The DNA topoisomerase VI–B Subunit OsMTOPVIB Is Essential for Meiotic Recombination Initiation in Rice

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Dear Editor,

In flowering plants, the life cycle alternates between diploid sporophyte and haploid gametophyte generations. Development of the male organ and germ cells is essential for successful plant fertility and crop yield. Male reproduction is a highly orchestrated process controlled by various intrinsic components. Although significant advances have been made in understanding male gametophyte formation, the molecular mechanisms controlling in this process are still largely unknown (Zhang et al., 2016).

To identify male sterility genes in rice, we have constructed a mutant library by treating the rice cultivar 9522 (Oryza sativa ssp. Japonica) with gamma ray radiation. This has led to the identification of two allelic male sterile mutants that we have designated Oryza sativa meiotic topoisomerase VIB–like (osmtopVIB-1 and osmtopVIB-2). The mutant plants exhibited normal vegetative growth but complete sterility during reproductive development. Iodine potassium iodide solution (I2-KI) staining showed that almost all the pollen grains were empty and shrunken (Figure 1A). Moreover, when mutant flowers were pollinated with wild-type pollen, no seeds set, indicating that the mega-gametogenesis was also affected in the mutant. Semi-thin sections were performed to clarify the cause of sterility in osmtopVIB mutants. No obvious phenotype was observed in osmtopVIB mutants until tetrad stage. Tetrads of abnormal size and shape were found in the anther locule of osmtopVIB mutants (Supplemental Figure 1), suggesting that osmtopVIB mutants may be defective during meiosis.

To confirm that OsMTOPVIB is required for normal meiosis, chromosome behavior was investigated in pollen mother cells (PMCs) of both wild-type and osmtopVIB mutants. In the wild type, chromosomes began to condense and become visible as thin
threads at leptotene. At zygotene, the chromosomes continued to condense as well as
synapsis initiation sites forming, bringing the homologs into close apposition. At
pachytene, synapsis concluded with the formation of the synaptonemal complex (SC).
Twelve bivalents appeared at diakinesis which then aligned along the equatorial plate
at metaphase I (Figure 1B). The homologous chromosomes then separated and were
pulled towards the opposite poles by the spindle from anaphase I to telophase I.
During the second meiotic division, sister chromatids of each chromosome segregated
and tetrads formed at the end of meiosis II (Supplemental Figure 2 A-C). In
osmtopVIB-1 meiocytes, chromosome morphogenesis was similar to the wild type
from leptotene to zygotene. However, homologous chromosomes did not pair with
each other and no synapsed homologs were observed. Due to the lack of homologous
pairing and synapsis, 24 univalents were observed at diakinesis (Figure 1B). At
metaphase I, these univalents could not align on the equatorial plate and thus
segregated randomly to opposite poles during anaphase I. As a result, abnormal
tetrads with several micronuclei were generated after the second meiotic division
(Supplemental Figure 2 D-F). The chromosome behavior in osmtopVIB-2 meiocytes
was similar to osmtopVIB-1 (Supplemental Figure 3 C-H). These results indicated that
osmtopVIB mutants were defective in homologous pairing and synapsis.

To investigate whether SC formation is affected in osmtopVIB-1, we checked the
localization of PAIR2, PAIR3 and ZEP1, which are SC components (Nonomura et al.,
2006; Wang et al., 2011; Wang et al., 2010). OsREC8, a conserved meiosis-specific
component of the cohesion complex, can be used as a marker for meiotic
chromosomes (Shao et al., 2011). In osmtopVIB-1, OsREC8 localization was
indistinguishable with wild type and therefore used as a maker for chromosome
behavior in rice meiosis. In osmtopVIB-1 meiocytes, PAIR2 and PAIR3 loaded
normally onto the chromosomes and colocalized with OsREC8, which is consistent
with the wild type (Supplemental Figure 4). In the wild type, ZEP1 appeared as
punctate foci on leptotene chromosomes and then formed continuous linear signals
along the entire chromosomes at pachytene (Figure 1C). However, no foci or linear
ZEP1 signals were observed in osmtopVIB-1 meiocytes from leptotene to pachytene, indicating that the central element is absent in the mutant (Figure 1C). Overall, these results suggest that although axial elements install normally, SC formation is severely disrupted in osmtopVIB-1.

Rice DMC1 mediates single-strand invasion during meiotic recombination and is essential for recombination dependent synapsis (Wang et al., 2016). MER3 is required for the formation of interference-sensitive COs, the major pathway in rice (Wang et al., 2009). Thus, DMC1 and MER3 were used as markers to monitor the process of meiotic recombination. We found that punctate foci of DMC1 and MER3 were observed in the wild-type meiocytes at zygotene. By contrast, no DMC1 and MER3 foci were observed in the osmtopVIB-1, suggesting that OsMTOPVIB is required for inter-homologue chromosome recombination during meiosis (Figure 1C).

In most organisms including rice, meiotic recombination and synapsis depends on the formation of double strand breaks (DSBs). To investigate whether abolishment of recombination in osmtopVIB-1 is caused by defects in DSB formation, we performed dual immunolocalization of OsREC8 and γH2AX. The histone H2A variant, H2AX, becomes rapidly phosphorylated to γH2AX when meiotic DSBs are formed. Therefore, γH2AX foci have been considered as suitable markers for DSB sites (Dickey et al., 2009). We detected numerous γH2AX signals during zygotene in wild type. However, no γH2AX signals were observed in osmtopVIB-1, which indicated that OsMTOPVIB is essential for DSB formation in rice (Figure 1D).

To identify the mutated gene, a map-based cloning approach was used. The mutated gene was mapped to chromosome 6 between markers FM603 and FM606, defining a 56-kb region (Supplemental Figure 5). Sequence analysis revealed that one base (T) deletion (osmtopVIB-1) and a base (C) deletion (osmtopVIB-2) in the second exon of LOC_Os06g49450 (http://www.gramene.org/) caused a frame shift and premature translational termination (Supplemental Figure 5).
OsMTOPVIB encodes a protein of 487 amino acids in length. OsMTOPVIB shares 39% sequence identity with the Arabidopsis orthologue MTOPVIB as well as showing homology with topoisomerase VIB from other flowering plants. The OsMTOPVIB protein sequence has no obvious similarity with known functional domains. However, HHpred analysis suggested that this protein shares structural homology with the two archaeal topo VIB proteins with known crystal structures. The OsMTOPVIB contains four characteristic domains of TOPVIB protein family, including the GHKL domain, the small domain (SmD), the transducer domain and the C-terminal domain (Supplemental Figure 6). TOPVIB is one of the two subunits of the archaeal type VI topoisomerase, which catalyzes a DNA topology change through generating double-strand breaks during DNA replication, transcription and recombination (Bergerat et al., 1994). Topo VI is a heterotetramer comprised of two TopVIA and TopVIB subunits each. TopVIA is responsible for DNA cleavage and TopVIB is involved in ATP binding and hydrolysis. The evolutionarily conserved DSB protein SPO11 is the eukaryotic orthologue of the archaeal TopVIA (Bergerat et al., 1997).

To define the spatial and temporal distribution of OsMTOPVIB in meiosis, dual immunolocalization was conducted using OsMTOPVIB and OsREC8 antibodies. In the wild type, OsMTOPVIB first appeared as punctate foci at leptotene. Then the number of OsMTOPVIB foci accumulated and reached a peak at late zygotene to early pachytene. The OsMTOPVIB foci started to diminish during pachytene and only a few residual foci were observed at late pachytene. After late pachytene, OsMTOPVIB signals were no longer detectable (Supplemental Figure 7). In wild-type meiocytes, γH2AX foci were mainly detected at zygotene. The OsMTOPVIB foci can be detected at pachytene, indicating that it may have other functions besides DSB formation during meiotic prophase I. The OsMTOPVIB signals only partially overlapped with the axis signals, which may represent a dynamic distribution of DSBs, and that pre-DSB sites originate in the chromosome loops before moving to the axes.
This result supports that OsMTOPVIB specifically functions during early Prophase I.

Most organisms contain one SPO11, however higher plants generally possess more than one SPO11 homolog. For example, three and five SPO11 homologs exist in the Arabidopsis and rice genome, respectively (Hartung et al., 2001; An et al., 2011). It is currently unclear which of the SPO11 homologs are required for meiotic DSB formation in rice. Previous studies indicate that the archaeal topoVIA associates with topoVIB to form a topo VI–like complex. A similar interaction of OsMTOPVIB with OsSPO11(s) involved in DSB formation may also occur in rice. A yeast two-hybrid assay revealed that OsMTOPVIB is able to interact with OsSPO11-1 and OsSPO11-4 respectively, but not with OsSPO11-2 and OsSPO11-3 (Figure 1E, Supplemental Figure 8). This result suggests that OsSPO11-1, OsSPO11-4 and OsMTOPVIB may associate together to form a heterotetrameric structure similar to that of the archaeal Topo VI. In addition, previous work has demonstrated that OsSPO11-1 is essential for homologous chromosome pairing and CO formation, while OsSPO11-4 possesses the double-strand DNA cleavage activity in vitro (Yu et al., 2010; An et al., 2011). Whether OsSPO11-1/4 are capable of interacting with OsMTOPVIB in planta and required for DSB formation needs further investigation.

In summary, we have identified an orthologue of archaeal TOPVIB in rice and demonstrated that OsMTOPVIB is essential for meiotic recombination initiation. It is well known that meiotic DSBs are catalyzed by SPO11, the eukaryotic equivalent of TOPVIA (Keeney et al., 1997). However due to sequence divergence, whether TopVIB is also required for meiotic DSB formation has long been a mystery until very recently when the orthologues of TopVIB were identified as the partner of SPO11 in Arabidopsis and mice (Robertet et al., 2016; Vrielynck et al., 2016). Our study proved the conserved function of TopVIB proteins in the model monocot rice, highlighting the evolutionary importance of TopVIB in meiotic DSB formation.
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**AUTHOR CONTRIBUTIONS**

W.L. and D.Z. designed the experiments. M.F. carried out most of the experiments. C.W., F.X. and M.C assisted in gene cloning and immunolocalization analysis. J.D.H. assisted in analyzing the data. M.F. J.D.H. and W.L. wrote the article.

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Figure Legend

Figure 1. OsMTOPVIB is required for meiotic DSB formation.
(A) Phenotypic characterization of wild type and osmtopVIB-1. From left to right are spikelets after moving the lemma and pelea (Scale bar, 500µm), pistils (Scale bar, 200µm), anthers (Scale bar, 200µm) and I2-KI staining of the pollen grains within the anther of wild type and osmtopVIB-1 (Scale bar, 100µm).
(B) Chromosome behaviors of male meiocytes of wild type and osmtopVIB-1 at meiotic prophase I. Scale bar, 5µm.
(C) Dual immunolocalization of REC8 and γH2Ax in wild type and osmtopVIB-1 male meiocytes. Scale bar, 5µm.
(D) Dual immunolocalization of ZEP1, DMC1 and MER3 with REC8 in wild type and osmtopVIB-1 male meiocytes. Scale bar, 5µm.
REC8 signals (green) were used to indicate the meiotic chromosome axes in C and D.
(E) Yeast two-hybrid assay to detect interactions of OsMTOPVIB with SPO11-1 and SPO11-4 respectively. Empty vector pGADT7 and pGBK7 were used as controls. The interactions were verified by the growth of yeast strains on the -Leu-Trp-His-Ade selection medium containing 10mM 3AT.

Supplemental Figure legends

Supplemental Figure 1. Transverse section analysis of osmtopVIB-1 anthers. The images are cross-sections of a single locule.
(A) to (E) Wild type anthers. (F) to (J) osmtopVIB-1 anthers. [A] and [F]: Stage 6 (pre-meiosis stage); [B] and [G]: Stage 7 (Meiosis Prophase I); [C] and [H]: Stage 8a (Dyad stage); [D] and [I]: Stage 8b (Tetrad stage); [E] and [J]: Stage 9 (Early microspore stage). Scale bar, 25µm.

Supplemental Figure 2. Meiotic chromosome behaviors of male meiocytes in wild type and osmtopVIB-1.
Chromosome behaviors of male meiocytes of wild type (A) to (C) and osmtopVIB-1 (D) to (F) at various stages. [A] and [D]: Metaphase I; [B] and [E]: Anaphase I; [F]: Telophase II. Scale bar, 5µm.

Supplemental Figure 3. Phenotypic observation and the chromosome behavior of
osmtopVIB-2.

I2-KI staining of the pollen grains within the anther of wild type (A) and osmtopVIB-2 (B). Scale bar, 100µm.

Chromosome behaviors of male meiocytes of osmtopVIB-2 (C) to (H) at various stages. [C]: Zygote; [D]: Pachytene; [E]: Diakinesis; [F]: Metaphase I; [G]: Anaphase I; [H]: Telophase II. Scale bar, 5µm.

**Supplemental Figure 4. Immunolocalization of PAIR2 and PAIR3 in wild type and osmtopVIB-1 male meiocytes.** Scale bar, 5µm.

**Supplemental Figure 5. Map-based cloning of OsMTOPVIB**

(A) Fine mapping of OsMTOPVIB on chromosome 6. Names and positions of the markers are noted. P0621D05, P0655A07, OJ1663_H12 and P0712G01 are rice genomic DNA accession numbers of bacterial artificial chromosome clones. cM, Centimorgan.

(B) A schematic representation of OsMTOPVIB. +1 indicates the putative starting nucleotide of translation, and the stop codon (TAG) is 6070. Black boxes indicate exons, and intervening lines indicate introns. Numbers indicate the exon length (bp). The deletion sites in osmtopVIB-1 and osmtopVIB-2 are shown.

**Supplemental Figure 6. Sequence and Structure of OsMTOPVIB.**

(A) Multiple sequence alignment of the plant MTOPVIB protein family showing the four conserved amino acid regions in OsMTOPVIB (b1 to b4). b1 and b2 are in the GHKL domain, b3 is in the small domain, and b4 is in the transducer domain. Identical amino acids are in red.

(B) Domain organization of the O. sativa MTOPVIB and the S. shibatae topoisomerase VIB subunit (S. shibatae TOPVIB). Homologous domains are shown in the same color: yellow, GHKL domain; purple, Helix-2Turn-Helix (H2TH) domain and small domain (SmD); blue, transducer domain; green, C-terminal (Cter) domain. The amino acid numbers indicate the domain borders.

Osat, Atha, Tcac, Grai, Ccle, Vvin, Rcom, Mdom, Mtru, Mgut, Alyr, Bstr, Crub, Acoe, Pvir, Phal, Svir, Sbic, Zmay, Bdis are the MTOPVIB sequences of Oryza sativa, Arabidopsis thaliana, Theobroma cacao, Gossypium raimondii, Citrus clementina, Vitis vinifera, Ricinus communis, Malus domestica, Medicago truncatula, Mimulus guttatus, Arabidopsis lyrata, Boechera stricta, Capsella rubella, Aquilegia coerulea,
Panicum virgatum, Panicum hallii, Setaria viridis, Sorghum bicolor, Zea mays, Brachypodium distachyon.

Supplemental Figure 7. Dual immunolocalization of OsREC8 and OSMOTOPVIB in the wild type meiocytes at various stages.
Leptotene. (B) Zygotene. (C) Early pachytene. (D) Late pachytene. Scale bar, 5µm.

Supplemental Figure 8. OsMTOPVIB do not interact with SPO11-2 and SPO11-3.
Yeast two-hybrid assay for interaction of OsMTOPVIB with SPO11-2 and SPO11-3. Empty vector pGADT7 and pGBK7 were used as controls. The interaction were verified by the growth of yeast strains on the -Leu-Trp-His-Ade+10mM 3-AT selection medium.

Supplemental Figure 9. Western blot assay of the OsMTOPVIB in wild type spikelets.
Lane 1: Protein standard.
Lane 2: Proteins isolated from WT spikelets at meiotic stages.

Reference


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