Rice MutLγ, the MLH1–MLH3 heterodimer, participates in the formation of type I crossovers and regulation of embryo sac fertility

Bigang Mao1,2†, Wenjie Zheng2†, Zhen Huang1, Yan Peng1, Ye Shao1, Citao Liu3, Li Tang1,2, Yuanyi Hu1, Yaokui Li1, Liming Hu2, Dan Zhang1, Zhicheng Yuan1, Wuzhong Luo1, Longping Yuan1,*†, Yaoguang Liu4,5,* and Bingran Zhao1,2,3,*†

1State Key Laboratory of Hybrid Rice, Hunan Hybrid Rice Research Center, Changsha, China
2Long Ping Branch, Graduate School of Hunan University, Changsha, China
3College of Agricultural, Hunan Agricultural University, Changsha, China
4State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, South China Agricultural University, Guangzhou, China

Summary

The development of embryo sacs is crucial for seed production in plants, but the genetic basis regulating the meiotic crossover formation in the macrospore and microspore mother cells remains largely unclear. Here, we report the characterization of a spontaneous rice female sterile variation 1 mutant (fsv1) that showed severe embryo sacs abortion with low seed-setting rate. Through map-based cloning and functional analyses, we isolated the causal gene of fsv1, OsMLH3 encoding a MutL-homolog 3 protein, an ortholog of HvMLH3 in barley and AtMLH3 in Arabidopsis. OsMLH3 and OsMLH1 (MutL-homolog 1) interact to form a heterodimer (MutLγ) to promote crossover formation in the macrospore and microspore mother cells and development of functional megaspore during meiosis, defective OsMLH3 or OsMLH1 in fsv1 and CRISPR/Cas9-based knockout lines results in reduced type I crossover and bivalent frequency. The fsv1 and OsMLH3-knockout lines are valuable germplasms for development of female sterile restorer lines for mechanized seed production of hybrid rice.

Introduction

The fertility of the female reproductive system determines the grain yield and the success of heterosis. Meiosis is a key process in rice reproductive development. In the past two decades, on account of a persistent barrier to mutant acquisition and character identification, the research addressing female sterility has been slower in rice. Therefore, it will be important to explore the genes related to plant female fertility and meiosis. In most angiosperms, the development of female gametophytes (FGs) generally includes meiosporogenesis and FG formation. From megaspore mother cell (MMC) to functional megaspore (FM) is the meiotic stage. Then, though three consecutive nuclear mitosis, the FM further develops into the seven-celled embryo sac (Drews and Koltunow, 2011; Nakajima, 2018). The main studies focusing on Arabidopsis, for example, show that SPL, WUS, MAC1 are involved in regulating the transformation of somatic cells to germ cells, MRP1 in regulation of the number of MMC, and SW1, ARP6 in regulation of meiosis of MMC (Boateng et al., 2008; Lieber et al., 2011; Nonomura et al., 2003; Qin et al., 2014; Sheridan et al., 1996; Yang et al., 1999). At FG genesis, RBR, LACHESIS, BLH1, MYB64 and MYB179 are involved in the regulation of mitosis of FM and the cellularization of FG (Gross-Hardt et al., 2007; Ingouff et al., 2009; Pagnussat et al., 2007; Rabiger and Drews, 2013). In addition, AGO proteins that mediate RNA silencing, DNA methylation and chromosome modification, also involve in the formation of FGs (Law and Jacobsen, 2010; Mallory and Vaucheret, 2010; Olmedo-Monfil et al., 2010; Rodríguez-Leal et al., 2015). However, there are few reports on the genes relating to FG formation in rice.

MutL proteins were first discovered in bacteria, where they are involved in post replicative DNA mismatch repair (MMR) and the control of genetic recombination. The eukaryotic MMR complex is composed of four heterodimers containing MutSβ (MSH2–MSH3), MutSα (MSH2–MSH6), MutLα (MLH1–PM51/PM52) and MutLγ (MLH1–MLH3). Research in yeast and mammals show that, as an endonuclease, MutLγ plays a major role in meiotic recombination by forming a complex with MutSγ (Msh4–Msh5) to promote meiotic crossovers (COs) (Cannavo et al., 2020; Flores-Rozas and Kolodner, 1998; Kadyrova et al., 2020; Kolodner, 2016; Lipkin et al., 2000; Nishant et al., 2010; Ranjha et al., 2014; Wang et al., 1999).

The proper chromosomal pairing, recombination, and segregation are central to meiosis and sexual reproduction (Bai et al., 1999). The meiotic recombination is initiated by the programmed formation of DNA double-strand breaks (DSBs). The processing of DSB ends form single-end invasion (SEI) intermediates (Manhart and Alan, 2016, Mimitou and Symington, 2009). The SEIs are further processed to form double Holliday junction (dHJ) intermediates. Subsequently, dHJs are resolved exclusively into COs (Borner et al., 2004; Manhart and Alan, 2016). In most eukaryotes, the repair of meiotic DSBs yields COs (Type I COs and Type II COs) or non-crossovers (NCOs) through three major pathways. Most SEIs produced NCOs via the synthesis-dependent strand annealing
(SDSA) pathway (Manhart and Alani, 2016; Mercier et al., 2015; Wang and Copenhagen, 2018; Wang and Kung, 2002). The major pathway formed the interference-sensitive Type I COs depend on ZMM group proteins (Zip1-4, Mer3, Msh4-5) in S. cerevisiae, as well as MLH1 and MLH3 in Arabidopsis (Borner et al., 2004; Mercier et al., 2015). The MutSγ stabilizes invasion intermediates and dHJs. MutLγ act with Exo1 and Sgs1 helicases to resolve recombination intermediates forming Type I COs (Rogacheva et al., 2014). In the Arabidopsis zmm mutants AtMsh4 and AtMsh5, COs are strongly reduced to about 15% of the wild-type level (Chelysheva et al., 2007; Chelysheva et al., 2012; Higgins et al., 2004; Higgins et al., 2008b). The rice Osmshd and Osmshs5 single mutants have a residual CO frequency of ~10% and 21.9%, respectively (Luo et al., 2013; Wang et al., 2016). Arabidopsis mhl1 and mhl3 mutants’ CO frequencies are reduced to ~50% of the wild-type level, and less affected than zmm mutants (Chelysheva et al., 2012; Jackson et al., 2006). An alternative pathway producing interference-insensitive Type II COs and NCOs relies on structure-specific endonucleases MUS81-MM54 (XPF), SLX1-SLX4 (URI-YIG), and YEN1 (GEN1) (Rad22XPG) (Boddy et al., 2001; Manhart and Alani, 2016; Osman et al., 2011; Schwartz and Heyer, 2011). About 85–90% of COs (Type I) arise from the ZMM-dependent pathway, while the remaining 10–20% of COs (Type II) are from the MUS81-MM54 pathway and other unknown routes in Arabidopsis (Higgins et al., 2008a; Lambing et al., 2017; Wang et al., 2017). At present, the MLH1 and MLH3 homologs in rice have not been functionally characterized.

Vegetative growth of AtMLH3 T-DNA insertion mutants is indistinguishable from wild-type plants, but with small siliques and a reduced seed number (~50%) per siliqua. The Atmlh3 mutant has a ~60% reduction in COs, and AtMLH1 fails to localize normally in mutant (Jackson et al., 2006). The loss of functional AtMLH1 gene leads to a significant reduction in fertility in both homozygotes and heterozygotes. A strong decrease (72%) in the frequency of homologous recombination is observed in the mutant (Dion et al., 2007). The barley des10 (Hvmhl3) mutants exhibit reduced recombination and chiasmatia counts of only 37% of the wild-type level, leading to chromosome mis-segregation. Unlike AtMlh3, des10’s normal synopsis progression is also disrupted, and meiotic recombination is skewed to the ends of chromosomes (Colas et al., 2016).

Although meiosis is evolutionarily conserved, many of the underlying mechanisms show species-specific differences. The roles of MLH3 homologs have been extensively studied in yeast, animals and Arabidopsis, but their biological functions remain largely unknown in rice. Here, we isolated a rice MutL-homolog 3 gene, named OsMLH3. Through the phenotype of OsMLH3 and OsMlh1 (MutL-homolog 1) knock out lines and protein interactions, we validated that the C-termini of OsMLH3 and OsMlh1 interact to form a heterodimer to participate in the MutSγ-MutLγ COs pathway and regulate male and female fertility. The OsMlh3 mutants exhibits slightly reduced pollen fertility and severe embryo sac abortion. Based on the special function and mutant phenotype of OsMlh3, the fsv1 and OsMlh3-knockout lines may be used as female sterile restorer lines for mechanized seed production of hybrid rice in the future.

Results

Characterization of a female sterile mutant

A spontaneous fsv1 mutant of rice was identified from a rice restorer line Gui99. The mutant showed normal vegetative growth (Figure 1a), and pollen fertility was slightly decreased (as revealed by l2-KI staining) (Figure 1b-c), but the seed-setting rate was just 13.7% (vs. 86.9% in the wild type) (Figure 1d-e). The reciprocal cross tests of the mutant and wild type with full pollination showed that when the wild type was a female parent and the mutant male parent, the hybrid seed-setting rate was 77.4%, but when fsv1 was as a female the seed-setting rate was 13.2% (Figure S1a-c), indicating that the female fertility of the mutant was defective, and was therefore classed as a female sterile mutant. In the f2 population, we found that the segregation ratio of plants with normal (593) and low (270) seed setting was about 3.1 (χ² = 0.454; P > 0.05), suggesting that the female sterile phenotype is controlled by a single recessive nuclear gene.

Map-based cloning of the FSV1 gene

To isolate the mutated gene that controls the female sterile phenotype, map-based cloning was used to isolate the fsv1 locus. The target gene was roughly mapped to an interval on the long arm of chromosome 9 between SSR markers RM24751 and RM24766 using 204 F2 mutant individuals. Then, further narrowed the FSV1-containing region to a 21.8-kb interval between the markers M2 and In9-22721 on BAC clone P0489D11. There were four open reading frames (ORFs1-4) in the target region. The sequencing analysis of four ORFs both in wild type and fsv1 showed that only ORF1 in fsv1 had a single-base transition (C to A) in the 13th exon (1,899bp on CDS), forming a premature stop codon (Figure 2a). ORF1 (LOC_Os09g37930) encodes a MLH3-like DNA MMR protein, consisting of 24 exons and 23 introns, with 4170 bp of full-length mRNA containing 3588 bp of the coding region together with a 340-bp 5’UTR and 242-bp 3’UTR. LOC_Os09g37930 is a MutL-homolog 3 gene, so we named it OsMlh3, and its mutant allele in fsv1 gene as Osmlh3-1.

To verify that the putative gene is responsible for the mutant phenotype, a 12.836-kb genomic DNA fragment containing the entire LOC_Os09g37930 gene region was transformed into the fsv1 mutant. Positive transgenic T1 plants displayed normal growth morphologies and seed settings (Figure 2b-e). Thus, OsMlh3 is the target gene.

OsMlh3 is a homolog of MutL proteins of the rice MMR system

OsMlh3 harbours 1195 amino acids (aa) with a predicted molecular mass of 135 kD, including three conserved functional domains, HATPase_c_3 (22–140 aa) and DNA_mis_repair (215–349 aa) in the N-terminus, and MutL_C (951–1113 aa) in the C-terminus. Ten MLH3 proteins with significant amino acid identities were detected in six Gramineae plants, Arabidopsis, human, mouse and yeast. Evolutionarily, these proteins can be arranged into three clades (Figure S2 a). So far, the Arabidopsis AtMLH3 and Hordeum vulgare HvMLH3 have been cloned in higher plants (Colas et al., 2016; Jackson et al., 2006). Compared to OsMlh3, the 350-aa N-terminus of AtMLH3 shares 47% identity and 62% similarity, and the 393-aa C-terminus shares 46% identity and 62% similarity. In H. vulgare, the N- (1–351 aa) and C- (960–1208 aa) termini of HvMLH3 have 79.8% and 80.4% identity with OsMlh3, respectively. The MLH3’s functional domains in six Gramineae plants are highly conservative; their identities range from 58.7% to 78.6%. The similarity of key functional domains in MLH3 proteins of the rice MMR system.
The three conserved domains are present in most species, particularly a highly conserved metal-binding domain DQHA(X)2E(X)4E at the C-terminus in all species. The meiotic function of MLH3 is fully dependent on the integrity of a putative nuclease motif DQHA(X)2E(X)4E in *S. cerevisiae* (Ranjha et al., 2014; Figure S2 b).

Expression pattern of *OsMLH3* and the protein sub-cellular localization

Total RNA from various tissues was used for quantitative reverse transcription- (qRT-) PCR analysis of *OsMLH3*. The highest expression level appeared in inflorescence and leaf blades, being sequentially weaker in roots and stems in the wild-type plant (Figure 3f). The expression pattern of the *OsMLH3* promoter was detected by using promoter-GUS assay. GUS gene expression results were consistent with the qRT-PCR data. GUS signals could be detected in roots, stems, leaves and florets, especially in the ovary of florets (Figure 3a-e). The results were consistent with the qRT-PCR results. To further elucidate the spatial expression patterns of *OsMLH3*, we performed RNA in situ hybridization with wild-type floral sections. The strong hybridization signal of *OsMLH3* was observed on stamens and pistil flower primordia (Figure S3 a-d). Strong signals were detected in the ovary during the late stage of spikelet formation (Figure S3 e-f). Sub-cellular
localization test shows that the fluorescent signal of OsMLH3-linker-GFP co-localized with the nuclear marker NLS-mKate in rice protoplast (Figure 3h–k), suggesting that OsMLH3 is a nuclear localized protein.

Abortion of most Osmlh3-1 embryo sacs at meiosporogenesis stage

We compared the embryo sac development at meiosporogenesis and FG genesis stages of wild-type Gui99 and Osmlh3-1 with whole-mount stain-clearing laser scanning confocal microscopy (WCLSM). In Gui99, the megasporocyte could undergo two meiotic divisions to produce four haploid spores (Figure 4a–c), among which three at the micropylar end degenerated, and the one remaining spore formed the FM (Figure 4d). Then, the mononucleate embryo sac went through three rounds of mitosis to form a seven-celled embryo sac, which contains an egg cell, two synergid cells, three antipodal cells and one central cell with two polar nuclei (Figure 4e–j). The megasporocyte developed normally in the Osmlh3-1 mutant (Figure 4k). However, few megaspore dyads and tetrads could be observed at meiosporogenesis stage (Figure 4L–M), failing to form FMs (Figure 4n) and mature embryo sacs (Figure 4o) in Osmlh3-1. We observed a large number of mature embryo sacs, and about 82.7% (86/104) of embryo sacs were abortive with no cell differentiation. A small number (14/104) of FMs formed mature embryo sacs, which can complete normal fertilization. These results confirm that most (86.5%) of the Osmlh3-1 mutant female gametes are non-functional, resulting in a low seed-setting rate.

Cytological analysis of Osmlh3-1 reveals defects in chromosomal recombination

Since the Osmlh3-1 mutant also showed slightly reduced pollen fertility (Figure 1c), we first analysed the chromosome behaviours of pollen mother cells (PMCs) in wild type and Osmlh3-1. In the wild type, condensing chromosomes became clearly visible at leptotene (Figure 5a), homologous chromosomes were partially synapsed and concentrated at zygotene (Figure 5b). Synaptonemal complex (SC) formation was complete at pachytene (Figure 5c). SC began to disassemble and homologous chromosomes remained paired at the chiasmata at diplotene (Figure 5d), and 12 bivalents were highly condensed at diakinesis (Figure 5e) before aligning on the equatorial plate at metaphase I (Figure 5f). The homologous chromosomes segregated equally to the opposite poles of the cell at anaphase I, thus reducing the chromosome number by a half (Figure 5g). During meiosis II, the sister
chromatids separated to produce tetrads, with each cell having 12 chromosomes (Figure 5h).

In the Osmlh3-1, early prophase I from leptotene through to pachytene was similar to that of the wild type, with apparently normal chromosome pairing and full synapsis at pachytene (Figure 5i-k). Some homologous chromosomes separated from each other at diplotene (Figure 5l). Condensed chromosome number was abnormal (>12) during diakinesis, with the presence of normal bivalents and a few univalents (Figure 5m). During metaphase I, the remaining bivalents aligned on the equatorial plane, whereas the univalents were distributed randomly (Figure 5n). The bivalents separated normally at anaphase I, but the univalents segregated randomly, resulting in an unequal distribution of chromosomes in the two daughter cells (Figure 5o). The second meiotic division subsequently occurred. A tetrad with uneven chromosomes was detected. We also observed some chromosome bridge at telophase II (Figure 5p) and micronuclei at the tetrad stage (Figure 5q). Thus, multiple aberrations in microspore development led to partial pollen sterility in the Osmlh3-1 mutant. These observations demonstrate that OsMLH3 is required for normal progression through meiosis and plays a crucial role in meiosis.

Random distribution of residual chiasmata in the Osmlh3-1 mutant

It is widely acknowledged that chiasmata play a critical role in the stability of bivalents (Ma, 2006). We quantified the chiasmata frequencies at metaphase to investigate differences in bivalent and CO between the Osmlh3-1 mutant and Gui99. The Osmlh3-1 mutant had a reduced number of bivalents compared with Gui99. Statistical analysis indicated a mean bivalent frequency of 9.7 per cell (n = 32) in the Osmlh3-1 mutant, in contrast to 12 per cell in Gui99. In the Osmlh3-1 mutant, the number of bivalents ranged from 8 to 12 bivalents per cell (Figure 5q). The mean bivalent frequency was reduced by about 19.2% in the Osmlh3-1 mutant. According to criteria previously, rod-shaped bivalents are scored as having one chiasma, whereas ring bivalents have two (Sanchez Moran et al., 2001). Mean chiasmata number in the Osmlh3-1 mutant ranged from 9 to 20, averaging 14.9 per cell (n = 32) (Figure 5s), compared with 20.0 per cell (n = 30) for Gui99 (Figure 5r), the mean chiasmata frequency of Osmlh3-1 was reduced by 28.9%. The range of cell chiasma frequencies in the Osmlh3-1 mutant was wide (9–20), but the range of cell chiasma frequencies was much narrower in Gui99 (19–24), suggesting that chiasma formation was less controlled in the Osmlh3-1 mutant. The number of remaining chiasma per cell in the Osmlh3-1 mutant was not a match to a Poisson distribution (Figure 5s), indicating that chiasma were not randomly distributed among cells, whereas the distribution in the wild type deviated significantly from a Poisson model (Figure 5r). All the results show that the cross-interference may still exist in Osmlh3-1.

OsMLH3 and OsMLH1 gene knockout lines have the same phenotype as fsv1

To confirm that the fsv1 mutant phenotypes were caused by the loss of function of OsMLH3, we generated OsMLH3-knockout lines (Osmlh3-2, Osmlh3-3 and Osmlh3-4) of an indica variety.
Huazhan (HZ) by using the CRISPR/Cas9 system (Ma et al., 2015) (Figure 6a). In addition, we also obtained knockout mutants (Osmlh1-1 and Osmlh1-2) of OsMLH1 (LOC_Os01g72880), the rice MutL-homolog 1 gene (Figure 6b). These knockout lines had a similar phenotype as fsv1 (Osmlh3-1) (Figure 6c), without apparent difference in vegetative growth, except that the seed-setting rate was reduced to 10–14% of the wild-type HZ (Figure 6d-e), fertile pollen decreased slightly, and there were more abortive pollen grains (Figure 6f). In the Osmlh3 and Osmlh1 knockout lines, most megaspores could not complete meiosis and form FMs, and embryo sacs were aborted without any cell structure (Table S1). A few FMs had formed abnormal embryo sacs, for instance, single polar nuclei (Figure S5 a, k), multiple polar nuclei (Figure S5 c, n, o), double embryo sac (Figure S5 h, l), degenerate embryo sac (Figure S5 d, e), no egg apparatus (Figure S5 g), small embryo sac (Figure S5 f) and polar nuclei positional abnormalities (Figure S5 b, i, j, m), which separated from the egg cell. Consequently, 77%–84% ovules could not form mature embryo sacs.

These OsMLH3 and OsMLH1 knockout lines showed mostly normal fertile pollen, but there are still abnormalities in the process of meiosis of PMCs. Comparing the meiosis behaviours of the wild-type HZ (Figure 7a–h), the knockout lines had more randomly distributed univalents during diakinesis and metaphase I (Figure 7m, u, n, v). There was an unequal distribution of chromosomes in the two daughter cells during anaphase I, with some chromosome bridge formation at telophase II (Figure 7o, w), and micronuclei at the tetrad stage (Figure 7p, x). The mean bivalent number was 9.6 in the Osmlh3-2 mutant (Figure 7 y1), which was about 19.3% less than in the wild type. The mean chiasma number in the Osmlh3-2 mutant ranged from 8 to 21, averaging 14.1 per cell (n = 30) (Figure 7 z2), compared with 19.5 (n = 32) for HZ (Figure 7 z1), the mean chiasma frequency of Osmlh3-2 was reduced about 27.8%. In the Osmlh1-1 mutation lines, the mean bivalent number was 9.5 (Figure 7 y2), and the mean chiasma number ranged from 8 to 22, averaging 15.2 per cell (n = 30) (Figure 7 z3). The mean bivalent and chiasma frequencies of Osmlh1-1 were reduced by about 20.6% and 22.5%, respectively. All the knockout lines had similar chromosome behaviours and CO distribution as fsv1 (Osmlh3-1) (Figure 7 z1-z3). The two gene knockout lines have the same phenotype as fsv1(Osmlh3-1) mutant. These results also prove that OsMLH3 is the candidate gene, and the DNA MMR genes OsMLH3 and OsMLH1 in the same pathway regulate the fertility of rice. OsMLH3 and OsMLH1 are involved in the formation of type I COs during meiosis, and affect the development of FGs, resulting in the abortion of most embryo sacs.

The OsMLH1-OsMLH3 heterodimer participates in the regulation of rice fertility

In yeast, meiotic CO requires resolution of Holliday junctions through actions of the DNA MMR factor MLH1-MLH3 (Rogacheva et al., 2014). The conserved C-terminal helix, encoded by MLH3 exon 7, is critical for the interaction between MLH1 and MLH3 proteins in mammals (Lipkin et al., 2000). However, an
interaction between the rice OsMLH1 and OsMLH3 has not been reported. The OsMLH1 N-terminus (274 amino acids) share 30% identity and 49% similarity, and the C-terminus (359 amino acids) share 26% identity and 44% similarity with OsMLH3 (Figure 8a). OsMLH3 is a larger protein with 1195 aa, and its full-length protein is transcriptionally activated. Therefore, we truncated OsMLH3 protein for four different lengths of peptide chains by functional domains, including OsMLH3-N (1–349 aa), OsMLH3-C1 (350–1195 aa), OsMLH3-C2 (470–1195 aa) and OsMLH3-C3 (805–1195 aa). The results indicated that OsMLH3-N was not self-activated, and OsMLH3-C3 could not grow on -LTH + 5Mm 3AT or -LTHA four SD-lacking solid medium. So, OsMLH3-N and OsMLH3-C3 could be selected as interaction studies (Figure S6 a). According to the key functional areas, different regions of the truncated OsMLH1A (1–723 aa), OsMLH1B (1–162 aa), OsMLH1C (152–452 aa) and OsMLH1D (442–723 aa) were prepared in the prey vector pGADT7 (AD). Yeast two-hybrid (Y2H) assays indicated that only OsMLH3-C3 could interact with the full-length OsMLH1A and truncated OsMLH1D (Figure 8b). The OsMLH3-N did not interact with OsMLH1 (Figure S6 b). This confirms that OsMLH1 and OsMLH3 interact through the C-terminal conserved domain.

We detected instantaneous expression and interaction between OsMLH3 and OsMLH1 using bimolecular fluorescent complementary (BiFC) assay in tobacco (Nicotiana benthami-ana). YFPn-OsMLH3 and OsMLH1-YFPc could get close and produced strong interaction signals in the same sub-cellular compartment of tobacco cells (Figure 8c). We also had constructed the HA-OsMLH3-C3 and OsMLH1D: myc vectors, then carried out a co-immunoprecipitation test; we found that OsMLH3 and OsMLH1 interacted in vivo (Figure 8d). All evidence suggests that OsMLH3 and OsMLH1 function as a heterodimer that regulates male and female fertility in rice.

Discussion

fsv1 is a spontaneous mutation in the MMR gene OsMLH3

The eukaryotic MMR proteins are essential for maintaining genome integrity during mitosis and meiosis. A previous investigation showed three MutL homologs (AtMLH1, SIMLH1 and AtMLH3) are also required for homologous recombination (HR) during prophase I of meiosis (Dion et al., 2007; Franklin et al., 2006; Jackson et al., 2006). Recently, a new MutL-homolog 3 (HvMLH3) has been characterized in barley (Colas et al., 2016). With classic map-based cloning, we confirmed that a female sterile mutant (fsv1) was a single-base mutation in exon 13 of rice
MutL-homolog 3 that formed a stop codon. The fsv1 mutant showed an extremely low seed-setting rate (13%). However, the defective mutation of the orthologous gene has a halved seed-setting rate in Arabidopsis and semi-sterile phenotype in H. vulgare (Colas et al., 2016; Jackson et al., 2006). Our qRT-PCR and promoter-GUS assays showed that OsMLH3 is a constituent expression gene; the highest expression level appeared in the inflorescence. However, AtMLH3 is expressed in bud tissue but not in vegetative tissues. The same-origin gene mutation causes different phenotypes. These data suggest that the MutL-homolog 3 in rice also participates in meiosis, but its function may differ from AtMLH3 and HvMLH3.

The Osmlh3 mutants possess a particular chiasmata phenotype

The presence of univalent in diakinesis and metaphase I indicates that in some cells there are an insufficient number of COs to ensure accurate chromosome segregation. Intriguingly, a similar proportion of wild-type CO was observed in des10 (37%) as in AtMLH3 knockouts (39%) (Colas et al., 2016; Jackson et al., 2006). However, our rice survey showed that the proportions of COs were 75% and 72% in Osmlh3-1 (fsv1) and Osmlh3-2, respectively, compared with the wild-type Gui99 and HZ. The results showed that the reduction in the number of COs caused
by the loss of function of OsMLH3 in rice was much less than those in Arabidopsis and barley, and also less severe than those found in classical ZMM mutants msh4 and msh5 (from 8.3% to 21.8%) in rice and Arabidopsis (Higgins et al., 2004; Higgins et al., 2008b; Luo et al., 2013; Wang et al., 2016; Zhang et al., 2014). The cell chiasma frequency distributions in the Osmlh3-2 and Osmlh1-1 mutants did not match to a Poisson distribution, indicating cross-interference was still present in these mutants.

Unknown CO formation pathway in the Osmlh3 mutant

In addition to the ZMM-MutLc pathway, another CO pathway depends on the eukaryotic structure-specific endonucleases, including MUS81-MMS4, SLX1-SLX4, and YEN1 (GEN1) (Argueso, 2004; Manhart and Alani, 2016; Schwartz and Heyer, 2011; Teresa de los Santos et al., 2003; Zakharyevich et al., 2012). The OsGen1 mutant is completely sterile and shows an approximately 6% reduction in chiasma formation in meiosis in rice. The type II COs do not exhibit interference and account for only 10% to 20% of total COs (Wang and Copenhaver, 2018; Wang et al., 2017). The OsMLH3 or OsMLH1 gene mutations result in only about 28% reduction of chiasmata frequency, but the reduction of COs in Atmlh3 mutant is about 60%. So, it is speculated that there is a yet undescribed bypass independent of MLH1/3 and unknown genes that compensate for OsMLH3 deficiency. On the other hand, considering the interaction between the MutLγ complex and MMS4-MUS81 in yeast (de los Santos et al., 2003; Wang and Kung, 2002), it is possible that the residual CO formation in the Osmlh3 mutant is mediated via the MMS4-MUS81 pathway, and beyond OsGEN1, there may be other unknown structure-selective endonucleases participating in JMs resolution and forming COs in rice. Thus, the main meiotic crossing over route remains poorly understood in rice.
The fertility effects on both males and females are incomplete in the Osmlh3 mutant

In the literature, we found no reports about development and chromosome meiosis of embryo sacs in Atmlh3 and de10 (Colas et al., 2016; Jackson et al., 2006). Only about 13.5% of florets could form mature embryo sacs in Osmlh3-1 mutants. OsMLH3 mutation can lead to serious abortion in female organ development. Because the meiosis of MMCs in the rice ovary is difficult to observe, we judge the FG development stage through morphological characteristics. At MMCs meiosis stage, it is difficult to observe the dyads, tetrads and FMs in the ovule. We speculated that MMCs meiosis process is abnormal. Finally, most of the embryo sacs abort. Unequal chromosome segregation affects both male and female fertility. However, the number of PMCs in a spikelet is large, while there is only one FG in the ovary. Therefore, more fertile pollen grains can still be found in each spikelet, but the number of fertile embryo sacs is less in Osmlh3-1. Unlike OsMsh4, OsMsl5, and many other meiosis genes, the fertility effects are incomplete in the Osmlh3 mutant.

OsMLH3-knockout rice can probably be used as a female sterile restorer line

The utilization of hybrid rice heterosis has led to a dramatic increase in rice production in China and other Asian countries (Lv et al., 2020; Wang et al., 2018; Yuan, 2014). But high seed production cost is a barrier to the use of hybrid rice. The cost of seed production can be reduced by mechanized seed production with mixed sowing and harvesting of the hybrid parents if the male parent (restorer line) is completely or partially female sterile. The main function of OsMLH3 is to participate in rice meiosis and regulate male and female fertility in rice. The Osmlh3-1 mutant shows severe embryo sac abortion and fertile pollens, which manifests as a female sterile phenotype. MLH1-MLH3 and MLH1-PMS1 have functional redundancy in DNA mismatch repair in yeast (Kolodner, 2016). So, the MMR function might not be affected in the Osmlh3-1 mutant. Thus, the OsMLH3-knockout lines and fsv1 can be used as male parents (restorer lines) that possess mostly fertile pollen grains. For example, we have performed a preliminary seed production test by mixed sowing of the OsMLH3-knockout restorer line (and Gui99 as a comparison) with a male-sterile line, and the hybrid seed-setting rates (22.7% and 24.4%) were similar between these crosses, while the self-pollinated seed-setting rate of OsMLH3-knockout restorer line was about 14%. We believe that this mixed-sowing and harvesting technology for hybrid seed production has the valuable potential for applying in hybrid seed production.

Experimental procedures

Plant materials

A spontaneous rice female sterile variation1 mutant (fsv1) was identified from the rice restorer line Gui99. I2-KI staining was used to evaluate pollen viability. Anthers were placed in I2-KI staining buffer containing 1% (w/v) I2 in 3% (w/v) KI to stain released pollen. fsv1 crossed with the Japonica rice variety 02428 to construct an F2 genetic analysis and mapping population. For a reciprocal cross test, wild type and mutant lines were crossed using the emasculation hybrid method. The genetic transformation materials were fsv1 mutant, Indica rice cultivar Huazhan (H2) and Japonica rice cultivar Taipei309 (TP309). All plant materials were grown in experimental fields of the Hunan Hybrid Rice Research Center in Changsha, China. Gui99, fsv1, 02428, TP309 and HZ grains were obtained from the State Key Laboratory of Hybrid Rice.

Fine mapping and cloning of the OsMLH3 gene

Genomic DNA of F2 plants was extracted and used for linkage analysis with available SSR markers (McCouch et al., 2002). Molecular markers for mapping are listed in Table S2. Sequences similar to that of OsMLH3 were searched using NCBI BLAST. Sequences were aligned with the BioEdit 7.0 software. The rooted phylogenetic tree was constructed with the MEGA7.0 software. To test inferred phylogeny, we used bootstraps with 1000 bootstrap replicates (Kumar et al., 2016). Pfam (https://pfam.xfam.org/search) was used for protein domain analysis.

Vector construction and rice transformation

A 12 836-kb genomic DNA fragment containing the entire OsMLH3 gene region, 4090 bp upstream of the ATG sequence, and 683 bp downstream of the TAG sequence was cloned to generate the pCAMBIA1300-OsMLH3 complementary construct. The construct was transformed into the fsv1 mutant. The positive transgenic plants were confirmed by the Hygromycin gene using primers HPT3F and HPT3R. The complemented plants showed a bimodal position at the mutation site by sequencing with primers exon13F and exon13R. All primer pairs are listed in Table S2. Constructing gene knockout vector pYLCRISPR/Cas9-MT(I)-OsMLH3 with target connector primers Cas9-OsMLH3-F and Cas9-OsMLH3-R, pYLCRISPR/Cas9-MT(I)-OsMLH3, with target connector primers Cas9-OsMLH1-F and Cas9-OsMLH1-R, the methods and the universal primers were as previously described (Ma and Liu, 2016), and Indica variety H2 was used for genetic transformation. The knockout sites were detected by sequencing with primer pairs Osmlh3F1 and Osmlh3R1 and Osmlh1F1 and Osmlh1R1. All primer pairs are listed in Table S2.

Quantitative real-time reverse transcription-PCR (qRT-PCR)

For qRT-PCR analysis, the total tissue RNA was extracted and reverse transcribed using an RNAPrep pure Plant Kit (TIANGEN, China) and a SuperScript II kit (TaKaRa, Japan). The OsMLH3 primer pairs OsMLH3F and OsMLH3R, as well as the ubiquitin gene (Os03g0234200) as a control, are listed in Table S2. PCRs were carried out using the SYBR premix Ex TaqTM kit (TaKaRa, Japan) amplified in a Roche 480II device. The 2^ΔΔCT method was used, as previously described (Livak and Schmittgen, 2001).

GUS histochemical staining

For the promoter-GUS assay, a 3995-bp genomic fragment upstream of the OsMLH3 translation start codon was amplified with primer pairs proOsMLH3F1 and proOsMLH3R1 (Table S2), and cloned into the pCAMBIA1305 vector to generate the pOsMLH3promoter-GUS expression construct, as described previously (Mao et al., 2012). Genetic transformation with Japonica variety TP309 and GUS histochemical staining were done as described previously (Jefferson, 1987).

RNA in situ hybridization

Young spikelets were fixed overnight in an FAA (RNase-free) fixative solution at 4°C, followed by dehydration in an alcohol series of ethanol and xylene, and then embedded in paraffin. An OsMLH3 cDNA fragment was amplified using primers InOsMLH3F1 and InOsMLH3R1 (Table S2) and cloned into the
pGEMT Easy vector. The probe was then transcribed in vitro using a DIG RNA Labeling Kit (SP6/T7) (Roche) following the manufacturer’s instructions. RNA hybridization and immunological detection were done as previously described (Kouchi and Hata, 1993).

Sub-cellular localization of OsMLH3

The 3585 bp of OsMLH3 CDS was amplified with the primers gfpF1 and gfpR1 (Table S2) and cloned into the pBWA(V)HS-ccdb-GLosgfpl vector to generate the OsMLH3-GFP expression vector, then co-transfected into rice protoplasts with marker plasmid mKate containing the NLS (MDPKKKRKV) (Chiu et al., 1996; Nelson et al., 2007), incubated in darkness at 28°C for 16 h, and observed using a confocal laser scanning microscope (Zeiss LSM 880).

Preparation of embryo sacs for WCLSM

Florets at various stages were collected carefully and fixed in FAA overnight at room temperature, then washed with 50% ethanol and stored in 70% ethanol at 4°C. Experimental method was as previously described (Huang et al., 2017). The oocytes were scanned with a confocal laser scanning microscope (Zeiss LSM 880). The excitation wavelength was 543 nm, and the emission wavelengths were 550–630 nm.

Meiotic chromosome examination

During the rice meiosis stage, everyday morning 8:00–10:00 or afternoon 16:00–18:00, young panicles (40–60 mm) of both wild type and mutants were harvested and fixed in Carnot’s solution (ethanol: glacial acetic acid = 3:1) at room temperature for 24 h, then washed with 70% ethanol and stored in 70% ethanol at 4°C. Experimental operation was as previously described (Wang et al., 2014). The male meiocyte chromosomes were observed using a fluorescence microscope (Zeiss Axio Imager M2).

Y2H assay

For Y2H screening, the OsMLH3-N and OsMLH3-C3 were cloned into the vector pGBK7 using primer pairs OsMLH3-F (EcoRI) and OsMLH3-NR1 (BamHI) and OsMLH3-C3F (EcoRI) and OsMLH3-C3-R (BamHI), respectively. OsMLH1A, OsMLH1B, OsMLH1C and OsMLH1D were cloned into the vector pGADT7 using primers OsMLH1-BF (EcoRI) and OsMLH1-DR (BamHI), OsMLH1-BF (EcoRI) and OsMLH1-DR (BamHI). The NH109 yeast strain (S. cerevisiae) was transformed with appropriate ‘bait’ and ‘prey’ plasmids according to the Clontech yeast transformation protocol. Yeast strains were grown on SD-Trap-Leu plates for 3 d at 30°C, and then spotted on the selective plates of SD-Trap-Leu-His-Adr plus X-a-gal. The plates were incubated for 5 d at 30°C. Positive ‘bait’–‘prey’ interactions were detected by blue colours revealed by the β-galactosidase reporter expression yeast. Yeast strains containing ‘bait’ or ‘prey’ plasmids combined with pGAD77 or pGBK7 were used as negative interaction controls. All primer pairs are listed in Table S2.

BiFC assay

The BiFC approach was done as described previously (Waadt et al., 2008). The full-length CDS of OsMLH3 was cloned into pCAMBIA1300-35S-YFPn to generate the YFPn-OsMLH3 construct using primers YN-OsMLH3-BamHI-F and YN-OsMLH3-Sall-R. The full-length CDS of OsMLH1 was cloned into pCAMBIA1300-35S-YFPC to generate the OsMLH1-YFPC construct with primers YC-OsMLH1-BamHI-F and YC-OsMLH1-Sall-R. Next, agrobacterium strains carrying the BiFC constructs were infiltrated into leaves of 5- to 6-week-old N. benthamiana plants, as previously described (Llave et al., 2000). YFP fluorescent signals were monitored using a laser confocal scanning microscope (Olympus FV1000). All primer pairs are listed in Table S2.

Co-immunoprecipitation assay

OsMLH3-C3 was cloned into pCAMBIA1300-35S-HA to generate the HA-OsMLH3-C3 construct using primers HA-OsMLH3-C3-EcoRIF and HA-OsMLH3-C3-SallR. OsMLH1D was cloned into pCAMBIA1300-35S-myc to generate the OsMLH1D-myc construct using primers OsMLH1D-myc-BamHI and OsMLH1D-myc-SallIR. The plasmid was transferred into Agrobacterium tumefaciens EHA105. When the concentration of the bacterial solution OD600 was 0.8, a mix of OsMLH1D-Myc-HA-OsMLH3-C3 (1:1) was injected into N. benthamiana. After being cultured for 72 h, all proteins were extracted from the injected leaf, co-precipitated by immunoprecipitation with 50 µL Myc medium, and detected by western blotting with HA antibody. HA-OsMLH3-C3 alone was used as a negative control. All primer pairs are listed in Table S2.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

B.Z., Y.L. and L.Y. designed the experiments. B.M. and W.Z. carried out most of the experiments. Z.H., Y.P., Y.S., C.L and L.T assisted in gene cloning and Y2H assay. Y.H., Y.L., L.H. and D.Z assisted in phenotypic identification and protein interaction tests. Z.Y. and W.L. assisted in field management. B.M. wrote the manuscript. Y.L. and B.Z. revised and approved the final version of the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.