Resolvase OsGEN1 Mediates DNA Repair by Homologous Recombination\[OPEN\]

Chong Wang, James D. Higgins, Yi He, Pingli Lu, Dabing Zhang, and Wanqi Liang*

Joint International Research Laboratory of Metabolic and Developmental Sciences, Shanghai Jiao Tong University-University of Adelaide Joint Centre for Agriculture and Health, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 20040, China (C.W., Y.H., D.Z., W.L.); Department of Genetics, University of Leicester, Leicester LE1 7RH, United Kingdom (J.D.H.); State Key Laboratory of Genetic Engineering and Collaborative Innovation Center of Genetics and Development, Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai 200433, China (P.L.); and School of Agriculture, Food and Wine, University of Adelaide, Urrbrae, South Australia 5064, Australia (D.Z.).

ORCID IDs: 0000-0001-9433-0112 (C.W.); 0000-0001-6027-8678 (J.D.H.); 0000-0002-5218-541X (Y.H.); 0000-0002-1764-2929 (D.Z.); 0000-0002-9938-5793 (W.L.).

Yen1/GEN1 are canonical Holliday junction resolvases that belong to the RAD2/XPG family. In eukaryotes, such as budding yeast, mice, worms, and humans, Yen1/GEN1 work together with Mus81-Mms4/MUS81-EME1 and Sīx1-Sīx4/SLX1-SLX4 in DNA repair by homologous recombination to maintain genome stability. In plants, the biological function of Yen1/GEN1 remains largely unclear. In this study, we characterized the loss of function mutants of OsGEN1 and OsSEND1, a pair of paralogs of Yen1/GEN1 in rice (Oryza sativa). We first investigated the role of OsGEN1 during meiosis and found a reduction in chiasma frequency by ~6% in osgen1 mutants, compared to the wild type, suggesting a possible involvement of OsGEN1 in the formation of crossovers. Postmeiosis, OsGEN1 foci were detected in wild-type microspore nuclei, but not in the osgen1 mutant concomitant with an increase in double-strand breaks. Persistent double-strand breaks led to programmed cell death of the male gametes and complete male sterility. In contrast, depletion of OsSEND1 had no effects on plant development and did not enhance osgen1 defects. Our results indicate that OsGEN1 is essential for homologous recombinational DNA repair at two stages of microsporogenesis in rice.

Homologous recombination (HR) is essential for maintaining genome stability by promoting the accurate repair of DNA lesions, such as double-strand breaks (DSBs) and stalled replication forks (Bzymek et al., 2010; Rass, 2013). During meiosis I, HR also facilitates the formation of chiasmata, the cytological manifestation of genetic crossovers, thus ensuring correct chromosome segregation at the first meiotic division. HR is initiated by a DSB that leads to the creation of 3’ single-stranded DNA tails, followed by Rad51-mediated strand-exchange between sister or homologous chromatids that form DNA joint molecules (JMs; Mimitou and Symington, 2009). Most JMs are repaired by the synthesis-dependent strand annealing pathway (Andersen and Sekelsky, 2010; Sarbajna et al., 2014). The remaining JMs may be ligated to form Holliday junctions (HJs), an important DNA intermediate consisting of four DNA strands of two homologous DNA helices (Mimitou and Symington, 2009). The resolution of HJs is crucial for the completion of recombination. In addition, HJs are toxic DNA structures if not processed appropriately because they can interfere with normal chromosome segregation as well as DNA replication. HJs are processed by two major mechanisms. One is through dissolution by the BLM-TopoisomeraseIIIa-RMI1-RMI2 complex (BTR in human, STR in yeast) to give rise to non-crossover (NCO) products (Wu and Hickson, 2003). Alternatively, HJs are resolved by structure-specific endonucleases to generate both crossover (CO) and NCO products (Mimitou and Symington, 2009).

HJ resolvases have been identified from a wide variety of organisms, including bacteriophages, archaea, bacteria, viruses, and eukaryotes. It has been demonstrated that Mus81-Mms4/MUS81-EME1 (MMS and UV-sensitive protein 81-Methyl Methane Sulfonate...
sensitivity4/Essential meiotic endonuclease1), Sxl1-Slx4/SLX1-SLX4 (Synthetic lethal of unknown function), and Yen1/GEN1 (Holliday junction resolvase YEN1/Gen endonuclease homolog1) are key structure-specific endonucleases involved in HJ resolution in eukaryotes (Boddy et al., 2001; Schwartz and Heyer, 2011). Mus81-Mms4 and Sxl1-Slx4 were identified from the same screen for proteins essential for the viability of yeast cells lacking the Slow growth suppressor1 gene (Mullen et al., 2001). Mus81-Mms4 belong to the XPF endonuclease family and are capable of cutting several branched DNA structures, including 3' flaps, nicked HJs, and D-loops (Boddy et al., 2001; Chen et al., 2001; Bastin-Shanower et al., 2003; Ciccia et al., 2003; Oğrünç and Sancar, 2003; Whitby et al., 2003; Taylor and McGowan, 2008). SLX1 is the catalytic subunit of the SLX1-SLX4 heterodimer endonuclease complex and belongs to the GIY-YIG nuclease family (Fricke and Brill, 2003; Dunin-Horkawicz et al., 2006). The SLX1-SLX4 complex prefers to cut 5' flap structures in vitro while it is also able to cleave HJs (Fricke and Brill, 2003; Fekairi et al., 2009; Svendsen et al., 2009; Wyatt et al., 2013). MUS81-EME1 and SLX1-SLX4 act in the same pathway and cooperatively introduce asymmetric cuts on HJs. The gapped and flapped DNA intermediates generated by the asymmetric incisions require further processing before ligation (Fricke and Brill, 2003; Castor et al., 2013; Wyatt et al., 2013). Recent reports showed that Saccharomyces cerevisiae Yen1 and its human ortholog GEN1 introduce symmetric nicks on HJs in a MUS81-EME1 and SLX1-SLX4 independent pathway (Wyatt et al., 2013). Yen1/GEN1 were considered to be the canonical HJ resolvases in eukaryotes as they cleave HJs in a manner analogous to that of the Escherichia coli resolvase RuvC (crossover junction endodeoxyribonuclease RuvC; Ip et al., 2008; Rass et al., 2010).

Yen1/GEN1 homologs belong to the fourth clade of the RAD2/XPG family of structure-specific nucleases and contain three functional domains: N-terminal XPG domain, internal XPG nuclease domain (XPGI), and helix-hairpin-helix (HhH) domain (Rass et al., 2010; Bauknecht and Kobbe, 2014). It was reported that Yen1/GEN1 homologs from human, yeast, Caenorhabditis elegans, Arabidopsis (Arabidopsis thaliana), and rice (Oryza sativa) have the ability to cleave 5'-flaps, replication forks (RFs), and HJs in vitro (Ip et al., 2008; Bailly et al., 2010; Yang et al., 2012; Bauknecht and Kobbe, 2014; Lee et al., 2015). The Yen1/GEN1 HJ resolution pathway shows a complicated interplay with other HJ resolution pathways and functional variation in different organisms. In S. cerevisiae the yen1 mutant lacks an obvious phenotype, while the yen1 mms4 double mutant is severely compromised in meiotic joint molecule resolution and fails to complete meiosis, suggesting that Yen1 acts in a Mus81-Mms4 independent pathway (Ip et al., 2008). In addition, although the yen1 mutant in budding yeast is proficient in DNA repair, the yen1 mus81 double mutant is extremely sensitive to a variety of DNA damaging agents and accumulates toxic recombination intermediates after MMS treatment, indicating that Yen1 can process recombination intermediates that arise in the absence of Mus81 after replication fork damage (Blanco et al., 2010). Kluyveromyces lactis mutants lacking a functional Mus81 are severely compromised in sporulation efficiency and crossover frequency, but lacking both Mus81 and Yen1 showed no further reduction in spor formation. Overexpression of Yen1 partially rescued the crossover defect in mus81 mutant and the DNA damage sensitivity of mus81 and sgs1 mutants. These results suggest that Yen1 is redundant with Mus81 and SGS1 in meiosis and DNA repair processes (Chen and Aström, 2012). In C. elegans, GEN-1 facilitates the repair of DNA DSBs only when other structure-specific nucleases are absent, and is not essential for homologous recombination during meiosis (Saito et al., 2012, 2013). Mutational analysis reveals that GEN-1’s function in DNA damage signaling is separated from its role in DNA repair. GEN-1 promotes germ cell cycle arrest and apoptosis via a pathway parallel to canonical DNA damage response pathways and acts redundantly with the 9-1-1 complex to ensure genome stability. This suggests that GEN-1 acts as a dual function Holliday junction resolvase that coordinates DNA damage signaling with a late step in DNA DSB repair (Bailly et al., 2010). In Drosophila, mus81 gen double mutants have elevated levels of apoptosis. In contrast to yeast, gen mutants are more hypersensitive to DNA damage than mus81 mutants, indicating that GEN plays a more dominant role than MUS81-MMS4 in responding to DNA damage (Andersen et al., 2011). Depletion of GEN1 by RNA interference-mediated gene silencing in human cells disrupts mitotic progression and increases the number of cells with multinuclei, increased apoptosis, and elevated levels of spontaneous DNA damage (Gao et al., 2012; Rodrigue et al., 2013). These phenotypes indicate that human GEN1 is required for DNA repair and recombination, which may indirectly lead to centrosome abnormalities (Rodrigue et al., 2013). Human cells depleted of GEN1 exhibit mild sensitivity to DNA damage after MMS and CPT treatment (Svendsen et al., 2009). Recent studies indicate that GEN1 and SLX-MUS81 act in two distinct pathways in sister chromatid exchange formation, chromosome abnormalities, and cell death (Wechsler et al., 2011; Garner et al., 2013; Wyatt et al., 2013). Human cells lacking SLX, MUS81, and GEN1 simultaneously exhibited impaired movement of the replication fork, endogenous checkpoint activation, chromosome instability, and multinucleation, indicating that GEN1 acts together with SLX4 and MUS81 to assure the timely and faithful completion of mitosis and to increase genome stability throughout the cell cycles (Sarbajna et al., 2014).

Although the roles of Yen1/GEN1 homologs have been extensively studied in yeast and animals, their biological functions remain largely unknown in plants. There is only one Yen1/GEN1 type of resolvase in yeast, animals, and moss. On the contrary, two Yen1/GEN1 homologs, named GEN1 and SINGLE-STRAND
DNA ENDONUCLEASE1 (SEND1), exist in the genome of most plants (Bauknecht and Kobbe, 2014; Olivier et al., 2016). It was reported that in Arabidopsis, SEND1 but not GEN1 acts as a functional Yen1/GEN1 homolog and plays an important role in the repair of toxic replication intermediates and telomere homeostasis, but not in meiotic recombination (Olivier et al., 2016), suggesting a functional diversification of these two paralogs. In rice, RNA interference-mediated gene silencing of rice GEN1 results in complete male sterility, which indicates that OsGEN1 plays an essential role in male microspore development in rice (Moritoh et al., 2005). OsSEND1 is induced by UV and DNA damage agents, indicating that it may play a role in DNA repair in somatic cells (Furukawa et al., 2003). However, it remains unclear how OsGEN1 regulates microspore development and whether OsSEND1 and OsGEN1 have redundant or divergent functions.

Here we describe the characterization of osgen1, ossend1 single mutants, and osgen1 ossend1 double mutants. Our data revealed that OsGEN1 but not OsSEND1 plays an essential role in male meiosis and mitotic DNA replication processes. We conclude that OsGEN1 is a functional homolog of Yen1/GEN1 in rice and that it has indispensible roles in chiasmata formation and DNA lesion repair in rice male gametophytes.

RESULTS

Identification of the osgen1 Mutant

We isolated a male sterile mutant designated osgen1 from our rice mutant library constructed by using 60Co γ-ray radiated O. sativa ssp. japonica cv 9522 (Chu et al., 2005; Chen et al., 2006). The osgen1 mutant showed normal vegetative growth and floral development (Fig. 1, A and B), except the stamen was smaller (Fig. 1C) and did not produce normal pollen (Fig. 1, D and E). When the osgen1 mutant was pollinated with wild-type pollen, they could set seeds normally and all F1 progeny displayed a normal phenotype, indicating that osgen1 female fertility is normal. F2 progeny segregated 75 normal and were able to complete meiosis and produce microspores, whereas the mutant microspores arrested at the early microspore stage (Zhang et al., 2011a) and then degenerated (Supplemental Fig. S1).

To isolate the mutated gene that controls the sterile phenotype, a F2 mapping population was generated by crossing the mutant plants with the Indica rice variety 9311. Sterile plants segregated in F2 populations were collected for genotyping. We initially mapped the mutation to chromosome 9. The mutation locus was further narrowed between two InDel markers (907-3 and 907-6), with a genetic distance of 78.5 and 78.8 centimorgans, respectively (Supplemental Fig. S2A). By resequencing the mutant genomic DNA, we found a 2 bp deletion in the seventh exon of OsGEN1 gene (LOC Os09g35000), leading to a frame shift and premature translation termination (Supplemental Fig. S2B). A genomic DNA fragment containing the OsGEN1 gene and 2 Kb upstream sequence was translationally fused to the β-glucuronidase (GUS) reporter and eGFP reporter separately and were introduced into osgen1 mutant plants. Both primary transgenic lines were fertile (Supplemental Fig. S4), indicating that OsGEN1 is responsible for the male sterile phenotype in osgen1. The full-length OsGEN1 protein sequence was used as a query to search the National Center for Bio-technology Information protein database and identified 41 homologs belonging to the RAD2/XPG family (Supplemental Fig. S2C). Of these, OsGEN1 clustered with 10 homologs in the Yen1/GEN1 subfamily, proteins that have similar structures but are variable in length and isoelectric points (Supplemental Fig. S3). The XPGI and N-terminal XPG domains are conserved in the Yen1/GEN1 subfamily (Moritoh et al., 2005; Yang et al., 2012), whereas the C terminus is highly variable. As the osgen1 mutation is present in the C terminus, the sterile phenotype suggests that this is important for OsGEN1 function.

We further investigated the spatio-temporal expression pattern of the OsGEN1 by quantitative RT-PCR (qRT-PCR) analyses. OsGEN1 is highly expressed in the shoot, young leaves, and anthers at stage 8b (tetrastage; Supplemental Fig. S5).

Figure 1. osgen1 plants exhibit normal vegetative growth but severe fertility defects. A, Wild-type and osgen1 plants after heading. B, Wild-type and osgen1 spikelets before anthesis. C, Wild-type and osgen1 spikelets after removal of the palea and lemma. D and E, I2-KI staining of the pollen grains within the anther of the wild type (D) and osgen1 (E). Bars = 5 cm in A, 1 mm in B and C, and 100 μm in D and E.
OsGEN1 Mutation Influences Meiotic Progression in the Pollen Mother Cell

It has been reported that OsGEN1 has HJ resolvase activity in vitro (Yang et al., 2012). To investigate a potential role for OsGEN1 in recombination, we analyzed the chromosome behavior in wild-type and osgen1 PMCs. During leptotene to pachytene of meiosis, chromosomes appeared normal in the osgen1 mutant, whereas at diakinesis, a small number of cells with univalents were observed (9.62%, n = 156; Fig. 2I). Randomly distributed DAPI bodies were also observed at later stages, including metaphase I (12.5%, n = 113; Fig. 2J), anaphase I (8.33%, n = 120; Fig. 2K), telophase I (9%, n = 111; Fig. 2L), prophase II (13.2%, n = 47; Fig. 2M), metaphase II (11.2%, n = 63; Fig. 2N), anaphase II (7%, n = 56; Fig. 2O), and tetrad (1.70%, n = 176; Fig. 2P). These observations suggested that the number of crossovers was affected in the mutant PMCs.

To further investigate whether OsGEN1 deficiency affects recombination, we quantified the frequency and distribution of chiasmata in osgen1 and the wild type by studying the shape of bivalents at diakinesis and metaphase I. At diakinesis, the X-shaped and ring-shaped bivalents were treated as having one and two chiasmata, respectively, and the 8-shaped bivalents were treated as having three chiasmata. At metaphase I, the rod-shaped bivalents were treated as having one chiasma and the ring-shaped having two or three chiasmata depending on the different characteristics as described in Sanchez Moran et al. (2001). The mean chiasma frequency in the wild type was 20.3 per cell (n = 168), while in osgen1 mutant the mean chiasma frequency was 18.8 per cell (n = 170; Fig. 3A). Thus, mutation of osgen1 led to an ~6% reduction in chiasma compared to the wild type. Because a small reduction in chiasma frequency could be more easily detected against a background with low numbers of residual chiasmata, we generated an osgen1 hei10 double mutant (Supplemental Fig. S6) and compared chiasma frequency to each of the single mutants. The chiasma frequency in the hei10 single mutant was 6.1 per cell (n = 167) A significant reduction in the number of chiasmata was observed in the osgen1 hei10 double mutant (reduced to 4.0 per cell, n = 149; I[314] = 7.2, P < 0.01; Fig. 3B). Furthermore, OsGEN1 mutation led to a decrease in the number of bivalents having two and three chiasmata, but higher numbers of univalents and bivalents with one chiasma in both the wild type and hei10 background (Fig. 3C).

OsGEN1 Is Dispensable for Formation of Synaptonemal Complex and Late Recombination Intermediates

To investigate whether OsGEN1 is required for synaptonemal complex (SC) formation, we performed dual-immunolocalization analysis of axis-associated proteins OsREC8, PAIR2, and PAIR3 and the transverse filament protein ZEP1 (Nonomura et al., 2006; Wang et al., 2010, 2011; Shao et al., 2011). In osgen1, no difference in the localization of OsREC8, PAIR2, PAIR3, and ZEP1 was observed in the mutant PMCs (Fig. 4; Supplemental Fig. S7), indicating that SC assembly is normal in osgen1.

We further analyzed male meiosis in osgen1 by using a panel of antibodies that mark recombination sites. DSB formation is the first step in meiotic recombination and in rice can be assayed indirectly by detection of phosphorylated γH2AX (Mahadeviah et al., 2001; Che et al., 2011). OsCOM1 and RPA2c are required for DSB end-processing and 3'-single-strand invasion after DSB generation (Ji et al., 2012; Li et al., 2013) and DMC1 and RAD51C indicate the number of potential strand invasion events (Neale and Keeney, 2006; Tang et al., 2014). Our analysis showed that the localization of γH2AX, OsCOM1, RPA2c, DMC1, and RAD51C in osgen1 was indistinguishable to wild-type male meioocytes (Fig. 5, A–H, J, and K; Supplemental Fig. S8, A–E). At zygotene, the average number of DMC1 foci was 64.9 (n = 18, range 48–98) in the wild type, and 61.3 (n = 18, range 54–84) in osgen1; the average number of RAD51C foci was 56.2 (n = 20, range 46–60) in the wild type, and 58.7 (n = 20, range 46–69) in osgen1. These results indicated that DSB formation and the initial steps of recombination were unaffected in the osgen1 mutant. HEI10 is the rice homolog of budding yeast Zip3 and C. elegans ZHP-3. It has been reported that HEI10 is required for Class I CO formation and used as a marker for COs during late prophase I in rice (Wang et al., 2012). At pachytene, the average number of HEI10 foci was 23.9 (n = 10, range 48–98).

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**Figure 2.** Meiotic progression in wild-type and osgen1 PMCs. Meiotic progression was assayed in wild-type (A–H) and osgen1 PMCs (I–P). A and I, Late diakinesis; B and J, metaphase I; C and K, anaphase I; D and L, telophase I; E and M, prophase II; F and N, metaphase II; G and O, anaphase II; H and P, tetrad. Bar = 5 μm.
21–27) in the wild type, and 23.2 (n = 10, range 22–28) in osgen1 (Fig. 5, I and L; Supplemental Fig. S8F), suggesting that HEI10 localization was not influenced by OsGEN1 mutation.

OsGEN1 Dynamically Localizes to Meiotic Chromosomes during Meiosis

To define OsGEN1 distribution during meiosis, dual immunolocalization assays were performed using OsGEN1 and OsREC8 antibodies. At leptotene, 147.5 punctate OsGEN1 foci were observed (n = 4, range 127–173; Fig. 6, A–C). At early zygotene, the numbers of OsGEN1 foci began to decline (79.7, n = 7, range 67–89; Fig. 6, D–F). At late zygotene, fewer OsGEN1 foci were observed (18.1, n = 10, range 13–24; Fig. 6, G–I) that continued to decline at pachytene ~7.18 (n = 11, range 5–13; Fig. 6, J–L) until diplotene (3.6, n = 8, range 3–5; Fig. 6, M–O).

OsGEN1 Does Not Colocalize with Recombination Proteins

To understand the relationship of OsGEN1 with other recombination proteins, we used dual-immunolocalization to investigate the colocalization of OsGEN1 with RPA2c, RAD51C, DMC1, and HEI10, respectively. At zygotene, the mean number of RPA2c foci per cell was 137.5 (n = 4, range 113–199) and OsGEN1 foci was 87 (n = 4, range 73–100), and two to
three merged signals were found. At early pachytene, RPA2c foci per cell was 102 (n = 4, range 83–134) and OsGEN1 foci was 34.25 (n = 4, range 30–39), and two to three merged signals were observed. Later, both RPA2c and OsGEN1 foci decreased and were hardly detected.

We also investigated the colocalization of OsGEN1 with DMC1 and RAD51C in PMCs. Different from MUS81 in Arabidopsis (Higgins et al., 2008), most of OsGEN1 foci do not colocalize with those of both proteins (Fig. 7, D–I). HEI10 is involved in the interference-sensitive CO pathway and marks these sites during diakinesis. To determine the relationship between HEI10 and OsGEN1, we counted the numbers of HEI10 and OsGEN1 foci in PMCs (Fig. 7, J–L). At zygotene, the mean number of HEI10 foci per cell was 170.25 (n = 4, range 141–201), and the mean number of OsGEN1 foci per cell was 116.25 (n = 4, range 88–160). At early pachytene, both HEI10 and OsGEN1 foci decreased, and the mean number of HEI10 foci per cell was 65.75 (n = 4, range 58–71), while the mean number of OsGEN1 foci per cell was 37.75 (n = 4, range 31–49). At these stages, only one to two foci colocalized, which may indicate that HEI10 and OsGEN1 are involved in different pathways during CO formation.

**OsGEN1 Is Required for DSB Repair Postmeiosis**

In Arabidopsis, AtMUS81 is the major resolvase involved in Class II CO formation. Mutation in atMUS81 caused a 10% decrease in Class II CO formation but only a slight decrease in pollen viability and seed number per silique (Berchowitz et al., 2007; Higgins et al., 2008). Therefore, we suspected that the complete male sterility of osgen1 is caused by developmental defects that occur in the microspore at later stages. To demonstrate this hypothesis, we observed the development of wild-type and osgen1 microspores. Wild-type microspores undergo two rounds of mitosis after the meiotic cell division and produce a male gametophyte containing a vegetative nucleus and two generative nuclei to ensure double fertilization (Fig. 8, E–H). However, osgen1 microspores arrest at mitosis I. In young osgen1 microspores, chromosomes remain highly compacted (Fig. 8, I–L) and DNA fragmentation was observed (Fig. 8K). In plants, after meiosis the microspore undergoes two rounds of mitosis to generate the functional male gametophyte. In the microspore, nuclear DNA is replicated before two rounds of mitosis. In the eukaryotic mitotic cell cycle, DSBs are often generated during DNA replication, which leads to the stalling of replication forks (Burgoyne et al., 2007). Under these circumstances, DSBs are repaired by HR repair. These processes are carefully managed and cell division does not proceed until DSB repair is complete (O’Connell and Cimprich, 2005). The MRE11/Mre11 complex works together with exonucleases EXO1 and Dna2 to initiate DSB repair during replication fork stalling (D’Amours and Jackson, 2002; Symington, 2016). It has been reported that OsGEN1 also has 5’-flap endonuclease activity besides resolvase activity, implying that OsGEN1 may act in a similar way as FEN-1, which is involved in the removal of primer regions for Okazaki fragment maturation during lagging strand synthesis (Yang et al., 2012). To monitor OsGEN1

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**Figure 4.** Dual immunolocalization of SC proteins OsZEP1 (magenta) and OsREC8 (green) in wild-type and osgen1 PMCs. A to C, The wild type; D to F, osgen1 mutant; A to F, pachytene. Bar = 2 μm.

**Figure 5.** Dual immunolocalization of recombination proteins (magenta) and OsREC8 (green) in wild-type and osgen1 PMCs. A to C and G to I, The wild type; D to F and J to L, osgen1 mutant. A and D, Leptotene. B, E, G, H, J, and K, Zygotene. C, F, I, and L, Pachytene. Bar = 2 μm.
function after meiosis, we detected OsGEN1 localization and found that OsGEN1 relocalized on chromosomes after the microspore released from the tetrad. OsGEN1 foci reached a peak when the microspore nucleus became loose (Fig. 8B) and released from chromosomes when the nucleus becomes condensed again (Fig. 8, C and D), indicating that OsGEN1 has an important function in DNA replication before the two rounds of mitosis.

To reveal the effect of osgen1 on DSB occurrence in osgen1 and wild-type microspores, we used γH2AX as a marker. In the wild type, γH2AX foci were rarely found in microspore nuclei, while in the osgen1 mutant γH2AX foci were up to 53.39 (n = 7, range 37–56; Fig. 9, A–F). In addition, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay detected a strong programmed cell death signal in the nucleus of osgen1 microspores (Fig. 9, G–L), suggesting that accumulation of DSBs results in cell death of the mutant microspore. Furthermore, disturbance of the cytoskeleton was found in osgen1 microspores. Tubulin and actin did not form grid structures and only showed fuzzy signals, confirming cell death of osgen1 microspores (Smertenko and Franklin-Tong, 2011; Supplemental Fig. S9). Taken together, these results suggest that OsGEN1 has an important role in DNA replication or DNA repair before two rounds of mitosis during early microspore development. The unrepaired DSBs induce cell death and abortion of the microspore.

OsSEND1 Is Not Essential for Rice Development

Two homologs of GEN1/Yen1 that are designated GEN1 and SEND1 exist across the plant kingdom (Olivier et al., 2016). It was reported that in Arabidopsis, AtSEND1 but not AtGEN1 acts as functional GEN1/Yen1 homolog. AtSEND1 plays important roles in repairing toxic replication intermediates and maintaining telomere homeostasis, but is not required for meiotic recombination (Olivier et al., 2016). OsSEND1 is induced by UV and DNA damage reagents, and consistently OsSEND1 plays a role in DNA repair in somatic cells (Furukawa et al., 2003). We used qRT-PCR to investigate the spatio-temporal expression pattern of the OsSEND1 and found that OsSEND1 is expressed at a lower level than that of OsGEN1 in the anther and in vegetative tissues (Supplemental Fig. S5). To understand the biological function of OsSEND1, we created

Figure 6. Dual immunolocalization of OsGEN1 (green) and OsREC8 (magenta) in wild-type PMCs. A to C, Leptotene; D to F, early zygotene; G to I, late zygotene; J to L, pachytene; M to O, diakinesis. Bar = 2 μm.

Figure 7. Dual immunolocalization of OsGEN1 (green) with recombination proteins (magenta). A to C, Early zygotene; D to L, zygotene. Bar = 2 μm.
two mutant alleles using the CRISPR/Cas9 system. Both alleles were created with a 1 bp insertion at 143 bp downstream of ATG (A in ossend1-1, T in ossend1-2), leading to a frame shift and premature translation termination. All of the homozygous ossend1-1 and ossend1-2 plants showed normal growth and fertility (Supplemental Fig. S10). We used DAPI staining to monitor ossend1 meiotic progression. The mutants showed no obvious abnormality when compared with the wild type (Fig. 10, A–H) and the ossend1 osgen1 double mutant is comparable to the osgen1 single mutant (Fig. 10, I–P). A small number of DAPI bodies were found (6.25%, n = 96) at diakinesis, metaphase I (9.32%, n = 236), anaphase I (10.53%, n = 114), telophase I (6.41%, n = 156), prophase II (10.18%, n = 226), metaphase II (11.36%, n = 88), anaphase II (7.4%, n = 54), and tetrad (2.72%, n = 147). These results suggest that OsSEND1 is not essential for rice development and meiotic progression even though it shows a similar expression pattern with OsGEN1.

DISCUSSION

OsGEN1 Is Irreplaceable for Male Reproduction in Rice

The resolution of JMs such as HJs arising from the HR process is critical for the assurance of genome stability and proper chromosome segregation. Enzymes capable of resolving HJs have been isolated from a wide variety of eukaryotic organisms including yeasts, worms, flies, mammals, and plants. It has been shown that three structure-specific endonucleases or enzyme complexes, Mus81-Mms4/MUS81-EME1, Sx1-Slx4/SLX1-SLX4, and Yen1/GEN1, function as HJ resolvases in eukaryotes. Previous studies indicate that Mus81-Mms4/MUS81-EME1 play a leading role in HJ resolution in most eukaryotes. By contrast, Yen1 has been reported to serve as a backup pathway of Mus81-Mms4 in yeast to deal with the processing of residue JMs that escape the activity of Mus81-Mms4 (Blanco et al., 2010; Ho et al., 2010). While in animals, GEN1 homologs define a distinct recombination intermediate resolution pathway to remove detrimental JMs together with MUS81-EME1 and SLX1-SLX4 pathways. Different from other eukaryotes, most plants possess two Yen1/GEN1 homologs, named GEN1 and SEND1, but only one Yen1/GEN1 homolog exists in the genome of more ancient mosses, implying that a gene duplication occurred during the early development of plants after separation from mosses (Bauknecht and Kobbe, 2014; Supplemental Fig. S2C). A very recent report showed that in Arabidopsis, SEND1 but not GEN1 acts as the functional Yen1/GEN1 homolog and plays an important role in toxic replication intermediate repair and telomere homeostasis in the absence of MUS81 (Olivier et al., 2016). In our study, we demonstrated that OsGEN1 but not OsSEND1 has indispensable roles in resolving meiotic and DNA replication intermediates in rice male reproductive cells.

In Arabidopsis, the single and double mutants of GEN1 and SEND1 show no observable developmental defects nor enhanced sensitivity to DNA damaging reagents. The activity of SEND1 is only required in plants lacking MUS81, suggesting that it provides a backup resolution pathway. However, disruption of GEN1 has no impact on the function of both SEND1 and MUS81. On the contrary, the loss of function of OsGEN1 in rice leads to complete male sterility. The abortion of mutant pollen appears to be caused by programmed cell death from the accumulation of deleterious recombination intermediates during DNA replication in young microspores. Although deficiency of OsGEN1 does not interfere with meiotic progression, a moderate decrease in CO frequency was observed in osgen1
pollen mother cells, indicating that OsGEN1 is also involved in the meiotic JM resolution. Furthermore, the osgen1 ossend1 double mutant shows no difference from the osgen1 single mutant. These results suggest that the two paralogs have acquired nonredundant biological functions after the gene duplication. Specifically, OsGEN1 has obtained essential roles in resolving recombinant intermediates in rice male germ cells. The biochemical activity and in vivo roles of OsSEND1 and the rice MUS81-MMS4 homologs remain to be determined. Previous studies showed that OsSEND1 is preferentially expressed in meristematic tissues and induced by DNA damaging agents (Furukawa et al., 2003). OsGEN1 is also highly expressed in vegetative tissues (Supplemental Fig. S5). Therefore, it could not be excluded that OsSEND1 and/or OsGEN1 have redundant functions with OsMUS81 in mitotic cells, especially under stress conditions.

Both OsGEN1 and OsSEND1 contain an XPG type amino-terminal domain, a central XPG nuclease domain, and an HhH DNA binding domain. The functional differences between OsGEN1 and OsSEND1 may be attributed to the highly polymorphic carboxyl-terminal region. Comparative analysis found that Arabidopsis SEND1 possesses a carboxyl-terminal chromodomain-like motif involved in DNA and histone recognition, which is not present either in GEN1 or in two rice paralogs. In our study, we found that a deletion of the carboxyl-terminal of OsGEN1 severely compromises the function of the protein. We used CRISPR technology to knock out OsGEN1. The CRISPR lines with truncated OsGEN1 lacking C terminus, HhH, and/or XPG1 domains show identical phenotypes with osgen1 mutant (Supplemental Fig. S11), which highlights the importance of the C terminus of OsGEN1 for its function. The activities of yeast Yen1 and human GEN1 are controlled by master cell cycle regulators and are regulated by phosphorylation or dephosphorylation. The phosphorylation sites reside in the C terminus of Yen1/GEN1 (Blanco et al., 2014; Chan and West, 2014; Eissler et al., 2014). We hypothesize that the function of OsGEN1 may be controlled by regulators through interaction with its C terminus.

OsGEN1 Is Required for CO Formation in Rice

During meiotic prophase I, crossover formation via homologous recombination is required to establish physical connections between chromosomes to ensure correct segregation. Meiotic crossovers are generated via two pathways (Higgins et al., 2004). In most eukaryotes, most COs are formed by the interference-sensitive pathway (Class I COs) in which one CO reduces the probability of a second CO nearby. The Class II COs are insensitive to interference and produce randomly distributed COs (Osman et al., 2011). In budding yeast, mammals, and Arabidopsis, Class I COs depend on the activity of ZMM proteins (Zip1-4, Mer3, Msh4, and Msh5; Börner et al., 2004; Bishop and Zickler, 2004;
OsGen1 Is Essential for Rice Pollen Development

Lynn et al., 2007; Guiraldelli et al., 2013) and Class II(197,255),(913,285)(197,266),(913,296) COs depend on HJ resolvase Mus81-Mms4 (de los Santos et al., 2003; Osman et al., 2003; Hollingsworth and Brill, 2004; Higgins et al., 2008). SLX4 has also been reported to play an important role in Class II CO formation in mice (Holloway et al., 2011).

It has been shown that there are two kinds of COs in rice: one appears to be sensitive to interference, and the other one is not (Wang et al., 2009). Rice homologs of ZMM components, including MER3, ZIP4, HEI10, OsMSH4, and OsMSH5, were reported to have conserved functions in Class I CO formation (Wang et al., 2009, 2012, 2015; Shen et al., 2012; Luo et al., 2013; Zhang et al., 2014c). It remains unknown which resolvase is required for Class II CO formation in rice. In Arabidopsis, Class II pathway accounts for only a small fraction of CO formation (Higgins et al., 2004, 2008; Berchowitz et al., 2007). In rice, disruption of Class I CO components cause 60 to 90% reduction in the CO number, suggesting that the Class II pathway accounts for no more than 10% CO formation. In our study, we showed that depletion of OsGEN1 leads to an ~6% decrease in chiasmata compared to the wild type, which resembled the small fraction of CO decrease observed in Class II mutants in Arabidopsis (Higgins et al., 2008). Furthermore, distribution of remaining chiasmata was also affected by the OsGEN1 mutation. Besides interference, having at least one CO per homologous chromosome pairs is another basic rule of meiotic CO formation. The Class I CO pathway is essential for obligatory CO formation. Disruption of the Class I CO pathway leads to the loss of most obligatory COs and the production of a large number of univalents. In het10, the proportion of chromosome pairs with at least one CO was substantially decreased (Fig. 3C). Previous studies and recent modeling analyses suggested that interference is an outcome of obligatory CO formation (Jones and Franklin, 2006; Zhang et al., 2014b). In osgen1, only a very small fraction of chromosome pairs (0.28%) lacked a crossover, suggesting that OsGEN1 has minor effects on obligatory CO formation and the interference pathway. On the contrary, OsGEN1 deficiency has a significant effect on non-obligatory CO formation. In the wild type, the proportions of chromosome pairs with two or three COs were 58.20% and 6.48% in the wild type (63 cells were counted), while in the osgen1 mutant it decreased to 51.91% and 2.82%, respectively (59 cells were counted). In contrast, the proportion of chromosome pairs having one CO increased from 35.32% in the wild type to 44.99% in the osgen1 mutant (Fig. 3C). On the other hand, chromosome pairing and synapsis are not affected by the dysfunction of OsGEN1 (Fig. 4). In addition, OsGEN1 proteins localize to meiotic chromosomes from leptotene to pachytene but do not colocalize with ZMM proteins (Figs. 6 and 7). Collectively, these results suggest that OsGEN1 is very likely responsible for the generation of Class II COs. OsGEN1 might be occasionally involved in resolving Class I double HJs and in its absence, could produce a low number of univalents.

In Arabidopsis, MUS81 has been shown to be the mediator of the interference-insensitive pathway. Mutation of MUS81 causes a reduction of ~10% of meiotic CO (Børner et al., 2007; Higgins et al., 2008). However, the single mutants or the double mutant of gen1 and send1 do not show any meiotic defects. Although 10% of telophase I and anaphase II nuclei in mus81 send1 meioocytes exhibit segregation defects, it is difficult to conclude that SEND1 is required for meiotic CO formation due to the severe mitotic defects that occurred at earlier stage (Olivier et al., 2016). But it could not be ruled out that SEND1 may also have a minor role in chiasmata formation in Arabidopsis.

Previous studies indicate that interference-sensitive COs arise from double HJs processed by ZMM complex (Børner et al., 2004; Lynn et al., 2007). The MSH4 and MSH5 heterodimers play an earlier role in stabilizing CO-specific recombination intermediates to ensure double HJs form and resolve (Shinohara et al., 2008). In contrast, interference-insensitive COs are generated by the action of MUS81/MMS4 on aberrant joint molecules, such as single or nicked HJs, that cannot be resolved by the ZMM proteins. Biochemical and genetic analysis in S. cerevisiae indicates that Mus81-Eme1 acts late in meiotic recombination (Oh et al., 2008). In vitro, OsGEN1 has the ability to cleave ligated or nicked double HJs. However, which of these structures are targeted by OsGEN1 in vivo is still unclear, but the observation that synopsis is completed and the localization of ZMM proteins is not affected in osgen1 (Figs. 4 and 5), suggests that OsGEN1 may act at a late stage to process JMs that are not resolved by the MSH4-MSH5 pathway. The exact substrates of OsGEN1 during meiosis and its interaction with other meiotic CO formation pathways need further investigation.

OsGEN1 Functions in DNA Repair in Germ Cells

In Arabidopsis, a 10% decrease in chiasmata in the attmus81 mutant leads to only a small reduction in pollen viability and seed set (Børchowitz et al., 2007; Higgins et al., 2008), indicating that the presence of a small subset of unresolved recombination intermediates during meiosis in osgen1 mutant is not enough to cause total male sterility. We found that after release from tetrad, OsGEN1 relocalized in microspore nuclei (Fig. 8, A–D). osgen1 was completely sterile and the OsGEN1+/− pollen were all fertile, suggesting that OsGEN1 proteins were synthesized in the male meioocytes and allocated to each microspore in the tetrad. osgen1 microspores do not undergo mitosis (Fig. 8, I–L) and accumulate DSBs that lead to programmed cell death and pollen abortion at a later stage (Fig. 9). This indicates that OsGEN1 also functions in pollen mitosis, which is a specific process in angiosperms where microspores freed from the tetrad undergo an asymmetric mitosis division to form bicellular pollen (Mascarenhas, 1989; McCormick, 1993).

Before mitosis, the microspore genome is replicated to ensure that the haploid state is maintained until mature pollen formation. During DNA replication,
HR-mediated repair is critical to remove DNA lesions that will lead to the failure of replication fork progression. It has been reported that Mus81-Mms4/MUS81-EME1, Yen1/GEN1, and Slx1-Slx4/SLX1-SLX4 can process failing RFs and/or the resolution of intermediates of HR-dependent RF recovery (Rass, 2013). The failure to repair damaged RFs may cause DSB accumulation in microspores. It has been reported that DSBs are extremely hazardous lesions and that cell division does not occur when DSBs are present (Burgoyne et al., 2007). Evidence suggests that the mitotic cell cycle is managed by a checkpoint mechanism that acts to delay cell division when DSBs are present at the end of G2. Previous studies indicate that GEN-1 has dual roles in both HR-mediated DNA repair and DNA damage signaling in C. elegans (Saito et al., 2012, 2013). OsGEN1 may not only participate in DNA repair, but also act as a cell-cycle controlling signal during the meiosis to mitosis cell-cycle transition.

OsGEN1 mutation may also result in unrepairred recombination intermediates during meiosis and lead to accumulation of DNA damage. However, compared with mutants of genes involved in DSB repair, such as oscom1, osrad51c, and osmre11 (Ji et al., 2012, 2013; Tang et al., 2014), only a low number of abnormal DAPI bodies appears in 8% meiocytes. In addition, we did not detect obvious DNA damage by TUNEL assay at tetrad stage. Therefore, we propose that there is no or only a very limited effect of OsGEN1 single mutation on meiotic DSB repair, which is unlikely to be causing strong DNA damage at later stage. The low number of abnormal DAPI bodies in the male meiocytes could suggest that there is a redundant pathway that can repair most of the recombination intermediates in the absence of OsGEN1, but not all. This redundant pathway would therefore not be present post-male meiosis.

CONCLUSION

In summary, we have determined that OsGEN1 has essential roles in processing recombination intermediates during male reproduction in rice. This is distinct from AtSEN1 and AtGEN1 that have essential overlapping roles with AtMUS81 in JM resolution in Arabidopsis, suggesting functional variations and divergence of the two Yen1/GEN1 paralogs in plant evolution. Further studies of mutants lacking a combination of osmus81/osgen1/ossend1 should provide additional insights into the mechanisms of recombination intermediate resolution during meiosis and mitosis in rice.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Molecular Cloning of OsGEN1

Rice (Oryza sativa) plants from the 9522 background (O. sativa ssp. japonica) were grown in the paddy field of Shanghai Jiao Tong University. F2 progenies for mapping were generated from a cross between cultivar 9311 (O. sativa ssp. indica) and the osgen1 mutant. One-hundred-forty-six male-sterile plants in the F2 population were selected for mapping, and bulked segregated analysis (Liu et al., 2005) was used to isolate OsGEN1.

Complementation of osgen1

For functional complementation, a 5,599-bp genomic sequence of OsGEN1 including the entire OsGEN1 coding region (3,623 bp) and 1,976-bp upstream sequence was amplified from wild-type rice genomic DNA and cloned into the binary vector pCAMBIA1301. We also exchanged the pCAMBIA1301 GUS reporter gene with eGFP, thus generating two marker constructs. Calli induced from young panicles of the homozygous osgen1 plants were used for transformation with Agrobacterium tumefaciens (EHA105), which carries the pCAMBIA1301-OsGEN1-GUS, pCAMBIA1301-OsGEN1-eGFP plasmid, or the control plasmid pCAMBIA1301. For transgenic plants, at least 10 independent lines were obtained from each construct and identified by PCR using the primers listed in Supplemental Table S1.

Mutant Phenotypic Analyses

Plant materials were photographed with a digital camera (Nikon; catalog no. E959) and a dissecting microscope (Motic; catalog no. K400). Transverse section observation was performed as described by Li et al. (2006). DAPI staining of microspores was performed as reported in Cheng (2013). Chromosomes were stained with DAPI (Vector Laboratories) and photographed using the Eclipse Ni-E microscope (Nikon).

Antibody Production

For preparation of OsGEN1 polyclonal antibody, a 627-bp DNA fragment encoding a 209-amino acid peptide of OsGEN1 (residues 284–492) was amplified from rice cDNA and cloned into pGEX-4T-1 (GE) with BamHI-XhoI. The recombinant protein was expressed in Escherichia coli DE3 (BL21; Novagen) and purified to produce polyclonal antibodies (prepared by Abclonal) specific to OsGEN1 (Supplemental Fig. S12). A rabbit DMC1 polyclonal antibody was prepared using the peptide MAPSKQYDEGGQLQLMDA (prepared by Abclonal). The polyclonal antibodies OsREC8, PAIR2, PAIR3, ZEP1, yH2AX, OsCOMT, RAD51c, and HET10 used in this study were prepared according to Wang et al. (2009, 2010, 2011, 2012), Ji et al. (2012), Miao et al. (2013), and Tang et al. (2014). The antibody against RPA2c was described in Li et al. (2013). Anti-β-tubulin antibody was obtained from Sigma-Aldrich (catalog no. T4026).

Immunolocalization Assays

Immunolocalization assays were performed according to Cheng (2013). Fresh young panicles at suitable phase were fixed in 4% (w/v) paraformaldehyde for 20 min at room temperature and wished thrice with PBS. Anters in the proper stages were squashed on a slide with PBS solution and soaked in liquid N and removed from the coverslip quickly with a blade. The slides were dehydrated through an ethanol series (70, 90, and 100%), then incubated in a 100% acetic acid for 20 min at room temperature and wished thrice with PBS. Anthers in the microsporocytes were fixed in 4% paraformaldehyde and 0.1% Triton X-100, then permeabilized with 0.1% Triton X-100 and treated with 0.1% proteinase K for 5 min at room temperature. The slides were then washed with 0.1% PBS for 5 min and incubated with a blocking solution (10% goat serum, 0.5% Tween 20, 1% BSA, and 2% chicken serum) for 1 h at room temperature. Primary antibodies against OsGEN1, OsREC8, PAIR2, PAIR3, ZEP1, yH2AX, OsCOMT, RAD51c, and HET10 were used in this study and were incubated with the slides at a 1:500 dilution. The slides were washed with PBS for 5 min followed by incubation with FITC-conjugated secondary antibodies (anti-rabbit) for 1 h at room temperature. The slides were then washed with PBS and mounted with DAPI (Life Technologies). Images were captured using a Leica DMBA2000 fluorescence microscope (Leica). For immunofluorescence microscopy, 1:400 dilution of DAPI (Life Technologies) was added to the coverslips before mounting. Images were captured using a Zeiss LSM 510 Meta confocal microscope. Immunochemistry was performed using the EZ1000 immunostaining system (Genomed Ltd., 2009). After the slides were analyzed with the Nikon Eclipse 80i fluorescence microscope (Nikon), they were counterstained with DAPI and analyzed with the Metaview advanced software (Improvision).
Fluorescence Microscopy Visualization of Actin Filaments

According to Zhang et al. (2011c), fresh free microspores were incubated in PEM buffer (100 mM PIPES, 10 mM EGTA, 5 mM MgSO\(_4\), and 0.3 mM mannitol, pH 6.9) that contains 2% (w/v) glycerol (Sigma-Aldrich) and 6.6 mM Alexa Fluor 488-phallolidin staining (Invitrogen). After 35 min incubation, pollen was observed using a TCS SP confocal laser scanning microscope (Leica).

CRISPR Knockout OsSEND1, HEI10, and OsGEN1

The sgRNA-Cas9 plant expression vectors were supplied by Professor Jiankang Zhu and constructed as previously described in Feng et al. (2013), Mao et al. (2013), and Zhang et al. (2014a). The primers for constructing the sgRNA vectors for OsSEND1 and HEI10 are listed in Supplemental Experimental Procedures. The constructed OsSEND1 sgRNA-Cas9 and HEI10 sgRNA-Cas9 plasmids were separately transformed into A. tumefaciens (EHA105) and then used to infect wild-type rice calli separately. The OsGEN1 CRISPR transgenic plants were produced by an outside company (Biogle). The transgenic plants identified by PCR using the primers for amplifying targeted regions of OsSEND1, HEI10, and OsGEN1 are listed in Supplemental Experimental Procedures and were sequenced directly. The third generation of the homozygous lines showing the same genotyping was used to observe the phenotype.

qRT-PCR Assay

Total RNA from the wild type was isolated using TRIZOL reagent (Sigma-Aldrich) from rice root, shoot, leaf, lemma/palea, and anthers. Rice anthers from different developmental stages as defined by Zhang et al. (2011a). Roots, shoots, and leaves were collected from 20-d-old seedlings. An amount of 0.2 mg of RNA per sample was reverse transcribed to synthesize cDNA using Primerscript RT reagent kit with genomic DNA eraser (Takara). qRT-PCR was performed as described by Fu et al. (2014).

Phylogenetic and Structure Analysis

The protein sequences were aligned using ClustalX (version 2.0; Larkin et al., 2007), and then adjusted manually using GeneDoc software (version 2.6.002; http://www.psc.edu/biomed/genedoc/). Neighbor-joining trees were constructed using the MEGA (version 4.0) software (Tamura et al., 2007) with the following parameters: Poisson correction, pairwise deletion, and bootstrap (1,000 replicates; random seed). The protein domain predictions were from InterProScan (http://www.ebi.ac.uk/interpro/search/sequence-search).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: OsGEN1 (Os09g0521900), OsSEND1 (Os08g0101600), OsGEN2 (Os02g0225200), OsREC8 (Os01g0578000), PAIR2 (Os09g0568800), PAIR3 (Os10g0405500), ZEP1 (Os04g0452500), OsCOM1 (Os06g0613400), RPA2c (Os06g0693300), DMC1 (Os11g016800), Os12g0143800, RAD51C (Os05g0580500), HEI10 (Os02g0232100), and HJAX (Os03g0721900).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Transverse section observation of the anther development in the wild type and osgen1.

Supplemental Figure S2. Map-based cloning of OsGEN1 and phylogenetic analysis of RAD2/XPG family members.

Supplemental Figure S3. Structural similarity and biochemical properties of Yen1/GEN1 homologs.

Supplemental Figure S4. Complementation of osgen1.

Supplemental Figure S5. Spatio-temporal analysis of OsGEN1 and OsSEND1 transcription by qRT-PCR.

Supplemental Figure S6. Phenotypes of HEI10 CRISPR lines.

Supplemental Figure S7. Dual immunolocalization of SC proteins OsZEP1 (magenta) and OsREC8 (green) in wild-type and osgen1 PMCs pachytene.

Supplemental Figure S8. Dual immunolocalization of recombination proteins (magenta) and OsREC8 (green) in wild-type and osgen1 PMCs.

Supplemental Figure S9. Tubulin arrays (red) and actin filaments (green) in wild-type and osgen1 PMCs.

Supplemental Figure S10. Phenotypes of OsSEND1 CRISPR lines.

Supplemental Figure S11. Phenotypes of OsGEN1 CRISPR mutation lines.

Supplemental Figure S12. Dual immunolocalization of OsREC8 (magenta) and OsGEN1 (green) in wild-type and osgen1 PMCs at zygotene.

Supplemental Table S1. Primers used in this study.

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LITERATURE CITED


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