg *et al.*, 1998; Yuan *et al.*, 2000; Yang *et al.*, 2006). The *C. elegans* HIM-3 protein localizes to the chromosome core and associates with both unsynapsed and synapsed chromosomes. In addition, HIM-3 plays important roles in chromosome pairing, synapsis, and segregation. Hence, HIM-3 protein is considered to be a meiotic chromosome core component (Zetka *et al.*, 1999). HTP-3 in *C. elegans* also colocalizes with HIM-3 throughout the meiotic prophase, indicating that HTP-3 is also a component of meiotic chromosome axes (Goodyer *et al.*, 2008; Severson *et al.*, 2009). Thus far, although several AE-associated proteins have been reported in maize, *Arabidopsis*, and rice, no AE core component has yet been identified in plants (Mercier and Grelon, 2008).

A previous study characterized the *PAIR3* gene and found that insertional mutations in its intron led to the elimination of synapsis in both male and female gametes (Yuan *et al.*, 2009). Here, we also identified two *pair3* mutants showing more severe phenotypes and demonstrated that *PAIR3* is required for bouquet formation, homologous pairing, normal recombination, and SC installation. In addition, it was also revealed that *PAIR3* localizes to the chromosome axis and is required for normal binding of *PAIR2* to chromosome axes.

RESULTS

Characterization of *pair3* mutants

We identified a tissue culture-derived mutant exhibiting complete sterility from the progeny of a *japonica* rice variety, Wuxiangjing 9. The mutant exhibited normal vegetative growth but showed complete sterility. Genetic analysis indicated that the mutant phenotype is controlled by a single recessive gene. The mutant failed to produce any viable pollen. In addition, the mutant could not set any seeds when pollinated with the wild-type pollens, indicating that the megagametogenesis was also affected by the mutation.

Molecular cloning of the *PAIR3* gene

To isolate the mutated gene controlling the sterile phenotype, a large F₂ mapping population was generated by crossing the heterozygous plants with *indica* rice variety 9311. F₂ and F₃ segregates (1100 in total) showing the sterile phenotype were used for gene mapping. Linkage analysis mapped the gene on the long arm of rice chromosome 10, which was further confined to a 30-kb region. By sequencing, one candidate gene, *Os10g0405500*, was found to be altered in the mutant. This gene was isolated recently and named *PAIR3*. A single nucleotide deletion at position 1044 in the coding region was found in the *pair3-1* mutant (Supplemental Figure S1), which resulted in a frameshift mutation.

We also obtained one other mutant showing the same defects as *pair3-1* from Nipponbare. Immunostaining with an antibody against *PAIR3* failed to find any *PAIR3* protein in the mutant. Thus, sequencing analysis was carried out and revealed that the mutant also contains a single nucleotide deletion at position 1538 in the coding region, and it was named *pair3-2* (Supplemental Figure S1). Both mutations resulted in the loss of predicted coiled-coil motifs. Additionally, we utilized an RNA interference (RNAi) approach to

down-regulate *PAIR3* in rice variety Yandao 8. Twenty transgenic plants were obtained, and 13 plants exhibited severe reduction of fertility. The results confirmed that the loss of function of the *PAIR3* gene led to the sterility phenotype.

Chromosome behavior in *pair3* mutants

To understand the cause of the complete sterility in *pair3* mutants, the meiotic chromosomes in pollen mother cells (PMCs) of both the wild type and *pair3-1* mutant were investigated. In the wild type, chromosomes began to condense and form thin lines at leptotene (Supplemental Figure S2A). In zygotene, pairing and synapsis occurred between homologous chromosomes (Supplemental Figure S2B). The synapsis was completed, and 12 fully synapsed chromosomes were clearly visible at pachytene (Supplemental Figure S2C). With further chromosome condensation occurring in diplotene and diakinesis, SCs were gradually disassembled and the paired homologous chromosomes were held together by chiasmata (Supplemental Figure S2D). At metaphase I, all bivalents were aligned along the equatorial plate (Supplemental Figure S2E). Subsequently, homologous chromosomes were separated from each other and moved to the opposite poles of the cell at anaphase I (Supplemental Figure S2F). The second meiotic division was very similar to mitosis and finally produced tetrad spores (Supplemental Figure S2, G–I).

Compared with the wild type, there was no obvious difference in *pair3-1* during leptotene (Figure 1A). However, pairing between homologous chromosomes was not observed from zygotene to pachytene (Figure 1, B and C). During diakinesis and metaphase I, many randomly distributed univalents were found (Figure 1D). We further quantified the chiasma frequency and showed that the *pair3-1* mutant formed an average of 3.25 chiasmata corresponding

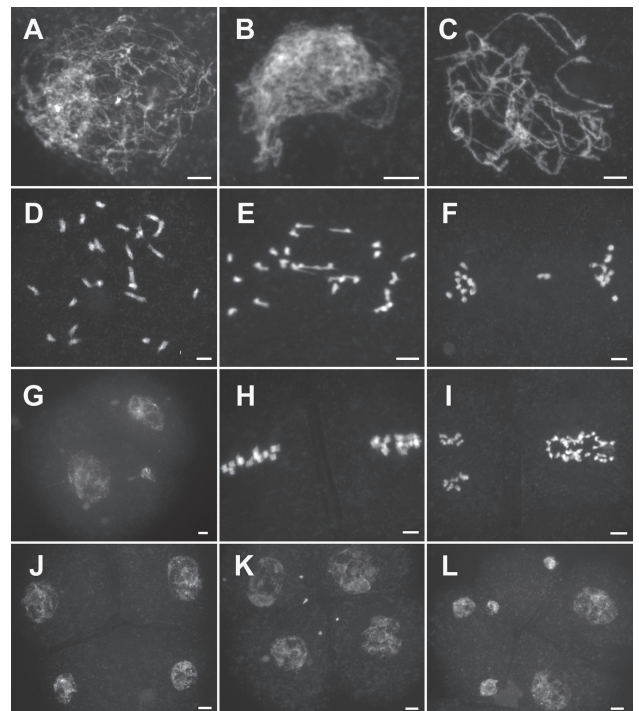


FIGURE 1: Meiosis in the *pair3* mutant. (A) Leptotene. (B) Zygotene. (C) Pachytene. (D) Diakinesis. (E) Anaphase I. (F) Telophase I. (G) Prophase II. (H) Metaphase II. (I) Anaphase II. (J) Telophase II. (K) Tetrads with several randomly distributed chromosomes. (L) Hexayads. Scale bars, 5 μ m.

to 3 bivalents per PMC ($n = 20$). In anaphase I, those univalents separated randomly toward two poles of the dyad (Figure 1E). In telophase I and prophase II, an uneven number of chromosomes could be seen in the two related cells (Figure 1, F and G). After the second division, tetrads and polyads with different numbers of chromosomes were formed (Figure 1, H–L). In addition, multiple micronuclei were frequently observed. These results implied that correct chromosome disjunction and cytokinesis were also affected by the mutation in the *PAIR3* gene. Such abnormality was also found in the *pair3-2* mutants but not in the previously reported *pair3* mutants (Yuan *et al.*, 2009). We suspected that the mutants identified in the present study contain alleles of *pair3* that result in more severe defects.

Bouquet is abolished in *pair3*

The clustering of telomeres into a “bouquet” is one of the mechanisms thought to facilitate these initial contacts leading to homologous chromosome alignment and subsequent pairing (Dawe, 1998; Harper *et al.*, 2004; Hamant *et al.*, 2006). To assess whether mutation of *PAIR3* affects telomere bouquet formation, we probed meiotic chromosomes at the leptotene stage with the clone pAtT4 that contains telomeric repeats in both the wild type and the *pair3-1* mutant. In the wild type, almost all telomeres attached to the nuclear envelope and clustered to a confined region, representing the typical bouquet configuration (Figure 2A). In the *pair3-1* PMCs ($n = 50$), telomeres remained scattered throughout the nuclei and never formed bouquets (Figure 2B), implying that *PAIR3* is required for normal bouquet formation in rice.

PAIR3 is required for homologous chromosome pairing

To further examine the defects of homologous pairing in *pair3*, we performed fluorescence in situ hybridization (FISH) analysis using 5S rDNA as a probe to monitor the pairing status. In the wild type, the two unpaired 5S signals were observed at leptotene and zygotene. With the pairing of homologous chromosomes, the two signals were well paired during pachytene (Figure 2C). By contrast, paired 5S

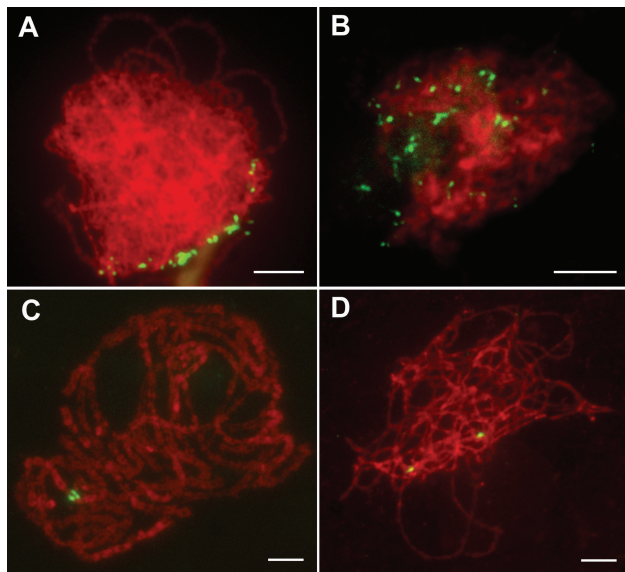


FIGURE 2: *PAIR3* is required for bouquet formation and homologous pairing. (A and B) Telomere bouquet formation analysis revealed by FISH using the telomere sequence as a probe (green) in both wild type (A) and the *pair3* (B) nuclei. Chromosomes (red) are stained with DAPI. (C and D) Immunofluorescence micrographs showing paired and unpaired 5S rDNA signals (green) in wild type (C) and *pair3* (D) mid-zygotene-stage nuclei, respectively. Bars, 5 μ m.

signals were never observed in all *pair3-1* cells ($n = 61$; Figure 2D), implying that the pairing was lost in *pair3-1*. Therefore, *PAIR3* is absolutely required for homologous chromosome pairing.

REC8 displays discontinuous signals during zygotene in *pair3* mutants

Meiosis-specific sister chromatid cohesion is essential for normal chromosome behavior in meiosis. The meiosis-specific cohesin *REC8* mediates most of the cohesion (Watanabe and Nurse, 1999). An antibody against *REC8* protein was used to discern whether the cohesion complex is normally installed on chromosomes in *pair3* mutants. In the wild-type meicytes, *REC8* presented as punctate foci in the premeiotic interphase and leptotene (Figure 3A). In late

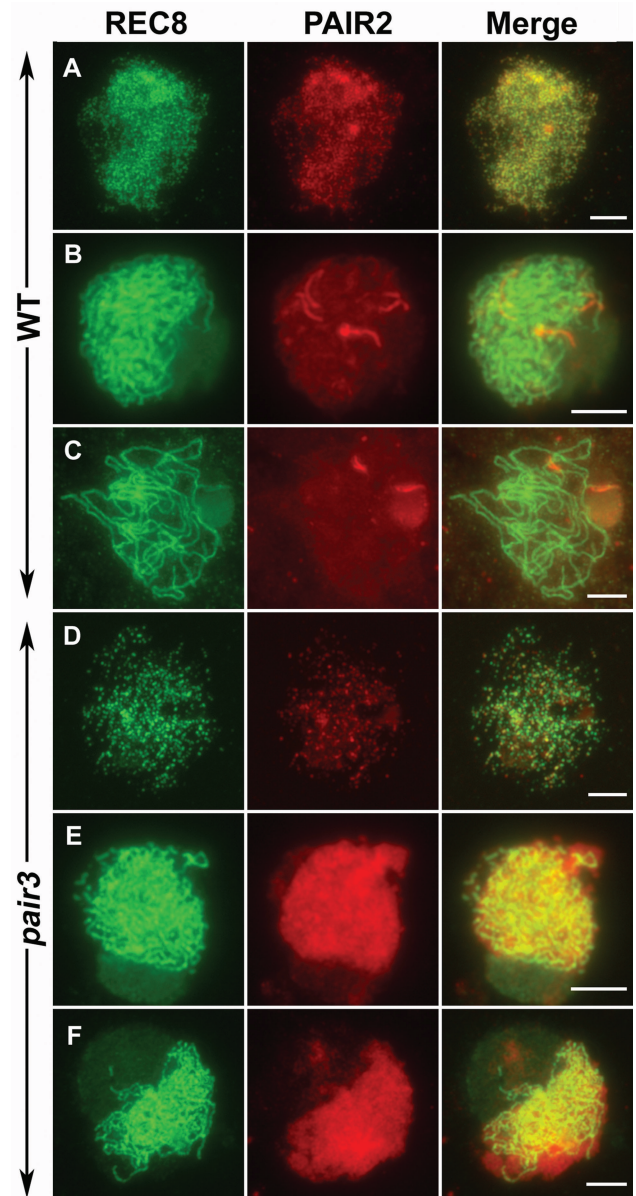


FIGURE 3: Dual immunolocalization of *REC8* and *PAIR2* in meicytes of the wild type (A–C) and *pair3* plant (D–F). (A) Leptotene. (B) Zygotene. (C) Pachytene. (D) Leptotene, *REC8* and *PAIR2* recruit normally. (E) Zygotene, *REC8* shows discontinuous signals along the chromosomes, while *PAIR2* exhibits diffused signals. (F) Pachytene, *REC8* shows continuous signals along the chromosomes, while *PAIR2* still appears as diffused signals. Bars, 5 μ m.

leptotene, those foci assembled to form short linear signals on the chromosomes. In zygotene and pachytene, REC8 was frequently seen as continuous threads along the chromosomes (Figure 3, B and C). In *pair3-1* meiocytes, REC8 proteins were also first visible as punctate foci (Figure 3D), which would also fuse and form short linear signals as those observed in the wild-type. Although REC8 linear signals were detected during zygotene, they were generally not continuous along the lengths of chromosomes, and frequent breaks/gaps were apparent (Figure 3E). At a stage corresponding to pachytene, when chromosomes were relatively condensed, REC8 signals became continuous and gaps/breaks were hardly detectable (Figure 3F). Thus, the loss of PAIR3 function may have only a minor effect on the normal progression of chromosome compaction or AE maturation.

PAIR2 requires PAIR3 to localize properly

PAIR2, a protein that associates with AEs in rice, is the homologue of yeast HOP1 and *Arabidopsis* ASY1 (Nonomura *et al.*, 2006). The signals of HOP1/ASY1 in *Arabidopsis* and maize are frequently used to detect the assembly of AEs (Chelysheva *et al.*, 2005; Golubovskaya *et al.*, 2006; Pawlowski *et al.*, 2009). In rice, PAIR2 proteins were visible as foci during interphase and leptotene in the wild type (Figure 3A). At zygotene, PAIR2 appeared as continuous lines on the chromosomes (Figure 3B). In pachytene, only a few residual PAIR2 signals could be observed on chromosomes. In *pair3-1* meiocytes, PAIR2 also appeared as foci in interphase and early leptotene, but those foci rarely elongated to form short linear signals at leptotene. Instead, PAIR2 signals became diffused in the nucleoplasm in cells later than leptotene (Figure 3, B and C). These results suggest that normal PAIR2 association with chromosomes depended on PAIR3.

Normal chromosome structures are formed in a *pair3* mutant

A previous study demonstrated that PAIR2 associates with AE in rice (Nonomura *et al.*, 2006). Here, the abnormal PAIR2 signals imply that the AEs in *pair3-1* might be severely disrupted; alternatively, it remains possible that AEs are normally formed but the association of PAIR2 with AEs is severely affected. With the aim of distinguishing between these two possibilities, we utilized a silver staining method to observe the chromosome axes in both the wild-type and *pair3-1* mutant. In the wild type, synapsed LEs were obviously observed during pachytene (Figure 4A). In the *pair3-1* mutant, apparently normal AEs were also observed (Figure 4B), suggesting that PAIR3 may not be required for the formation of chromosome axes.

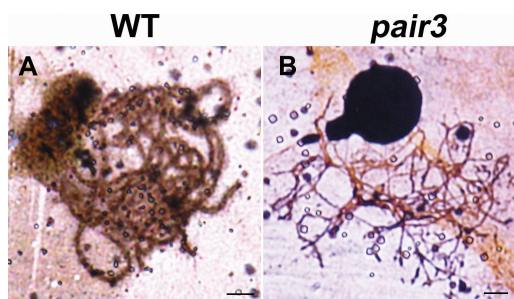


FIGURE 4: Silver staining of spread nuclei at pachytene stage in wild type and *pair3* mutant. AE is formed in both wild type (A) and *pair3* mutant (B). Bars, 5 μ m.

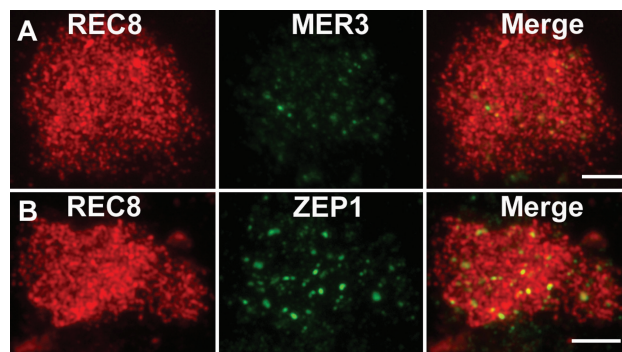


FIGURE 5. The localization of MER3 and ZEP1 in *pair3* mutant. (A) Only a few faint MER3 foci are detected in the *pair3* mutant. (B) Only a few ZEP1 aggregates are observed in the *pair3* mutant. Bars, 5 μ m.

PAIR3 is required for normal recombination and SC installation

In the wild-type rice, MER3 foci form on the chromosomes at early leptotene, and those foci can be used as a marker to monitor most recombination events in rice (Wang *et al.*, 2009b). To investigate whether normal recombination was also affected in the *pair3-1* mutant, we observed the distribution of MER3 by immunostaining. In the wild type, MER3 appeared as foci from leptotene to zygotene. By contrast, only a few faint MER3 foci were detected on the chromosomes in the *pair3-1* mutant (Figure 5A). This observation is consistent with a severe defect in recombination in *pair3-1* mutants.

Lack of bivalent formation implies that chromosome synapsis was abolished in *pair3-1* meiocytes. To determine whether initial assembly of SCs was affected in *pair3-1* mutants, we performed dual-label immunostaining using antibodies against ZEP1 and REC8 raised in mouse and rabbit, respectively. ZEP1 is a major component of the CE in rice SCs. In the wild-type PMCs, ZEP1 first appeared as punctate foci at early zygotene and then elongated quickly to form short linear signals along the chromosomes. At pachytene, ZEP1 signals were located along the entire chromosomes. In most *pair3-1* PMCs, only a few ZEP1 foci were observed (Figure 5B). However, short/fragmented ZEP1 signals were also found in a small number of nuclei. Together, the findings suggest that PAIR3 is important for proper ZEP1 assembly.

PAIR3 localizes along meiotic chromosomes during prophase I

To investigate the spatial and temporal pattern of PAIR3 in wild-type microsporocytes, a polyclonal antibody against PAIR3 protein was raised in mouse. PAIR3 signals were first seen as foci in preleptotene. Those foci then elongated and formed filamentous structures on the chromosomes at leptotene (Supplemental Figure S3A). During zygotene, pachytene, and diplotene (Supplemental Figure S3, B–D), continuous PAIR3 signals were distributed along the entire length of the chromosomes. PAIR3 signals began to decrease at diakinesis (Supplemental Figure S3, E and F), and no signals were visible in subsequent stages.

To compare the PAIR3 and PAIR2 localization patterns, specific antibodies were used to double label meiotic chromosomes of wild-type PMCs at different stages. During interphase, discrete foci of PAIR2 and PAIR3 were extensively colocalized (Figure 6A). The total number of PAIR2 foci was slightly fewer than that of PAIR3 foci. That is, some PAIR3 foci failed to colocalize with a PAIR2 signal, while all PAIR2 signals also corresponded with PAIR3 staining (Figure 6A). At leptotene, all PAIR2 and PAIR3 stainings showed complete

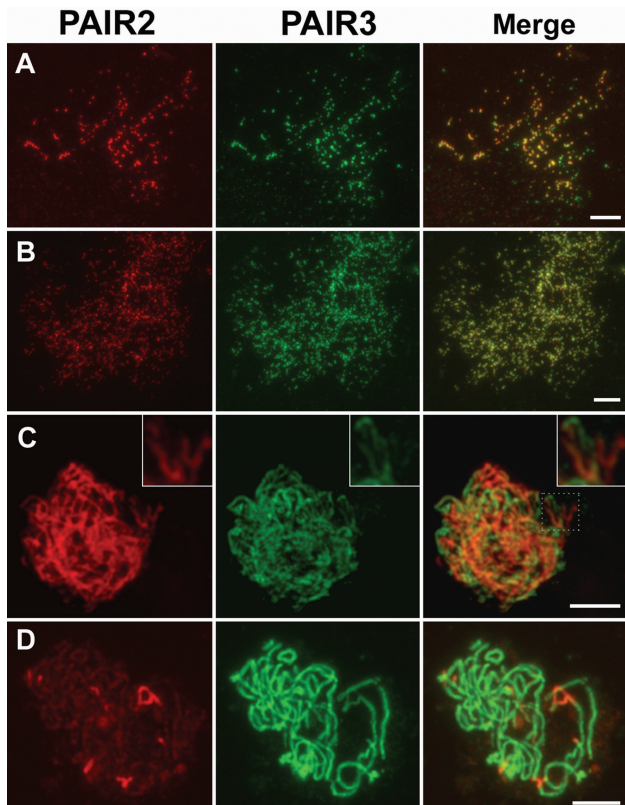


FIGURE 6. Dual immunolocalization of PAIR2 and PAIR3 in the wild-type prophase I. PAIR3 shows high colocalization with PAIR2 during interphase (A) and leptotene (B). (C) Zygotene, the magnified inset shows the complementary staining pattern between PAIR2 and PAIR3 protein. (D) Pachytene. Bars, 5 μm .

colocalization (Figure 6B). During zygotene, with the synapses of homologous chromosomes, PAIR2 proteins began to release from the chromosomes. Interestingly, remnant PAIR2 proteins often showed complementary staining patterns with PAIR3 proteins at this time (Figure 6C). Usually, one protein stains prominently, while the other is faint. Therefore, the merged images often exhibited red or green rather than yellow coloring. During pachytene, only a few PAIR2 fragments were observed, but PAIR3s were still distributed along the entire length of the chromosomes (Figure 6D).

As the localization of PAIR3 was similar to that of REC8, we also conducted dual immunostaining experiments using antibodies against PAIR3 and REC8. The results clearly showed that PAIR3 signals completely overlapped with REC8 signals from leptotene to diplotene (Supplemental Figure S3, A–D). The only difference observed was that the disappearance of PAIR3 was slightly earlier than that of REC8. That is, at diakinesis, REC8 signals were visible in all cells, while PAIR3 signals could be observed in some but not all cells (Supplemental Figure S3, E and F). However, no PAIR3 signals were found in any of the *pair3* PMCs observed, indicating that the mutants identified in this study may carry severe alleles.

PAIR3 localizes normally in *pair2*, *mer3*, and *zep1* mutants

To determine whether PAIR3 localization was affected by meiotic recombination, the distribution of PAIR3 was also investigated in two recombination mutants, *pair2* and *mer3*. In both mutants, PAIR3 localized on chromosomes normally and overlapped well with REC8 (Figure 7, A and B). Thus, PAIR3 localization is not affected by the recombination defects in the *pair2* and *mer3* mutants.

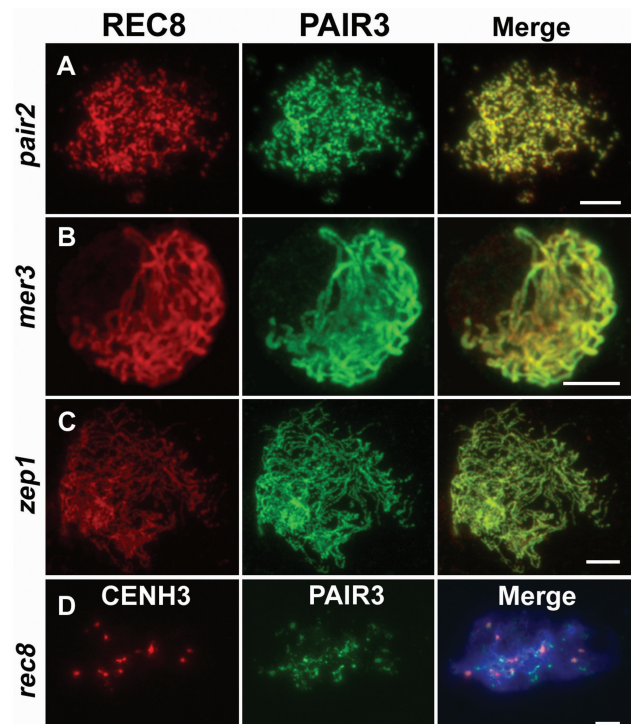


FIGURE 7. The localization of PAIR3 in the *pair2*, *mer3*, *zep1*, and *rec8* mutant. PAIR3 localizes on chromosomes normally in *pair2* (A), *mer3* (B), and *zep1* (C). (D) PAIR3 localization is defective in *rec8* mutant. Chromosomes are stained with DAPI (blue). Bars, 5 μm .

To determine whether the PAIR3 localization depended on the SC, we evaluated *zep1* mutant cells and found that PAIR3 still associated with the chromosome axis in the absence of SC (Figure 7C), indicating that ZEP1 is not required for the normal PAIR3 localization.

PAIR3 localization is defective in *rec8* mutants

In plants, sister chromatid cohesion has been shown to be required for the formation of meiotic chromosome axes. We next investigated whether PAIR3 localization was affected in *rec8* mutants using an anti-CenH3 antibody to identify the PMCs. In the *rec8* mutant, PAIR3 localization was severely disrupted, and only a few discrete PAIR3 signals were observed on the chromosomes (Figure 7D). This result indicated that the structural platform provided by cohesins may be essential for the localization of PAIR3.

DISCUSSION

PAIR3 localizes on chromosome axes during meiosis in rice

AEs play important roles in chromosome pairing and SC assembly. In addition, the recruitment of AEs is essential for chromosome condensation, pairing, and recombination. Although the identification of meiotic chromosome core proteins still remains elusive in plants, three axis-associated proteins have been described so far: ASY1/PAIR2/HOP1, REC8/SYN1/AFD1, and SCC3 (Mercier and Grellon, 2008). ASY1 is first observed as punctate foci in meiotic preleptotene and is maintained during pachytene, suggesting that ASY1 signals in *Arabidopsis* can indicate the structure of both AEs and LEs. PAIR2, the rice homologue of ASY1, also associates with AEs during preleptotene and leptotene. During zygotene, PAIR2 is released from the chromosomes during SC installation, suggesting that PAIR2 signals mainly reflect the structure of AEs, but not LEs (Nonomura *et al.*, 2006). Additionally, normal AEs are observed in *pair2*-null mutant meiocytes, indicating that PAIR2 is not directly

required for AE formation in meiosis (Nonomura *et al.*, 2006). The cohesion proteins REC8 and SCC3 are components of the complex responsible for sister chromatid cohesion (Chelysheva *et al.*, 2005; Golubovskaya *et al.*, 2006; Zhang *et al.*, 2006). Cohesins act as frameworks for the assembly of AEs/LEs, so cohesion signals during prophase I can reflect the location of both these elements. Mutations of cohesin genes always lead to chromosome condensation defects, formation of univalents, and chromosome fragmentation.

Here, the analyses of *pair3* mutants indicated that PAIR3 is essential for bouquet formation, homologous pairing, and normal recombination as well as SC assembly. In addition, abnormal chromosome segregation and cytokinesis, which are universal defects of asynaptic mutants, were also observed in the *pair3* mutants. The immunocytological experiments showed that PAIR3 appeared as foci in preleptotene. Those foci then fused to form short linear signals at leptotene. From zygotene to pachytene, the signals appeared as continuous signals. Dual immunostaining results showed that PAIR3 displayed extensive overlapping staining patterns with REC8 from preleptotene to pachytene, suggesting that PAIR3 localized to the axial cores of both synapsed and unsynapsed chromosomes, corresponding to LE and AE, respectively.

In the *pair3* mutant, the ZEP1 protein failed to assemble along the chromosomes, suggesting that PAIR3 was required for SC assembly. Furthermore, PAIR3 localized normally to the unsynapsed chromosomes in the *zep1* mutant. Therefore, this observation further confirmed that PAIR3 assembles before and independently of synapsis.

PAIR3 is required for PAIR2 localization to chromosomes

In the *pair3* mutant, REC8 associated with chromosomes normally, suggesting that REC8 localization to the chromosome is independent of PAIR3. In the *rec8* mutant, PAIR3 localization was severely disrupted, suggesting that REC8 is required for PAIR3 loading onto chromosomes. Similar dependence was also found in budding yeast, where the loading of Pds5 onto chromosomes requires the function of Rec8, but the association of Rec8 with chromosomes is independent of Pds5 (Zhang *et al.*, 2005b; Jin *et al.*, 2009). Both results are consistent with the fact that REC8 is required for cohesion and for formation of AEs/LEs.

A previous study demonstrated that PAIR2 associated with chromosome axes, and mutation of the *PAIR2* gene caused the elimination of homologue pairing and synapsis and resulted in 24 completely unpaired univalents in the *pair2* mutant during pachytene and diakinesis (Nonomura *et al.*, 2004). In this study, the *pair3* mutant displayed almost the same phenotype as the *pair2* mutant, and it is likely that the PAIR2 and PAIR3 proteins work coordinately in the same pathway or process.

In the *pair2* mutant, PAIR3 localized normally as in the wild type, so PAIR2 is not required for the assembly of PAIR3. In the *pair3* mutant, PAIR2 signals appeared in leptotene as in the wild type, suggesting that PAIR3 is not required for initial recruitment of PAIR2. However, from late leptotene to pachytene, PAIR2 in the *pair3* mutant did not localize onto chromosomes normally and its foci did not extend to form long-stretched signals. These results imply that PAIR3 recruitment to meiotic chromosome axes is a prerequisite for subsequent PAIR2 association. Thus, the loss of normal recombination and synapsis in the *pair3* mutant may be due to the loss of normal anchoring of PAIR2 during meiosis.

Both PAIR2 and PAIR3 localize on the chromosome axes, but their duration is markedly different. Associated with synapsis during zygotene in the wild type, PAIR2 signals are released from the chromosome axes and are hardly detectable at pachytene

(Nonomura *et al.*, 2004). By contrast, PAIR3 signals can still be clearly observed on chromosomes during pachytene. The dependence of PAIR2 localization on PAIR3, as well as the longer persistence of PAIR3 signals on chromosomes, leads us to postulate that PAIR3 might function to provide a platform for PAIR2 proteins. Studies in *Arabidopsis* revealed that ASY1 acts as the interface between the developing chromosome axes and the recombination machinery, where it is required to ensure interhomologue recombination (Sanchez-Moran *et al.*, 2007). Similarly, we propose that PAIR3 may work as the interface between the chromosome axes and PAIR2 protein, which is essential for normal functioning of the recombination machinery.

The dependence between AE-localized proteins has also been found in other species. For example, Red1 is essential for the recruitment of Hop1 to the chromosomes (Smith and Roeder, 1997), and SYCP3 is required for the normal binding of SYCP2 to the chromosomes (Peltari *et al.*, 2001). Meanwhile, HTP-3 is indispensable for the localization of HIM-3 to the chromosomes (Goodyer *et al.*, 2008). In contrast to those AE mutants, however, the *pair3* mutant in our study displayed normal assembly of chromosome axes. In addition, no chromosome condensation defect or chromosome fragmentation was detected. Those results indicated that the PAIR3 protein may not be a structural component of the chromosome axes but may belong to a structure that runs along the axes and associates with them.

Three identified axis-associated proteins in plants, namely, PAIR2/ASY1/HOP1, REC8/AFD1/SYN1, and SCC3, are all evolutionarily conserved among eukaryotes. However, PAIR3 shows little sequence similarity with other known proteins in *Arabidopsis* and other model organisms (Yuan *et al.*, 2009), suggesting that PAIR3 may be a newly evolved meiotic protein in rice. Studies in other organisms have shown that, although the ultrastructural appearance of the SC is highly conserved, many of its components are ill conserved at the amino acid sequence level (de Boer and Heyting, 2006). Therefore, it is more likely that proteins with poor homology at the amino acid sequence level, but with similar structure, perform similar functions in other model organisms.

The PAIR3 protein contains putative coiled-coil motifs in its C-terminal domain. Similarly, the SYCP3/COR1 protein also has a predicted coiled-coil motif in the C-terminal half. In addition, both PAIR3 and SYCP3/COR1 assemble and disassemble in the same time as the SCs and label the AE/LE in a continuous manner. Mutation of either gene results in the defects in pairing, synapsis, and chiasma formation. However, SYCP3 is essential for the formation of LEs, while PAIR3 is not. Several lines of evidence suggest that the AE/LE seen by staining is only a small component of the axial structure by both morphological and functional criteria. In addition, it is likely that axial components may well occur in multiple (partially overlapping) layers (Zickler and Kleckner, 1999). Therefore, it is also possible that PAIR3 is an AE/LE component and localizes on one layer of the AE/LE.

In conclusion, our study strongly supports that the PAIR3 protein is associated with chromosome axes during meiosis and is required for the binding of PAIR2 to the chromosomes. Further investigation of the interaction between those meiotic proteins will promote the understanding of meiotic prophase chromosome cores in plants.

MATERIALS AND METHODS

Materials

The *pair3-1* and *pair3-2* mutants were identified from Wuxiangjing 9 (*japonica*) and Nipponbare (*japonica*), respectively. All plant materials were grown in individual lines in paddy fields. In summer, all

materials were grown in Beijing, while in winter all materials were planted in Hainan Island in southern China.

Molecular cloning of *PAIR3*

To fine map *PAIR3*, InDel markers 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/nucleotide>. The primer sequences of InDel markers were as follows: P1 (5'-TACGTGTCCTTGTGCCTGA-3' and 5'-ACAAATGAACTGAGAACGTG-3'), P2 (5'-CTTCCGATTAATTCCTTTC-3' and 5'-GGGTCTAGCTATTTGCTCT-3'), P3 (5'-TTCTACGAGCTTCCTACG-3' and 5'-CTTAGACTCATTATAGTAC-3'), P4 (5'-CATATAGTGTGCATATGCACC-3' and 5'-GTCGTGTGTTGCCAACAT-3'), P5 (5'-CACTTCAGTGCTGGGTGTGC-3' and 5'-TCAAAGGGCAAGTTAACGAC-3'), P6 (5'-GCGCATCCATGCATATCAA-3' and 5'-GACAAGGTGTTGCCCAAGAA-3'), P7 (5'-GTCAATGTGAATGGGAGACC-3' and 5'-AGCAGCTCAAGGCAGCGA-3'), and P8 (5'-TTTCAGTGGCTCTGTTCCG-3' and 5'-CAAAGGAAATGAGTGAGGCT-3').

Construction of RNAi and rice transformation

A 472-bp fragment of *PAIR3* was amplified by PCR with primers P31F (5'-ACTCGAGATAATACGCCAGCACCT-3') and P31R (5'-CAGATCTGTACGTCCTGGTCAATAC-3'); this fragment was cloned into the pGEM-T vector (Promega, Madison, WI) and sequentially cloned into the *Bam*HI/*Sall* and *Bgl*II/*Xho*I sites of the pUC-RNAi vector. Subsequently, the stem loop fragment was cloned into the pCAMBIA2300-Actin vector. The resulting RNAi construct was transformed into an *A. tumefaciens* strain and then into mature embryo-derived callus of rice.

Antibody production

The antibodies against REC8, *PAIR2*, *MER3*, and *ZEP1* were produced as described (Wang *et al.*, 2009b, 2010). To generate the antibody against *PAIR3*, a 561-bp fragment of *PAIR3* cDNA (amino acids 248 to 393) was amplified with primers P3GSTF (5'-GTGAATCCCAGACCTATTACTCGTTCGCT-3') and P3GSTR (5'-AGTCTC-GAGTCATCGTGTCCACCCTCTCT-3'). The fragments were digested with *Eco*RI/*Xho*I and ligated into the expression vector pGEX-4T-2. The expression vector was transformed into BL21 (DE3) and was induced by the addition of 0.3 mM isopropyl β -D-1-thiogalactopyranoside to the culture medium. The *PAIR3*-fusion peptides, mainly expressed in the soluble fraction, were purified using the MagneGST Protein Purification System (Promega). The specificity of *PAIR3* antibody was checked by immunofluorescence.

Meiotic chromosome preparation

Young panicles of both wild type and *pair3* 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 micro-CCD camera. For silver staining, the slides were stained under coverslips in a moist chamber at 60° with a 2:1 (vol/vol) mixture of 50% aqueous silver nitrate to developer (2 g gelatin, 1 ml formic acid, 100 ml distilled water). The slides were washed off under running tap water, rinsed twice in distilled water, air dried, and photographed using an Olympus BX61 microscope. The chiasma

frequency was quantified as described (Sanchez Moran *et al.*, 2001; Wang *et al.*, 2009b).

Fluorescence in situ hybridization

FISH analysis was conducted as described by Zhang *et al.* (2005a). Two repetitive DNA elements were used as the FISH probes: pTa794 has 5S ribosomal RNA genes from wheat (Cuadrado and Jouve, 1994), and pAtT4 contains telomeric repeats. Digoxigenin-11-dUTP-labeled probes were detected using an anti-digoxigenin conjugated with rhodamine antibody (Roche Diagnostics, Indianapolis, IN). Chromosomes were counterstained with DAPI in Vectashield antifading solution (Vector Laboratories). Original images were observed under Olympus BX61 fluorescence microscopy and captured with a DVC1412 CCD camera using IPLab software. The final images were merged using Adobe Photoshop CS2 software.

Immunofluorescence

Fresh young panicles were fixed in 4% (wt/vol) paraformaldehyde for 30 min at room temperature. Anthers in the proper stage were squashed on a slide with phosphate-buffered saline (PBS) solution and covered with a coverslip. After the slide was soaked in liquid nitrogen and the coverslip removed, the slide was dehydrated through an ethanol series (70%, 90%, and 100%) before being used in immunostaining. Slides were then incubated in a humid chamber at 37° for 4 h 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).

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