OsHUS1 Facilitates Accurate Meiotic Recombination in Rice

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Abstract

Meiotic recombination normally takes place between allelic sequences on homologs. This process can also occur between non-allelic homologous sequences. Such ectopic interaction events lead to chromosome rearrangements and are normally avoided. However, much remains unknown about how these ectopic interaction events are sensed and eliminated. In this study, using a screen in rice, we characterized a homolog of HUS1 and explored its function in meiotic recombination.

Introduction

Meiosis is a highly dynamic process in which chromosomes undergo dramatic structural changes and movements [1,2]. During the course of meiosis, intimate interactions develop between homologous chromosomes. Among these interactions, homologous recombination (HR) and pairing are the core events that occur during the production of functional gametes [3,4]. Meiotic recombination is a powerful determinant that creates genetic diversity and provides mechanical stability for the accurate separation of homologous chromosomes. Therefore, meiotic recombination has a strong bias towards homologous chromosomes rather than sister chromatids and is mediated by a complex mechanism [5,6]. After DNA replication in the premeiotic S phase, a proteinaceous axis is assembled between two chromatids. Homologous recombination then occurs along the chromosomes, beginning with the formation of programmed double strand breaks (DSBs). In conjunction with the initiation of recombination, homologous chromosomes begin to align in pairs.

Studies have shown that for most species, homologous pairing depends on homologous recombination [7]. However, recombination not only occurs between allelic DNA sequences on homologs, but it also frequently occurs between dispersed non-allelic DNA segments that share high sequence similarity [8]. The latter recombination pattern is usually referred to as ectopic recombination (ER, also known as non-allelic homologous recombination). As eukaryotic genomes are rich in repeated DNA sequences, ER can produce chromosomal rearrangements, which in humans result in numerous genomic disorders [9,10]. Despite the adverse impact of ER on genome integrity, ER occurs relatively frequently; in budding yeast, the frequency of ER is roughly on par with that of allelic recombination [11,12]. To avoid the deleterious consequences of ER, cells have evolved multiple strategies to suppress ER formation [13]. One strategy is preventing DSB formation in or near DNA repeats. In budding yeast (Saccharomyces cerevisiae), suppression of DSBs in rDNA repeats depends strongly on silent information regulator 2 (Sir2), which encodes a histone deacetylase that promotes the formation of a closed, compact chromatin structure in the rDNA and other regions. Sir2 may suppress DSBs in rDNA in part through the deleterious consequences of ER, cells have evolved multiple strategies to suppress ER formation [13]. One strategy is preventing DSB formation in or near DNA repeats. In budding yeast (Saccharomyces cerevisiae), suppression of DSBs in rDNA repeats depends strongly on silent information regulator 2 (Sir2), which encodes a histone deacetylase that promotes the formation of a closed, compact chromatin structure in the rDNA and other regions. Sir2 may suppress DSBs in rDNA in part through the formation of a nucleosomal conformation that is not permissive for SPO11 activity [14]. The second strategy is preventing the use of non-allelic homologous templates for recombination and/or favoring the use of allelic templates. In budding yeast, homologous alignment and synopsis restrict the ability of ectopically located sequences to find each other and recombine [15]. There are also reports on the competition between normal allelic recombination and ER [16]. As both mechanisms involve preventing ectopic...
Role of Rice HUS1 in Meiosis

Meiosis is a special type of cell division that generates gametes for sexual reproduction. During meiosis, recombination not only occurs between allelic sequences on homologs, but also between non-allelic homologous sequences at dispersed loci. Such ectopic recombination is the main cause of chromosomal alterations and accounts for numerous genomic disorders in humans. To ensure genomic integrity, those ectopic recombinations must be quickly resolved. Despite the importance of ectopic recombination suppression, the mechanism underlying this process still remains largely unknown. Here, using rice as a model system, we identified the rice HUS1 homolog, a member of the RAD9-RAD1-HUS1 (9-1-1) complex, and elucidated its roles in meiotic recombination. In OsHUS1, vigorous ectopic interactions occur between nonhomologous chromosomes, and the number of crossovers is reduced. We suspect that OsHUS1 participates in regulating ectopic interactions during meiosis, probably by forming the canonical RAD9-RAD1-HUS1 (9-1-1) complex.

These mutants were found to be mutated in the functional homolog of fission yeast and mammalian HUS1. In the Oshus1 mutants, meiotic homologous pairing took place normally during prophase I, while nonhomologous chromosomes interacted vigorously as well. Multivalents were frequently found to be arranged on the equatorial plate at metaphase I. Chromosome bridges and fragments occurred at anaphase I and telophase I, rendering the mutants completely sterile. These results suggest that OsHUS1 might specifically function in sensing and removing aberrant associations between non-allelic sequences during meiosis, probably via the 9-1-1 complex.

Results

Cloning of OsHUS1

Among our rice sterile mutant libraries, 16 lines with phenotypes meeting the criteria mentioned above were isolated. One of the mutant lines, S7678, which was derived from Nipponbare (a japonica cultivar) tissue culture, was selected for further study. Based on information about its mutation (see below), the mutant was named Oshus1-1. The Oshus1-1 plants did not exhibit defects in vegetative growth under natural growth conditions, except for total male sterility (Figure S1). Fertile plants and sterile plants from the progeny of Oshus1-1+/− produced a 3:1 segregation ratio (fertile: 214; sterile, 66), which established this mutant as a single recessive mutant (χ² = 0.30; P = 0.5). When we pollinated the mutant flowers with wild-type pollen, the mutant did not set seed, indicating that female fertility is also affected in this mutant.

We isolated Oshus1 by map-based cloning. A mapping population was constructed by crossing Oshus1-1+/− plants to Nanjing 11 (an indica cultivar) plants. The mutant gene locus was mapped to a physical region of approximately 100 kb on the long arm of chromosome 4. According to information obtained from the public database (Rice Genome Annotation Project, http://rice.plantbiology.msu.edu), we sequenced several genes in this region. As a result, a point mutation (A to T) was found in the gene Os04g44620, which introduced a stop codon (AAG to TAG) in the second exon. We named the mutant Oshus1-1 based on the homology of the protein sequence.

Next, we isolated another mutant from Huanghuazhan (an indica cultivar), which has the same phenotype as that of Oshus1-1. Using map-based cloning and DNA sequencing, we found that this mutant carries a ten-nucleotide deletion in the fourth exon of OsHUS1, causing frame shift and premature stop codon formation. We named this allele Oshus1-2. The chromosome behavior in Oshus1-2 meiocytes was the same as Oshus1-1 (Figure S2A).

We generated a gene-specific p35S OsHUS1-RNAi construct and used it to transform Yandao 8 (a japonica cultivar) rice. Most Oshus1-RNAi lines showed a severe reduction in fertility (93%, n = 30), and the chromosome behavior in the male meiocytes of these lines mirrored that of Oshus1 (Figure S2B). From these results, we conclude that the mutation in the Oshus1 gene led to the sterility phenotype.

Characterization of Oshus1

There are three full-length cDNA sequences of Os04g44620 published in the Rice Genome Annotation Project website, including AK107445, AK101159, and AK060420. Using RT-PCR and RACE (rapid amplification of cDNA ends) on young panicles, we found that AK060420 is the correct sequence for this gene. Alignment of the cDNA sequence with the genomic sequence revealed that OsHUS1 is composed of six exons and five introns (Figure S3). The open reading frame of OsHUS1 has a

Author Summary

Meiosis is a special type of cell division that generates gametes for sexual reproduction. During meiosis, recombination not only occurs between allelic sequences on homologs, but also between non-allelic homologous sequences at dispersed loci. Such ectopic recombination is the main cause of chromosomal alterations and accounts for numerous genomic disorders in humans. To ensure genomic integrity, those ectopic recombinations must be quickly resolved. Despite the importance of ectopic recombination suppression, the mechanism underlying this process still remains largely unknown. Here, using rice as a model system, we identified the rice HUS1 homolog, a member of the RAD9-RAD1-HUS1 (9-1-1) complex, and elucidated its roles in meiotic recombination. In OsHUS1, vigorous ectopic interactions occur between nonhomologous chromosomes, and the number of crossovers is reduced. We suspect that OsHUS1 participates in regulating ectopic interactions during meiosis, probably by forming the canonical RAD9-RAD1-HUS1 (9-1-1) complex.
length of 981 bp, encoding a 326 amino-acid peptide. Using BLASTp, we found that OsHUS1 shares some similarity (approximately 25% identity and 45% similarity) with the HUS1 protein in mammals and fission yeast (Figure S4). Reciprocal BLAST searches further confirmed that the isolated protein is the closest relative of HUS1 in rice (Figure S5).

As shown above, there were several defects during meiosis in Oshus1. We then examined the spatial and temporal expression patterns of OsHUS1. Using quantitative RT-PCR, we found that OsHUS1 could be detected as early as the seedling stage. In adult-stage rice, Oshus1 was expressed not only in young panicles but also in vegetative organs such as leaves, roots, and internodes (Figure S6), with the highest expression observed in leaf blades.

Chromosome behavior in Oshus1-1

The behavior of meiotic chromosomes was revealed by 4′,6-diamidino-2-phenylindole (DAPI) staining. In wild-type pollen mother cells (PMCs), meiosis began with chromosome condensation and the appearance of chromosomes as thin, thread-like structures at leptotene (Figure 1A). As zygotene progressing, homologous chromosomes underwent pairing and synapsis (Figure 1B). During pachytene, homologous pairing culminated in the formation of synaptonemal complexes (SCs; Figure 1C).

After the disassembly of the SC at diplotene, the resulting 12 bivalents were further condensed, revealing the presence of chiasmata at diakinesis (Figure 1D). At metaphase I, the bivalents were aligned in the middle of the cell (Figure 1E). Homologous chromosomes separated and migrated toward opposite poles at anaphase I and telophase I (Figure 1F and 1G), generating dyads at early pachytene. At first glance, almost all homologous chromosomes were aligned perfectly and exhibited “bubble-like” structures (Figure 2C). During middle pachytene stage, associations between nonhomologous chromosome were observed in all PMCs (n = 252), which caused the chromosomes to stick to each other (Figure 2D). At late pachytene, this type of association became more prominent (Figure 2E–H). At diakinesis, multivalents were detected in all PMCs (n = 521). These multivalents ranged in size from associations of four chromosomes to the extreme case of 24 chromosomes (Figure 3A, 3E); the average number of bivalents per cell was only 1.6. At metaphase I, multivalents and bivalents were located on the equatorial plate due to the drag force exerted on centromeres by spindle fibers (Figure 3B, 3F). During anaphase I, the multivalents and bivalents fell apart, and extensive chromosome bridges and segments were observed (Figure 3C, 3G). At telophase I, two masses of chromosomes arrived at opposite poles of the nuclei, and several distinct dot-like chromosome fragments still remained on the equatorial plate (Figure 3D, 3H). In a few cells (4%, n = 881), up to 10 or 11 homolog pairs could be individualized at diakinesis and metaphase I. We also found some cells with a small amount of chromosome bridges and fragments at anaphase I and telophase I (Figure 3E, F). These types of defects were maintained during meiosis II, and no normal tetrad was produced.

Synapsis is incomplete in most Oshus1-1 meiocytes

By performing DAPI staining of pachytene chromosomes, we found that in Oshus1-1, homologous chromosomes could pair normally. To validate whether normal SCs were affected by the mutation of Oshus1, we performed immunofluorescent examination using antibodies against ZEP1 in Oshus1-1 PMCs. ZEP1 is the transverse filament protein of SC in rice and hence, a perfect tool to mark the course of synapsis [30]. In leptotenic and zygotenic Oshus1-1 PMCs, the ZEP1 patterns appeared as dots and short fragments, which were identical to those of the wild-type (Figure 4A and 4B). During pachytene, only approximately 10% (n = 300) of the meiocytes showed full-length ZEP1 signals along the homologous chromosomes (Figure 4C). In the remaining 90% of meiocytes, linear ZEP1 signals extended and could be detected along almost the entire chromosomes, with the exception of a few discontinuities/gaps, some of which exhibited the “bubble-like” structures mentioned above (Figure 4D and 4E). The discontinuities/gaps of ZEP1 signals indicate that the SC integrity might be slightly affected by the mutation of OsHUS1.

Oshus1 mutants show a reduced number of bright HEI10 foci

Many incidents during meiosis are believed to be interdependent, e.g., pairing is recombination-dependent in mammals and higher plants. The nearly normal SC assembly observed in Oshus1 meiocytes is reminiscent of the proper loading of important factors involved in SC assembling and homologous recombination. To further verify the relationship between OsHUS1 and several other meiotic recombination factors, immunodetection was carried out in Oshus1-1 using antibodies against PAIR3, PAIR2, OsZIP4, OsMER3, and HEI10.

PAIR2 is the rice homolog of yeast HOP1 and Arabidopsis ASY1, which associates with unpaired chromosome axes at early meiosis I. PAIR3 is also an axis-associated protein that can bind both unpaired chromosomes and paired chromosomes. Both PAIR2 and PAIR3 are usually utilized to mark the meiotic chromosome axis, and they also play fundamental roles in the recombination process [31–33]. OsMER3 and OsZIP4 are members of the ZMM protein family and are essential for early meiotic HR in rice [34,35]. In the Oshus1-1 mutant, PAIR2 appeared as foci at leptotene and associated with the chromosome axis as linear

Figure 1. Meiosis in the wild type. (A) Leptotene. (B) Zygote. (C) Pachytene. (D) Diakinesis. (E) Metaphase I. (F) Anaphase I. (G) Telophase I. (H) Dyad. (I) Tetrad. Bars, 5 μm. doi:10.1371/journal.pgen.1004405.g001

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signals at early zygotene (Figure 5A). PAIR3 signals were first observed as dots at early leptotene and then elongated gradually along the entire lengths of the chromosomes during zygotene (Figure 5B). The appearance of OsMER3 and OsZIP4 commenced at early leptotene, and the number of OsMER3 foci (average 257±15, n = 44, range 221–281) and OsZIP4 foci (average 299±22, n = 35, range 289–328) reached its peak at early zygotene (Figure 5C and 5D); similar results were obtained in the wild-type. At pachytene, both OsMER3 and OsZIP4 decreased rapidly and no signals were found in the later stages in the wild-type and Oshus1-1. The normal loading patterns of these four meiotic factors showed that early HR in Oshus1-1 is not disturbed.

Previous studies suggest that the interference-sensitive pathway accounts for most of the crossovers (COs) in rice [34–36]. We thus wanted to know whether interference-sensitive COs were affected by the mutation of OsHUS1. The HEI10 prominent foci correspond to the interference-sensitive CO sites in rice [36]. We counted the number of HEI10 foci (average 16.9±1.9, n = 17, range 13–20) in Oshus1-1 (Figure 5E) and compared that with the corresponding data for the wild-type (average 24.5±1.8, n = 30, range 22–28). We found that the mean number of HEI10 bright foci of Oshus1-1 was significantly reduced compared with that of the wild-type ($t_{45} = 13.8, P < 0.01$). Therefore, the number of interference-sensitive COs is reduced in Oshus1-1 due to the loss of OsHUS1.

### Ectopic associations in Oshus1-1 are dependent on PAIR1 but independent of OsRAD51C

Meiotic recombination is initiated by the formation of DSBs, which is catalyzed by SPO11 proteins; these proteins have been identified in budding yeast, Arabidopsis, and animals [37]. However, to date, no spo11 mutants have been isolated in rice [38]. Recently, three new proteins that are also implicated in DSB formation.
formation were reported in *Arabidopsis*, i.e., PRD1, PRD2, and PRD3 [39,40]. Among these, PRD3 is thought to be the homolog of rice PAIR1. Furthermore, the phenotype of the pair1 mutant (asynaptic, with no bivalent formation) is reminiscent of the phenotype observed in a mutant lacking DSBs [41]. We isolated an asynaptic mutant (Figure 6A–D), and it was proven to be a new allele of pair1. Then, pair1 Osus1-1 double mutants were generated using this new pair1 allele. The double mutants showed a typical pair1 phenotype, i.e., an absence of bivalents and lack of chromosome fragments at anaphase I (Figure 6E–H). Therefore, ectopic interactions, as well as chromosome fragmentations in Osus1-1, require the formation of DSBs.

To learn whether OsHUS1 is involved in DSB repair pathway in rice meiosis, we generated Osrad51c Osus1-1 double mutants. OsRAD51C, like its functional homolog AtRAD51C, is essential for meiotic DSB repair [42–44]. In the Osrad51c mutant, homologous pairing and synapsis were defective at zygotene and pachytene, and univalents were observed at diakinesis and metaphase I (Figure 6I–K). In anaphase I, all of the univalents broke into fragments without any chromosome associations and scattered randomly in the nucleus (Figure 6L). These defects are consistent with the role of Osrad51c in meiotic DSB repair. In the Osrad51c Osus1-1 double mutant, a cumulative effect of the two single mutations was detected; homologous pairing was disrupted, and ectopic chromosome associations were detected in all meiocytes observed. (Figure 6M–O; n = 322). At anaphase I, extensive chromosome fragments were also produced (Figure 6P). Therefore, the occurrence of ectopic interactions in Osrad51c Osus1-1 suggests that ectopic interactions between nonhomologous chromosomes do not require OsRad51C.

OsCOM1 functions both in promoting homologous recombination and in resolving chromosome entanglements [45]. In the Ocom1 mutant, both homologous pairing and synapsis were abolished at pachytene (Figure 6Q), and aberrant nonhomologous associations were detected. From diakinesis to metaphase I, the most obvious phenotype was an entangled chromosome mass (Figure 6R, S). At anaphase I, chromosome fragments were generated (Figure 6T). We also generated Ocom1 Osus1-1 double mutants. The phenotype of the Ocom1 Osus1-1 double mutant could not be distinguished from that of the Osus1 single mutant (Figure 6U–X), suggesting that OsHUS1 might function after OsCOM1 during meiosis. Of course, we cannot exclude the possibility that the ectopic interaction phenotype of Osus1 might be hidden by the severe chromosome entanglement of Ocom1.

**Ectopic associations in Osus1-1 are independent of OsMER3 and ZEP1**

Since most COs in rice are derived from the interference-sensitive pathway, we set out to study the relationship between ectopic interactions and interference-sensitive COs. To this aim, the Osmer3 Osus1-1 double mutant was generated, and its homologous behaviors were investigated. In Osmer3, fully aligned chromosomes were detected during pachytene (Figure 7A), indicating the homologous pairing is not affected by the mutation of OsMER3. However, during diakinesis and metaphase I, the mutant cells showed a mixture of both univalent and bivalent chromosomes (Figure 7B, C). In anaphase I, the bivalents separated normally but the scattered univalents segregated randomly (Figure 7D). Intriguingly, in the Osmer3 Osus1-1 mutant, homologous pairing was not observed at pachytene stage (Figure 7E). FISH experiments further confirmed that homologous pairing was disrupted in Osmer3 Osus1-1 meiocytes (n = 101, Figure S7). In diakinesis and metaphase I, both multivalents with ectopic interactions and univalents were detected in all meiocytes (n = 122, Figure 7F, G). The multivalents contained an average of 7.0 associated chromosomes (ranging from 2 to 22); the average number of univalents per cell was 8.2 (ranging from 0 to 16). At anaphase I, both univalents and multivalents were pulled toward two poles of the nucleus. Additionally, chromosome bridges and fragments were also found at this stage (Figure 7H). These results suggest that ectopic interactions in Osus1 arise independently from the OsMER3-mediated pathway.

To determine whether the defects in Osus1 are affected by synapsis, we also generated the zep1 Osus1-1 double mutant. In
the zep1 mutant, synapsis was totally disrupted, but 12 bivalents were present at metaphase I and segregated normally at anaphase I (Figure 7I–L). In the zep1 Oshus1-1 double mutant, homologous chromosomes aligned along the entire length of the chromosome, but the SC was not assembled (Figure 7M). However, ectopic interactions were still clearly observed in all meiocytes (n = 298, Figure 7N–P). These results indicate that ectopic interactions are likely independent of synapsis in Oshus1-1.

OsHUS1 localizes to meiotic chromosomes during early prophase I

To further elucidate the role of OsHUS1 in meiosis, we prepared polyclonal antibodies in mice against the entire length of recombinant, His-tagged OsHUS1. Using antibodies against OsREC8 and OsHUS1, we performed dual immunofluorescence staining in rice PMCs. OsREC8, the cohesin protein in rice, was used to indicate the meiotic chromosome axes in this study [34,46]. During leptotene, OsHUS1 proteins appeared as discrete foci in the nuclei and were loaded on the chromosome axes, as indicated by their full colocalization with OsREC8 (Figure 8A). The intensity of OsHUS1 then reached its peak at early zygotene, but this protein still appeared as foci rather than short lines (Figure 8B). At late zygotene, the number of OsHUS1 foci decreased, and many of them fell off the chromosomes (Figure 8C). At pachytene, the OsHUS1 immunostaining signal was completely absent in the nuclei (Figure 8D). No OsHUS1 signal was observed in male meiocytes of Oshus1-1, which confirmed the specificity of the OsHUS1 antibody (Figure 8E).

To further investigate the function of OsHUS1 protein, the immunolocalization pattern of OsHUS1 was investigated in Osmer3, zep1, and pair1 mutants. The localization pattern of OsHUS1 was not obviously affected in Osmer3 or zep1 (Figure S8A, B). This result is consistent with the observation that no ectopic interaction was found in either of the mutants. On the contrary, in the pair1 mutant, we failed to detect any OsHUS1 signals (Figure S8C), implying that the function of OsHUS1 depends on the formation of DSBs.

Oshus1-1 seedlings are hypersensitive to mitomycin C

In yeast and mammals, HUS1 protein is implicated in various DNA damage response pathways [47–51]. In rice, OsHUS1 has the highest expression abundance in leaves, suggesting that this protein, like its counterparts in yeast and mammals, is potentially involved in the mitotic DNA damage response. To address this possibility, we tested whether Oshus1 plants showed higher
sensitivity to mitomycin C (MMC), a DNA cross-link agent, than wild-type plants. Surface-sterilized seeds from wild-type and Oshus1-1/+ plants were sown on solid 1/2 MS medium containing 0 or 20 μg/ml MMC. When planted on medium lacking MMC, the development of wild-type seedlings was identical to that of progeny derived from an Oshus1-1/+ plant. However, when treated with MMC, the development of wild-type seedlings was only slightly suppressed, while approximately one-quarter of the progeny derived from the Oshus1-1/+ plants showed severe growth retardation (Figure 9). Using a PCR genotyping assay, we determined that all of the severely growth-retarded seedlings were Oshus1-1/2 (n = 20). These data demonstrate that Oshus1-1 rice is hypersensitive to MMC, indicating that OsHUS1 plays an important role in somatic DNA damage repair.

### Discussion

OsHUS1 is involved in somatic DNA damage responses

HUS1 is thought to form a PCNA-like complex with its two partners, RAD9 and RAD1 [23]. HUS1 has been intensively investigated in yeast and mammals, with studies primarily focusing on the mitotic DNA damage response. A mutation in MEC3 (the HUS1 counterpart in budding yeast) results in delayed entry into the S phase and slow DNA replication in response to DNA damage-inducing agents [52]. Fission yeast lacking HUS1 also fails to arrest the cell cycle after DNA damage or the blocking of DNA synthesis [53]. Targeted disruption of mouse HUS1 causes embryonic lethality due to the accumulation of chromosome breaks [49].

In this study, we found that rice hus1 seedlings were hypersensitive to the genotoxin MMC, suggesting that OsHUS1 has a conserved function in somatic DNA repair. Expression data for OsHUS1 show high accumulation of its transcript in somatic tissues, which further supports the somatic role of this protein. These findings are also in agreement with the hypothesis that OsHUS1 in rice is the functional homolog of fungal and animal HUS1. By performing a BLASTp search, we found that the homologs of S. pombe RAD9 and RAD1 also exist in rice. In addition, RAD9 is also involved in the regulation of DNA damage repair in the model plant Arabidopsis [54]. Therefore, it is highly possible that OsHUS1 in rice, like its yeast and animal counterparts, also participates in somatic DNA repair responses by forming the 9-1-1 complex.

OsHUS1 may be required for the suppression of ectopic interactions during meiosis

Studies in yeast and humans have revealed parallels between meiotic ER and allelic recombination, such as the observation that both processes occur during prophase I and are initiated by programmed DSBs. ER also results in crossover formation, which can affect genome stability during gametogenesis [12,13,55]. Therefore, ER should be inhibited, and/or its intermediates must be quickly eliminated, to ensure accurate homolog segregation during meiosis.

The function of the 9-1-1 complex in suppressing ER was first suggested in yeast [26]. However, to our knowledge, this function has not been reported in higher organisms, likely due to the lack of cytological evidence. Here, in Oshus1 meiocytes, we noticed that at late pachytene, one homolog pair frequently adhered or fused to another homolog pair at several sites, forming cross-like shapes. At the pachytene to diplotene transition (in which homologous pairs began to separate partially due to SC disassembly), the associations became more pronounced. The most remarkable defects observed in Oshus1 meiosis were multivalents at metaphase I and subsequent chromosome fragmentation.
The chromosome behaviors observed in the pair1 Oshus1-1 double mutant indicate that ectopic interactions rely on meiotic DSBs in Oshus1-1, which supports the notion that ectopic and allelic interactions share a common mechanism [36]. DSB formation is essential for homologous chromosome pairing in meiosis [3]. Here, although strong ectopic interactions occurred in Oshus1, homologous pairing took place normally. The nearly perfect ZEP1 signals along the entire lengths of chromosomes at pachytene indicated that synapsis was not severely disturbed in Oshus1. In addition, OsZIP4 and OsMER3 localized normally in Oshus1. It is likely that the early ectopic intermediate-preventing system may function well, and excessive ectopic interaction initiations are prevented in a timely manner in Oshus1. Intriguingly, unlike the Oshus1 and Osmer3 single mutants, the Oshus1 Osmer3 double mutant exhibited disrupted homologous pairing. In light of the competition between allelic and ectopic recombination [57] and the important roles they play during homologous pairing [3], it is attractive to consider that the increase in ectopic interactions and the decrease in allelic associations reduce the chance of homolog recognition and subsequent homolog alignment. Since homolog alignment mainly occurs at zygotene stage, it is reasonable to postulate that ectopic interactions initiate during or prior to zygotene. This hypothesis is consistent with the view that in yeast, ectopic recombination occurs concurrently with allelic recombination during meiosis [13].

Studies in budding yeast have revealed that ER occurs frequently during meiosis [11,12]. However, it remains unknown whether ER also occurs frequently during plant meiosis. Here, we observed that all meiocytes showed the presence of multivalents in Oshus1. We therefore propose that in wild-type meiocytes, early ectopic interactions, accompanied by allelic interactions, may inevitably occur during homolog searching and homolog recognition. Once homolog recognition is accomplished, those ectopic interaction intermediates might be quickly detected and resolved by the surveillance mechanism. OsHUS1 is likely to be an important component of the surveillance mechanism that specifically eliminates ectopic interaction intermediates during meiosis.

Figure 7. DAPI staining of Osmer3, Osmer3 Oshus1-1, zep1 and zep1 Oshus1-1 mutant. (A–D) Osmer3. (E–H) Homologous pairing is disrupted while ectopic associations are remained in Osmer3 Oshus1-1. (I–L) zep1. (M–P) The zep1 Oshus1-1 double mutant exhibiting occurrence of ectopic association. Bars, 5 μm.

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Ectopic associations are independent of the interference-sensitive CO pathway

In budding yeast, a physical assay revealed that levels of ER increase from 1% in wild-type to 3–5% in rad17, rad24, and mec1-1 single mutants. HR is also reduced approximately two-fold in these mutants, from 25–30% in wild-type to 15% in rad17, rad24, and mec1-1. These data indicate that the increase in ER does not quantitatively account for the decrease in HR. Therefore, ER and HR likely occur via different pathways [26]. Here, we demonstrated that the loss of OsMER3 function did not affect ectopic interactions (through characterization of Osmer3 Oshus1-1), implying that these ectopic interactions do not arise from the interference-sensitive crossover formation pathway. In this study, we also observed that the average number of bright HEI10 foci was reduced in the Oshus1-1 mutant, showing that the number of interference-sensitive COs was reduced in the absence of OsHUS1. Thus, the similar alterations in ectopic and allelic interactions between yeast and rice imply that the function of HUS1 may be conserved among different organisms.

Interference plays a role in both controlling and constraining the final distribution of COs. Although the mechanism underlying these processes remains unclear, it has been postulated that spreading interference signals are transmitted along the length of the chromosome axes [58]. Therefore, one possible explanation for the decrease in interference-sensitive CO number is that the spreading interference signals may also be transmitted through associated nonhomologous chromosome axes in Oshus1. Alternatively, it is possible that partial allelic interactions are redirected into ectopic interactions or resolved toward sister chromatids in the absence of OsHUS1.

Possible functions of OsHUS1 during meiosis

Studies in yeast and mammals have shown that the 9-1-1 complex is involved in multiple DNA repair courses by binding to numerous partners, including base excision repair proteins and mismatch repair proteins [23]. Among these, the mismatch repair protein MSH2 is postulated to be involved in the intermediate elimination of ER [13]. In yeast and humans, MSH2-MSH6 heterodimer (MutSα) and MSH2-MSH3 heterodimer (MutSβ) are mismatch recognition factors that function in the mismatch repair pathway. Recent studies have revealed that each subunit of the 9-1-1 complex can interact with both the MSH2/MSH3 and MSH2/MSH6 complexes. In addition, the 9-1-1 complex can also stimulate the DNA binding activity of MutSα [59]. The biochemical properties of the 9-1-1 complex are likely similar during mitosis and meiosis. We therefore postulate that OsHUS1 may also function as a component of the 9-1-1 complex to sense ectopic interaction and further recruit MutS to eliminate ectopic interaction intermediates. The characterization of RAD9, RAD1, and MSH2 homologs in rice will deepen our understanding of the ER-eliminating mechanism.
Materials and Methods

Plant materials

Oshus1-1 was derived from Nipponbare (a japonica cultivar) induced by tissue culture. Oshus1-2 was derived from Huanghazhan (an indica cultivar) induced by 60Co−γ ray radiation. The new pair1 mutant allele was obtained from Nipponbare through tissue culture. In this allele, a retrotransposon was inserted in the 7th exon of PAIR1. The new Osrad51c allele was derived from an indica rice variety Zhongxian 3037, induced by 60Co−γ ray radiation and found to have a premature stop codon in the 9th exon of OsRAD51C. The Osmom1 and zfp1 alleles employed in this study is Osmom1-3 and zfp-1, respectively [30,45]. Nipponbare was used as the wild type in the related experiments.

Molecular cloning of OsHUS1

STS markers were developed based on sequence differences between japonica variety Nipponbare and indica variety 9311, which were used for map-based cloning of OsHUS1. Primers sequence were listed in Supporting information, Table S1. The cDNA sequence for OsHUS1 was verified by 3′RACE. Total RNA was extracted from rice young panicles (6–8 cm) using TRIZOL reagent (Invitrogen). A measure of 3 μg RNA was reverse-transcribed with Oligo-Adaptor primer (CTGATCTAGGTACCGGATCC-d(T)16) using the superscript III RNaseH reverse transcriptase (Invitrogen). Two rounds of PCRs were carried out using Adaptor primer (CTGATCTAGGTACCGGATCC), gene specific primers RACE1F (TGACTCTCTTCTATGGTATTTC) and RACE2F (CTAGACTGACGGACAAGTCC). The product was cloned into pMD19-T vector (TaKaRa) and sequenced.

Generating OsHUS1 RNAi transgenic plants

A 261bp fragment from the exons of OsHUS1 was amplified by PCR with the primer pair OsHUSiRNAF (AAGGATCCCT-GACAGTATGCTTACTC) and OsHUSiRNAR (AGGTC-GACACCATAAGGTACGTCG). The product was introduced into rice protoplasts using pUCRRNAi vector. The OsHUS1 RNAi construct was introduced into Agrobacterium tumefaciens strain EHA105 and transformed the japonica cultivar Yandao 8.

Quantitative RT-PCR analysis

Total RNA was extracted from the internode, leaf, root, panicle and seedling of Nipponbare, and was reverse-transcribed into cDNA. Quantitative RT-PCR analysis was performed using the CFX96 Real Time system (Bio-Rad) and Eva Green (Biotium). Primer sequences are listed in Supporting information, Table S1. The cDNA was reverse-transcribed with UBIQUITIN II- and UBIQUITIN I sites of the pUCRRNAi vector. The OsHUS1 RNAi construct was introduced into Agrobacterium tumefaciens, strain EHA105 and transformed the japonica cultivar Yandao 8.

Sensitivity test

Husked seeds from the wild-type plants and the heterozygous Oshus1+− plants were surface sterilized. Then they were sown on solid 1/2 MS medium containing 20 μg/ml MMC (Solarbio) in a light incubator. Genotype and phenotype assays of the seedlings were assayed 14 days later.

Antibodies

To generate the antibody against OsHUS1, the coding region of it was amplified from Nipponbare leaf cDNA with primer pair OsHUS1PETF (ATGGATCCATGTAAGTTCAAGGCCTTC) and OsHUS1PETR (ATCTCGAGACGGATCCAGGTCGACCAGC), and then ligated to the BamHI-XhoI site of the expression vector pET-30a (Novagen). The expression vector was transformed into E. coli strain BL21 (DE3) and was induced for 3 h at 37°C by addition of 0.3 mM IPTG. His-tagged OsHUS1 were accumulated in the inclusion bodies and they were washed and subjected to SDS-PAGE. The main band of His-tagged OsHUS1 on the gel was cut off and powdered and used as an antigen against mice. The OsREC8, PAIR2, PAIR3, OsMER3, OsZIP4, HEI10, and ZEP1 polyclonal antibodies were used as described before [30,32,34,35].

Cytology

Young panicles of at meiosis stage were harvested and fixed in Carnoy’s solution (ethanol/glacial acetic acid = 3:1) for chromosome spreading. Meiotic chromosome preparation and immunofluorescence were performed as previously described [34]. The FISH procedure was performed as described [60]. Microscopy was conducted using a ZEISS A2 fluorescence microscope with a microCCD camera. Image capture and analysis was carried out using IPLab software (BD Biosciences).

Supporting Information

Figure S1 Phenotype of the Oshus1-1 mutant. (A) A wild-type plant; (B) A Oshus1-1 plant; (C) Comparison of a wild-type (left) and a Oshus1-1 panicle (right); (D, E) I2-KI staining of pollen grains in the wild type (D) and Oshus1-1 mutant (E). Bars, 50 μm. (TIF)

Figure S2 Meiotic chromosomes at Metaphase I in Oshus1-2 and OsHUS1 RNAi plant. (A) Oshus1-2. (B) An OSHUS1 RNAi line. Scale bars, 5 μm. (TIF)

Figure S3 Structure of the OsHUS1 gene. Exons are represented by black boxes. Gray boxes show the untranslated regions. The position of the OsHUS1 mutation is indicated by an arrow. (TIF)

Figure S4 Alignment of HUS1 homologues. Identical amino acids are shaded in black whereas similar amino acids are shaded in gray. (TIF)

Figure S5 Phylogenetic tree of the 20 homologs defined by OsHUS1. The tree is constructed using MEGA 4.0 based on the neighbor-joining method. Numbers next to branches are clade value of panicle is set as 1. (TIF)

Figure S6 Relative expression level of OsHUS1 in different tissues analyzed by quantitative RT-PCR. Values are means ±SEM (standard error of mean) of three independent experiments and value of panicle is set as 1. (TIF)

Figure S7 Detection of homologous chromosome pairing revealed by FISH in Oshus1-1, Osmer3 and Osmer3 Oshus1-1. (A-C) Pachytene; (D) Diakinesis; FISH signals of 58 tDNA are in green, signals of the BAC clone a0065A15 on the long arm of chromosome 9 are in red, and chromosomes are in blue stained with DAPI. Bars, 5 μm. (TIF)

Figure S8 Dual immunolocalization of OsREC8 and OsHUS1 in Osmer3, zfp1 and pair1 PMCs. (A) Osmer3 shows a normal role of rice HUS1 in meiosis.
localization of OsHUS1. (B) cebp displays a normal localization of OsHUS1. (C) OsHUS1 is absent in pair I. Bars, 5 μm. (TH)

Table S1  Primers used for OsHUS1 map-based cloning. (DOCX)

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Author Contributions

Conceived and designed the experiments: ZC MG KW. Performed the experiments: LC DT XC. Analyzed the data: YL QH YS KW. Contributed reagents/materials/analysis tools: QL HY. Wrote the paper: ZC LG KW.
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