The Novel Gene **HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS** of Rice Encodes a Putative Coiled-Coil Protein Required for Homologous Chromosome Pairing in Meiosis

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We have identified and characterized a novel gene, PAIR1 (HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS1), required for homologous chromosome pairing and cytokinesis in male and female meiocytes of rice (*Oryza sativa*). The pair1 mutation, tagged by the endogenous retrotransposon Tos17, exhibited meiosis-specific defects and resulted in complete sterility in male and female gametes. The PAIR1 gene encodes a 492-amino acid protein, which contains putative coiled-coil motifs in the middle, two basic regions at both termini, and a potential nuclear localization signal at the C terminus. Expression of the PAIR1 gene was detected in the early stages of flower development, in which the majority of the sporocytes had not entered meiosis. During prophase I of the pair1 meiocyte, all the chromosomes became entangled to form a compact sphere adhered to a nucleolus, and homologous pairing failed. At anaphase I and telophase I, chromosome nondisjunction and degenerated spindle formation resulted in multiple uneven spore production. However, chromosomal fragmentation frequent in plant meiotic mutants was never observed in all of the pair1 meiocytes. These observations clarify that the PAIR1 protein plays an essential role in establishment of homologous chromosome pairing in rice meiosis.

**INTRODUCTION**

Sexual reproduction is required for generating gametes containing a haploid chromosome complement from diploid somatic cells. Meiosis is a crucial event in this process, in which two successive rounds of chromosome segregation follow a single round of DNA replication. Meiosis has evolved to achieve two contrary purposes: the maintenance of genome stability and the creation of genetic diversity. To achieve these two purposes, the most important processes are homologous chromosome pairing and recombination.

Chromosome pairing becomes evident by the close parallel association of homologous pairs during meiotic prophase, which is divided into several substages according to the state of synapsis and condensation. Homologous chromosomes have begun to condense at leptotene and to become partially synapsed at zygotene, fully synapsed at pachytene, and desynapsed but held together by chiasmata at diplotene and diakinesis. Synapsis guarantees a strong and close connection of the homologous pairs facilitated by a network of longitudinal and transversal protein fibers called the synaptonemal complex (SC; Moens, 1969; Westergaard and von Wettstein, 1972; Gillies, 1975), whereas the SC function on early prophase has not yet been elucidated.

On the other hand, presynaptic alignment of homologous chromosomes is believed to be important in facilitating a homology search before SC formation in many organisms (Zickler and Kleckner, 1998). Recent analyses that used the fluorescent in situ hybridization (FISH) technique clearly revealed the meiosis-specific clustering of telomeres in the nucleus (the so-called bouquet formation), in some cases of the centromeres as well, contributes to presynaptic alignment (Chikashige et al., 1994; Scherthan et al., 1996; Bass et al., 1997; Martinez-Perez et al., 1999; Armstrong et al., 2001). In addition, chromosome aggregation adhered to the nucleolus (the so-called synizetic knot) is believed to occur as part of the homology search in early meiotic prophase (Loidl, 1988). To elucidate the relationship between presynaptic chromosome conformation and homologous pairing, genetic analyses using meiotic mutants are necessary. In *Schizosaccharomyces pombe*, loss of a telomere binding protein TAZ1 (Cooper et al., 1998; Nimmo et al., 1998) and a spindle pole body (SPB)–associated protein KMS1 (Shimanuki et al., 1997; Niwa et al., 2000) impair telomere clustering and decrease the frequency of meiotic recombination.

In plants, *PAM1* of maize (*Zea mays*) is reported that obviously affects bouquet formation (Golubkovskaya et al., 2002). In
addition, several interesting genes and mutations affecting homology search have been identified in plant meiosis. The \textit{phs1} mutation of maize causes nonhomologous synapsis, delay of DNA double-strand break (DSB) repair, and dramatically reduced RAD51 foci on meiotic chromosomes, although it has little effect on bouquet formation (Pawlowski et al., 2004). The AHP2 of Arabidopsis \textit{(Arabidopsis thaliana)} is homologous to \textit{Saccharomyces cerevisiae} \textit{HOP2} (Leu et al., 1998) and \textit{S. pombe} MEU13 (Nabeshima et al., 2001), which are known to act in monitoring homology between pairing partner at meiosis, and its mutation causes some early meiotic aberrations, such as chromosome fragmentation and unbalanced chromosome segregation (Schommer et al., 2003). The \textit{SYN1/DIF1} (Peirson et al., 1997; Bai et al., 1999) and \textit{SWI1} (Mercier et al., 2001, 2003) of Arabidopsis are required for precise reductive division. The loss-of-function of both genes resulted in precocious separation of sister chromatid cohesion and chromosome fragmentation during early meiosis. However, genetic and biochemical interaction among these genes is still unclear. In addition, the \textit{PHS1} and \textit{SWI1} encode unknown proteins, suggesting that identification of key players in early plant meiosis is not completed yet.

Initiation and repair of DSBs are also known to affect presynaptic chromosome alignment (Loidl et al., 1994; Weiner and Kleckner, 1994), and in some species they influence recombination and SC formation as well (Giroux et al., 1989; Celerin et al., 2000; Grelon et al., 2001). In \textit{S. cerevisiae}, meiotic recombination is initiated by DSBs (Sun et al., 1989; Cao et al., 1990) that are catalyzed by \textit{SPO11}, a type II topoisomerase (topoisomerase VI, subunit A; Bergerat et al., 1997). Although meiotic DSB formation has only been proven experimentally in yeast, this process is considered to be extensively conserved in mammals and plants (Grelon et al., 2001). In the \textit{spo11} mutant of Arabidopsis, stages typical of pachytene and SC formation are seldom observed (Grelon et al., 2001), thereby indicating the relationship between presynaptic events and DSB initiation also exists in the plant kingdom. However, recent analysis using \textit{mei1/ spo11} double mutants revealed a \textit{SPO11}-independent initiation/repair pathway of DSBs in meiosis of Arabidopsis (Grelon et al., 2003). To build a comprehensive understanding of the molecular mechanism promoting early meiosis in plants, further studies using meiotic mutants are necessary.

A number of spontaneous or induced synopsis mutants have been reported in rice (\textit{Oryza sativa}; Kitada and Omura, 1983; Kitada et al., 1983; Nonomura et al., 2004). However, the \textit{PAIR2} gene, which is the ortholog of \textit{S. cerevisiae} \textit{HOP1} (Hollingsworth et al., 1990) and Arabidopsis \textit{ASY1} (Caryl et al., 2000; Armstrong et al., 2002), is the only reported case of cloning rice meiotic genes (Nonomura et al., 2004). Herein, we report the second case of the isolation and characterization of a novel meiotic gene \textit{PAIR1 (HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS1)}, encoding a putative coiled-coil protein in \textit{O. sativa} ssp \textit{japonica} \textit{(2n = 24)}. A targeted \textit{pair1} mutation by the retrotransposon \textit{Tos17} exhibited complete male and female sterility and a complex phenotype during rice meiosis I, in which an unusual entangled configuration of chromosomes at early prophase resulted in a defect of bivalent formation. Spindle formation and sporulation were also affected. Such a broad phenotype from early to late stages of meiosis is usual in plant synaptic mutants. Here, we clarify that the \textit{PAIR1} protein of rice plays an essential role in establishment of homologous chromosome pairing in early meiotic prophase.

RESULTS

Isolation of the \textit{pair1} Mutant

We screened meiotic mutants from \textit{Tos17} insertion lines induced by somatic culture and regeneration (Hirochika et al., 1996; Yamazaki et al., 2001). In the ND0016 of 600 sterile lines, complete male- and female-sterile plants segregated but with normal plant morphology (Figure 1A). The sterile phenotype segregated as a single recessive mutation (fertile:sterile = 241:76, \(x^2 = 0.178\) for 3:1). The mutant showed complete pollen sterility (Figure 1C) and no obvious construction of female spores in the ovule (Figure 1G), in contrast with normal spores in wild-type siblings (Figures 1B and 1F). Pollination of the mutant flowers with wild-type pollen did not result in seed production (data not shown), thus supporting the notion that the female gamete was not functional in the mutant. No noticeable abnormalities were observed in the morphology of anther walls and ovules of the homozygous \textit{pair1} mutant (Figures 1D to 1G). These results clearly indicate that the \textit{pair1} mutation only affected male and female sporogenesis.

\textit{Tos17} Insertion Caused \textit{pair1} Mutant Phenotype

Segregants \((n = 317)\) of the third generation of regenerated plants (R3) of the ND0016 line were screened for the presence of a \textit{Tos17} insertion tag of the \textit{PAIR1} gene. Approximately 10 copies of transposed \textit{Tos17} were detected in those plants, whereas the original Nipponbare cultivar contained two \textit{Tos17} copies (data not shown). Of these transposed copies, a 4.5-kb Xbal fragment was completely linked to the \textit{pair1} sterile phenotype. A genomic 2.0-kb fragment flanking the \textit{Tos17} copy was cloned from the 4.5-kb fragment, and the same blot was reprobed with this fragment. This probe led to the detection of two allelic bands of 6.5 kb and 4.5 kb (part of this result is shown in Figure 2A); the former was linked to the wild-type phenotype, whereas the homozygous latter bands were linked to the sterile phenotype. The sterile phenotype was reproduced in the next generation of fertile R3 plants carrying heterozygous bands (H in Figure 2A). Thus, we concluded that segregation mode of tagged site was completely consistent with that of sterile phenotype in ND0016. An identical sequence to the tagged site was included in the bacterial artificial chromosome clone OSJNBA0009C08, which mapped to the short-arm end of chromosome 3 (0.0-centimorgan position; http://rgp.dna.affrc.go.jp/).

We were unsuccessful in our attempt to isolate the cDNA clone against the tagged sequence, despite screening >70,000 clones derived from young panicles. On the other hand, three independent trials of rapid amplification of 5'-cDNA ends (5'-RACE) and 3'-RACE reactions (see Methods) successfully amplified a 1.7-kb cDNA. In all 5'-RACE trials, the cDNA extension stopped at the same position (underlined in Figure 2B). A TATA box,
a potential binding site of transcription factors (White and Jackson, 1992), was found 37 bp upstream of the 5'-RACE end (Figure 2B). In addition, a translation initiation codon of the gene was predicted 100 bp downstream of the 5'-end by the RiceGAAS gene annotation software (http://ricegaas.dna.affrc.go.jp/). From these results, we concluded that the isolated cDNAs would cover the entire mRNA of the tagged gene.

Figure 1. Defects of the pair1 Mutation Observed Only in Male and Female Sporocytes.

(A) The plant morphology of the homozygous pair1-1 mutant (−/−) was normal, whereas complete sterility resulted in erected panicles and prolonged greening of leaves compared with the heterozygous wild-type sibling (+/−).
(B) Fertile pollens in a heterozygous sibling, stained by iodine potassium iodide solution.
(C) Completely sterile pollen in the homozygous pair1-1 mutant.
(D) and (E) In anther wall development, no discernible difference was observed between the wild type (D) and the pair1-1 mutant (E), respectively. Ep, epidermis; En, endothecium; Mi, middle layer; Ta, tapetum cells; PMC, pollen mother cell. Bar = 20 μm.
(F) and (G) In the wild-type ovule, three of four female spores were degenerated (arrows and bottom left illustration, smaller arrows for degenerated spores; [F]), whereas no obvious structure of spores was observed in the mutant ovules (G). Bar = 20 μm.

To confirm that the Tos17 insertion in this locus resulted in the mutant phenotype, a 8.2-kb XhoI-BamHI fragment including the entire coding region and a 3.4-kb upstream sequence (Figure 2C)
PAIR1 Encoded a Novel Unknown Protein

The PAIR1 gene encoded a peptide sequence of 492 predicted amino acids with a molecular mass of 54 kD (Figure 3A). A database search revealed significant similarity only to the hypothetical protein T1N6.6 (832 amino acids) of Arabidopsis, which is 32.6% identical to PAIR1 in a 507–amino acid overlap (data not shown). The fact that few proteins share the similarity with PAIR1 suggests that the peptide sequence of PAIR1 might be less conserved among organisms.

α-Helical coiled-coil motifs containing 13 heptad repeats were predicted in the middle of the peptide (166 to 207 amino acids [cc1], 229 to 249 amino acids [cc2], and 274 to 301 amino acids [cc3]) by the online network protein sequence analysis (Combet et al., 2000). Three clusters of heptad repeats were rich in hydrophobic residues at the first and fourth heptad (highlighted in Figure 3A). Generally, the first and fourth hydrophobic residues of the heptad are located on the same side of the α-helix and contribute to protein dimerization (Landschulz et al., 1988; Moitra et al., 1997). In the cc1 sequence, a helix-turn-helix structure was also predicted according to Chou and Fasman (1978) (dotted in Figure 3A). All three alleles contained Tos17 insertions in the coiled-coil region (arrows in Figure 3A). The pl value was estimated at 10.32 for the whole PAIR1 peptide, and three basic regions with a higher pl value than the average were predicted (0 to 60 amino acids, 330 to 430 amino acids, and 460 to 492 amino acids; Figure 3A). The basic region at the C terminus contained a KRRRR peptide alignment (Figure 3A), which was predicted as a potential nuclear localization signal (Chelsky et al., 1989). A transient assay by bombardment of a 3SS-GFP (green fluorescent protein)-PAIR1 fusion construct into Allium cepa (onion) epidermal cells revealed that the fusion protein was localized in the nucleus in contrast with 3SS-GFP alone (Figure 3B), suggesting that PAIR1 also acts in the nucleus of the rice meiocytes. The N terminus including a basic region was rich in S/T-P-X-X or S/T-S/T-X-X motifs, characteristic of DNA binding proteins (Suzuki, 1989), are boxed.

Figure 3. Primary and Secondary Structure Prediction of PAIR1.

(A) The vertical arrows indicate the position of Tos17 insertion in the respective three alleles. The α-helical regions were predicted according to Chou and Fasman (1978). The pl values of 20 amino acids were estimated in each 10–amino acid interval along the PAIR1 peptide and plotted on the graph. The coiled-coil structure with three heptad clusters (cc1, cc2, and cc3), separated by two nonhelical sequences, was predicted at the middle of the peptide (shaded bars and wavy lines). The coiled-coil regions contained 13 heptad repeats in total, whose first and fourth residues were frequently hydrophobic (highlighted). The cc1 sequence was predicted to make a helix-turn-helix structure (turned at the residues with dots). Three basic amino acid clusters were detected (hatched bars and underline). The C-terminal basic region including a KRRRR sequence was predicted to act as a nuclear localization signal (NLS; closed box and double underline). The S/T-P-X-X or S/T-S/T-X-X motifs, characteristic of DNA binding proteins (Suzuki, 1989), are boxed. (B) Nuclear localization of GFP-PAIR1 fusion protein during transient expression in Allium cepa epidermal cells.
Aberrant Kinetics of Homologous Chromosomes in *pair1* Meiocytes

The chromosome behavior in the meiocytes of wild-type siblings and *pair1-1* mutants was characterized. In the wild type, the 24 chromosomes appeared as very thin threads at leptotene (Figure 4A) and underwent synapsis at zygotene (Figure 4B). During zygotene, chromosomes formed a synizetic knot (Figure 4B), followed by a relaxed formation of fully synapsed bivalents in pachytene (Figure 4C). After synapsis was relieved at diplotene (Figure 4D), 12 bivalents became tightly condensed at diakinesis (Figure 4E) and arranged at the metaphase plate subsequent to nuclear envelope breakdown (Figure 4F). Reductive division of bivalents occurred at anaphase I (Figure 4G), and subsequent equational division produced tetrads (Figure 4H).

In the *pair1-1* mutant, chromosome configuration appeared normal at leptotene (Figure 4J), and the first visible abnormality was detected at zygotene. Throughout this stage, very thin threads aggregated around the nucleolus in the mutant (Figure 4K). The normal appearance of thick threads was not observed at pachytene and diplotene (Figures 4L and 4M). In addition, the chromosome configuration with a synizetic knot was maintained up to late diplotene or early diakinesis (Figure 4M), whereas the knot was released at pachytene in the wild type (Figure 4C). There were 24 completely unpaired univalents observed at diakinesis (Figure 4N). Even after synizetic configuration eventually diffused, we observed several univalents connected by a chromatin bridge (arrowheads in Figure 4N). Despite a complete absence of homologous pairing, all the univalents frequently arranged at the metaphase plate (Figure 4O). The univalents were divided unequally at anaphase I, and several lagging univalents were observed (Figure 4P). Though considerable synchronous segregation of sister chromatids was observed at anaphase II (Figure 4Q), several chromatids often moved independently (Figure 4Q, arrowheads). Throughout meioses I and II, precocious sister chromatid separation and

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**Figure 4.** Homologous Chromosome Pairing Is Defective in Male and Female Meiocytes of the Wild Type and *pair1-1* Mutant.

(A) to (H) Chromosomal spreads from wild-type male meiocytes in various stages: leptotene (A), zygotene (B), pachytene (C), diplotene (D), diakinesis (E), metaphase I (F), metaphase II (G), and anaphase II (H).

(I) Homologous chromosomes at diakinesis in wild-type female meiocytes.

(J) to (Q) Chromosomal spreads from *pair1-1* male meiocytes in various stages. No noticeable aberration was observed at leptotene (J) and synizetic zygotene (K). However, synizetic conformation still remained at pachytene and diplotene in the mutant (L) and (M). At late diakinesis, 24 complete univalents were observed in the mutant (N), in which several univalents were frequently connected by a thin-chromatin thread (arrowheads). Though all univalents aligned on the division plane (O), nondisjunction and delayed univalents (arrows) were observed in the mutant (P). Most of the chromatids were synchronously divided to the opposite poles, whereas several chromatids independently moved (arrowheads; Q).

(R) Approximately 24 univalents were observed in the mutant female meiocytes at diakinesis. Bar = 20 μm.
detectable fragmentation of chromosomes were not observed in pair1-1 meiocytes (Figure 4P). In female meiocytes of pair1-1, unpaired univalents were also observed at diakinesis (Figure 4R).

Ratios of characteristic meiocytes in 0.6- to 0.9-mm anthers were compared between the wild type and the mutant (Figure 5). In the wild type, 80% of the meiocytes entered into zygotene (Figure 5, light blue bars) in 0.6-mm anthers and completely shifted to pachytene or later stages in 0.9-mm anthers. Of the 86 mutant meiocytes observed, however, zygotene cells with synizetic configuration were constantly observed, even in 0.9-mm anthers. A normal appearance of pachytene and diplotene was seldom observed. The FISH foci of the 25S rDNA locus were counted on male meiocytes with 0.6- to 0.8-mm anthers to assess homologous chromosome pairing at early prophase (Figure 6). In the wild type, separated foci at leptotene (1.55 foci averaged) or zygotene (1.30 foci) converged into a single focus (1.21 foci) at pachytene, whereas foci in the mutant did not converge (1.52 foci, even in 0.8-mm anther), indicating synopsis was completely defective in the mutant (Table 2). Thus, we concluded that the crucial events for meiosis, such as the timing of the release the synizetic knot and subsequent homolog synopsis at zygotene and pachytene, were defective both in male and female meiocytes of the pair1 mutant.

Abnormal Spore Formation in the pair1 Mutant

The pair1 mutation was abnormal not only in chromosome division but also in cytokinesis of meiocytes. In the male meiocytes at dyad stage, all of the wild-type dyads shared an equal volume of nuclei and cell mass (Figure 7A). By contrast, 46.7% of the pair1-1 dyads shared an unequal nuclei and/or cell mass (Figures 7B and 7C). In addition to various sizes of nuclei, multiple micronuclei were frequently observed. Unusual triad formation was observed in 8.3% of the pair1-1 dyads (Figure 7D).

At tetrad stage, 70% of the spore sets of the pair1-1 mutant showed abnormal morphology (Table 1). Only 1.9% of the tetrads observed had wild-type morphology. Most of the mutant tetrads exhibited various sizes of nuclei and/or cell mass, such as unequal nuclei distribution with multiple micronuclei (Figure 7F) and spores without a nucleus (Figure 7G). Polyads, tetrads with more than four spores, were also observed in 8.1% of the mutant spore sets (Figures 7H and 7I; Table 1). These results indicate that the loss-of-function mutation in PAIR1 gene also affects correct chromosome disjunction and cytokinesis during sporulation.

Abnormal Spindle Formation at Meiosis I in pair1 Mutants

Spindle morphogenesis was observed at meioses I and II by indirect immunofluorescent staining using anti-a-tubulin antibody. The negative control without the primary antibody gave no signal (data not shown). In the wild type, the network of cytoplasmic microtubules appeared to surround the nuclear envelope at diakinesis (Figure 8A). After nuclear envelope breakdown, a bipolar short array of microtubules associated to bivalents arranged at the metaphase plate (Figure 8B) and formed an obvious structure of bipolar spindles at metaphase I (Figure 8C). Each set of 12 univalents synchronously segregated toward opposite poles by the anaphase I spindles (Figure 8D). At late telophase I, the phragmoplast formed between the daughter pronuclei (Figure 8E). The phragmoplast is a cytoskeletal structure held by two arrays of microtubule bundles and is required for determining the position of the cytokinetic plane (Verma, 2001). As the cell plate expanded, the phragmoplast depolymerized in the center and repolymerized along the edge of the growing cell plate (Figure 8F). In meiosis II, the shape and density of the spindles were almost the same as that of meiosis I (Figures 8G and 8H).

Apparent abnormalities were not observed at diakinesis (Figure 8I) and early metaphase I (Figure 8J). The first noticeable abnormality appeared at metaphase I in the pair1-1 mutant. In all of the 10 meiocytes observed, the spindles at metaphase I and
PAIR1-1

Genotype Dyad (%) Triad (%) Normal Aberrant Tetrap "Pentayad (%) Hexayad (%)

+/- 1 (9.9) 0 109 (98.2) 1 (0.9) 0 0

-/- 47 (12.7) 36 (9.7) 7 (1.9) 262 (70.6) 16 (4.3) 3 (0.8)

*Contains an uneven volume of cytoplasm, various sizes of nuclei, and/or several micronuclei.

DISCUSSION

We identified a rice pair1 mutant with complete male and female sterility that exhibited a complex phenotype during meiosis I. The mutant phenotype was limited to germ cells (Figure 1), and the PAIR1 gene was expressed mainly at the premeiotic or early meiotic stages (Figure 2D). The mutant phenotype was first apparent at zygote, the stage of synizetic knot formation (Figures 4J and 5). In the mutant meiocytes, no pairing of 25S rDNA foci was detected through zygote to pachytene (Figure 6; Table 2). The rDNA locus in japonica rice is close to the short-arm end of chromosome 9. Because synopsis is generally initiated subtelomERICally in many organisms (von Wettstein et al., 1984), unpairing of rDNA foci may represent asynapsis of whole extent of homologous chromosomes. Thus, we conclude that the normal PAIR1 product has an essential role in establishment of homologous chromosome pairing in early meiosis.

We previously identified and characterized the mutation in rice PAIR2 gene (Nonomura et al., 2004), the ortholog of Arabidopsis ASY1 that is an important factor for synopsis and functions by localizing along lateral elements of the SC (Caryl et al., 2000; Armstrong et al., 2002). The pair2 mutant, as well as the pair1, completely lacked chromosome pairing in early meiosis. However, no tangled chromosomes appeared in the pair2, indicating that tangled chromosomes observed in the pair1 mutant are not simply the result of the failure of SC formation. Rather, premeiotic expression suggests that the PAIR1 is involved in or before presynaptic homolog alignment.

The newly identified PAIR1 gene encoded a putative nuclear protein of 492 amino acids containing an α-helical coiled-coil structure (Figure 3). The S/T-P-X-X or S/T-S/T-X-X motifs, which are present in many DNA binding proteins (Suzuki, 1989), were abundant in the N terminus of PAIR1. In addition, a nonhelical basic region was also identical at the N terminus. A basic amino acid cluster juxtaposed with Leu-zipper motifs is called a bZIP domain and is often shared in transcription factors binding to sequence-specific DNA (Fassler et al., 2002). Thus, we speculate that PAIR1 forms a coiled-coil dimer and acts on the chromosomes during meiosis I. Indeed, the SMC (structure maintenance of chromosomes) family of proteins contains a coiled-coil structure, and their heterodimers function directly in sister chromatid cohesion or in the condensation pathway during mitosis and meiosis (Hirano, 2000). In addition, SC is known to be composed of many coiled-coil proteins (Meuwissen et al., 1992; Offenberg et al., 1998; Tung and Roeder, 1998; Yuan et al., 1998).
Figure 8. Immunostaining of Spindles in Wild-Type and pair1-1 Male Meiocytes.

(A) to (H) Male meiocytes of the wild type.

(I) to (T) Male meiocytes of pair1-1 mutant.

(A) Tubulin fibers were present throughout the cytoplasm at diakinesis. Chromosomes were stained by PI (red), and microtubules were immunologically stained by Alexa488 (green).

(B) Bipolar-oriented microtubules had developed at early metaphase I.

(C) Mature spindles were formed at metaphase I.

(D) Bivalents were separated by the spindle into two sets of 12 univalents at anaphase I.

(E) The phragmoplast had begun to form between daughter pronuclei at telophase I, in which diminished staining in the midzone indicated the cytokinetic plane (arrow).

(F) A division plate with a phragmoplast ring had grown to separate the daughter cells.

(G) Two sets of univalents aligned at metaphase-II plates.

(H) Two sets of univalents were separated to the opposite poles by spindles.

(I) and (J) No obvious abnormality was observed in the microtubule array in pair1-1 mutant at diakinesis (I) and early metaphase I (J).

(K) The mutant spindle is composed of wavy, thin bundles of microtubules.

(L) Several delayed univalents (arrowheads) were always observed at anaphase I.

(M) Extra microtubule fibers (arrows) extended to a different direction from the poleward axis at telophase I.

(N) The ectopic extension of microtubule fibers (arrow) also appeared at late telophase I.

(O) Several micronuclei (arrowheads) were observed in the mutant dyad.

(P) Daughter nuclei were enclosed by the microtubule corona, in which micronuclei were clearly detected outside the corona at the dyad stage.

(Q) to (T) During meiosis II, the spindles exhibited normal appearance but not around the micronuclei (arrowheads). Bar = 10 μm.
However, some coiled-coil members are known to act on the SPB (I kemoto et al., 2000) or microtubule fibers (Yamashita et al., 1997) in yeast meiosis. An antibody against PAIR1 protein, which is now under construction, would permit clarification of its localization in rice meiocytes.

Recently, the PHS1 gene has been known to act in early meiosis of maize (Pawlowski et al., 2004). The phs1 mutation exhibits interesting meiotic defects of nonhomologous synapsis, delay of DSB repair, and dramatically reduced RAD51 foci. These defects result in tangled chromosomes compacted into a small sphere in pachytene, unpaired univalents in diakinesis, and abnormal polyads, closely resembling the pair1 phenotype. The PHS1 is also conserved in rice genome (Pawlowski et al., 2004). Although the status of DSB repair and RAD51 recombination machinery has not yet been examined in this study, phenotypic similarity between maize and rice mutants suggests the relation of both meiotic proteins.

The pair1-like defects in early meiosis, such as tangled chromosomes and homolog unpairing, were also observed in syn1/dif1 (Peirson et al., 1997; Bai et al., 1999) and ahp2 mutants (Schommer et al., 2003) of Arabidopsis. The SYN1 is a homolog of a meiotic cohesin REC8 in S. pombe and is required for sister chromatid cohesion during meiosis I (Cai et al., 2003). AHP2 is a homolog of S. cerevisiae HOP2 (Leu et al., 1998) and S. pombe MEU13 (Nabeshima et al., 2001) and is proposed to function in monitoring sequence homology to either promote homolog pairing or destabilize the pairing between nonhomologs. However, a significant difference lay between these Arabidopsis mutants (syn1 and ahp2) and monocot mutants (phs1 and pair1).

In the Arabidopsis mutants, fragmented chromosomes were detected from diakinesis to anaphase I (Bai et al., 1999; Schommer et al., 2003) but never in both monocot mutants. In this study, a complete set of 24 univalents was clearly visible in all meiocytes at anaphase I (Figure 4P). Chromosome fragmentation is thought to result from the accumulation of unrepaired DSBs of DNA (Schommer et al., 2003). These facts lead to a possibility that the PAIR1 promotes homolog pairing independent of DSB initiation/repair pathway. Indeed, in the maize pbs1 mutant, the DSBs are initiated and repaired, even though delayed (Pawlowski et al., 2004).

On the other hand, the At-spo11 mutant also exhibits pair1-like phenotype in early meiosis. This indicates that the homolog pairing should be coupled with DSB formation. Thus, as another possibility, we favor that the PAIR1 might act in presynaptic homolog alignment before loading DSB initiation machinery on meiotic chromosomes, although it is possible that the PAIR1 is a component of SC or involved in DSB initiation step.

The pair1 mutation also affected spindle and phragmoplast formation at anaphase I and telophase I (Figures 8M and 8N). There was further indirect evidence in support of this observation by the appearance of polyads and several micronuclei at dyad and tetrad stages (Figures 7 and 8P). Abnormal spindle formation has also been reported in the maize desynaptic mutants dys1 and dys2 (Chan and Cande, 1998). Although there are few reports of striking aberrations in spindle formation, most of the Arabidopsis meiotic mutants described so far produce polyads; the spo11 and mei1 in DSB initiation (Grelon et al., 2001, 2003), the dmc1 in recombination steps (Couteau et al., 1999), the syn1/dif1 in sister chromatid cohesion (Bai et al., 1999; Bhatt et al., 1999), the asy1 in chromosome synapsis (Caryl et al., 2000), and ma5 in chromosome division (Glover et al., 1998). Abnormal formation of nonbipolar spindles or polyads in those synaptic mutants assumes the involvement of microtubule organization in early meiotic events, such as homolog alignment, synapsis, and recombination, whereas the abnormal phenotype may simply be the result of unpaired and missegregated univalents.

Interestingly, in many organisms, treatment of meiotic tissues with microtubule depolymerizing agents inhibited cells in spindle formation and in homologous chromosome pairing (Shepard et al., 1974; Loidl, 1988). Induction of chromosome intertwining was also observed in colchicine-treated Triticum aestivum (wheat) meiocytes (Thomas and Kaltksikes, 1977). At meiotic prophase of S. pombe, it is known that all telomeres cluster near the SPB moving along an oscillatory path, and this is probably for efficient chromosome pairing (Chikashige et al., 1994; Hiraoka, 1998; Niwa et al., 2000). This movement is principally led by the pull of microtubules that connect the SPB to microtubule-anchoring sites on the cell cortex (Yamamoto and Hiraoka, 2001). Although the yeast cell system is critically different from plant cells, these findings strongly suggest that microtubule organization is important for early meiotic events common in eukaryotes. Analysis of PAIR1 function will contribute toward elucidating the relationship between meiotic chromosome behavior and microtubule organization in plant cells.

### Table 2. Distribution of the FISH Foci for the 25S rDNAs in Male Meiocytes

<table>
<thead>
<tr>
<th>PAIR1-1 Genotype</th>
<th>Anther Length (mm)</th>
<th>No. of Meiocytes Observed*</th>
<th>Rate of Pachytene</th>
<th>Average No. of Foci</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/−</td>
<td>0.6</td>
<td>143</td>
<td>4.1%</td>
<td>1.55</td>
<td>0.055</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>272</td>
<td>45.2%</td>
<td>1.30</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>255</td>
<td>64.9%</td>
<td>1.21</td>
<td>0.040</td>
</tr>
<tr>
<td>−/−</td>
<td>0.6</td>
<td>153</td>
<td>ND†</td>
<td>1.49</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>141</td>
<td>ND</td>
<td>1.46</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>110</td>
<td>ND</td>
<td>1.52</td>
<td>0.060</td>
</tr>
</tbody>
</table>

*Only the meiocytes at leptotene, zygotene, and pachytene were examined.
†ND indicates not determined because no typical pachytene is observed in the mutant.
METHODS

Plant Materials

Callus derived from the *japonica* cultivar Nipponbare was employed for regeneration after 5 months of suspension culture, as described by Hirochika et al. (1996). From a total of 27,998 regenerated plants, reduction of seed fertility was observed in 4032 lines, both in the R1 generation and its segregant progenies (R2 or R3 generation). From those sterile lines, 600 lines segregating sterile plants in the R2 were randomly selected for meiotic chromosome observation. Crossing of the wild-type sterile lines, 600 lines segregating sterile plants in the R2 were randomly generation and its segregant progenies (R2 or R3 generation). From those regeneration after 5 months of suspension culture, as described by

Young (30- to 40-mm) panicles using the RNeasy plant mini kit (QIAGEN, 1997) on the DNA Data Bank of Japan (DDBJ) and National Center for

Tos17

The linkage relationship between the sterile phenotype and transposed

Genetic Analyses

The linkage relationship between the sterile phenotype and transposed *Tos17* fragments was analyzed by DNA gel blot hybridization or PCR (see below) in the R3 population, which also included 317 pair1 heterozygous R2 plants. The DNA extraction, DNA gel blot analysis, and cloning of the *Tos17*-tagged sequence steps were performed as described by Nonomura et al. (2003). A rice genomic sequence corresponding to the *Tos17* flanking sequence was identified using BLASTN (Altschul et al., 1997) on the DNA Data Bank of Japan (DDBJ) and National Center for

Biototechnology Information Web sites.

To determine the cDNA sequence, total RNAs were extracted from young (30- to 40-mm) panicles using the RNeasy plant mini kit (QIAGEN, Hilden, Germany). Poly(A)+ RNAs were isolated using Oligotex-dT30 Super (Takara Bio, Shiga, Japan). The cDNA synthesis was performed by 5′-RACE and 3′-RACE using the Marathon cDNA amplification kit (BD Biosciences, Franklin Lakes, NJ). In the 5′- and 3′-RACE analysis, a specific antisense 497 primer, 5′-CACCAGATTGCTTATATCTGC-3′, and a sense 496 primer, 5′-CTCCGACCTTGCACCTTGAGAC-3′, were used, respectively (Figure 2C). Both RACE fragments were ligated at the EcoRI site, and the resultant full-length cDNA was inserted into the pGEM plasmid (Promega, Madison, WI). The cDNA sequence was analyzed using GENETYX-MAC 12.0 software (Genetyx, Tokyo, Japan).

PCR genotyping for the pair1 segregated population was performed using a set of primers: 465, 5′-TACGAGCTTTCTAGCTTCCCTG-3′, and 471, 5′-TACGAGCTTTCTAGCTTCCCTG-3′, for the pair1-1, pair1-2, and pair1-3 alleles, and of F05, 5′-CAGAATAGCAGTCATGCCTG-3′, and F06, 5′-AGGATTGTGTGTGTGTGTG-3′, for the pair1-4 allele (Figure 2C). The PCR conditions were an initial condition of 94°C incubation for 2 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min.

Complementation of pair1 Phenotype

The genomic library constructed using a λEMBL3/BamHI vector (Stratagene, La Jolla, CA) was screened by plaque hybridization probed with *PAIR1* cDNA according to Sambrook et al. (1989). An 8.2-kb *Xho1*-BamHI fragment (Figure 2C) was inserted into the pPZP2H-lac binary vector (Fuse et al., 2001) and transformed into pair1-1 homozygous callus culture. We obtained 24 pair1-1 homozygous callus cultures from the 96 R3 seeds, which were harvested from heterozygous R2 plants and genotyped by PCR using the primers 465 and 471. A sample of callus transformed with the empty vector was used as a negative control. The *PAIR1* plasmid and the empty vector were introduced into the callus culture by *Agrobacterium tumefaciens*–mediated transforma-

tion, and the cultures were regenerated as described by Hiei et al. (1994).

RT-PCR

Total RNA was isolated from roots, shoots, adult leaves, and young panicles according to Sambrook et al. (1989), with a slight modification. One microgram of total RNA was reverse transcribed by SuperScript II RNaresh RT (Invitrogen, Carlsbad, CA) with the oligo(dT)20 primer. In the PCR step, two specific primers within the *PAIR1* cDNA were used, a sense 496 primer in the sixth exon and an antisense F09 primer, 5′-GTT-ACATTTAATTGCTAGTGCGGAAC-3′, in the 11th exon (Figure 2C). The PCR products were loaded on an agarose gel, and a DNA gel blot analysis was performed using a nested PCR probe to confirm the precise amplification from *PAIR1* mRNA. As a control, the same RT products were provided for PCR using primers in the rice actin (RAc): 5′-AATGGGAT-GATATGGAGAA-3′ and 5′-CCTCCAATCCGACACTGTA-3′.

Transient Assay of GFP-PAIR1 Localization

The *PAIR1* cDNA was amplified by PCR with two primers tagged with an *NcoI* site, 5′-CATGCGATGGAAGCTTTAGATGAC-3′ and 5′-CATGCGATGGAAGCTTTAGATGAC-3′, and inserted to an *NcoI* site of pCMV35s-EGFP (B657-NO53) plasmid (Chiu et al., 1996) in frame with the C terminus of a *PAIR1* peptide. The plasmid was amplified in DH10B cells and isolated by QIAGEN plasmid maxi kit. The bombardment of plasmid DNAs was performed as described by Chiu et al. (1996), using a PDS-1000/He biolistic particle delivery system (Bio-Rad, Hercules, CA). The cells were stained with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI), and signals were observed at 6 h after the bombardment using an Olympus FLUOVIEW confocal laser-scanning microscopy (CLSM) system (Tokyo, Japan) for GFP and an Axioskop20 (Zeiss, Jena, Germany) and Nikon 600CL camera system (Pixera, Los Gatos, CA) for DAPI.

Meiotic Chromosome Observation

The young (40- to 60-mm) panicles, including flowers in meiosis, were fixed with 3:1 ethanol:acetic acid (EAA) and stored at 4°C until observation. Chromosome spreads of pollen mother cells were prepared according to Nonomura et al. (2003). They were stained with 4% Giemsa solution (Sigma, St. Louis, MO) diluted in 33 mM phosphate buffer, pH 6.8, for 30 min and photographed using a BX50 light microscope with a DP50 system (Olympus).

To observe female meiosis, flowers fixed in EAA were stained with 1.0 mg/mL of propidium iodide (PI) in 0.1 ML-Arg, pH 12.4, according to Nonomura et al. (2003). Stained pistils in meiosis were dissected from flowers, whole-mounted on the slide with a drop of Vectashield (Vector Laboratories, Burlingame, CA), and viewed using a CLSM system (Olympus). Images were merged and enhanced using Photoshop 7.0 (Adobe, Mountain View, CA) software.

Optical Tissue Sectioning

To observe the morphology of anther walls and ovules, Kasten’s fluorescent periodic acid–Shiff methods were employed according to Vollbrecht and Hake (1995). Flowers in meiosis were fixed with EAA. After hydrolyzation in 50% and then 30% ethanol for 20 min, flowers were treated in 0.5% periodic acid in 70% ethanol/20 mM NaOAc at room temperature for 25 min and then rinsed twice for 5 min in water. They were then stained in 0.25% acriflavine in 37.5 mM K2S2O5/0.017 N HCl at room temperature for 20 min. Stained tissue was rinsed copiously with water until very little acriflavine leaked out (1 to 2 h) and then dehydrated through a series of ethanol solutions for 20 min each. Dehydrated tissue
was treated by methyl salicylate series, 3:1, 1:1, and 1:3 of ethanol:methyl salicylate, for at least 1 h each step and in 100% methyl salicylate twice for 1 h each step. The anthers and pistils were dissected, whole-mounted in methyl salicylate under a coverslip, and viewed using a CLSM system (Olympus).

**FISH Analysis for Nucleolar Organizing Region**

A probe for 25S rDNA locus (Takaiwa et al., 1985) was amplified by PCR using the primers 5'-CTGTGAAAGGCTTGGATG-3' and 5'-CTACTACCAAGAGATCTG-3', in which the usual reaction buffer contained a nucleotide mixture with 50% digoxigenin-labeled dUTP (Roche, Indianapolis, IN) of unlabeled thymidine-5'-(triphosphate as an addition. The young (40- to 60-mm) panicles were fixed with 4% (w/v) paraformaldehyde in PMEG buffer (25 mM Pipes, 5 mM EGTA, 2.5 mM MgSO4, 4% glycerol, and 0.2% DMSO, pH 6.8) in a 50-mL tube. After vacuum infiltration, they were shaken at a medium speed at room temperature for 3 h. The panicles were washed six times in PMEG at medium shaking for 20 min each. They could be stored in PMEG at 4°C at least for 2 months. A single anther from the six in a flower was provided to determine its meiotic stage by 1 μg/mL of DAPI in Vectashield (Vector Laboratories) and viewed by fluorescence microscope Axioscop 20 (Zeiss). The anthers at appropriate stages were provided for cell-wall digestion in an enzyme mixture at 37°C for 20 min and at 4°C for 10 min, in which 1.5 volumes of 50 mg/mL cytolysinase (Sigma) was mixed with 20 volumes of 2% cellulase Onozuka RS (Yakult Honsha, Tokyo, Japan), 0.3% pectolyase Y-23 (Kikkoman, Chiba, Japan), and 1.5% macerozyme R200 (Yakult Honsha) in PMEG. The anthers were squashed in 20% acetic acid on the polv-L-Lys-coated slide and collapsed by a coverslip. The coverslip was removed on dry ice. After air drying, the slide was treated with 100 μg/mL RNaseA at 37°C for 1 h, rinsed twice with 2× SSC for 5 min and once with 50 mM Tris-HCl, pH 7.5, for 5 min, treated with 5 μg/mL of Proteinase K in 50 mM Tris-HCl at 37°C for 30 min, and rinsed twice with water at 4°C for 10 min for each step. The male meiocytes on the slides were refixed with EAA for 10 min, rinsed twice with 95% ethanol for 10 min, and air dried. The hybridization procedure was performed according to Schwarzacher and Heslop-Harrison (2000). The labeled probe was detected by FITC-conjugated anti-digoxigenin antibody (Roche). Chromosomes were counterstained with 1 μg/mL of DAPI, and photos were taken using an Axioscop20 (Zeiss) and Penguin 600CL camera system (Pixera).

**Indirect Immunofluorescence**

To observe the spindle morphology in male meiocytes, we adopted the method described by Chan and Cande (1998), with slight modifications. Fixation and digestion steps of the cells and cell walls were performed in an identical manner to the FISH method. After fixed anthers were rinsed five times with PMEG for 5 min each wash and squashed in 10 μL of PMEG, the cell suspension was transferred to a 1.5-mL microtube. An equal volume of preserved (20°C) 3% SeaPrep agarose (BioWhittaker Molecular Applications, Rockland, ME) in PMEG was added to the suspension. After gently mixing the solution completely, it was then cooled to 4°C for 30 min. The agarose block was then rinsed with 500 μL of PBS (137 mM NaCl, 8.1 mM Na2HPO4, 2.68 mM KCl, and 1.47 mM KH2PO4, pH 7.4) twice at room temperature for 5 min each wash. The agarose block was then incubated with 50 μL of monoclonal antibody against the rat-α-tubulin subunit, OBT06145S (Oxford Biotechnology, Oxfordshire, UK), diluted with PBS (1:100) at room temperature overnight. It was rinsed with PBS twice for 1 h and then incubated in 50 μL of PBS-diluted (1:200) Alexa488 conjugated antibody against goat anti-rat IgG (Invitrogen) at room temperature overnight. The agarose block was rinsed with PBS twice for 1 h, incubated in 50 μL of 30 μg/mL of PI in PBS at room temperature for 2 h, and washed with PBS twice for 5 min each wash. The agarose block was placed with a drop of Vectashield (Vector Laboratories) to the inside of a 18 × 18-mm square drawn by rubber cement on the slide, heated to 55°C for 3 min, and overlaid with a coverslip. The male meiocytes were observed using the FLUOVIEW CLSM system (Olympus). Five or six photos of optical sections from a meiocyte were merged and enhanced using Photoshop 7.0 (Adobe).

**Accession Numbers**

The PAIR1 cDNA sequence described in this article has been deposited in DDBJ under accession number AB158462. Accession numbers for the other sequences mentioned are GenBank AC107224 (bacterial artificial chromosome clone OSJNba0009C08) and GenBank AC009273 (hypothetical protein T1N6.6).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AB158462, AC107224, and AC009273.

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Rice PAIR1 for Homolog Pairing in Meiosis
The Novel Gene *HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS1* of Rice Encodes a Putative Coiled-Coil Protein Required for Homologous Chromosome Pairing in Meiosis
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