Running head: OsGEN1 is essential for Rice pollen development

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OsGEN1 is required for DNA repair by homologous recombination during male meiosis and microspore development in Rice

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Summary: The canonical Holliday Junction resolvase OsGEN1 plays an important role in rice pollen development via influence interference-insensitive Crossover formation and DNA repair during pollen mitosis processes.
Footnotes:

C.W., J.D.H. and W.L. designed the experiments. C.W. and Y.H. carried out the experiments. C.W., J.D.H., P.L., and W.L. analyzed the data. C.W., J.D.H., D.Z. and W.L. wrote the article.

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ABSTRACT
Yen1/GEN1 are canonical Holliday Junction (HJ) 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 Slx1-Slx4/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. We first investigated the role of OsGEN1 during meiosis and found a reduction in chiasma frequency by ~6% in osgen1 mutants, compared to wild type. The remaining chiasmata deviated from a Poisson distribution, suggesting a possible involvement of OsGEN1 in the formation of interference-insensitive crossovers. Post-meiosis, 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.

Key words: OsGEN1, structure-specific endonuclease, homologous recombination, meiosis, crossover, Holliday junction
INTRODUCTION

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). The majority of JMs are repaired by the synthesis-dependent strand annealing (SDSA) pathway (Andersen and Sekelsky, 2010; Sarbajna and West, 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-TopoisomeraseIIIα-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 (SSEs) to generate both crossover (CO) and non-crossover (NCO) products (Mimitou and Symington, 2009).

HJ resolvases have been identified from a wide variety of organisms, including bacteriophages, archaeabacteria, bacteria, viruses and eukaryotes. It has been demonstrated that Mus81-Mms4/MUS81-EME1 (MMS and UV-sensitive protein 81–Methyl Methane Sulfonate sensitivity 4/Essential meiotic endonuclease 1), Slx1-Slx4/SLX1-SLX4 (Synthetic lethal of unknown function) and Yen1/GEN1 (Holliday junction resolvase YEN1/Gen endonuclease homolog 1) are key structure-specific endonucleases involved in HJ resolution in eukaryotes (Boddy et al., 2001; Schwartz and Heyer, 2011). Mus81-Mms4 and Slx1-Slx4 were identified from the same screen for proteins essential for the viability of yeast cells lacking the SGS1 (Slow growth
suppressor 1) 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; Ogrunc 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 S. 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 E. 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 (XPGN), internal XPG nuclease domain (XPGL) and helix-hairpin-helix (HhH) domain (Rass et al., 2010; Bauknecht and Kobbe, 2014). It was reported that Yen1/GEN1 homologs from human, yeast, C. elegans, Arabidopsis and rice 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.,
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 following replication fork damage (Blanco et al., 2010). *K. lactis* mutants lacking a functional Mus81 are severely compromised in sporulation efficiently and crossover frequency, but lacking both Mus81 and Yen1 showed no further reduction in spore 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 Astrom, 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; Saito et al., 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 predominant 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 multi-nuclei, 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 (SCE) 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 multi-nucleation, 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 SEND1 (SINGLE-STRAND DNA ENDONUCLEASE 1), 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 important roles 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 an osgen1 ossend1 double mutant. 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 homologue of Yen1/GEN1 in rice and that it has indispensable roles in class II chiasmata formation and DNA lesion repair in rice male gametophytes.
RESULTS

Identification of the \textit{osgen1} Mutant

We isolated a male sterile mutant designated \textit{osgen1} from our rice mutant library constructed by using $^{60}$Co $\gamma$-ray radiated \textit{O.sativa} ssp.\textit{japonica} cv.9522. (Chu et al., 2005; Chen et al., 2006). The \textit{osgen1} mutant showed normal vegetative growth and floral development (Figure 1A and 1B), except the stamen was smaller (Figure 1C) and did not produce normal pollen (Figure 1D and 1E). When the \textit{osgen1} mutant was fertilized with wild-type pollen, normal seed set was observed and all F1 progeny were indistinguishable from wild type, thus indicating that \textit{osgen1} is female fertile. F2 progeny segregated 75 sterile plants from 288 total plants ($x^2=0.1667$, P>0.05), indicating mono-factorial recessive inheritance of the mutation. To identify the stage responsible for the male fertility defect in \textit{osgen1}, we prepared transverse sections of pollen mother cells (PMCs) in the mutant and wild type. The wild-type PMCs appeared 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 Figure 1).

To isolate the mutated gene that controls the sterile phenotype, an F2 mapping population was generated by crossing the mutant plants with the \textit{Indica} rice variety 9311. Sterile plants segregated in the 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 Figure 2A). By re-sequencing the mutant genomic DNA, we found a two base pair deletion in the seventh exon of \textit{OsGEN1} gene (LOC Os09g35000), leading to a frame shift and premature translation termination (Supplemental Figure 2B). A genomic DNA fragment containing the \textit{OsGEN1} gene and 2Kb upstream sequence was translationally fused to the beta-glucuronidase (GUS) reporter and eGFP reporter separately and were introduced into \textit{osgen1} mutant plants. Both of the primary transgenic lines were fertile (Supplemental Figure 4), indicating that \textit{OsGEN1} is responsible for the male sterile phenotype in \textit{osgen1}. The full length OsGEN1 protein sequence was used as a query to search the
National Center for Biotechnology Information protein database and identified 41 homologues belonging to the RAD2/XPG family (Supplemental Figure 2C). Of these, OsGEN1 clustered with 10 homologues in the Yen1/GEN1 sub family, proteins which have similar structures but variable in length and isoelectric points (Supplemental Figure 3). The XPGI and XPGN 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 (Tetrad stage) (Supplemental Figure 5).

OsGEN1 Mutation Influences Meiotic Progression in the Pollen Mother Cell

It has been reported that OsGEN1 has Holliday Junction 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 pollen mother cells (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) (Figure 2I). Randomly distributed univalents were also observed at later stages, including metaphase I (12.5%, n=113) (Figure 2J), anaphase I (8.33%, n=120), (Figure 2K), telophase I (9%, n=111) (Figure 2L), prophase II (13.2%, n=47) (Figure 2M), metaphase II (11.2%, n=63) (Figure 2N), anaphase II (7%, n=56) (Figure 2O) and tetrad (1.70%, n=176) (Figure 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 wild type by studying the shape of bivalents at diakinesis and metaphase I. At diakinesis, the “X” shape and ring-shaped bivalents were treated as having one and two chiasmata respectively, and the “8” shape 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 characteristic as described previously (Sanchez Moran et al., 2001). The mean chiasma frequency in wild type was 20.3 per cell (n=168), while in osgen1 mutant the mean chiasma frequency was 18.8 per cell (n=170). Thus, mutation of osgen1 led to an approximately ~6% reduction in chiasma compared to wild type. In wild-type PMCs, the frequency of chiasma per cell deviated from a predicted Poisson distribution ($\chi^2_{20}=114$, $P<0.001$) (Figure 3A), which was also the case with osgen1 ($\chi^2_{18}=144$, $P<0.001$) (Figure 3B), suggesting that the majority of chiasmata were not randomly distributed among cells.

Since 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 Figure 6) 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) and the remaining chiasmata fitted a Poisson distribution ($\chi^2_{13}=19$, $P>0.1$) (Figure 3C) which is consistent with the previous report (Wang et al., 2012). A significant reduction in the number of chiasmata was observed in osgen1 hei10 double mutant (reduced to 4.0 per cell, n=149; $t_{314}=7.2$, $P<0.01$). In addition, the remaining chiasmata also fitted a Poisson distribution ($\chi^2_{8}=13$, $P>0.05$) (Figure 3D), suggesting that the further reduction in chiasma in the osgen1 hei10 double mutant are a random subset of the residual chiasmata present in the hei10 single mutant. Furthermore, OsGEN1 mutation led to a decrease in the number of bivalents having two and three chiasmata, but increased number of univalents and bivalents with one chiasma in the wild type and hei10 background (Supplemental Figure 7).

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; Shao et al., 2011; Wang et al., 2011). In osgen1, no difference in the localization of OsREC8, PAIR2, PAIR3 and ZEP1 was observed in
the mutant PMCs (Figure 4 and Supplemental Figure 8), 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 (Mahadevaiah 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 meiocytes (Figure 5A to 5H, 5J, 5K and Supplemental Figure 9A to 9E).

At zygotene, the average number of DMC1 foci was 64.9 (n=18, range 48-98) in wild type, and 61.3 (n=18, range 54-84) in osgen1; the average number of RAD51C foci was 56.2 (n=20, range 46-80) in 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 21-27) in wild type, and 23.2 (n=10, range 22-28) in osgen1 (Figure 5I, 5L and Supplemental Figure 9F), 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) (Figure 6A to 6C). At early zygotene, the numbers of OsGEN1 foci began to decline (79.7, n=7, range 67-89) (Figure 6D to 6F). At late zygotene, fewer OsGEN1 foci were observed (18.1, n=10, range 13-24) (Figure 6G to 6I) that continued to decline at pachytene ~7.18 (n=11, range 5-13) (Figure 6J to 6L) until diplotene, (3.6, n=8, range 3-5) (Figure 6M to 6O).
OsGEN1 does not Co-Localise with Recombination Proteins

In order to understand the relationship of OsGEN1 with other recombination proteins, we used dual-immunolocalization to investigate the co-localization 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 2-3 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 also 2-3 merged signals were observed. Later, both RPA2c and OsGEN1 foci decreased and were hardly detected. We also investigated the co-localization of OsGEN1 with DMC1 and RAD51C in PMCs. Different from MUS81 in Arabidopsis (Higgins et al., 2008), most of OsGEN1 foci do not co-localize with those of both proteins (Figure 7D to 7I). HEI10 is involved in the interference sensitive CO pathway and marks these sites during diakinesis. In order to determine the relationship between HEI10 and OsGEN1, we counted the numbers of HEI10 and OsGEN1 foci in PMCs (Figure 7J to 7L). 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 1-2 foci co-localized, which may indicate that HEI10 and OsGEN1 are involved in different pathways during CO formation.

OsGEN1 is Required for DSB Repair post-Meiosis

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 siliqae (Berchowitz et al., 2007; Higgins et al., 2008). Therefore we suspected that the completely 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 following the meiotic cell division and produce a male gametophyte containing a vegetative nucleus and two generative nuclei to ensure double fertilization (Figure 8E to 8H). However, osgen1 microspores arrest at mitosis I. In young osgen1 microspores, chromosomes remain highly compacted (Figure 8I to 8L) and DNA fragmentation was observed (Figure 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 prior to 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 function after meiosis, we detected OsGEN1 localization and found that OsGEN1 re-localized on chromosomes after the microspore released from the tetrad. OsGEN1 foci reached a peak when the microspore nucleus became loose (Figure 8B) and released from chromosomes when the nucleus becomes condensed again (Figure 8C and 8D), 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 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 to 56) (Figure 9A to 9F). In addition, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay detected a strong programmed cell death (PCD) signal in the nucleus of osgen1 microspores (Figure 9G to 9L), 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 Figure 10). 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 which 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 quantitative RT-PCR (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 as well as vegetative tissues (Supplemental Figure 5). In order to understand the biological function of OsSEND1, we created two mutant alleles using the CRISPR/Cas9 system. Both alleles were created with a one base pair 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 (Supplementary Figure 11). We used DAPI staining to monitor ossend1 meiotic progression. The mutants showed no obvious abnormality when compared with wild type (Figure 10A to 10H) and the ossend1 osgen1 double mutant is comparable to the osgen1 single mutant (Figure 10I to 10P). A small number of univalents 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 joint DNA intermediates (JMs) such as Holliday junctions (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, Slx1-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 which 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 Figure 2C). 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 JMs resolution. Furthermore, the osgen1 ossend1 double mutant shows no difference from the osgen1 single mutant. These results suggest that the two paralogs have acquired non-redundant 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 Figure 5). 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 XPGI domains show identical phenotypes with osgen1 mutant (Supplemental Figure 12), which highlights the importance of the C-terminus of OsGEN1 for its function. The activity of
yeast Yen1 and human GEN1 are controlled by master cell cycle regulators and are regulated by phosphorylation or de-phosphorylation. 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, the majority of 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) (Borner et al., 2004; Bishop and Zickler, 2004; Lynn et al., 2007; Guiraldelli et al., 2013) and class II CO 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 important roles 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; Shen et al., 2012; Wang et al., 2012; Luo et al., 2013; Zhang et al., 2014b; Wang et al., 2015). 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; Berchowitz et al., 2007; Higgins et al., 2008). In rice, disruption of Class I CO components cause 60-90% reduction in the CO number, suggesting that Class II pathway accounts for no more than 10% CO formation. In our study, we showed that depletion of OsGEN1 leads to ~6% decrease in chiasmata compared to 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 chias mata deviates significantly from a Poisson distribution, suggesting they are non-random. On the other hand, chromosome pairing and synapsis are not affected by the dysfunction of OsGEN1 (Figure 4). In addition, OsGEN1 proteins localize to meiotic chromosomes from leptotene to pachytene but do not co-localize with ZMM proteins (Figure 6 and Figure 7). Collectively, these results suggest that OsGEN1 is responsible for the generation of class II COs, although we could not exclude the possibility that OsGEN1 may be involved in the class I CO formation.

In Arabidopsis MUS81 has been shown to be the mediator of the interference insensitive pathway. Mutation of MUS81 causes a reduction of approximate 10% of meiotic CO (Berchowitz 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 meiocytes exhibit segregation defects, it is difficult to conclude that SEND1 is required for meiotic CO formation due to the severe mitotic defects 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 (Borner et al., 2004; Lynn et al., 2007). The MSH4 and MSH5 heterodimers play an earlier role in stabilizing CO-specific recombination intermediates to ensure dHJs 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 act late in meiotic recombination (Oh et al., 2008). In vitro, OsGEN1 has the ability to cleave ligated or nicked dHJs. However, which of these structures are targeted by OsGEN1 in vivo is still unclear, but the observation that synapsis is completed and the localization of ZMM proteins is not affected in osgenl (Figure 4 and Figure 5), suggests that OsGEN1 may act at a late stage to process JM s 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 atmus81 mutant leads to only a small reduction in pollen viability and seed set (Berchowitz 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 (Figure 8A to 8D). osgen1 microspores do not undergo mitosis (Figure 8I to 8L) and accumulate DSBs which lead to PCD and pollen abortion at a later stage (Figure 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 bi-cellular 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 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 damaging signaling in C. elegans (Saito et al., 2012; Saito et al., 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.
CONCLUSION

In summary, we have determined that \textit{OsGEN1} has essential roles in processing recombination intermediates during male reproduction in rice. This is distinct from \textit{AtSEND1} and \textit{AtGEN1} that have essential overlapping roles with \textit{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 \textit{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 \textit{OsGEN1}

Rice plants from the 9522 background (\textit{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 (\textit{O.sativa ssp. Indica}) and the \textit{osgen1} mutant. 146 male sterile plants in the F2 population were selected for mapping and bulked segregated analysis (Liu et al., 2005) was used to isolate \textit{OsGEN1}.

Complementation of \textit{osgen1}

For functional complementation, a 5599bp genomic sequence of \textit{OsGEN1} including the entire \textit{OsGEN1} coding region (3623bp) and 1976bp 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 \textit{osgen1} plants were used for transformation with \textit{Agrobacterium tumefaciens} (EHA105), which carries the pCAMBIA1301-\textit{OsGEN1-GUS}, pCAMBIA1301-\textit{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.
Mutant Phenotypic Analyses

Plant materials were photographed with a Nikon E995 digital camera and a Motic K400 dissecting microscope. Transverse section observation was performed as described previously (Li et al., 2006). DAPI staining of microspores was performed as reported (Cheng, 2013). Chromosomes were stained with DAPI (Vector Laboratories, Burlingame, CA) and photographed by the Nikon’s Eclipse Ni-E microscope.

Antibody Production

For preparation of OsGEN1 polyclonal antibody, a 627bp DNA fragment encoding a 209 amino acid peptide of OsGEN1 (residues 284 to 492) was amplified from rice anther cDNA and cloned into pGEX-4T-1 (GE) with BamH1-Xho1. The recombinant protein was expressed in E.coli DE3 (BL21) (Novagen) and purified to produce mouse polyclonal antibodies (prepared by Ablonal of China) and specific to OsGEN1 (Supplemental Figure 13). A rabbit DMC1 polyclonal antibody was prepared using the peptide MAPSKQYDEGGQLQLMDA (prepared by Ablonal of China). The polyclonal antibodies OsREC8, PAIR2, PAIR3, ZEP1, γH2AX, OsCOM1, RAD51C and HEI10 used in this study were prepared according to previous reports (Wang et al., 2009; Wang et al., 2010; Wang et al., 2011; Ji et al., 2012; Wang et al., 2012; Miao et al., 2013; Tang et al., 2014). The antibody against RPA2c was described before (Li et al., 2013). Anti-β tubulin antibody was obtain from Sigma-Aldrich (Catalog no.T4026).

Immunolocalization Assays

Immunolocalization assays were performed according to the previous study (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. Anthers in the proper stages were squashed on a slide with PBS solution and soaked in liquid nitrogen and remove the coverslip quickly with a blade. The slides were dehydrated through an ethanol series (70, 90, and 100%), then incubated in a humid chamber at 37°C for 4 h
with different antibody combinations diluted 1:500 in TNB buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.5% blocking reagent). After three rounds of washing in PBS, Alex 555-conjugated goat anti-rabbit antibody (Life technologies) and Dylight 488-conjugated goat anti-mouse antibody (Abbkine) (1:1000) were added to the slides. Finally, after incubation in the humid chamber at 37°C for 1 h, the slides were counterstained with DAPI. The slides were photographed using the Nikon’s Eclipse Ni-E microscope, and analysis by the NIS-Elements AR software (Nikon).

**TUNEL Assay**

Microsporocytes at appropriate stages were collected and added onto a slide. Anthers were dissected from the spikelet and broken using forceps that released the microsporocytes into 10 ml of enzyme digestion mixture (includes 0.1 g cytohelicase, 0.0375 g sucrose, 0.25 g polyvinyl pyrrolidone MW40,000 (all Sigma)) to hydrolyze 20 min at room temperature. 5 µl 0.1% Triton X-100 was added to slides with 10 µl 4% (w/v) paraformaldehyde and allowed to air dry. Slides were washed with 1 × PBS for 5 minutes followed by use of the TUNEL detection kit (Dead End Fluorometric TUNEL system; Promega).

**Fluorescence Microscopy Visualization of Actin Filaments**

According to a previous study (Zhang et al., 2011b), fresh free microspore were incubated in PEM buffer (100 mM PIPES, 10 mM EGTA, 5 mM MgSO4 and 0.3 M mannitol, pH 6.9) that contains 2% (w/v) glycerol (Sigma-Aldrich) and 6.6 mM Alexa Fluor 488-phalloidin staining (Invitrogen). After 35 min incubation, pollen were observed using a Leica TCS SP5 confocal laser scanning microscope.

**CRISPR knockout OsSEND1, HEI10 and OsGEN1**

The sgRNA-Cas9 plant expression vectors were supplied by Professor Jiankang Zhu and constructed as previously described (Feng et al., 2013; Mao et al., 2013; 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 plasmid were separately transformed into Agrobacterium tumefaciens (EHA105) and then used to infect wild-type rice calli separately. The OsGEN1 CRISPR transgenic plants were produced by company (Biogle, China). 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 which shows the same genotyping was used to observe the phenotype.

**Quantitative RT-PCR Assay**

Total RNA from wild type was isolated using TRIZOL reagent (Sigma) from rice root, shoot, leave, lemma/palea and anthers. Rice anthers from different developmental stages as defined previously (Zhang et al., 2011a). Root, shoot and leave were collected from 20 day old seedlings. 0.2 mg of RNA per sample was reverse transcribed to synthesize cDNA using Primescript RT reagent kit with genomic DNA eraser (Takara). qRT-PCR was performed as described previously (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 (1000 replicates; random seed). The protein domain prediction were predicted 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), OsSDS (Os03g0225200), OsREC8 (Os05g0580500), PAIR2
(Os09g0506800), PAIR3 (Os10g0405500), ZEPI (Os04g0452500), OsCOMI
(Os06g0613400), RPA2c (Os06g0693300), DMCl (Os11g0146800, Os12g0143800),
RAD51C (Os01g0578000), HEI10 (Os02g0232100), H2AX (Os03g0721900).

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vector.

Figure Legends

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 wild type (D) and osgen1
(E). Bars = 5 cm in (A), 1 mm in (B) and (C), 100 µm in (D) and (E).

Figure 2. Meiotic Progression in Wild-type and osgen1 PMCs.

Meiotic progression was assayed in wild type (A to H) and osgen1 PMCs (I to 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=5µm.

Figure 3. Chiasma Distribution Is non-Random in Wild-type and osgen1
Compared to hei10, and osgen1 hei10.

Chiasma distribution was calculated in the wild type (A) (n=168), osgen1 (B) (n=170),
hei10 (C) (n=167) and osgen1 hei10 (D) (n=149) PMCs. Red lines with triangles
indicate observed distributions, whereas blue lines with circles show predicted Poisson
distribution.

Figure 4. Dual Immunolocalization of SC Proteins OsZEP1 (magenta) and OsREC8 (green) in Wild-type and osgen1 PMCs.
A to C 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.

Figure 6. Dual Immunolocalization of OsGEN1 (green) and OsREC8 (magenta) in Wild-type PMCs.

Figure 7. Dual Immunolocalization of OsGEN1 (green) with Recombination Proteins (magenta).
A to C early zygotene. D to L zygotene. Bar = 2 μm.

Figure 8. OsGEN1 is Essential for Microspore Development.
A to D OsGEN1 localization on microspore chromosomes, OsGEN1 foci are shown in green. E to L Microspore development in wild type (E to H) and osgen1 (I to L) after meiosis. The arrow in K shows DNA fragmentation. Bar = 2 μm.

Figure 9. Accumulation of DNA Damage causes Cell Death of osgen1 PMCs.
A to C and G to I wild-type, D to F and J to L osgen1. A to F show the distribution of γH2Ax foci. G to L TUNEL assays in wild-type and osgen1 microspores. Bar = 1 μm.

Figure 10. Meiosis in ossend1 and ossend1 osgen1 PMCs.

**Supplemental Figure 1. Transverse Section Observation of the Anther Development in Wild-type and osgen1.**

Anther locules from the wild-type (A) to (D), (I) to (L) and *osgen1* (E) to (H), (M) to (P). A and E stage 6 (pre-meiosis stage); B and F stage 7 (meiosis); C and G stage 8a (dyad stage); D and H stage 8b (tetra stage); I and M Stage 9 (young microspore stage); J and N stage 10; K and O stage 11; L and P stage 13. Bars =15 μm.

**Supplementary Figure 2. Map-based Cloning of OsGEN1 and Phylogenetic Analysis of RAD2/XPG Family Members.**

A, Map-based cloning of the *OsGEN1* gene. The names and positions of the molecular markers are indicated. AP006859 and AP005864 are genomic DNA accession numbers. The *OsGEN1* locus was mapped between two molecular markers (907-3 and 907-6). B, A schematic diagram of the exon and intron organization of *OsGEN1*. Black boxes indicate exons, lines indicate introns and grey boxes indicate 5’-UTR and 3’-UTR. The mutant sequence has two base pair deletion in the seventh exon, which is boxed. +1 Indicate the starting nucleotide of translation (ATG), and +1890 indicates the stop codon (TAG). C, Bootstrap neighbor-joining tree was constructed using MEGA with full length of each protein. Os, *Oryza sativa*; At, *Arabidopsis thaliana*; Pp, *physcomitrella patens*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*.

**Supplementary Figure 3. Structural Similarity and Biochemical Properties of Yen1/GEN1 Homologues.**

The representative endonucleases were selected from humans (*Homo sapiens*), budding yeast (*Saccharomyces cerevisiae*), fruit fly (*Drosophila melanogaster*), mouse (*Mus
musculus), worm (Caenorhabditis elegans), Arabidopsis (Arabidopsis thaliana) and rice (Oryza sativa). The conserved domains of each protein are highlighted in different colored boxes. The XPGN domain is indicated as red box; The XPGI domain is indicated as blue box; The HhH domain is indicated as yellow box. For each protein, the length in amino acids, isoelectric points, molecular weight and similarity with OsGEN1 are indicated at right.

Supplemental Figure 4. Complementation of osgen1.
A to B Images of inner floral organs of osgen1 transgenic lines carrying the construct of OsGEN1 promoter: gDNA: GUS (A), and OsGEN1 promoter: gDNA: eGFP (B). Bar=1mm. C to D I2-KI-stained pollen grains from A and B. Bars = 100μm. E, Schematic diagram of the OsGEN1 genomic fragment used for complementation of osgen1. The fragment consists of the 1976-bp promoter sequence upstream ATG and the 3623-bp genomic DNA spanning coding region of OsGEN1. The upper one shows a translational fusion of the GUS reporter with OsGEN1 and the lower one shows a translational fusion of the eGFP reporter with OsGEN1.

Supplemental Figure 5. Spatio-temporal analysis of OsGEN1 and OsSEND1 transcription by qRT–PCR.
Red indicate OsGEN1 expression pattern and blue indicate OsSEND1 expression pattern. S3-6 to s13 indicate rice anther development stages. Stage 3-6: archesporial cells differentiation and microspore mother cells formation; stage 7: first meiosis division process; stage 8a: dyad formation; stage 8b: tetrad formation; Stage 9: free microspore stage; stage 10: microspore vacuolation; stage 11: two nuclei microspore stage; stage 12: three nuclei microspore stage; stage 13: mature pollen formation and anther dehiscence.

Supplemental Figure 6. Phenotypes of HEI10 CRISPR lines.
A, A schematic diagram of the exon and intron organization of HEI10. Black boxes indicate exons, lines indicate introns and white boxes indicate 5’-UTR and 3’-UTR.
The mutant sequence had one base pair deletion in the fifth exon, which produced a truncated protein 134 amino acid in length. +1 Indicate the starting nucleotide of translation (ATG), and +915 indicates the stop codon (TAG). B, Sequencing results of the HEI10 gene in wild-type and HEI10 CRISPR lines. C, hei10-3 spikelets after removal of the palea and lemma. D, I2-KI staining of the pollen grains within the anther of hei10-3. E and G, hei10-3 and osgen1 hei10-3 PMCs diakinesis. F and H, hei10-3 and osgen1 hei10-3 PMCs metaphase I. Bars = 1 mm in (C), 100μm in (D), 5μm in (E) (F) (G)and (H).

Supplemental Figure 7. Chiasmata frequency in wild-type (n=63), osgen1 (n=59), hei10 (n=50) and osgen1 hei10 double mutant (n=59).

Supplemental Figure 8. Dual Immunolocalization of SC Proteins OsZEP1 (magenta) and OsREC8 (green) in Wild-type and osgen1 PMCs pachytene. Bar = 2 μm.

Supplemental Figure 9. Dual Immunolocalization of Recombination Proteins (magenta) and OsREC8 (green) in Wild-type and osgen1 PMCs. A is leptotene. B, D and E is zygotene. C and F is pachytene. Bar = 2 μm.

Supplementary Figure 10. Tubulin arrays (red) and Actin filaments (green) in Wild-type and osgen1 PMCs. A to D wild-type, E to H osgen1 mutant. Bar=2μm.

Supplementary Figure 11. Phenotypes of OsSEND1 CRISPR lines. A, A schematic diagram of the OsSEND1 protein. Arrows indicated the mutation site. B, Sequencing results of the OsSEND1 gene in wild-type and OsSEND1 CRISPR lines. C, Morphology of the wild-type and the ossend1-1 plants (T1). (Bar = 5 cm, 1cm and 100μm).
Supplementary Figure 12. Phenotypes of OsGEN1 CRISPR Mutation Lines.

A, A schematic diagram of the OsGEN1 protein. Arrows indicated the mutation site. osgen1-2 and osgen1-3 had a T insertion at 338bp and 603bp respectively, both caused a frame shift and premature translation termination. B, Sequencing results of the OsGEN1 gene in wild-type and OsGEN1-2 CRISPR lines. C, Sequencing results of the OsGEN1 gene in wild-type and OsGEN1-3 CRISPR lines. D and F, osgen1-2 and osgen1-3 spikelets after removal of the palea and lemma. E and G, I2-KI staining of the pollen grains within the anther of osgen1-2 and osgen1-3. H to O is meiotic progression in osgen1-2 and osgen1-3 PMCs. Bars = 1 mm in (D) and (F), 100μm in (E) and (G), 5μm in (H) to (O).

Supplemental Figure 13. Dual Immunolocalization of OsREC8 (magenta) and OsGEN1 (green) in Wild-type and osgen1 PMCs at zygotene.

(A) to (C) is wild-type, (D) to (F) and osgen1.

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A to D OsGEN1 localization on microspore chromosomes, OsGEN1 foci are shown in green. E to L Microspore development in wild type (E to H) and osgen1 (I to L) after
Figure 9. Accumulation of DNA Damage causes Cell Death of *osgen*1 PMCs.

A to C and G to I wild-type, D to F and J to L *osgen*1. A to F show the distribution of γH2Ax foci. G to L TUNEL assays in wild-type and *osgen*1 microspores. Bar = 1 μm.
Figure 10. Meiosis in *ossend1* and *ossend1 osgen1* PMCs.