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OsDMC1 is required for homologous pairing in *Oryza sativa*

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Abstract *OsDMC1* is the rice homologue of the yeast *DMC1 gene*. Here, we analyzed the function of *OsDMC1* in meiosis using an RNA interference approach. The OsDMC1–RNAi lines grew normally during their vegetative phase but showed spikelet and pollen sterility. The sterility phenotypes were associated with down-regulated OsDMC1 transcript and protein levels mediated by RNAi. Further cytological observations of male meiocytes revealed that knock-down of *OsDMC1* led to defects in bivalent formation and subsequent unequal chromosome segregation and irregular spore generation, and induced changes in male meiotic progression. Fluorescent in situ hybridization experiments revealed that the OsDMC1–RNAi lines were defective in homologous pairing. These data indicate that *OsDMC1* is essential for rice meiosis and plays an important role in homologous pairing.

Keywords *OsDMC1* · Male meiocytes · Meiosis · Homologous pairing · *Oryza sativa*

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Introduction

Meiosis allows diploid sporophytic cells to produce haploid cells, which develop further into gametophytes or gametes, thus being essential not only for the maintenance of genomic stability but also for the creation of genetic diversity. Unlike mitosis, meiosis involves two sequential rounds of nuclear divisions after a single round of DNA replication. During the second meiotic division (meiosis II), sister chromatids separate just as they do in mitosis. However, the first meiotic division (meiosis I) is unique and involves the segregation of homologous chromosomes. To ensure accurate segregation, homologous chromosomes come together and form stable bivalents through a complex series of processes which include pairing, synapsis, and recombination (Pawlowski et al. 2004). During the early prophase of meiosis I, homologous chromosomes recognize each other and pair side by side. The pairing of homologous chromosomes is then stabilized by formation of synaptonemal complex. Coincidently, recombination occurs and results in exchange of genetic material between homologous chromosomes.

Recently, cytological and molecular genetic studies in yeast have made considerable progress in understanding the mechanisms underlying these prophase I events (Pawlowski and Cande 2005). Pairing, synapsis, and recombination are strictly coupled processes, and tight coordination of the three events is a scaffold for faithful segregation of homologous chromosomes at meiosis I (Ma 2005; Pawlowski and Cande 2005; Hamant et al. 2006). The yeast Dmc1 protein was identified as a eukaryotic homologue of RecA, a recombinase in *Escherichia coli* (Bishop et al. 1992; Masson and West 2001). Similar to RecA, yeast Dmc1 can bind to single-strand DNA (ssDNA) to form nucleoprotein filaments, catalyze the invasion of ssDNA

into homologous duplex DNA and promote DNA strand exchange (Hong et al. 2001). Consistent with its biochemical activities, the yeast Dmc1 protein has been shown to be necessary for recombination, pairing, and synapsis during meiosis. In the yeast SK1 strain background, *dmc1* mutations result in accumulation of unrepaired double-strand breaks (DSBs) and cause failure in formation of the synaptonemal complex (Bishop et al. 1992). Another yeast *dmc1* mutant in a BR2495 background also exhibits decreased pairing and delayed synapsis (Rockmill et al. 1995). These defects in early events of meiosis eventually induce activation of the pachytene checkpoint, which leads to meiotic cell cycle arrest (Roeder and Bailis 2000). Meiotic arrest in yeast *dmc1* cells is alleviated by mutation in checkpoint genes such as *MEC1*, *RAD17* and *RAD24* (Lydall et al. 1996; Grushcow et al. 1999). These *dmc1 checkpoint* double mutants can complete both meiotic nuclear divisions but show chromosome fragmentation caused by the failure to repair DSBs (Lydall et al. 1996). These observations suggest the essential role of yeast Dmc1 in meiosis and the involvement of the protein in homologous pairing, synapsis, and recombination.

Homologues of the yeast *DMC1* gene have been identified in many higher eukaryotes, including mouse (Habu et al. 1996), human (Habu et al. 1996), *Arabidopsis* (Sato et al. 1995; Klimyuk and Jones 1997; Doutriaux et al. 1998), lily (Kobayashi et al. 1994) and rice (Ding et al. 2001; Shimazu et al. 2001; Kathiresan et al. 2002). Similar to yeast *dmc1* mutants, mouse Dmc1-deficient germ cells show defects in pairing, synapsis, and resultant meiotic arrest, followed by apoptosis (Pittman et al. 1998; Yoshida et al. 1998). In plants, *Arabidopsis dmc1* (*atdmc1*) mutants form univalents instead of bivalents at meiotic prophase I (Couteau et al. 1999). Immunolocalization analysis of the Dmc1 protein in lily meiocytes shows that Dmc1 foci appear on chromosomes at leptotene, accumulate to a high level at zygotene and disappear at pachytene stage (Terasawa et al. 1995). Taken together, these observations suggest a possible role of Dmc1 in homologous pairing and/or synapsis in plants; however, conclusive evidence identifying pairing and synapsis is not yet available. In addition, *atdmc1* mutants were not identified to have the phenotypes of meiotic arrest and/or chromosome fragmentation, which have been observed in yeast and mouse *dmc1* mutants (Couteau et al. 1999). The phenotypic differences possibly result from different mechanisms of cell cycle control between plants and yeast/mouse (Couteau et al. 1999). As well, plant Dmc1 could be unimportant for repair of DSBs in meiotic recombination (Li et al. 2004). Inconsistent with this suggestion, recent in vitro biochemical assays have demonstrated the recombination activity of the rice Dmc1 protein (Kant et al. 2005; Rajanikant et al. 2006). Therefore, further studies involving other model organisms are necessary to determine the roles of plant Dmc1 in meiotic pairing, synapsis, and recombination and to understand the mechanisms underlying prophase I in higher plants.

Here, we dissected the function of *DMC1* in plant male meiosis by using rice (*Oryza sativa*), which represents an excellent system in addition to *Arabidopsis* for studying the mechanisms of meiosis because of its mid-sized genome and synchronous meiosis within an anther and well-established cytological techniques allowing for its analysis (Chen et al. 2005; Itoh et al. 2005; Zhang et al. 2006). OsDMC1-deficient rice plants generated by RNA interference (RNAi) showed aberrances in meiotic chromosome behaviors at prophase I and subsequent defects in chromosome segregation and spore formation. Furthermore, to determine the underlying causes of these meiotic defects, we monitored dynamic changes in homologous pairing in the OsDMC1-RNAi male meiocytes by using fluorescent in situ hybridization (FISH). Our results indicate the involvement of *OsDMC1* in homologous pairing.

Materials and methods

RNA interference constructs

A 600-bp fragment specific to OsDMC1 cDNA (nucleotides 516–1115) was amplified from the full-length OsDMC1 cDNA (Ding et al. 2001) first by use of the primer pairs PF1 (5′-TGCATCTAGACCGGCCTGAACG AATTGT-3′, *Xba*I) and PR1 (5′-CCGCACTAGTCCAAA CCTATATGGCCTA-3′, *Spe*I), and then by the pairs PF2 (5′-AGCTGTCGACCCAAACCTATATGGCCTA-3′, *Sal*I) and PR2 (5′-TGAAGGTTACCCCGGCCTGAA CGAATTGT-3′, *Bst*EII). After digestion with the corresponding restriction enzymes, the two amplified fragments were inserted in opposite directions into the binary RNAi tool vector pWTC605 (Zhang et al. 2006), derived from pCAMBIA1300, to generate the OsDMC1–RNAi plasmid pOsDMC1i.

Plant transformation

pOsDMC1i was introduced into an *Agrobacterium tumefaciens* strain EHA105 by a freeze-thaw method (Hofgen and Willmitzer 1988). Rice calli were induced from mature embryos on 2N6 medium (Hiei et al. 1994) and transformed by the strain carrying pOsDMC1i in AAM medium (Toriyama and Hinata 1985), then selected on 2N6 media containing the antibiotics hygromycin B, cefotaxime, and carbenicillin. Resistant calli were transferred onto MS medium (Murashige and Skoog 1962) supplemented with

NAA and 6-BA to obtain regenerated seedlings. The regenerated plants (T0 generation) were transplanted to soil and grown under greenhouse conditions. The ratoons (R1 generation), which refer to the plantlets generated from buds in basal stalks of their T0 lines, were maintained under standard growth conditions. Seeds from T0 lines were selected on 1/2 MS medium containing 40 mg/L of hygromycin B. The surviving T1 plantlets were transferred to soil and grown under standard growth conditions.

PCR and Southern blotting

For PCR screening of OsDMC1–RNAi lines, two pairs of primers, P190F (5′-CGGGAACTACAAGACACGTGC-3′, located in the spacer sequence between the two inserted OsDMC1 cDNA fragments) and PR2, PF1, and P190R (5′-TAACCTTCGGGCATGGCAC-3′, located in the spacer sequence), were used to amplify genomic DNA extracted from each of the possible transgenic lines as described (Edwards et al. 1991).

For Southern blotting analysis, genomic DNA was isolated from PCR-positive lines by CTAB method (Buchholz et al. 1998), then digested with *Eco*RI, which cuts only once within pOsDMC1i. Southern blot hybridization was performed with the digested DNA as previously described (Zhang et al. 2006).

Semi-quantitative RT-PCR

Total RNA was isolated with TRIZOL Reagent (Invitrogen, USA) from young panicles. First-strand cDNA was synthesized from 5 μg of total RNA using ReverTra Ace (TOYOBO, Japan), then used as a template for PCR amplification with OsDMC1 cDNA-specific primers PF3 (5′-AAGCGGAAATCTGTTGTT-3′) and PR3 (5′-TCAGT CTTTCGCATCCAT-3′). The amplified tubulin TubA cDNA was used as an internal control to normalize the cDNA input (Zhang et al. 2006).

Antibody production and Western blotting

For antibody production, the complete open reading frame of *OsDMC1* was PCR amplified from the first-strand cDNA synthesized from panicle RNA by use of Pyrobest DNA Polymerase (TaKaRa, Japan) and the two gene-specific primers PF4 (5′-TATAGGATCCATGGCGCCGTCC AAGCA-3′, *Bam*HI) and PR4 (5′-TCAGC TCGAGTC AGTCTTTCGCATCCAT-3′, *Xho*I). The amplified product was double-digested with *Bam*HI/*Xho*I and ligated into a linearized prokaryotic expression vector pET28a (+) (Novagen, USA). The construct was confirmed by sequencing (TaKaRa, Japan) and then transformed into *E. coli* strain BL21 (DE3). After induction, the polyhistidine-tagged fusion protein (391 amino acids, 43 kDa) was purified by use of Ni-NTA agarose column (QIAGEN, Germany) under denaturing conditions according to the manufacturer's protocols. The purified protein was partially renatured by sequential dialysis at 4°C against a PBS buffer (pH 7.0) with decreasing urea concentrations, and then condensed with use of a Microcon YM-10 membrane (Millipore, USA). Finally, the resultant protein was verified by ESI Q-TOF MS/MS (Micromass, England) and used to produce polyclonal rabbit antisera.

Total proteins were extracted from young panicles of wild-type and OsDMC1–RNAi plants as described (Nelson 1984), then subjected to SDS-PAGE on 8% gels. Western analysis involved use of the antibody against OsDmc1 or tubulin (Sigma, Germany) as previously described (Zhang et al. 2006), except that the transfer buffer used was Tris–glycine–methanol solution (40 mM Tris-base, 39 mM glycine, 20% methanol).

Small RNA isolation and detection

Total RNA was extracted from young panicles and used to enrich low-molecular-weight (LMW) RNA. Using the SP6/T7 Transcription Kit (Roche, USA), a ^{32}P-labeled antisense RNA probe was generated by in vitro transcription from a linearized T-vector (Promega, USA) carrying the 600-bp fragment used in the OsDMC1–RNAi construct. Enrichment of LMW RNA and northern blot analysis were performed as described previously (Hamilton and Baulcombe 1999; Mette et al. 2000).

Cytological analysis

To assess the viability of mature pollen grains, flowers were randomly collected from each OsDMC1–RNAi line and wild-type plant at the heading stage. Anthers of the sampled flowers were dissected and immersed in Alexander's solution (Alexander 1969). Stained anthers were crushed and observed under light microscopy.

Anthers from young flowers (2–5 mm long) fixed in Carnoy's solution were used for preparation of nuclear spreads. Male meiotic spreads were prepared and stained with 4′,6-diamidino-2-phenylindole (DAPI) as described (Ross et al. 1996; Chen et al. 2005). The meiotic stage of individual nuclei was determined from anther length (Itoh et al. 2005), chromosome morphology (Ross et al. 1996; Chen et al. 2005) and nucleolus position (Bass et al. 1997).

FISH was performed as described previously (Jones and Heslop-Harrison 2000). The digoxigenin-labeled centromeric probe pRCS2 (Dong et al. 1998) was a gift from Zhukuan Cheng (Institute of Genetics and Development

Biology, Chinese Academy of Sciences). The 25S rDNA probe was labeled with digoxigenin by PCR (Nonomura et al. 2004).

Results

Generation and molecular characterization of OsDMC1–RNAi lines

Previous studies have shown that *OsDMC1* is the rice homologue of the yeast *DMC1* gene (Ding et al. 2001; Kathiresan et al. 2002) and the OsDmc1 protein displays a recombination activity in vitro (Kant et al. 2005; Rajanikant et al. 2006). Here, we analyzed the function of *OsDMC1* by an RNAi approach, which is effective in defining functions of a gene (Waterhouse et al. 1998; Chuang and Meyerowitz 2000). To guarantee the specificity of RNAi, the OsDMC1–RNAi vector pOsDMC1i was constructed with a 600-bp cDNA fragment of OsDMC1, which shares no similarity to any other sequences in the present rice genome database. We obtained 12 independent transformants from independent hygromycin-resistant calli transformed with pOsDMC1i.

Integration of the transgene in the genome was examined by PCR with primer pairs localized to the spacer and the inserted cDNA sequences on pOsDMC1i. Eight of the 12 lines were positive for the insertion of the transgene, and the remaining 4 were negative. For convenience, the 8 PCR-positive (OsDMC1–RNAi) lines were designated L1–L8, respectively, with the negative ones used as a non-transgenic control (NT) and designated NT1–NT4. Furthermore, we examined the copy number of the transgene in the 8 OsDMC1–RNAi lines by Southern blot hybridization and found that L1, L3, L4, L5, and L8 had only one copy of the insertion; L2, L6, and L7 had two copies; and their integrated regions differed from each other.

OsDMC1–RNAi lines are sterile

All the 8 OsDMC1–RNAi lines grew and developed normally during their vegetative stage, but their seed production was severely disrupted as compared with wild-type plants (Fig. 1A, B). To quantify the extent of sterility, we evaluated the mean percentage of filled grains per panicle (MPFG) of these lines at seed maturity. In contrast to wild-type plants, with an MPFG of approximately 85%, RNAi lines L3, L5, and L6 were fully sterile, and the other five lines (L1, L2, L4, L7, and L8) showed partial sterility ($0\% < \text{MPFG} < 20\%$). For the three fully sterile lines, their sterile phenotypes were further examined by use of R1 plants derived from T0 plants, whereas for the 5 partially sterile lines, the phenotypes were checked in their R1 and T1 generations. The MPFG and the sterile phenotypes were stable in R1 and/or T1 generations (Fig. 1C). As a control, the MPFG of the non-transgenic plants (NT1–NT4) was comparable to that of wild-type plants (Fig. 1C). These results suggest that *OsDMC1* is involved in spikelet fertility.

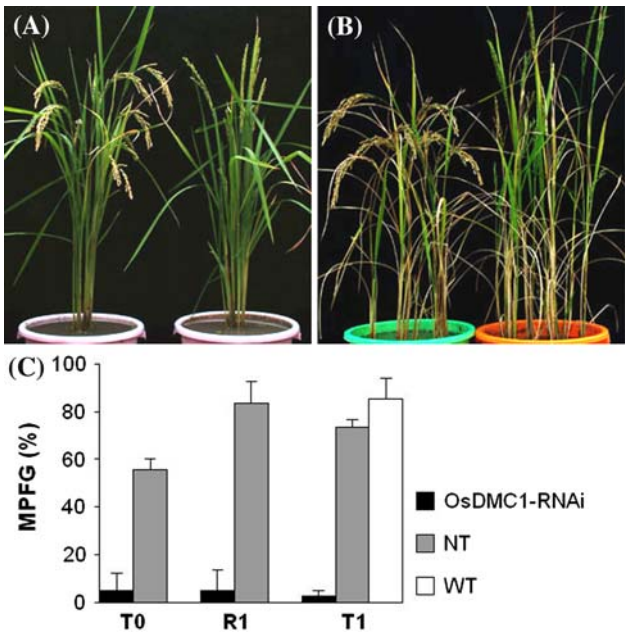

Fig. 1 OsDMC1–RNAi rice lines are disrupted in fertility. (**A**) The plant morphology of wild-type (left) and OsDMC1–RNAi (right, R1 generation) rice. In OsDMC1–RNAi rice, the sterile panicles remained upright at maturity, whereas the filled panicles of the wild-type bent with their own weight. (**B**) The plant morphology of wild-type (left) and OsDMC1–RNAi (right, T1 generation) rice. (**C**) The mean percentage of filled grains per panicle (MPFG %) in the T0, R1 and T1 generation of OsDMC1-RNAi lines, with non-transgenic (NT) and wild-type (WT) plants as controls

To further investigate whether the sterile phenotype involved pollen abortion, we compared the viability of mature pollen grains from the OsDMC1–RNAi lines (L1–L8) with the pollen grains from the non-transgenic (NT1–NT4) and wild-type plants by Alexander staining (Alexander 1969), whereby viable pollen grains are stained red and non-viable ones stained green. Anthers of non-transgenic and wild-type plants showed red-colored pollen grains (240 anthers tested) (Fig. 2A). Most of the wild-type pollen grains were ''viable'' and regular in size (45 ± 2 μm diameter); only less than 10% were aberrant (Fig. 2D). In the partially sterile lines (L1, L2, L4, L7, and L8), more than 50% of the pollen grains (300 anthers recorded) were ''non-viable'' (Fig. 2B, E). However in the fully sterile lines (L3, L5, and L6), pollen grains with a wild-type appearance were rarely observed in the 180 examined anthers (Fig. 2C). More than 95% of pollen

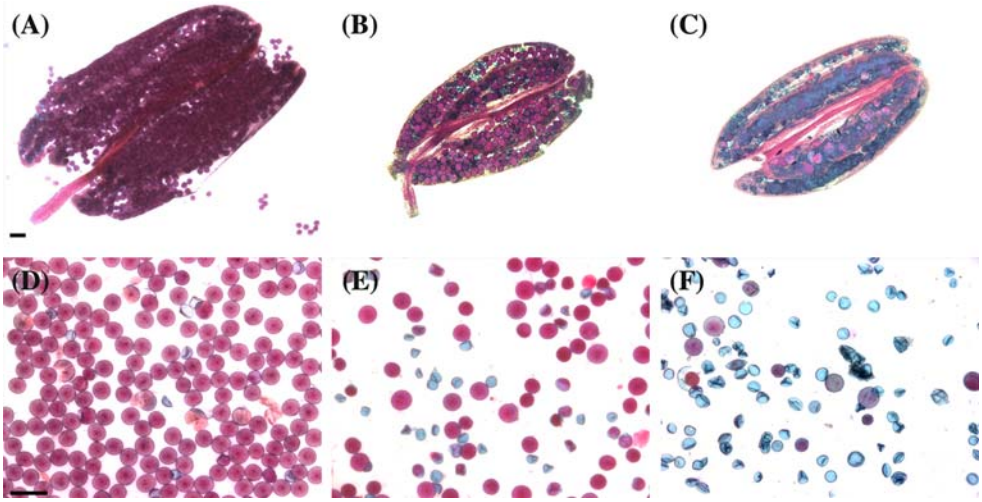

Fig. 2 Pollen viability of OsDMC1–RNAi plants. Alexander staining of anthers (**A**–**C**) and mature pollen grains (**D**–**F**) from wild-type (**A** and **D**) plants, partially sterile (**B** and **E**) and fully sterile OsDMC1–RNAi lines (**C** and **F**). Scale bar = 100 μm in (**A**) for (**A**)–(**C**), and in (**D**) for (**D**)–(**F**)

grains from the three lines were ''non-viable,'' shrunken, and variable in size (25–62 μm diameter) and shape (Fig. 2F). Hence, pollen viability appeared to be compatible with the spikelet fertility in these OsDMC1–RNAi lines, suggesting that pollen abortion in these RNAi lines related to their sterile phenotypes.

Knock-down of OsDMC1 transcript and protein is associated with sterility in OsDMC1–RNAi plants

In plants in which RNA interference is active, exogenous hairpin RNAs transcribed from the sense-spacer-antisense sequence in RNAi constructs are digested into 21- to 23-nt small RNAs and finally diminish or eliminate the endogenous transcript and protein of a target (Hamilton and Baulcombe 1999). To investigate whether endogenous OsDMC1 transcript and protein expression was knocked down in OsDMC1-RNAi lines, we used semi-quantitative RT-PCR and Western blotting, respectively, to examine the RNAi lines (L1, L3, L4, L5, L6, L7, and L8) and wild-type plants. The endogenous OsDMC1 transcript and protein in the seven RNAi lines were greatly down-regulated as compared to that of the wild-type control (Fig. 3A, B). The three fully sterile lines (L3, L5, and L6) showed more down-regulated levels of both the transcript and protein than the partially sterile lines (L1, L4, L7, and L8) (Fig. 3A, B).

To confirm whether the down-regulation of OsDMC1 transcript and protein was achieved by RNAi, we detected small RNAs specific to OsDMC1 in the RNAi lines (L1, L3, L4, L5, L6, L7, and L8) and wild-type plants by Northern hybridization. Small RNAs of about 23 nt, specific to OsDMC1, were detectable in all these RNAi lines but not in the wild-type plants (Fig. 3C), which indicates that the integrated RNAi constructs were transcribed and functioned in these RNAi lines. In addition, the small RNAs were at the highest levels in the three fully sterile lines, which exhibited the greatest decrease in OsDMC1 transcript and protein levels, and at relatively low levels in the other four partially sterile lines (Fig. 3A–C). Therefore,

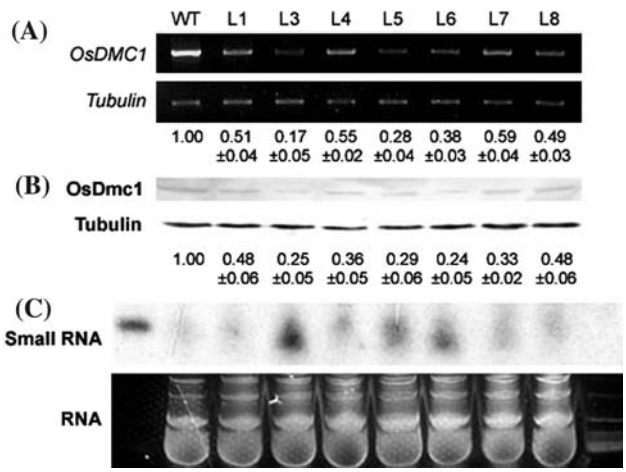

Fig. 3 OsDMC1 transcript and protein level reduced in OsDMC1-RNAi lines. (**A**) Semi-quantitative determination of OsDMC1 mRNA by RT-PCR. Total RNAs prepared from panicles of OsDMC1–RNAi (L1 and L3–L8) and wild-type plants was used to synthesize the first-strand cDNA. Constitutively expressed tubulin was used as an internal control. Results shown below the image represent quantified values of OsDMC1 mRNA levels (ratios of normalized data by use of the tubulin signals for RNAi lines vs. wild-type), and the data are mean ± SD of triplicates. (**B**) Western blot detection of OsDmc1 protein in RNAi and wild-type plants with tubulin as an internal control. Results shown below the image represent estimated OsDmc1 protein levels (ratios of normalized data by use of the tubulin signals for RNAi lines vs. wild-type), and the data are mean ± SD of triplicates. (**C**) Northern hybridization detection of small RNAs specific to *OsDMC1*. About 10 picomoles per lane of 28-mer sense (PF1) and antisense (PR1) DNA primers (sequences indicated in Materials and methods) were loaded on the first and the last lane, respectively, as size markers and indicators of the hybridization specificity. The *OsDMC1*-specific antisense RNA probes hybridized only to the sense DNA primer but not to the antisense primer

these data indicate that down-regulation of the transcript and protein levels relates to the sterile phenotypes in these RNAi lines.

OsDMC1–RNAi plants undergo aberrant meiosis

To evaluate whether aborted pollen grains resulted from defects in meiosis, we carried out detailed cytological analysis of meiosis in male meiocytes from the three fully sterile OsDMC1–RNAi lines (L3, L5, and L6) and wild-type control. From premeiotic interphase to middle zygotene stage, no obvious differences were detected between wild-type (Fig. 4A–D) and OsDMC1–RNAi (Fig. 4AA–DD) meiocytes. At late zygotene and pachytene stages, the chromosome threads of OsDMC1–RNAi nuclei (Fig. 4EE–GG) appeared more tenuous than those of the wild-type (Fig. 4E–G). Differences between OsDMC1–RNAi and wild-type meiosis became more evident when chromosomes condensed at late diplotene (Fig. 4H, HH) and diakinesis stages (Fig. 4I, J, II, JJ). In the wild-type, 12 bivalents were formed at diakinesis (Fig. 4I, J). However, 46.7% of OsDMC1–RNAi nuclei (n = 632) had more than 12 distinguishable chromosomes (Fig. 4II, JJ), which indicates the presence of univalents. At metaphase I, these univalents did not align on the metaphase plate but, rather, dispersed throughout the nucleus (Fig. 4KK). At anaphase I, lagging chromosomes and unequal chromosome segregation were observed (Fig. 4LL). At telophase I, these lagging chromosomes remained in the middle when other chromosomes had reached the opposite poles of the cell (Fig. 4MM).

These defects led to aberrant chromosome behavior and unequal separation of chromosomes in meiosis II (Fig. 4NN–QQ). Eventually, most OsDMC1–RNAi meiocytes (51.1%, n = 552) generated dyads (8.0%; Fig. 4RR), triads (26.6%; Fig. 4S, SS), polyads (2.9%; Fig. 4TT), or abnormal tetrads with daughter cells of different sizes (13.6%; Fig. 4T), whereas tetrads with four daughter cells of equal size were formed in wild-type meiocytes (Fig. 4R).

In addition, a comparison of meiotic progression between OsDMC1–RNAi and wild-type male meiocytes revealed that meiocytes of one OsDMC1–RNAi anther showed asynchronous cell cycle progression during meiotic prophase I. Generally, in wild-type rice, male meiosis progresses synchronously in hundreds of male meiocytes from the same anther (Zhang and Zhu 1987); thus, meiocytes in the same prophase I anther were at the same substage (66.7% of the 57 examined prophase I anthers) or two nearby substages (26.3%). However, such synchronization was impaired in the OsDMC1–RNAi lines. In the 89 examined OsDMC1-RNAi anthers with prophase I meiocytes, 33.7% contained meiocytes spanning 3–7 meiotic stages and 9.0% included meiocytes spanning 2 discrete stages. Although meiotic behavior and progression were abnormal in the OsDMC1–RNAi lines, most of the OsDMC1-RNAi meiocytes passed through every stage of meiosis and formed almost as many pollen grains as the wild-type; only a few meiocytes in which meiotic processes were severely disrupted arrested at a special stage. For example, abnormal meiocytes blocked at metaphase I were occasionally observed (2–20 arrested cells per anther) in 11 of 20 examined anthers at uninucleate stage (Fig. 5).

Meiotic asynchrony was also observed in two daughter cells of dyads. In the wild-type, the two daughter cells of one dyad showed synchronous nuclear division (Fig. 4P, Q). However, in about half of OsDMC1–RNAi dyads (101 well-spread nuclei at anaphase II recorded), one of the two daughter cells was at a more advanced stage of meiosis II (Fig. 4PP, QQ, SS).

OsDMC1–RNAi lines are defective in homologous pairing

To address events underlying the aberrant meiosis in OsDMC1–RNAi lines, we performed FISH to trace the progression of homologous pairing in male meiocytes from the three fully sterile RNAi lines (L3, L5, and L6) and wild-type plants. The digoxigenin-labeled telomeric probe 25S rDNA, which can bind exclusively to the short-arm end of chromosome 9 (Takaiwa et al. 1985) was used to hybridize with the well-spread male meiotic nuclei. In wild-type plants, all the meiocytes at premeiotic interphase had two distinct signals (Fig. 6A, U); by leptotene stage, the percentage of cells with two signals decreased greatly to 52% and later decreased further to 37% at early zygotene, 27% at mid-zygotene, and 16% at late zygotene (Fig. 6U). Eventually, all the meiocytes had one signal at the beginning of pachytene stage (Fig. 6K, U), when pairing and synapsis were completed. Similarly, all OsDMC1–RNAi meiocytes had two signals at interphase (Fig. 6F, U). However, when meiosis progressed, a higher proportion of cells showed two signals, with 66% at leptotene, 48% at early zygotene, 45% at mid-zygotene, and 42% at late zygotene (Fig. 6U). Even at pachytene stage, 39% of the RNAi meiocytes still contained two separate signals (Fig. 6L–N, P–S, U). When homologous chromosomes had desynapsed along the chromosome arm at metaphase I, two signals located at the two separate ends of the same bivalent were seen in wild-type meiocytes (Fig. 6O). However, in RNAi meiocytes at metaphase I, some homologous chromosomes existed as univalents (as indicated by the cytological results); thus, the two signals were situated at

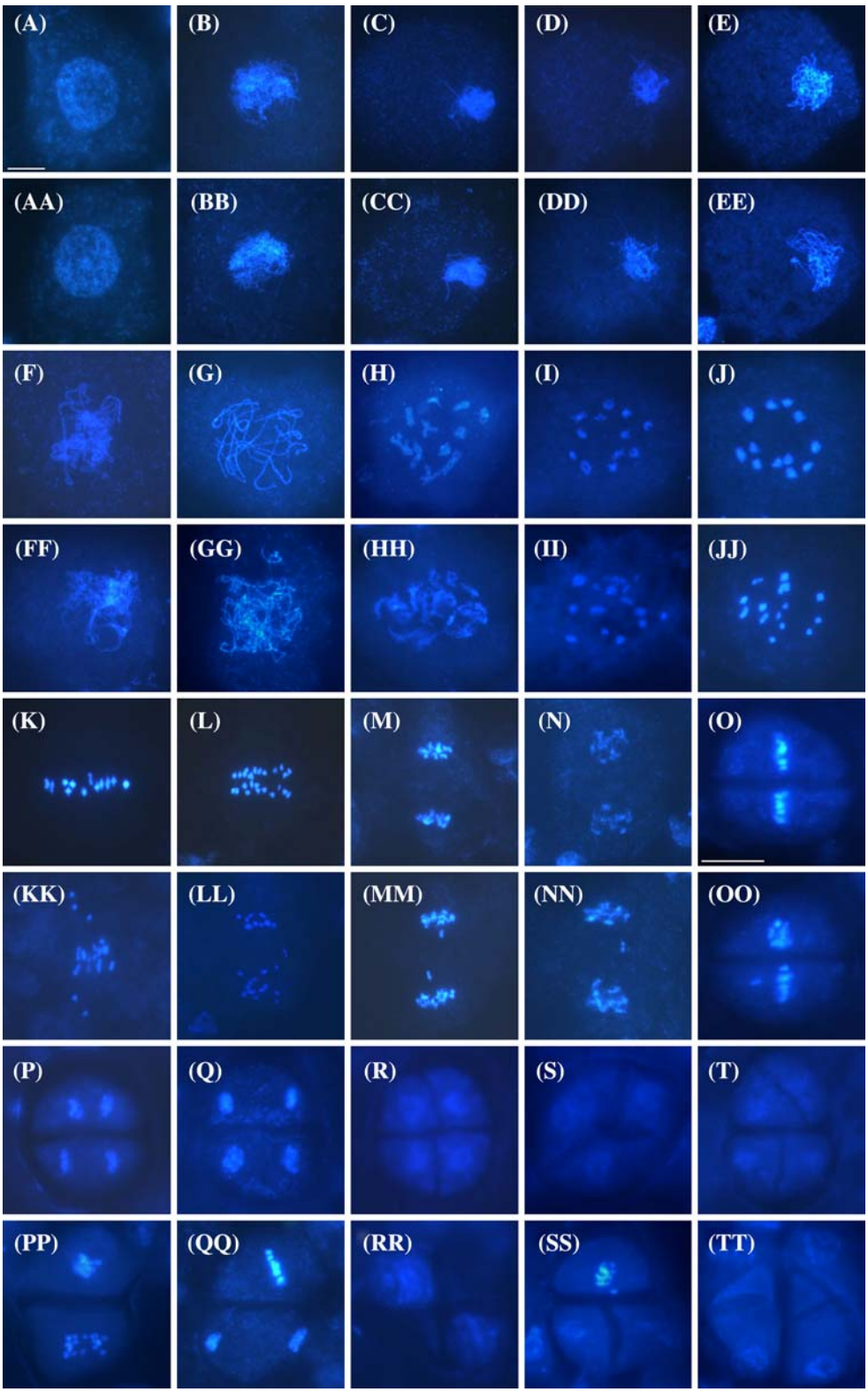

Fig. 4 Male meiosis in OsDMC1–RNAi lines. Male nuclear spreads were prepared from wild-type (A–R) and OsDMC1–RNAi (S, T and AA–TT) plants and then counterstained with DAPI. Stages include interphase (A and AA), leptotene (B and BB), early zygotene (C and CC), middle zygotene (D and DD), late zygotene (E and EE), early pachytene (F and FF), late pachytene (G and GG), late diplotene (H and HH), early diakinesis (I and II), late diakinesis (J and JJ), metaphase I (K and KK), anaphase I (L and LL), telophase I (M and MM), prophase II (N and NN), metaphase II (O and OO), anaphase II (P and PP), telophase II (Q and QQ), tetrad stage (R: normal tetrad, RR: dyad, S and SS: triad, T: abnormal tetrad with daughter cells of different sizes, TT: polyad). Scale bar = 10 μm in (A) for (A)–(N) and (AA)–(NN), and in (O) for (O)–(T) and (OO)–(TT)

the ends of two univalents (34% of the 41 recorded RNAi meiocytes at metaphase I; Fig. 6T). These observations indicated that OsDMC1-deficiency affected homologous pairing along chromosome arms.

We also investigated pairing by using the centromeric probe pRCS2, which contains four copies of a 168-bp tandem repeat sequence and localizes to centromere regions of all 24 rice chromosomes (Dong et al. 1998). In the

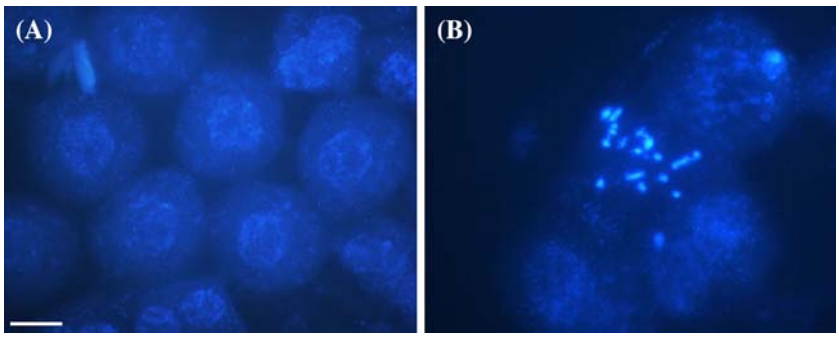

Fig. 5 Microspores at uninucleate stage of wild-type (**A**) and OsDMC1–RNAi (**B**) plants. Scale bar = 10 μm in (**A**) for (**A**) and (**B**)

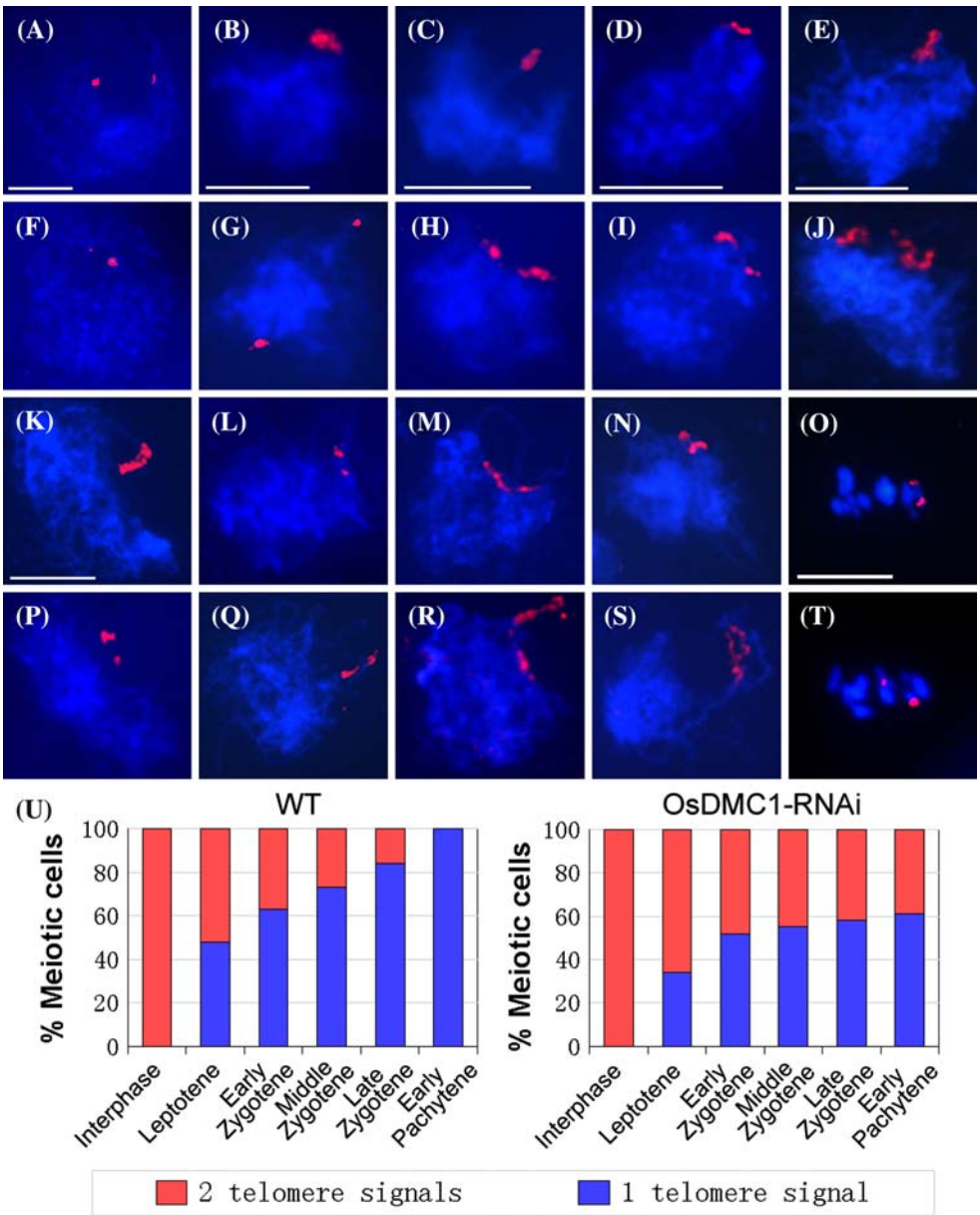

Fig. 6 FISH results with the telomeric probe 25S rDNA in wild-type (A–E, K, and O) and OsDMC1–RNAi (F–J, L–N, and P–T) male meiocytes. Meiotic chromosomes spreads were probed with the 25S rDNA probes (red) and then counterstained with DPAI (blue). Stages include premeiotic interphase (A and F), leptotene (B and G), early zygotene (C and H), middle zygotene (D and I), late zygotene (E and J), early pachytene (K–N and P–S) and metaphase I (O and T). Bar =10 μm; meiotic nuclei at the same stages share the same scale. Statistical results are shown in (U) ($50 < n < 100$ for wild-type; $100 < n < 250$ for OsDMC1–RNAi plants)

wild-type, 13–24 signals were observed in all examined male meiocytes at interphase (Fig. 7A, P). As meiocytes progressed through the stages of leptotene, zygotene, and pachytene, the proportion of cells with 13–24 signals decreased rapidly, with an increase in the proportion of cells having 8–12 signals. Finally, all the meiocytes at early

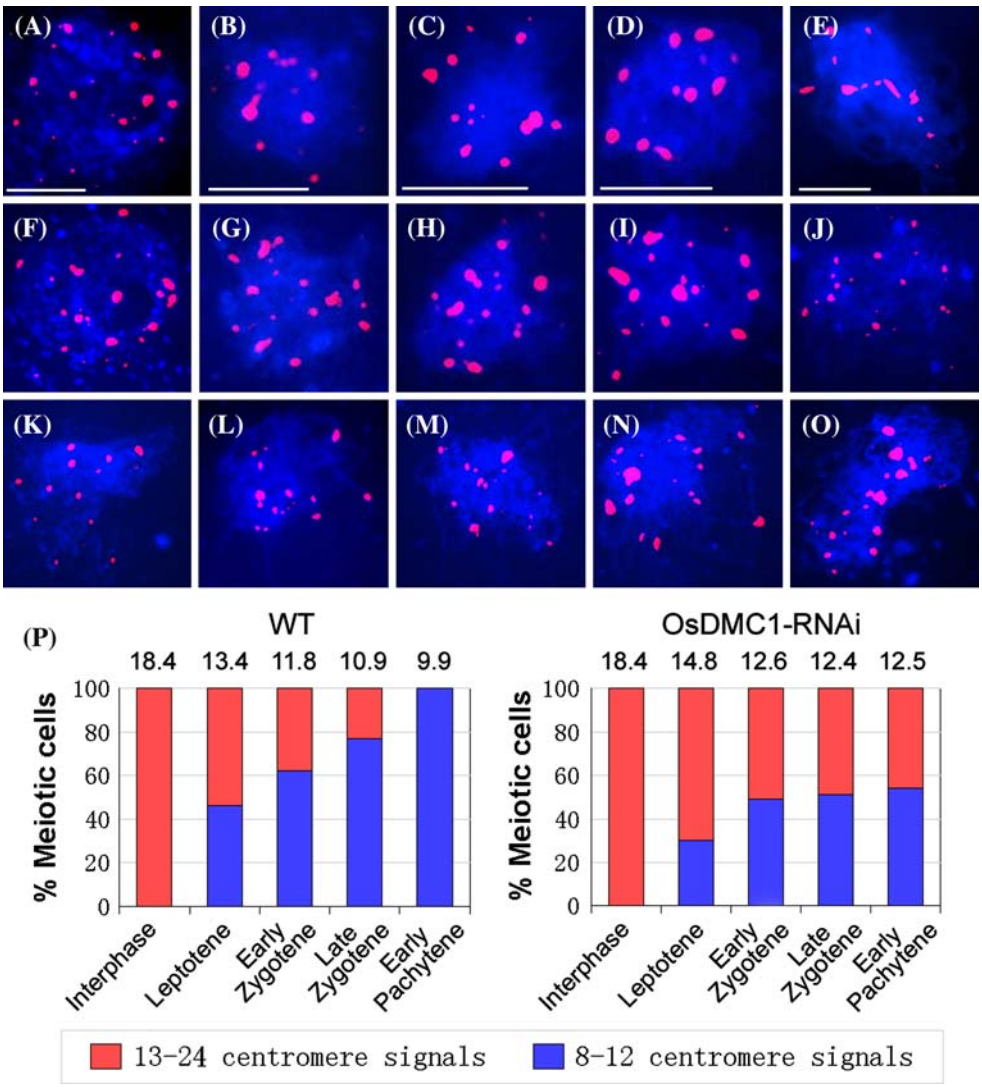

Fig. 7 FISH results with the centromeric probe pRCS2 in wild-type (A–E) and OsDMC1–RNAi (F–O) male meiocytes. Meiotic chromosome spreads were probed with the pRCS2 probes (red) and then counterstained with DPAI (blue). Stages include premeiotic interphase (A and F), leptotene (B and G), early zygotene (C and H), late zygotene (D and I), and early pachytene (E, J, and K–O). Bar =10 μm; meiotic nuclei at the same stages share the same scale. Statistical results are shown in (P) ($50 < n < 200$); the number at the top of each bar denotes the average number of the centromere loci detected per nucleus at each stage

pachytene stage showed 8–12 signals (9.9 per nucleus, on average; Fig. 7E, P). Consistent with wild-type controls, all the OsDMC1–RNAi male meiocytes at interphase had 13–24 signals (Fig. 7F, P). However, from leptotene to late zygotene stages, the proportion of cells with 8–12 signals was lower than that of the wild-type at each corresponding stage (Fig. 7P). The proportion of cells with 8–12 signals did not change from early zygotene to early pachytene stages (Fig. 7P). Finally at early pachytene stage, only 54% of the RNAi meiocytes contained 8–12 signals (Fig. 7J–O, P), while 100% of wild-type meiocytes had 8–12 signals (Fig. 7E, P). This suggests defects in pairing around centromeric regions in OsDMC1-RNAi lines. Taken together, these results clearly showed that knock-down of *OsDMC1* disrupted homologous pairing.

Discussion

OsDMC1 is essential for homologous pairing

Homologous pairing, synapsis, and recombination are essential to ensure that homologous chromosomes remain in an organized and individualized state to facilitate the exchange of genetic materials between homologous chromosomes and subsequent accurate segregation of the chromosomes in yeast and mammalian (Kleckner 2006). The yeast Dmc1 protein has been shown to be necessary for pairing and synapsis; similar roles have also been identified for the mouse Dmc1 protein (Bishop et al. 1992; Rockmill et al. 1995; Pittman et al. 1998; Yoshida et al. 1998). In plants, previous observations in male

meiotic spreads of *Arabidopsis dmc1* mutants (Couteau et al. 1999) and a comparison of nuclear distribution of the Dmc1 protein among diploid, triploid, and hybrid lilies (George et al. 2002) suggest the possible involvement of Dmc1 in pairing. Our FISH experiments revealed that knock-down of *OsDMC1* in rice caused defects in pairing. Together with the evidence that OsDmc1 is able to promote interaction between homologous DNA (Kant et al. 2005), we conclude that plant Dmc1 is also crucial for homologous pairing. These findings indicate that Dmc1 is functionally conserved among eukaryotes through evolution.

Possible links between plant Dmc1 and meiotic cell cycle control

In yeast and mouse, the precise progression of meiotic prophase I is controlled by a pachytene checkpoint; therefore, the failure of pairing, synapsis, and recombination caused by mutations in *DMC1* or other genes involved in prophase I can lead to activation of this checkpoint, which results in meiotic arrest and subsequent apoptosis (Roeder and Bailis 2000). However, complete meiotic arrest or apoptosis is not observed in plants, which suggests that plants might have a different mechanism of meiotic cell cycle control (Couteau et al. 1999; Yang et al. 2003). Recently, studies of *Arabidopsis* meiotic genes such as *TAM*, *ATM*, and *MMD1* (Magnard et al. 2001; Garcia et al. 2003; Yang et al. 2003) have demonstrated that mutations in these genes can alter meiotic cell cycle progression. These findings suggest that meiotic checkpoints may exist in plants, but the checkpoint regulation in plants seems to be much less stringent than that in yeast and mouse; thus abnormal meiocytes are able to continue through meiosis after a brief arrest (Yang et al. 2003). Because the effect of *dmc1* mutation on meiotic progression was not evaluated in *Arabidopsis*, whether the *atdmc1* mutation could trigger meiotic checkpoints or induce meiotic cell cycle alterations is unclear. Through detailed cytological analysis of male meiosis in OsDMC1–RNAi rice plants, we showed that knock-down of *OsDMC1* seemed to disrupt meiotic cell cycle progression, as evidenced by the following: (1) male meiocytes within an anther exhibited asynchronous meiotic prophase I; (2) meiosis II was asynchronous for the two daughter cells of a dyad; and (3) a few severely disrupted meiocytes were arrested at metaphase I even when normal ones entered into the uninucleate stage. Similarly, mutations in the rice *PAIR1* gene, which is required for pairing, also cause changes in proportion of each meiotic prophase I substage (Nonomura et al. 2004). These observations suggest that failure of pairing may trigger the meiotic checkpoint in plants, analogous to the situation in yeast and mouse.

Cell-cycle studies in yeast and mouse have identified two checkpoints at meiosis I: one monitors pairing, synapsis, and recombination before the pachytene stage, and the other supervises chromosome alignment on the metaphase plate prior to chromosome segregation (Page and Orr-Weaver 1997; Roeder 1997). Rice OsDMC1-deficient plants (our study) and *pair1* mutants (Nonomura et al. 2004) begin to display aberrances at zygotene stage and show changes in meiotic progression. In contrast, Arabidopsis *mmd1* mutants start to exhibit defects at diakinesis and show delayed meiotic progression and apoptosis-like phenotypes (Yang et al. 2003). Therefore, plants could have the two meiotic checkpoints as well. This hypothesis can explain the alteration of male meiotic progression in OsDMC1–RNAi lines. Activation of the first meiotic checkpoint by defects in pairing does not block meiotic division but does slow the meiotic progression. As a result, most abnormal meiocytes pass through every stage of meiosis and produce irregular spores. However, in some severely disrupted male meiocytes, these abnormalities also trigger the second meiotic checkpoint, which leads to metaphase I arrest.

Plant Dmc1 and meiotic recombination

Besides functioning in pairing and synapsis, yeast Dmc1 has a crucial role in DSB-induced recombination (Bishop et al. 1992; Pawlowski and Cande 2005). Recent biochemical studies have revealed that the rice Dmc1 protein can catalyze DNA strand exchange in vitro (Kant et al. 2005; Rajanikant et al. 2006). However, chromosome fragmentation, considered a clue for the failure to repair DSBs (Li et al. 2004), was not observed in OsDMC1–RNAi rice plants or *Arabidopsis dmc1* mutants (Couteau et al. 1999). In *Arabidopsis*, mutations in *RAD51*, another eukaryotic homologue of RecA, result in seriously disrupted meiosis and chromosome fragmentation (Li et al. 2004). The phenotypic dissimilarities indicate that in plants, Rad51 may play a major role in repair of DSBs, whereas Dmc1 may be more important for other events in recombination (Li et al. 2004). This notion seems to be supported by the fact that the two proteins colocalize on leptotene and zygotene chromosomes in lily, which suggests that their collaboration may be necessary for meiotic recombination in plants (Terasawa et al. 1995). Nevertheless, further experiments are necessary to elucidate the function of Dmc1 in meiotic recombination in plants.

In summary, our results clearly demonstrate that OsDMC1 deficiency mediated by RNAi caused abnormalities in homologous pairing in rice. These defects led to alteration in meiotic cell cycle progression but did not block meiotic division. Our findings suggest that *OsDMC1* is essential for pairing, and meiotic defects at prophase I may

induce activation of meiotic checkpoints. Therefore, this study has provided a novel insight into functional conservation and divergence of the Dmc1 protein through evolution among eukaryotes and extended our knowledge of the mechanisms underlying meiosis in plants.

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