REPRODUCTIVE BARRIERS AND GENE INTROGRESSION IN RICE SPECIES

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REPRODUCTIVE BARRIERS AND GENE INTROGRESSION IN RICE SPECIES

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Editorial: Reproductive Barriers and Gene Introgression in Rice Species

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Keywords: rice, reproductive barriers, wide hybridization, gene introgression, wild species

Editorial on the Research Topic

Reproductive Barriers and Gene Introgression in Rice Species

Rice (*Oryza sativa* L.) is one of the most widely consumed cereals both in developing and developed countries with the third-highest worldwide production (FAOSTAT, http://www.fao.org/faostat/en/#data). Rice production is constantly subject to multiple environmental stresses, both biotic and abiotic. In addition, the effect of such stresses changes in response to environmental conditions. The increasing global food demand, together with rapid population growth forces rice geneticists and breeders to speed up and push forward the improvement of resistance and/or avoidance to such stresses as well as productivity (International Rice Research Institute, https://www.irri.org/world-food-day-2020).

To achieve this complex task, the use of distant relatives of rice as donor of genetic diversity has been attempted to improve the resistance together with productivity in rice varieties, because of the limited genetic variation within current rice varieties (Brar and Khush, 1997, 2018). However, the introgression of useful genes from distant relatives of rice is often prevented by interspecific or intersubspecific reproductive barriers (Oka, 1988).

Various types of reproductive barriers can occur at different stages during the life cycle (Rieseberg and Willis, 2007). Depending on the developmental stage, reproductive isolation can be categorized as prezygotic and postzygotic reproductive isolation (Smith, 1989). Prezygotic reproductive isolation prevents the formation of hybrid zygotes, while postzygotic reproductive isolation results in hybrid incompatibility, including hybrid necrosis, weakness, sterility, and breakdown (Stebbins, 1950). Postzygotic reproductive barriers are the major obstacles for transferring favorable genes from wild and the cultivated relative to cultivated rice. Understanding the causes and consequences of reproductive isolation, and providing a rational breeding strategy, may improve cultivated rice for various traits of importance to rice improvement, such as yield, quality, biotic and abiotic resistance.

In the last few decades, much progress has been made in these research areas, especially in mapping genes associated with reproductive barriers and resistance to environmental stresses (Brar and Khush, 1997, 2018; Ouyang and Zhang, 2018). The Research Topic entitled "Reproductive Barriers and Gene Introgression in Rice Species" is meant to gather together current knowledge in this field and share it with the scientific community to accelerate rice genetic improvement.

In this Research Topic, Sakata et al. isolated two novel loci *S22A* and *S22B* for hybrid pollen sterility between *O. sativa* and *O. glumaepatula*. Both genes encoded proteins containing DUF1668 domains that were expressed gametophytically in anthers, which is Poaceae species specific DUF. Functional diversifications of duplicated genes in different rice species could lead to hybrid sterility or breakdown as observed in cases of *S27*/*S28*, *DPL1*/*DPL2,* and *DGS1*/*DGS2* loci (Mizuta et al., 2010; Yamagata et al., 2010; Nguyen et al., 2017). The only common feature among these hybrid

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incompatibility systems is that they are caused by gene duplication/reciprocal loss-of-function mutations, but their encoding proteins are different. In *S22A* and *S22B*, the duplicated DUF1668-domain genes may have provided genetic potential to induce hybrid incompatibility by consequent mutations after the wild species' divergence. Unraveling the molecular mechanism of the *S22* locus will help us understand the nature of hybrid sterility.

Hybrid sterility is the major bottleneck for introgressing favorable genes from wild relatives of rice into modern varieties (see Li et al. for a review). Many scientists have devoted their careers in developing strategies to overcome hybrid sterility in rice such as the bridging-parent strategy, using wide-compatibility genes, and gene-editing approaches. Kuniyoshi et al. discovered a new approach for enhancing the hybrid fertility via pollen culture (see also Koide et al.). Abnormal meiosis in the first division restitution (FDR) and/or the second division restitution (SDR) resulted in various ploidy levels of the regenerated plants. Early rescue of microspores carrying diploid gametes led to increased fertility. This study provided a new vision to overcome hybrid sterility.

Furthermore, researchers contributed various kinds of knowledge about other reproductive barriers and reproductive biology: transmission ratio distortion (Zhang et al.), hybrid incompatibility (with special emphasis on autoimmunity in hybrids, Calvo-Baltanás et al.), hybrid breakdown (Munguambe et al.; Matsubara), male and female development genes—*OsMFS1*/*OsHOP2* (Lu et al.), and self-incompatibility (Lian et al.) in this Research Topic.

Chromosome segment substitution line libraries (CSSLs) are a powerful genetic resource for systematic genetic dissection of agronomic traits and varieties improvement in rice. Singh et al. produced six interspecific introgression libraries derived from crossing three wild species as the donor parents with two cultivated varieties as the recurrent parents. The three wild AA genome species originated from different regions and represented abundant genetic diversity; the popular *indica* cv. IR 64 and *japonica* cv. Cybonnet constituted the domesticated *O. sativa* backgrounds. This study contributed to understand the effects of wild introgressions in *indica* and *japonica* backgrounds, respectively. The genes for pericarp color, hull color, shattering, flowering time and seed production were confirmed using introgression libraries. Further, it is great news that seeds from the six CSSLs are available from the Genetics Stocks-Oryza (GSOR) center at the Dale Bumpers National Rice Research Center in Stuttgart, AR, United States. Mussurova et al. introduced the concept of platinum standard reference genome sequences—a new standard by which contiguous near-gap free reference genomes of wild relatives. Such genomic resources will much facilitate the introgression of useful genes from wild relatives into cultivated rice.

Molecular marker assisted (MAS) selection promotes faster breeding of rice lines carrying introgressions of rice resistant genes into recipient parents (see Ramalingam et al.). For enhancing resistance to bacterial blight, blast, and sheath blight in rice, a pyramiding scheme was adopted where 1) three genes for bacterial blight, *xa5*, *xa13*, and *Xa21*, were introgressed into recurrent parents by a first cycle of MAS, and 2) the *Pi54* gene for blast and three QTLs for sheath blight (*qSBR7-1*, *qSBR11-1*, and *qSBR11-2*) were introduced into the improved lines by the second cycle of MAS. Such rapid-stacking, pyramiding strategies are now widely used to produce commercial and released varieties both in the private sector and, increasingly, in the public sector (e.g., at IRRI and AfricaRice through the Bill and Melinda Gates Foundation and USAID funded projects). Uddin and Fukuta reviewed quantitative trait loci related to differentiation between lowland and upland ecotypes. Such

FIGURE 1 | In memory of Dr. Darshan Singh Brar. From top left to bottom right: Dr. Brar (1) the cytologist, (2) the geneticist, (3) the wise, (4) the contemplator, (5) the mentor (with Dr. Kofi Bimpong and Ms. Joie M. Ramos), (6) the breeder, (7) the teacher (with Mr. Jiawu Zhou). Pictures 1–5 are from the IRRI archives (see http://news.irri.org/2020/03/dr-darshan-brar-leading-irri-breeder.html). Pictures 6 and 7 are from a Sept 29, 2009 field trip to Chuxiong, Yunnan (taken by K.L. McNally). Later that day, Dr. Brar received the Yunnan Provincial Government Friendship Award for Foreign Experts at a special provincial banquet held in Kunming in celebration of the 60th anniversary of the founding of the People's Republic of China.

information must be also very useful for improvement of rice adapted in different environments.

The African domesticated species, *O. glaberrima,* possesses many valuable traits for tolerance to biotic and abiotic stresses and comprises an elite gene pool for improving Asian cultivated rice. Dong et al. identified *Pi69*(t), a novel gene for rice blast resistance that was mapped to a 0.9 cM region on the long arm of chromosome 6. Lines carrying *Pi69*(t) were resistant to 148 of 151 blast strains collected from 6 countries, suggesting that *Pi69*(t) confers broad-spectrum resistance to blast and is a valuable gene for blast-resistance breeding. This is the first blast resistance gene identified from *O. glaberrima*. In summary, this Frontier hot issue provides an up-to-date account of some of the exciting areas of current research related to reproductive barriers and introgression in rice, particularly by virtue of the effort of young researchers. Both the research and review publications shed light on the nature of reproductive barriers and provide new insights for rice variety improvement.

Lastly, we dedicate this issue to the memory of Dr. Darshan Singh Brar (March 7, 1944–March 11, 2020) (**Figure 1**). Not only was Dr. Brar a globally recognized plant breeder and cytogeneticist, but he was also a respected colleague, teacher, mentor, friend. His career at IRRI (1987–2012) centered around using wild species as donors for important traits for rice improvement, understanding reproductive barriers, and rice diversity. He never sought the limelight, preferring to focus diligently on his work. His insight and tutelage inspired many, his humble and kind nature are sorely missed, and his humor made the days glow. Dr. Brar, we will never forget you; our lives were enriched by the time we were fortunate to have had with you.

ETHICS STATEMENT

Written informed consent was obtained from the relevant individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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OsMFS1/OsHOP2 Complex Participates in Rice Male and Female Development

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Meiosis plays an essential role in the production of gametes and genetic diversity of posterities. The normal double-strand break (DSB) repair is vital to homologous recombination (HR) and occurrence of DNA fragment exchange, but the underlying molecular mechanism remain elusive. Here, we characterized a completely sterile *Osmfs1* (male and female sterility 1) mutant which has its pollen and embryo sacs both aborted at the reproductive stage due to severe chromosome defection. Map-based cloning revealed that the OsMFS1 encodes a meiotic coiled-coil protein, and it is responsible for DSB repairing that acts as an important cofactor to stimulate the single strand invasion. Expression pattern analyses showed the *OsMFS1* was preferentially expressed in meiosis stage. Subcellular localization analysis of OsMFS1 revealed its association with the nucleus exclusively. In addition, a yeast two-hybrid (Y2H) and pull-down assay showed that OsMFS1 could physically interact with *OsHOP2* protein to form a stable complex to ensure faithful homologous recombination. Taken together, our results indicated that *OsMFS1* is indispensable to the normal development of anther and embryo sacs in rice.

Keywords: meiosis, sterile, *OsMFS1*, *OsHOP2*, rice

INTRODUCTION

Meiosis is indispensable for sexual reproduction in eukaryotes, and is a specialized cellular division process. In this process, the sexually reproducing cells undergo a round of DNA replication and two successive rounds of nuclear division, and ultimately form genetically recombined haploid generative cells (Dawe, 1998; Kleckner, 2006). During the first division (meiosis I), homologous pairing and synapsis form a synaptonemal complex (SC, a tripartite proteinaceous structure), then carry on genetic recombination and chromosomal segregation. During the second division (meiosis II), the sister chromatids separate equally to form four genetically different haploid cells. Compared with mitosis, these two chromosome segregations of meiosis possess a great amount of innovations (Ma, 2006; Mercier et al., 2015; Gray and Cohen, 2016).

Although there are many reports on meiosis (Keeney et al., 1997; Fernandez-Capetillo et al., 2003; Zhang et al., 2017), the molecular mechanism of homologous recombination (HR) remains uncovered (Wang and Copenhaver, 2018). HR possesses an important biological significance within the coexistence of high-risk chromosomal behavior which can lead to chromosomal developmental defects and endanger the survival of the organisms. Therefore, it is crucial to ensure the smooth progress of HR. HR initiates with double-strand breaks (DSBs), which are induced by Spo11 (a conserved topoisomerase-like protein) during the early stages of meiosis (Bergerat et al., 1997; Keeney et al., 1997; Shehre-Banoo et al., 2007). Subsequently, the DSBs are processed to form recombinogenic single-stranded tails with 3′ termini (Farah et al., 2005; Mimitou and Symington, 2009), which serves as a probe for researching a homologous repair template. The extensive single-stranded DNAs (ssDNA) were bound by RPA (heterotrimeric replication protein) complexes with high affinity to protect them from nuclease (Paques and Haber, 1999; Lee et al., 2003; Aklilu et al., 2014). *RAD51* and *DMC1* are two homologs of the bacterial recombinase RecA (Sung and Robberson, 1995; Shinohara et al., 1997; Hong et al., 2001). DMC1 and RAD51 proteins were recruited to replace the RPA complex (Ellen et al., 2006) and form a right-handed helical nucleoprotein filament on the single strands in an ATP-dependent manner to promote the search for homology and catalyze the single strand invading homologous duplex DNA (Tashiro et al., 1996; Hong et al., 2001; Masson and West, 2001; Cloud et al., 2012; Marie-Therese et al., 2012). The strand invasion leads to some or all DSBs forming homologous joint molecules, which were termed the SC (Schwacha and Kleckner, 1997; Masson et al., 1999; Masson and West, 2001; Sheridan et al., 2008), and finally produce crossovers (COs) or non-crossover (non-COs) due to different resolutions (Allers and Lichten, 2001; Hunter and Kleckner, 2001; Borner et al., 2004; Mimitou and Symington, 2009).

The molecular, genetic, and molecular phylogenetic analysis indicated that many meiotic proteins are conserved among plants, animals, and fungi (Gray and Cohen, 2016; Loidl, 2016). The HR pathway requires auxiliary proteins to assist the strand invasion of the recombinase DMC1 and RAD51, with the proteins MND1 (meiotic nuclear division protein 1) and HOP2 (homologous-pairing protein 2) acting as vital cofactors (Leu et al., 1998; Patrick et al., 2003; Yi-Kai et al., 2004; Zierhut et al., 2004; Neale and Scott, 2006). The MND1 protein was first identified in screening for genes in budding yeast, and the disruption of MND1 would lead to meiotic arrest, aberrant synapses, and defects in SC formation (Rabitsch et al., 2001; Zierhut et al., 2004). Previous studies reported that MND1 could physically interact with HOP2 to form a heterodimeric complex (Hideo and Shirleen, 2002; Li et al., 2004; Yi-Kai et al., 2004; Julien et al., 2007; Zhao et al., 2014). MND1 and HOP2 could be coimmunoprecipitated from crude extracts of meiotic yeast cells (Hideo and Shirleen, 2002). The *Arabidopsis mnd1* and *hop2* mutant plants were disrupted in both male and female meiosis due to them failing to form bivalents (Claudia et al., 2006; Stronghill et al., 2010). Biochemical studies have shown that the MND1/HOP2 complex can condensate dsDNA (double-stranded DNA) and physically interact with DMC1 and RAD51 that efficiently stabilize the presynaptic filament and stimulate strand invasion activity (Petukhova et al., 2003; Yi-Kai et al., 2004; Johnson and O'Donnell, 2005; Vignard et al., 2007; Pezza et al., 2010). However, neither MND1 nor HOP2 can facilitate this reaction alone (Pezza et al., 2010). In budding yeast, the *hop2* and *mnd1* mutants exhibited the same defects in meiosis and suggested that MND1/HOP2 complex was the central role during the HR process (Leu et al., 1998; Gerton and Derisi, 2002; Petukhova et al., 2003; Zierhut et al., 2004). However, related research in monocots has not been reported.

Here we identified a completely sterile *Osmfs1* (male and female sterility 1) mutant at the heading stage. Our results suggested that the heterodimeric complex OsMFS1/*OsHOP2* is vital for the repairment of DSBs in rice.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

We identified one completely sterile mutant by screening ethyl methanesulfonate (EMS)-mutagenized M_2 rice lines. The F_2 mapping population was generated from a cross between the heterozygous mutant and N22 (*indica*). The F_2 population was grown in the Tu Qiao Experiment Station of Nanjing Agricultural University. At the mature stage, plants with the phenotype of complete sterility were selected as homozygous mutants for mapping.

Preparation of Embryo Sacs

We obtained embryo sacs at different stages by fixing WT and mutant florets in Carnoy's solution (75% ethanol and 25% acetic acid), and the dissected ovaries were preexisted in 70% ethanol. Subsequently, the samples were hydrated sequentially in 50, 30, and 15% ethanol and distilled water, stained with 1% eosin-Y for about 8 h, and washed several times in distilled water until colorless. The samples were treated for 8 h in citric acid-disodium hydrogen phosphate buffer (0.1 mol/l, pH 5.0) and followed by Hoechest staining (25°C in darkness for 24 h). The samples were washed three times in distilled water, and processed through an ethanol series (15, 30, 50, 70, 85, 95, and 100%) for dehydration. Then, the samples were treated in 1:1 ethanol and methylsalicylate for 1 h, cleared three times in methylsalicylate (2, 2, 15 h), and finally saved in methylsalicylate. We applied a laser confocal scanning microscope (Zeiss Microsystems LSM 700) to compare the various developmental stages of embryo sacs between the WT and the *Osmfs1*.

Meiotic Chromosome Examination

Young panicles (40∼60 mm) of WT and the mutants were fixed in Carnoy's solution (ethanol: glacial acetic 3:1) and stored at −20°C until needed. Firstly, we selected one of six anthers to stain with 1% acetocarmine solution on glass slides, and then judged the development stage with optical microscopy. Finally, the appropriate anthers at the meiotic stage were squashed under a cover slip in 40% acetic acid. The slides were frozen in liquid nitrogen for 5 min and dried at room temperature after quickly

removing the cover slips. After that, the samples were treated with 20 μl of 0.1 mg ml^{-1} propidium iodide to stain chromatin for 20 min. The male meiotic chromosomes were observed using a fluorescence microscope.

Scanning (SEM) and Transmission Electron Microscopy (TEM)

The anthers of WT and mutants were fixed in 2.5% glutaraldehyde, rinsed three times using distilled water dehydrated through an ethanol series (30, 50, 70, 85, 90, 100, and 100%), fixed in 1% OsO_4 for 2-hour, dehydrated through an ethanol series, and subjected to critical point drying with CO_2. The anthers were coated with gold by an E-100 ion sputter and observed with a scanning electron microscope (S3400; Hitachi). For TEM, mature anthers were fixed in 1% glutaraldehyde and 1% OsO_4 for 1-hour and dehydrated through an ethanol series. The samples were embedded in Spurr's medium prior to thin sectioning. Sections were double-stained with 2% uranyl acetate and 2.6% aqueous lead citrate solution, and examined with a JEM-1230 transmission electron microscope (Jeol) at 80 kV (Ren et al., 2014; Wang et al., 2015).

Map-Based Cloning

We identified the *Osmfs1* mutant, which was mutagenized from Ninggeng 4 (a *japonica* variety) by ethyl methanesulfonate (EMS). The F_2 population was generated from a cross between *Osmfs1* heterozygous plants and N22 (an *indica* variety), and finally 111 completely sterile plants were selected from 1500 F_2 population. The DNA of these plants were isolated from leaves using the modified CTAB method (Murray and Thompson, 1980), and the gene was fine-mapped to a 282-kb genomic region between the markers NN9S-L4 and NN9S-L7. Sequence analysis showed that the *Osmfs1* mutants existed a premature translational termination. For fine mapping of the *Osmfs1* locus, bulked-segregated analysis was used and molecular markers were designed by comparison of the local genomic sequence differences between N22 (*O. sativa*, *indica*) and Nipponbare (*O. sativa*, *japonica*), available at the National Center for Biotechnology Information (NCBI)[1]. Primers used in the mapping are listed in **Supplementary Table S1**.

CRISPR-Cas9 Vector Construction

For the CRISPR-Cas9 vector, two 20-bp target sites specific for *MFS1* and *HOP2* were synthesized[2], fused with the *Aar*I linearized intermediate vector SK-Grna, and then introduced into CRISPR-Cas9 binary vector pCAMBIA1305 (Sun et al., 2017) to generate a knock-out construct. The sequencing confirmed plasmids were transformed into A. tumefaciens strain EHA105, and we used *Agrobacterium tumefaciens*-mediated transformation of rice callus (T65, *O. sativa*, *japonica*) to generate transgenic rice plants (Jeon et al., 2000). We mixed two kinds of *Agrobacterium* (*MFS1* and *HOP2*) in equal amounts to generate double mutant plants. Primers used in the plasmid construction are listed in **Supplementary Table S1**.

[1]http://www.ncbi.nlm.nih.gov/

[2]http://cbi.hzau.edu.cn/cgi-bin/CRISPR

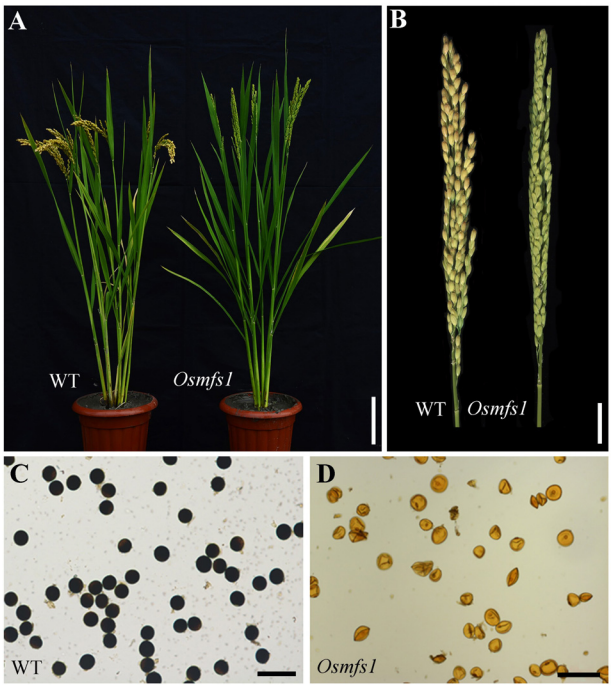

FIGURE 1 | Phenotype analysis between the WT and the *Osmfs1* mutant. **(A)** Comparison between a WT plant (left) and an *Osmfs1* mutant (right) plant at the heading stage. Bars = 10 cm. **(B)** Comparison between a WT panicle (left) and an *Osmfs1* mutant panicle (right) at the harvest stage. Bars = 2 cm. **(C)** Viable pollen grains in a WT plant. **(D)** Inviable pollen grain in an *Osmfs1* mutant plant. Bars = 100 μm in panels **(C,D)**.

β-Glucuronidase (GUS) Histochemical Staining

We amplified a putative 2.6-kb genomic promoter fragment by PCR and cloned the fragment into the binary vector pCAMBIA1305 digested with *Bam*HI and *Hin*dIII by In-Fusion (Takara Bio, Japan) to drive *GUS* reporter gene expression (primers used in the plasmid construction are listed in **Supplementary Table S1**). The construct was transformed into T65 (*O. sativa, japonica*) by *Agrobacterium tumefaciens*-mediated method. Young spikelets of T_1 transgenic plants were incubated in GUS staining solution as described previously (Jefferson, 1987). The samples were observed under the stereomicroscope.

Subcellular Localization of OsMFS1 Protein

To investigate the cellular localization of the OsMFS1 protein, the *OsMFS1* cDNA was fused with GFP and inserted in the pAN580-GFP vector or pCAMBIA1305.1-GFP between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator (primers sequences are listed in **Supplementary Table S1**). The *35S-OsMFS1-GFP* plasmid was transformed into rice protoplasts and *N. benthamiana* epidermal cells according to the protocols described (Zhao et al., 2013).

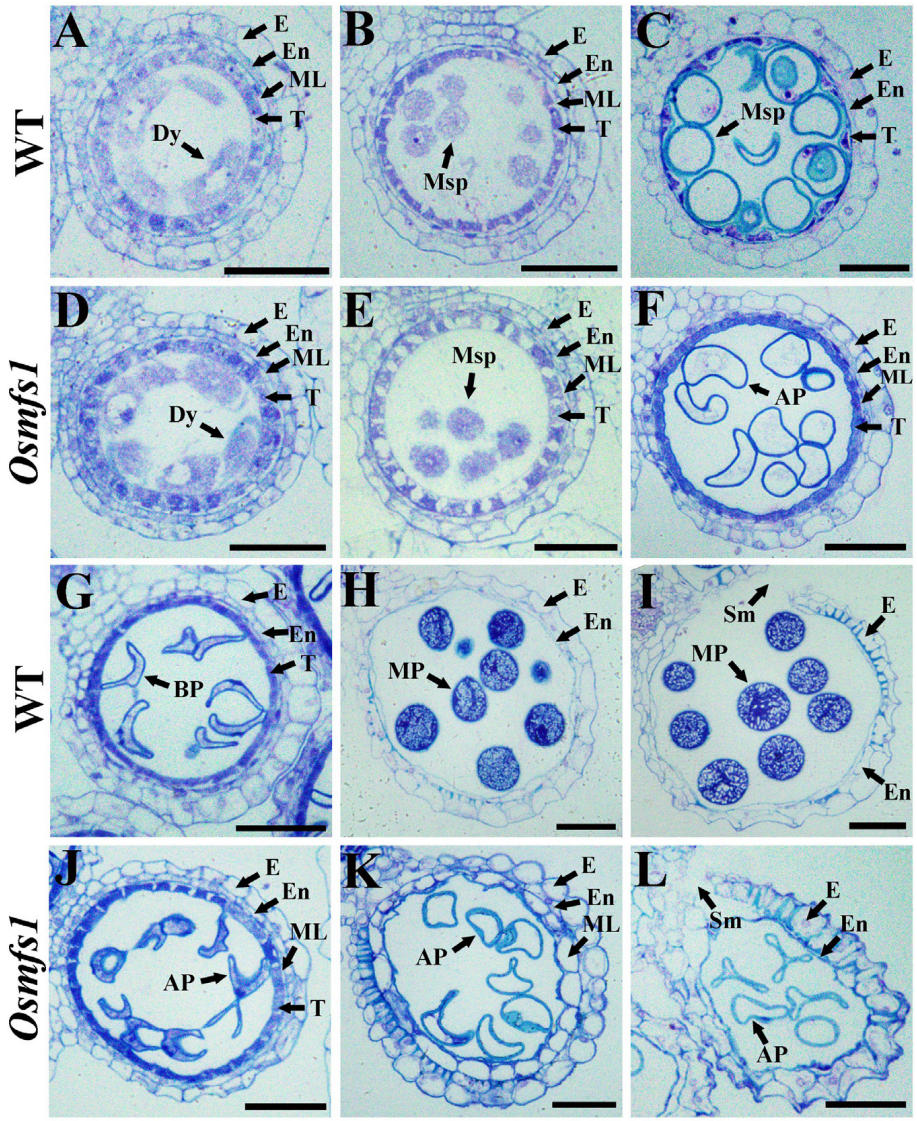

FIGURE 2 | Semi-thin section comparison of anther development in the WT and the *Osmfs1* mutant. Transverse section of WT and *Osmfs1* anthers were stained with toluidine blue. The images are of cross sections through single locules and pictures are captured with a 40× ordinary optical microscope. **(A–C)** and **(G–I)** the WT anthers; **(E,F,J,L)** the *Osmfs1* anthers; **(A,D)** Meiosis stage. **(B,E)** Early microspore stage. **(C,F)** Vacuolated pollen stage. **(G,J)** Bicellular pollen stage. **(H,K)** Mature pollen stage. **(I,L)** Anther dehiscence stage. E, epidermis; En, endothecium; ML, middle layer; T, tapetum; Dy, dyad cell; Msp, microspore; BP, bicellular pollen; AP, abnormal pollen; MP, mature pollen; Sm, stomium. Bars = 1 μm.

RNA Isolation and Quantitative real-time reverse transcription-PCR (RT-qPCR)

Total RNA was extracted using a RNeasy Plant Mini Kit (Qiagen) from various tissues and anthers at different stages. The first-strand cDNA was synthesized using 1 μg RNA and QuantiTect® Reverse Transcription Kit (Qiagen). For RT-qPCR analyses, 20 μl reaction volumes containing 0.4 μl of cDNA, 0.2 μM of gene-specific primers, and SYBR Premix Ex Taq Kit (TaKaRa), running on ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions and three biological repeats were performed. The rice *Ubiquitin* gene was used as the internal control. Primers used for RT-qPCR are listed in **Supplementary Table S1**.

Yeast Two-Hybrid (Y2H) Assay

Full-length cDNA of genes were amplified and cloned into the Y2H prey vector pGADT7 or bait vector pGBDT7 (Clontech). Then the two plasmid types were co-transformed into Gold yeast (*Saccharomyces cerevisiae*), and transformant plants were grown on SD–Leu/–Trp plates at 30°C for 3 days. Subsequently, six individual clones were selected and mixed into 60 μl of 0.9% NaCl and diluted 10, 100, and 1000-fold, each concentration absorbing

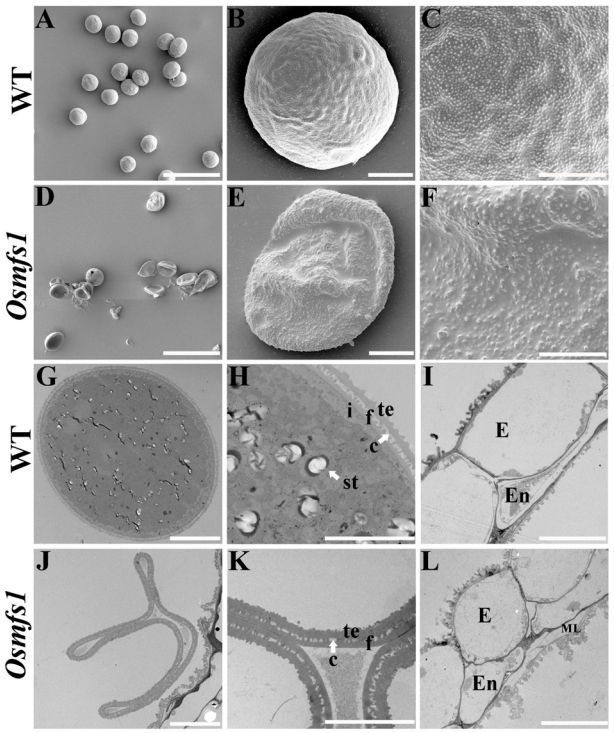

FIGURE 3 | Scanning electron micrography (SEM) and transmission electron micrography (TEM) of pollen from the WT and *Osmfs1* mutant. **(A)** Scanning electron microscopy image of the mature WT pollen grain. **(B)** Magnified single pollen grain from panel **(A)**. **(C)** Further magnification showing the exine from panel **(B)**. **(D)** Scanning electron microscopy image of the mature *Osmfs1* pollen grain. **(E)** Magnified single pollen grain from panel **(D)**. **(F)** Further magnification showing the exine from panel **(E)**. **(G)** Transmission electron microscopy image of the WT pollen. **(H)** Magnification of the pollen wall from panel **(G)**. **(I)** Transmission electron microscopy image showing the WT anther wall. **(J)** Transmission electron microscopy image of the *Osmfs1* pollen. **(K)** Magnification of the pollen wall from panel **(J)**. **(L)** Transmission electron microscopy image showing the *Osmfs1* anther wall. c, columella; st, starch; i, intine; f, foot layer; te, tectum; E, epidermis; En, endothecium; ML, middle layer. Bars = 100 μm in panels **(A,D)**, 10 μm in panels **(B**,**E**,**G**,**J**,**I,L)**, and 5 μm in panels **(C**,**F**,**H,K)**.

6 μl. The activation ability was assayed on selective media -LTHA (SD–Leu/–Trp/–His/–Ade) containing X-α-gal (40 μg ml^{-1}).

Pull-Down (*in vitro*) Assay

The coding regions of *OsMFS1* and *OsHOP2* were amplified and *OsMFS1* was cloned into the pMAL-c2X vector while *OsHOP2* was cloned into the pCOLD-His vector, which carried an N-terminal His Tag. Then we obtained MBP-OsMFS1 and pCOLD-His-*OsHOP2* plasmids. Then the two plasmids transformed into *Escherichia coli* BL21 cells. MBP- and MBP-OsMFS1-coupled beads were used to capture pCOLD-His-*OsHOP2*. The western blot was applied to analyze the results of pull-down.

Accession Numbers

Sequence data from this article for the mRNA, cDNA, and genomic DNA can be found in the GenBank/EMBL/Gramene data libraries or Web site under accession numbers: *OsMFS1*, *Os09g0280600*; *OsHOP2*, *Os03g0710100*; *OsDMC1A*, *Os12g0143800*, *OsDMC1B*, *Os11g0146800*, *OsRAD51-A1*, *Os11g0615800*; *OsRAD51-A2*, *Os12g0497300*; *OsRPA2b*, *Os02g0633400*; *ATMND1*, and *AT4G29170*.

RESULTS

Morphological Characterization of the *Osmfs1* Mutant

At the seedling, tillering, and flowering stages, the *Osmfs1* mutant showed no obvious differences from the WT. At anthesis, however, mutants exhibited complete sterility compared with WT plants (**Figures 1A,B**), while heterozygous plants appeared fully fertile. I_2-KI staining indicated that the *Osmfs1* mutant pollen grains lacked starch and were completely non-viable when compared with the WT (**Figures 1C,D**). Then we detected the female fertility of the *Osmfs1* mutant by pollinating the WT pollens, and the mutant failed to set seeds (**Supplementary Figure S1F**). Therefore, we concluded that the *Osmfs1* mutant is sterile in both male and female instances.

Abnormal Development of Male Gametes in the *Osmfs1* Mutant

To characterize the developmental defects of pollen in the *Osmfs1* mutant, we examined anther development with semi-thin sections. During the early microspore and meiosis stage, the male meiocytes seemed to undergo normal development and no obvious difference appeared between the WT and the mutant (**Figures 2A,B,D,E**). During the vacuolated pollen stage, almost all wild-type vacuolated microspores regularly arranged in anther locule, and the microspores were spherical and filled with cell sap (**Figure 2C**). Nevertheless, the microspores of the *Osmfs1* mutant appeared with an irregular shape and no vacuoles (**Figure 2F**). At the bicellular pollen stage, pollen grains were shown to be crescent-shaped and cell sap ran off, preparing for the next starch filling and indiscernible difference between WT and *Osmfs1* mutant (**Figures 2G,J**). Mature pollen grain from the WT filled with starch granules and stained densely (**Figures 2H,I**), whereas mature pollen grains of the *Osmfs1* mutant lacked starch granules and showed an irregular shape (**Figure 2K**). The development processes of epidermis, endothecium, and tapetum were indistinguishable between the WT and the *Osmfs1* mutant (**Figures 2A–G,J**). At the vacuolated pollen stage, the middle layer completely degraded in WT (**Figure 2C**), while it slowly delayed in the mutant until the stage of anther dehiscence (**Figures 2F,J,K,L**).

In order to get insight into abnormalities of the *Osmfs1* pollens, SEM and TEM observation were performed. SEM observation showed that the mature pollen grains of WT were spherical and the exine of mature pollen was covered with sporopollenin (**Figures 3A–C**). However, pollen grains of the mutants were irregular, shrunken, and coated with less sporopollenin compared with that of WT (**Figures 3D–F**). The pollen wall was made up of exine and intine, and then the

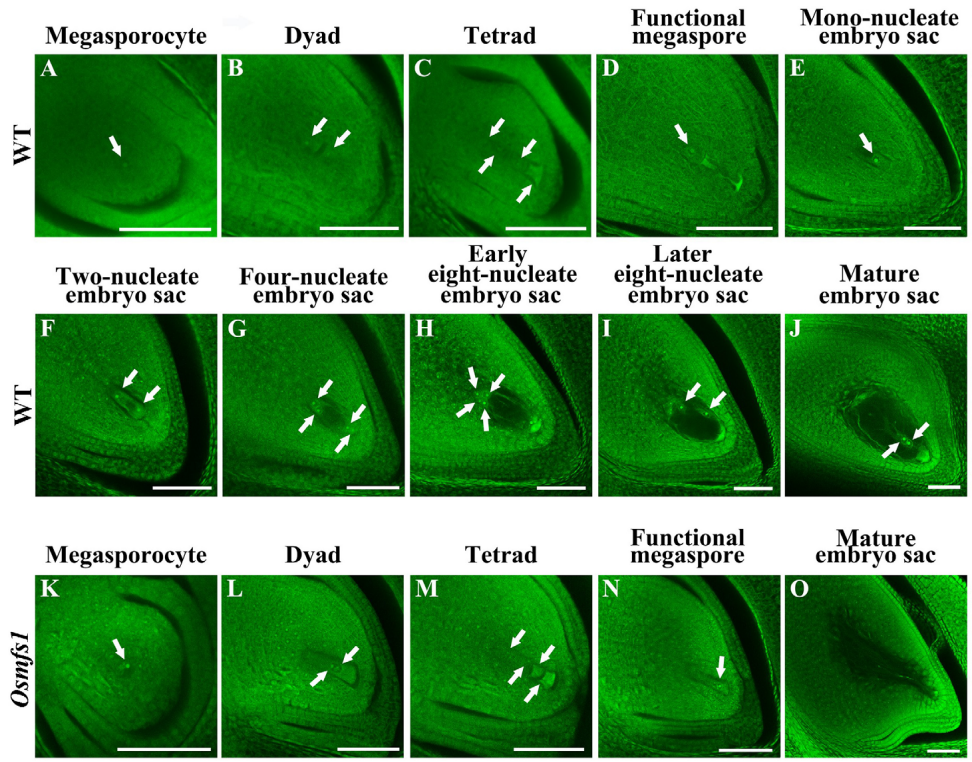

FIGURE 4 | Embryo sac development in the WT and the *Osmfs1* mutant. **(A–J)** the WT, **(K–O)** the *Osmfs1*, **(A,K)** Megasporocyte, **(B,L)** Dyad, **(C,D)** Tetrad, **(D,N)** Functional megaspore, **(E)** Mono-nucleate embryo sac, **(F)** Two-nucleate embryo sac, **(G)** Four-nucleate embryo sac, **(H,I)** Eight-nucleate embryo sac, **(J,O)** Mature embryo sac. The mature embryo sacs of the WT eventually formed viable egg cells when mutant showed hollow. Scale bar = 20 μm.

exine was divided into the tectum and foot layer linked by the columella. TEM indicated that the mature pollen grains of WT were filled with starch granules (**Figures 3G,H**). In contrast, there is no starch granule accumulation in pollen grains of the *Osmfs1* mutant instead of several degrading substances. Meanwhile, the pollen wall of *Osmfs1* mutant was dysplastic, with a thicker tectum and foot layer, and columella was degrading (**Figures 3J,K**). In addition, consistent with semi-thin sections, a residual middle layer still exists in the *Osmfs1* mutant while not in WT (**Figures 3I,L**). In conclusion, the *Osmfs1* mutant was male sterile due to the abnormal pollen development.

Arrested Development of Embryo Sac in the *Osmfs1* Mutant

When *Osmfs1* mutant pollinated with WT pollen, it failed to set seed and indicated that the embryo sac was dysplastic (**Supplementary Figure S1**). To further verify the cause of abortion of *Osmfs1* mutant embryo sacs, whole-mount stain-clearing confocal laser microscopy (WCLSM) was applied to investigate the development of embryo sacs of the WT and *Osmfs1* mutant. In the WT, the megasporocyte underwent meiotic divisions to produce four haploid megaspores, and three micropylar megaspores degenerated while the chalazal-most megaspore became a functional megaspore (**Figures 4A–D**). The size and vacuoles of the functional megaspore increased during the mono-nucleate embryo sac stage (**Figure 4E**), then the mono-nucleate embryo sac underwent cell division without cytokinesis to form a two-nucleate embryo sac (**Figure 4F**), and the two daughter nuclei migrated to the poles and a central vacuole formed (**Figure 4G**). Associated with the vacuole increasing in size, eight-nucleate embryo sacs were formed after two rounds of mitosis (**Figure 4H**). At the eight-nucleate embryo sac stage, four nuclei near the chalaza differentiated into the polar nuclei and three antipodal cells while four nuclei at the micropylar end turned into the polar nuclei, an egg cell, and two synergid cells (**Figure 4H**). Thereafter, the two polar nuclei expanded and migrated to the center of the embryo sac and formed a diploid central nucleus, then the central cell moved toward the egg apparatus (**Figure 4I**). At the mature embryo sac stage, the starch granules around the egg cell disappeared and the largest volume appeared in the embryo sac (**Figure 4J**).

The normal megasporocyte could be detected in the *Osmfs1* mutant (**Figure 4K**), and the megasporocyte went through meiotic divisions to produce dyad and tetrad (**Figures 4L,M**), whereas three chalazal-most megaspores degenerated at tetrad stage and the micropylar megaspore could be observed at the functional megaspore stage (**Figure 4N**). Thereafter, the abnormal functional megaspore was unable to undergo mitotic divisions and completely degenerated before the mono-nucleate embryo sac stage, resulting in the failure to form seven cells

and eight nuclei embryo sacs (**Figure 4O**). Therefore, our results suggested that the development of embryo sacs of *Osmfs1* was also disturbed during the meiosis stage and resulted in the embryo sacs abortion.

Osmfs1 Mutant Shows Disrupted Chromosome Behavior

To further investigate male sterility in the *Osmfs1* mutant, meiotic chromosome behavior was observed. In WT, condensing chromosomes were seen in single threads at leptotene when DSBs were prepared for homologous recombination (**Figure 5A**). At zygotene, homologous chromosomes started to pair with synapsis and were concentrated to one side of the nucleus (**Figure 5B**). During pachytene, all the synapsed chromosomes were scattered in the nucleus when the SC accomplished, meanwhile the strand exchange occurred between the sister chromatids of homologous chromosomes (**Figure 5C**). At diakinesis, chromosomes further condensed and appeared to have rod-like structures, ultimately forming twelve bivalents in the nucleus (**Figure 5D**). At metaphase I, the twelve bivalents aligned on the equatorial plate (**Figure 5I**). Subsequently, homologous chromosomes separated and migrated to opposite poles at anaphase I (**Figure 5J**). At telophase I, chromosomes reached opposite poles and started to condense, then cell division led to the formation of dyads (**Figure 5K**). Finally, four daughter cells came out after the second meiotic division (**Figure 5L**).

In the *Osmfs1* mutant, no obvious differences were found at leptotene compared with the WT plants (**Figure 5E**). However, some unpaired chromosomes could be detected during zygotene and pachytene (**Figures 5F,G**). The disruption was more remarkable at diakinesis, and more than 12 chromosomes caused by incomplete pairing of chromosomes dispersed among the cells, compared with 12 chromosomes in the WT (**Figures 5D,H**). A majority of univalents could be observed within the nucleus (**Figure 5H,M**), and a lot of chromosomes would not be drawn toward the poles by spindle fibers instead of scattered in cells randomly (**Figures 5N,O**). Finally, chromosomes were unequally separated and this led to the abnormal tetrad (**Figure 5P**). Therefore, we confirmed that the disorganized chromosome behavior in the meiosis stage was the key reason for the abnormal microspores.

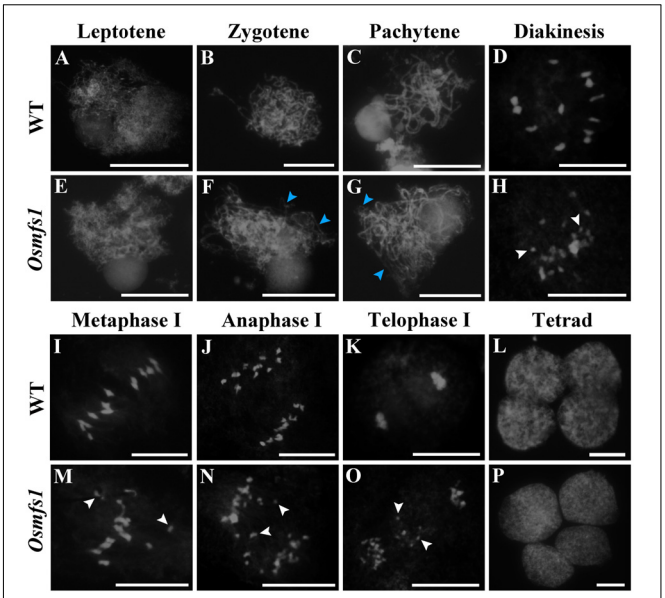

FIGURE 5 | Meiotic chromosomes spreads of pollen mother cells in the WT and the *Osmfs1* mutant. **(A–D, I–L)** WT. **(E–H, M–P)** *Osmfs1*. **(A,E)** Leptotene. **(B,F)** Zygotene. **(C,G)** Pachytene stage. **(D,H)** Diakinesis. **(I,M)** Metaphase I. **(J,N)** Anaphase. **(K,O)** Telophase. **(L,P)** Tetrad. The blue arrows point to the unpaired chromosomes, the white arrows point to univalents. Bars = 100 μm.

Isolation of *OsMFS1* Gene by Map-Based Cloning

To determine the molecular mechanism of the mutant, we isolated the *OsMFS1* gene using a map-based cloning approach. An F_2 population was generated from a cross between *Osmfs1* heterozygous plants and N22, and the *OsMFS1* locus was preliminarily mapped to a region between two simple sequence repeat (SSR) markers RM23662 and RM23916 on the chromosome 9. Subsequently, the location was narrowed to a 282-kb genomic region between the markers NN9S-L4 and NN9S-L7 by using 1500 F_2 plants (**Figure 6A**). Sequencing and comparison analysis revealed a single nucleotide substitution of guanine (G) to adenine (A) in the 9th exon of *LOC_Os09g10850*, which is predicted to encode a meiotic coiled-coil protein. The mutation caused a frameshift that resulted in premature translational termination (**Figure 6A**). The *LOC_Os09g10850* gene contains ten exons and nine introns and expects a 23.9 KDa protein with 207 amino acids. We obtained sequences from the NCBI database and aligned them using Bioxm software, and the result showed that the OsMFS1 protein was conserved in several species (**Supplementary Figure S2**). To further verify that *LOC_Os09g10850* was the objective gene, we performed targeted mutagenesis of the *LOC_Os09g10850* in the genetic background of T65 using the CRISPR/Cas9 technology. We obtained two independent Cas9-free homozygous mutants, which were mutated at different sites. Two contained 1-bp insertion in the extron (*Osmfs1-1* and *Osmfs1-2*), and both had premature terminations of protein translation (**Figures 6B–D**). Like the *Osmfs1* mutant, we did not observe any significant differences between the transgenic lines and WT plants during vegetative growth, and all lines were completely sterile at the harvest stage (**Supplementary Figure S1**). Taken together, our results demonstrated that *LOC_Os09g10850* was the target gene of causal mutation of *Osmfs1*.

Expression Pattern and Subcellular Localization of *OsMFS1*

Quantitative RT-PCR (RT-qPCR) was performed to examine the *OsMFS1* expression in various tissues of the WT including leaves, leaf sheaths, internodes, stems, panicles, and roots. The results showed that *OsMFS1* was constitutively expressed in the above-mentioned tissues (**Figure 7A**). Interestingly, *OsMFS1*

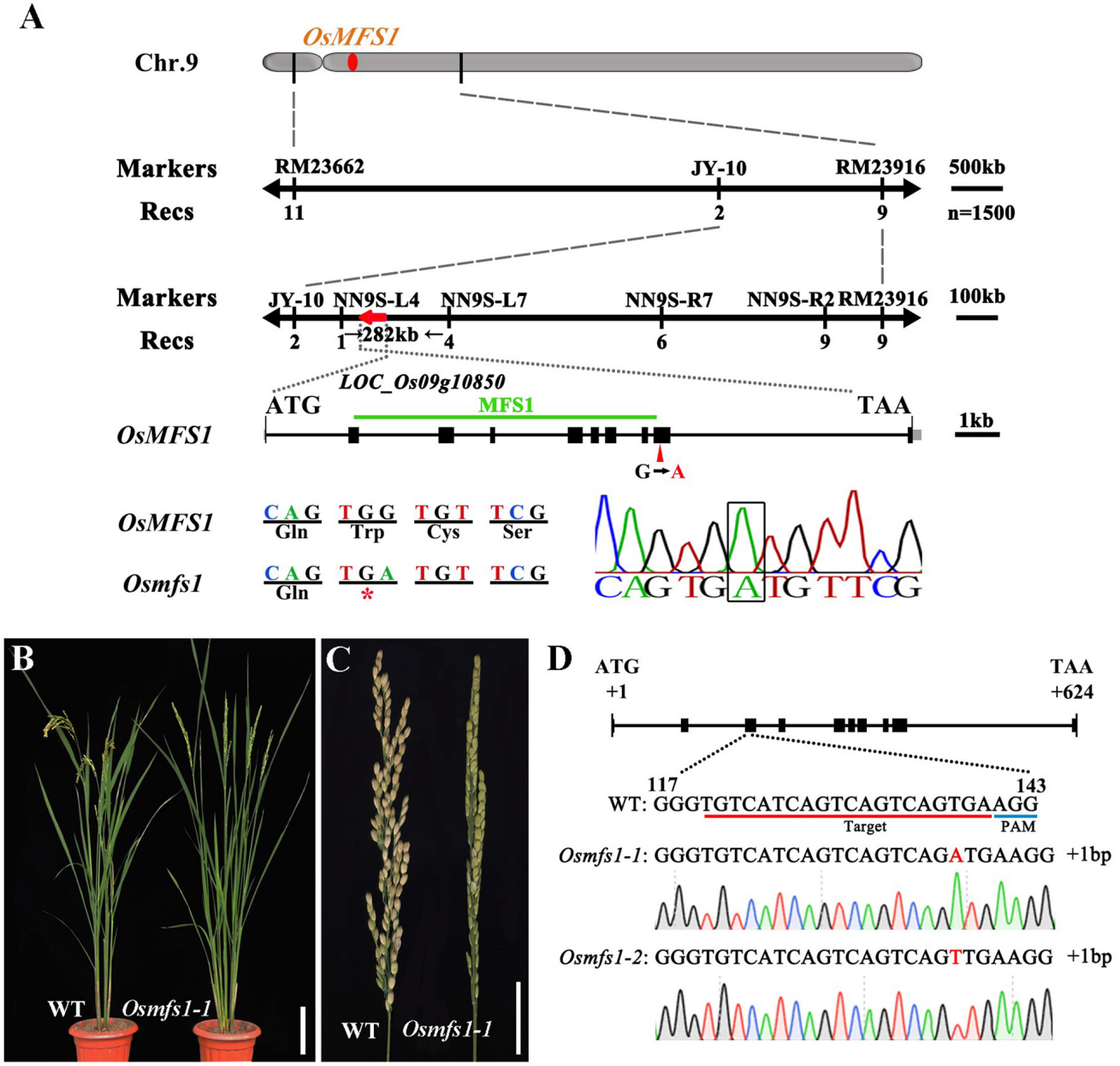

FIGURE 6 | Map-based cloning of the *OsMFS1* gene and phenotype comparisons between the WT and the *Osmfs1* mutant (The positive T_3 plants of CRISPR/Cas9). **(A)** Primary mapping was performed using markers RM23662 and RM23916 on chromosome 9, and further fine-mapped to a 282-kb genomic region between the markers NN9S-L4 and NN9S-L7, the *OsMFS1* gene was located in the region. **(B)** Comparison between a WT plant (left) and a positive plant of *Osmfs1* (right) at the heading stage. Bars = 10 cm. **(C)** Comparison between a panicle of WT (left) and a panicle of *Osmfs1* (right) at the harvest stage. Bars = 2 cm. **(D)** A schematic representation of the gene structure of *OsMFS1*, two lines with different mutation types were obtained, the sequences of both *Osmfs1-1* and *Osmfs1-2* have a single nucleotide insertion.

expressions were highly expressed in the anthers during the meiosis stage and then declined toward maturation (**Figure 7B**). Subsequently, we obtained transgenic plants which carried an *OsMFS1* pro:GUS vector, and the GUS signals were strongly detected in the anthers of young panicles and peaked at the meiosis stage (S7∼S9) in the transgene plants (**Figure 7C**), and these results were consistent with the data of RT-qPCR. In conclusion, we confirmed that the *OsMFS1* was highly expressed during the meiosis stage. In order to determine the subcellular localization of the OsMFS1 protein, we constructed a GFP (green fluorescent protein) fusion vector named OsMFS1-GFP, and the OsMFS1-GFP fusion protein was localized in the nucleus, indicating that OsMFS1 was a nuclear localization protein (**Figures 7D–K**).

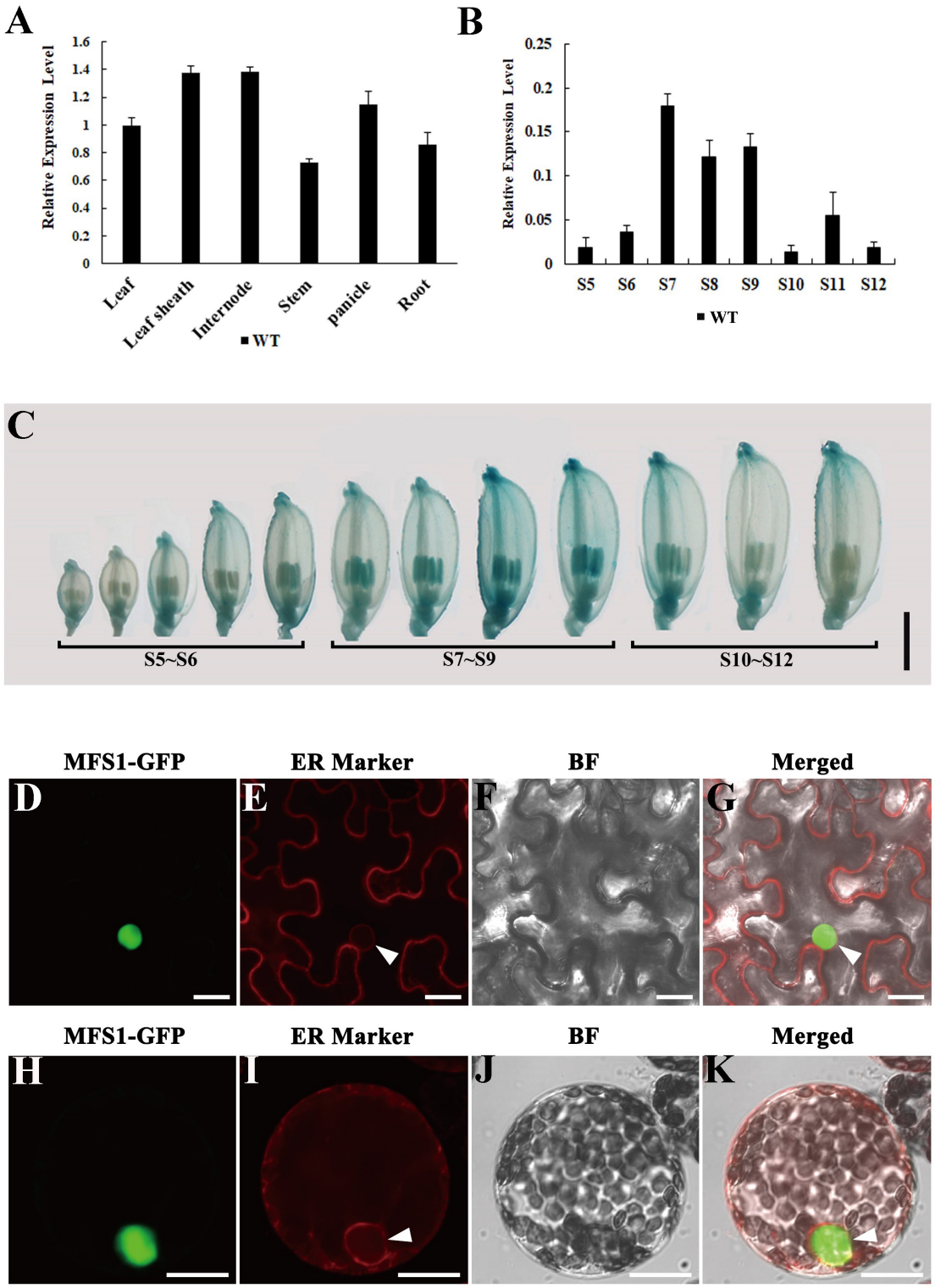

FIGURE 7 | Expression pattern analysis of *OsMFS1* and subcellular localization. **(A)** The result of quantitative RT-PCR of the *OsMFS1* in various different tissues of the WT. **(B)** The result of quantitative RT-PCR of the *OsMFS1* in different stage of WT anther. The results of panels **(A,B)** were performed with three biological repeats. **(C)** Indicating the expression stages of the panicles of *OsMFS1* by GUS staining. Bars = 2 mm. **(D–K)** Subcellular localization of OsMFS1 protein in *N. benthamiana*. **(D–G)** Subcellular localization of the OsMFS1-GFP protein in the cells of leaf epidermal. **(H–K)** Subcellular localization of the OsMFS1-GFP protein in protoplasts. **(D,H)** OsMFS1-GFP is detected in the nucleus. **(E,I)** Localization of the ER marker. **(F,J)** images of Bright field. **(G,K)** Merged the images of panels **(D–F, H–J)**, respectively. Bars = 10 μm.

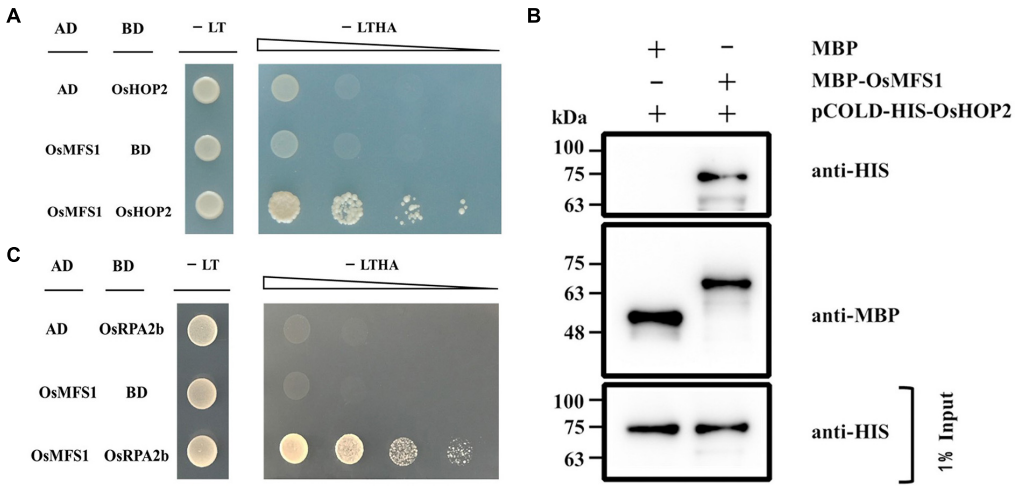

FIGURE 8 | Yeast-two-hybrid (Y2H) assays and *in vitro* pull-down assay to test interactions of OsMFS1, *OsHOP2*, and OsRPA2b. **(A)** Examination of the interaction between OsMFS1 and *OsHOP2*. **(B)** *in vitro* pull-down assay was conducted to confirm the interaction between OsMFS1 and *OsHOP2*, MBP-MFS1 and pCOLD-His-HOP2 interaction *in vitro*, but not MBP itself. **(C)** Examination of the interaction between OsMFS1 and OsRPA2b. The interactions were verified by growing the yeast on the selective medium, Full-length OsMFS1 was inserted in the prey vector pGADT7 (AD). Full-length *OsHOP2* and OsRPA2b were cloned into the bait vector pGBKT7 (BD). -LT, selective medium (SD–Leu/–Trp); -LTHA, selective medium (SD–Leu/–Trp/–His/–Ade).

OsMFS1 Protein Physically Interacts With *OsHOP2*

It was previously reported that MND1 specifically interacted with HOP2 in yeast and *Arabidopsis*, and their complex could efficiently condensate double-stranded DNA and support strand invasion (Pezza et al., 2010). OsMFS1 and MND1 are homologous gene families (**Supplementary Figure S3**), and to test the interactive relationship between *OsMFS1* and *OsHOP2* in rice, we performed protein interaction between OsMFS1 and *OsHOP2* by a Y2H assay. The results demonstrated that the OsMFS1 could physically interact with the *OsHOP2* (**Figure 8A**). To further confirm the interaction, an *in vitro* pull-down assay was conducted and proved the interaction between the OsMFS1 and *OsHOP2* (**Figure 8B**). In addition, we found a novel interaction between OsMFS1 and OsRPA2b by a Y2H assay (**Figure 8C**), the OsRPA2b was another meiotic gene, and the interaction needed further verification.

Subsequently, to determine the link between OsMFS1 and *OsHOP2*, meiotic chromosome behavior of *Oshop2* and *Osmfs1/Oshop2* mutants were studied. We generated loss-of-function single and double mutants by CRISPR/Cas9. Fortunately, we obtained the homozygous *Oshop2-1* mutants and *Osmfs1/Oshop2* double mutants (**Figures 9A,G**), and phenotypic analysis results showed that the *Oshop2-1* and *Osmfs1/Oshop2* exhibited complete sterility at the harvest stage, and the pollens were inviable (**Figures 9B–F**). The *Oshop2-1* and *Osmfs1/Oshop2* shared the same chromosomal defects with *Osmfs1-1*, and the univalents were easy to distinguish (**Figures 10A–L**), indicating that OsMFS1 and *OsHOP2* probably act in the same pathway during meiotic DSB repair. All in all, the mutation of *OsMFS1* and *OsHOP2* led to meiotic dysplasia, and no normal pollen grains were formed. Consequently, these mutants showed complete sterility. Thus, our results confirmed that the OsMFS1 is a rice homolog of MND1 and physically interacts with *OsHOP2* to participate in DSB repair in the meiosis.

DISCUSSION

In this study, we showed the interaction between OsMFS1 and *OsHOP2* by Y2H and pull-down assay in rice, which were also observed in yeasts, mammals, and *Arabidopsis thaliana* (Hideo and Shirleen, 2002; Petukhova et al., 2005; Claudia et al., 2006). The *Oshop2* mutants shared the same chromosomal defects with *Osmfs1*, suggesting that the OsMFS1 and the *OsHOP2* acted in the same pathway forming heterodimeric complex to stimulate DSB repair. Therefore, *MFS1* is conserved in higher eukaryotes with common functions in different species (**Supplementary Figure S2**). Several studies indicated that the MND1 always interact with HOP2 to form a stable heterodimer in various species, including yeasts, *Arabidopsis thaliana*,and mammals, and both *MND1* and *HOP2* are predicted to have coiled coil domain that promotes the connection by coiled-coil interaction (Burkhard et al., 2001). The complex also physically interacts with DMC1 and RAD51 to ensure the proper synapsis of homologous chromosomes (Petukhova et al., 2005; Claudia et al., 2006), suggesting the central role of the MND1/HOP2 complex in meiotic HR. In *S. cerevisiae*, the HOP2 protein is expressed specifically during meiosis and the *hop2*-null mutant shares the same serious meiotic defects with the *mnd1* mutant: DSBs unrepaired and arrests at the meiotic pachytene stage (Leu et al., 1998; Gerton and Derisi, 2002; Zierhut et al., 2004). In mice, strand invasion activity of HOP2 protein is strong and capable of promoting D-loop formation, but no such activity is detected in the MND1/HOP2 complex, and the recombinational activity of HOP2 protein is most likely suppressed or quenched by MND1, suggesting a novel regulatory

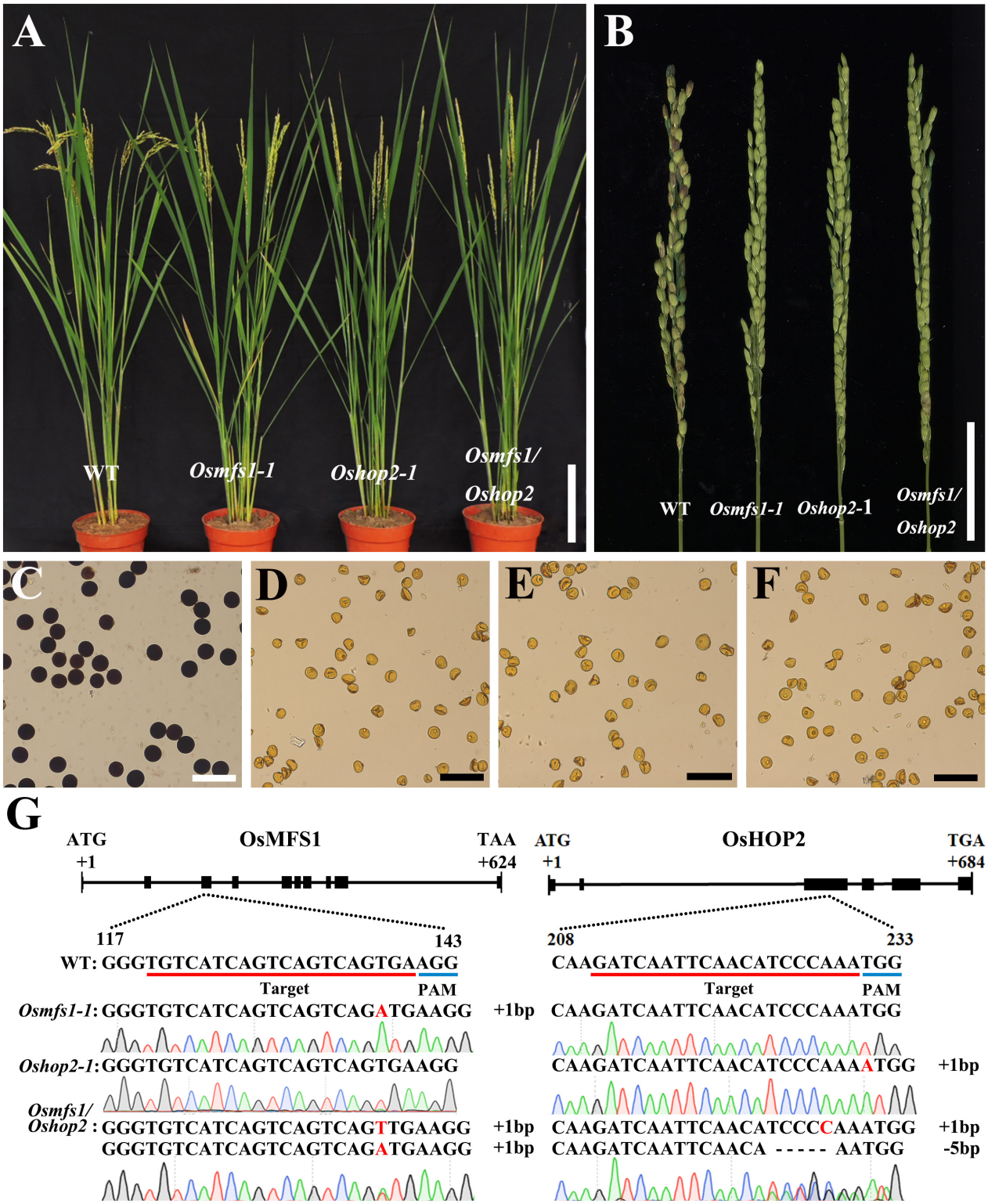

FIGURE 9 | Phenotype analysis of *Osmfs1-1*, *Oshop2-1*, *Osmfs1/Oshop2,* and WT. **(A)** Comparison between a WT plant and an *Osmfs1-1* plant, an *Oshop2-1* plant, an *Osmfs1/Oshop2* plant at the heading stage. Bars = 10 cm. **(B)** Comparison of panicle between the WT and the *Osmfs1-1*, the *Oshop2-1*, the *Osmfs1/Oshop2* at the harvest stage. Bars = 4cm. **(C)** Viable pollen grains in a WT plant. **(D–F)** Inviable pollen grain in the *Osmfs1-1*, *Oshop2-1,* and *Osmfs1/Oshop2*. Bars = 100 μm in panels **(C–F)**. **(G)** The schematic representations of the gene structure of *OsMFS1* and *OsHOP2*, the knockout sites of *Osmfs1-1*, *Oshop2-1*, *Osmfs1/Oshop2* were verified by sequencing.

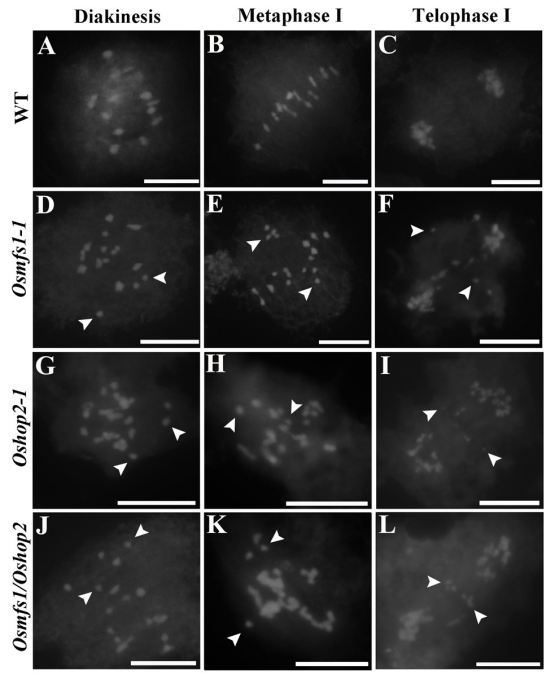

FIGURE 10 | Analysis of meiotic chromosome behavior in *Osmfs1-1*, *Oshop2-1*, *Osmfs1/Oshop2,* and WT. **(A,D,G,J)** pollen mother cell in diakinesis. **(B,E,H,K)** pollen mother cell in metaphase I. **(C,F,I,L)** pollen mother cell in telophase I. The mutant *Oshop2-1* and *Osmfs1/Oshop2* shared the same chromosomal defects with *Osmfs1-1*. The white arrows point to free univalents. Bars = 50 μm.

mechanism (Petukhova et al., 2005). We speculated that the recombinational activity of HOP2 in the complex might be an effective driving force to interact with and stimulate DMC1- and RAD51-mediated single-stranded DNA (ssDNA) invasion into homologous chromosomes to form a synaptonemal complex.

Previous studies showed that the MND1/HOP2 complex was the key factor to activate the invasion vitality of both DMC1 and RAD51 D-loop formation, but not the individual proteins, and the foci of DMC1 and RAD51 accumulate on single strands and the subsequent process was arrested in the *mnd1* or *hop2* mutant (Petukhova et al., 2003; Bugreev et al., 2014). Although the MND1 abrogated the recombinase activity of HOP2, it formed a new molecular interface to interact with DMC1 and RAD51. However, the function of the MND1/HOP2 complex is indispensable. In the mutant of either *mnd1* or *hop2*, the homologous chromosome pairing and DSB repairing were both abnormal and disordered (Leu et al., 1998; Gerton and Derisi, 2002; Zierhut et al., 2004). In our study, we investigated the chromosome behavior of the *Osmfs1* mutants and found that the mutants were defective in the chromosome pairings and the unpaired chromosomes were clearly observed, eventually resulting in a large number of free univalents. Due to *OsMFS1* gene mutation, the complex OsMFS1/*OsHOP2* would lose their function and the DSB would not be repaired at all. The mutants were completely sterile, and the I_2-KI staining also showed that the mutant's pollen was completely aborted. The MND1/HOP2 complex promoted DMC1 and RAD51 to complete the invasion of single strands whereas itswas not active itself in the formation of the D-loop (Petukhova et al., 2005). DMC1 and RAD51 have the potential ability to form D-loop. Neither of them can promote the formation of D-loop, and the MND1/HOP2 complex is needed to activate such vitality to function (Hideo and Shirleen, 2002; Petukhova et al., 2005; Bugreev et al., 2014). Although both DMC1 and RAD51 take part in forming D-loop, these two approaches are not equal. During WT meiosis, DMC1-mediated interhomolog (IH) DNA repair and the pathway appears to be predominant, whereas the pathway of RAD51-mediated intersister (IS) DNA repair has just a supportive role (Bishop et al., 1992; Cloud et al., 2012; Valerie et al., 2012). Meiotic *RAD51* is negatively regulated by *DMC1*. In *Arabidopsis*, in the presence of DMC1, the pathway DMC1-mediated would be sufficiently activated by the complex MND1/HOP2 (Marie-Therese et al., 2012; Clemens et al., 2013), whereas the pathway RAD51-mediated would only have a limited back-up function. In the absence of DMC1, the suppression of RAD51 is relieved, and the complex MND1/HOP2 is dispensable for inter-sister (IS) DNA repair (Clemens et al., 2013). Taken together, we speculated that neither DMC1-mediated nor RAD51-mediated pathways are activated during the meiosis in the *Osmfs1* mutant. This also implied that the chromosomal defects may be only part of the developmental abnormalities that caused by the mutation. How *Osmfs1* causes such defects needs further investigation. Fortunately, we also obtained *Oshop2-1* mutants and *Osmfs1*/*Oshop2* double mutants. The phenotypic identification of these mutant plants revealed that their phenotypes were consistent with *Osmfs1*, and the pollen was completely non-viable. Both the two mutants showed complete sterility. Using PI staining, it was found that the plants of both lines showed severe chromosomal defects (**Figure 9**); we speculate that OsMFS1 plays an important role in mediating the maturation of CO.

Recently, the interaction of *OsHOP2* and OsZIP1 was reported, indicating that *OsHOP2* play a key role in facilitating pairing of synapsis and the formation of CO (Shi et al., 2019). Obviously, not all *OsHOP2* form a complex with OsMFS1. Here we identified that the OsMFS1 protein could interact with another meiotic protein OsRPA2b through Y2H, which had not been reported before (**Figure 8C**). The RPA (Replication protein A) is a kind of ssDNA binding protein (SSB) and plays an essential role in multiple processes of eukaryotic DNA metabolism, including DNA replication, DNA repair, and homologous recombination in human and yeast DNA (*Saccharomyces cerevisiae*) (Johnson and O'Donnell, 2005; Machida et al., 2005). Heterotrimeric protects ssDNA and preserves the formation of hairpin (Ellen et al., 2006). The stable heterotrimer have three subunits: *RPA1* (∼70 kDa), *RPA2* (∼32 kDa), and *RPA3* (∼14 kDa). These *RPA* subunits have multiple copies in *Arabidopsis* and rice (Chang et al., 2009), *OsRPA2b* is one copy of *OsRPA2* in rice. OsMFS1 physically interact with OsRPA2b rather than other subunits, and it suggests the unknown and positive connection between OsMFS1 and OsRPA. These results indicate that these two proteins, OsMFS1 and *OsHOP2*, not only form a complex, but also possess

independent functions. Our results confirmed that the phenotype of the *Osmfs1* mutant is induced by the loss of function of the OsMFS1/*OsHOP2* complex and the DSB fails to be repaired. Nevertheless, in-depth study is underway on the individual features of OsMFS1 involved in homologous recombination.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

JW supervised the project. JW, ZZ, CW, and JL conceived and designed the research plans. JL mapped *MFS1* and wrote the manuscript. JL, CW, and HW performed the experiments and collected and analyzed the data. HZ and WB performed the semi-thin sections. DL, YT, and YX performed real-time PCR. SY, QW, and XY generated the transgenic plants. SL, XL, and LC conducted the fieldwork. ZZ, JL, and CW supervised and complemented the writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00518/full#supplementary-material

FIGURE S1 | Phenotype analysis of WT, *Osmfs1-1*, *Osmfs1-2* (The positive plants of CRISPR/Cas9). **(A)** Comparison between a WT plant and an *Osmfs1-1* plant and an *Osmfs1-2* plant at the heading stage. Bars = 10cm. **(B–D)** Analysis of meiotic chromosome behavior in WT, *Osmfs1-1* and *Osmfs1-2* in metaphase I. The white arrows point to free univalents. Bars = 50 μm in panel **(B)**. **(E)** Seed setting rates of WT (91.80%, *n* = 174, 230, 169, respectively), *OsMFS1/Osmfs1* (90.57%, *n* = 170, 142, 165, respectively), *Osmfs1* (0%, *n* = 492), *Osmfs1-1* (0%, *n* = 156, 196, 160, respectively), *Osmfs1-2* (0% *n* = 177, 142, 127, respectively) at the heading stage, three panicles were counted. **(F,G)** Female fertility analysis of WT and *Osmfs1*. **(F)** The saturated pollination results showed that the seed setting rate of WT was 73.91% (*n* = 92), and the *Osmfs1* mutant was completely sterile (0%, *n* = 104), one panicle was counted. **(G)** Hoechest staining of embryo sacs showed the WT (100%, *n* = 32) develops normally while the mutant was completely sterile (0%, *n* = 27). n indicates the numbers of spikelet or embryo sac; Nd indicates No detection.

FIGURE S2 | Amino acid alignment of OsMFS1 and its homologs. The sequences obtained from NCBI database and compared using Bioxm. Five species were selected including *Oryza sativa*, *Arabidopsis thaliana*, *Zea mays*, *Glycine max*, and *Nicotiana*. Amino acid similarities are shaded in pink.

FIGURE S3 | Phylogenetic tree of the OsMFS1 proteins. Phylogenetic tree was constructed by MEGA 7.0 using Neighbor-Joining method, including *Sorghum bicolor*, *Zea mays*, *Panicum hallii*, *Setaria italica*, *Oryza sativa*, *Brachypodium distachyon*, *Glycine max*, *Gossypium hirsutum*, *Arabidopsis thaliana*, *Brassica napus*, *Nicotiana tabacum*, *Solanum tuberosum*, *Mus musculus*, *Homo sapiens*, *Saccharomyces cerevisiae*.

TABLE S1 | Primers used in map-based cloning, RT-qPCR and plasmid construction.

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frontiers in Plant Science

REVIEW
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A Region on Chromosome 7 Related to Differentiation of Rice (*Oryza sativa* L.) Between Lowland and Upland Ecotypes

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Due to global population expansion and climate change impacts, the development of a stable yielding variety that adapts well to unfavorable conditions for rice cultivation, can contribute to sustainable and stable production in rice (*Oryza sativa* L.). Understanding genetic differentiations to ecotypes for rice cultivations, such as upland, rainfed lowland, and irrigated lowland, is very important to develop the breeding materials for adapting to each environmental condition. The upland landrace variety basically has low tiller/panicle numbers and a large panicle, and the plant architecture is different from that of the lowland variety. The tiller and panicle numbers have been considered as one of the most difficult traits for genetic changes artificially in rice breeding. A low tiller recessive gene *ltn2* originated from a New Plant Type variety, IR 65600-87-2-23, harboring segments from an upland variety, Ketan Lumbu (Tropical Japonica Group), was found on chromosome 7, and the other QTLs for culm length, culm weight, panicle length, panicle weight, seed fertility, harvest index, and soil surface rooting were also detected in the same chromosome region. These low tiller genes and the other QTLs were estimated to play an important role in developing the architecture for upland rice. Some QTLs for root growth angle, *DRO3* and *qSFR7*, were also found in the same chromosome region from upland varieties categorized into the Tropical Japonica Group, and the QTLs may also be relevant to upland adaptation together with other traits. Previous studies using high throughput re-sequencing (whole genome variation data) of a large batch of rice accessions could identify the ecotype differentiated genomic regions (EDRs) and Ecotype differentiated genes (EDGs) such as *Os07g0449700*, a type response regulator, which is critical in upland adaptation in the same region of chromosome 7. Two selective loci, *E3735* and *E4208*, for upland and lowland differentiation, and their corresponding genes *Os07g0260000* and *Os07g0546500* were also detected on chromosome 7 by drought-responding EST-SSRs. These findings indicate that the region on chromosome 7 is highly possible to related to the plant shoot and root architecture in the upland rice variety that has an important role and differentiates between upland and lowland ecotypes.

Keywords: differentiation, upland, lowland, ecosystems, plant architecture, rice (*Oryza sativa* L.)

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INTRODUCTION

Climate change is the main causal element or factor of biotic and abiotic stresses, which have negative effects on global food production including rice (*Oryza sativa* L.) (Raza et al., 2019). Recently, one study estimated a 15% decrease of rice yield in irrigated conditions of developing countries and a 12% increase in rice price as a result of climate change by 2050 (IFPRI, 2009). By contrast, current estimates showed that rice production had to increase by over 20% before 2030 to satisfy the demands of the world's growing population (Purevdorj and Kubo, 2005; FAO RMM, 2018) and to avoid food crises.

The development of a stable yielding variety that adapts well to unfavorable conditions for rice cultivation, such as rainfed lowland and upland ecosystems, can contribute to sustainable rice production and ensure global supply. The respective characteristic traits of rice varieties have been modified under both natural and artificial selections, which lead to phenotypical adaptation to the respective environment and genetic differentiation at the same times between ecotypes (Lyu et al., 2014). Under the upland or rainfed lowland ecosystems, rice cultivation needs to reduce water use in rice production and increase the water use efficiency, and from an environmental perspective, emission of methane is substantially lower in this condition (Xia et al., 2014). Understanding the genetic differentiation among upland, rainfed lowland, and irrigated lowland rice is very important for rice breeding in unfavorable conditions. Especially, rice varieties between upland and irrigated lowland differ significantly in phenotypical and physiological traits. Particularly, the upland variety basically has low tiller/panicle numbers, tick and long culm, and a large panicle, and the plant architecture is different from that of lowland varieties.

Several studies have been conducted to fine the genetic factor(s) for differentiation of ecotypes between lowland and upland. Ishikawa et al. (1992) found that the allele of isozyme gene *Pgd-1* locating chromosome 11 (Wu et al., 1988) was related with the differentiation between Japanese upland varieties and Japanese lowland ones. Zheng et al. (2000) detected three QTLs, *qPRN7* for root penetration ability, *qRN7* for root numbers, and *qRPI* for root penetration index on chromosome 7 in a wax-petroleum layer system simulated to Asian upland soil. Thick root and high penetration ability are important for drought resistance in upland soil. Two deeper rooting QTL, *DRO3* by Uga et al. (2015) and *qRDR7* by Luo et al. (2015) which regulate root-growth-angle of upland rice were also detected on chromosome 7. Uddin et al. 2016a; 2016b) described *qPN7* for panicle number and a gene for low tiller number, *ltn2*, which constrict plant architecture in upland on chromosome 7. Monkada et al. (2001) and Lafitte et al. (2004) reported *qDTH7, qSF7* and *qGW7*, which extended the heading date and increased the spikelet fertility and per grain weight in upland. Xu et al. (2020) recently reported upland specific QTLs, *qHD7*, *qGY7*, and *qHI*, which plays important role in reproduction and yields in upland. Three candidate genes; *Os07g0449700* by Lyu et al. (2014), *Os07g0260000* and *Os07g0546500* by Xia et al. (2014), were also reported as ecotype differentiation genes on chromosome 7 which regulate the root system and plant height in upland rice varieties and help with drought tolerance or resistance. Thus, some chromosome regions were estimated to related with the genetic differentiation between lowland and upland ecosystems, and many QTLs genes for agronomic traits differentiating between ecotypes in lowland and upland have been detected (**Table 1**).

TABLE 1 | List of genes and QTLs on chromosome 7 for ecotype differentiation.

QTL or gene detected	Physical position (Mb) of the closest marker*	Regulatins/Functions	Origin	Ecotype	Reference
DRO3	24.2	Root angle	Kinandang Patong	Upland	Uga et al. (2015)
qSOR1	25.6	Surface rooting	Gemdjah Beton	Lowland	Uga et al. (2012)
qPN7^{b,d}	24.5	Panicle No.	IR65600-87-2-2-3	Upland	Uddin et al. (2016a)
qTN7 and *ltn2*	25.1	Low tiller No.	IR65600-87-2-2-3	Upland	Uddin et al. (2016b)
qSF7	26.0	Spikelet fertility & Root depth	Azucena	Upland	Lafitte et al. (2004)
qGW7	26.0	Weight per Grain & Root depth			
qSFR7	23.6	Soil Surface rooting	IR65600-87-2-2-3	Upland	Tomita and Fukuta (2019)
qHD7	25.5	Heading date	B61144F-MR-6	Upland	Xu et al. (2020)
qGY7	25.5	Grain yield			
qHI7	25.5	Harvest index			
qRDR7	26.0	Deep rooting	IRAT 109	Upland	Luo et al. (2015)
qBRT^{a}	19.0	Root thickness		Lowland	Li et al. (2011)
qBRT^{b}	25.9	Root thickness		Lowland	Li et al. (2011)
qRN^{a}	29.6	Root number		Upland	Li et al. (2005)
qDTH7	24.8	Heading date	*O. rufipogon (IRGC #105491)*	upland	Monkada et al. (2001)
qPRN7	17.8	Penetrated root numbers	Azecena	Upland	Zheng et al. (2000)
qTRN7	14.4	Total root numbers			
qRPI	28.4	Root penetration index			
Os07g0449700	16.1	Root and shoot development	Panel of upland varieties	Upland	Lyu et al. (2014)
Os07g0260000 Os07g0546500	9.1	Drought tolerance	Panel of upland varieties	Upland	Xia et al. (2014)

**Physical position of the closest markers were retrieved from the genome database in Gramene (http://www.gramene.org/) and Oryzabase (https://shigen.nig.ac.jp/rice/oryzabase/). DRO, deeper rooting; SOR, soil surface rooting; BRT, basal root thickness; RDR, ratio of deep rooting; PN, panicle number; TN, tiller number; HI, harvest index; GW, grain weight; SF, spikelet fertility; GW, grain weight; DTH, days to heading; DH, heading date; RP, root penetration; RN, root number; PRN, penetrated root number.*

Interestingly, most of the research findings indicated that a region on chromosome 7 was related to the variations of unique traits between the two ecotypes, such as drought tolerance/avoidance and upland adaptation. This article review examines the involvement of chromosome 7 in the genetic differentiation of upland and lowland varieties, and then discusses how this information can be used in breeding a rice variety that adapts well to unfavorable conditions for sustainable production in rice.

A LOW TILLER GENE CONTRIBUTING TO PLANT ARCHITECTURE

Plant architecture with a low tiller and high-density grain is desirable in limited water conditions to maintain a proper plant density, to avoid episodic drought, and to keep the stable yield production based on the minimizing of reproductive tillers under the serious conditions, such as rainfed lowland and upland (Vergara et al., 1990; Farooq et al., 2011). The control for numbers of tiller and panicle may be the important breeding target for increasing the adaptability and seed production in the growing stages from productive to reproductive stages of rice against upland and serious environmental conditions.

Several artificial mutant genes for tiller numbers, *tdr2* (Hasegawa et al., 2005) and *rcn9* (Jiang et al., 2006) on chromosome 1, *OsTB1* on chromosome 3 (Takeda et al., 2003), *HTD1* on chromosome 4 (Zou et al., 2005), and *D3* (Ishikawa et al., 2005), *MOC1* (Li et al., 2003), and *rcn8* (Jiang et al., 2006) on chromosome 6, have been found in rice. These genes are associated with branching mechanisms of the tiller. As the natural variations, many QTLs for numbers of panicles and tillers have been reported. Lin et al. (1996) found them on chromosomes 2, 4, 5, and 6; Wu et al. (1998) were on chromosomes 1, 3, and 5; Yan et al. (1998) were on chromosomes 1, 2, 3, 4, 5, 6, 7, 8 and 12; Nagata et al. (2002) were on chromosome 1; Hittalmani et al. (2003) were on chromosomes 1, 3, 4, and 12; Miyamoto et al. (2004) were on chromosomes 2, 5, 6, and 8; Liu et al. (2008; 2010; 2012) were on chromosomes 1, 2, 3, 4, 6, 7, 8, 9, and 12, and Bian et al. (2013) were on chromosomes 1, 2, 4, 5, 6, 7, 8, and 10. A low tiller-number gene, *Ltn* was found, on chromosome 8 in a Japanese Japonica Group variety, Aikawa 1 (Fujita et al., 2010). Thus, several genes and many QTLs have been identified almost rice chromosomes except for chromosome 11, as the genetic factors for controlling the tiller and panicle numbers in rice. These QTLs for tiller or panicle numbers by Yan et al. (1998), Liu et al. (2008; 2010; 2012), and Bian et al. (2013) were detected in the similar region of long arm on chromosome 7.

International Rice Research Institute (IRRI) developed the New Plant Type (NPT) varieties characterizing as the low tiller numbers and a high productive tiller ratio by introducing the chromosome segments from the Tropical Japonica Group varieties, as one of the traits for genetic improvements to increase rice yield production (Khush, 2000). Fujita et al. (2009) reported 334 introgressions lines (INLs) with the genetic background of an Indica Group variety IR 64 and harboring several chromosome segments from NPT varieties which were bred by the crosses between Indica Group and Tropical Japonica Group upland varieties. In the other words, these chromosome segments in INLs were mainly originated from the Tropical Japonica Group upland varieties. Uddin et al. (2016a) found 18 QTLs for yield component traits including a QTL for low panicle numbers (as an indicator of tiller number), *qPN7*, on chromosome 7, by association analyses with 35 INLs harboring chromosome segments from the NPT variety, IR 65600-87-2-2-3 among them (**Figure 1**). IR 65600-87-2-2-3 harbors the chromosome segments of the Tropical Japonica Group upland rice variety, Ketan Lumbu. The other QTLs for culm length, panicle length, panicle weight, harvest index (panicle weight/total weight), and fertility rate were also found in the same region detected *qPN7* on chromosome 7. To confirm the detailed chromosome position of the QTLs detected,

FIGURE 1 | Phenotypic characteristics of an introgression line for *ltn2* originated from an IR 65600-87-2-2-3. Photo of rice plants at maturity stage. Rice was cultivated in lowland field at Tropical Agricultural Research Front, JIRCAS, Ishigaki, Okinawa, Japan, in 2015. **(A)** IT 6600-87-2-2-3, **(B)** YTH16, **(C)** YTH34, **(D)** IR 64.

advanced genetic studies were conducted using two hybrid populations. A total of 88 F_3 family lines derived from a cross between IR 64 and YTH34 which was one of 35 INLs harboring the segment on chromosome 7 from the NPT variety, IR 65600-87-2-2-3. The values of panicle numbers, spikelet numbers, and dry weight of whole plant in YTH34 were lower than those of IR 64 under the cultivation in irrigated lowland condition, and remarkable reductions of these values were observed under upland cultivation (**Table 2**) and an F_3 population consisting of 72 plants, which was self-pollinated from an F_2 plant, F_2-JII-IV-10, harboring the heterozygote chromosome region for *qPN7* (Uddin et al., 2016b). These segregation analyses under upland conditions confirmed the single gene segregations. It was found a QTL for low tiller and designated as a recessive gene, *ltn2*, originated from the Tropical Japonica Group rice (**Figure 2**). Linkages were also found with some Simple Sequence Repeats (SSR) markers RM505, MRG5344, and RM21950. The genetic distances between RM505 and *ltn2* were 3.4 cM, and between RM21950 and *ltn2* they were 1.1 cM, and *ltn2* was mapped most closely with RM21950. Advanced QTL analysis also confirmed other QTLs for culm length, culm weight, panicle length, panicle weight, seed fertility, and harvest index in the same chromosome region. These QTLs decreased the traits' values with the YTH34 allele. The INLs, YTH34, with IR 64 genetic background, is

TABLE 2 | Agronomic traits of IR 64 and YTH34 under irrigated lowland and upland.

Conditions	variety	Panicle No./ plant	Culm length (cm)	Panicle length (cm)	Spikelet No./ panicle	Seed fertility (%)	Panicle weight(g)	Dry weight (g)	Harvest index(%)	Days to heading
Irrigated	IR 64	22.0	70.3	25.3	130.6	48.2	25.1	78.5	31.9	107.6
lowland		15.0 (68)	70.8 (100)	25.9	109.1	85.4	32.2	66.6 (84)	48.4	108.3
	YTH34			(102)	(83)	(177)	(128)		(151)	(100)
Upland	IR 64	47.0	51.3	25.9	138.0	63.4	51.9	140.0	38.0	140.0
		17.0	39.9	22.8	102.0	66.6	14.6	48.0 (34)	30.0	151.0
	YTH34	(36)	(77)	(88)	(74)	(105)	(28)		(78)	(107)

Rice varieties were cultivated at Tropical Agricultural Research Front, JIRCAS, Ishigaki, Okinawa Japan, in 2013 (Date were refereed from Uddin et al., 2016b).
Mean values among 12 plants were calculated in each trait.
( ) = Relative value (%) in compared with that of IR 64.

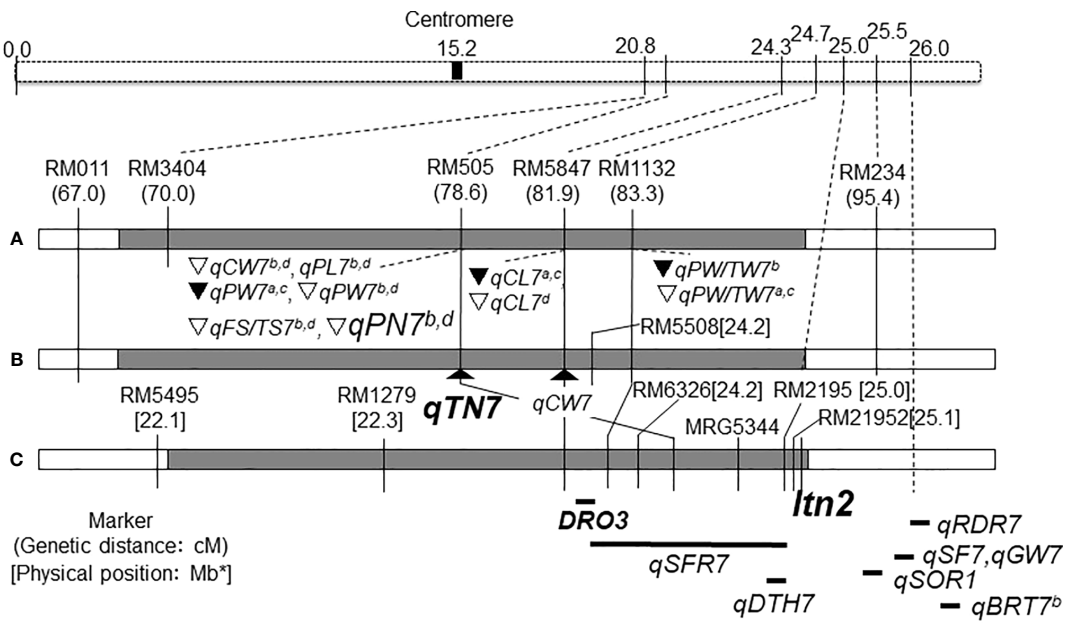

FIGURE 2 | Choromosome location of low tiller number gene, *ltn2* and QTLs detected on chromosome 7. **(A)** Uddin et al. (2016a); **(B, C)** Uddin et al. (2016b); *DRO3*: Uga et al. (2015); *qSFR7*: Tomita and Fukuta (2019); qBRT7[a,b]: Li et al. (2011); *qRDR7*:Luo et al. (2015); *qSF7, qGW7*: Lafitte et al. (2004); *qSOR1*: Uga et al. (2012); *qDTH*: Monkada et al. (2001). ∇: YTH34 allele decreased value in upland.▼:YTH34 allele decreased value in irrigated lowland. CW, culm weight; PW, panicle weight; PW/TW, ratio of panicle weight to total plant weight; CL, culm length; PL, panicle length; PN, panicle number; FS/TS, ratio of fertile spikelet per panicle; BRT, Basal root thickness; RDR, Ratio of deep rooting; SOR, Soil surface rooting; SF, Spikelet fertility; GW, Grain weight; DTH, Days to heading. [a]Irrigated lowland in 2011, [b]Upland in 2011, [c]Irrigated lowland in 2012, and [d]Upland in 2012. *Physical position of the Chromosome 7 was redrawn from the genome database in Oryzabase (https://shigen.nig.ac.jp/rice/oryzabase/) using the Nipponbare reference genome. IR 64, YTH 34, Flanked region of QTL.

introduced only the genetic factor(s) for tiller/panicle numbers and those for the other traits locating the other chromosomes were not harbored. These results indicated that *ltn2* originated from a tropical Japonica Group upland rice variety, Ketan Lumbu, had a strong genetic effect for occurring of tiller and panicle with negative effects for the other traits. The ecotype of upland landrace rice is characterized such as traits; low panicle/tiller numbers, long and thick culm, and large panicle. These traits are controlled by several genetic factors which were distributed on the rice genome chromosomes in each, and these were accumulated in the genetic background of upland ecotype rice. The tiller/panicle numbers have been known as the most difficult trait to detect genetically, because of large environmental error (Sasahara, 1997). In the other words, these were easy to be influenced and changed by environmental conditions. To control the tiller/panicle numbers, the genetic factor(s) with strong effects may be needed under the serious and unfavorable environmental conditions of upland. The low tiller gene and the other QTLs detected on chromosome 7, which were originated from the Tropical Japonica Group variety, were estimated to play the important role for developing shoot architecture of upland rice with strong effects.

GENETIC FACTORS FOR ROOT ARCHITECTURE

One major QTL for soil-surface roots, *qSOR1*, was found using 124 recombinant inbred lines derived from a cross between an Indonesian lowland rice cultivar, Gemdjah Beton, with soil-surface roots and a Japanese lowland rice cultivar, Sasanishiki, was detected and localized on the long arm of chromosome 7 by Uga et al. (2012).

A QTL for deep root-growth-angle, *DRO1*, was found on chromosome 9, using 26 chromosome segment substitution lines (CSSLs) which were developed from the cross combination between an upland variety, Kinandang Patong (Tropical Japonica Group), and IR 64 as the recurrent parent (Uga et al., 2015). And another QTL, *DRO3*, was also found on the long arm of chromosome 7 using the progenies derived from a cross between IR 64 and a CSSL harboring *DRO1*. *DRO3* contributed to the deep root growth angle trait with the allele of Kinandang Patong. The nearest marker RM5508 for *DRO3* locates in the same chromosome regions of *ltn2* with the distance of 0.7 cM. Another QTLs for deep rooting, *qRDR7*, was also reported in the same region of chromosome 7 and the allele of an upland variety IRAT109 contributed to deep rooting in the genetic background of Zhenshan 97B which was a shallow rooting parent by Luo et al. (2015).

Tomita et al. (2017) tried to clarify the genetic variation for the root angle distributions using 97 accessions, and these were classified into two cluster groups, A and B. The accessions of cluster group A showed shallow rooting including the soil-surface root type, but and the numbers of accession were few. An INL, YTH16, by Fujita et al. (2009) harboring chromosome segments from a common NPT variety, IR65600-87-2-2-3, was also included. The accessions in cluster group B showed a wide variation from shallow to deep rooting types in both Indica and Japonica Groups, lowland and upland ecotypes, and landraces and improved types. There were no relationships between the root vertical angle and total root numbers among them. These findings indicated that the root angle distributions were not related with the differentiations between Japonica Groups and Indica Groups, among ecotypes for lowland and upland, and among degrees of genetic improvement, and the accessions with soil-surface root were rare and unique among natural variations. Tomita and Fukuta (2019) clarified that the soil surface-rooting in YTH16 was controlled by three QTLs on chromosomes 2, 5, and 7, and one QTL, *qSFR7*, on chromosome 7 had the biggest effect and played a main role. *qSFR7* was detected in the same region as *ltn2* by Uddin et al. (2016b). The QTL for soil surface-rooting on chromosome 7 is also one of the unique characters originated from the Tropical Japonica Group variety in rice. The genetic factor(s) for root architecture may also be relevant to upland adaptation together with other traits.

ECOTYPE DIFFERENTIATED GENES

With the basis of the rice genome sequence and annotation databases, NCBI: http://www.ncbi.nlm.nih.gov/gene/; TIGR: http://rice.plantbiology.msu.edu/, we found the physical locations of *MRG5433* on chromosome 7 and three putative and expressed genes close to the target region of the Japonica Group rice genome (cultivar: Nipponbare), such as Os07 g0607500, Os07g0607700, and Os07g0607800, were found that were related to abiotic stress tolerance (Sottosanto et al., 2007; Zhang et al., 2008; Huang et al., 2009; Kera et al., 2012).

Using the entire genome resequencing data from a large panel of 84 upland and 82 irrigated lowland rice accessions, Lyu et al. (2014) reported some selective deviations in rice chromosomes, which were called as the ecotype differentiation genomic regions (EDRs). In the EDRs, several individual ecotype's differentiated genes (EDGs) that are critical in upland adaptation or the phenotypical differentiation between the two ecotypes, lowland and upland, were also detected. A type-A response regulator (ARR) gene, *Os07g0449700*, on chromosome 7, is of special interest as it belongs to the ARR gene family induced by cytokinin, plays important roles in cytokinin signaling, and has impacts on root and shoot development. This ecotype differentiation of this ARR gene, *Os07g0449700*, may explain some differences of root and plant height between upland and irrigated rice varieties. Further reciprocal transgenic experiments between upland and irrigated rice will clarify the effects of *Os07g0449700* in the adaptation of upland rice.

Xia et al. (2014) reported seven selective loci that are ecotype preferable alleles expressed under drought stress by the analyses for 47 drought-responding expressed sequenced tags-simple sequence repeats (EST-SSRs) markers, using 377 rice landraces collected from China. These EST-SSRs markers are selected from the DNA transcribe regions and are closely related to the functional gene expressed under drought stress. Among the seven,

two loci, *E3735* and *E4208*, were related to the differentiation between upland and lowland varieties, and these corresponded to *Os07g0260000* and *Os07g0546500*, which were detected on the rice chromosome 7, which may have an important effect on drought resistance or drought tolerance in rice.

CONCLUSION

Crops adapted to different agro-ecosystems always promote the variation of agriculturally important genes (Xia et al., 2014). The knowledge and information regarding the genetic differentiation for eco-typical variations of traits and adaptations will greatly contribute to harnessing the genetic resources for the breeding and cultivation of rice under upland as well as unfavorable environments. We reviewed the involvement of a region on chromosome 7 in the genetic differentiations for rice ecotypes to lowland and upland ecosystems.

The *ltn2* region on chromosome 7 showed the unique reactions: low tiller, low dry matter production, short panicle length, and short culm lengths (Uddin et al., 2016b), and soil surface rooting (Tomita et al., 2017; Tomita and Fukuta, 2019). Usually, the upland landrace varieties are shown the unique characters: low tiller, heavy and big panicle, and short and early maturation, and these agricultural traits might contribute to avoid the risk of short water supply and to keep the stable yield production based on the minimalizing reproductive tillers under rainfed conditions. This unique shoot architecture of low tiller and panicle may be an important trait for adaptation to serious upland environmental conditions (Uddin et al., 2016b). YTH34 had a lower plant height, spikelet numbers, dry matter productions, and longer time to heading, and did not show good performances compared with that of IR 64 under the upland condition (**Table 2**). These results suggest that the genetic background of *ltn2* is efficient in the upland variety. This *ltn2* related to one of the genetic factors for the adaptation of rice cultivar to upland conditions might be very useful for the genetic improvement of rice cultivars under upland or unfavorable conditions with the other genetic factors.

YTH34 harboring *ltn2* and the genetic information of the low tiller gene including linked SSR markers as well as the proposed candidate genes will be useful for the genetic modification of plant architecture in the rice variety, and for understanding the genetic mechanism of differentiation between lowland and upland ecotypes. The model plant type for adaptation to upland conditions will be able to be reconstructed by *ltn2*. The information of gene pyramiding based on *ltn2* will help us to understand the architecture of upland rice cultivars.

The ecotype differentiated genes such as *Os07g0449700* for root and shoot development (Lyu et al., 2014), EST-SSRs based selective loci E3735 and E4208 and their corresponding genes *Os07g0260000* and *Os07g0546500* for drought resistance (Xia et al., 2014), and other detected QTLs (Uga et al., 2015; Uddin et al., 2016a; Uddin et al., 2016b) on the rice chromosome 7 also play key roles in upland adaptation and result in the phenotypical differentiation. Clarification of the relationships among these candidate genes, *ltn2*, and QTL for the soil surface root on chromosome 7, will be investigated in future studies.

This review will not only help the geneticists to understand the underlying molecular basis of adaptive divergence, but also provide to breeder and agronomist valuable information for rice domestication and adaptation, especially on the upland/water stress and drought torelant in rice.

AUTHOR CONTRIBUTIONS

MNU prepared the manuscript basically, and YF made the plan for the conformance of the manuscript. The paper was prepared based on the PhD course study for MNU under the supervision of YF in University of Tsukuba.

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Identification and Fine Mapping of *Pi69*(t), a New Gene Conferring Broad-Spectrum Resistance Against *Magnaporthe oryzae* From *Oryza glaberrima* Steud

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The discovery and deployment of new broad-spectrum resistance (*R*) genes from cultivated rice and its wild relatives is a strategy to broaden the genetic basis of modern rice cultivars to combat rice blast disease. *Oryza glaberrima* possessing many valuable traits for tolerance to biotic and abiotic stresses, is an elite gene pool for improvement of Asian cultivated rice. An introgression line IL106 derived from *O. glaberrima* (Acc. IRGC100137) confers complete resistance to *Magnaporthe oryza*e in blast nursery. Genetic analysis using 2185 BC_6F_2 progenies derived from a cross between IL106 and the recurrent parent Dianjingyou 1 showed that IL106 harbors a single dominant resistance gene against *M. oryzae* strain 09BSH-10-5A. This gene was preliminarily mapped on the long arm of chromosome 6 of rice in a region of ca. 0.9 cM delimited by two SSR markers (RM20650 and RM20701). In order to finely map this gene, 17,100 additional progenies were further analyzed. As a result, this gene was further narrowed down to a region flanked by two molecular markers STS69-15 and STS69-7, and co-segregated with 3 molecular markers, RM20676, STS69-21 and STS69-22 on the long arm of chromosome 6. Based on reference genome sequences, this *R* gene was mapped in silico in 76.1-Kb and 67.7-Kb physical intervals, and containing 4 and 3 NBS-LRR candidate genes in *O. sativa* cultivar Nipponbare and *O. glaberrima* cultivar CG14, respectively. Because no blast resistance gene was finely mapped in this physical interval before, this *R* gene was considered as not described yet and designated as *Pi69*(t), which is the first identified and finely mapped blast *R* gene from *O. glaberrima*, as far as we know. Evaluation of IL106 with 151 blast strains collected from 6 countries in Asia showed that 148 strains are avirulent on IL106, suggesting that *Pi69*(t) is a broad-

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spectrum blast *R* gene, and a promising resistant resource for improvement of Asian cultivated rice.

Keywords: *Oryza glaberrima*, introgression line, *Magnaporthe oryzae*, *Pyricularia oryzae*, resistance gene, fine mapping

INTRODUCTION

The African cultivated rice, *Oryza glaberrima* Steud., is well adapted for cultivation in West Africa (Linares, 2002; Sarla and Swamy, 2005), and possesses many valuable traits for tolerance to abiotic stresses, such as salinity, drought and strong weed competitiveness (Sarla and Swamy, 2005). *O. glaberrima* is also reported to have high level of resistance against several diseases and insect pests, such as *Rice yellow mottle virus* (Ndjiondjop et al., 1999; Pidon et al., 2017), bacterial leaf blight (Djedatin et al., 2011), blast (Silué and Notteghem, 1991; Rama Devi et al., 2015), green rice leafhopper (Fujita et al., 2010), as well as rice gall midge (Ukwungwu et al., 1998). Although it contains a narrow genetic base compared with other *Oryza* species (Wang et al., 2014; Meyer et al., 2016; Ndjiondjop et al., 2017), *O. glaberrima* is considered as an excellent gene reservoir for improvement of Asian cultivated rice, due to its useful traits of agronomic importance (Linares, 2002; Sarla and Swamy, 2005).

Rice blast, caused by the ascomycete fungus *Magnaporthe oryzae* (syn., *Pyricularia oryzae*) (Couch and Kohn, 2002), is one of the most destructive diseases for rice, and is responsible for significant yield losses under favorable environmental conditions worldwide (Ou, 1980; Savary et al., 2019). Rice-*M. oryzae* interactions follow the gene-for-gene relationship (Silué et al., 1992; Jia et al., 2000). Utilization of resistance (*R*) genes is one of the most economical, effective and environment-friendly approaches for blast control. However, the *R* genes of rice cultivars are often overcome shortly after their release, due to the emergence of strains of the pathogen virulent on certain *R* genes (Zeigler et al., 1994). Thus, it is necessary to mine new genes with broad spectrum of resistance against *M. oryzae* from diversities of rice species and use them in appropriate management strategies for durable control of blast in rice production (Zhu et al., 2000; Raboin et al., 2012; Sester et al., 2014). To date, over 100 blast *R* genes have been identified and mapped on different chromosomal regions of rice, through broad genetic and linkage analysis in the past decades (Ballini et al., 2008; Ashkani et al., 2016). These *R* genes have mainly been identified from *O. sativa*, and only 5 *R* genes originated from wild species of genus *Oryza*, including *Pi40* (*O. australiensis*), *Pi54rh* (*O. rhizomatis*), *Pi54* (*O. officinalis*), *Pi57*(t) (*O. longistaminata*), and *Pid3-A4* (*O. rufipogon*) (Jeung et al., 2007; Das et al., 2012; Lv et al., 2013; Devanna et al., 2014; Dong et al., 2017). *O. glaberrima* was domesticated from its wild progenitor *O. barthii* independently from *O. sativa* (Sweeney and McCouch, 2007). Although several studies previously reported that *O. glaberrima* expressed high level of resistance against rice blast (Silué and Notteghem, 1991; Rama Devi et al., 2015), no blast *R* gene locus was further identified and mapped yet. Whether blast *R* genes in *O. glaberrima* are different from *R* genes identified from other *Oryza* species so far remains unknown.

In order to discover useful genes of agronomic importance from *O. glaberrima*, a set of BC_5F_4 introgression lines (ILs) was constructed through successive backcross strategy between IRGC100137, an accession of *O. glaberrima* and *O. sativa* cultivar Dianjingyou 1 (DJY1), an *O. sativa* subsp. *japonica* cultivar, used as male recurrent parent (Xu et al., 2014). The ILs were evaluated for blast resistance in blast nursery in the field and by artificial inoculation with *M. oryzae* isolates in the greenhouse. Twelve ILs showing complete resistance to *M. oryzae* compared with the susceptible recurrent parent DJY1 were obtained. In this study, we describe the identification and fine mapping of a new blast resistance locus *Pi69*(t) from *O. glaberrima*.

MATERIALS AND METHODS

Rice Materials and Mapping Population Construction

Resistant introgression line IL106 derived from *O. glaberrima* (accession No. IRGC100137) was crossed with a susceptible recurrent parent Dianjingyou 1 (DJY1) to generate BC_6F_1 seeds, the BC_6F_1 seeds were further sown and grown in a greenhouse to generate BC_6F_2 population for linkage and genetic analysis for resistance to rice blast. Resistant donor IL106, 10 monogenic lines (IRBLZ-Fu (*Piz*), IRBLZ5-CA (*Pi2*), IRBLzt-T (*Piz-t*), IRBL9-W (*Pi9*), IRBL5-M (*Pi5*), IRBLKH-K3 (*Pikh*), IRBL1-CL (*Pi1*), IRBL7-M (*Pi7*), IRBL20-IR24 (*Pi20*), and IRBLTA2-PI (*Pita2*)), as well as susceptible control cultivar Lijiangxintuanheigu (LTH) were used to test resistant/susceptible phenotypes to 151 *M. oryzae* strains.

M. oryzae Cultivation and Spore Production

The *M. oryzae* isolate 09BSH-10-5A that is avirulent to IL106 and virulent to DJY1 was cultured on oatmeal medium (20 g of oatmeal, 15 g of agar, 10 g of sucrose, and 1 L of distilled water) for 7 days in the dark at 25°C. Then aerial mycelia were washed off by gentle rubbing with distilled water and paintbrush. The colony was then successively exposed to fluorescent light for 3 days to induce sporulation at 25°C. Conidia were harvested by softly scraping and flooding the medium surface with distilled water containing 0.01% Tween 20 detergent. The concentration of conidial suspension was adjusted to 50,000 conidia/ml for inoculation (Dong et al., 2017).

Plant Planting and Pathotesting

The BC_6F_2 population seeds derived from the cross between IL106 and DJY1 were sown in plastic trays of 20×12×5 cm filled with paddy soil, and each tray was sowed with 95 germinated seeds. Seedlings were inoculated with *M. oryzae* strain 09BSH-10-5A by spraying at 4-leaf stage with 20 ml conidial suspension per tray. The inoculated rice plants were incubated overnight in a dark chamber at 25°C for 24 h with over 95% relative humidity, and then transferred back to the greenhouse. Lesion types on rice leaves were observed 6–7 days after inoculation and scored according to a standard reference scale (Silué et al., 1992). Plants scored from 1 to 3 were considered to be resistant and plants scored from 4 to 6 were considered to be susceptible. Furthermore, 151 *M. oryzae* isolates from 6 countries were used to test the resistant spectrum of *Pi69*(t) gene carrying in IL106 and 10 known blast *R* genes carrying in monogenic lines.

Marker Development and Genetic Map Construction

Genomic DNA was extracted from fresh leaves of each plant following the method described by Edwards et al. (1991). A total of 229 SSR markers distributed evenly across all 12 rice chromosomes (McCouch et al., 2002) were used for identification of introgressed regions from *O. glaberrima*. Sequence-tagged site (STS) markers were developed within the critical region based on the sequence alignment of the genomic sequences of Nipponbare (*O. sativa*) and CG14 (*O. glaberrima*, http://plants.ensembl.org/Oryza_glaberrima/Info/Index).

PCR amplification conditions consisted of a denaturing step of 94°C/3 min, followed by 35 cycles of 94°C/30 s, annealing temperature 55°C/30 s, and 72°C/1 min, ending with an extension step of 72°C/7 min. Amplicons were separated by 8% polyacrylamide gel electrophoresis and detected by silver staining. Information of all primers used for gene mapping in this study is listed in **Table 1**. The genetic and linkage map of polymorphic markers was constructed using MAPMAKER/EXP 3.0 (Lander et al., 1987). The Kosambi mapping function was used to transform recombination frequency to genetic distance (cM).

Physical Map Construction *In Silico* and Candidate Gene Annotation

To construct physical map of *Pi69*(t) *in silico* based on the reference genome sequence of *O. sativa* subsp. *japonica* cultivar Nipponbare, all molecular markers were anchored on chromosome 6 of Os-Nipponbare-Reference-IRGSP-1.0 pseudomolecules by BLAST (https://blast.ncbi.nlm.nih.gov/). To annotate the candidate *R*

TABLE 1 | Summary of PCR primers used for linkage analysis.

Marker	Forward primer (5'-3')	Genomic position (bp) of Nipponbare[a]	Expected size (bp)[b]	Genomic position (bp) of CG14[c]	Expected size (bp)[d]	Annealing temperature (°C)
RM30	F: TGGGGTGGTTAGGCATCGTC	27253291-27253310	85	20426541-20426560	–	55
	R: CCTCACCACACGACACGAGC	27253375-27253356		not available		
RM345	F: ATGCAACCTCCTCTTCTCCA	30865862-30865881	136	23089611-23089630	143	55
	R: ATTGGTAGCTCAATGCAAGC	30865997-30865978		23089753-23089734		
RM20625	F: GGAGGGAGGAATGGGTACACG	28533451-28533471	182	21297908-21297928	107	55
	R: TTGAGAGTGAAACGAGAACCAACC	28533632-28533609		21298014-21297991		
RM20650	F: CGAGTGGATCAGCAAATCTACAGC	29161210-29161233	111	21803046-21803069	107	55
	R: CAGCATCAGGCTTGTGTTAATGG	29161320-29161298		21803152-21803130		
RM20676	F: GATCTCCACCACCTCCATCTCC	29885931-29885952	192	22383635-22383656	129	55
	R: CCTACATCAAGGCTCGCTACTGC	29886122-29886100		22383763-22383785		
RM20701	F: GAGAAGAAATTCAGAGAGCAGAGC	30349781-30349804	164	22767158-22767181	155	55
	R: CAACCACATGATCCATATGACG	30349944-30349923		22767312-22767290		
RM20661	F: GAACACATGACACCACCTTTGC	29479730-29479751	152	22031105-22031126	143	55
	R: GCGTTTCTCATTCTGTTCTTGC	29479881-29479860		22031247-22031226		
RM20674	F: CAACCCAACCCAACATCTGC	29782053-29782072	195	22299523-22299542	184	55
	R: CCTCTTGTCTTTGGAGGCCTTACC	29782247-29782224		22299706-22299683		
RM20678	F: CCGACCCATCAAACACAAATAGG	29976869-29976891	142	22466394-22466416	136	55
	R: CTTCTTCGGCTTCGCCTTCC	29977010-29976991		22466529-22466510		
STS69-21	F: GGTAGACAAGTTAACACCCAACCATGA	29899071-29899097	158	22395527-22395553	195	55
	R: GCACAGACAGGGGAGGAAGCAAAC	29899228-29899205		22395721-22395698		
STS69-7	F: ATCGGCCTGGTCTACTACGAGTAATC	29948504-29948529	136	22437870-22437895	131	55
	R: CCATTGATCAAATTCTACATGAATC	29948639-29948615		22438000-22437976		
STS69-15	F: CCTGTGTACGTGTGTTCTGTATGC	29872500-29872523	184	22370311-22370334	165	55
	R: CATCCACAAGCAGAGCTGGTC	29872683-29872663		22370475-22370455		
STS69-22	F: GCGCTGCGACGGAAAGAATA	29934780-29934799	147	22416918-22416899	150	55
	R: TCCGGCCTCTATATCCACAAAG	29934926-29934905		22417067-22417046		

F, forward; R, reverse.

[a]*genomic position of each marker along chromosome 6 of O. sativa subsp. japonica cultivar Nipponbare (IRGSP1.0).*

[b]*expected size of PCR products in Nipponbare.*

[c]*genomic position of each marker along chromosome 6 of O. glaberrima cultivar CG14.*

[d]*expected size of PCR products in CG14.*

genes, both the 76.1-Kb and 67.7-Kb target regions in Nipponbare and CG14 respectively were analyzed by using the FGENSH platform (http://www.softberry.com/).

RESULTS

Genetic Analysis for Blast Resistance in IL106

The resistant donor IL106, recurrent parent DJY1, BC_6F_1 plants from IL106/DJY1 and BC_6F_2 population were inoculated with 09BSH-10-5A (**Supplementary Figures S1A, B**). The resistant donor IL106 and BC_6F_1 plants showed complete resistance, and recurrent parent DJY1 was susceptible to 09BSH-10-5A. The segregation of resistant and susceptible progenies among 2185 BC_6F_2 individuals fitted with an expected 3:1 ratio (resistant/susceptible: 1664/521, $\chi^2 = 1.556$, $P = 0.212$), indicating that a single dominant *R* gene from IL106 confers complete resistance to *M. oryzae* strain 09BSH-10-5A.

Identification and Mapping of *R* Gene Locus in IL106

To identify and map the *R* locus in IL106, a total of 217 SSR markers distributed evenly across all 12 rice chromosomes were used to determine the polymorphism between resistance donor IL106 and recurrent parent DJY1. As expected, a large majority of markers were monomorphic between IL106 and its recurrent parent. Three SSR markers, RM345 on chromosome 6, RM6329 on chromosome 3, and RM3702 on chromosome 8, showed polymorphism between IL106 and DJY1, suggesting that three introgression fragments from *O. glaberrima* possessed in IL106. In order to verify the linkage relationship between these 3 SSR loci and *R* gene in IL106, 94 random susceptible individuals from BC_6F_2 population inoculated with 09BSH-10-5A were genotyped with these 3 SSR markers. The results showed that the severe segregating distortion (92 homozygotes of susceptible allele to 2 heterozygotes) was only detected for RM345, implying linkage between the *R* gene and this marker which located on chromosome 6.

To determine the *O. glaberrima* introgression length of the *R* gene region in IL106, 27 SSR markers located on the long arm of chromosome 6 were selected to survey the polymorphism between IL106 and DJY1. The result showed that the introgression fragment was located between SSR markers RM30 and RM345, and 4 SSR markers (RM20625, RM20650, RM20676, and RM20701) within this interval were also polymorphic. Subsequently, the mapping population consisting of 2,185 BC_6F_2 plants was genotyped with the two SSR markers RM30 and RM345, and the recombinants were further genotyped with 4 SSR markers (RM20625, RM20650, RM20676, and RM20701) to map the *R* gene location. Taken together, the *R* locus was mapped to a 0.9 cM region flanked by RM20650 and RM20701 on the long arm of chromosome 6, and co-segregated with RM20676 (**Figure 1A**).

Fine Mapping of *R* Gene Locus in IL106

To further map this *R* gene locus, 17,100 additional BC_6F_2 plants were genotyped with two flanking markers RM20650 and RM20701. All the 312 recombinants were then phenotyped for resistance to *M. oryzae* strain 09BSH-10-5A. As a result, 167 and 145 recombinants were detected between phenotypes (R or S) and RM20650 and RM20701 genotypes, respectively (**Figure 1B**). Meanwhile, new SSR markers located in RM20650-RM20701 interval were surveyed for polymorphism between IL106 and DJY1, and 3 polymorphic SSR markers RM20661, RM20674, and RM20678 were obtained. Then, the 312 recombinants were genotyped with these new SSR markers and RM20676. The recombination events btweeen the *R* locus and RM20661, RM20674, RM20676, and RM20678 were 121, 7, 0, and 13, respectively (**Figure 1B**), The *R* locus was linked to RM20661 and RM20674 by a genetic distance of ca. 0.71, and 0.041 cM, co-segregated with RM20676, and linked to RM20678 by 0.076 cM on the other side, respectively.

To further finely map the *R* locus, 24 STS markers were developed based on the genome sequences of Nipponbare and CG14. Five STS markers polymorphic between IL106 and DJY1 were used to genotype the 7 and 13 recombinants at RM20674 and RM20678 loci, respectively. As a result, 4 recombinants were detected at STS69-15 locus on RM20674 side, and 4 recombinants were detected at STS69-7 locus on RM20678 side. No recombinants were identified at RM20676, STS69-21, and STS69-22 loci. These results indicated that the *R* gene locus was narrowed down to the region flanked by STS69-15 and STS69-7, and co-segregated with 3 molecular markers RM20676, STS69-21, and STS69-22 (**Figure 1B**). The genotypes and phenotypes of 8 recombinants between STS69-15 and STS69-7 are shown in **Figure 1C**.

Differentiation Between *R* Gene Carrying in IL106 and *Pi-tq1* in Teqing

The blast resistant gene *Pi-tq1* from *indica* cultivar Teqing was previously mapped to a 4.24 Mb physical interval flanked by two RFLP markers, RZ682 and RZ508 (Tabien et al., 2000) that spanned over the *R* locus described here on the long arm of chromosome 6. To distinguish these two genes, IL106 and Teqing were inoculated with 3 *M. oryzae* strains (09BSH-10-5A, BS139, and HN09-1C-7) that are avirulent on IL106, but virulent on DJY1. The result showed that Teqing was resistant to both 09BSH-10-5A and BS139, but susceptible to HN09-1C-7 (**Supplementary Figure S1C**), suggesting that the *R* gene in IL106 could be different from *Pi-tq1*, because of their distinct reactions to HN09-1C-7. To demonstrate that the resistance of HN09-1C-7 is controlled by the same *R* gene in IL106, this strain was inoculated to 191 F_2 progenies from the cross between IL106 and DJY1. The numbers of resistant and susceptible individuals were 149 and 42, respectively and fitted to a 3:1 ratio (P=0.337), confirming that IL106 possesses a single resistance gene to the strain HN09-1C-7. Genotyping of 42 susceptible and 4 resistant individuals with 4 molecular markers (RM30, RM345, STS69-7, and STS69-15) linked with *Pi69*(t), identified 4 and 3 recombinants at RM30 and RM345 loci, respectively, and no recombinants at both STS69-7 and STS69-15 loci (**Supplementary Figure S2**), indicating that the gene conferring resistance to HN09-1C-7 in IL106 was mapped to the same chromosomal region as that *R* gene conferring

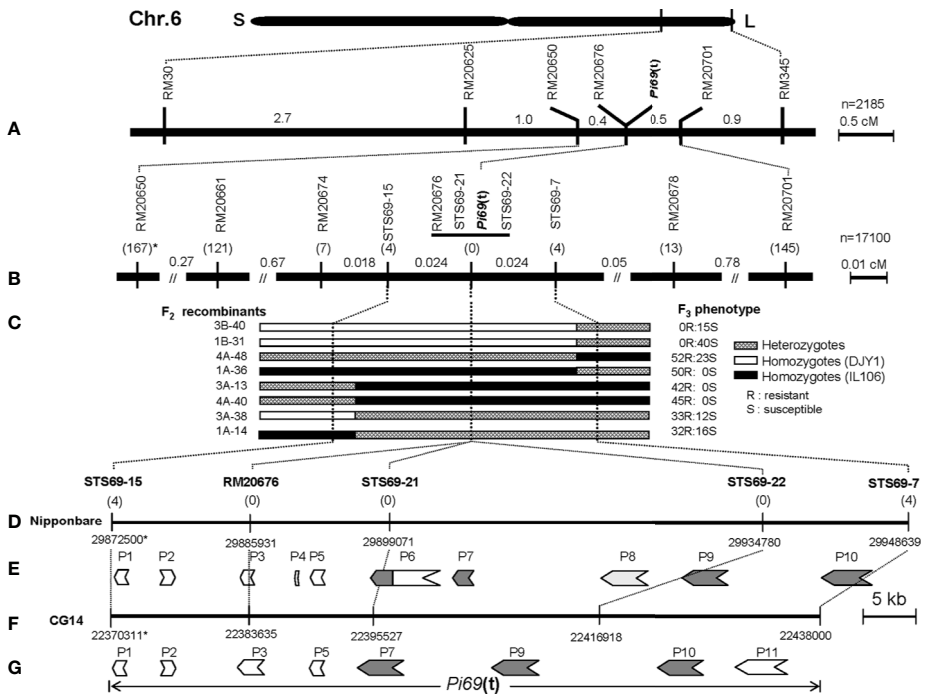

FIGURE 1 | Genetic and physical maps of *Pi69*(t) locus on rice chromosome 6. **(A)** A genetic map of *Pi69*(t) locus. Map distances are in cM. **(B)** An integrated fine genetic map of *Pi69*(t) locus on chromosome 6, *: the numbers in parentheses under the markers present the number of recombinants between marker loci and *Pi69*(t); **(C)** The genotyping and phenotyping of key recombinants located between two markers STS69-15 and STS69-7. **(D)** Physical map of the *Pi69*(t) locus based on the reference genome sequence of *O. savita* cultivar Nipponbare. *: chromosomal position of markers on genomic sequence of chromosome 6 of Nipponbare; **(E)** Predicted candidate *R* genes for *Pi69*(t) in Nipponbare (*O. sativa*). **(F)** Physical map of the *Pi69*(t) locus based on the reference genome sequence of *O. glaberrima* accession CG14. *: chromosomal position of markers on genomic sequence of chromosome 6 of CG14; **(G)** Predicted candidate *R* genes for *Pi69*(t) in CG14 (*O. glaberrima*).

resistance to 09BSH-10-5A, and the resistance to both 09BSH-10-5A and HN09-1C-7 is controlled by the same *R* gene in IL106. Taken together, all these data confirmed that *R* locus in IL106 is different from *Pi-tq1*, due to their distinct reactions to *M. oryzae* strain HN09-1C-7. Because no blast *R* gene was finely mapped in chromosomal region flanked by STS69-15 and STS69-7 on chromosome 6 of rice to date, this major *R* gene carrying in IL106 from *O. glaberrima* was considered as a new gene and was tentatively designated as *Pi69*(t).

Resistance Spectrum of *Pi69*(t)

To determine the resistance spectrum of *Pi69*(t), IL106, and other 10 monogenic lines carrying broad-spectrum *R* genes were inoculated and assessed with 151 *M. oryzae* strains from Cambodia (16 strains), Laos (20 strains), Myanmar (4 strains), Thailand (20 strains), Vietnam (18 strains), and China (77 strains). IL106 was resistant to all strains from Cambodia and Laos, and susceptible to only 3 strains from China, Thailand and Vietnam (YX162, TH451, and VN4118; **Supplementary Table S1**). IL106 also showed broader resistant spectrum compared with nine monogenic lines carrying different known *R* genes, except for the line IRBL9-W carrying *Pi9* that was resistant to all tested strains. These results suggest that *Pi69*(t) gene could confer broad-spectrum resistance against *M. oryzae* in IL106.

In Silico Physical Map Construction of *Pi69*(t) Gene Locus

To construct the physical map of *Pi69*(t) locus *in silico*, all the molecular markers closely linked to *Pi69*(t) were anchored to the genome sequences of both *O. sativa* subsp. *japonica* cultivar Nipponbare (IRGSP1.0) and *O. glaberrima* cultivar CG14 (AGI1.1) through BLAST analysis (http://plants.ensembl.org/). The two flanking markers and 3 co-segregating markers were anchored to the target region (**Figures 1D, F**). The physical distance between two closest flanking markers STS69-15 and STS69-24 was about 76.1 Kb (genomic position: 29872500-29948639) in Nipponbare, and 67.7 Kb (genomic position: 22370311- 22438000) in CG14.

Both the target genome sequences from Nipponbare and CG14 were annotated through the bioinformatics platform FGENSH (http://www.softberry.com). The annotation showed that 8 and 10 genes (named tentatively from *P1* to *P11*) were predicted in Nipponbare and CG14, respectively (**Figures 1E, G**). Among all these annotated genes, *P4* and *P8* genes were absent in CG14, while *P11* gene was absent in Nipponbare. Among these predicted genes, both *P1*(*LOC_Os6g49300*) and *P3*(*LOC_Os6g49320*) encode

the putative genes homologous with glycosyltransferase; *P2* (*LOC_Os6g49310*) encodes a gene homologous to MATE efflux family protein; *P4* (*LOC_Os6g49330*) annotated in Nipponbare only is an uncharacterized protein; *P5* (*LOC_Os6g49340*) encodes a F-box and DUF domain containing protein; P6 (*LOC_Os6g49350*) and *P11* encode a retrotransposon in Nipponabre and CG14 respectively; the remaining four genes (*P7* (*LOC_Os6g49360*), *P8* (*LOC_Os6g49380*), *P9* (*LOC_Os6g49390*), and *P10* (*LOC_Os6g49420*)) were predicted to be typical *R* genes encoding protein with the conserved structure of nucleotide-binding site and leucine-rich repeat (NBS-LRR; **Figures 1E, G**). In comparison with *P7* in CG14, there are two genes (*P6* and *P7*) in Nipponbare caused by an insertion of retrotransposon. Amino acids analysis among P7 in CG14, P6, and P7 in Nipponbare showed that the amino acid sequence on the 3' side of P6 in Nipponbare has high similarity with those in P7 of CG14, and *P7* in Nipponbare encoded only a truncated NBS-LRR protein compared with its P7 allele in CG14.

Evidence of *O. glaberrima* Genome Fragment Integration

To validate whether the fragment carrying *Pi69*(t) in IL106 was integrated from *O. glaberrima*, the introgression line IL106, the original *O. glaberrima* accession IRGC100137, the recurrent parent *japonica* cultivar DJY1, as well as two *indica* cultivars R498 and Teqing, were genotyped with 13 molecular markers used for mapping *Pi69*(t). The results showed that the size of all the DNA fragments amplified from IL106 were the same as those from IRGC100137 (**Figure 2**). Meanwhile, these molecular markers were polymorphic among line IL106, and all rice cultivars (DJY1, R498 and Teqing).

DISCUSSION

The discovery and deployment of broad-spectrum *R* genes from a large number of cultivated rice varieties and its wild relatives is an effective strategy to broaden the genetic basis of resistance of modern rice cultivars, to cope with the diversity and variability over time of pathogen population in rice production (Jeung et al., 2007; Su et al., 2015; Deng et al., 2017). Several incompatibility barriers such as pre- and post-fertilization barriers, hybrid sterility between Asian cultivated rice and its wild and cultivated relatives, however, have been hindering the utilization of favorable genes controlling important agronomic traits (Brar and Khush, 1997; Xu et al., 2014; Brar and Khush, 2018). Construction of introgression lines of these relatives with Asian cultivated rice cultivars has been proved to be one of the effective measures for further discovery and use of favorable genes from the wild species for rice breeding, and several genes conferring resistance to biotic stresses from wild species were identified in introgression lines (Brar and Khush, 1997; Gutierrez et al., 2010; Rama Devi et al., 2015; Brar and Khush, 2018). Extensive studies on exploiting and identification of blast resistance genes have been conducted, mainly focusing on the *O. sativa* and wild relatives. Relatively few investigations have been performed to identify new blast *R* genes in *O. glaberrima* (Silué and Notteghem, 1991; Rama Devi et al., 2015). In this study, we have successfully identified and finely mapped *Pi69*(t), the first blast *R* gene from *O. glaberrima* by using an introgression line IL106 derived from *O. glaberrima*. *Pi69*(t) confers a broad-spectrum resistance to *M. oryzae* diverse strains from 6 Asian countries, indicating that *Pi69*(t) is a promising resistance resource for improvement of Asian cultivated rice for resistance to rice blast.

Over 20 blast major *R* genes have been identified and mapped on rice chromosome 6, and the majority of them were mapped proximal to the centromere. The cloned *R* genes *Piz-t*, *Pi2*, *Pi9*, *Pi50*, and *Pigm* are members of the multigene family *Pi2*/*Pi9* locus located on the short arm, while the *Pid-2*, *Pid3*/*Pi25*(t), and *Pid3-A4* are located on the long arm (Chen et al., 2006; Shang et al., 2009; Chen et al., 2011; Lv et al., 2013). Using recombination inbred lines derived from a cross between Lemont and Teqing, Tabien et al. (2000) identified and mapped 3 blast resistance genes from Teqing (*Pi-tq1*, *Pi-tq2*, and *Pi-tq3*). Among them, *Pi-tq1* was also mapped to chromosome 6 of rice but located proximal to telomeric side. Two flanking markers RZ682 and RZ508 defined a larger physical region of *Pi-tq1* locus of around 4.24 Mb covering *Pi69*(t) locus. Although *Pi69*(t) could be differentiated from *Pi-tq1* by using *M. oryzae* strain HN09-1C-7, due to their distinct reactions to this strain, whether *Pi69*(t) is allelic, or closely linked to *Pi-tq1* remains to be determined through allelism test or fine mapping of *Pi-tq1*.

Pyramiding of *R* genes with different resistance specificity in the same cultivar is one effective measure to broaden the resistance spectrum against *M. oryzae*, and development of polymorphic molecular markers is the prerequisite to stack target genes into one cultivar with marker-assisted selection method (Hittalmani et al., 2000). The molecular markers developed in this study, tightly linked to *Pi69*(t), showed good polymorphisms among 5 tested rice lines/cultivars belonging to *indica* or *japonica* types. These markers are good candidates for pyramiding of *Pi69*(t) with

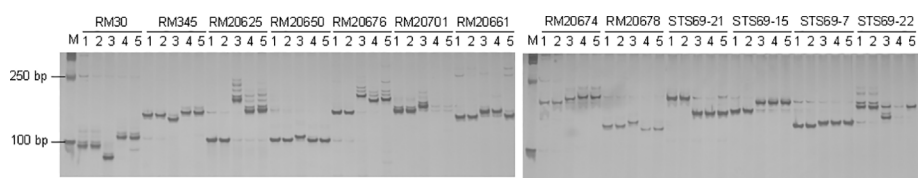

FIGURE 2 | Confirmation of the origin of the introgression of the *Pi69*(t). Thirteen molecular markers linked with *Pi69*(t) were used to amplify the DNA fragments of the *O. glaberrima* (IRGC100137) donor parent (1), the introgression line IL106 (2), the recurrent parent DJY1 (3), and two *indica* cultivars: R498 (4), and Teqing (5). M, molecular weight marker DL2000. The PCR products were separated by 8% polyacrylamide gel.

other *R* genes for improvement of Asian cultivated cultivars in disease-resistant rice breeding program.

Most of cloned resistance genes from plants encode NBS-LRR like proteins that directly or indirectly recognize the pathogen effectors to trigger host defense responses (Dangl and Jones, 2001). Almost all the cloned rice *R* genes to blast encode NBS-LRR like proteins, except for *Pid2*, *pi21*, and *Ptr* genes, which encode a B-lectin receptor kinase, a proline-rich protein, and an atypical protein with amardillo repeat domain, respectively (Lv et al., 2013; Su et al., 2015; Ashkani et al., 2016; Deng et al., 2017; Zhao et al., 2018). *O. glaberrima*-derived *Pi69*(t) gene was located in a region containing a cluster of NBS-LRR like genes. These genes are potential or promising candidates for *Pi69*(t).

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**

AUTHOR CONTRIBUTIONS

QY, DaT, LD, SL, PX, and DiT: Conceived idea and designed research. QY, DaT, and DiT wrote the manuscript. LD, SL, MK, QY, WD, XL, YB, LZ, JL, and JZ: Performed experiments and analyzed data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01190/full#supplementary-material

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Fertile Tetraploids: New Resources for Future Rice Breeding?

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Ploidy manipulation is an efficient technique for the development of novel phenotypes in plant breeding. However, in rice (*Oryza sativa* L.), severe seed sterility has been considered a barrier preventing cultivation of autotetraploids since the 1930s. Recently, a series of studies identified two fertile autotetraploids, identified herein as the PMeS (Polyploid Meiosis Stability) and Neo-Tetraploid lines. Here, we summarize their characteristics, focusing on the recovery of seed fertility, and discuss potential future directions of study in this area, providing a comprehensive understanding of current progress in the study of fertile tetraploid rice, a classical, but promising, concept for rice breeding.

Keywords: rice, tetraploid, fertility, pollen, seed

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INTRODUCTION

The phenomenon of polyploidy, which refers to the multiplication of chromosome sets within cells, often doubling a normal (diploid) set into a quadruple (tetraploid) set, is a widespread and distinctive feature of the higher plants (Stebbins, 1950). Polyploids are traditionally classified into autopolyploids, which originate from a single parent species (xx to xxxx), and allopolyploids, which originate from two hybridizing species (xx + yy to xxyy) (Clark and Donghe, 2018). In plants, polyploidization occurs frequently, and 25%–30% of flowering plants are suggested to be current polyploids, which have not yet diploidized (Van de Peer et al., 2017). The adaptive potential of polyploidy has long been discussed. Van de Peer et al. (2017) summarized the adaptive potential of polyploids with respect to the effects of interaction between species (i.e., changes in modes of reproduction and changes in interactions with pollinators and herbivores), environmental robustness (i.e., increasing tolerance to abiotic stresses), and species diversification. These changes in adaptive potential are reported to be caused by changes in the genomes/transcriptomes of the polyploid, including structural changes in genomes/genes, and transcriptional and functional alterations in duplicated genes (for a detailed review of these changes, see Renny-Byfield and Wendel (2014)]. Because the function of a duplicated gene should be redundant at the time of polyploidization, it can accumulate mutations, which may affect its function without reducing the fitness of the individual (Blanc and Wolfe, 2004). It is therefore widely accepted that polyploids have greater mutational robustness than diploids (Van de Peer et al., 2009). Fawcett et al. (2009) showed that the majority of the most recent duplication events in flowering plants are clustered in time and seem to coincide with the period of the most recent mass extinction. This suggests that polyploid plants coped better than diploid plants with the markedly changed environments of that period (Van de Peer et al., 2009).

In crop species, it has been frequently observed that polyploidization causes plants to grow larger, more quickly, and with higher yields, compared to their diploid relatives (Renny-Bayfield

and Wendel, 2014), although some studies suggest that polyploidization did not have a selective advantage over diploid genomes during the domestication process (Hilu, 1993). In addition to improved crop yield, recent studies have shown that polyploidy generates novel or diversified characteristics such as changes to grain threshing properties (Zhang et al., 2011) and grain hardness (Chantret et al., 2004) in wheat, flowering time in sunflower (Blackman et al., 2010), growing condition in coffee (Combes et al., 2012), and quality of fiber in cotton (Jiang et al., 1998). These studies prompted us to survey the potential capacity for polyploidization in rice, a staple food for four billion people globally (CGIAR, http://ricecrp.org/), as a means of providing new genetic materials for future crop improvement. In this review, we focus on autotetraploid rice, especially its fertility, because low seed fertility has been regarded as a barrier obstructing its use. In the course of summarizing two pioneering studies on autotetraploid rice with high seed fertility, we will discuss potential future directions for ploidy manipulation in rice, a classical but promising concept.

HISTORY OF THE STUDY OF TETRAPLOID RICE

Asian cultivated rice (*Oryza sativa* L.) is a diploid species with two sets of 12 chromosomes (2n = 24). [Although Asian cultivated rice may have experienced whole genome duplication more than 96 million years ago (Guo et al., 2019), this species is regarded as diploid, as it did not experience a "recent" polyploidization. In this review, we therefore regard Asian cultivated rice as a diploid species and tetraploid rice as containing two sets of 24 chromosomes]. As far as we know, no autotetraploid or allotetraploid rice varieties are used in agriculture. The first report of spontaneously autotetraploid rice was by Nakamori (1933), 2 decades after the determination of its chromosome number (Kuwada, 1910). Soon after the 1933 report, Ichijima (1934) reported artificially induced tetraploids. Some subsequent studies concerning the artificial induction of tetraploidy in rice have been reported (Cua, 1950; Oka, 1953). According to He et al. (2010), research on polyploid rice breeding was initiated in 1953 in China.

LOW SEED FERTILITY: A BARRIER PREVENTING TETRAPLOID RICE USE

In general, polyploidization confers greater stress tolerance by fostering slower development, delayed reproduction, longer life span, improved defense against pathogens and herbivores, larger seeds, and lower reproductive effort with greater emphasis on vegetative reproduction [Hilu, 1993, see also review by Levin (1983)]. In rice, large grain and leaf size are often observed in autotetraploid plants. In addition, Tu et al. (2014) reported higher salt tolerance in autotetraploid rice than in diploid rice. These studies suggest the potential usefulness of autotetraploid rice for breeding. However, low numbers of spikelets per panicle and low fertility have frequently observed in tetraploid rice throughout its known existence. Oka (1954) surveyed the phenotypes of a diverse set of autotetraploid rice varieties. Although greater vegetative than reproductive growth was not specifically indicated (**Table 1**), he showed that seed fertility in tetraploid varieties ranged from 0% to 55% (Oka, 1954; Oka, 1955). This low seed set rate has been the main barrier preventing use of autotetraploid for rice breeding (Cai et al., 2007).

Lower polyploid fertilities have been considered to be due to abnormal behavior of the chromosomes during meiosis (Bomblies et al., 2016). In the first division of meiosis, a pair of homologous chromosomes forms a bivalent, which later segregates. However, in polyploids, especially autopolyploids, the proper formation of bivalents is often inhibited, because homology among three or more chromosomes means that they fail to provide the special intrinsic cues necessary for normal, diploid-like segregation (Bomblies et al., 2016). In autotetraploid rice, chromosomal behavior at meiosis has been surveyed by Cai et al. (2007) and Wu et al. (2014). Cai et al. (2007) found that a tetraploid line, Dure-4X, displays abnormal meiotic behaviors including a higher rate of multivalents, univalents, and trivalents during prophase, lagging chromosomes during metaphase, and micronuclei during anaphase and telophase. In addition to such abnormalities, asynchrony of chromosomes and abnormal cell shape are also observed in meiosis in pollen mother cells in tetraploids with low fertility (Wu et al., 2014). These studies suggested that abnormal chromosomal behavior is one of the reasons for low seed fertility in autotetraploid rice. Recent studies have revealed the differences of epigenetic state and transcriptomes between the diploid and tetraploid rice (Xu et al., 2014; Zhang et al., 2015; Li et al., 2018). Zhang et al. (2015) suggested that the increased methylation level of transposable elements (TE) alters the expression of genes near by the TE in autotetraploid rice. Transcriptomic differences, including the expression of small RNAs, long non-coding RNAs, meiosis-related genes, and carbohydrate metabolism-related genes have been suggested to be related to meiotic abnormalities and the subsequent reduction of pollen fertility in autotetraploid rice (Li et al., 2016; Li et al., 2017; Chen et al., 2018; Li et al., 2020).

TABLE 1 | General characteristics of tetraploid rice lines (summary of Oka, 1954).

Traits	Characteristics in tetraploids
Panicle length	Increased (0.95 to 1.30)
Grain length	Increased (1.15 to 1.30)
Awn	Developed
Plant height	Reduced (0.65 to 1.00)
Spikelet number per panicle	Reduced (0.50 to 0.90)
Panicle number per plant	Reduced (0.40 to 0.70)
Seed fertility	0% to 55%
Pollen fertility	55% to 95%

Numbers in parenthesis indicate ratio of phenotype value of tetraploids compared to diploids.

PMES (POLYPLOID MEIOSIS STABILITY) AND NEO-TETRAPLOIDY: EMERGING FERTILE AUTOTETRAPLOID RICE

Although its low seed fertility has long been a barrier preventing the use of autotetraploid rice in breeding, two new autotetraploid rice series with high seed and pollen fertilities, identified herein as the PMeS and Neo-Tetraploid lines, were developed recently (**Table 2**). The PMeS lines have been developed and analyzed by Cai et al. (2007) and subsequent studies (He et al., 2010; He Y. C. et al., 2011; Tu et al., 2014). They derive from the progenies of crosses between *indica* and *japonica* rice subspecies. Unlike other autotetraploid rice lines, PMeS lines do not show abnormal chromosomal behavior at meiosis. In addition, their pollen development pattern appears normal at all stages, lacking the many abnormalities seen in other autotetraploid rice lines (He et al., 2010). These results suggest that stable meiosis, timely tapetum degradation, and normal mitochondrial development were the critical factors ensuring the high pollen fertility of PMeS lines (He et al., 2010). Recently, Xiong et al. (2019) revealed that the rice homolog of the gene *MND1*, which plays a role in meiosis in yeast and *Arabidopsis*, also plays a crucial role in improving the seed set rate in PMeS lines. They showed that the *OsMND1* expression level in panicles of a PMeS line was higher than in the diploid lines or in the other autotetraploid line with low seed set rate and *OsMND1* overexpression improved pollen fertility and seed set. The meiotic behavioral observations suggested that the effects of *OsMND1* might be maintaining the balance of synapsis and recombination (Xiong et al., 2019). However, it is still unknown why the expression level of *OsMND1* was lower in autotetraploid line with low seed set rate than the PMeS line. More research is necessary for a full understanding of the molecular mechanism of normal meiosis in PMeS lines (Xiong et al., 2019).

The other autotetraploid rice varieties with high seed and pollen fertilities were named the Neo-Tetraploid lines by Guo et al. (2017). These lines derived from the progenies of crosses between T44 (96025) and T45 (Jackson-4X) and showed a seed set rate of more than 80%, while their parents (T44 and T45) showed less than 32% (Guo et al., 2017; Bei et al., 2019). Cytological observation of the meiotic stages in Neo-Tetraploid rice lines showed that fewer abnormalities in these lines than their autotetraploid parents, which displayed different chromosomal configurations at diakinesis (Bei et al., 2019). Surprisingly, a total of 324 genes in the Neo-Tetraploid rice genome showed new mutations, which do not exist in its parents' genomes (Bei et al., 2019). By means of a transcriptome analysis, Bei et al. (2019) suggested that genomic structural reprogramming, DNA variations, and differential expression of some important meiosis- and epigenetics-related genes might be associated with the high fertility of Neo-Tetraploid lines.

AUTOTETRAPLOID RICE AND HYBRID STERILITY

Autotetraploid rice varieties with high seed fertilities are also considered to be important resources for hybrid rice breeding (Guo et al., 2017). Interestingly, both PMeS and Neo-Tetraploid lines produce F1 progenies with high seed setting rates when crossed with other autotetraploid rice lines (**Table 2**, He Y. C. et al., 2011; Guo et al., 2017). In PMeS lines, He Y. C. et al. (2011) showed that the seed set rate of an F1 hybrid of Balilla-4X × HN2026-4X (a PMeS line) was 67.18%, while it was 37.26% in the F1 of Balilla-4X × NJ11-4X (a non-PMeS line). They suggested that the normal meiotic behavior in lines with a PMeS background led to the high frequency of successful fertilization, normal embryo development, and high seed set rate observed in its F1 progeny.

In Neo-Tetraploid lines, the agronomic traits of various autotetraploid hybrids were examined by Guo et al. (2017). They found that the seed setting rate in the hybrids of Neo-Tetraploid rice was significantly higher than in the hybrids generated from other autotetraploid lines. Their transcriptome analysis revealed that genes expressed differently by the hybrid and its parents included meiosis stage-specific- and meiosis-related genes, such as *RAD51* and *SMC2*. Because *RAD51* and *SMC2* are involved in recombination between homologous chromosome and control of chromosomal structure, respectively, during meiosis (Aya et al., 2011), their transcriptional changes should affect to the fertility observed in autotetraploid hybrids. However, the understanding of the cause of transcriptional changes in hybrids still remains a challenging.

The seed fertility of autotetraploid hybrids is also controlled by several hybrid sterility loci. At such loci in rice, several alleles exist; some of them interact in the heterozygous state to cause abortion of gametes (see the review of Koide et al., 2008b). At this locus, the allele that does not cause abortion of gametes is called the neutral allele (Ikehashi and Araki, 1986; Koide et al., 2008a). He et al. (2011b) and Wu et al. (2015) showed that interaction

TABLE 2 | Comparison of two fertile tetraploid rice series.

	Neo-Tetraploid lines	PMeS lines
Line name	Huaduo1, 2, 3, 4, 5, 8[a,b,c,d]	Sg99012, HN-2026-4X[e,f]
Cross combination	Inter-subspecific (*japonica* and *indica*)	Inter-subspecific (*japonica* and *indica*)[f]
Seed set	more than 68%[a,b]	Over 80%[e,f]
Pollen fertility	more than 92%[a]	84.15% (stained), 78.52% (germinated)[g]
Meiotic behavior in male gametes	Few abnormality were detected[a]	Normal[e]
Seed set of F1	83.07% (when crossed with *japonica* varieties)[a] 76.45% (when crossed with *indica* varieties)[a]	67.18% (when crossed with Ballila-4X)[e]

[a]*Bei et al., 2019*, [b]*Guo et al., 2017*, [c]*Chen et al., 2019*, [d]*Ghaleb et al., 2020*, [e]*He Y. C. et al., 2011*, [f]*Cai et al., 2007*, [g]*He et al., 2010*.

between three hybrid sterility loci (*Sa*, *Sb*, and *Sc*) has significant effects on the pollen fertility of autotetraploid hybrids, and that pollen fertility further decreased with increasing allelic interaction. They also found abnormal chromosomal behavior in the hybrids with low pollen fertility and suggested that the gene interactions of hybrid sterility loci tend to increase the chromosomal abnormalities that cause the partial abortion of male gametes, leading to the decline in seed set of the autotetraploid rice hybrids (He et al., 2011b). Such abnormalities in chromosomal behavior were reduced in hybrids with neutral alleles (Sa^n and Sb^n) at loci *Sa* and *Sb*, respectively (Wu et al., 2017). Chen et al. (2019) developed hybrids between the Neo-Tetraploid line and autotetraploid varieties with neutral alleles. They found an improvement of seed set rate and suggested that meiosis-related and meiosis-specific genes, and those related to saccharide metabolism and starch synthase, were involved in these heterozygote-specific phenotypes (Chen et al., 2019).

DISCUSSIONS AND CONCLUSIONS

These two series of pioneer studies on the PMeS and Neo-Tetraploid lines have opened the door to the use of autotetraploid varieties in rice breeding and cultivation. To facilitate the development of fertile autotetraploid lines, further research aimed at understanding the origin of the genes/quantitative trait loci (QTLs) responsible for high fertility in these tetraploid rice varieties will be necessary. Both the PMeS and Neo-Tetraploid rice lines have been developed from the progenies of crosses between the *indica* and *japonica* rice subspecies. Since the parental autotetraploid varieties show seed sterility, one possibility for the origin of high-fertility genes/QTLs in the new varieties' progenies is the nature of interactions between genes (or genomic regions) derived from different autotetraploid parents. If so, a QTL analysis of high seed fertility could be performed by developing recombinant inbred lines derived from crosses between autotetraploids. The Neo-Tetraploid lines show different expression of some important meiosis stage specific- and meiosis-related genes (Guo et al., 2017; Bei et al., 2019). Genes/QTLs responsible for high seed fertility might control the expression of these. Interestingly, the PMeS and Neo-Tetraploid lines show high seed fertility not only when selfing, but also in hybrids with other autotetraploid rice strains (He Y. C. et al., 2011; Guo et al., 2017). These results suggest that the genes/QTLs responsible for high seed fertility in PMeS and Neo-Tetraploid lines act dominantly, suppressing unstable chromosomal behavior during meiosis in hybrids. If this is so, the question arises as to why the initial hybrids of *indica* and *japonica* used for developing the PMeS and Neo-Tetraploid lines did not show high seed fertility. Another possibility for the origin of genes/QTLs responsible for high seed fertility is the new appearance of these genes/QTLs during the line selection process. The finding of new mutations, absent from the parental genome, in a Neo-Tetraploid line (Bei et al., 2019) may support this scenario. The activation of TEs followed by alteration of the epigenetic state (Zhang et al., 2015; Guo et al., 2017; Bei et al., 2019) may cause new appearance of genes/QTLs in tetraploid rice. Further study is necessary to unclear why many such mutations would occur in the autotetraploid lines.

Although their origin is unclear, identifying the genes responsible for high seed fertility in the PMeS and Neo-Tetraploid lines will enable the transfer of this technique to other autotetraploid rice varieties through marker-assisted selection or genome editing. A diverse set of fertile autotetraploid rice varieties will greatly increase their potential usefulness in future agriculture. Ploidy manipulation, a classical concept, will revive in the field of rice breeding in the near future.

AUTHOR CONTRIBUTIONS

YKo, DK, and YKi contributed to conceptualization of this review. YKo wrote original draft. DK and YKi reviewed and edited the original manuscript.

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Potential of Platinum Standard Reference Genomes to Exploit Natural Variation in the Wild Relatives of Rice

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As the world's population expands from 7.6 billion to 10 billion over the next 30 years, scientists and farmers across the globe must explore every angle necessary to provide a safe, stable and sustainable food supply for generations to come. Rice, and its wild relatives in the genus *Oryza*, will play a significant role in helping to solve this 10 billion people question due to its place as a staple food for billions. The genus *Oryza* is composed of 27 species that span 15 million years of evolutionary diversification and have been shown to contain a plethora of untapped adaptive traits, e.g., biotic and abiotic resistances, which can be used to improve cultivated rice. Such traits can be introduced into cultivated rice, in some cases by conventional crossing, and others *via* genetic transformation and gene editing methods. In cases where traits are too complex to easily transfer to cultivated rice [e.g., quantitative trait loci (QTL)], an alternative strategy is to domesticate the wild relative that already contains the desired adaptive traits – i.e., "neodomestication". To utilize the *Oryza* genus for crop improvement and neodomestication, we first need a set of genomic resources that can be used to efficiently identify, capture, and guide molecular crop improvement. Here, we introduce the concept of platinum standard reference genome sequences (PSRefSeq) – a new standard by which contiguous near-gap free reference genomes can now be produced. By having a set of PSRefSeqs for every *Oryza* species we set a new bar for how crop wild relatives can be integrated into crop improvement programs.

Keywords: neodomestication, rice wild relatives, platinum reference genomes, *Oryza coarctata*, *Oryza*

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INTRODUCTION

As the world population grows and climate change dictates new conditions for agriculture worldwide, finding new solutions to ensure a sustainable food supply is crucial. Rice is the staple food in the regions of the world with the largest projected population growth. Moreover, these regions and their available arable land are expected to be most negatively affected by climate change in the future (Milovanovic and Smutka, 2017). Cultivated rice, as for most modern crops, has undergone domestication and genetic bottlenecks over millennia, giving this crop limited genetic potential to adapt to rapid environmental changes. In

contrast, the wild relatives (WRs) of rice have been shown to possess the genetic potential to enhance yield, nutrient content, and resistance to several environmental stressors (Brar and Khush, 2018).

For decades, rice breeders have utilized genetic traits from the WRs of the genus *Oryza*, first *via* conventional crossing and now more recently using plant transformation and gene editing tools, to produce new, more resilient varieties of cultivated rice (Brar and Khush, 2018). Although these WRs contain novel alleles and variations with the potential to improve cultivated rice, issues such as hybrid-viability, -sterility, and -weakness hinder their introgression into cultivated rice, especially from the more distant relatives (Nadir et al., 2018). However, a toolbox of high-quality WR genome assemblies and gene-editing tools could help address these issues by providing technology for the precise transfer of newly discovered and advantageous wild alleles and genes into cultivated backgrounds.

Gene editing would also enable researchers to leverage these WRs with adaptive traits through neodomestication, i.e., the domestication of plants that have not previously been used for agriculture (Zsögön et al., 2018). As most traits in domesticated ideotypes arose from loss-of-function mutations, inducing such mutations in the orthologs of domestication-related genes in the WRs could result in a neodomesticated plant that maintains its desired adaptive trait(s) (e.g., abiotic and biotic resistance) (Doebley et al., 2006; Fernie and Yan, 2019). Though very far from a trivial task, neodomestication of the WRs of rice could potentially solve the challenge of feeding the planet's rapidly growing population in the face of climate change. Neodomestication untangles the relationships between plant architecture, physiological mechanisms, and cellular processes that produce the complex and polygenic traits necessary to provide resistance or tolerance to environmental stressors.

Thus, in this review, we will discuss the following topics:

1. The untapped pool of genetic diversity buried within the genomes of the wild relatives of rice
2. The use of high-quality near gap-free reference genome sequences to aid in crop improvement
3. Barriers to genetic introgression and alternative strategies to introgression *via* neodomestication

GENETIC DIVERSITY OF THE WILD RELATIVES OF RICE

The *Oryza* genus contains 11 different genome types across 27 species, both diploid and polyploid, with a 3.6-fold difference in genome sizes (from around 300 Mb in the diploid FF genome of *O. brachyantha* to 1,283 Mb in the HHJJ polyploid genome of *O. ridleyi*, with the AA genome of cultivated rice falling in the 400Mb range). There are four species complexes within the *Oryza* genus: (1) *O. sativa*, (2) *O. officinalis*, (3) *O. ridleyi*, and (4) *O. meyeriana* (Vaughan, 1989). The WRs *O. rufipogon* and *O. barthii* have been identified as the progenitors of the domesticated rice species, *O. sativa* and *O. glaberrima*, respectively (Fuller et al., 2010; Wang et al., 2014; Chen et al., 2019). According to the classifications of gene pools initially defined by Harlan and de Wet, 1971, species in the *O. sativa* complex fall within the primary gene pool (Nadir et al., 2018). Despite reproductive barriers such as low cross-fertility, low F1 seed germinability and F2 hybrid weakness, common introgression within the *O.sativa* complex was observed and gene transfer requires traditional breeding methods (Jones et al., 1997; Jena, 2010; Zheng and Ge, 2010; Pusadee et al., 2016; Wang et al., 2017; Nadir et al., 2018). The secondary gene pool is represented by the *officinalis* complex and *O. brachyantha*, which is defined with regards to the primary gene pool as cross-incompatible with non-homologous chromosome pairing and require special techniques such as embryo rescue to achieve gene transfer (Jena, 2010). The tertiary gene pool complexes include the *O. meyeriana*, *O. ridleyi*, and unclassified *O. schlecteri* and *O. coarctata* species, which are highly cross-incompatible with *O.sativa*, producing anomalous, sterile or lethal hybrids (Jena, 2010; Brar and Khush, 2018). Taking into account their evolutionary history and known polyploidization events (both ancient and recent) (Ge et al., 1999; Lu et al., 2009), the WRs across the genus have successfully adapted to a wide range of habitats, which is reflected in their genomic content and associated phenotypes. A subset of these adaptive traits have been utilized for crop improvement, particularly the genes that control major biotic and abiotic stress resistances as well as yield-enhancement (Zhang K. et al., 2019).

The WRs of rice show a remarkable adaptive plasticity to a diverse set of habitats, often with extreme or suboptimal conditions (such as lack of freshwater, high temperatures, and flooding). This adaptivity has resulted in a range of beneficial traits that have been selected to "fine-tune" existing accessions of cultivated rice, which are crucial for adapting to changes in environmental conditions (**Table 1A**). For example, the first disease resistance gene cloned in rice was *Xa21* from the AA genome WR - *O. longistaminata* (Song et al., 1995). This gene confers resistance to rice blast, a devastating disease of rice. Another gene, *Bph18*, which provides resistance to the brown planthopper, was cloned from a more distant EE genome WR – *O. australiensis* (Ji et al., 2016). Both of these resistance genes have been introduced into *O. sativa* through marker-assisted selection to produce at least 13 resistant varieties grown in India, Philippines, China, and Korea (Sanchez et al., 2013). Abiotic traits such as salt stress tolerance in *O. coarctata*, heat tolerance *via* evening flowering in *O. australiensis*, early morning flowering (EMF) during the cooler morning hours in *O. officinalis*, as well as genes involved in low temperature adaptation have been identified in *O. rufipogon* are also strong candidates for introgression into cultivated rice (Zeigler et al, 2014; Bheemanahalli et al., 2017; Biswal et al., 2019).

The WRs of rice have also been used as a source of other agronomic traits, such as yield. For example, *O. rufipogon*, the closest WR of Asian cultivated rice, was found to contain yield-enhancing QTL (*yld1* and *yld2*). These QTL were found to be associated with increased yield (18% and 17%) relative to the high-yielding Chinese hybrid rice V64 (Xiao et al., 1996). Subsequent studies have identified other QTL in *O. rufipogon* linked to yield component traits, including increased grain per panicle (e.g., *gpp3.1* identified in crosses with *O. rufipogon* and Jefferson which is a US japonica cultivar), and increased grain weight (e.g., *gw1.1* and *gw1.2* in crosses with Brazil Caiapo japonica cultivar) (Moncada et al., 2001; Thomson et al., 2003). Besides yield-component traits, a

TABLE 1A | Wild Species in the genus *Oryza* and available resources.

Species	2n	Genome Type	Distribution	Abiotic Traits of Interest	Ref	Biotic Traits of Interest	Ref
***Oryza sativa* complex**							
O. rufipogon sensu lato	24	AA	Asia, Oceania	Iron, acid, drought, submergence and aluminum tolerance	2,4	Resistance to BB, SB, tungro, and YSB	2, 3,5
O. nivara	24	AA		Drought Tolerance	4	Resistance to GSV and BB	2,3,5
O. glaberrima Steud.	24	AA	(cultivated)	Transpiration efficiency, heat, iron, acid, aluminum, salt, and drought tolerance	1,4	Resistance to BB and nematodes	5
O. barthii A. Chev.	24	AA	Africa	Heat and drought tolerance	1	Resistance to BB	5
O. glumaepatula Steud.	24	AA	Central/South America	Submergence Tolerance	4		
O. meridionalis Ng	24	AA	Oceania	Transpiration efficiency, heat tolerance	1,4		
O. longistaminata Chev. et Roehr	24	AA	Africa	Transpiration efficiency, heat and drought tolerance, and temperature plasticity	1	Resistance to GSV, BB, and YSB	2,3,5
***O. officinalis* complex**		multiple					
O. punctata Wall ex Watt, diplo.	24	BB	Africa	Drought tolerance and temperature plasticity	1	Resistance to BPH and ZLH	3
O. schweinfurthiana Wall ex Watt, tetra.	48	BBCC	Africa	Drought tolerance and temperature plasticity	1	Resistance to BPH and ZLH	3
O. minuta J.S. Presl ex C.B. Presl.	48	BBCC	Asia, Oceania			Resistance to BPH, GSV, BB, SB, and blast	2,3,5
O. officinalis Wall ex Watt	24	CC	Asia, Oceania	Drought and heat tolerance, and moisture plasticity	1,4	Resistance to BPH, GSV, BB, GLH, WPH, and blast	2,3,5
O. rhizomatis D.A. Vaughan	24	CC	Sri Lanka	Drought and submergence tolerance	4	Resistance to BB	5
O. eichingeri Peter	24	CC	Africa, Sri Lanka	Submergence tolerance	4	Resistance to BPH, WBPH, and GLH	3
O. latifolia Desv.	48	CCDD	Central/South America	Flooding tolerance, moisture plasticity	1	Resistance to GSV, BB, and BPH	2,3
O. alta Swallen	48	CCDD	Central/South America			Resistance to stem borer	3
O. grandiglumis (Doell.) Prod	48	CCDD	South America	Flooding and submergence tolerance	1,4		
O. australiensis Domin	24	EE	Australia	Transpiration efficiency, temperature and drought tolerance	1	Resistance to BPH, GSV, BB, and blast	2,3
***O. ridleyi* complex**		HHJJ					
O. ridleyi Hook	48	HHJJ	Asia, Oceania	Low radiation and moisture plasticity, and flooding tolerance	1	Resistance to blast, BB and stem borer	2,3
O. longiglumis Jansen	48	HHJJ	New Guinea			Resistance to BPH, BB and blast	2,3
***O. granulata* complex**		GG					
O. granulata Nees et Arn ex Watt	24	GG	Asia, Oceania	Cold tolerance, low radiation, temperature, moisture plasticity, and aerobic soil adaptation	1,3		
O. meyeriana (Zoll. et Mor. ex Steud.) Baill	24	GG	Asia, Oceania		1		
Others		multiple					
O. brachyantha Chev. et Roehr.	24	FF	Africa			Resistance to GSV, BB and yellow stem borer	2,3
O. coarctata	48	KKLL	Asia	Salt Tolerance	3,4		
O. *schlechteri* Pilger	48	HHKK	New Guinea	Flooding Tolerance	1		

BB, Bacterial blight; RYMV, ice yellow mottle virus; BPH, brown planthopper; WPH, whitebacked planthopper; GSV, grassy stunt virus; YSB, yellow stem borer; ZLH, zigzag leafhopper; GLH, Green leafhopper; 1 – Atwell et al., 2014; 2 – Toriyama, 2005; 3 – Jena, 2010; 4 – Menguer et al., 2017; 5-Vikal et al., 2007.

knowledge gap exists regarding WRs that may have superior nutritional and/or taste qualities. For example, seed from the KKLL genome WR *O. coarctata* is known for its sweet palatable taste and is consumed as a local delicacy in Bangladesh (Kabir and Humayun, 2012). Overall, such traits could potentially be utilized in cultivated rice to address the nutritional needs and flavor preferences of a growing population.

Beyond the association of traits to genes-of-interest, polyploidy in crops often confers greater genome plasticity and adaptation. The polyploid WRs of rice are likely to be more adaptive than their extinct diploid progenitors and may exhibit higher adaptability to extreme habitats, different climates, soil types, drought/submergence conditions, and biotic stresses (Van de Peer et al., 2017). In rice, hormone responses and differential expression of stress-response genes due to polyploidy confer autotetraploid rice with better drought-tolerance characteristics than diploid rice. Autotetraploid *O. sativa* (produced by colchicine-treatment to cause genome doubling) was less affected under severe drought stress, with less effect on net photosynthetic rates and peroxidation levels of its cell membranes demonstrated higher activity of the enzymes (superoxide dismutase, peroxidase, catalase) implicated in decreasing the amount of reactive oxygen species, which limited membrane lipid peroxidation (Yang et al., 2014). The authors hypothesized that the gene dosage effects due to polyploidy are the reason for drought tolerance in autotetraploid *O. sativa*. Similarly, polyploidy was found to confer rice growth and survival advantages under salt stress. When exposed to salt stress, four pairs of polyploid accessions, created from diploid progenitors, presented higher salt tolerance *via* dry weight, chlorophyll a/b content, and mortality rates of the tetraploids (Jiang et al., 2013). In addition, root ultrastructure imaging revealed decreased membrane damage and increased stability of nuclei and membrane organelles in the roots of tetraploid rice compared to diploid rice under salt stress (Tu et al., 2014). As the salt-induced production of reactive oxygen species affects membrane integrity *via* lipid/protein peroxidation, it is hypothesized that increased stability of membranes, nuclei and membrane organelles is an indication of normal metabolism under salt stress in roots of the tetraploid rice (Tu et al., 2014). Previously, tetraploid rootstocks in citrus have been demonstrated to be more tolerant to salt stress as compared to the corresponding diploid rootstocks (Saleh et al., 2008). These examples show that polyploidy can be advantageous due to potential dosage effects. However, a better understanding of genome content and structure is required, besides the trait-associated genes and QTL, to identify advantageous traits in the WRs for their introgression into cultivated backgrounds.

USE OF HIGH-QUALITY REFERENCE GENOMES TO AID IN CROP IMPROVEMENT

For crop improvement of the genus *Oryza*, breeders require access to a comprehensive set of genomic tools that can bridge the 27 species and 11 genome types that *Oryza* has accumulated over 15 million years of evolutionary history. The International *Oryza* Map Alignment Project (I-OMAP), established in 2003, set out to create a genus-level comparative genomics platform for the interrogation of the genus *Oryza* and provide information on rice-related genome evolution and organization, comparative genomics, physiology, biochemistry, and crop improvement. I-OMAP began with the generation of a set of publicly available deep-coverage BAC libraries and manually edited physical maps for cultivated rice and WRs used in active breeding programs (Jacquemin et al., 2013). These resources led to many early discoveries on the genome biology of *Oryza*, and grasses in general, and facilitated the generation of several draft genome assemblies, including that of African rice (Wang et al., 2014; Stein et al., 2018).

Recently, I-OMAP generated a set of 15 PSRefSeqs from one representative accession for each of the 15 sub-populations of *O. sativa*, based on the 3,000 rice genome (3K-RG) dataset, to be used as a reference guide to characterize all standing natural variation within Asian cultivated rice (Zhou et al., 2020). A PSRefSeq is defined as a high-quality near gap-free chromosome-level reference genome validated with optical maps. The *O. sativa* PSRefSeq dataset is composed of 12 newly sequenced genomes (Zhou et al., 2020), and 3 previously published genomes for Minghui 63 (MH63), Zhenshan 97 (ZS97) and N22 (Zhang et al., 2016a; Zhang et al., 2016b; Stein et al., 2018). The average number of contigs for these 15 assemblies is 113 contigs (i.e., 912, 237, and 181 for N22, MH63, and ZS97, respectively, and an average of 30 contigs for the remaining 12). The average number of gaps across the 15 assemblies is 46, with 8 out of 15 having less than 10 gaps (Zhou et al., 2020).

For ZS97 and MH63, genome completeness was estimated at ~92.7% (ZS97), ~94.8% (MH63) using the CEGMA pipeline with a core set of 248 eukaryotic genes (Parra et al., 2009). BUSCO evaluations for the remaining 13 genomes, which interrogate a much larger core gene set (N=956 for N22 and N=1,427 for the 12 newly sequenced assemblies), averaged 98.6%, and demonstrates the high contiguity and completeness of the majority of the *O. sativa* PSRefSeq dataset (Simão et al., 2015). When compared with previously released rice genomes, the *O. sativa* PSRefSeq data set, described here, provides more contiguous and accurate sequence data which, thereby, will improve further downstream analysis such as TE (transposable element), centromere/telomere and gene annotations.

The *O. sativa* 15 PSRefSeq dataset, combined with the original IRGSP RefSeq (International Rice Genome Sequencing Project, 2005; Kawahara et al., 2013), presents a multiple reference pan-genome template for the primary gene pool of Asian cultivated rice for mapping resequencing data that can accurately characterize genetic variation at the subpopulation level (Zhou et al., 2020). Given that this PSRefSeq dataset is now publicly available, the next logical step is to produce PSRefSeqs that represent the secondary and tertiary gene pools of the genus *Oryza*, and when combined, will create an unprecedented pan-genome for the entire genus – i.e., a "pan-genus-genome" or *Oryza*-PGG. Currently, I-OMAP is producing a set of PSRefSeqs from the representatives of all 25 WRs and *O. glaberrima*, using a combination of long-read sequencing technologies and optical maps (Udall and Dawe, 2018), with a target release date of December 2020.

The *Oryza* PGG will provide the full range of short, medium, and long-range structural variations that exist across the genus.

Moreover, the *Oryza* PGG will confirm previously discovered structural variants (SVs) and the presence/absence of variants (PAVs). Zhou et al. (2020) recently showed that the majority of PAVs in the subpopulations of *O. sativa* are comprised of transposable elements (TEs). The *Oryza* PGG will allow us to better annotate and understand TEs in the WRs of rice and shed light on the contribution of TEs to genome size variation and genome plasticity of adaptive traits. For instance, the TE content of *O. coarctata* was estimated to be the lowest among *Oryza* species, despite a predicted genome size of 665 Mb (Zuccolo et al., 2007; Mondal et al., 2018).

Analyses of the *Oryza* PGG will also reveal the vast majority of single gene and gene family content and evolution across the genus. Previously, Zhang L. et al. (2019) leveraged the genomic data available for 13 *Oryza* species (domesticated and wild) to study the formation of *de novo* protein-coding genes. They identified 175 candidate *de novo* genes and estimated the rate of *de novo* gene origination as 51.5 *de novo* genes per MYA. *De novo* originated genes can provide novel biological functions and have previously been shown to play a key role in pathogen resistance in plants (Chen et al., 2013). For instance, *OsDR10* is an *Oryza*-lineage specific *de novo* originated gene that confers broad-spectrum bacterial blight resistance when suppressed in rice (Xiao et al., 2009). Currently, *OsDR10* is characterized as an "orphan" gene and there is currently little information on its evolution in the *Oryza* lineage. Once the *Oryza* PGG becomes available, the evolutionary mechanisms contributing to the generation of *OsDR10* may be revealed and thus, provide insights into new gene evolution across the genus.

Finally, PSRefSeqs of each species will also aid conservation efforts by serving as "platinum standard" templates for characterizing the diversity of seed bank materials. This information will guide the management of genetic resources through *in-situ* conservation efforts to capture the genetic diversity in the wild or *ex-situ* collections of the newly identified wild populations (Brozynska et al., 2016). Some of the WRs in the genus are endangered or, as in the case of *O. neocaledonica* (Molla et al., 2018) and *O.schlecteri* absent from known genebank collections (Germplasm Resources Information Network, n.d.). From an evolutionary perspective, gained or lost SVs and copy number variations (CNVs) can be indicative of local adaptation of wild populations to unique habitats. The information gained from PSRefSeqs will help in the long-term maintenance of wild populations in their native habitats.

BARRIERS TO GENETIC INTROGRESSION AND ALTERNATIVE STRATEGIES TO INTROGRESSION *VIA* NEODOMESTICATION OF WR RICE SPECIES

For the successful introgression of desired traits from the WRs of rice, incompatibility barriers between the secondary, tertiary, and primary *Oryza* gene pools must be overcome (Harlan and de Wet, 1971; Vincent et al., 2013). The introgression of desirable traits within the AA genomes is relatively straightforward compared to the introgression of traits from more distant complexes. For instance, crossing WRs from the secondary/tertiary gene pools is more laborious and can require embryo rescue and chromosome doubling to produce interspecific hybrids and alien introgression lines (AILs). For the closely related *O. officinalis* complex species, monosomic alien addition lines (MAALs) and interspecific hybrids, with *O. sativa* as the recurrent parent, have been successfully produced to carry the following biotic stress-resistance traits: brown planthopper resistance, whitebacked planthopper, and bacterial blight resistances from *O. officinalis*, *O. minuta*, and *O. latifolia*, respectively (Brar and Khush, 2018).

Despite the successes achieved by introgression from the *O. officinalis* complex, crossing *O. sativa* with more distant WRs is difficult and time-consuming, and can result in progeny depression or complete progeny sterility. Moreover, linkage drag of loci, related to negative traits such as low yield, and shattering can also occur (Xiao et al., 1998). For the *meyeriana* complex, breeding efforts resulted in 40 derived lines of *O. sativa* x *O. granulata*, some of which were found to contain partial alien chromosome introgressions from two out of six *O. granulata* chromosomes analyzed. However, no favorable traits (such as cold and low radiation tolerances) were found to be introgressed from *O. granulata* (Brar et al., 1996). Similarly, attempts to produce hybrids from *O. sativa* x *O. ridleyi* and *O. sativa* x *O. coarctata* crosses failed due to necrosis in the progeny or progeny sterility, respectively, thereby demonstrating the difficulty of hybrid formation between distant WRs of rice with *O. sativa* (Brar and Khush, 2018).

"Neodomestication" represents an alternative strategy to conventional trait introgression from a WR into a domesticated species. This method preserves adaptations to biotic/abiotic stresses while exhibiting the traits of a domesticated crop (e.g., loss of shattering, erect growth, larger seeds). For example, homologues of genes involved in the domestication of rice in the WRs could be targeted for neodomestication to produce a domesticated ideotype in a WR. Shapter et al. (2013) presented an early example of an accelerated neodomestication process using random EMS mutagenesis to initiate the domestication of weeping rice grass (*Microlaena stipoides*), which resulted in the generation of two non-shattering mutant plants in the orthologs of *qSH1* and *sh4*. Now, with gene editing technology such as CRISPR-Cas9 (Bortesi and Fischer, 2015), it is possible to precisely edit homologues of well-studied domestication genes in crop WRs, such as those listed in **Table 1B**, to activate genes, modify alleles, introduce targeted base substitutions, delete large genomic segments, or introduce complete genes previously unavailable in a given species (Fernie and Yan, 2019). Besides targeting orthologues of domestication genes for neodomestication of WR *Oryza* candidates, CRISPR/Cas9 gene editing could be used to introduce WR candidate genes implicated in favourable traits such as abiotic stress tolerances into the cultivated rice background by overcoming the reproductive *Oryza* gene pool barriers (Harlan and de Wet, 1971).

As a proof-of-concept study for neodomestication using gene editing, Zsögön et al. (2018) targeted six key domestication traits for

TABLE 1B | Domestication genes in *Oryza sativa*.

Gene Name	RAP Gene ID	*Oryza* specie	Protein category	Domestication Trait	Ref
SH4	Os04g0670900	*O.sativa indica* x *O.nivara*	Myb3 transcription factor	Loss of seed shattering: the absence of abscission layer formation	Li et al., 2006
qSH1	Os01g0848400	*O.sativa* Nipponbare x *O.sativa* Kasalath	BEL1-type transcription factor	Loss of seed shattering: the absence of abscission layer formation	Konishi et al., 2006
PROG1	Os07g0153600	*O. rufipogon* x *O.sativa*	Cys(2)-His(2) zinc-finger protein	Erect growth and high yield	Tan et al., 2008; Wu et al., 2018
OsLG1	Os04g0656500	*O. rufipogon* x *O.sativa*	SBP-domain transcription factor	Panicle shape: closed panicle	Ishii et al., 2013
Rc	Os07g0211500	*O. rufipogon* x *O.sativa*	bHLH-protein	Seed pericarp color: white pericarp	Sweeney et al., 2006
Bh4	Os04g0460000	*O.sativa, O.rufipogon, O.nivara, O.glaberrima, O.barthii*	Amino acid transporter	Seed hull color: straw-white seed hull	Zhu et al., 2011; Vigueira et al., 2013
LABA1	Os04g0518800	*O. rufipogon* x *O.sativa*	Cytokinin-Activating Enzyme	Awn morphology: short, barbless awns	Hua et al., 2015
Ehd4	Os03g0112700	*O.sativa* Kita-ake	CCCH-type zinc finger protein	Flowering time: photoperiodic control of flowering	Gao et al., 2013
Hd6	Os03g0762000	*O.sativa* Nipponbare x *O.sativa* Kasalath	CK2alpha protein kinase subunit	Flowering time: delay of flowering under long-day conditions	Yamamoto et al., 2000
Hd3a	Os06g0157700	*O.sativa* Nipponbare x *O.sativa* Kasalath	Rice ortholog of *Arabidopsis* FT (mobile flowering signal)	Flowering time: transition to flowering under short-day conditions	Yamamoto et al., 1998; Komiya et al., 2008

loss of function in the wild tomato *S. pimpinellifolium* (i.e., *SP, SELF-PRUNING; O, OVATE; FW2.2, FRUIT WEIGHT; LYCOPENE BETA CYCLASE, FASCIATED, MULTIFLORA*). Four of the six genes (i.e., all except *FASCIATED* and *MULTIFLORA*) were successfully targeted and contained indel mutations, which resulted in a WR with a domesticated "phenotype", i.e., an altered morphology, a three-fold increase in fruit size, a ten-fold increase in fruit number compared to the WR, and a 500% increase in lycopene accumulation in the fruit, relative to the cultivated *Solanum lycopersicum* (Zsögön et al., 2018).

Similarly to wild tomato, neodomestication of the WRs of rice is theoretically possible, albeit non-trivial. *O. coarctata* is an attractive candidate for neodomestication because it is the only halophyte in the *Oryza* genus and can thrive in salinity levels of up to 40 E.CedS m^{-1} (i.e., brackish water salinity levels) along the coastal regions from Pakistan to Myanmar (Bal and Dutt, 1986). In India, the habitat of this species is similar to that of mangrove forests where *O. coarctata* can be found submerged in saline water for up to 12 h a day due to lunar tides (Bal and Dutt, 1986; Mondal et al., 2018). For decades, rice breeders have tried to introgress the halophyte characteristics of *O. coarctata* into cultivated rice with limited or no success (Brar and Khush, 2018; Prusty et al., 2018). Thus, *O. coarctata* is an ideal candidate for neodomestication and, if successful, would allow farmers to grow rice on land that otherwise is unable to support conventional rice farming practices. A proposed methodology for the neodomestication of *O. coarctata* includes the targeting of orthologues of domestication genes in *O. coarctata* (**Table 1B**) with precise gene editing. An *O. coarctata* PSRefSeq will enable less off-target CRISPR/Cas9 enzyme effects (not attributed to the enzyme mode of action) as the contiguity and fidelity of the PSRefSeq allows for better precision for single guide RNA (sgRNA) selection (Biswal et al., 2019). Moreover, *O. coarctata*, unlike *S. pimpinellifolium*, is not a direct progenitor of the cultivated *O. sativa* and is a polyploid which, besides affecting the number of domestication-related genes, also translates to a vast difference in the genome structure, thereby making gene editing more challenging due to the potential linkage of domestication genes to non-desirable traits (i.e., linkage drag). Given the paucity of available genome assembly resources, a PSRefSeq for *O. coarctata* would play a vital role in the precise identification of the orthologues of domestication genes that could be targeted for neodomestication.

AUTHOR CONTRIBUTIONS

SM and NA-B contributed equally to this work and share co-first authorship. SM and NA-B wrote the manuscript. AZ contributed to writing the manuscript, editing and organizing the structure of the manuscript. RW reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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New Insights Into the Nature of Interspecific Hybrid Sterility in Rice

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Interspecific and intraspecific hybrid sterility is a typical and common phenomenon of postzygotic reproductive barrier in rice. This is an indicator of speciation involved in the formation of new species or subspecies, and it significantly hampers the utilization of favorable genes from distant parents for rice improvement. The *Oryza* genus includes eight species with the same AA genome and is a model plant for studying the nature of hybrid sterility and its relationship with speciation. Hybrid sterility in rice is mostly controlled by nuclear genes, with more than 50 sterility loci genetically identified to date, of which 10 hybrid sterility loci or pairs were cloned and characterized at the molecular level. Comparing the mapping results for all sterility loci reported indicated that some of these loci from different species should be allelic to each other. Further research revealed that interactions between the multiple alleles at the hybrid sterility locus caused various genetic effect. One hypothesis for this important phenomenon is that the hybrid sterility loci are orthologous loci, which existed in ancient ancestors of rice. When one or more ancestors drifted to different continents, genetic divergence occurred because of adaptation, selection, and isolation among them such that various alleles from orthologous loci emerged over evolutionary time; hence, interspecific hybrid sterility would be mainly controlled by a few orthologous loci with different alleles. This hypothesis was tested and supported by the molecular characterization of hybrid sterility loci from *S1*, *S5*, *Sa*, *qHMS7*, and *S27*. From this, we may further deduce that both allelic and non-allelic interactions among different loci are the major genetic basis for the interspecific hybrid sterility between *O. sativa* and its AA genome relatives, and the same is true for intraspecific hybrid sterility in *O. sativa*. Therefore, it is necessary to raise the near-

isogenic lines with various alleles/haplotypes and pyramided different alleles/haplotypes from sterile loci in the same genetic background aiming to study allelic and non-allelic interaction among different hybrid sterility loci in the AA genome species. Furthermore, the pyramiding lines ought to be used as bridge parents to overcome hybrid sterility for rice breeding purposes.

Keywords: allelic variation, hybrid sterility, interspecific hybrid, rice, Oryza sativa

INTRODUCTION

Reproductive barriers are very common and important phenomena in biology, being widely observed in animal and plant populations. Reproductive isolation was classified into pre-zygotic and post-zygotic isolation mechanisms (Smith, 1989). The former prevents the formation of hybrids, and the latter acts after the formation of a hybrid and includes hybrid necrosis, weakness, hybrid sterility, and lethality (Stebbins, 1950).

Hybrid sterility is generally thought to be the most pervasive post-zygotic isolating mechanism, which provides an initial driving force for genetic differentiation and thus plays a key role in speciation (Orr and Presgraves, 2000). One of the major challenges in biology is to understand the origin of species, so the relationship between the hybrid sterility and the formation of new species, or subspecies, is a subject of major interest in evolutionary biology (Darwin, 1859; Coyne and Orr, 2004). Yet, the introgression of favorable genes from distantly relatives through wide crossing and heterosis utilization between subspecies or species are hindered by the hybrid sterility.

Various causes have been ascribed to explain hybrid sterility, such as meiotic irregularities (Yao et al., 1958), chromosomal aberrations (Henderson, 1964), and cytoplasmic-nuclear interactions (Shinjyo, 1975). Recent reports have also speculated that structural variation at particular trait loci may contribute to the intersubspecific hybrid sterility between the *O. sativa* L. ssp. *indica* and *O. sativa* L. ssp. *japonica* accessions (Shen et al., 2017; Wang et al., 2018).

Nevertheless, most known cases of sterility arise from a disharmonious interaction between nuclear genes derived from their respective parents (Stebbins, 1958; Grant, 1981). Genes for hybrid sterility have been reported frequently in fungi, animals, and plants (Brideau et al., 2006; Lee et al., 2008; Bikard et al., 2009). This prominence indicates that gene interactions are critically involved in generating hybrid sterility.

Hybrid sterility occurs widely in hybrids of Asian cultivated rice species, especially between the Asian and African rice species, and between the cultivated rice species and their wild relatives. Because *Oryza* genus had broad genetic diversity and well-characterized genetic base, it is perhaps one of the best model plants to study the nature of hybrid sterility. Based upon published reports of more than 50 sterility loci genetically identified, and 10 hybrid sterility loci or pairs cloned and characterized at the molecular level, this review examines the links between the evolutionary relationship of the *Oryza* genus and hybrid sterility, the genetic models of hybrid sterility, the allelic variation of orthologous loci for hybrid sterility, the non-allelic interactions for hybrid sterility, and strategies for overcoming hybrid sterility in rice improvement.

HYBRID STERILITY AND THE CLASSIFICATION, ORIGIN, AND EVOLUTIONARY RELATIONSHIPS OF THE *ORYZA* GENUS

The *Oryza* genus has eight AA genome species of diploid chromosome, of which six are wild and two are cultivated (Vaughan, 1989). One of the cultivated species, *O. sativa* L., has a global distribution and is now classified as two subspecies: *O. sativa* L. ssp. *japonica* and *O. sativa* L. ssp. *indica*. The *japonica* subspecies is further classified as three subpopulations, *tropical*, *temperate*, and *aromatic*, while the *indica* subspecies contains two subpopulations, *indica* and *aus* (Garris et al., 2005) or six subpopulations (Wang et al., 2018). Another cultivated species is *O. glaberrima* Steud., which is localized in West Africa and commonly referred to as African rice (Morishima et al., 1963). The six wild species are *O. rufipogon* Griff. and *O. nivara* Sharma et Shastry from Asia; *O. longistaminata* A. Chev. et Roehr. and *O. barthii* A. Chev. from sub-Saharan Africa; *O. meridionalis* Ng from Australia, and *O. glumaepatula* Steud. from South America (Vaughan et al., 2003). Hybrid sterility in rice was frequently observed between and within the AA genome species and was thought to serve as an important indicator for studying these species' relationships, besides their morphological, physiological, ecological traits, and molecular markers (Kato et al., 1928; Chu et al., 1969; Morishima, 1969; Lu et al., 1998; Naredo et al., 1998; Lu et al., 2000; Vaughan et al., 2003).

The origin and evolutionary relationship of the AA genome nevertheless remain quite contentious, with different opinions and arguments being advanced. Most researchers now believe that the domestication of rice from wild to cultivated species occurred independently in Africa and Asia, respectively (Londo et al., 2006; Vaughan et al., 2008). Recent studies confirmed that *O. glaberrima* was domesticated directly from an *O. barthii* subgroup in a single domestication center along the Niger river (Wang et al., 2014).

One hypothesis is that *japonica* was derived from *indica* (Chang, 1976; Oka, 1988), while according to an alternative hypothesis both *indica* and *japonica* were originated independently from their wild ancestors (Second, 1982; Bautista et al., 2001; Cheng et al., 2003). Based on an analysis of their whole chloroplast genome sequences, the maternal genome of *japonica* may have been derived from *O. rufipogon* and that of *indica* may

have originated from *O. nivara*, which would support the independent domestication theory (Wambugu et al., 2015). By employing the *indica-japonica* specific insertion/deletion markers to evaluate the genetic relationships within the genus *Oryza*, Chin et al. (2017) recently provided more evidence of *indica* and *japonica* evolving independently.

All the above studies concerned with rice's origin and evolutionary history relied on evidence from either archaeological analyses or genetic markers. Given that the strength of reproductive isolation is significantly and positively correlated with parental divergence (Coyne and Orr, 2004), comparative analyses of the timing and evolutionary progression of genetic changes underlying reproductive isolation genes could us better understand the mechanisms of speciation (Moyle and Payseur, 2009). With the advent of molecular biology and in recent years genomic studies especially, the hybrid sterility genes, also called "speciation genes", provide an unique angle of view for understanding the genetic, origin and evolution of the *Oryza* genus. For instance, the molecular evolution analysis of the *S5* locus, one of the important female sterility loci in *indica-japonica* hybrids, revealed that *indica* and *japonica* subspecies of the Asian cultivated rice *O. sativa* were domesticated independently from wild species (Du et al., 2011). This result thus supported the independent origin theory of *indica* and *japonica* and was consistent with the finding reported by Wambugu et al. (2015). The cloning of *S1* locus, which is the most important genetic factor causing the hybrid sterility between two cultivated species, provides experimental evidence for the independent origin of Asian cultivated rice and African cultivated rice (Xie et al., 2019b).

GENETIC MODELS FOR THE GENETIC MECHANISM OF HYBRID STERILITY IN RICE

Two genetic models are generally accepted for explaining the genetic mechanism underpinning hybrid sterility in rice according to the Mendelian inheritance: allelic interaction (Kitamura, 1962) and epistatic interaction (Oka, 1957; Oka, 1974). The former proposes that a genetic interaction between two divergent alleles on a single locus causes the abortion of gametes carrying a specific allele, while the latter proposes that epistatic interactions between two loci cause hybrid sterility.

To date, more than 50 hybrid sterility loci in rice have been identified from gene mapping populations generated from various germplasm lines (**Supplementary Table 1**). Some of these loci cause pollen sterility, some cause female gamete abortion, and a few cause the abortion of both. Yet most of these loci seem to act independently on hybrid sterility, which could be explained by the one-locus interaction model; for those showing epistatic interactions, they fit the two-loci interaction model.

From the mapping loci, 10 hybrid sterility loci or pairs of epistatic interaction were cloned and characterized at the molecular level, for which the genetics of seven loci—*S1*, *S5*, *S7*, *Sa*, *Sc*, *hsa1*, and *qHMS7*—follow the one-locus interaction model (Chen et al., 2008; Long et al., 2008; Yang et al., 2012; Kubo et al., 2016; Yu et al., 2016; Shen et al., 2017; Xie et al., 2017; Koide et al., 2018; Yu et al., 2018; Xie et al., 2019b). Most loci contain two or more closely linked genes interacting to cause gamete sterility, which can take these recognized forms: a single Mendelian factor, such as two genes at *Sa*, *qHMS7* and *hsa1* locus (Long et al., 2008; Kubo et al., 2016; Yu et al., 2018); three genes at *S1* and *S5* loci (Yang et al., 2012; Xie et al., 2017; Koide et al., 2018; Xie et al., 2019b); and genomic structural variation at the *Sc* locus (Shen et al., 2017). All can be regarded as interaction between different alleles from a single Mendelian factor to control hybrid sterility.

The other three pairs (*S27/28*, *DPL1/DPL2*, and *DGS1/DGS2*) follow the two-loci interaction model (Mizuta et al., 2010; Yamagata et al., 2010; Nguyen et al., 2017). These gamete-essential genes were interchromosmal duplicated segment from their ancestral loci. The reciprocal loss of one of the duplicated genes caused the sterility in hybridization in the divergent species. Therefore, genetic segregation and recombination gave rise to gametes lacking any duplicated functional genes in the hybrids, thus leading to hybrid sterility (Mizuta et al., 2010; Yamagata et al., 2010; Nguyen et al., 2017).

However, the molecular mechanisms underlying each of the loci are in fact complex though the genetic basis of reproductive isolation seems simple according to classical genetic analysis. Not only are the genes involved in hybrid sterility characterized by very different functional categories, the way in which they interact to cause sterility is also distinct, and this topic has been reviewed before (Ouyang and Zhang, 2013; Xie et al., 2019a). However, it is necessary to recheck hybrid sterility from different perspectives.

SEVERAL HYBRID STERILITY LOCI FROM DIFFERENT SPECIES SHOULD CONFER MULTIPLE ALLELES

In reviewing the progress made in the genetic mapping study of hybrid sterility loci/QTL between *O. sativa* and its AA genome relatives, six "hot spots" from different species were frequently identified (**Figure 1**). Comparing the mapping results for all these sterility loci suggested that some sterility loci arising from multiple species should be allelic to each other, since they were located to the same chromosome region and were identified as having similar genetic activity.

The interspecific hybrid sterility locus *S1* on chromosome 6 was first identified in the cross between *O. sativa* (T65*wx*) and *O. glaberrima* (W025) (Sano et al., 1979). It is frequently reported by a large number of studies between the two cultivated rice species, *O. sativa* (*indica*/*japonica*) and *O. glaberrima* (Koide et al., 2008; Garavito et al., 2010; Zhou et al., 2010; Li J. et al., 2012). The *S1* locus functions as a "gamete eliminator": both male and female gametes carrying the allele of *O. sativa* are aborted in the heterozygote, for which the type of pollen abortion was of an empty abortion phenotype. The *S10* locus, inducing both male and

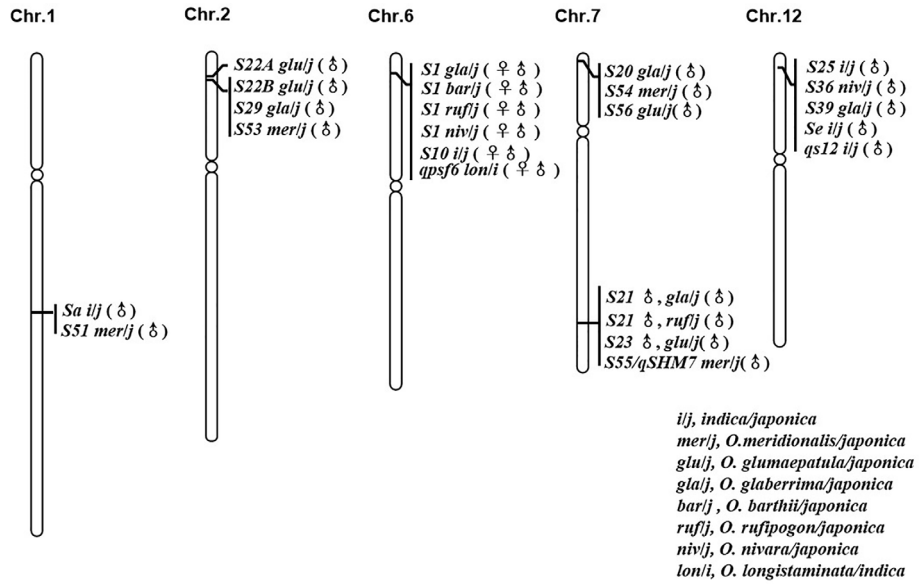

FIGURE 1 | Loci with good co-linear relationship and similar genetic action for hybrid sterility in rice.

female gametes' abortion in an intraspecific hybrid cross between T65*wx* (*japonica* type) and PTB 10 (*indica* type), was mapped onto the similar chromosome position as *S1* on chromosome 6 (Sano et al., 1994), with some later studies indicating that *S1* and *S10* are allelic to each other (Heuer and Miézan, 2003; Zhu et al., 2005). Chen et al. (2009) detected one main-effect QTL, *qpsf6*, for pollen and spikelet fertility on the short arm of chromosome 6 close to the SSR marker RM587, from the cross between an *indica* rice cultivar RD23 of *O. sativa* and an accession of *O. longistaminata*. Comparing their position and effect indicated that this QTL coincides with the gamete eliminator *S1* (Chen et al., 2009). Recently, a genetic study found that *S1* also was a major sterility locus in the hybrid combinations obtained from *O. sativa* crossed with *O. rufipogon*, *O. nivara*, and *O. barthii*, respectively (Yang et al., 2016), which strongly suggests there was an orthologous hybrid sterility locus controlling hybrid sterility between *O. sativa* and its AA genome relatives in this area on rice chromosome 6.

The pollen killer locus *S22*, initially derived from the cross between *O. glumaepatula* and *O. sativa*, was subsequently identified as two closed but linked loci, *S22A* and *S22B*. Since the sterile alleles *S22A* and *S22B* are closely linked and contributed to hybrid sterility in the one-locus allelic interaction model, the *S22A* and *S22B* loci were regarded as a single Mendelian locus in their initial mapping experiments (Sobrizal et al., 2000a; Sakata et al., 2014). A pollen killer locus *S29(t)*, which induced hybrid sterility in the cross between *O. glaberrima* and *O. sativa*, was mapped onto the similar position to *S22* on chromosome 2, whose comparative mapping indicated that *S29(t)* corresponded to *S22B* (Hu et al., 2006; Sakata et al., 2014). *S53*(t), caused pollen sterility in an *O. sativa*–*O. meridionalis* hybrid, was identified on the same chromosome region harboring *S22B* (Li et al., 2018b). The male gametes carrying the *O. sativa* allele were viable whereas those carrying the *O. glumaepatula/O. meridionalis* allele were aborted in the *S22B* and *S53*(t) loci; hence, these loci from different species should be allelic to each other with multiple alleles. This implies there might be another orthologous hybrid sterility locus between the Asian cultivated rice *O. sativa* and the African rice *O. glaberrima*, and its wild relatives *O. glumaepatula*, *O. meridionalis* on rice chromosome 2.

The major locus *Sa* conferring the *indica-japonica* hybrid male sterility was identified on chromosome 1 with the genetic background of a *japonica* variety (Zhuang et al., 1999). Work by Li et al. (2018b) identified a pollen killer *S51*(t) on chromosome 1 from *O. meridionalis*. The sterile pollen in the heterozygotes of *S51*(t) and *Sa* showed a similarly empty abortion phenotype under I_2-KI staining. Additionally, both *S51*(t)^j and *Sa*^{j}, the *japonica* alleles, were aborted and were incapable of being transmitted to the progeny *via* the male gametes. According to a comparison of the location and the genetic mode of locus action, the *S51*(t) can be allelic to *Sa* (Li et al., 2018b).

The hybrid sterility locus *S20* was identified on the distal end of the short arm of chromosome 7, from the cross between *japonica* rice (Taichung 65) and African rice Acc. IRGC104038 (Doi et al., 1999). An examination of map positions also revealed that *S20* was highly likely to be allelic to *S54*(t) and *S56*(t), which were respectively identified in the hybridization crossed combinations between *O. sativa* (*japonica*) and *O. meridionalis* (Li et al., 2018b) and between *O. sativa* (*japonica*) and *O. glumaepatula* (Zhang et al., 2018). Just like *S20*, the *japonica* alleles of *S54*(t) and *S56*(t) were aborted and could not be inherited in progeny *via* male gametes, so plants with normal pollen fertility were homozygous for *O. meridionalis* and *O.*

glumaepatula allele in the inbreeding population raised from the semisterile individuals (Li et al., 2018b; Zhang et al., 2018).

The hybrid sterility locus *S21* was identified on the distal end of the long arm of chromosome 7, from the cross of *japonica* rice (Taichung 65) and African rice Acc. IRGC104038 (Doi et al., 1999). But a new allele of *S21* was identified from *O. rufipogon* Acc. IRGC105715 (Miyazaki et al., 2007). Using the *O. glumaepatula* introgression lines with the background of *O. sativa* (Taichung 65), the pollen semi-sterile locus *S23*(t) was also located on the long arm of chromosome 7 and was thought to be allelic to *S21* from *O. glaberrima* and *O. rufipogon* after comparing their map positions (Sobrizal et al., 2000b; Miyazaki et al., 2007). The pollen killer locus *S55*(t)/*qHMS7*, from *O. meridionalis*, has a map position similar to that of *S21* identified from *O. rufipogon*, *O. glaberrima*, and *S23*(t) identified from *O. glumaepatula* (Doi et al., 1999; Sobrizal et al., 2000b; Miyazaki et al., 2007; Li et al., 2018b; Yu et al., 2018). This congruence pointed to a major pollen sterility locus on the long arm of chromosome 7 that is capable of inducing sterility in the hybridization combinations arising from *O. sativa* crossed with *O. glaberrima*, *O. rufipogon*, *O. glumaepatula*, and *O. meridionalis*, respectively, whose sequence analysis was based on the cloning of *S23* and *qHMS7* (Yu et al., 2018; Fang et al., 2019).

S36 and *S25* are two F_1 pollen sterility loci, both found on the distal end of the short arm of chromosome 12 from the interspecific cross of *O. sativa* (*japonica*) and *O. nivara*, and the intersubspecific cross of *O. sativa*, respectively (Kubo et al., 2001; Win et al., 2009). The pollen sterility locus *Se* and hybrid male sterility QTL *qS12*, both from the intersubspecific cross between *japonica* and *indica*, were mapped onto the short arm of chromosome 12 (Zhu et al., 2008; Zhang H. et al., 2011). Further, the pollen killer *S39*(t) identified from the interspecific cross between *O. sativa* ssp. *japonica* and *O. glaberrima* was likewise mapped onto the same chromosome region of *S36* and *S25* (Xu et al., 2014). Those pollen grains carrying the *japonica* allele were sterile in the heterozygous state. Having a similar map position and same genetic activity, *S25*, *S36*, *S39*, *qS12*, and *Se* might therefore be multiple alleles on the same locus responsible for hybrid sterility in rice.

The six hot spots mentioned above each includes at least two hybrid sterile alleles from different interspecific hybrid crosses, and researchers believe these loci should be allelic to each other, though their allelic relationship has yet to be tested. Nonetheless, the existence of these multiple alleles implies that the hybrid sterility among AA genome species of genus *Oryza* could be governed by a few orthologous loci. It is thus imperative to clarify the allelic relationships of these loci, although any robust confirmation of this allelism is difficult to obtain due to their diverse genetic backgrounds. Based upon this consideration, the near-isogenic lines (NILs) of 5 hybrid sterility loci (*S1*, *S39*, *S44*, *S53*, and *S21*/*qHMS7*) have been developed in the same genetic background, using 35 rice accessions from eight species of AA genome as donors and one *japonica* cultivar (Dianjingyou 1) as the recurrent parent. And the allelic relationship confirmation is ongoing.

THE ALLELIC INTERACTION AMONG DIFFERENT ALLELES OF ORTHOLOGOUS LOCI AND NON-ALLELIC INTERACTION AMONG DIFFERENT LOCI INDUCE INTERSPECIFIC HYBRID STERILITY BETWEEN *O. SATIVA* AND AA GENOME SPECIES

One hypothesis for multiple alleles from different species is that these hybrid sterility loci are orthologous loci, which existed in ancient ancestors. When one or more ancestors drifted to different continents, genetic divergence occurred because of adaptation, selection, and isolation among them. With this accumulated genetic differentiation, the various alleles may divide the original function, lose it altogether, or introduce a new function (Lynch and Force, 2000). Hence, interspecific hybrid sterility would be mainly controlled by a few orthologous loci with different alleles/haplotypes. This hypothesis was proven by the molecular characterization of cloned hybrid sterility loci in rice.

The hybrid sterility locus *qHMS7*, which confers pollen semi-sterility in the hybrid between *O. meridionalis* and *O. sativa* ssp. *japonica* Dianjingyou 1 (DJY1), was cloned and molecularly characterized (Yu et al., 2018). The *O. sativa* allele of *ORF2* (*ORF2-D*) and *ORF3* (*ORF3-D*) respectively encoded the toxin and antidote, while *ORF2-mer* was non-functional and *ORF3* was absent in *O. meridionalis*, which induced the pollens carrying the *O. meridionalis* allele to be aborted (Yu et al., 2018). The analyzed gene structure of the *qHMS7* locus indicated that *ORF2* was present in all sequenced accessions, with a total 27 haplotypes of *ORF2* identified, yet only one haplotype of *ORF3* (*ORF3-D*) was identified in parts of *O. rufipogon* and most of Asian-cultivated rice accessions, but none in *O. meridionalis*, *O. longistaminata*, *O. barthii*, and *O. glaberrima* (Yu et al., 2018). So, *qHMS7* was not only detected in the cross of *O. sativa*–*O. meridionalis* (Yu et al., 2018; Li et al., 2018b) but also in the crosses of *O. sativa*–*O. glaberrima* and *O. sativa*–*O. rufipogon* as *S21* (Doi et al., 1999; Miyazaki et al., 2007). The pollen semi-sterility locus *S23* in *O. sativa*–*O. glumaepatula* has a similar map position to *qHMS7* (Sobrizal et al., 2000b; Fang et al., 2019). Sequence analysis revealed that *S23* was allelic to *qHMS7*, the *ORF2* and *ORF3* of *S23* have the same "Toxin-Antidote" function when compared with *qHMS7*; however, the genetic effect of *S23* differs from *qHMS7* in two ways (Fang et al., 2019). On the one hand, F_1 pollen fertility was semi-sterility (51.16 ± 1.29%) for *qHMS7* and no homozygote for *O. meridionalis* was obtained, indicating the pollen grains carrying *O. meridionalis* allele had aborted completely; whereas, for *S23*, its F_1 pollen fertility was higher (63.15 ± 13.49%) than that of *qHMS7* and a few homozygotes of *O. glumaepatula* was found available, indicating that the pollen grains carrying the *O. glumaepatula* allele were not completely sterile (Yu et al., 2018; Fang et al., 2019). On the other hand, the pollen sterility of F_1 hybrid could only be observed in a short photoperiods, but not in a long photoperiods at the *S23* locus, yet no phenotypic differences were observed in different environments at the *qHMS7* locus (Li et al.,

2018b; Yu et al., 2018; Fang et al., 2019). The allele on *O. sativa* interacted with the respective alleles from *O. meridionalis* and *O. glumaepatula*, resulting in hybrid sterility with different genetic effects; accordingly, the allele on *O. glumaepatula* should differ from that on *O. meridionalis*. More research should reveal the nature of interactions occurring between the different haplotypes in rice.

The *Sa* locus was the first cloned and molecularly characterized hybrid sterility locus, from the *indica-japonica* cross, and it comprises two adjacent genes, *SaF* and *SaM* (Long et al., 2008). Most *indica* cultivars have the allele of $SaM^{+}SaF^{+}$, whereas all *japonica* cultivars contain $SaM^{-}SaF^{-}$. The alleles $SaM^{+}SaF^{+}$ and $SaM^{+}SaF^{-}$ were variably present in *Oryza* species having the GG, FF, CC, EE, and AA genomes, including *O. barthii*, *O. nivara*, and *O. sativa* subsp. *indica*. Three alleles in particular $SaM^{+}SaF^{+}$, $SaM^{+}SaF^{-}$, and $SaM^{-}SaF^{-}$ were found in the *O. rufipogon* populations. The mutation of *SaM* most likely arose in an *O. rufipogon* population with the allele $SaM^{+}SaF^{-}$ and then generated the allele $SaM^{-}SaF^{-}$, whereas the variation in *SaF* occurred before the evolutionary split and gave rise to most of the current *Oryza* species (Long et al., 2008). We noticed that eight AA genome species possess the alleles of $SaM^{+}SaF^{+}$ and $SaM^{+}SaF^{-}$, but not *O. sativa* subsp. *japonica*, so it is readily inferred that the hybrid sterility locus *S51*(t) detected from *O. meridionalis–japonica* hybrid was one allele of *Sa* (Li et al., 2018b), with neutral allele *Sa-n* being identified from the *indica* rice and wild rice *O. rufipogon* (Long et al., 2008). It is reasonable to anticipate that, with further research, a tri-allelic system consisting of three types of *Sa* alleles ($SaM^{+}SaF^{+}$, $SaM^{+}SaF^{-}$, and $SaM^{-}SaF^{-}$) will emerge, which is able to control hybrid male sterility and fertility not only in *O. sativa* but also among AA genome species of *Oryza*.

The hybrid sterility locus *S5*, controlling female semi-sterility in hybrids between *indica and japonica*, consists of three closely linked genes *ORF3*, *ORF4*, and *ORF5* that function together in a "Killer–Protector" system (Yang et al., 2012). The typical *indica*-like ($ORF3^{+}ORF4^{-}ORF5^{+}$), *japonica*-like ($ORF3^{-}ORF4^{+}ORF5^{-}$), and neutral ($ORF3^{+}ORF4^{+}ORF5^{+}$ and $ORF3^{-}ORF4^{-}ORF5^{-}$) alleles were found in wild rice accessions of *O. rufipogon* and *O. nivara*; this suggested the *S5* locus already existed in wild relatives and the ancestors of *indica* and *japonica* rice, thus probably originating before domestication (Du et al., 2011; Yang et al., 2012). Presently, *S5* has only been identified in the intersubspecific hybrid cross between *indica* and *japonica*, but according to the distribution of alleles in AA genome we think *S5* also had effect for interspecific hybridization crosses.

Interspecific hybrid sterility locus *S1* is also a tripartite "Killer-Protector" complex (Xie et al., 2019b). The African rice *S1* allele is composed of three adjacent genes (*S1A4*, *S1TPR*, *S1A6*), while the Asian *S1* allele includes only one gene, *S1TP*, which is a truncated form of *S1TPR* (Xie et al., 2017; Koide et al., 2018; Xie et al., 2019). Based on the analyzed gene structure of the *S1* locus, the function structure *A4-TPR-A6* exists in all examined accessions of *O. glaberrima*; and the nonfunction or dysfunction structureis present in all analyzed accessions of *O. sativa*, *O. longistaminata*, and *O. rufipogon*. The accessions of *O. meridionalis* and *O. barthii* possess both of the structures (Xie et al., 2019b).Total 7 haplotypes and 22 allelic variations of the *S1* locus were detected in the *Oryza* species (Xie et al., 2019b). Further studying the relationship between hybrid sterility and the multiple allelic interactions is needed.

Taken together, the research findings above indicate the interaction between divergent alleles/haplotypes may lead to hybrid sterility, even their interaction mechanisms and genetic effects may be different. It can be further deduced that not only the allelic interactions among different alleles of orthologous loci, but also the non-allelic interactions among different loci, constitutes the major genetic basis for interspecific hybrid sterility between *O. sativa* and other AA genome species.

Benefit from the clone of *S27* and *S28*, a pair of epistatic interaction loci caused F_1 hybrid sterility in the hybrid pairs *O. sativa–O. glumaepatula* and *O. sativa–O. nivara* (Yamagata et al., 2010; Win et al., 2011), we can trace the interaction between different alleles that induce hybrid sterility. The *S27* allele of *O. sativa* is composed of two tandem mitochondrial ribosomal protein L27 genes (*mtRPL27a* and *mtRPL27b*), and the *S28* allele of *O. sativa* contains a loss-of-function allele for *mtRPL27a*. In brief, the *S27* has normal functioning and *S28* has no function in the *O. sativa* allele, whereas only the *S28* has normal functioning and *S27* is absent in the *O. glumaepatula* allele. The epistatic interaction induces the abortion of pollen grains carrying *S27* and *S28*, both of which are inactive in F_1 progeny of *O. sativa* and *O. glumaepatula*. Judging from a comparison of their genomic sequences, *mtRPL27a* at *S28* on chromosome 4 is thought to be the most ancestral locus. With an interchromosomal duplication, a new copy of *mtRPL27a* at *S27* on chromosome 8 was generated, and likewise *mtRPL27b*, also at *S27* (Ueda et al., 2006). Among the eight AA genome species, the duplicated segment of *S27* was widely observed, occurring in all except *O. glumaepatula*, and only part of the accessions of *O. barthii* and *O. longistaminata* lacked this segment (Yamagata et al., 2010). Further study showed that the interaction between *S27* and *S28* loci also causes the pollen sterility in the F_1 hybrid of *O. sativa* and *O. nivara*, which inherited the duplicated segment of *S27* (Win et al., 2011). Sequence analysis revealed that the structure of *S27* allele in *O. nivara* is different from that of *O. glumaepatula*; in the former it is composed of two inactive genes (*mtRPL27a*, *mtRPL27b*) while in the latter species these two genes are absent. In short, *S27-niv* is a loss-of-function allele of *S27*. These results highlight that the mechanisms for hybrid sterility are likely different because of epistatic interactions between *S28* loci and different alleles of *S27* in divergent rice species.

The multiple alleles causing hybrid sterility in rice were analyzed at the molecular level for *S5*, *Sa*, *qHMS7/S21/S23/S55*, and *S27*. Meanwhile the cloning of hybrid sterility loci also proves the data support for the allelic relationship of these hybrid sterility loci mentioned above with same molecular location and similar genetic action. According to the latest research, at each hybrid sterility locus, the number of interacting alleles does not seem to be limited to three, of which one is neutral. It has been reported that four alleles

characterize the *S7* locus, *S-7*^{kn}, *S-7*^{cp}, *S-7*^{ai}, and *S-7*^{n}, which cause female sterility in the *indica-japonica* hybrid (Yanagihara et al., 1992). The genotype of *S-7*^{ai}/*S-7*^{cp} shows semi-sterility, in that the female gametes carrying *S-7*^{cp} are aborted. But in the *S-7*^{ai}/*S-7*^{kn} genotype, only some of the female gametes possessing *S-7*^{ai} are aborted. On the other hand, the *S-7*^{ai}/*S-7*^{n}, *S-7*^{n}/*S-7*^{cp}, and*S-7*^{n}/*S-7*^{kn} genotypes did not exhibit sterility as female gamete abortion (Yanagihara et al., 1992). The initial loci existing in the ancestral species likely differentiated into multiple alleles/haplotypes due to the emergence of genetic differentiation, which accumulated in each population; importantly, this variation was not harmful in the populations in which they arose. Of course, the variation that may affect fitness has been eliminated with the death of the individual. After hybridization between divergence populations, due to the incompatible interaction between two or more functionally diverged genes, the gametes carrying the weak alleles will be aborted, which may be nonfunctional, inactivated, or lack of corresponding protectors. This also explains why the cloned hybrid sterility loci involved in the one-locus systems are mostly composed of more than two linked genes. These closely linked genes, which are distinguished by minimal recombination, form a complex acted as a single Mendelian factor that can avoid inducing the suicidal killer/nonprotector allele in a hybrid that would result in a breakdown of the system (Xie et al., 2019a). The evolutionary history of cloned hybrid sterility genes has been described by many researchers (Long et al., 2008; Yamagata et al., 2010; Du et al., 2011; Kubo et al., 2016; Yu et al., 2018). Ouyang and Zhang (2013) proposed three evolutionary genetic models, those of parallel divergence, sequential divergence, and parallel-sequential divergence, to illustrate the process going from essential genes in the ancestral species to diverged genes based on the cloning data of hybrid sterility genes (Ouyang and Zhang, 2018). By disentangling the interaction between various alleles of these "speciation genes" which underpin the timing and evolutionary progression of genetic changes, we can understand the processes and patterns underlying the speciation and origin of AA genome species of rice further.

STRATEGIES FOR OVERCOMING HYBRID STERILITY IN RICE IMPROVEMENT

During the process by which cultivated rice was domesticated from wild ancestor thousands years ago, the cultivated rice's genetic diversity has been gradually eroded. More importantly, the frequent use of a few elite parents in rice genetic improvement programs has exacerbated this decline in genetic diversity (Simmonds, 1976; Moncada et al., 2001). These challenges hinder our ability to attain further improvement in yield of new varieties while also making rice more susceptible to disease epidemics and pest outbreaks (Tanksley and McCouch, 1997). Thus, the relatives of *O. sativa*, which contain favorable genes (alleles), could be a valuable genetic resource for rice improvement *via* hybridization techniques. On the other hand, the utilization of heterosis in the grain yield of rice has boosted rice production in the past few decades. Current rice hybrids are mostly derived from the *indica* lines. Given their limited genetic diversity, intra-subspecific hybrid vigor now only provides rice breeders with limited yield increases, whereas the distant hybrids derived from intersubspecific even interspecific crosses performed stronger heterosis. However, severe reproductive isolation, such as that incurred by interspecific and intersubspecific hybrid sterility, limits the extensive utilization of these rice distant hybrids (Ikeda et al., 2009; Bolaji and Nwokeocha, 2014). Therefore, hybrid sterility studies should also aim to overcome the reproductive barrier, so as to enable breeders to utilize the valuable extant genetic resources and obtain the strong vigor of these hybrids.

Since hybrid sterility is partly caused by the negative interaction between divergent alleles in the background of the hybrid, replacing the divergent allele with the same or neutral alleles can eliminate the hybrid sterility on the corresponding locus. In this respect, the neutral alleles *S5*-n, *Sa*-n, *Sb*-n, *Sd*-n, and *Se*-n in the intersubspecific hybrid of *indica-japonica* (Ikehashi and Araki, 1986; Long et al., 2008; Li J. Q. et al., 2012), as well as *S38*-n, *S39*-n in the interspecific hybrid of *O. sativa* and *O. glaberrima* (Li et al., 2018a), have been identified. The use of these neutral alleles will give a strong promises on overcoming the interspecific and intersubspecific hybrid sterility. In most cases, as small number of sterility loci are involved in the hybrid cross, so it is necessary to pyramid several sterility loci alleles by using molecular marker assistant backcrossing methods. For example, Guo et al. (2016) polymerized four *indica* alleles of pollen sterility loci (*Sb*, *Sc*, *Sd*, *Se*) and the neutral allele of *S5* locus, imparting the embryo sac sterility to the *japonica* variety, with eight *indica*-compatible *japonica* lines with different *japonica* backgrounds thus obtained. These *indica*-compatible *japonica* lines are highly compatible with the *indica* and effectively overcome the hybrid sterility in the intersubspecific hybrid. In the same vein, two neutral alleles of hybrid sterility loci were introgressed into *indica* rice HJX74 to develop HJX74 wide-compatible lines that were compatible with both *japonica* and *indica* testers (Guo et al., 2016).

Another promising strategy is to create hybrid-compatible lines with artificial neutral alleles generated by advanced molecular approaches, such as RNA interference technologies and gene-editing technology. For example, the neutral allele *S1*^{mut} was created by mutagenesis and the heterozygous hybrids harboring *S1*^{mut}/*S1*^{g} and *S1*^{mut}/*S1*^{s} did not exhibit sterility (Koide et al., 2018). Artificial neutral *S1* alleles could also be obtained by disrupting any one of the three genes in the *S1*-g (*S1A4-S1TPR-S1A6*), through a CRISPP/Cas9 knockout (Xie et al., 2019). The same is true for *qHMS7* (Yu et al., 2018). The introduction of both *Sa*-n and *Sc*-n *via* CRISPR/Cas9 plant genome editing can assist in overcoming unwanted hybrid male sterility (Shen et al., 2017; Xie et al., 2017). A combination of these strategies may provide better assurance of hybrid fertility. With the further discovery and creation of neutral loci and the generation of intersubspecies and interspecies bridge parents, rice improvement will step into a new era.

CONCLUDING REMARKS

The Bateson-Dobzhansky-Muller model posits that hybrid sterility arises from a disharmourose interaction between functionally diverged genes from the hybridizing distant parents (Bateson, 1909; Dobzhansky, 1937; Muller, 1942). This model was first proposed in tomato by Rick (1966) and supported by many other reports (Kitamura, 1962; Ikehashi and Araki, 1986; Sano, 1990). According to the model, independent mutations occur in diverging populations but without a reduction in fitness, becoming fixed; consequently, multiple alleles, including a neutral allele, present at the causal orthologous loci, which then interact negatively in the background of the hybrid (Nei et al., 1983). One locus allelic interaction model underpins the negative interaction occurring at a single locus as a consequence of the independent evolution of two alleles, thereby causing a significant reduction in the fitness of the heterozygote when compared with their homozygote parents. Such negative interaction can also occur between two independent loci affecting gamete development, the gametes carrying the recessive alleles at both loci aborted during gamete development, but gametes of other genotypes remained normal. In this review, we rechecked and updated current genetic models for hybrid sterility in rice. The multiple alleles should exist at each orthologous hybrid sterility locus including, but not limited to, the neutral allele. The major genetic basis for interspecific hybrid sterility between *O. sativa* and AA genome species in genus *Oryza* should be the interaction between various alleles at a single locus, and various alleles with other loci, and the same is true for intersubspecific hybrid sterility in *O. sativa*. Even some of the sterility loci identified in individual combinations/species existed like an orphan, but whether they harbor various alleles in other AA genome species or not requires more study.

Currently, our understanding of hybrid sterility remains limited to that within Asian cultivated rice and between Asian cultivated rice and other species of AA genome, along with a few reports on hybrid sterility among different ecotypes within *O. sativa* that are available. Meanwhile, the availability of information is very limited due to different backgrounds of the genetic materials used by various researchers. To better understand these processes, it is essential to identify, clone, and functionally characterize more hybrid sterility loci in the genus *Oryza*. Efforts should also extend to investigating both the genetic diversity and geographical distribution of the alleles of various loci at the species level, including extant wild relatives. Since allelic and non-allelic interactions among major sterility loci are the foundation for understanding the relationship between hybrid sterility and speciation, it is necessary to raise near-isogenic lines with various alleles/haplotypes and pyramided different loci, with the same genetic background, based on the cloning and molecule characteristics. Then, taking these data together, it would become possible to provide more evidence illuminating the origin of genes responsible for hybrid sterility and the evolutionary processes for the establishment of new species in *Oryza* genus and shed light on overcoming hybrid sterility in rice improvement.

AUTHOR CONTRIBUTIONS

DT proposed the insights. JL conceived and wrote the paper. JZ, YZ, YY, and QP reviewed and edited the manuscript.

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SUPPLEMENTARY MATERIAL

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How Hybrid Breakdown Can Be Handled in Rice Crossbreeding?

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In crosses between genetically divergent parents, traits such as weakness and sterility often segregate in later generations. This hybrid breakdown functions as a reproductive barrier and reduces selection efficiency in crossbreeding. Here, I provide an overview of hybrid breakdown in rice crosses and discuss ways to avoid and mitigate the effects of hybrid breakdown on rice crossbreeding, including genomics-assisted breeding.

Keywords: genomics-assisted breeding, *Oryza*, reproductive barrier, sterility, weakness

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INTRODUCTION

Breeders and researchers alike have been interested in hybridization and introgression between divergent genotypes as the potential driver leading to the ecological divergence of progeny (Rieseberg et al., 2007; Arnold et al., 2012). Conversely, hybridization can sometimes involve a reduced hybrid fitness, such as weakness and/or sterility in F_1 and later generations, even in cases of hybridization between members of the same species.

Reduced hybrid viability and/or fertility segregating in F_2 or later generations are referred to as hybrid breakdown (HB), in which recessive alleles are necessarily associated. This reproductive barrier has been observed for a long time in both plants and animals (Dobzhansky, 1970; Grant, 1971). Many of the genetic analyses of this barrier have revealed that it is accomplished by a complementary effect between and/or among loci with differentiated alleles, commonly called the Bateson–Dobzhansky–Muller (BDM) incompatibility (Rieseberg and Willis, 2007). HB necessarily involves intrinsic postzygotic reproductive barriers, such as hybrid inviability (including weakness, necrosis, and chlorosis) and hybrid sterility (in the male, female, or both gametes); therefore, some researchers may not distinguish HB from inviability and sterility in the F_1 progeny. Nevertheless, I believe that HB is a convenient classification for reproductive barriers because it implies their underlying genetic basis (i.e., the involvement of recessive alleles).

Recent studies using the *Arabidopsis* model plant species have provided a better understanding of HB regarding its molecular mechanisms (Vaid and Laitinen, 2019). Conversely, although rice (*Oryza* species) is a model crop, the current understanding of the genetic basis of HB remains limited in this species, probably because HB is not a reproductive barrier in F_1 hybrids, in which a higher grain production is expected compared with the parental inbred lines, and probably because inferior plants that segregate in F_2 and later generations can be easily selected out based on the phenotype from the breeding population. The difficulty in genetic mapping caused by recessive inheritance can also be behind this limitation.

Here, I outline HB in rice crosses while referring to information provided by *Arabidopsis* studies, and discuss how HB is handled in rice breeding.

THE GENETIC BASIS OF HYBRID BREAKDOWN IN RICE

It is considered that cultivated rice, *O. sativa japonica* and *indica*, forms a species complex with their putative progenitor (*O. rufipogon*) and wild species; however, multiple reproductive barriers, including HB, are observed in crosses among them (Oka, 1988; Vaughan et al., 2003).

To the best of my knowledge, Dr. Oka was the first to describe the genetic basis of HB in intersubspecific crosses between *japonica* and *indica*, which was accounted for by two complementary genes (Oka, 1957). However, the responsible genes were not mapped on chromosomes, because molecular markers were not available at that time.

In rice hybrids, HB has often been described in intersubspecific (*O. sativa* ssp. *japonica* × ssp. *indica*) and interspecific (*O. sativa* × *O. nivara* and *O. sativa* × *O. glumaepatula*) crosses (Oka, 1957; Sato and Morishima, 1988; Wu et al., 1995; Li et al., 1997; Fukuoka et al., 1998, 2005; Kubo and Yoshimura, 2002, 2005; Matsubara et al., 2007a,b, 2015; Yamamoto et al., 2007; Ichitani et al., 2012;for intersubspecific crosses; Sobrizal et al., 2001; Miura et al., 2008 for interspecific crosses) (**Table 1**). Most of these HB cases were caused by two-locus BDM incompatibility and recessive alleles, whereby 1/16 of the F_2 progeny that was homozygous for recessive alleles at both loci showed the HB phenotype; however, 4/16 of the progeny that was heterozygous at only one locus showed the HB phenotype, depending on the cross combination (**Table 1** and **Figure 1A**). In the backcross hybrids, one locus was already fixed with the alleles from the recurrent parent; therefore, 1/4 of the BC_nF_2 progeny exhibited the HB phenotype (**Table 1** and **Figure 1B**).

The HB phenotype in rice hybrids varies according to the cross combination. For example, the *hbd2*/*hbd2 hbd3*/*hbd3* genotype shows weakness, but no obvious seed sterility. However, the *hbd4*/*hbd4 hbd5*/*hbd5* genotype exhibits both weakness and seed sterility (Matsubara et al., 2007a, 2015; Yamamoto et al., 2007; **Table 1**). There are also complicated cases in which HB showing both weakness and seed sterility are observed (*hwe1*/*hwe1 hwe2*/*hwe2* genotype) or HB showing only seed sterility (*hsa1*/*hsa1 hsa2*/*hsa2 hsa3*/*hsa3*) is found, despite the same cross combination, i.e., Asominori × IR24 cross (Kubo and Yoshimura, 2002, 2005; **Table 1**). As these HBs have independent genetic bases, at least 5/64 of F_2 progeny would show the HB phenotype in this cross.

To date, several sets of loci responsible for HB have been mapped to particular genomic regions using DNA markers in crosses between the *japonica* and *indica* rice varieties (Chrs 7 and 10, Fukuoka et al., 1998; Chrs 1 and 12, Wu et al., 1995; Kubo and Yoshimura, 2002; Matsubara et al., 2015; Chrs 6 and 11, Fukuoka et al., 2005; Chrs 8, 9, and 12, Kubo and Yoshimura, 2005; Chrs 2 and 11, Matsubara et al., 2007a; Yamamoto et al., 2007, 2010; Chrs 11 and 12, Ichitani et al., 2012; **Table 1** and **Figure 1C**). The results of these studies revealed that loci underlying rice HB are shared in some crosses, but differ in other crosses, which is suggestive of their diversification in rice genomes. For example, in some cases the HB allele carried by *O. nivara*, a species related closely to *incica* varieties, share the same locus with an *indica* variety; however, the HB allele carried by *O. glumaepatula*, a species closely related to cultivated varieties, does not share the locus with any other variety (**Table 1** and **Figure 1C**). It should be noted that the HB cases described above are caused by a set of genes with major effects; however, there are also cases caused by a set of genes with minor effects, e.g., slightly reduced seed fertility, which cannot be overlooked in rice breeding, that have not yet been detected.

MOLECULAR MECHANISMS UNDERLYING HYBRID BREAKDOWN

In *Arabidopsis* hybrids, several molecular mechanisms underlying the BDM type of HB have been experimentally demonstrated, e.g., autoimmune response (Bomblies et al., 2007; Alcázar et al., 2009) and reciprocal silencing of duplicated genes (Bikard et al., 2009; Vlad et al., 2010; Agorio et al., 2017; Blevins et al., 2017).

Autoimmune response: Bomblies et al. (2007) reported first that the autoimmune response that an *NB-LRR* disease-resistance gene or *R* gene is associated with *Arabidopsis* HB in intraspecific crosses, although this autoimmune response is mainly expressed as necrosis in F_1 plants, and HB in F_2 progeny seemed to be conditioned by temperature. In *Arabidopsis* hybrids, several lines of evidence of HB caused by the autoimmune response have been described (Alcázar et al., 2009, 2010). The involvement of *NB-LRR* genes in HB suggests that multiple genomic regions can be associated with the *Arabidopsis* HB; in fact, the extensive survey carried out by Chae et al. (2014) supports this idea.

Also in rice hybrids, HB caused by an autoimmune response has been reported in a *japonica* × *indica* cross (Yamamoto et al., 2010). In this case, HB occurs in F_2 plants when the hybrid breakdown 2 (*hbd2*) gene, which encodes casein kinase I and is carried by the *indica* variety is combined with a cluster of *R* genes carried by the *japonica* variety. As these causative alleles are likely to behave as partially recessive ones, the gene products can be involved (Matsubara et al., 2007a; Yamamoto et al., 2007, 2010).

Reciprocal silencing of duplicated genes: this notion was first theoretically proposed as a genetic basis for hybrid incompatibility (Werth and Windham, 1991; Lynch and Force, 2000). In the model, one population loses function at one locus and retains it at the other, whereas the other population experiences the opposite effect. Consequently, 1/16 of the F_2 zygotes do not have functional genes (Lynch and Force, 2000). The HB in *Arabidopsis* hybrids described by Bikard et al. (2009) and Vlad et al. (2010) is a good example of this phenomenon. In this scenario, cases involving epigenetic silencing have also been reported (Agorio et al., 2017; Blevins et al., 2017).

As rice has experienced both whole-genome and segmental duplication (Wang et al., 2007; Guo et al., 2019), reciprocal silencing could be prevalent as a cause of hybrid incompatibility. However, to date, there is no evidence of HB caused by reciprocal silencing of duplicated genes in rice.

TABLE 1 | Genetic basis of the hybrid breakdown reported in rice crosses.

Cross combination						Genotype of weak and/or sterile plant	Expected segregation ratio in F_2 population (Normal, weak, and/or sterile)	References
Parent 1			Parent 2					
Variety or accession	Species	Genotype	Variety or accession	Species	Genotype			
Sasanishiki	*j*	*Hwd1/Hwd1 hwd2/hwd2*	Col. No.15	*i*	*hwd1/hwd1 Hwd2/Hwd2*	*Hwd1/hwd1 hwd2/hwd2* *hwd1/hwd1 Hwd2/hwd2* *hwd1/hwd1 hwd2/hwd2*	11 : 5	Fukuoka et al., 1998
Taichung 65	*j*	*Hwf1/Hwf1*	IRGC 105668[a]	*O. glu*	*hwf1/hwf1*	*hwf1/hwf1*	3 : 1	Sobrizal et al., 2001
Asominori	*j*	*Hwe1/Hwe1 hwe2/hwe2*	IR24	*i*	*hwe1/hwe1 Hwe2/Hwe2*	*hwe1/hwe1 hwe2/hwe2*	15 : 1	Kubo and Yoshimura, 2002
Tachisugata	*j/i*	*hbd4/hbd4 Hbd5/Hbd5*	Hokuriku 193	*i*	*Hbd4/Hbd4 hbd5/hbd5*	*hbd4/hbd4 hbd5/hbd5*	15 : 1	Matsubara et al., 2015
Asominori	*j*	*Hsa1/Hsa1 hsa2/hsa2 Hsa3/Hsa3*	IR24	*i*	*hsa1/hsa1 Hsa2/Hsa2 hsa3/hsa3*	*hsa1/hsa1 hsa2/hsa2 hsa3/hsa3*	63 : 1[b]	Kubo and Yoshimura, 2005
Sasanishiki	*j*	*Hwg1/Hwg1 hwg2/hwg2*	ARC10303	*i*	*Hwg1/Hwg1 hwg2/hwg2*	*Hwg1/hwg1 hwg2/hwg2* *hwg1/hwg1 Hwg2/hwg2* *hwg1/hwg1 hwg2/hwg2*	11 : 5	Fukuoka et al., 2005
Koshihikari	*j*	*Hbd1/Hbd1*	Nona Bokra[a]	*i*	*hbd1/hbd1*	*hbd1/hbd1*	3 : 1	Matsubara et al., 2007b
Koshihikari	*j*	*Hbd1/Hbd1*	IRGC 105444[a]	*O. niv*	*hbd1/hbd1*	*hbd1/hbd1*	3 : 1	Miura et al., 2008
Sasanishiki	*j*	*Hbd2/Hbd2 hbd3/hbd3*	Habataki	*i*	*hbd2/hbd2 Hbd3/Hbd3*	*hbd2/hbd2 hbd3/hbd3*	15 : 1	Matsubara et al., 2007a
Koshihikari	*j*	*Hbd2/Hbd2 hbd3/hbd3*	Habataki	*i*	*hbd2/hbd2 Hbd3/Hbd3*	*hbd2/hbd2 hbd3/hbd3*	15 : 1	Yamamoto et al., 2007
J-147	*j*	*hca1/hca1 Hca2/Hca2*	IR24	*i*	*Hca1/Hca1 hca2/hca2*	*hca1/hca1 hca2/hca2*	15 : 1	Ichitani et al., 2012

j, O. sativa subspecies japonica. i, O. sativa subspecies indica. j/i, a variety derived from a cross between j and i. O. glu, O. glumaepatula. O. niv, O. nivara. [a]*These parents were used as donors to develop chromosomal segment substitution lines of a japonica variety (Taichung 65 or Koshihikari).* [b]*For convenience, semi-sterile segregants were categorized as normal.*

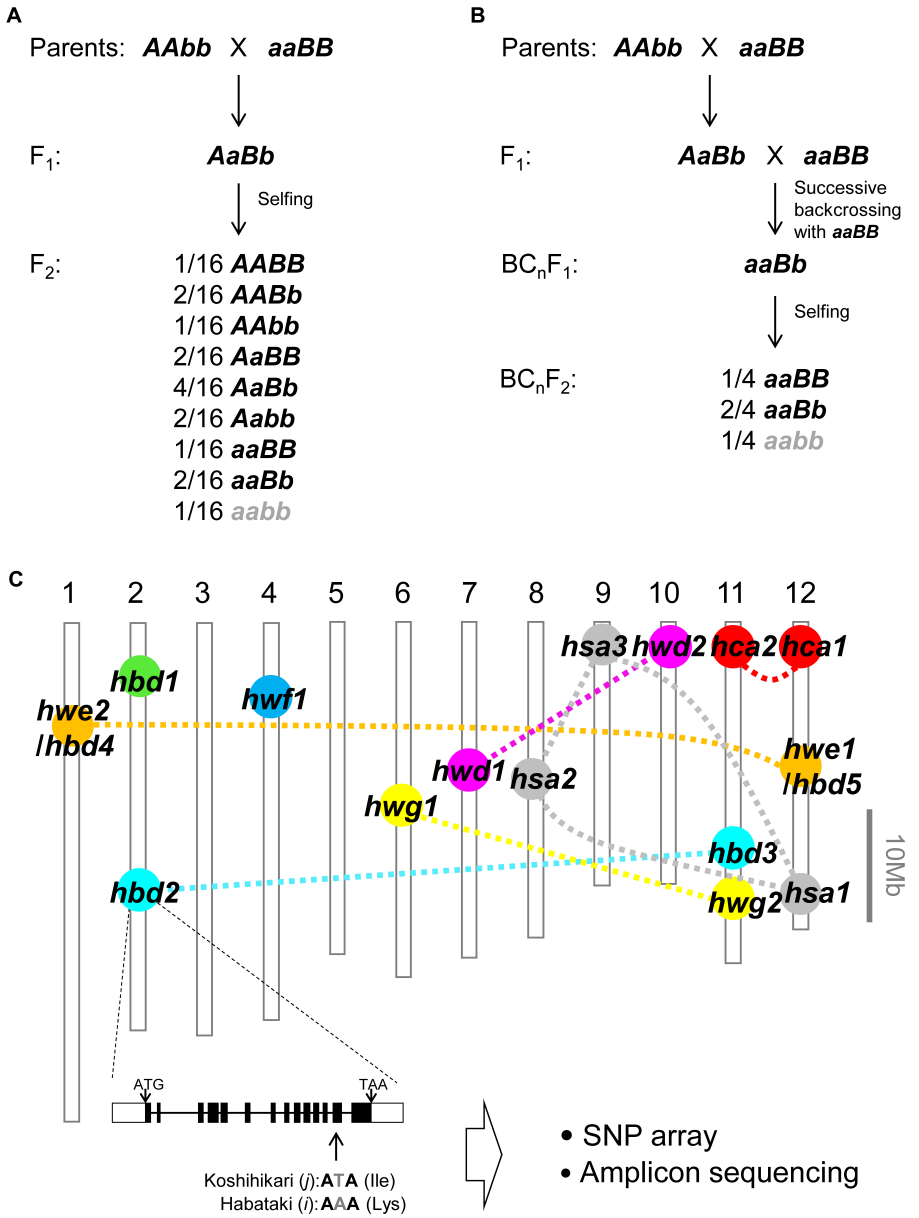

FIGURE 1 | The genetic basis of hybrid breakdown (HB). **(A)** Schematic representation of the HB genotype that segregates in the F_2 progeny. When the parental genotypes are *AAbb* and *aaBB*, 1/16 of the F_2 progeny without dominant allele, which is indicated by gray character (i.e., *aabb*), show reduced viability and/or fertility, although the other genotypes are normal. In some rice crosses, the F_2 progeny with only one dominant allele (i.e., *Aabb*, *aaBb*) also show reduced viability and/or fertility. **(B)** Schematic representation of the HB genotype that segregates in the BC_nF_2 progeny. Further, 1/4 of the BC_nF_2 progeny shows reduced viability and/or fertility. **(C)** Chromosomal location of genes underlying HB in rice crosses. The genes are roughly mapped based on the results of gene mapping in each study. A set of complementary genes are connected by the dotted line. Complementary genes with *hbd1* and *hwf1* have not been reported. The example of the functional nucleotide polymorphism causing HB published by Yamamoto et al. (2010) is shown, and this information will allow us to conduct a survey by SNP array or amplicon sequencing.

Independent of the autoimmune response and reciprocal silencing, an interaction between a *DEAD-box RNA helicase 18* and another gene (*MORPHEUS MOLECULE 1* as a likely candidate) that cause HB in *Arabidopsis* hybrids has been identified (Plötner et al., 2017; Vaid et al., 2020). Interestingly, the HB phenotype reduces between the F_3 and F_4 generations, implying epigenetic regulation of gene expression.

In rice hybrids, among the three *hybrid sterility-a* (*hsa*) loci, Kubo et al. (2016) recently showed that the *hsa1* locus consists of two genes, and that these genes encode a DUF1618 protein and an uncharacterized protein with some similarity to a nucleotide-binding protein, respectively. The molecular features of the remaining complementary genes, *hsa2* and *hsa3*, have not been reported.

HYBRID BREAKDOWN IN CONVENTIONAL RICE BREEDING

For decades, the bulk-population method was widely employed in conventional rice breeding. In this method, after crossing, the F_4 or F_5 population is raised by self-fertilization in bulk (rather than by the single-seed descent method) without artificial selection, but the early generation population is subjected to natural and viability selection (Allard, 1960; Ikehashi and Fujimaki, 1980 for details). In the bulk-population method, many weak and/or sterile genotypes can be expected to be eliminated from the population before the establishment of an advanced-generation population; therefore, this method may provide limited information about HB.

For the introgression or accumulation of desirable traits from donor(s) to a variety, rice breeders have often performed backcrossing or multiple parental crossing, followed by the bulk-population method. Empirically, it has long been known that these crossing methods allow the mitigation of the loss of selection candidates by reproductive barriers, because these methods often reduce the segregation of disruptive combinations of alleles associated with HB in a hybrid progeny. However, the mitigation of reproductive barriers by these crossing methods is inevitably dependent on the HB genotype of the parents.

HYBRID BREAKDOWN IN GENOMICS-ASSITED BREEDING

The publication of reference crop genome sequences and the development of next-generation sequencing technologies have accelerated the progress of the molecular breeding of crops (Kole et al., 2015). In this context, genomics-assisted breeding, such as genomic selection based on genotypes of genome-wide DNA markers, has been considered in crop breeding (Spindel and Iwata, 2018).

In genomics-assisted breeding of self-pollinated crops, advanced-generation populations, such as recombinant inbred lines, are often used as reference populations, from which genome-wide genotype and phenotype data are obtained. Subsequently, selection based only on marker genotypes is carried out in the progeny of early generations (e.g., F_2). In this selection scheme, it should be noted that the reference population does not usually provide the information of genomic region for HB, because weak and/or sterile progeny should have been eliminated in the early generations after crossing. Therefore, if such selection scheme is adopted, we will have to abandon some important selection candidates. Alternatively, we may select undesirable candidates such that HB becomes apparent in later generations.

DISCUSSION

Despite the limited number of studies on this subject, it seems that the loci underlying HB in rice crosses are diversified rather than shared (**Table 1** and **Figure 1**). As described above, the information on the distribution of HB-associated alleles among cross parents should be a prerequisite for rice breeding, particularly for maximizing the effectiveness of genomics-assisted breeding. Therefore, the additional detection of HB in rice crosses and the mapping of responsible genes in the rice genome are needed. In *Arabidopsis* hybrids, such data about the *R* genes and their interacting genes has been extensively surveyed (Alcázar et al., 2010, 2014; Chae et al., 2014). The reporting of the *hbd2* gene by Yamamoto et al. (2010) in a rice hybrid is a good example of an extensive survey performed using SNP arrays or amplicon sequencing (**Figure 1C**). The *hbd3* and *hsa1* genes are also candidates for this type of survey. Eventually, the development of criteria that allow us to predict HB based on the genomic information of the parental lines will serve as an important tool for genomics-assisted breeding. Even if a causal factor has not been identified as a single gene, closely linked markers, such as single-nucleotide polymorphisms, will be effective for classifying HB-associated haplotypes, because the linkage disequilibrium of cultivated rice has been estimated to extend to 100–200 kb, although that of wild species (such as *O. rufipogon*) may extend over several tens of kb (Mather et al., 2007; McNally et al., 2009; Huang et al., 2010). This information will enable the design of a more efficient and effective cross combination.

Bulked segregant analysis followed by next-generation sequencing can be useful for the mapping of HB-associated loci, as well as conventional linkage mapping (such as quantitative trait locus analysis), because HB segregants often show distinguishable features (about traits such as plant height, tiller number, and fertility) from normal growth segregants in each cross population. The acquisition of imaging data using a digital camera and drone loading may also play an important role in phenotyping in the laboratory and the paddy field. These efforts will provide valuable information not only to rice breeders, but also to evolutionary biologists.

Furthermore, if an HB-associated allele is identified, gene disruption through ion beam or genome editing may help overcome HB, as exemplified by F_1 hybrid sterility in rice (Koide et al., 2018; Xie et al., 2019).

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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I would like to dedicate this paper to the memory of Dr. Darshan Brar. He was an eminent rice breeder who vigorously introduced useful genes carried by wild species into cultivars beyond reproductive barriers.

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ORIGINAL RESEARCH
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Genetic Dissection and Validation of Chromosomal Regions for Transmission Ratio Distortion in Intersubspecific Crosses of Rice

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Transmission ratio distortion (TRD) refers to a widespread phenomenon in which one allele is transmitted by heterozygotes more frequently to the progeny than the opposite allele. TRD is considered as a mark suggesting the presence of a reproductive barrier. However, the genetic and molecular mechanisms underlying TRD in rice remain largely unknown. In the present study, a population of backcross inbred lines (BILs) derived from the cross of a *japonica* cultivar Nipponbare (NIP) and an *indica* variety 9311 was utilized to study the genetic base of TRD. A total of 18 genomic regions were identified for TRD in the BILs. Among them, 12 and 6 regions showed *indica* (9311) and *japonica* (NIP) alleles with preferential transmission, respectively. A series of F_2 populations were used to confirm the TRD effects, including six genomic regions that were confirmed by chromosome segment substitution line (CSSL)-derived F_2 populations from intersubspecific allelic combinations. However, none of the regions was confirmed by the CSSL-derived populations from intrasubspecific allelic combination. Furthermore, significant epistatic interaction was found between *TRD1.3* and *TRD8.1* suggesting that TRD could positively contribute to breaking intersubspecific reproductive barriers. Our results have laid the foundation for identifying the TRD genes and provide an effective strategy to breakdown TRD for breeding wide-compatible lines, which will be further utilized in the intersubspecific hybrid breeding programs.

Keywords: rice, transmission ratio distortion, backcross inbred lines, chromosome segment substitution line, allele frequency, epistatic interaction

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INTRODUCTION

Reproductive isolation is recognized as a powerful driving force for maintaining species identity (Ouyang et al., 2010; Dagilis et al., 2018). An important genetic factor maintaining reproductive isolation is transmission ratio distortion (TRD), which is defined as the allele inheritance in progenies of hybrids that show a statistically significant deviation from the expected Mendelian segregation ratios, also leading to deviations in genotype frequencies (Koide et al., 2012; Leppala et al., 2013; Li et al., 2019). TRD acts as one of the selfish genetic elements, distorting segregation or non-Mendelian transmission of alleles that can alter allele transmission in hybrids among progenies of heterozygotes, since the gametes carrying competing alleles increase their transmission over other alleles (Masly et al., 2006; Moyle and Graham, 2006; Koide et al., 2008a;

Phadnis and Orr, 2009; Phadnis, 2011). TRD has been described in intersubspecific and intrasubspecific hybrids in various animal and plant species, such as *Drosophila* (Mcdermott and Noor, 2012; Corbett-Detig et al., 2013), mouse (Bauer et al., 2005, 2007), mosquito (Shin et al., 2012), rice (Yang et al., 2012; Reflinur et al., 2014; Shen et al., 2017; Xie et al., 2019), *Arabidopsis* (Bikard et al., 2009; Leppala et al., 2013), and lettuce (Giesbers et al., 2019).

Multiple genetic factors could affect TRD such as chromosome segregation during meiosis (Fishman and Willis, 2005; Fishman and Sweigart, 2018) as well as prezygotic and postzygotic isolation (Harushima et al., 2001; Koide et al., 2008b). In addition, environmental fluctuations can result in allelic frequency changes at a single locus. For example, allelic frequency can change as conditions shift from drought to rainfall (Allard et al., 1992). Multiple loci have been proposed to explain reproductive isolation (Sano, 1990; Orr, 1996; Orr and Turelli, 2001; Phadnis and Orr, 2009). Several well-known reproductive isolation systems have been identified and characterized (Kusano et al., 2001; Kusano et al., 2002; Hu et al., 2017). For example, in mouse (*Mus musculus* L.), the *t* haplotype system including the *t* complex responder (*Tcr*) and the *t* complex distorter (*Tcd*) genes correspond to reduced fertility, and preferentially transmit the *Tcr* (*t*-haplotype) alleles in the heterozygous male progeny (Schimenti, 2000; Willison and Lyon, 2000). In *Drosophila* (*Drosophila melanogaster*), the segregation distorter (*SD*) system containing the distorter *SD/SD* + causes preferential transmission of male alleles due to the dysfunction of *SD* + spermatids (Merrill et al., 1999; Kusano et al., 2001, 2002). Three genes in fission yeast (*Schizosaccharomyces kombucha* and *Schizosaccharomyces pombe*), *cw9*, *cw27*, and *wtf4*, from the self-driver *wtf* (for with *Tf*) gene family encode both a gamete-killing poison and an antidote to the poison such that non-protected *S. pombe* (*Sp*) gametes are killed, and thus there is a loss of transmission of the *Sp* allele from heterozygotes (Hu et al., 2017; Nuckolls et al., 2017).

In rice, the *S5* killer-protector system includes three tightly linked genes on chromosome 6 that regulate both segregation distortion and hybrid embryo-sac fertility. *ORF4*+ and *ORF5*+ constituted a killer system that could kill the female gametes with *ORF3*−, resulting in distorted allele frequency in heterozygotes, but the *ORF3*+ allele could rescue these alleles (Yang et al., 2012). Recently, a toxin–antidote system of *qHMS7* was identified for male sterility that contains two tightly linked genes, *ORF2* and *ORF3*, encoding toxic and antidote genetic elements, respectively (Yu et al., 2018). The African rice *S1* allele (*S1-g*) including three closely linked genes (*S1A4*, *S1TPR*, and *S1A6*) that constitute a killer-protector system can eliminate gametes carrying the Asian allele (*S1-s*), leading to significant preferential transmission of the *S1-g* allele (Xie et al., 2019). Overall, these findings indicate that the genetic and molecular mechanisms regulating TRD are involved in allelic interactions at either a single locus or multiple loci. Therefore, it is critical to identify the genomic regions for TRD since it is the first step toward dissecting the molecular basis of such a complex trait.

The *indica* and *japonica* are the two major subspecies of Asian cultivated rice (*Oryza sativa* L.) (Huang et al., 2012). Reproductive barriers such as hybrid incompatibility and hybrid weakness are commonly observed in crosses between *indica* and *japonica*, and intersubspecific hybrids have more serious reproductive isolation than intrasubspecific crosses (Ouyang and Zhang, 2013). Meanwhile, reproductive isolation causes poor utilization of heterosis in hybrid rice (Ouyang et al., 2010). The objective of the present study is to identify the TRD regions to investigate the genetic basis of TRD in rice. We conducted TRD analyses by using backcross inbred lines (BILs), which were derived from an intersubspecific cross of the *japonica* cultivar Nipponbare and the *indica* variety 9311. As a result, a number of genomic regions were identified for TRD in the intersubspecific mapping BILs and validated in CSSL-derived F_2 populations. Furthermore, interaction effects of two regions on TRD were explored. These findings could provide new insights into the genetic basis of TRD in rice.

MATERIALS AND METHODS

BIL Population and CSSL-Derived Populations

The BIL population and chromosome segment substitution line (CSSL-derived) population were used to dissect the genetic basis of TRD in rice. The BILs, which included 437 lines, were developed by single-seed descent from the cross between two genome-sequenced rice cultivars, the *japonica* variety Nipponbare (NIP) and the elite *indica* restorer line 9311 (Yuan et al., 2019). Briefly, NIP (male) was crossed with 9311 (female), and the F_1 was then backcrossed to 9311 (female) to obtain BC_1F_1 lines. Subsequently, 437 BC_1F_8 lines were produced by the single-seed descent method and were genotyped for TRD analysis. To validate the effect of the TRD regions, three types of CSSLs were derived using the backcross scheme with marker-assisted selection approach (Sun et al., 2015; Zhang et al., 2020). First, 12 CSSLs, each carried the introduced NIP segment surrounding the TRD region of interest within the 9311 background, were developed by using NIP (male) as the donor and 9311 (female) as the recurrent parent, named as NY lines. Second, nine CSSLs in the ZS97 background were developed using NIP (male) as the donor and an *indica* cultivar Zhenshan 97 (ZS97, female) as the recurrent parent, then named as NZ lines. Third, five CSSLs each carrying the introduced MH63 segment in the ZS97 background were developed using an *indica* Minghui 63 (MH63, male) as the donor and ZS97 (female) as the recurrent parent, named as MZ lines. These 26 CSSLs were then crossed with the recurrent parent (9311 or ZS97) to generate 26 relevant F_2 populations (hereafter named as the NY-, NZ-, and MZ-derived populations, respectively). The other line harboring two introduced NIP segments surrounding *TRD1.3* and *TRD8.1* was used to generate an additional F_2 population. The BIL population and CSSLs were grown at the experimental field of Huazhong Agricultural University (HAU) in Wuhan (30.48N, 114.2E), China, and each line was planted in a row with 10 individual spacings of 16.7 × 26.6 cm. The CSSL-derived F_2 populations, comprising a various number of individuals were planted in the same field and used for genotype analysis. Field management was carried out according to the local standard practices.

Genotype Analyses

Genomic DNA was extracted from young seedling leaves using the CTAB method as described previously (Murray and Thompson, 1980). The 437 BILs were genotyped using a genotyping-by-sequencing (GBS) strategy (Yuan et al., 2019). The GBS library were generated as described in a previous report (Elshire et al., 2011), where the genomic DNA from each of the BILs was digested with *Mse*I, followed by end blunting, dATP addition at the 3′ end, and ligation of Y-adapters. Fragments of 300–550 base pairs (bp) in size were isolated using a Gel Extraction kit (Qiagen, Valencia, CA, United States). These fragments were then purified using the Agencourt AMPure XP System, then diluted for sequencing. Sequencing was performed on the selected tags using Illumina HiSeq (Illumina, San Diego, CA, United States) with the paired-end mode and 150-bp read length (Huang et al., 2009; Elshire et al., 2011). The Burrows Wheeler Aligner software (V0.7.15) was used to map the clean reads for each sample on the reference genome (MSU7.0[1]) (Li and Durbin, 2009). Single-nucleotide polymorphism (SNP) calling was performed by Genome Analysis Toolkit (McKenna et al., 2010). In total, 49,890 high-quality SNPs were identified for the BILs after filtering out the low-quality SNPs using three criteria: (1) minor allele frequency $\geq$ 5%, (2) heterozygous genotype $\leq$ 20%, and (3) missing genotype $\leq$ 20%. The heterozygous genotypes were set as missing data. Due to 37 lines with >20% missing data, a high-density bin map was generated based on SNP genotypes of 400 BILs as previously described with a minor modification (Huang et al., 2009; Li et al., 2017). Briefly, the genotype of each line was scanned with a sliding window of 15 SNPs and a step size of 1. An "a/b" ratio of 12:3 or higher was recognized as "a," and 3:12 or lower was recognized as "b." The missing genotypes were coded as "—". Adjacent windows with the same genotype were combined into a block, and the recombinant breakpoint information was assumed to be at the boundary of adjacent blocks with different genotypes. The interval between two adjacent crossovers in the entire population was defined as a recombination bin. A genetic linkage bin map was constructed for further analysis using the "MAP" function in IciMapping (Li et al., 2007). The insertion/deletion (Indel) and simple sequence repeat (SSR) polymorphic markers that could separate the paired parents (ZS97 vs. NIP, 9311 vs. NIP, ZS97 vs. MH63) were selected to genotype the CSSL-derived populations. To determine the digenic interaction between *TRD1.3* and *TRD8.1*, two polymorphic markers, SD1C and SD8C, that were linked with *TRD1.3* and *TRD8.1*, respectively, were used to classify the nine genotypes in the F_2 population. All the Indel and SSR markers were separated using 4% polyacrylamide gel electrophoresis and silver stains for visualization. The bin genotypes of the BILs are provided in **Supplementary Table S1**.

Transmission Ratio Distortion Analysis

For BILs, the genotype and allele frequencies are the same values for each bin since the BILs had only two homozygous genotypes (NIP and 9311). Deviation of observed allele frequency of each bin in the BILs from the theoretical Mendelian segregation ratio (3:1 for both allele and genotype frequency) was investigated by a chi-square test. The chi-square (χ^2) and *P*-values were determined by using R function *chisq.test*[2]. The bin showing non-Mendelian segregation ($P < 0.0001$) was initially considered to have a significant TRD effect. The TRD region analysis in the BILs was performed using the single-marker analysis (SMA) model in QTL IciMapping (v4.0) (Li et al., 2007). A significance level of LOD > 4.5 was set as the threshold to declare the presence of the putative TRD effect in a given bin. The nomenclature of the TRD region or locus followed the principles suggested by a previous report (McCouch, 2008). When several adjacent bins exhibited TRD effects with the same allele transmission pattern, a TRD region was presented with peak bin and its interval defined as the first and last bins that showed TRD effects (Li et al., 2017, 2019). The statistical χ^2 and *P*-values of the observed allele and genotype frequency of each polymorphic marker distorted from the expected segregation ratios in the CSSL-derived populations, and nine genotype ratios in the additional F_2 population were also determined by using R function *chisq.test*.

RESULTS

Bin Markers Displaying Transmission Ratio Distortion

The BIL population (n = 400) was used to evaluate the genetic basis of TRD. The high-density bin map was generated with 3,235 bins as markers, which were evenly distributed on all of the 12 chromosomes, and the bin lengths ranged from 30 kb to 3.0 Mb with an average of 115 kb in these 400 BILs. There were 745 out of 3,235 bins showing significant distortion from the expected Mendelian allele segregation ratio (3:1), which were distributed on all of the chromosomes except for chromosomes 7, 9, 10, and 11 (**Supplementary Figure S1** and **Supplementary Table S2**). Among all the distorted markers, 179 and 566 markers were significantly skewed toward NIP and 9311, respectively.

Detection of Genomic Regions for Transmission Ratio Distortion in Backcross Inbred Lines

To detect the distorted genomic regions more precisely, the SMA method in QLT IciMapping was conducted in the BILs (Li et al., 2007). A total of 18 genomic regions (91.9 Mb, approximately 24.8% of genome coverage based on the Nipponbare genome) were identified for TRD in the BILs (**Figure 1** and **Supplementary Table S3**). Among them, chromosomes 1, 8, and 12 each contained three TRD loci or regions, whereas one region was identified on chromosome 5. Twelve and six regions with the alleles were significantly skewed toward 9311 and NIP, respectively (**Supplementary Table S3**). These results were consistent with the allele segregation ratios determined by using bin markers in the BILs.

Among the identified TRD regions, *TRD1.3* in Bin390 (38.3–38.4 Mb) on chromosome 1 had the most significant effect

[1]http://rice.plantbiology.msu.edu/index.shtm

[2]http://www.r-project.org/

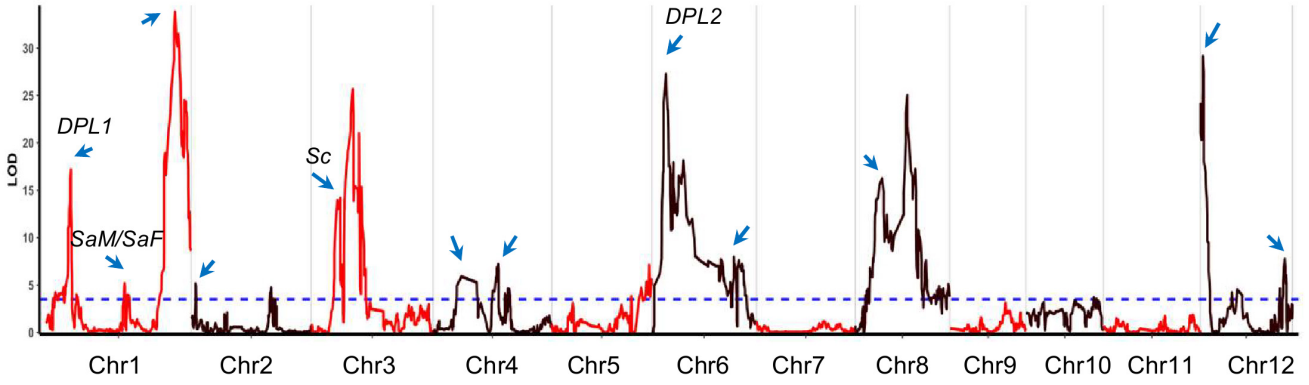

FIGURE 1 | The transmission ratio distortion (TRD) regions identified in backcross inbred lines (BILs). The *x*-axis represents the physical location along each numbered chromosome. The *y*-axis represents the logarithm of odds (LOD) values. Horizontal dashed line indicates the declaration threshold. Blue arrows represent the regions or genes associated with TRD or segregation distortion in previous studies. Four reported genes are highlighted.

(LOD = 33.8; χ^2 = 179.4) and was significantly skewed toward the NIP allele (**Supplementary Table S3**). Four regions (*TRD3.2*, *TRD6.1*, *TRD8.2*, and *TRD12.1*) showed a moderate effect (LOD $\geq$ 20.0), and all of them were significantly skewed toward 9311. For the remaining 13 regions with relative minor effects (LOD < 20.0), eight regions (*TRD1.1*, *TRD1.2*, *TRD3.1*, *TRD4.1*, *TRD6.2*, *TRD8.1*, *TRD8.3*, and *TRD12.3*) in which alleles were significantly skewed toward 9311, and five regions (*TRD2.1*, *TRD2.2*, *TRD4.2*, *TRD5*, and *TRD12.2*) were significantly skewed toward NIP (**Supplementary Table S3**). These data indicate that 9311 gametes of most TRD regions carried competing alleles, thus gaining a transmission advantage over NIP alleles in the BILs.

Validation of the Transmission Ratio Distortion Regions in Chromosome Segment Substitution Line-Derived Populations

To validate the TRD effects, a total of 12 CSSLs (NY) that each carried a particular introduced NIP segment (*TRD1.2*, *TRD1.3*, *TRD2.1*, *TRD2.2*, *TRD3.1*, *TRD3.2*, *TRD4.1*, *TRD5*, *TRD6.2*, *TRD8.1*, *TRD8.3*, or *TRD12.2*) were selected and crossed with 9311 to produce the 12 NY-derived populations (**Supplementary Table S4**). Meanwhile, nine CSSLs that each carried the introduced NIP segment encompassing a corresponding locus (*TRD1.3*, *TRD2.1*, *TRD2.2*, *TRD3.1*, *TRD3.2*, *TRD4.1*, *TRD5*, *TRD8.1*, or *TRD12.2*) were crossed with ZS97 to produce the relevant NZ-derived populations (**Supplementary Table S5**). Five CSSLs that each carried the introduced *indica* MH63 segment harboring a target locus (*TRD1.2*, *TRD2.1*, *TRD3.2*, *TRD8.1*, or *TRD12.2*) were also crossed with ZS97 to produce the relevant intrasubspecific MZ-derived populations (**Supplementary Table S6**). In addition, 2–11 polymorphic SSRs and Indel markers (**Supplementary Table S7**) that were evenly distributed in every relevant TRD region were used to investigate the genotype of the F_2 populations.

TRD analysis in the 12 NY-derived populations confirmed that six regions (*TRD1.2*, *TRD4.1*, *TRD5*, *TRD6.2*, *TRD8.1*, and *TRD8.3*) exhibited a significant effect on TRD (**Figure 2**). For example, the NY(*TRD1.2*)-derived population (n = 188) was genotyped using seven polymorphic markers that were evenly distributed in the *TRD1.2* region to validate the TRD effect (**Figure 2** and **Supplementary Table S4**). Six out of seven markers, including ID01C061, ID01C063, ID01C065, ID01C067, ID01C068, and ID01C071 were significantly distorted from both the expected ratio (1:2:1) for genotype frequency and the expected ratio (1:1) for allele frequency. These six distorted markers were all skewed toward 9311 (**Supplementary Table S4**), indicating that the 9311 alleles at *TRD1.2* were transmitted to the progeny at higher frequencies than NIP alleles in heterozygotes. The NY (*TRD8.1*)-derived population (n = 272) was genotyped using seven polymorphic markers, and these markers were all significantly distorted from the expected Mendelian genotype and allele segregation ratios, and skewed toward 9311 (**Figures 2**, **3** and **Supplementary Table S4**). Using the same approach, *TRD4.1*, *TRD5*, *TRD6.2*, and *TRD8.3* effects were also confirmed in the relevant NY-derived populations. The genotypes and alleles at the three regions (*TRD4.1*, *TRD6.2*, and *TRD8.3*) were all skewed toward 9311, and the NIP alleles had preferential transmission at *TRD5*, leading to the genotype skewed toward NIP in the progeny (**Figure 2** and **Supplementary Table S4**). Thus, five TRD regions (*TRD1.2*, *TRD4.1*, *TRD6.2*, *TRD8.1*, and *TRD8.3*) in which the alleles were significantly skewed toward 9311, indicating that the 9311 alleles were transmitted to the progeny at higher frequencies than NIP alleles in the F_2 populations.

Three regions (*TRD4.1*, *TRD5*, and *TRD8.1*) also exhibited significant effects on TRD in NZ-derived populations (**Figure 2**). *TRD4.1* and *TRD8.1* were both confirmed in NY-derived and NZ-derived populations. Nine assayed markers at *TRD4.1* were all skewed toward *indica* alleles (9311 or ZS97). The TRD effect of *TRD8.1* was also validated in the NZ (*TRD8.1*)-derived population (n = 175), and the markers within the region were significantly skewed toward ZS97, while the *japonica* (NIP) alleles at *TRD5*

	NY-derived		NZ-derived		MZ-derived	
	TRD	Towards	TRD	Towards	TRD	Towards
TRD1.2	Y	9311	NA	NA	N	E
TRD1.3	N	E	N	E	NA	NA
TRD2.1	N	E	N	E	N	E
TRD2.2	N	E	N	E	NA	NA
TRD3.1	N	E	N	E	NA	NA
TRD3.2	N	E	N	E	N	E
TRD4.1	Y	9311	TRD	ZS97	NA	NA
TRD5	Y	NIP	TRD	NIP	NA	NA
TRD6.2	Y	9311	NA	NA	NA	NA
TRD8.1	Y	9311	TRD	ZS97	N	E
TRD8.3	Y	9311	NA	NA	NA	NA
TRD12.2	N	E	N	E	N	E

FIGURE 2 | Validation of 12 genomic regions for TRD in the chromosome segment substitution line (CSSL)-derived F_2 populations. NY-derived and NZ-derived indicate the populations developed using Nipponbare (NIP) as the donor in 9311 and Zhenshan 97 (ZS97) backgrounds, respectively. MZ-derived represent the F_2 population developed using Minghui 63 (MH63) as the donor in the ZS97 background. "N" and "Y" indicate the absence and presence of TRD effect in a given region, respectively. "Toward" indicates that the allele in the heterozygote was preferentially transmitted to progeny. "E" indicates that the two alleles in the heterozygote were transmitted equally to progeny. NA, data not available.

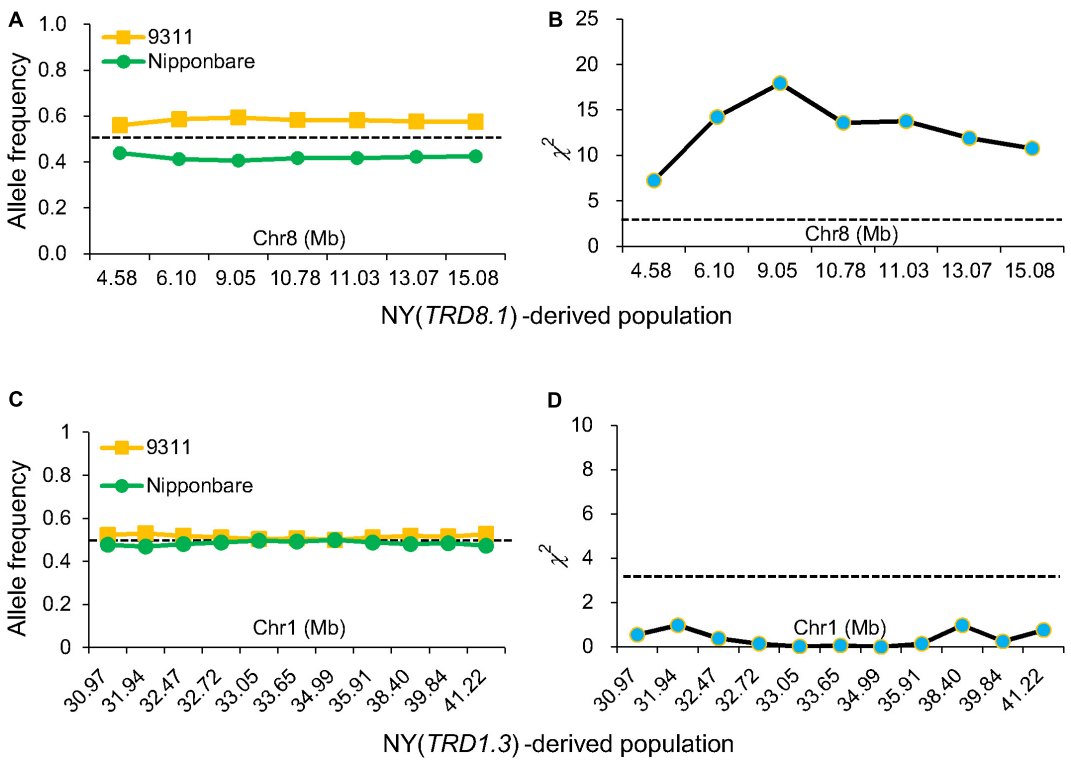

FIGURE 3 | Validation of *TRD1.3* and *TRD8.1* effects on TRD. Allele frequencies **(A)** and chi-square (χ^2) test for allele frequencies **(B)** at seven markers in the *TRD8.1* region in the NY(*TRD8.1*)-derived population (n = 272). Allele frequencies **(C)** and chi-square test for allele frequencies **(D)** at 11 markers in the *TRD1.3* region in the NY (*TRD1.3*)-derived population (n = 131). Horizontal dashed lines indicate the theoretical segregation ratio (1:1) in **(A,C)** and the significant threshold at $P < 0.05$ in **(B,D)**. The x-axis represents the physical locations of the used markers along the numbered chromosome.

were transmitted to the progeny at higher frequencies than either 9311 or ZS97 alleles (**Figure 2** and **Supplementary Table S5**). These results indicated that either 9311 or ZS97 alleles at *TRD4.1* and *TRD8.1* revealed preferential transmission to the progeny over the NIP alleles (**Figure 2** and **Supplementary Tables S4, S5**).

Meanwhile, five regions (*TRD1.2*, *TRD2.1*, *TRD3.2*, *TRD8.1*, and *TRD12.2*) with TRD effects detected in the BILs were not validated in the MZ-derived populations (**Figure 2**). Three regions of *TRD2.1*, *TRD3.2*, and *TRD12.2* had no effects on TRD in either NY-derived population or NZ-derived population (**Figure 2** and **Supplementary Table S6**). Consistently, these distorted regions were not detected in the previous study with an F_2 population derived from the same combination of MH63 and ZS97 (Li et al., 2017).

Epistatic Interaction Effect Between *TRD1.3* and Other Genomic Regions

To validate the *TRD1.3* effect in the BILs, NY(*TRD1.3*)-derived population (n = 131) and NZ (*TRD1.3*)-derived population (n = 157) were used (**Supplementary Tables S4, S5**). Unexpectedly, all assayed markers in the *TRD1.3* region showed normal-Mendelian segregation ratios in both F_2 populations (**Figures 3C,D**). These results indicated that the *TRD1.3* effect was dependent on other loci within the backgrounds of 9311 or ZS97. To detect the regions that interacted with *TRD1.3*, the BILs were separated into two subpopulations that had a fixed NIP (named as SubN, n = 172) or 9311 genotypes (named as SubY, n = 181) at the *TRD1.3* region, respectively. The other 47 lines were removed due to missing data (n = 17) and heterogeneous recombination (n = 30) in the *TRD1.3* region. A total of 14 genomic regions were identified in these two subpopulations and overlapped with the TRD regions detected in the BILs (**Supplementary Table S8**). Among them, 9 and 11 regions were detected in SubN and SubY, respectively. Six regions (*TRD1.1*, *TRD3.1*, *TRD3.2*, *TRD6.1*, *TRD8.3*, and *TRD12.1*) were identified in common in both the subpopulations, suggesting that they were not affected by the effect of *TRD1.3*. However, there were five regions (*TRD1.2*, *TRD4.1*, *TRD5*, *TRD6.2*, and *TRD8.1*) that could only be identified in SubY, but not in SubN (**Figure 4** and **Supplementary Table S8**), implying that these five regions may interact with *TRD1.3*. These results indicate that genetic interactions play a role in affecting TRD in the BILs.

As *TRD8.1* was the most significant region among the mentioned five regions that were identified only in SubY and was validated in CSSL-derived populations of both 9311 and ZS97 backgrounds (**Figures 2**, **3A,B**), it was interesting to determine whether there was an interaction between *TRD1.3* and *TRD8.1*. One line that carries two introduced NIP segments containing both TRD regions was selected and crossed with 9311 to generate additional F_2 population. In this F_2 population (n = 524), two markers (SD1C and SD8C), tightly linked with *TRD1.3* and *TRD8.1*, respectively, were used to classify the nine genotypes. The nine genotype ratios did not fit the expected Mendel's

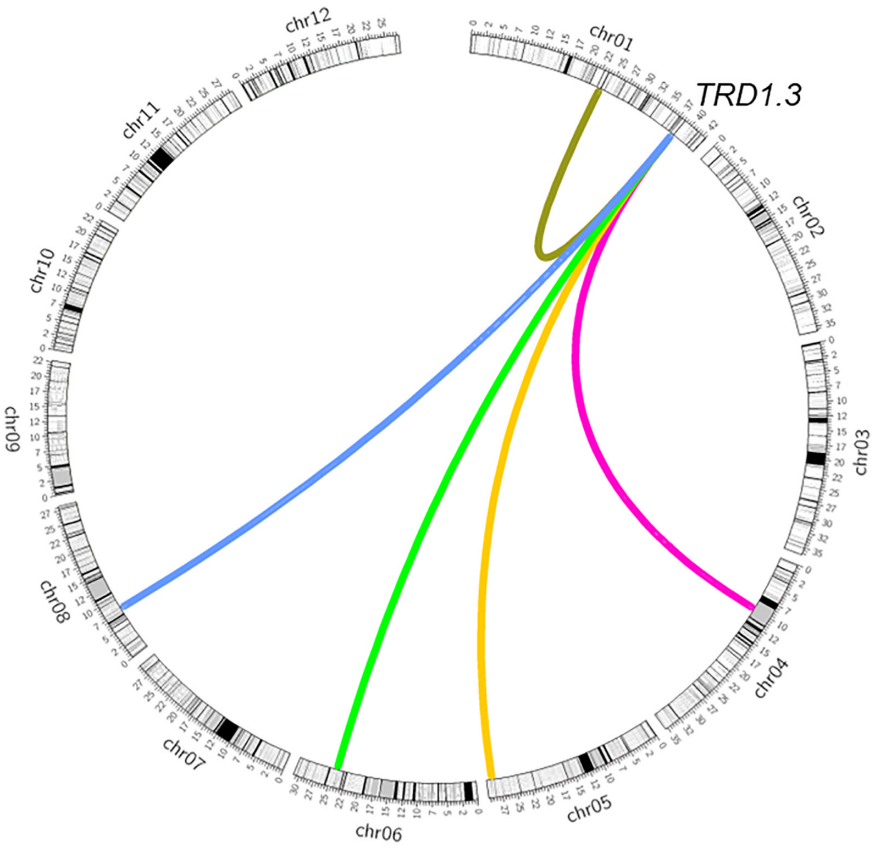

FIGURE 4 | Epistatic interaction of *TRD1.3* with other chromosomal regions affecting transmission ratio distortion. Rice chromosomes with bins are indicated in the outer circle. The colored connection lines represent the epistatic interactions of *TRD1.3* with the regions (*TRD1.2*, *TRD4.1*, *TRD5*, *TRD6.2*, *TRD8.1*).

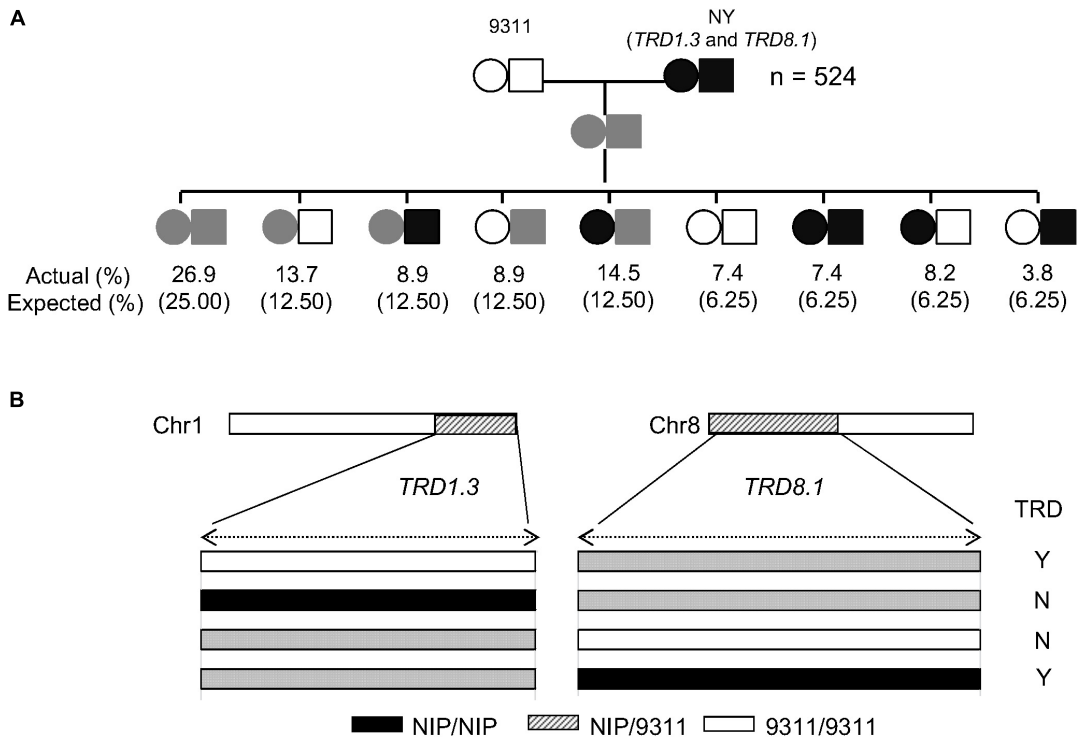

FIGURE 5 | Validation of the epistatic interaction between *TRD1.3* and *TRD8.1*. **(A)** Segregation ratio of the nine genotypes at *TRD1.3* and *TRD8.1* in the F_2 population (*n* = 524). "NY(*TRD1.3/TRD8.1*)" represent the line harboring two introduced NIP segments surrounding *TRD1.3* and *TRD8.1* within the 9311 background. The circle and rectangle indicate *TRD1.3* and *TRD8.1*, respectively. The white and black represent the homozygous 9311 and NIP genotype, respectively. The gray (streak) represents the heterozygous genotype. "Actual (%)" and "expected (%)" represent actual segregation ratio and theoretical Mendelian segregation ratio for nine genotypes. **(B)** Schematic diagram of the *TRD1.3* and *TRD8.1* interaction. "N" and "Y" indicate absence and presence of significant TRD effect in a given heterozygous region. The white, black, and gray bars represent homozygous genotypes of 9311 and NIP, and heterozygote, respectively.

segregation laws in this F_2 population (**Figure 5A**), suggesting that the two regions had a significant digenic interaction ($\chi^2 = 24.1$; $P = 2.2 \times 10^{-3}$). Moreover, the two alleles (NIP and 9311) of *TRD8.1* were equally transmitted to the progeny when *TRD1.3* carried the NIP allele, but *TRD8.1* had a significant effect on TRD given that *TRD1.3* had the 9311 allele (**Figure 5B** and **Supplementary Table S9**). These results confirmed that *TRD1.3* and *TRD8.1* had significant interaction effects on TRD in the segregation populations.

DISCUSSION

Identification and characterization of the TRD regions are essential for better understanding of the genetic basis of TRD. In the present study, we have identified 18 TRD regions in which the alleles segregated abnormally in the BIL population that was derived from the intersubspecific cross of NIP and 9311. Among them, 12 regions (75.1%) were significantly skewed toward the *indica* (9311) alleles, suggesting that the gametes carrying the *indica* alleles were transmitted at higher frequencies to the progeny than the *japonica* (NIP) alleles. These findings are consistent with previous results that the majority of *indica* alleles are transmitted as the favored alleles in intersubspecific crosses (Wang et al., 2009; Reflinur et al., 2014).

We used the CSSLs that contained a particular *japonica* (NIP) segment within two *indica* backgrounds (9311 and ZS97) to further test the TRD effects (**Supplementary Tables S4, S5**). Using NY-derived segregating populations, 6 out of 12 regions were validated, whereas 3 out of 9 regions were confirmed using NZ-derived segregating populations (**Figure 2**). There were three TRD regions (*TRD4.1*, *TRD5*, *TRD8.1*) in the 9311/NIP BILs consistently validated in both the two *indica* backgrounds, in which the different type of alleles preferentially transferred to the progeny, indicating complex mechanisms across intersubspecific hybrids (**Supplementary Tables S4, S5**). In addition, by comparing the regions detected in the BILs with those identified in the MH63/ZS97 F_2 population (Li et al., 2017), we found that only *TRD4.1* was colocalized in the overlapping region of *Sd4*, suggesting that *TRD4.1* was robust in both inter- and intrasubspecific populations.

It is notable that some TRD regions detected in the BILs were not fully validated in CSSL-derived populations (**Figure 2**). For example, six genomic regions of *TRD1.3*, *TRD2.1*, *TRD2.2*, *TRD3.1*, *TRD3.2*, and *TRD12.2* showed significant effects on TRD in the BILs, but could not be validated in their corresponding CSSL-derived F_2 populations. These results suggest that epistatic interaction hidden in genetic backgrounds could result in allelic frequency alteration as reported previously (Leppala et al., 2013;

Li et al., 2017). We found that *TRD1.3* exhibited a significant epistatic interaction with *TRD8.1* (**Figure 5**). Moreover, a different allele-transmitted ability was observed in *TRD1.3* and *TRD8.1*. The NIP and 9311 alleles of *TRD8.1* were equally transmitted to the progeny if *TRD1.3* had the NIP allele, but the 9311 alleles at *TRD8.1* were preferentially transmitted to the progeny when *TRD1.3* was the 9311 allele. However, the NIP alleles at *TRD1.3* was preferentially transmitted to the progeny given that *TRD8.1* was the NIP allele, but the two alleles (NIP and 9311) of *TRD1.3* were equally transmitted to the progeny given that *TRD8.1* was the 9311 allele (**Supplementary Table S9**). In this case, the *japonica* alleles at *TRD8.1* could help the *japonica* alleles at *TRD1.3* have a higher chance of being transmitted to the progeny. This finding suggests that many specific TRD alleles could positively contribute to breaking intersubspecific reproductive barriers.

By comparing the TRD regions detected in this study with the ones from previous studies, we found that at least 12 regions were colocalized in the same or overlapping regions that harbored genes/loci associated with TRD or hybrid sterility (Wan et al., 1996; Li et al., 2017, 2019; Shen et al., 2017) (**Figure 1** and **Supplementary Table S3**). Six regions (*TRD1.1*, *TRD1.3*, *TRD4.1*, *TRD6.1*, *TRD12.1*, and *TRD12.3*) were mapped in the same regions reported previously for segregation distortion (Mizuta et al., 2010; Li et al., 2017). Intriguingly, two regions (*TRD1.1* and *TRD6.1*) were located in the same regions covering the known genes *DPL1* and *DPL2* in which divergent alleles were involved in the allele TRD by pollen incompatibility in rice hybrids (Mizuta et al., 2010). *TRD3.1* in peak Bin879 (8.67–8.82 Mb) is located surrounding the *Sc* gene that has been reported to affect allele TRD (Shen et al., 2017). The other six regions (*TRD2.1*, *TRD3.1*, *TRD4.2*, *TRD8.2*, and *TRD12.1*) contain previously reported QTLs/genes for hybrid sterility that lead to reproductive barriers (**Supplementary Table S3**) (Wan et al., 1996; Long et al., 2008; Mizuta et al., 2010; Li et al., 2017; Shen et al., 2017). *TRD1.2* with peak Bin233 (23.38–23.47 Mb) was mapped around the two adjacent genes *SaM* and *SaF* that are involved in *indica*/*japonica* hybrid male sterility (Long et al., 2008). Therefore, the mechanisms underlying hybrid sterility also caused different probabilities of allelic transmission. In addition, we also identified six novel regions (*TRD2.2*, *TRD3.2*, *TRD5*, *TRD8.1*, *TRD8.3*, and *TRD12.2*) that have not been reported before (**Supplementary Table S3**). In particular, *TRD2.2* was found in peak Bin654 (approximately 200 kb) on chromosome 2. *TRD3.2* is located in peak Bin910 (12.33–12.56 Mb) with an approximately 200-kb size. *TRD5* was found in peak Bin1676 (approximately 100 kb) on chromosome 5. *TRD8.1* was located in peak Bin2207 (8.32–8.38 Mb) with an approximately 69-kb size. These novel TRD regions with a fine resolution can be further functionally exploited to improve the utilization of subspecific hybrid breeding programs.

Transmission ratio distortion can result from gametic or zygotic selections. We found that the allele frequencies at six regions (*TRD1.2*, *TRD4.1*, *TRD5*, *TRD6.2*, *TRD8.1*, and *TRD8.3*) in the relevant CSSL-derived F_2 populations were significantly skewed toward NIP or 9311 allele, indicating that the gametic factors were involved in these regions. However, the heterozygous genotypes at the six regions in the F_2 populations all showed the normal segregation ratio (approximately 0.5), and the observed frequency of the three genotypes exhibited no significant difference compared with the theoretical genotype frequency (**Supplementary Tables S4, S5**). These results indicate that the gametic factor is involved in the TRD regions. Meanwhile, other six regions (*TRD1.3*, *TRD2.1*, *TRD2.2*, *TRD3.1*, *TRD3.2*, and *TRD12.2*) in corresponding F_2 populations showed normal allele segregation ratios (**Supplementary Tables S5, S6**), which did not provide enough information to distinguish gametic or zygotic selections.

CONCLUSION

Eighteen TRD regions were identified in the BIL population that was derived from the intersubspecific cross of 9311 and NIP with a high-density SNP map. Among them, three TRD regions were validated in CSSL-derived secondary populations within both the two *indica* genetic backgrounds. Furthermore, digenic interaction between *TRD1.3* and *TRD8.1* was found affecting TRD. The identification of the TRD regions can be utilized in further map-based cloning of the causal genes for TRD, which will facilitate understanding of the genetic basis of reproductive barriers. In addition, our data reveal that TRD, in some cases, can positively contribute to breaking the intersubspecific reproductive barrier, therefore providing an attractive strategy of introgression breeding in intersubspecific hybrids in rice.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in DRYAD 10.5061/dryad.sf7m0cg44.

AUTHOR CONTRIBUTIONS

SY designed and conceived the research. CZ, DW, LT, QS, ZY, XT, and JW developed the population and NILs. CZ and DW conducted the experiments. CZ and JW analyzed the data. CZ, HH, and SY wrote the manuscript. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.563548/full#supplementary-material

Supplementary Figure S1 | Allele frequencies at the bins in backcross inbred lines (BILs). The cyan and orange colors indicate 9311 and Nipponbare alleles, respectively. The *x*-axis represents the physical location of the bins along each numbered chromosome. The *y*-axis represents the allele frequencies. Horizontal dashed lines indicate the theoretical allele segregation ratio (3:1) in the BILs.

Supplementary Table S1 | Bin genotypes of the backcross inbred line population derived from the cross of 9311 and Nipponbare.

Supplementary Table S2 | The number of bins with allele transmission ratio distortion in the backcross inbred line population derived from the cross of 9311 and Nipponbare.

Supplementary Table S3 | Genomic regions detected for allele transmission ratio distortion in the backcross inbred lines.

Supplementary Table S4 | Twelve transmission ratio distortion regions tested in NY-derived populations in the 9311 background.

Supplementary Table S5 | Validation of nine transmission ratio distortion regions in NZ-derived populations in the ZS97 background.

Supplementary Table S6 | Validation of five transmission ratio distortion regions in MZ-derived populations in the ZS97 background.

Supplementary Table S7 | Primers used for genotyping F_2 populations.

Supplementary Table S8 | The TRD regions detected for allele transmission ratio distortion in the two subpopulations.

Supplementary Table S9 | Epistatic interaction of *TRD1.3* and *TRD8.1* regions in the F_2 population derived from NY(*TRD1.3*/*TRD8.1*) and 9311.

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Diploid Male Gametes Circumvent Hybrid Sterility Between Asian and African Rice Species

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In F_1 hybrids of *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice), heterozygosity leads to a complete gamete abortion because of allelic conflict at each of the 13 *hybrid sterility* (*HS*) loci. We systematically produced 19 plants from the F_1 hybrids of both the rice species by the anther culture (AC) method. Five of the 19 interspecific hybrid plants were partially fertile and able to produce seeds. Unlike ordinal doubled haploid plants resulting from AC, these regenerated plants showed various ploidy levels (diploid to pentaploid) and different zygosities (completely homozygous, completely heterozygous, and a combination). These properties were attributable to meiotic anomalies in the interspecific hybrid F_1 plants. Examination of the genetic structures of the regenerated plants suggested meiotic non-reduction took place in the interspecific hybrid F_1 plants. The centromeric regions in the regenerated plants revealed that the abnormal first and/or second divisions of meiosis, namely the first division restitution (FDR) and/or second division restitution (SDR), had occurred in the interspecific hybrid. Immunohistochemical observations also verified these phenomena. FDR and SDR occurrences at meiosis might strongly lead to the formation of diploid microspores. The results demonstrated that meiotic anomalies functioned as a reproductive barrier occurred before the *HS* genes acted in gamete of the interspecific hybrid. Although such meiotic anomalies are detrimental to pollen development, the early rescue of microspores carrying the diploid gamete resulted in the fertile regenerated plants. The five partially fertile plants carrying tetraploid genomes with heterozygous alleles of the *HS* loci produced fertile diploid pollens, implying that the diploid gametes circumvented the allelic conflicts at the *HS* loci. We also proposed how diploid male gametes avoid HS with the killer–protector model.

Keywords: anther culture, division restitution, hybrid sterility, interspecific hybrid, meiosis, rice, tetraploid, diploid gamete

INTRODUCTION

Although cultivated rice species *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice) both have AA genomes, the first filial generation (F_1) between these two species does not produce fertile seeds (Morinaga and Kuriyama, 1957; Oka, 1957). This type of reproductive isolation, designated as hybrid sterility (HS), is associated with abnormal gamete development and sterility (Morinaga and Kuriyama, 1957; Oka, 1957; Sano et al., 1979). To date, 13 *HS* loci have been reported to be involved in HS in F_1 hybrids between *O. sativa* and *O. glaberrima* (*sat–gla*) (Sano, 1983, 1990; Doi et al., 1998, 1999; Taguchi et al., 1999; Ren et al., 2006; Zhang et al., 2006; Li et al., 2011; Ouyang and Zhang, 2013; Xu et al., 2014; Yu et al., 2018). In particular, pollen sterility is noticeable in these hybrids, and fertility is completely lost; in contrast, female gametes do not exhibit such severe sterility, as seeds are produced when fertile pollen grains are crossed (Jones et al., 1997; Garavito et al., 2010). Microspores in the process of completing meiosis and developing into pollen are capable of differentiating into plants. If pollen destined for abortion can be rescued during early developmental stages, it could create hybrid plants. Not only are these individuals useful as genetic resources, but they also have a high potential in elucidating the mechanism of hybrid sterility.

In the 1960s, Gopalakrishnan et al. (1964) and Oka (1968) created individuals producing fertile seeds in F_1 tetraploid hybrids of *sat–gla*. In 1980, Woo and Huang reported that anther culture (AC) of an F_1 hybrid of *sat–gla* gave rise to tetraploid, diploid, and haploid plants (Woo et al., 1978). Unfortunately, these significant findings were given scant attention, being published too early to be of wide interest. The results described in those studies have thus not been validated, and the fertility of such F_1 tetraploid hybrids has not been analyzed in detail. Furthermore, the mechanism responsible for the formation of polyploids following AC of the interspecific hybrids has not been studied subsequently. In these interspecific hybrids, detailed observations are required to determine if pairing between genomes occurs during meiosis and whether distributions of homologous chromosomes in the first meiotic division and/or sister chromatids in the second meiotic division take place.

In general, AC can duplicate haploid genomes derived from male gametes to form a doubled haploid (DH) individual with complete homozygosity (Guha and Maheshwari, 1964; Niizeki and Oono, 1968; Germana, 2011). The differentiated individual from AC is, therefore, a complete pure line (Yan et al., 2017). In the present study, 19 plants were differentiated from the calli induced from anther-containing microspores of an F_1 hybrid of *sat–gla*. These differentiated individuals were mostly polyploids and exhibited heterozygosity in many genomic regions, which might cause allelic conflicts of *HS* loci. Nonetheless, some tetraploid individuals produced fertile seeds and the offspring in the next generation. Many studies on HS between *sat–gla* have mainly focused on *HS* genes, but little attention has been paid to other genetic factors. These polyploids were mainly a consequence of meiotic anomalies attributable to a failure during first or second meiotic divisions, namely the first division restitution (FDR) and second division restitution (SDR). The diploid gametes of the fertile tetraploids could circumvent the allelic conflicts at heterozygous *HS* loci that cause hybrid sterility. Anomalous meiosis in the F_1 hybrid that preceded the action of the *HS* genes should be added as one of the causes of hybrid sterility. Here, we demonstrate that diploid gametes can circumvent HS between *sat–gla* and thus allow partially fertile individuals to be regenerated. We also examine the relationship between meiotic anomalies and HS and discuss the defeat of HS by polyploidization.

MATERIALS AND METHODS

Plant Materials and AC

The calli derived from AC in this study originated from the same materials obtained by Kanaoka et al. (2018). Interspecific F_1 hybrid individuals were produced by crossing *O. glaberrima* Steud. with *O. sativa* L. ssp. *japonica*. The seed parent *O. glaberrima* accession IRGC 104038 from Senegal (designated as WK21) was kindly provided by the International Rice Germplasm Center of the International Rice Research Institute (Philippines) and conserved at Kyushu University. Nipponbare (Nip) was used as the pollen parent. Callus induction from AC was carried out according to Kanaoka et al. (2018) and is described as follows. After sterilization with 70% ethanol, panicles with spikelets at the booting stage (uninucleate stage) were incubated at 10°C (low temperature treatment) in the dark for 4–10 days. Approximately 70 anthers per dish were plated onto RI-13 callus-induction medium (Woo et al., 1978) prepared in a ø 90 mm × H 15 mm plastic dish. The plated anthers were then cultured at 25°C in the dark for 4 months. Grown calli were transplanted to fresh medium to promote further growth. To induce plant regeneration, calli grown to a diameter of 2 mm were moved to N6 medium (Chu, 1978) and incubated under light conditions at 25°C. When plantlets developed and roots emerged in the medium, the plantlets were transplanted to sterile soil, which included equal amounts of peat moss, vermiculite, and compost. The rice plants were grown under shade conditions in the greenhouse. Phenotypic traits in terms of pollen fertility, seed set rate, seed length, seed width, plant height, leaf length, leaf breadth, ligule length, and pistil color, were measured with four, six, and five individuals from the self-pollinated progenies of three fertile tetraploid lines, RP2-25 (from #25), RP2-38 (from #38), and RP2-80 (from #80), respectively. For counting precise seed numbers, the panicles of these materials were sacked to capture shattering seeds after heading.

Pollen Observation

The anthers for pollen observation were collected based on a distance between the auricles of flag leaf and penultimate leaf. To estimate microspore stages, microspore was collected when then the two auricles were separated by the following distances: −1.0 to +1.0 cm for the uninucleate stage and +2.0 to +6.0 cm for the binucleate state. In addition, mature pollen was collected after heading. These distances were almost all the same among the plant materials used. Collected anthers were fixed with formalin–acetic acid–alcohol fixative and then

prepared for microscopic observation. For observation at each microspore developmental stage, anthers were squashed on a microscope slide. After addition of 10 μl acetocarmine (Wako 1st grade, Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) or Lugol's iodine staining solution [0.15% (w/v) I_2, 1.5% (w/v) KI], the slide was covered with a cover slip and observed to respectively determine the pollen developmental stage or fertility of mature pollen.

Chromosome Counting

For chromosome number estimation, mitosis was observed using cells from root tips of regenerated plant #20, which were pretreated using 2 mM 8-hydroxyquinoline for 2–2.5 h at 20°C. After fixation, 1 mm of each root tip was cut off and macerated in enzyme solution consisting of 6.0% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical, Tokyo, Japan), 6.0% (w/v) Pectolyase Y-23 (Kyowa Chemical Products, Kagawa, Japan), and 75 mM KCl for 60 min at 37°C. The root tips were washed with a drop of distilled water for 5 min on a glass slide. To spread cells, each root tip was thoroughly squashed using a needle with 10 μl ethanol-acetic acid [3:1 (v/v)], and the slide was then flame-dried. The spread cells were stained for 30 min with Giemsa solution (Kanto Chemical Co., INC., Tokyo, Japan) diluted 30 times with Sorensen's phosphate buffer (pH 6.8). After washing with distilled water, the number of chromosomes was counted under an optical microscope (Olympus BX-50 F, Olympus, Tokyo, Japan).

Ploidy Analysis

Ploidy levels of materials were examined by measuring relative nuclear DNA amounts by flow cytometry as described in Miyashita et al. (2011). Nuclear suspensions obtained by extraction of small pieces of leaf tissue with nuclear extraction buffer (Quantum Stain NA 2A, CytoTechs, Ibaraki, Japan) were filtered through a 30-μm nylon mesh (Partec Celltrics, Lincolnshire, IL, United States). The fluorescent intensity of nuclei stained with DAPI (pH 7.5) was measured using a flow cytometer (Partec PA, Partec GmbH, Münster, Germany). The ploidy level of each examined individual was estimated using the fluorescent intensity of diploid tissue as a standard.

Genotyping

PCR detection of polymorphisms between WK21 and Nip was based on comparison of their complete genome sequences. The complete genome sequence of Nip was obtained from IRGSP-1.0 (RAP-DB), while that of WK21 was sequenced and deposited into the DDBJ under accession number DRS049718. Genomic DNA of regenerated plants from WK21/Nip F_1 individuals were extracted from mature leaves of well-grown regenerants. For genotyping of regenerated plants, we used 57 markers designed using simple sequence repeat (SSR) or InDel polymorphisms between WK21 and Nip (**Supplementary Figure 1**). Among the 57 markers, 22 were randomly distributed on each of 12 chromosomes (McCouch et al., 2002), and 24 were located near the centromere of each chromosome. Each centromere location was based on the Rice Genome Annotation Project database[1]. In addition, 12 markers linked to *HS* loci were used to test zygosity. PCR amplifications for genotyping were performed using GoTaq Green Master Mix (Promega, Madison, WI, United States), with the resulting products subjected to 3% agarose gel electrophoresis (**Supplementary Figure 2**). Three genotyping analyses were independently performed.

[1]http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/

Immunohistochemical Staining

To visually detect FDR and SDR in pollen mother cell (PMC) extracted from Nip, WK21, and the WK21/Nip F_1 samples (**Supplementary Table 4**), we performed immunohistochemical staining with anti-*O. sativa* centromeric histone H3 (OsCenH3) antibody and anti-α-tubulin mouse antibody and observed with a fluorescence microscope, BZ-X800 (Keyence, Osaka, Japan). PMC samples from Nip, WK21, and the F_1 hybrid were soaked for 20 min in a fixative consisting of microtubule-stabilizing buffer (5 mM PIPES, 0.5 mM $MgSO_4$, and 0.5 mM EGTA, pH 7.0) containing 3% (w/v) paraformaldehyde and 0.1% (v/v) Triton X-100 and then rinsed twice in 1× PBS buffer for 10 min. In the primary reaction, two primary antibodies were used: anti-OsCenH3 rabbit antibody and anti-α-tubulin mouse antibody (T6199, Sigma-Aldrich, St. Louis, MO, United States) (Nagaki et al., 2004). A primary antibody solution containing the two antibodies was diluted 200 times with a blocking buffer [0.4 M Tris–HCl (pH 7.5), 3.5% (w/v) NaCl, and 2% (w/v) BSA]. Fixed anthers were gently dissected on a glass slide using tweezers. Cells from the dissected anthers were suspended in 20 μl of 1× PBS, and covered with a coverslip, and then stored in a freezer (−80°C). After freezing, the coverslip was removed, 100 μl of the primary antibody solution was applied, and the solution was covered by a piece of parafilm (55 × 26 mm) to spread the solution. The samples were placed in a moisture chamber to prevent drying and kept at 4°C for 14 h. After the primary reaction, the samples were rinsed three times with 1× PBS for 10 min. Two secondary antibodies were used: Alexa Fluor 488-labeled anti-mouse antibody (#A-11001: Invitrogen, Carlsbad, CA, United States) and Alexa Fluor 555-labeled anti-rabbit antibody (#A20739: Invitrogen). The secondary antibody solution was diluted 200 times with the same blocking buffer used in the primary reaction. After the washing, the PBS buffer was removed from the slides, and then 100 μl of secondary antibody solution was applied, and the solution was covered by a piece of parafilm. The slides were placed in a moisture chamber and incubated at 37°C for 1 h. After the secondary reaction, the samples were rinsed using the same procedure applied after the primary reaction and then dried. To stain DNAs with minimal fading, 20 μl of ProLong Diamond Antifade Mountant with DAPI (Invitrogen) was applied to each slide before observation.

RESULTS

Pollen Sterility of Interspecific F_1 Hybrids

Interspecific hybrids between *sat–gla* are well known to exhibit severe HS that possibly involves more than a dozen *HS* genes (Sano et al., 1979; Sano, 1983, 1990; Doi et al., 1998, 1999;

Taguchi et al., 1999; Ren et al., 2006; Zhang et al., 2006; Li et al., 2011; Xu et al., 2014; Yu et al., 2018). An interspecific F_1 hybrid of *O. sativa* L. ssp. *japonica* Nipponbare (Nip) and *O. glaberrima* Steud. accession IRGC 104038 from Senegal (designated as WK21) produced panicles with sterile seeds as a consequence of aborted pollen and a partially fertile embryo sac (**Figure 1A**). The mature pollen grains from WK21/Nip F_1 were less strongly stained by Lugol's solution, indicating their sterility and inability to accumulate polysaccharides (**Figure 1A**). To explore the progression of this pollen sterility, we observed developing pollen grains in Nip, WK21, and WK21/Nip F_1 (**Figure 1B**). As development continued, pollen grains of both parents first showed evidence of acetocarmine staining at the early binucleate stage and were fully stained at the trinucleate stage (**Figure 1B**). During the early uninucleate stage of pollen development, most microspores from WK21/Nip F_1 plants exhibited no prominent differences in size or shape compared with the parents (**Figure 1B**), but some had abnormal structures, such as a fused form or a larger size than that of normal microspores (**Figure 1C**). The proportion of standard-shaped microspores in WK21/Nip F_1 plants decreased as they developed into pollen (**Figure 1B**). At the mature stage, normal, round pollen grains had disappeared, and the number of cavitated pollen grains had increased (**Figure 1B**).

Plant Regeneration From Calli of Interspecific F_1 Hybrids

In previous research, Kanaoka et al. (2018) successfully rescued microspores at the late uninucleate stage in interspecific hybrid plants (WK21/Nip F_1 and its reciprocal cross hybrid Nip/WK21 F_1) to induce calli by the AC method with RI-13 medium. In that study, 98 calli were obtained from 28,181 anthers, which corresponded to induction frequencies of approximately 11 calli from 14,724 Nip/WK21 anthers and 87 calli from 13,457 WK21/Nip anthers (**Supplementary Table 1**). In the present study, we used the 87 calli derived from WK21/Nip F_1 for plant regeneration. The 11 Nip/WK21 calli (the opposite cross combination to WK21/Nip) were not used because only a single plantlet was generated. Distinct frequencies of callus generation between the two reciprocal hybrids were used to infer whether certain sporophytic influences were due to cytoplasmic or maternal effects of the parental plants. Regeneration of plants from calli was attempted using N6-based medium. We obtained 19 regenerated plantlets from the WK21/Nip F_1-anther derived calli (**Supplementary Table 1** and **Supplementary Figure 3**). Thirteen plantlets were regenerated from 23 Nip calli induced with SK-1 medium, whereas no plantlets were regenerated from WK21 calli in this study (**Supplementary Table 1**). The 19 plantlets from the WK21/Nip F_1-anther derived calli were grown in soil; 17 became mature plants, while two died (**Supplementary Figure 3**). Two phenotypic traits typically different between *sat–gla*, namely, leaf smoothness and awn presence, were segregated in the 17 regenerated plants and both parents (**Figures 2A,B**).

Genotyping of Regenerated Plants

The 19 plantlets grown as seedlings from callus were genotyped with 22 SSR markers located on the 12 rice chromosomes and polymorphic between the two parents (**Supplementary Figures 1**, **2**). In general, the DH plants obtained via AC had completely homozygous genomes as a result of the doubling of the male gametic genome. Any heterozygotes may have been due to DNA of somatic tissues (e.g., from anther walls) of the F_1 hybrid plants, but we could not rule out the possibility of allopolyploids involving both parental genome sets. As shown

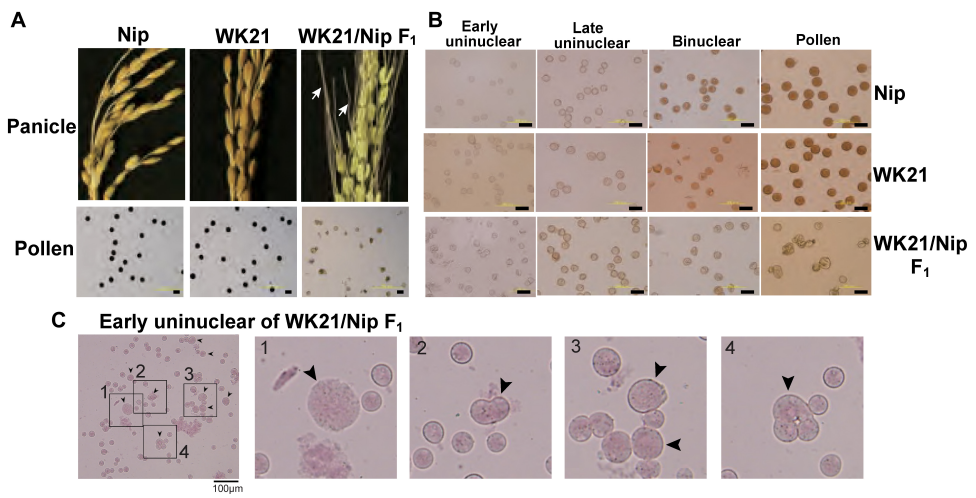


FIGURE 1 | Images of microspores at different developmental stages in Nip, WK21, and WK21/Nip F_1 plants. **(A)** Panicles and pollen grains of Nip, WK21, and WK21/Nip F_1 plants. Panicles in Nip and WK21 were fertile, while the panicle in WK21/Nip F_1 was sterile. Awns developed in the interspecific F_1 hybrid (white arrows) but not in the parents. Pollen grains from Nip and WK21, which were stainable with Lugol's iodine solution, exhibited potential fertility, whereas pollen from WK21/Nip F_1 was sterile, as reflected by the absence of staining. **(B)** Microspores at early uninucleate, late uninucleate, binucleate, and trinucleate stages. Microspores were stained with acetocarmine. The black bar in each panel corresponds to 100 μm. **(C)** Abnormal microspores in WK21/Nip F_1 plants at the early uninucleate stage. Microspores were stained with acetocarmine. Black arrows indicate abnormally shaped microspores. The black bar in each panel corresponds to 100 μm.

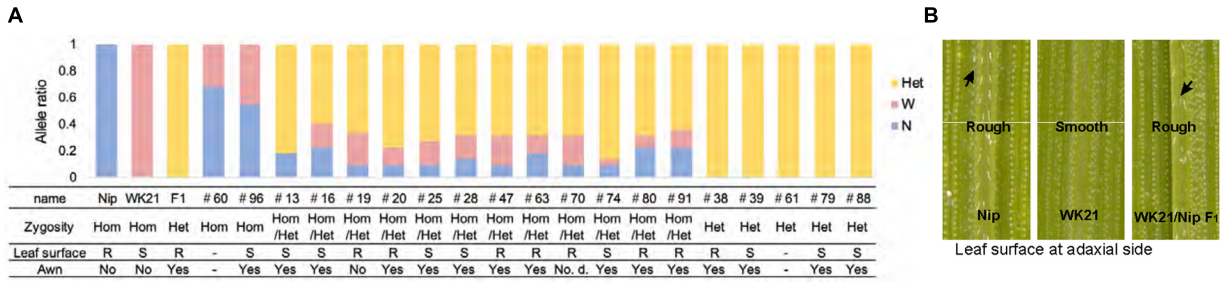


FIGURE 2 | Characteristics of the 19 regenerated plants. **(A)** Origins and ratios of the alleles in 19 plant genomes regenerated from the calli of WK21/Nip F_1 hybrids. The origins of the alleles are indicated by blue (N) for homozygous allele of *Oryza sativa* (Nip), pink (W) for homozygous allele of *O. glaberrima* (WK21), and yellow (Het) for the heterozygous allele. The allelic ratio was calculated with 22 markers used for genotyping (one or two selected from each of 12 chromosomes) detailed in **Supplementary Figure 1** and **Supplementary Table 2**. The state of the leaf surface [rough (R) or smooth (S)] and the presence (yes) or absence (no) of awns (**Figure 1A**) are also indicated. **(B)** Leaf surfaces of Nip, WK21, and WK21/Nip F_1 plants. Surfaces of adaxial sides of Nip and F_1 leaves were rough because trichomes were present (black arrows), whereas those of WK21 were smooth because trichomes were lacking.

in **Figure 2A**, genotyping of the 19 plantlets revealed that two plantlets (#60 and #96) were completely homozygous (Hom) for either genotype at each marker locus, while five plantlets (#38, #39, #61, #79, and #88) were heterozygous (Het) at all loci. The remaining 12 individuals had mixed genomes (Hom/Het) containing both homozygous and heterozygous loci (**Figure 2A** and **Supplementary Table 2**). The coexistence of homozygous and heterozygous loci in the plantlets derived from AC has two possible causes: an abnormality of meiosis in the parental plants or fusions between cells containing homozygotes and/or heterozygotes during callus culture. These results are in contrast to the observations of Morinaga and Kuriyama (1957), who did not detect any meiotic anomalies in their cytological study of interspecific hybrids between *sat–gla*.

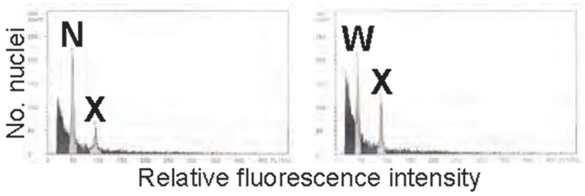
Ploidy Analysis of Regenerated Plants

To examine ploidy levels of the 19 regenerated plantlets obtained from AC, we performed a flow cytometric analysis (**Figure 3A**). Ploidy levels of the analyzed samples were based on relative fluorescence intensity comparisons with the parental diploid. As shown in **Figure 3B**, 5 of the 19 plantlets were diploid, and 12 regenerated plants—eight tetraploids, three triploids, and a pentaploid—were polyploid. No haploids were obtained. No apparent relationship was observed between ploidy level and degree of homo- or heterozygosity, but the three triploids were commonly Het plantlets (**Figure 2**). Among the 12 Hom/Het plantlets, five were diploid, one was pentaploid, and six were tetraploid (**Figure 2**). Microscopic observation also supported the results of the flow cytometric analysis: root tip cells from the examined plantlet (#20) had a chromosome number larger than 40, compared with 24 chromosomes in the parental *sat–gla* diploid (**Figure 3C**). As both the observations for chromosomal numbers did not seem to be perfectly accurate, we could not rule out the presence of aneuploid in the regenerated plants. Unlike AC of intraspecific hybrids, which usually produces DH plants, AC of the interspecific *sat–gla* F_1 hybrid resulted in many polyploid regenerants (12/19). These results led us to consider whether microspores from the F_1 hybrid were directly responsible for the aberrant ploidy levels.

Origin of the Hom/Het Plants

We considered three possible causes for the polyploidy of the regenerants. First, the 12 Hom/Het plants (#13, #16, #19, #20, #25, #28, #47, #63, #70, #74, #80, and #91) were expected to result from the generation of abnormal tetrads through incomplete meiotic reduction. These meiotic anomalies involve two major arrests of meiotic reduction: FDR and SDR (Jauhar, 2007; De Storme and Geelen, 2013; Han et al., 2018; **Figure 4A**). FDR is the halt in division of homologous chromosomes after recombination during meiosis I, while SDR is the arrest of the separation of paired sister chromatids during meiosis II (**Figure 4A**). Either meiotic division restitution produces microspores carrying diploid Hom/Het genomes. Diploid microspores with Hom/Het genomes may be duplicated during callus formation or regeneration processes, resulting in tetraploid Hom/Het plants. In regard to possible causes of incomplete meiotic reduction, we could test whether FDR or SDR was responsible for the Hom/Het plants. Hom/Het plants arising by FDR were expected to exhibit heterozygosity (i.e., both parental sequences) around centromeric regions (De Storme and Geelen, 2013). Because centromeric regions rarely undergo recombination, centromeric regions in paired homologous chromosomes between *sat–gla* remained heterozygous after meiosis I (**Figure 4A**). In contrast, Hom/Het plants generated by SDR would have homozygous centromeric regions (i.e., either parental sequence) because of the cancelation of sister-chromatid separation during meiosis II (De Storme and Geelen, 2013; **Figure 4A**). To distinguish between these two possibilities, the 12 chromosomes of the 12 regenerants were genotyped using centromeric-region-specific SSR and insertion/deletion polymorphism (InDel) primers (McCouch et al., 2002; **Supplementary Figure 2** and **Supplementary Table 3**). Genotyping of the centromeric regions yielded homozygous bands for the Hom plants and heterozygous bands for the Het plants (**Figure 4B** and **Supplementary Table 3**). Genotyping of the 12 Hom/Het plants uncovered two clear patterns: eight individuals (#13, #19, #20, #25, #47, #63, #74, and #80) were heterozygous for all the markers in centromeric regions, while the remaining four individuals (#16, #28, #70, and

A



B

Plant ID	Relative ploidy to Nipponbare	Relative ploidy to WK21	Relative ploidy (Average)	Relative ploidy to #28	Estimated ploidy level
#16	1.94	2.05	2.00	-	4x
#20	1.91	2.08	2.00	-	4x
#25	1.93	2.08	2.01	-	4x
#28	1.96	2.08	2.02	-	4x
#38	1.94	2.08	2.01	-	4x
#39	1.46	1.55	1.51	-	3x
#47	1.93	2.01	1.97	-	4x
#74	2.44	2.58	2.51	-	5x
#79	1.44	1.56	1.50	-	3x
#80	1.94	2.12	2.03	-	4x
#88	1.48	1.53	1.51	-	3x
#96	1.91	2.06	1.98	-	4x
#13	-	-	-	0.50	2x
#19	-	-	-	0.49	2x
#63	-	-	-	0.50	2x
#70	-	-	-	0.49	2x
#91	-	-	-	0.51	2x

C

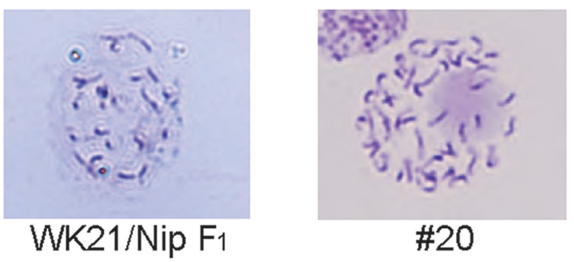


FIGURE 3 | Ploidy analyses of somatic cells of regenerated plants based on flow cytometry (FCM) and Giemsa staining. **(A)** FCM-based ploidy analysis. Left: FCM histogram of samples of Nip and plant #20 showing two peaks—N (derived from the Nip genome) and X (derived from the #20 genome). Right: FCM histogram showing two peaks—W (from WK21) and X. The ploidy level of #20 was determined by comparing peak X with peaks N and W from the diploid parental lines. **(B)** Ploidy levels of regenerated plants estimated from fluorescence intensity peak ratios. Ploidy levels of regenerated plants were based on relative fluorescence intensities of nuclei in Nip and WK21 cells. Diploid ploidy levels estimated by this method were validated by comparison with regenerated plant #28, which was determined to be tetraploid. **(C)** Giemsa staining of mitotic cells. Left: mitotic cell from the root tip of a WK21/Nip F_1 plant. The number of chromosomes in the cell appears to be a half that of a #20 plant. Right: mitotic cell from the root tip of a #20 plant regenerated from WK21/Nip F_1. More than 40 chromosomes are visible.

#91) were homozygous (**Figure 4B** and **Supplementary Table 3**). These results suggest that the first eight Hom/Het plants resulted from FDR and that the latter four plants were derived from SDR. In **Figure 1C**, the unusual shapes of microspores with a possible association with anomaly meiosis were shown. To verify the occurrence of abnormalities at meiosis in the interspecific F_1 hybrid between *sat–gla*, we observed PMCs from Nip, WK21, and these interspecific F1 hybrid (**Figures 4C,D** and **Supplementary Table 4**). Certain numbers of PMCs (4/20 for meiosis I and 3/25 for meiosis II) in the interspecific F_1 hybrid retained abnormal meiotic divisions, but not in the parents (**Supplementary Table 4**). Normal bivalent chromosomes observed at diplotene in meiosis I are necessary for reduction division, which leads to meiosis II, whereas univalent chromosomes in meiosis I are unable to undergo normal division, resulting in loss of meiosis I. As shown on the left side of **Figure 4C**, immunochemical staining with anti-OsCenH3 rabbit antibody revealed a numerous pairs of centromeric signals, which implies alignment of bivalent chromosomes at diplotene in PMCs. In the interspecific F_1 hybrid, the diplotene sample exhibited unpaired centromeric signals that were given by the presence of univalent chromosomes (right side of **Figure 4C**). During anaphase II, we also observed unequal division, in which spindle fibers with α-tubulin were not equally formed in dividing cells (right side of **Figure 4D**) relative to normal division (left side of **Figure 4D**). These observations in PMCs of the interspecific F_1 hybrid support the occurrence of FDR and SDR in meiosis I and II, respectively.

Second, five Het plants corresponding to three triploids (#39, #79, and #88), one tetraploid (#38), and one missing (#61) obviously contained both parental genomes (**Figure 2A** and **Supplementary Table 2**). PMCs that failed to undergo both divisions at meiosis I and II may not have formed tetrads. The occurrence of both division restitutions in a single meiocyte may therefore have given rise to tetraploid Het plants; however, making an assumption about whether the heterozygotic status of the triploids was due to simple aberrant meiosis or a complex process mediated by other factors is difficult. Third, in the Hom plants (#60 and #96), #96 with tetraploid genome may have arisen by haploid gamete doubling, but we could not ascertain exactly when doubling occurred during the AC procedure (**Figure 2A**).

Fertility and *HS* Locus Genotypes of Regenerated Plants

Among the 19 plantlets obtained from AC, 17 grew to maturity, while two (#60 and #61) died at the seedling stage. Of the surviving regenerated plants, the five tetraploid ones (#20, #25, #38, #47, and #80) generated seeds (**Figure 5**). More specifically, the five partially fertile tetraploid plants comprised four Hom/Het plants and one Het plant (**Figures 2A**, **5**). The partial fertility of each of these five plants could not be determined because they had inherited the shattering trait from their parent *O. glaberrima* WK21; however, two regenerated plants, #38 and #80, produced a relatively higher number of seeds. To confirm that HS had been overcome in the regenerants, the 17 regenerants were genotyped using 12 SSR primers linked to known *HS* loci (Kanaoka et al., 2018). As shown in **Figure 5**, plant #96 was homozygous for alleles from either of the two parents at each SSR locus. In the four Het plants (#38, #39, #79, and #88), the *HS* locus-specific SSR markers were all heterozygous (**Figure 5** and **Supplementary Table 5**). The Hom/Het plants were mixed, carrying both homozygotic and heterozygotic loci (**Figure 5** and **Supplementary Table 5**). The three fertile tetraploid plants, #25, #38, and #80, were heterozygous at more than eight *HS* loci, a situation that would have caused sterility if these plants had been

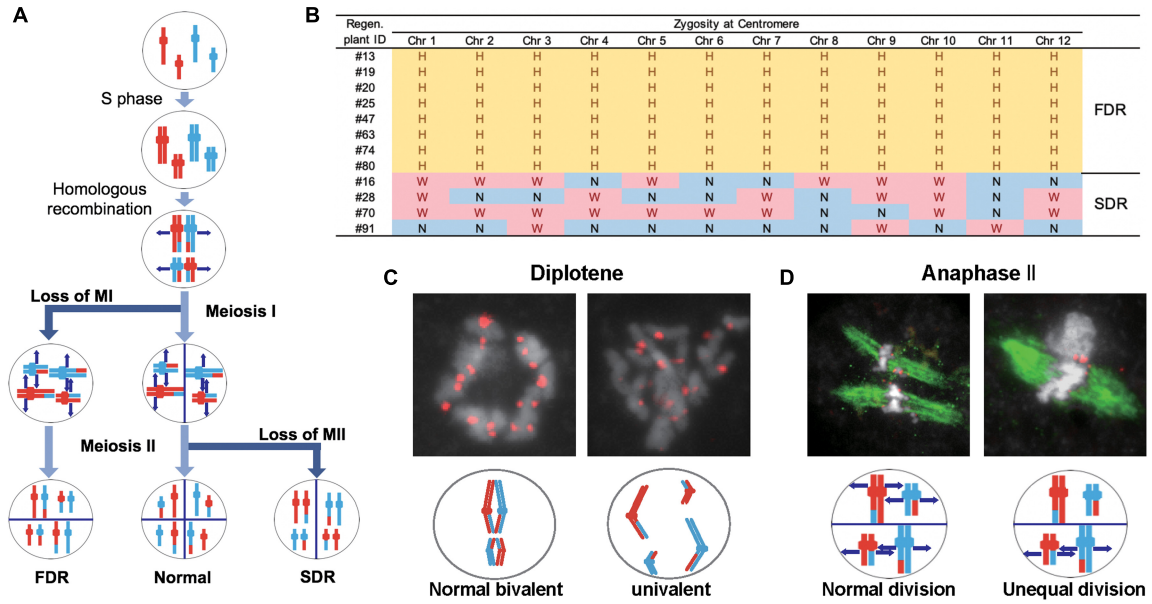


FIGURE 4 | Meiotic anomalies associated with FDR and SDR in WK21/Nip F_1. **(A)** Schematic diagram of chromosomal separations following normal, FDR, and SDR meiotic events. The three different chromosomal separation pathways following normal, FDR, and SDR events during meiotic division are based on De Storme and Geelen (2013). In the normal situation, bivalent homologous chromosomes separate after recombination at the end of meiosis I, with the sister chromatids remaining attached at the beginning of meiosis II and then separating. In FDR, homologous chromosomes fail to separate at the end of meiosis I, resulting in homologous chromosomes in the gametes. SDR bypasses meiosis II, and sister chromatids are distributed into gametes. FDR and SDR lead to centromeric regions (shown as knobs) that are respectively heterozygous or homozygous between homologous chromosomes. Red and blue are used to indicate the parental origin of chromosomal regions. **(B)** Genetic zygosities of centromeric regions of the 12 chromosomes of 12 regenerated plants and detection of FDR and SDR. Markers in centromeric regions (two of each chromosome) used in this analysis are detailed in **Supplementary Figure 1** and **Supplementary Table 3**. In the table, the zygosities of the 12 centromeres are indicated by "N" for homozygous centromeres from *O. sativa* (Nip), "W" for homozygous centromeres for *O. glaberrima* (WK21), or "H" for heterozygous centromeres. **(C)** Immunohistochemical detection of normal and anomalous gametes during meiosis I in WK21/Nip F_1. Using anti-OsCenH3 antibody, centromeric regions were observed in chromosomes at diplotene in meiosis I in PMCs from WK21/Nip F_1. Left: detection of paired signals (red spots) from centromeres at diplotene in a PMC, implying normal bivalent chromosomes (white portions). Right: non-aligned, dispersed centromeric signals, indicative of univalent chromosomes. **(D)** Immunohistochemical detection of normal and anomalous gametes during meiosis II of WK21/Nip F_1. Using anti-α-tubulin mouse antibody, spindle fiber formation (green zone) was observed at anaphase II in PMCs from WK21/Nip F_1. During normal anaphase II, sister chromatids (white zone) prepared to move toward opposite poles of the cell to generate haploid gametes. Left: normal division, showing movement of sister chromatids to the poles via the spindle fibers in both compartments as monitored using α-tubulin antibody. Right: unequal division in a PMC. In the upper compartment, no α-tubulin was observed, and sister chromatids were unable to separate and move to the poles; in contrast, the movement of sister chromatids along spindle fibers was apparent in the lower compartment.

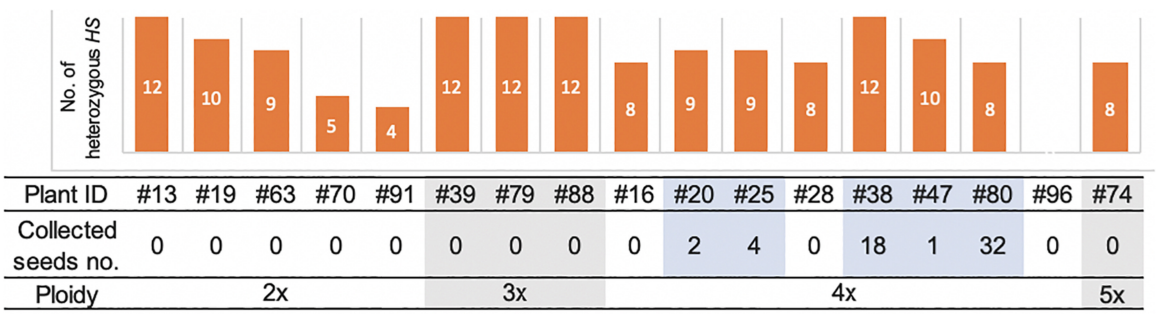


FIGURE 5 | The numbers of heterozygous loci of the 12 *HS* loci in the genomes of 17 regenerated plants and collected seeds and ploidy. The numbers of the heterozygous *HS* loci were examined by genotyping with the DNA markers of the 12 *HS* loci. The details of markers used in this analysis are listed in **Supplementary Figure 1** and **Supplementary Table 5**. The number of collected seeds and ploidy of each regenerated plant are indicated below. Five fertile plants highlighted with pale blue had seeds, which were all tetraploid.

diploid. Our results are in agreement with the observations of Gopalakrishnan et al. (1964) and Oka (1968) that the tetraploidy of the interspecific hybrid allowed escape from HS.

Phenotypes of Fertile Tetraploids

The self-pollinated progenies of the three fertile tetraploid lines, RP2-25 (from #25), RP2-38 (from #38), and RP2-80 (from #80), were examined in terms of 20 traits (**Table 1**). Averages of pollen fertilities in the RP2 three lines were ranged from 5.8 to 39.0% (**Table 1**), and most of the individual plants could produce somehow partially normal pollens except one plant (**Supplementary Table 6**). In terms of the seed set rates, their averages were accounted for further low from 0.1 to 8.0% with large standard deviations (**Table 1**). These seed set rates resulted from only one or two individuals with fertility (0.5–40%) and the other plants were sterile (**Supplementary Table 6**). The *HS* loci might explain the difference between fertile and sterile plants, as shown in a partially fertile plant (#6) in the RP2-38 line, which possessed the WK21 homozygous allele at the *S34(t)* locus in the tetraploid genome (**Supplementary Table 6**). However, it is difficult to explain why parental tetraploid plants were partially fertile for this reason.

Seed sizes from #80 plants were larger than those of the parents: 1.42- and 1.34-fold longer and 1.33- and 1.38-fold wider relative to Nip and WK21, respectively (**Table 1**). Likewise, fertile plants in the next generation (RP3) also produced bigger seeds (**Table 1**). Of the five fertile tetraploid plants, the fertilities of plants from #25, #38, and #80 were passed along to subsequent generations. These plants thus appeared to have overcome the HS between *sat–gla*. Self-pollinated progenies of three fertile tetraploid lines, RP2-25 (from #25), RP2-38 (from #38), and RP2-80 (from #80), were obtained and phenotypically compared with their parental lines, Nip and WK21. The following characters were measured: seed length, seed width, plant height, leaf length, leaf breadth, ligule length, and pistil color (**Table 1**). Most phenotypes in the second generation derived from the three tetraploid regenerated lines were larger than those of their parents (**Table 1**), thus reflecting typical tetraploid vigor. In regard to pistil color, the blackish purple pistils of WK21 were expressed in the F_1 generation and RP2-25 and RP2-38 lines, while the white pistils of Nip were inherited by the RP2-80 line (**Table 1**).

DISCUSSION

Production of Plants From Hybrids Between *sat–gla* by AC

Because of gamete sterility, progenies cannot be generated from interspecific hybrids of *sat–gla* (Oka, 1957; Sano et al., 1979). In this study, we successfully regenerated plants from callus induced by culturing sterile microspores of interspecific hybrid plants without the recombinant DNA techniques. Five of the 19 regenerated plants produced seeds. According to Kanaoka et al. (2018), the essential factor for obtaining plants from interspecific hybrids with strong HS is the use of callus obtained by culturing anthers with microspores at the uninucleate stage. Uninucleate-stage microspores are required for embryogenesis not only in rice but also in wheat (Hassawi and Liang, 1990). In grape (Gribaudo et al., 2004), barley (Hoekstra et al., 1992), and *Brassica napus* (Telmer et al., 1992), embryoid bodies can also be differentiated directly from uninucleate microspores. Microspores appear to lose their embryogenic (or callus formation) ability after the uninucleate stage, and differentiation into pollen then irreversibly progresses (Kinoshita et al., 2000). Even in the interspecific hybrid between *sat–gla* exhibiting HS, microspore decay had not yet begun in uninucleate microspores (**Figures 1B,C**, **6A**). This stage is a crucial point for rescuing microspores to obtain plants from AC of interspecific hybrids (Kinoshita et al., 2000; **Figure 6A**).

Diploid plants differentiated through AC usually have complete homozygosity because haploid male gametes are spontaneously doubled during the differentiation process. In this study, only two DH lines were detected among the 19 regenerated plants (**Figure 2A**). The other individuals differed in terms of zygosity and ploidy level from ordinal diploid DH lines (**Table 2**). We could thus readily infer that abnormalities occurred during male gametophyte formation in the interspecific hybrid. We therefore examined anomalies related to male gametophyte formation from two perspectives, genomic zygosity and ploidy level.

Variations in Zygosity

Individuals derived by AC of the interspecific hybrid were divided into three groups on the basis of zygosity: (1) Hom individuals having completely homozygous genomes, (2) Het individuals with complete heterozygosity, and (3) Hom/Het plants having both homozygous and heterozygous genomic regions (**Figure 2A**). The first group presumably originated from cases in which the haploid genome of a gamete spontaneously doubled during callus formation or regeneration in an AC-derived rice plant (Rout et al., 2016; Naik et al., 2017). The complete heterozygosity of plants in the second group had two possible causes (Huang et al., 1997): (a) callus formation of the F_1 somatic cells, such as anther wall cells, and (b) callus formation occurring in the PMC harboring the paired genomes before the first meiotic division. The third group, which included both homozygous and heterozygous regions, may have emerged after meiotic recombination (Pinson and Rutger, 1993). Tetraploid Hom/Het plants may have been derived from microspores in which the diploid genome was doubled during callus development, while diploid Hom/Het plants may have arisen from microspores formed from callus without genome doubling. AC of rice intraspecific hybrids rarely produced Hom/Het plants, which were derived from diploid microspores (Grewal et al., 2011).

Meiotic Anomalies

In AC of rice, plant differentiation occurs via callus. The most active period of callus formation during pollen development corresponds to the middle to late uninucleate microspore stage. We observed abnormal forms of microspores at the uninuclear stage in the interspecific hybrid, such as microspores that were twice the size of normal ones and fusions of two microspores (**Figure 1C**). Flow cytometry and chromosome observations

TABLE 1 | The features of the progenies (RP2−25, −38, −80) from the fertile regenerated plants, 25, 38, and 80, respectively.

Plant materials	Pollen fertility (%)	Seed set rate (%)	Seed size[2] (mm)		Seed size[3] (mm)		Plant height (cm)	Leaf length (cm)				Leaf breadth (cm)				Ligule length (cm)				Color of pistils
			Length	Width	Length	Width		Flag leaf	Flag-1[4]	Flag-2	Flag-3	Flag leaf	Flag-1	Flag-2	Flag-3	Flag leaf	Flag-1	Flag-2	Flag-3	
RP2-25[1] (*n* = 4)	5.8 ± 1.8	0.1 ± 0.2	n.d.		n.d.		122.9 ± 6.6	28.0 ± 7.7	59.0 ± 13.2	77.7 ± 8.6	81.6 ± 4.2	2.13 ± 0.07	1.71 ± 0.09	1.47 ± 0.13	1.50 ± 0.07	0.65 ± 0.16	1.97 ± 0.42	3.43 ± 1.08	2.94 ± 0.86	Blackish purple
RP2-38 (*n* = 6)	16.5 ± 20.1	8.0 ± 14.5	n.d.		8.7 ± 0.5	2.9 ± 0.3	130.7 ± 13.9	26.7 ± 14.2	55.6 ± 21.1	78.5 ± 6.8	81.5 ± 8.7	1.94 ± 0.25	1.56 ± 0.29	1.51 ± 0.13	1.49 ± 0.11	0.82 ± 0.29	1.63 ± 0.34	2.62 ± 0.67	2.58 ± 0.41	Blackish purple
RP2-80 (*n* = 5)	39.0 ± 20.7	0.6 ± 1.3	9.1 ± 0.5	3.6 ± 0.3	9.0 ± 0.2	4.0 ± 0.3	120.3 ± 3	16.3 ± 8.4	43.4 ± 7.3	75.1 ± 5.7	78.8 ± 6.8	1.64 ± 0.61	1.86 ± 0.26	1.87 ± 0.10	1.67 ± 0.16	0.47 ± 0.14	0.96 ± 0.29	1.70 ± 0.31	1.52 ± 0.21	White
WK21 (seed parent)	90.4 ± 1.6	91.5 ± 1.1	6.8 ± 0.4	2.6 ± 0.3	–	–	115.1	18.6	40.2	69.2	61.3	1.60	1.40	1.25	1.45	0.40	0.50	0.55	0.70	Blackish purple
Nipponbare (pollen parent)	89.7 ± 1.3	85.0 ± 1.1	6.4 ± 0.4	2.7 ± 0.2	–	–	97.9	18.4	33.2	56.7	69.5	1.45	1.43	1.30	1.20	0.40	0.85	1.00	1.30	White
WK21/Nip[5] F_1	0.0	0.0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Blackish purple

[1]RP2 is the next generation of the regenerated plants from AC. [2]The seeds were ones (RP2) from the fertile regenerated plants. [3]The seeds were ones (RP3) from the seeds of the progeny (RP2) of the regenerated plants. [4]Flag-1, Flag-2, and Flag-3 mean one, two, and three leaves previous to the flag leaf emerged, respectively. The measurements of the 20 characteristic items were performed with four, six, and five progenies from the three tetraploid plants, RP2-25, RP2-38, and RP2-80, respectively. [5]Three traits of F_1 were references of those we have previously observed plants, but we did not measure plants with the same conditions as listed in this table.

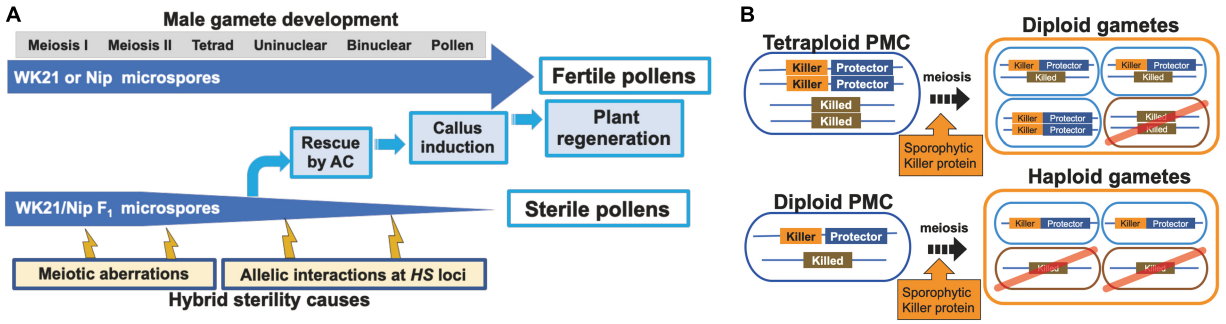


FIGURE 6 | Models of processes of hybrid sterility and its circumvention. **(A)** A new model of hybrid sterility and plant regeneration by AC during microspore development in WK21/Nip F_1. Microspores of WK21/Nip F_1 do not develop into pollen because of HS due to 1) meiotic aberrations and 2) allelic interactions at *HS* loci. **(B)** Higher rates of fertile gametes in tetraploids compared with diploids according to the killer–protector model. Under the killer–protector model of HS, the killer protein has a sporophytic effect on gametes during microspore development after meiosis, but the gamete expressing the protector protein is not killed. In the case of a heterozygous tetraploid plant, which contains two killer–protector alleles and two killed alleles, three-quarters of the gametes possess protector alleles. In the case of a heterozygous diploid plant, only half of the gametes carry a killer–protector allele. In theory, a heterozygous tetraploid thus produces 25% more surviving gametes than does a heterozygous diploid plant.

TABLE 2 | The genetic properties and fertility of the 19 regenerated plants.

Regenerated plant ID	Ploidy	Genetic zygosity		Types of meiotic aberration	Collected seed number
		Non-centromeric region	Centromeric region		
#60	–	Hom	Hom	Haploid gamete	0
#96	4x	Hom	Hom	Haploid gamete	0
#13	2x	Hom/Het	Het	FDR	0
#19	2x	Hom/Het	Het	FDR	0
#20	4x	Hom/Het	Het	FDR	2
#25	4x	Hom/Het	Het	FDR	4
#47	4x	Hom/Het	Het	FDR	1
#63	2x	Hom/Het	Het	FDR	0
#74	5x	Hom/Het	Het	FDR	0
#80	4x	Hom/Het	Het	FDR	32
#16	4x	Hom/Het	Hom	SDR	0
#28	4x	Hom/Het	Hom	SDR	0
#70	2x	Hom/Het	Hom	SDR	0
#91	2x	Hom/Het	Hom	SDR	0
#38	4x	Het	Het		18
#39	3x	Het	Het		0
#61	–	Het	Het		0
#79	3x	Het	Het		0
#88	3x	Het	Het		0

Hom, the genome comprising complete homozygous genotypes; Het, the genome comprising complete heterozygous genotypes; Hom/Het, the genome containing both homozygous and heterozygous genotypes; FDR, the first division restitution; SDR, the second division restitution, gray-shaded regenerated individuals indicate those producing fertile seeds. #60 and #61 died at the seeding stage before analysis of FCM.

demonstrated that many regenerated plants were tetraploid, triploid, or pentaploid (**Figure 3**). These observations suggest that meiotic anomalies of interspecific hybrids lead to insufficient microspore separation and occasional fusion at the tetrad stage. Our genomic analysis revealed that 12 of 19 regenerated plants resulted from abnormalities in division after meiotic recombination (**Figure 4B** and **Supplementary Table 3**). Meiotic divisions were also observed by immunohistochemical staining for OsCenH3 and α-tubulin (**Figures 4C,D** and **Supplementary Table 4**). Both anomalies in the first and second divisions during meiosis were only detected in the PMC samples from the interspecific F_1 hybrid, but not in the PMC samples from the parents (**Supplementary Table 4**). In the interspecific F1 hybrid, the meiotic anomalies involved cancelation of either the first or second division, thereby leading to diploid gametophyte generation and subsequent polyploid formation during plant regeneration from callus (**Figure 4**; Jauhar, 2007; De Storme and Geelen, 2013; Han et al., 2018). In some cases, neither the first nor the second division occurred, and the tetraploid gametes were able to develop into callus and differentiate directly

into plants. We were able to deduce the mechanisms associated with the occurrence of tetraploidy based on meiotic anomalies in the interspecific hybrid. Although a detailed explanation for how triploid and pentaploid plants were generated from AC of the hybrid could not be determined, we assumed that triploid and pentaploid plants might be attributable to the unusual fusion of microspores at the tetrad stage after the second division in meiosis.

Because most male gametes in F_1 hybrids between *sat–gla* should decay during pollen development, determination of the genome harbored by each gamete has not been possible. In this study, we demonstrated that genetic characterization of male gametes in hybrid plants between *sat–gla* is feasible by rescuing abortive microspores with AC and allowing them to differentiate into plants. More than a dozen *HS* loci between *sat–gla* can act on male and/or female gametes and, in particular, cause male gametes to become sterile (Koide et al., 2008; Garavito et al., 2010; Kanaoka et al., 2018). Although *HS* genes are widely known to be responsible for HS, our study has clearly shown that meiotic anomalies occur before these genes act (**Figure 6A**). Alternatively, meiotic anomalies may also be one of the causes of HS that collapses the gamete genome (**Figure 6A**). Future required work includes a detailed analysis of meiotic anomalies occurring in PMCs in hybrids and clarification of the relationship between the mechanism of non-segregation of the first and second divisions and gamete decay.

Ploidy Levels and HS Avoidance Mechanisms

Among the plants derived from AC, all five plants that produced seeds had tetraploid and heterozygous genomic regions. Four of these five fertile plants were Hom/Het, and one was a completely Het individual. The four Hom/Het plants also had many alleles of *HS* loci as heterozygote. Gametes possessing a killed allele at an *HS* locus will not survive (Sano et al., 1979; Jones et al., 1997). The existence of multiple *HS* loci reduces the number of surviving gametes by one-half per each additional locus. More than a dozen *HS* loci have been found between *sat–gla*, and most of the hybrid gametes are sterile or die (Sano et al., 1979; Sano, 1983, 1990; Doi et al., 1998, 1999; Taguchi et al., 1999; Ren et al., 2006; Zhang et al., 2006; Li et al., 2011; Xu et al., 2014; Yu et al., 2018). In the present study, partially fertile plants were obtained from a tetraploid with heterozygous *HS* alleles. Except for backcross lines with either parent, we never obtained such fertile plants from self-pollinated interspecific F_1 hybrids between *sat–gla* (**Figure 5** and **Table 2**). Among the 19 regenerated plants from AC, in contrast, we obtained five partially fertile plants, all of which were tetraploids. Polyploidization may thus be a way to remove the barrier between the two species.

The *HS* genes responsible for the *S1* locus between *sat–gla* have recently been isolated (Xie et al., 2017, 2019; Koide et al., 2018), thus allowing the mechanism of the killer–protector system to be elucidated. In this system, a killer gene is linked to a protector gene that protects gametes from the action of the former (Yang et al., 2012; Ouyang and Zhang, 2013; Zhu et al., 2017; Xie et al., 2019). When a protector gene is present in the same gamete, the killer allele is protected against the killer protein itself. If so, the killer gene at the *HS* locus appears to sporophytically act on other gametes (not encased in the same membrane) that do not have a protector after separation into a tetrad. Tetraploids from *sat–gla* hybrids are likely fertile because three-quarters of diploid gametes from a tetraploid plant contain both killer and protector alleles (**Figure 6B**). In contrast, a diploid plant derived from hybrids between *sat–gla* produces haploid gametes, a half of which may contain both killer and protector alleles (**Figure 6B**). Although the different killer–protector allele ratios in gametes may reflect the distinct seed fertilities of the tetraploid vs. the diploid, the killer–protector system is not the only explanation for these observations.

Characteristics of Fertile Plants Obtained From AC

Five lines of partially fertile tetraploids, #20, #25, #38, #47, and #80, were obtained by AC of the interspecific hybrid of *sat–gla* (**Figure 5** and **Table 2**). Fertility in these regenerated plants was a critical issue of the tetraploid lines, which also appeared in the RP2 generation. Partial fertility and complete sterility segregated into the plants of the RP2-25, RP2-38, and RP2-80 lines (**Table 1** and **Supplementary Table 6**). The relationships between these fertilities and *HS* loci were not clear in this study, although tetraploidy could mitigate sterility in a certain genetic background (**Supplementary Table 6**). The seed sizes of RP2-38 and RP2-80 lines were respectively 1.3 to 1.4 times larger than those of the parental lines and were inherited by the next generation (**Table 1**). Plant heights, flag-leaf lengths, flag-leaf widths, and ligule lengths of plants grown from the seeds of RP2-25, RP2-38, and RP2-80 were superior to the parental traits (**Table 1**). This typical biomass enlargement may have been due to tetraploidization; alternatively, heterosis may have occurred, as the genomes of these strains were heterozygous. In the *sat–gla* diploid F_1 hybrid, however, the values of these traits were often intermediate between those of the parents, and the tetraploid vigor was thus unlikely the result of heterosis (**Table 1**). Even if heterosis was a factor—given that these tetraploid plants retained heterozygous genomes—the maintenance of heterotic traits in the progeny would be difficult.

In a tetraploid plant with two different alleles at a locus, 10 generations are theoretically required to reduce the proportion of heterozygotes to less than one-quarter of a population; in a diploid plant, this percentage is achieved by the third generation. This characteristic implies that the number of generations during which recombination can take place in a heterozygous tetraploid is much larger compared with a diploid (Pecinka et al., 2011). Tetraploid hybrid plants therefore have the potential to create highly variable allelic combinations by repeated recombination during meiosis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DK and YKi conceived and planned the work. DK, IM, YKa, and YS-K performed the experiments and analyzed the data. YO directed the anther culture procedure. HY arranged the materials. DK, TY, and KN performed immunochemical staining. YH assisted with the ploidy analysis. YKo assisted with the data analyses. IT supported the mitotic observations. DK and YKi wrote and improved the manuscript. YKi supervised DK's Ph.D. study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.579305/full#supplementary-material

Supplementary Figure 1 | Information of chromosomal locations (above) and primers (below) for the genotyped loci.

Supplementary Figure 2 | A genotyping profile of the 19 regenerated plants using 10-C2 located on centromere in the chromosome 10.

Supplementary Figure 3 | Seventeen regenerated plants obtained from AC of WK21/Nip F1.

Supplementary Table 1 | Numbers of the induced calli and regenerated plants by AC from the interspecific rice hybrids and their parents.

Supplementary Table 2 | Genotypes of the 19 regenerated plants from the calli of WK21/Nip F1 hybrids.

Supplementary Table 3 | Genetic zygosities of centromeric regions of the 12 chromosomes of 12 regenerated plants and detection of FDR and SDR.

Supplementary Table 4 | Numbers of PMCs indicating normal and anomaly in meiosis I (prophase I) and meiosis II (anaphase II to telophase II).

Supplementary Table 5 | Genotyping of the 12 HS loci in the 17 regenerated plants.

Supplementary Table 6 | Pollen fertility, seed set rate, and genotypes of HS loci for the self-pollinated progenies of the three fertile tetraploid lines, RP2-25, RP2-38, and RP2-80.

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A Coordinated Suite of Wild-Introgression Lines in *Indica* and *Japonica* Elite Backgrounds

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Rice, *Oryza sativa* L., is a cultivated, inbreeding species that serves as the staple food for the largest number of people on earth. It has two strongly diverged varietal groups, *Indica* and *Japonica*, which result from a combination of natural and human selection. The genetic divergence of these groups reflects the underlying population structure of their wild ancestors, and suggests that a pre-breeding strategy designed to take advantage of existing genetic, geographic and ecological substructure may provide a rational approach to the utilization of crop wild ancestors in plant improvement. Here we describe the coordinated development of six introgression libraries (n = 63 to 81 lines per library) in both *Indica* (cv. IR64) and *Japonica* (cv. Cybonnet) backgrounds using three bio-geographically diverse wild donors representing the *Oryza rufipogon* Species Complex from China, Laos and Indonesia. The final libraries were genotyped using an Infinium 7K rice SNP array (C7AIR) and analyzed under greenhouse conditions for several simply inherited (Mendelian) traits. These six interspecific populations can be used as individual Chromosome Segment Substitution Line libraries and, when considered together, serve as a powerful genetic resource for systematic genetic dissection of agronomic, physiological and developmental traits in rice.

Keywords: *Oryza sativa*, crop wild relatives, *Oryza rufipogon* Species Complex, chromosome segment substitution line, pre-breeding resources

INTRODUCTION

Modern-day plant breeders seldom return to wild gene pools to rigorously explore crop wild relatives as a source of variation for plant improvement (Hajjar and Hodgkin, 2007; Warschefsky et al., 2014; Dempewolf et al., 2017). This is because using wild or exotic materials is disruptive to established breeding programs; when they are crossed to elite breeding lines, the influx of new alleles throughout the genome disturbs carefully constructed gene complexes that are the basis of highly valued traits in commercial varieties (Dempewolf et al., 2012). Even as favorable alleles are introduced, they are frequently accompanied by linked deleterious ones that require effort to eliminate. Sterility barriers may also hamper the generation of fertile offspring, adding significant time and cost to variety development (Zamir, 2001;

Haussmann et al., 2004). Furthermore, it is difficult to predict the genetic potential of exotic materials because quantitatively inherited phenotypes are often masked until the accessions have been repeatedly backcrossed into well-adapted varieties (Frey et al., 1984; Eshed and Zamir, 1995; Tanksley and McCouch, 1997). Instead, for over half a century, plant breeders have been able to make reliable genetic gain in staple food crops by crossing and recombining variation within elite gene pools, fine-tuning varieties to fit target environments and relevant agricultural practices. Indeed, the combination of improvements in breeding and management has enabled steady increases in global production of staple crops such as maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.) for over 60 years (Food and Agriculture Organization of the United Nations, 2020).

Despite the steady results hitherto achieved with this approach, new challenges lie on the horizon. Paleoclimatic evidence suggests that we are coming to the end of an unprecedented period of global climate stability that ushered in the era of agriculture during the Pleistocene-Holocene transition (Feynman and Ruzmaikin, 2018). Increased variability in temperature and rainfall patterns are forecasted to escalate occurrences of extreme drought, flooding, wind, salt incursion, and pest and disease infestation (Rosenzweig et al., 2014; Fita et al., 2015). By 2050, an additional two billion people will share our planet (United Nations Department of Economic and Social Affairs, 2017), placing even greater pressure on plant-based food, fiber, feed and fuel production (Tilman et al., 2002; Godfray et al., 2010). These upheavals create new impetus for crop improvement programs to respond to a multitude of challenges, and new varieties must address the changing biophysical and economic constraints of growers and producers as well as the evolving demands and aspirations of consumers. Never has there been a more urgent call for plant breeders to take on so many significant challenges at once.

As the far-reaching effects of climate change are increasingly recognized and the need to reconcile short term gains in productivity with the long term viability of agricultural ecosystems becomes more apparent, plant breeders, policy makers and agriculturalists seek new ways of incorporating an expanded palette of genetic variation in farmers' fields (Dempewolf et al., 2014; Pilling et al., 2020). There is long-standing interest in wild and exotic germplasm as a source of genetic resilience and potentially as a source of novel nutritional and/or quality traits (Redden et al., 2015; Dempewolf et al., 2017; Li et al., 2018; Burgarella et al., 2019; Rosyara et al., 2019). However, efforts to explore the range of genetic variation found in wild and exotic ancestors have been largely *ad hoc* and are undertaken without a clear path to commercial variety release. This is not to discount the many examples of successful pre-breeding, where wild alleles been shown to confer adaptive advantages in advanced breeding lines (Tanksley and McCouch, 1997; Zamir, 2001; Gur and Zamir, 2004; McCouch et al., 2007; Dwivedi et al., 2008; Mammadov et al., 2018), but rather to point out that these efforts are costly, time consuming and, in a majority of cases, fail to traverse the "last mile" to find their way into farmers' fields. A more comprehensive understanding of how to systematically explore diverse sources of natural variation, predict genetic value and efficiently incorporate exotic variation into elite cultivated genetic backgrounds is the first step toward enhancing the value of plant genetic resources in the context of crop improvement (Warschefsky et al., 2014; Street et al., 2016; Migicovsky and Myles, 2017; Prohens et al., 2017; Li et al., 2018).

In rice, the *Oryza rufipogon* Species Complex (ORSC) is the progenitor of *O. sativa*. It is found dispersed throughout a wide range of habitats across South, Southeast and Eastern Asia (Oka, 1988; Vaughan et al., 2008). Over the last million years, the ORSC has evolved and diversified on both sides of the Himalayan Mountains, successfully adapting to highly variable ecosystems and environmental conditions (Guo and Ge, 2005; Stein et al., 2018). An evaluation of genome-wide variation in a collection of 286 ORSC accessions originating from 15 countries revealed six subpopulations reflecting both perennial and annual types of rice distributed across the geographical range of the species complex. Three of the ORSC subpopulations were found to be genetically related to the three most divergent *O. sativa* subpopulations, *Indica*, *Aus*, and *Japonica*, raising interesting questions about cross-compatibility, wild-cultivated combining ability, and shared allele complexes. To explore these questions, we undertook the development of a coordinated set of wild × cultivated chromosome segment substitution line (CSSL) libraries by systematically recombining distinct subpopulations of both wild and cultivated forms of rice.

The objective of this study was to develop and make available a suite of wild × cultivated rice CSSLs to stimulate both scientific discovery and translation in breeding, while helping to bridge the gap between genebanks and crop improvement. We provide a resource that can be rigorously evaluated in diverse environments using advanced phenotyping technologies, genetically dissected and/or expanded through crossing and/or genome manipulation, and shared widely with researchers and breeders with a common interest in exploring the genetic potential of crop wild relatives. Toward this goal, we generated six introgression libraries in elite *Indica* (cv. IR64) and *Japonica* (cv. Cybonnet) backgrounds using three genetically and bio-geographically diverse ORSC donors from China, Laos and Indonesia. This coordinated set of CSSL libraries is the first of its kind; it will enable investigators to begin to systematically examine the impact of wild introgressions in two very different genetic backgrounds, to mix and match introgressions from the same or different wild donor genomes, and to compare and contrast the phenotypic impact of introgressions in homozygous or heterozygous combinations. These materials provide the basis for predicting whether some wild subpopulations combine more productively with *Indica* versus *Japonica* varieties and offer insights about the allelic series in diverse germplasm. Deeper understanding about which combinations of wild by elite parents are most likely to give rise to superior offspring will help provide a roadmap to design pre-breeding programs that enable rice

breeders to make more efficient use of wild and exotic plant genetic resources.

MATERIALS AND METHODS

Parental Selection and Initial Crossing

A total of six libraries of inter-specific CSSLs were constructed in two *O. sativa* (*Indica* and *Japonica*) backgrounds using three wild (ORSC) donor accessions. To help select wild donors for CSSL development, genetic relationships were examined among 286 ORSC accessions and 54 *O. sativa* varieties initially using 50 Simple Sequence Repeat (SSR) markers and subsequently using a set of 113,739 GBS SNPs (Kim et al., 2016). A rooted Neighbor Joining (NJ) dendrogram using an *Oryza officinalis*, Wall ex Watt accession set as the outgroup was constructed with 100 bootstrap replicates (Tamura and Nei, 1993) in Geneious v10.0.9. ORSC donors were selected to represent geographical, phenotypic and genomic diversity, and donor selection was also influenced by cross compatibility with the cultivated recurrent parents (RPs). Three donor plants per wild accession were grown out for crossing with multiple individuals from the two RPs in the Guterman Greenhouse at Cornell University (Kim et al., 2016). Efforts to develop purified donor lines from the ORSC individuals used for crossing were not successful, and therefore additional plants were grown from the original donor seed stocks and used as proxies in some of the subsequent analyses.

CSSL Development

Three criteria were used to select lines for generation advance over the course of development: (1) presence of a wild introgression in a target region, i.e., a region that contributed to the goal of providing coverage of the entire donor genome with independent, overlapping substituted segments, (2) minimum number of wild introgressions in non-target regions, contributing to the goal of creating a library consisting of a set of near isogenic lines, each carrying a single wild introgression in an RP genetic background and (3) recovery of a "lost introgression," in cases where a heterozygous donor segment present in one generation was not recovered in the subsequent generation, requiring the retrieval of a line from an earlier generation. The breeding scheme is presented in **Supplementary Figure S1**.

Marker-assisted selection (MAS) was carried out at every generation. Foreground selection was performed in the first three generations using 384 Oligo Pool Assays (OPAs) to establish sets of lines that covered the donor genome (Thomson et al., 2012). Subsequent generations utilized 6 K or 7 K SNP arrays (the C6AIR and C7AIR; Thomson et al., 2017; Morales et al., 2020), to eliminate non-target introgressions (negative selection) and increase the proportion of recurrent parent background. Length of each donor segment was determined based on the physical position of the marker genotypes. For a given introgression line, the preferred range for a target donor introgression was 4–5 Mb (13–17 cM), with maximum recurrent parent background. Each introgression line was selected such that there was a 1–2 Mb overlap within its neighboring introgression line on both sides of the introgressed segment. Donor segment selection was given preference over background recovery. Therefore, if donor segments were lost during backcrossing, plants from an earlier generation with less recurrent parent recovery were selected for the final library. For each population, the set of lines representing the final library were analyzed using CSSL Finder v.0.9.722[1]. The target segment size was calculated in Microsoft Excel, whereas the number of donor and background segments, and the percent recurrent parent genome, were calculated using CSSL finder. The libraries were visualized using the CSSL finder or GGT (van Berloo, 2008) software during population development.

Genotyping

Genomic DNA was extracted from leaf tissue from individual plants in each backcross generation using a modified CTAB protocol (Dellaporta et al., 1983; Fulton et al., 1995). As new technology became available, various genotyping methods were employed for MAS during library development. These platforms were SSRs, 384-OPA, the Cornell_6K_Array_Infinium_Rice (C6AIR) and the C7AIR (McCouch et al., 2002; Orjuela et al., 2010; Thomson et al., 2012, 2017; Morales et al., 2020). Details on each of these genotyping methods are provided in **Supplementary Data 1**. Genotyping of the final libraries was carried out using the C7AIR SNP array (Morales et al., 2020). The "ACGT" nucleotide file from Genome Studio was exported in the PLINK format and uploaded on TASSEL GUI[2]. The taxa (samples) were divided into six groups, each representing one of the six CSSL populations. In each library, SNPs were called based on major vs. minor allele, where it was assumed that the minor allele was inherited from the wild donor parent. SNPs with ≥39% missing data or heterozygous calls, monomorphic SNPs, and singleton SNPs were removed from each dataset. Specific SNPs were removed when it was known that one of the parents could carry either the major or the minor allele at that variant due to heterogeneity of the parental accessions. In addition to genome-wide assays, all five parental accessions in addition to CSSLs that had red or red/brown pericarp were analyzed using functional InDel markers for *Rc* developed by Sweeney et al. (2006) and sequencing primers for *Rd* described by Furukawa et al. (2007). Likewise, donors and RPs were sequenced at black hull genes, *Ph* and *BH4* using primers described in Yu et al. (2008) and Zhu et al. (2011).

Phenotyping

Morphological variation for 16 traits was documented for donor accessions and RPs based on observations of two to five plants per accession. Parent plants were grown in 4 inch pots in the Guterman greenhouse at Cornell University (Ithaca, NY, United States) during summer 2015 and evaluated as described in **Supplementary Table S1**. The phenol reaction of the grain was determined on the parents following the method of

[1]http://mapdisto.free.fr/CSSLFinder/

[2]https://tassel.bitbucket.io/

Chen et al. (2014). CSSLs were also evaluated for pericarp color, hull color, seed shattering, seed set, and delayed flowering (noted as very late flowering under long (∼15 h) days.

CSSL Statistics

Custom R scripts[3] were written to calculate per-library and per-line statistics and to make figures of marker distribution and graphical genotypes. Percent donor genome, percent heterozygosity, and percent missingness were computed based on the total number of informative markers per library. To map QTL in the interspecific CSSL population, stepwise regression within IciMapping v4.0 software[4] (RSTEP-LRT option) can be implemented as described in Wang et al. (2017) and Balakrishnan et al. (2020).

Germplasm Availability

Seeds from the CSSLs and the RPs, IR64 and Cybonnet, are available from the Genetics Stocks-*Oryza* (GSOR) center in Stuttgart, AR, United States[5]. As is standard practice, five seeds per genetic stock will be provided for researchers to amplify. For partially sterile and low-yielding CSSLs, seeds from sibling or progeny lines may be provided in lieu of the original CSSL seed. Seeds from genebank accessions of the three ORSC donors are available as follows: OrA (W1944) from the National Institute of Genetics, Japan; OrB (IRGC106148) and OrC (IRGC105567) from the International Rice Germplasm Collection in the Philippines.

[3]https://www.R-project.org/

[4]http://www.isbreeding.net/software/?type=detail&id=14

[5]https://www.ars.usda.gov/GSOR

RESULTS AND DISCUSSION

Selection of Wild and Cultivated Parents

Genetic relationships among a collection of 286 accessions from the ORSC and a set of 54 *O. sativa* control samples were examined using a rooted NJ dendrogram and a previously generated set of 113,739 GBS SNPs (Kim et al., 2016; **Figure 1**). Based on geographical, phenotypic and genomic diversity, as well as cross-compatibility, three ORSC accessions were selected for crossing with both *Indica* and *Japonica* elite varieties. They were (1) NIAS W1944 (hereafter referred to as OrA) from China, classified in the Institute of Genetics in Japan as *O. rufipogon* Griff.; (2) IRGC106148 (hereafter referred to as OrB) from Laos, identified as *Oryza nivara* Sharma and Shastry; and (3) IRGC105567 (hereafter referred to as OrC) from Indonesia, classified as *O. rufipogon* Griff (**Table 1**). These accessions represented wild subpopulations W6, W4/W1 admix, and W1, respectively, as reported by Kim et al. (2016). Genetically, this suggests that OrA is more closely related to the *Japonica* varietal group, OrB shares ancestry with *Aus* and *Indica*, and OrC represents a group of ancestors that do not share close ancestry with any *O. sativa* subpopulation.

Recurrent parents were selected to represent both the *Japonica* and the *Indica* varietal groups, with emphasis on elite, publicly available breeding material commonly used in public-sector breeding programs and widely grown in different parts of the world. Cultivars Cybonnet (CV-122, PI636726, GSOR301380) and IR64 (IRGC117268, GSOR301401) were selected as the RP (**Table 1**). Cybonnet is an elite *tropical japonica* from the United States adapted to the sub-tropics, and was released by the Arkansas Agricultural Experiment Station in 2004. It is early maturing, high-yielding, and semi-dwarf, with long-grain, improved milling yield and good blast resistance

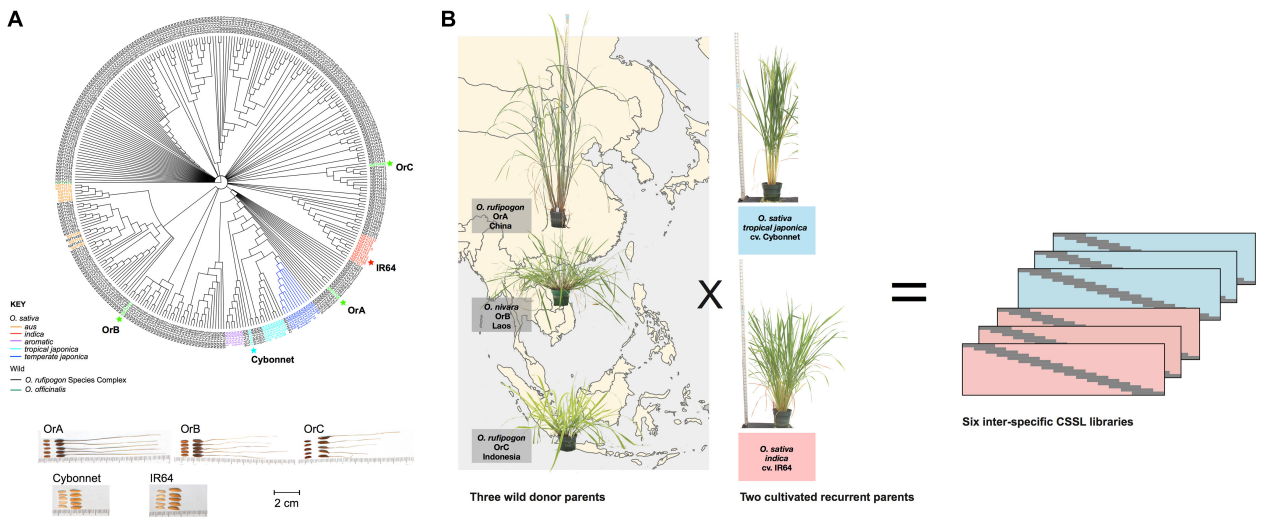

FIGURE 1 | Parental selection of six inter-specific Chromosome Segment Substitution Line (CSSL) libraries. Six libraries of CSSL libraries were constructed in two elite *O. sativa* backgrounds (cv. Cybonnet and IR64) using three wild *Oryza rufipogon* species complex (ORSC) donor accessions (OrA, OrB, and OrC). Seed morphology and genetic relationships of parental lines with each other and other wild and cultivated accessions are shown in panel **(A)**. Geographical origin and plant architecture variation of the wild donors are shown in panel **(B)**.

TABLE 1 | Parents used to construct six CSSL libraries.

Parent	Gene bank ID[1]	Name	Species[2]	Country	Nuclear genome[3]	Subpopulation[4]	Chloroplast haplotype[3]
Donor	NIAS W1944	OrA	*O. rufipogon*	China	W6	–	I-1
	IRGC 106148	OrB	*O. nivara*	Laos	W4/W1	–	I-1
	IRGC 105567	OrC	*O. rufipogon*	Indonesia	W1	–	VIII-2
Recurrent	PI 636726	Cybonnet	*O. sativa*	United States	–	*tropical japonica*	In-12
	IRGC 117268	IR64	*O. sativa*	Philippines	–	*indica*	I-5

[1]*IRGC = International Rice Germplasm Collection, IRRI, Philippines; NIAS = National Institute of Genetics, Mishima, Japan distributed by National BioResource Project: Rice; PI-Plant Introduction, distributed by the National Small Grains Collection (NSGC) at Aberdeen, ID, United States, a component of the National Plant Germplasm System (NPGS) of the United States Department of Agriculture - Agricultural Research Service.*
[2]*Species designation as presented in IRGC/NIAS/NSGC.*
[3]*As per Kim et al. (2016). W6 is Japonica-like; W4/W1 is Indica admix (aus/indica); W1 did not share close ancestry with any O. sativa subpopulation.*

(Gibbons et al., 2006). IR64 is an elite *Indica* developed by the International Rice Research Institute (IRRI) in the Philippines and released in 1985. It is a semi-dwarf variety with high yield potential, short growth duration, enhanced resistance to diseases and insect pests and is well-known for its superior eating quality (Khush, 2005; Mackill and Khush, 2018). IR64 is adapted to the tropics and is widely grown throughout South and SE Asia. It is still commonly used as a parent for breeding of both inbred and hybrid varieties (Virmani and Kumar, 2004; Toriyama and Kazama, 2016).

Phenotypic Diversity of Donor and Recurrent Parents

The wild donors and the elite RPs were evaluated in the greenhouse for 16 characters, with emphasis on plant and seed morphological traits, flowering time, and seed set (**Table 2** and **Supplementary Table S1**). OrA from China was readily differentiated from the other two donors with its tall, upright plant type, compact panicles, lack of shattering, lack of stolons and elbows, as well as its early flowering. OrB from Laos had short stature, abundant stolons and elbows, spreading culm habit, open panicle type and abundant shattering, with intermediate flowering time (**Figure 1**). OrC from Indonesia was also short, flowered later than the other donors, had open panicles that shattered readily, and very poor seed set. All donors had black hull, long awns and positive phenol reaction. Conversely, neither recurrent parent displayed awns, elbows or a shattering habit during any greenhouse or field observations, and both had a high rate of seed production. Cybonnet had erect culm angle, compact panicles and straw colored grains. IR64 had erect culm angle with intermediate panicle type and golden hull color. Cybonnet seeds showed no reaction to phenol while IR64 seeds had a positive phenol reaction whereby seeds turned a dark purple-black color when exposed to phenol.

Characterization of CSSL Libraries

Table 3 provides a summary of the six CSSL libraries in terms of number of lines, donor contribution, marker coverage, and percent heterozygosity. Individual libraries are comprised of 63–81 lines each, with an estimated percent donor contribution per line ranging from 2.9 to 6.9%, based on the total number of informative markers per library. Libraries constructed using OrA gave rise to populations with the largest number of spurious background introgressions per line compared to libraries constructed using OrB or OrC, though the reason for this is not clear (**Figures 2**, **3**).

The number of informative SNPs per population ranged from 1,496 to 2,962, and was correlated with the degree of genetic relatedness between the parents. Thus, in the Cybonnet × OrA library, the number of SNPs detected was lower than in the Cybonnet × OrB or OrC libraries, and the reverse was true for the IR64 libraries. This was consistent with the ORSC subpopulation relationships reported by Kim et al. (2016) and with the phylogenetic analysis summarized in **Figure 1**. The average inter-marker distance is negatively correlated with marker number, but does not adequately portray the patterns of marker distribution observed in the different libraries; these are summarized in **Figure 4**. The large gaps in marker coverage are most pronounced in the IR64 populations (with maximum sizes of 8.3, 9.3, and 8.8 Mb in the OrA, OrB, and OrC populations, respectively), but are also apparent in the Cybonnet populations (with maximum sizes of 4.8, 3.3, and 1.9 Mb in the OrA, OrB, and OrC populations, respectively) (regions highlighted in green in **Figure 4**). On chromosome 4, a marker gap at 12,954,360–21,271,163 bp in the IR64 × OrA population was found to be partially shared with a gap at 14,482,427–23,294,656 bp in the IR64 × OrC and also with a gap from 20,352,551 bp until the end of the chromosome at 35,502,694 bp in the Cybonnet × OrA population. Other examples include the gaps on chromosome 8 observed from 9,289,473 to 17,516,403 bp in IR64 × OrA, 8,897,653–18,185,078 bp in IR64 × OrB, and 15,439,243–18,108,496 bp in IR64 × OrC. These regions that are devoid of informative markers are referred to as "SNP deserts," and are not believed to be the result of major bias in the SNP assays, based on the fact that marker coverage in the Cybonnet × OrB and Cybonnet × OrC populations is fairly uniform and that apparent gaps in one population are invariably well covered regions in another. They are indicated as white spaces in the CSSL maps in **Figures 2**, **3**. Overall, the amount of missing data after SNP filtering was very low, ranging from 0.03 to 0.32%, and the level of heterozygosity is <1% in all libraries.

We hypothesize that the "SNP deserts" may be due to one or more of the following: (1) these regions are common by state (cbs) and inferred to be common by descent (cbd) in the different cross combinations, thereby resulting in swaths of

TABLE 2 | List of traits evaluated under greenhouse conditions in the CSSL donors and recurrent parents.

Trait name	ORSC[a] donor accessions			*O. sativa* recurrent accessions	
	OrA	OrB	OrC	Cybonnet	IR64
Culm angle	Erect (5–10°)	Spreading (>60–80°)	Open (~60°)	Erect (4–8°)	Erect (12–17°)
Awn presence*	9, Long and fully awned	9, Long and fully awned	9, Long and fully awned	0, Absent	0, Absent
Panicle type*	1, Compact	3, Open	3, Open	1, Compact	2, Intermediate
Rhizome/stolon formation*	1, Vegetative crown	3, Vegetative crown and weak rhizomes	3, Vegetative crown and weak rhizomes	1, Vegetative crown	1, Vegetative crown
Elbows*	1, Absent	2, Present	2, Present	1, Absent	1, Absent
Plant height	avg. 197 cm	avg. 89.1 cm	avg. 99 cm	avg. 116 cm	avg. 98 cm
Days to flowering in Ithaca, NY (~15 h daylength)	avg. 89 days	avg. 110 days	avg. 135 days	avg. 85 days	avg. 94 days
Seed production	Moderate	Moderate	Low	High	High
Seed shattering or hilum abscission*	0	9 (>50%)	9 (>50%)	0	0
Lemma and Palea: Hull color*	8, Black	8, Black	8, Black	2, Straw	3, Gold
Caryopsis: Grain color*	5, Brown	5, Red	5, Red	2, Light brown	2, Light brown
Hull length or grain length (mm)	8.3	7.3	8.4	9.3	9.8
Hull width or grain width (mm)	2.6	2.3	2	2.5	2.7
Grain length or caryopsis length (mm)	6.4	5.4	5.9	7.1	7.2
Grain width or caryopsis width (mm)	2.1	1.9	1.7	2.1	2.2
Phenol reaction[b]	1, Positive	1, Positive	1, Positive	0, Negative	1, Positive

Numbers refer to the Standard Evaluation System (SES) for rice; see also* ***Supplementary Table S1* *for descriptions.*
[a] *Oryza rufipogon Species Complex (ORSC).*
[b] *Determined using the method of Chen et al. (2014).*

monomorphic SNPs; (2) sterility challenges led to irrecoverable introgressions, whereby target donor segments in one generation could not be propagated into future generations, resulting in an apparent lack of polymorphism in these regions in the final library dataset; and/or (3) heterogeneity in recurrent parent accessions used for crossing gave rise to polymorphic regions in final libraries that were difficult to attribute to either donor or recurrent parent and thus markers in these regions were dropped during SNP filtering (see section "Materials and Methods"). It is difficult to explicitly attribute marker gaps to cbd (hypothesis one) because the exact individuals used for crossing may not have been the same ones used for genotyping; however, given our current understanding of the genetic relationships between wild and cultivated rice subpopulations, we postulate that it is unlikely the gap on chromosome 4 shared between IR64 × OrA, IR64 × OrC, and Cybonnet × OrA libraries is a result of cbd. As an example of hypothesis two, we report efforts to maintain a line containing an introgression covering the region on chromosome 11 from 14 to 25.7 Mb in the Cybonnet × OrA population. The line was eventually lost due to sterility, and its absence from the final library led to apparent monomorphism in the genotyping dataset, explaining the marker gap in this region for that library (**Figure 4**). As for hypothesis three, within-line genetic heterogeneity is common in many improved varieties of rice, and is an essential characteristic of landrace and wild accessions found in genebanks (Olufowote et al., 1997). In this study, three different individuals per wild accession were used as pollen donors in crosses with multiple individuals from the two RPs. Regions in the genome where heterogenity is prevalent among individuals of an accession pose difficulties downstream in determining whether donor introgressions are present. Future attempts to carry out deep sequencing on these lines may likewise run into similar challenges in attributing alleles to donor or recurrent parent.

TABLE 3 | Summary statistics on CSSL libraries.

Recurrent parent	Cybonnet			IR64		
Donor parent	**OrA**	**OrB**	**OrC**	**OrA**	**OrB**	**OrC**
Number of lines	63	76	77	68	81	69
Average donor contribution* (%)	6.9	3.3	2.9	6.1	3.3	4.1
Number of informative SNPs	2116	2693	2521	2962	1496	1558
Average inter-marker distance (kb)	172	137	145	124	245	233
Average missingness* (%)	0.32	0.16	0.06	0.12	0.03	0.06
Average heterozygosity* (%)	0.99	0.46	0.17	0.73	0.17	0.22

**Average donor contribution, heterozygosity and missingness metrics are computed based on the number of markers (not physical distances or positions).*

Wild Phenotypes Displayed in CSSL Lines

Depending on the specific donor segments carried by each of the CSSLs, wild characteristics are sometimes observed in the final lines. Classic domestication traits that help differentiate wild and cultivated rice include pericarp color, hull color, degree of seed shattering and flowering time. Some of these characteristics make the CSSLs difficult to grow and/or subject them to regulatory

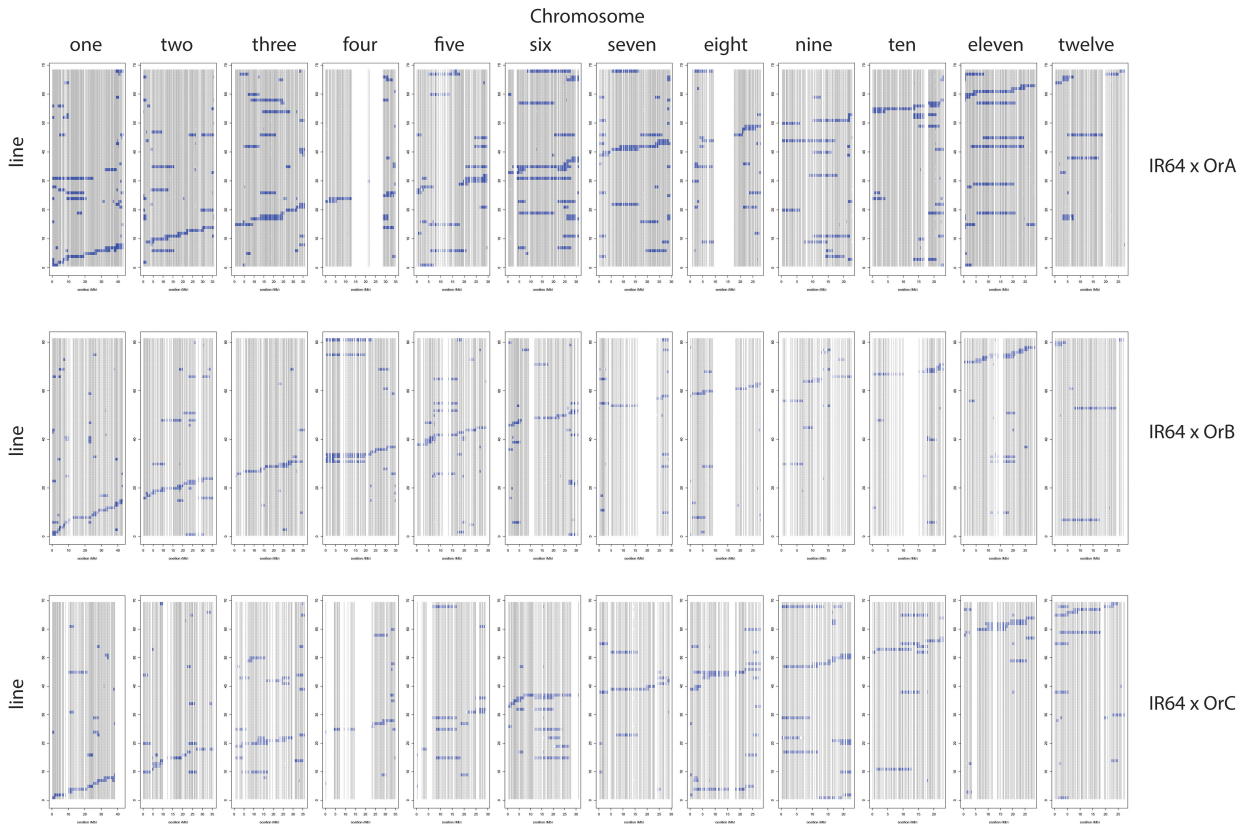

FIGURE 2 | IR64 CSSL libraries using OrA (top), OrB (middle), and OrC (bottom) donors. The 12 chromosomes are identified along the top and the markers for each of the 12 chromosomes are represented as a "column" based on physical distance. The genotype of each CSSL (line) is represented as a "row" within the given library identified on the left side.

oversight, and thus knowing which lines carry "wild" traits is helpful in designing experiments to further evaluate the lines.

Pericarp Color

Out of the total 434 CSSLs comprising the six introgression libraries, 16 had pericarp color typically associated with wild rice (i.e., red or red/brown) (**Supplementary Figure S2**); 10 of these were lines in the Cybonnet background, and six were in the IR64 background. Five of the six libraries presented ILs with colored pericarp; the Cybonnet × OrC library was comprised only of white pericarp lines. Pericarp color is dependent upon the combination of alleles at the *Rc* (Os07g0211500) and *Rd* (Os01g0633500) loci, located on rice chromosomes 7 and 1, respectively (**Table 4** and **Figure 4**). Both *Rc* and *Rd* must carry a dominant allele to produce red pericarp, whereas a dominant *Rc* allele and a recessive *rd* allele will produce brown pericarp. Any other combination (*rc/rd*, *rc/Rd*) will produce rice with white pericarp. All three donor accessions in these libraries had red pericarp and both RPs had white pericarp (**Supplementary Figure S2**). PCR results showed that both cultivated parents were homozygous recessive at the *Rc* locus; both carried the 14 bp deletion in exon 2 associated with lack of function for *Rc*, while all three wild parents carried the dominant (functional) allele at *Rc*. We next examined the *Rd* gene and found that Cybonnet carried the recessive (non-functional) allele but IR64 had the dominant *Rd* allele. OrB and OrC both carried the dominant (functional) allele at the *Rd* locus, but OrA was heterogeneous; some individuals carried the dominant allele and had red seeds, and others had brown seeds due to a recessive allele at *Rd*.

Supplementary Figure S2 shows CSSLs that have donor introgressions at the *Rc* and/or *Rd* genes, and indicates the pericarp color observed in the grain. Of the 16 CSSLs that carried pigmented pericarp, eight were from the Cyb × OrA library, and none were from the Cyb × OrC library. The lines were genotyped using the methods described above and the observed pericarp color was consistent with genetic predictions in the libraries derived from OrB and OrC in both the Cybonnet and the IR64 backgrounds. On the other hand, sequencing of the *Rd* gene in the CSSLs derived from OrA revealed two separate single nucleotide substitutions (mut#1 = A and mut#2 = G) in exon 2, each of which have previously been shown to render the gene non-functional (Sun et al., 2018). In the Cyb × OrA library, some CSSLs carried the wild type allele at *Rd* while others carried a non-functional *rd* allele; all lines with a non-functional allele carried mut#2 in exon2, while a few also carried mut#1. We observed a single line, Cyb_ OrA_4, with brown pericarp due to the fact that it carried a donor introgression at *Rc* on chromosome 7 and a second donor introgression on chromosome 1 carrying the *rd* allele with

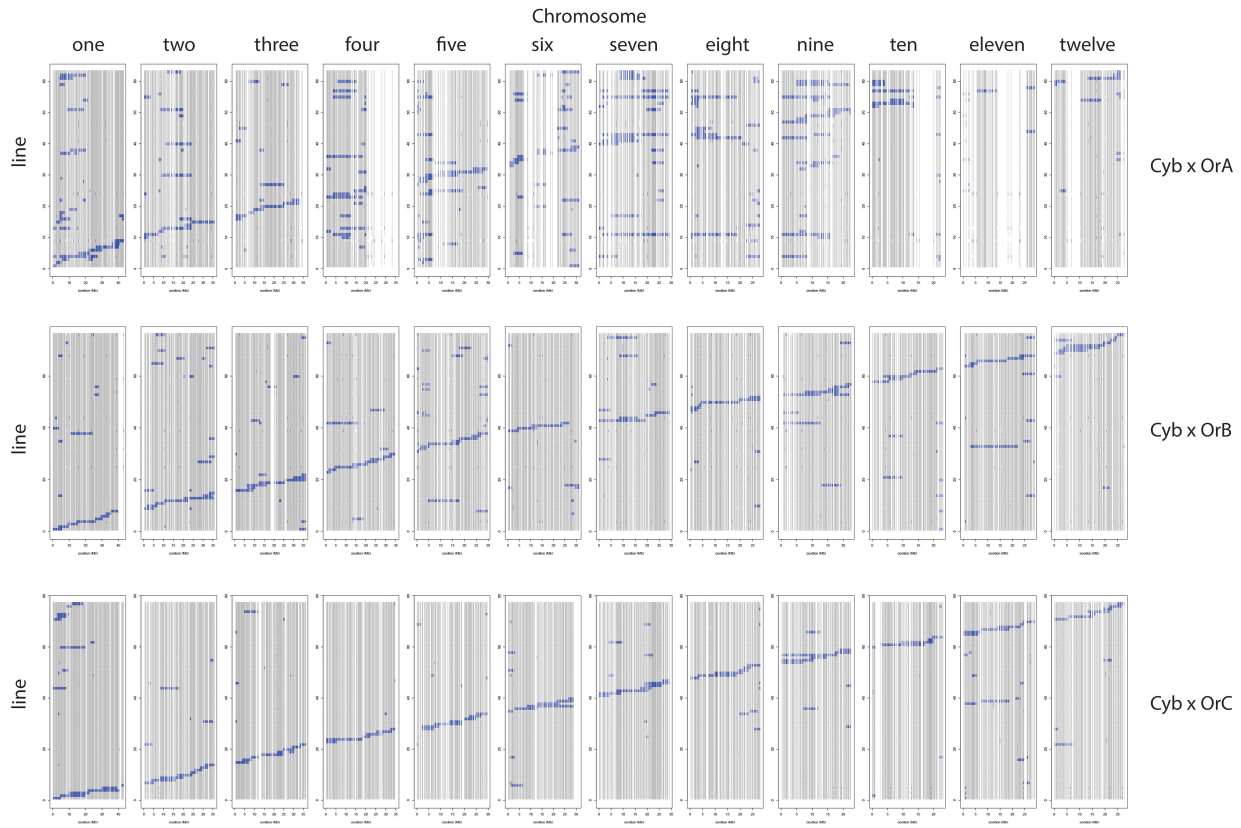

FIGURE 3 | Cybonnet CSSL libraries using OrA (top), OrB (middle), and OrC (bottom) donors. The 12 chromosomes are identified along the top and the markers for each of the 12 chromosomes are represented as a "column" based on physical distance. The genotype of each CSSL (line) is represented as a "row" within the given library identified on the left side.

both mut#1 and mut#2. Other lines (Cyb_OrA_6, IR64_OrA_5) carrying the same donor allele at *rd* (with the two mutations) had white pericarp because they carried *rc* alleles from their respective RPs on chromosome 7 (**Supplementary Figure S2**).

Hull Color

The black hull phenotype is controlled by at least two complementary genes that are linked in a cluster of domestication-related genes on rice chromosome 4 (Fukuda et al., 2012). The gene Black Hull 4 (BH4) encodes a tyrosine transporter (Zhu et al., 2011), and Phenol Reaction (Ph) encodes the polyphenol oxidase (PPO) enzyme which uses tyrosine as a substrate (Yu et al., 2008; **Table 4** and **Figure 4**). A third gene identified as OsbHLH016 is located between the other two genes on chromosome 4, and encodes a bHLH transcription factor. It is known to positively regulate the biosynthesis of anthocyanin and has been tentatively associated with black hull (Sun et al., 2018). Other genes for black hull have been reported on chromosomes 4, 5, and 7 (Morishima and Oka, 1981; Maekawa, 1984; Gu et al., 2005). Functional alleles at both BH4 and Ph are necessary to generate the black hull phenotype, but straw-white hull color has been selected under domestication. Multiple functional mutations have been documented in both genes, with clear divergence between the *Indica* and *Japonica* gene pools, resulting in straw-gold hull color in a majority of cultivated germplasm (Yu et al., 2008; Zhu et al., 2011; Fukuda et al., 2012).

In this study, the three donor parents all had black hulls, while both RPs had straw-gold hulls (**Figure 1** and **Table 2**). To confirm which of the parental lines carried a functional *Ph* gene, seeds of the donors and RPs were tested for phenol reaction. All three wild donors and the IR64 RP displayed positive phenol reaction, while Cybonnet seeds showed no discoloration. Based on this evidence, we determined that Cybonnet carried the non-functional *ph* allele, while IR64 carried the functional *Ph* allele, consistent with a majority of *Indica* varieties. We further confirmed this result by sequencing the *Ph* locus. Sequencing demonstrated that Cybonnet carried a 1-bp insertion in exon 1 (chr 4: 31,749,302 bp) causing a frameshift that rendered the protein non-functional. This was somewhat unexpected, as a well known 18 bp loss-of-function deletion in exon 3 is more characteristic of *japonica* varieties (Yu et al., 2008). Sequencing of the *BH4* gene confirmed that all three wild donor parents carried functional *BH4* alleles, while Cybonnet and IR64 both carried non-functional alleles caused by a common 22 bp deletion in exon 3 (Zhu et al., 2011). We next assessed hull color in the CSSLs and found that only three lines, all from the IR64 × OrB library, carried the

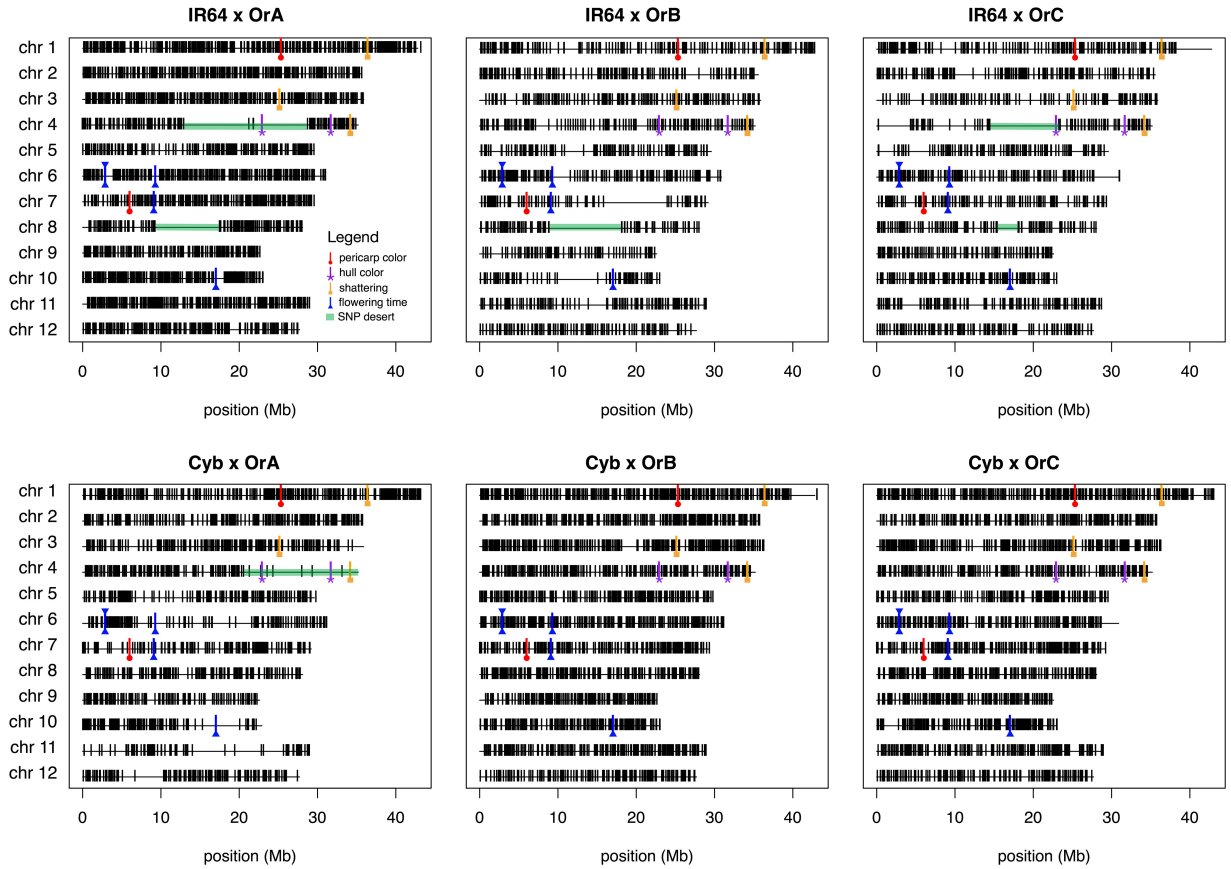

FIGURE 4 | Physical distribution of markers using the C7AIR across the six finalized CSSL libraries. Total number of polymorphic markers in the populations ranged from 1496 (IR64 × OrB) to 2962 (IR64 × OrA). Visual gaps in marker distribution are due to either lack of polymorphism or poor SNP calling for which markers were filtered. Genes discussed in this manuscript are annotated using symbols shown in the legend: red line with circle for pericarp color genes (*Rc*, *Rd*); purple line with asterisk for hull color genes (*Ph*, *BH4*); orange line with square for shattering genes (*SH4*, *qSH1*, *Sh1*); and blue line with triangle flowering time genes (*RFT*, *Hd1*, *Hd3a*, *Ehd1*, *Ghd7*). Also indicated are marker gaps in green highlight that are discussed in the text.

black hull phenotype (individuals IR64_OrB_34, IR64_OrB_35, and IR64_OrB_81). Each of the black hulled lines contained a donor introgression at the *BH4* locus, but the introgression alone was not predictive of the phenotype; two lines from the same library carried donor introgressions at *BH4* (individuals IR64_OrB_15 and IR64_OrB_21), but did not have black hulls (**Supplementary Table S2**). These results suggest that if functional alleles at *Ph* and *BH4* alone were sufficient to cause black hull, any CSSL in the IR64 background (*Ph/bh4*) would need only a single introgression carrying a functional copy of *Bh4* to manifest the black hull phenotype, while a CSSL in the Cybonnet background (*ph/bh4*) would require an extended donor introgression covering both genes (∼8.8 Mb region). As summarized in **Supplementary Table S2**, we identified four CSSLs in the Cybonnet × OrA population carrying an introgression that potentially encompasses both *Ph* and *BH4*; however, these lines did not have black hulls, suggesting that none of them carried both genes. The paucity of markers in the region (**Figure 4**) precludes explicit definition of the introgression breakpoints. Sequencing the *Ph* and *BH4* regions of the five previously mentioned IR64 × OrB CSSLs potentially carrying the *BH4* introgression would further elucidate the mechanism underlying the black hull phenotype.

Shattering

Several major shattering genes have been identified in rice (Cai and Morishima, 2000; Onishi et al., 2007). *Shattering 4 (SH4)* is a member of the trihelix family of transcription factors and lies near the end of the long arm of chromosome 4, linked to the two major *black hull* loci described above (Li et al., 2006; Lin et al., 2007). *qSH1* is a BEL1-type homeobox-containing protein on chromosome 1 (Konishi et al., 2006). *OsShattering 1 (OsSh1)* is a YABBY transcription factor that was originally mapped as an ortholog of the *Shattering1* gene in sorghum (Lin et al., 2012); it is co-located with *qSH3*, a shattering QTL on chromosome 3 (Inoue et al., 2015; Ishikawa et al., 2017; **Table 4** and **Figure 4**).

While shattering was not measured quantitatively in this study, 14 CSSLs were noted to have an unusually high degree of shattering such that they required bagging of panicles to ensure seed harvest (**Supplementary Table S2**). Thirteen of the 14 shattering CSSLs carried a donor introgression at *SH4* (**Supplementary Table S2**). The exception was CSSL

TABLE 4 | Gene reference Table with fsnps added.

Gene_ID	Gene symbol synonym(s)	CGSNL Gene name	Gene name synonym(s)	Chr. No.	RAP ID	RAPDB start (bp)	RAPDB end (bp)	MSU ID
Rc	*RC, OsbHLH017, OsbHLH17*	*BROWN PERICARP AND SEED COAT*	*Brown pericarp and seed coat, basic helix loop helix 17*	7	Os07g0211500	6,062,889	6,069,317	LOC_Os07g11020.1
Rd	*RD, DFR, OsDFR, OS-DFR*	*RED PERICARP AND SEED COAT*	*Red pericarp and seed coat, dihydroflavonol 4-reductase, dihydroflavonol-4-reductase*	1	Os01g0633500	25,382,714	25,384,678	LOC_Os01g44260.1
Bh4	*Bh4, OsATL14, ATL14*	*BLACK HULL 4*	*Black hull 4, amino acid transporter-like 14*	4	Os04g0460200	22,969,845	22,971,859	LOC_Os04g38670.1; LOC_Os04g38660.1
Ph	*BHC, Bhc(Po), Bhc, Po, Ph, Bh3, PPO, Phr1, BH1*	*BLACK HULL C*	*Phenol staining, polyphenol oxidase, PPO enzyme, BLACK HULL1*	4	Os04g0624500	31,749,141	31,751,604	LOC_Os04g53300.1
OsbHLH016	*OsbHLH016, Kala4, OsB2*		*Basic helix-loop-helix protein 016 Key gene for black coloration by anthocyanin accumulation on chromosome 4*		Os04g0557500	27,915,598	27,939,357	LOC_Os04g47059.1
SH4	*Sh4, SHAT2, SHA1, qSH4, OsSh4,*	*SHATTERING 4*	*Shattering 4, grain shattering quantitative trait locus on chromosome 4, SHATTERING ABORTION2*	4	Os04g0670900	34,231,186	34,233,221	LOC_Os04g57530.1
qSH1	*qSH1, qsh1, Qsh1, qSH-1, RIL1, OsRIL1*	*Shattering (QTL)-1*	*QTL of seed shattering on chromosome 1, RI-LIKE1, erticillate rachis-like 1*	1	Os01g0848400	36,445,456	36,449,951	LOC_Os01g62920.1
OsSh1	*YAB2, OsYAB2, OsSh1, Sh1, SH1, OsFIL2, OsSH1, Osh1, qSH3*	*SHATTERING1*	*YABBY2, Os YABBY2, Shattering1, FIL homolog 2*	3	Os03g0650000	25,197,057	25,206,948	LOC_Os03g44710.1
RFT1	*FT-L 3, OsFTL3, FT-L3, RFT, OsRFT1*	*RICE FLOWERING-LOCUS T 1*	*Rice Flowering-locus T 1, FT-like gene 3, RFT*	6	Os06g0157500	2,926,823	2,928,474	LOC_Os06g06300.1
Hd3a	*HD3A, FT, OsHd3a, qHD3(t)*, FT-L 2, OsFTL2*	*HEADING DATE 3A*	*Heading date-3a, Heading date (QTL)-3(t), Flowering locus T, FT-like gene 2*	6	Os06g0157700	2,940,004	2,942,452	LOC_Os06g06320.1
Hd1	*Se1, Se1(Lm,Lf,Rs,Fl), HD1, K, Se-1, Hd1(t), qHD1(t), OsA, OsBBX18, BBX18, Hd1/OsA, OsCCT21*	*PHOTOSENSITIVITY 1*	*Photosensitivity1, Heading date, HEADING DATE 1, Arabidopsis CONSTANS(CO) gene ortholog, B-box-containing protein 18, CCT domain-containing gene 21, CCT (CO, CO-LIKE and TOC1) domain protein 21*	6	Os06g0275000	9,336,376	9,338,569	LOC_Os06g16370.1
Ghd7	*Ghd2, OsCCT26, OsCMF8, Osl, OsEH7 Ghd7/Hd4, EH7-1/Hd4, Ghd7-0a,*	*HEADING DATE 7*	*heading date 7, "\Grain number, plant height, and heading date7\," CCT domain-containing gene 26, CCT (CO, CO-LIKE and TOC1) domain protein 26, Early heading 7*	7	Os07g0261200	9,152,377	9,155,030	LOC_Os07g15770.1
Ehd1	*Ef1, Ef2, Ehd1, Eh1(t)*, qEHD-10- 1(t) (qEhd1), OsRR30, OsRR22*	*EARLINESS 1*	*Earliness1, EARLY HEADING DATE 1*	10	Os10g0463400	17,076,098	17,081,344	LOC_Os10g32600.1
Pi-ta	*PITA, Pita (sl, Pi4a, Pi-ta), Pita2, Pi4a, Pi-4, OsTRXh6, OsTrx30*	*PYRICULARIA ORYZAE RESISTANCE TA*	*Pyricularia oryzae resistance-ta, Magnaporthe grisea resistance-ta, Blast resistance ta, Thioredoxin H-type 6, Thioredoxin 30*	12	Os12g0281300	10,606,359	10,611,917	LOC_Os12g18360.2 LOC_Os12g18360.1
GS3	*LK3, lk3(t), SG3, SG3-GS3, OsGS3, OsGW3, RGG3, GGC1 Mi, OsGGC1,*	*LONG KERNEL 3*	*long kernel 3, grain size, grain length and weight protein, GRAIN SIZE 3, G gamma subunit GS3, Heterotrimeric G Protein gamma Subunit*	3	Os03g0407400	16,729,501	16,735,109	Not annotated
ALK	*alk, OsSSIIa, SSIIa, SSS2A, SS2a, OsSSII-3*	*ALKALI DEGENERATION*	*ALKALI DEGENERATION, Soluble starch synthase 2–3, chloroplast precursor, Soluble starch synthase 2–3, chloroplastic/amyloplastic, Soluble starch synthase II- 3, Starch synthase IIa, soluble starch synthase Iia*	6	Os06g0229800	6,748,398	6,753,302	LOC_Os06g12450.1
Waxy	*WX1, Wx, WX-B, OsWx, GBSS, OsGBSSI, GBSS1, GSS*	*GLUTINOUS ENDOSPERM*	*glutinous endosperm, WAXY, Granule-bound starch synthase 1/ chloroplastic/ amyloplastic, Granule- bound starch synthase I, UDP-glycogen synthase, Granule-bound starch synthase/ chloroplast precursor, glycogen [starch] synthase, UDPG-glycogen transglucosylase, uridine diphosphoglucose-glycogen glucosyltransferase*	6	Os06g0133000	1,765,622	1,770,653	LOC_Os06g04200.4 LOC_Os06g04200.3 LOC_Os06g04200.2 LOC_Os06g04200.1

"IR64_OrB_29" which had a shattering phenotype but did not carry donor alleles at *SH4*; however there was a donor introgression at the *OsSh1* locus on chromosome 3. Nine CSSLs from three different libraries (five from the IR64 × OrA library, two from the IR64 × OrC library, and two from the Cybonnet × OrC library) carried donor introgressions at *SH4* but were not observed to be shattering, and between 3 and 6 lines from each of the six libraries carried introgressions at *OsSh1* and/or *qSH1* with no obvious effect on the phenotype (**Supplementary Table S2**). We found no highly shattering phenotypes in the Cybonnet × OrA library, nor did we find any black hull phenotypes. This may be due to the fact that there was a paucity of SNP markers across the *BH4–SH4* region of chromosome 4 in this library, relative to all other libraries, which made it virtually impossible to select for the presence of donor introgressions in that region during CSSL development (**Figure 4**).

Seed shattering is the result of a developmentally programmed weakening or degradation of the abscission cell layer at the base of the grain where it attaches to the pedicel, and abscission layer formation is known to be regulated by multiple genes that control the timing and degree of seed shattering (Inoue et al., 2015). Large variation in the degree of seed shattering is observed in rice cultivars, with *Indica* varieties typically exhibiting higher degrees of shattering than *Japonica*. Consistent with this observation, 12 of the shattering lines in this study were from the IR64 libraries while only two were from the Cybonnet libraries. These results suggest that the major-effect genes described above interact with other genes in the genetic background to determine the extent and degree of shattering, and that the pattern of interacting background alleles in IR64 (*Indica*) differs from Cybonnet (*Japonica*). Thus, despite the presence of donor introgressions at *OsSh1/qSH3, qSH1* and *SH4* in many of our shattering CSSLs, it is not surprising that these loci, alone, are not predictive of the phenotype. These results open the door to further research using these materials to gain a deeper understanding of the genetic determinants of seed shattering in rice.

Flowering Time

Eight CSSLs in the Cybonnet background were observed to have modified phenology and displayed later-than-expected flowering under the long days of summer in the greenhouse in Ithaca, NY (12–16 h of daylight). Four of the lines derived from crosses with OrA, which itself had the earliest flowering of the three wild donors, and two lines each from the other Cybonnet libraries. Data on flowering time under long day conditions is not available for the IR64 libraries. Studies of flowering time in rice have demonstrated that a combination of large- and small-effect genes define the genetic architecture of the trait, and adaptation to a wide range of daylength and temperature regimes is mediated by complex gene × gene interactions in both wild and cultivated populations. To date, more than a dozen flowering time genes have been cloned and characterized (OGRO database)[6] (Yamamoto et al., 2012) and numerous additional small-effect QTLs have been extensively documented (Bentley et al., 2013; Itoh and Izawa, 2013; Hori et al., 2015).

In this study we identified lines carrying donor introgressions at five major flowering time loci located on chromosomes 6, 7, and 10 (**Figure 4**; **Table 4**; and **Supplementary Table S2**). The genes belong to two independent signaling pathways, as summarized in Hori et al. (2015). The first pathway, referred to as the *OsGI-Hd1-Hd3a* (*rice GIGANTEA*, *Heading data 1*, *Heading date 3a*) pathway, corresponds to the *GI-CO-FT* (*GIGANTEA*, *CONSTANS*, *FLOWERING LOCUS T*) pathway in *Arabidopsis*. *Hd1* is a homolog of *Arabidopsis CO* and it promotes heading under short-day and represses it under long-day conditions (Yano et al., 2000). *HD1* promotes the expression of both *Hd3a* and its tandemly duplicated paralog, *Rice flowering locus T 1* (*RFT1*), under short-day conditions, but inhibits *Hd3a* and *RFT1* under long-day conditions (Kojima et al., 2002). *Hd3a* and *RFT1* function as florigens, or floral inducers (Tamaki et al., 2007; Ogiso-Tanaka et al., 2013). These three genes are all located on chromosome 6 in rice. The other pathway represents an independent signaling cascade that also regulates the florigens but is unique to rice. It includes *Early heading date 1* (*Ehd1*) and *Grain number, plant height and heading date 7* (*Ghd7*). *Ehd1* is expressed only under short-days and it promotes flowering by inducing transcription of *Hd3a* and *RFT1*, functioning independently of *Hd1* (Doi et al., 2004). *Ghd7* represses *Ehd1*, *Hd3a*, and *RFT1* under LD conditions, and does not affect *Hd1* mRNA levels (Xue et al., 2008). *Ehd1* is located on chromosome 10 and *Ghd7* on chromosome 7 (**Figure 4**).

Chromosome segment substitution lines containing introgressions at these five loci are summarized in **Supplementary Table S2**. For the four late-flowering lines from the Cyb × OrA library, we observed no relationship between the phenotype and the presence of donor introgressions at any of the above-mentioned flowering time loci. However, for the Cyb × OrB and Cyb × OrC libraries, the presence of a donor introgression at the *Hd1* locus was perfectly predictive of late flowering. In the case of Cyb_OrB_39, which was also late flowering, we see that the line carries an introgression across the *Hd3a* and *RFT1* genes, but does not include *Hd1*. This suggests that donor alleles at other genes are responsible for the phenotype in this line.

Seed Production in CSSLs

During development of the CSSL libraries, we observed instances of low seed set (less than 200 seeds per plant under greenhouse or field conditions) in several lines per population (**Supplementary Table S2**). Overall, 28 lines were noted to have low levels of fertility (four in the Cyb × OrA, six in the Cyb × OrB, four in the Cyb × OrC, four in the IR64 × OrA, six in the IR64 × OrB, and four lines in the IR64 × OrC populations). Six lines in the Cybonnet × OrB and OrC libraries had low or no seed set in the field (Lafayette, LA, United States, 2019) but it was possible to obtain some seed set from the same plants grown under greenhouse conditions in Ithaca, NY, United States. Over the course of development, we also noticed that some lines were fertile in the heterozygous condition, but we were unable to harvest seed from homozygous offspring. In some of these

[6]http://qtaro.abr.affrc.go.jp/ogro

cases, we were able to select sib lines that contained segment(s) of interest and produced seed when selfed, but over time, it became more difficult to find lines containing specific regions of introgression; in some cases, we lost segments entirely due to sterility (**Supplementary Table S3**). For example, in both the Cyb × OrB and IR64 × OrB libraries, a ∼650 kb region on chromosome 1 (41,985,829–42,630,129 bp) was only maintained as a heterozygous region and the seed set was always very low in the CSSLs carrying this introgression, indicative of the presence of a sterility gene(s). This region was implicated only in the OrB libraries, as sterility was not a problem in the corresponding lines developed from the OrA or the OrC donors. At this time, we have chosen to maintain some lines as backcross populations as an insurance policy to avoid losing additional segments. A case in point is Line Cyb_OrB_4 which contains six introgressions in chromosomes 1, 3, and 11, and is currently propagated by backcrossing to Cybonnet.

There are hundreds of loci known to contribute to varying levels of gametic and zygotic incompatibilities distributed throughout the 12 chromosomes of rice as noted in Oryzabase[7] (Kurata and Yamazaki, 2006) and the OGRO database[6]; (Yamamoto et al., 2012). Most are population-specific, and this makes it difficult to associate the low fertility phenotypes with particular genes or introgressions in the CSSLs. Our objective in documenting CSSLs with low levels of seed set (as well as very late flowering) is to alert researchers which lines may be difficult to propagate and therefore require special attention. In some cases, we also alert users of these genetic resources that seeds of heterozygous backcross progeny or bulked seeds harvested from multiple generations of pure-line sibs may be distributed for particular lines.

Functional SNPs on the C7AIR and Predicted Phenotypes of the CSSLs

The C7AIR includes eight functional SNPs in its design that target important agronomic or physiological traits (Morales et al., 2020). They tag causal variants in genes conferring blast disease resistance (*Pi-ta*; Bryan et al., 2000), grain size (*GS3*; Fan et al., 2006; Takano-Kai et al., 2009), and eating and cooking quality (*ALK*, *WAXY*; Okagaki and Wessler, 1988; Wang et al., 1995; Gao et al., 2003). The parents of all six libraries were polymorphic for these variants, and CSSLs carrying donor SNPs at *Pi-ta*, *GS3*, *ALK*, and *WAXY* were documented (**Supplementary Table S2**). At *GS3*, both Cybonnet and IR64 carried the allele for long grain, while the three wild parents carried the wild type allele conferring short-medium grain length. At *Pi-ta*, the two RPs carried the allele for resistance while the wild parents carried the ancestral *pi-ta* allele conferring blast susceptibility (Huang et al., 2008). The evolution of resistance at the *Pi-ta* locus is believed to have been the result of a mutation that coincided with domestication, so it is not entirely surprising that none of the wild donors used in this study were found to carry the resistant allele (Lee et al., 2009; Yoshida and Miyashita, 2009). We were unable to predict grain quality phenotypes in the CSSLs because the traits determined by *ALK* and *WAXY* are quantitative, epistatic, and post-transcriptionally regulated (Bligh et al., 1998; Tian et al., 2009; Huggins et al., 2019). Evaluating these lines for novel grain and nutritional quality attributes is an exciting area for future research.

[7]https://shigen.nig.ac.jp/rice/oryzabase/

CSSL Identifier

To facilitate downstream use and evaluation of our six libraries by the greater rice genetics and breeding community, we developed a simple, user-friendly R Shiny Application that enables searching for lines of interest across user-selected libraries by inputting a genomic region of interest. The web application returns a comprehensive list of individual lines across the selected libraries that harbor introgressions either fully or partially across the region of interest from the donor accessions so that users may shortlist lines that they may potentially want to evaluate. Users may access the C7Air genotype data in **Supplementary Dataset 2** or may also download C7AIR genotype data directly from the application for their filtered list of lines. This application is available at http://cssl-identifier.rcac.purdue.edu/ and is hosted by Purdue University Research Computing.

Utility

The design and development of these CSSLs represents only the first step in an ambitious conceptualization of a public pre-breeding program that serves to develop, test, and deploy novel variation, with the long-term goal of enabling breeders to more readily tap into the hidden potential of wild and exotic plant genetic resources. The materials developed here are available for distribution and offer researchers new opportunities to undertake complementary multi-year, multi-location trials for yield and agronomic performance, response to abiotic and biotic stresses, and quality traits important to the rice community. To maximize the potential for wide utility and interpretation, phenotypes must be collected using controlled vocabularies, with accompanying climatic metadata and management details to provide environmental context. Further, experimental data describing phenotypic, genotypic and environmental variation should be deposited into a common database or data management system to make those data findable, accessible, interoperable and re-usable (Wilkinson et al., 2016) so that benefits can be collectively realized.

Pre-breeding is inevitably a long-term value proposition. Benefits will continue to accrue over many years if efforts are carried out in partnership with applied breeding programs dedicated to finished variety development. A partnership model that leverages public and private sector resources would enable the large scale experiments that are needed to drive recombination via rapid-cycle recurrent selection in order to overcome linkage drag and give rise to novel sources of useful variation. Favorable new materials could then be extracted by breeders periodically for crossing with elite lines, while the essential cauldron of diverse alleles were continuously managed, simmered and stirred. The potential of a well-designed, thoughtfully implemented pre-breeding program that is fueled by complementary partnerships, creative benefit-sharing agreements, and strategic deployment of resources has far-reaching consequences for plant breeders and for agricultural communities world-wide. Such a pre-breeding program can drive innovation, motivate new thinking, and unleash strategies

that iteratively create value by reinvesting in the deployment of natural variation across the landscape, bringing rewards for decades to come.

DATA AVAILABILITY STATEMENT

The genotypic datasets presented in this study can be found in **Supplementary Data 2**—Genotype information (C7AIR) for CSSL populations. Sequencing data for grain and hull color genes from the wild and cultivated parents and selected CSSLs is available in GenBank with the following Accession IDs: *Bh4* (MW310629 - MW310643), *Rd* (MW310644 - MW310658), *Rc* (MW310659 - MW310674), *Ph* (MW310675 - MW310688).

AUTHOR CONTRIBUTIONS

NS, LA, HK, KA, SH, J-WK, ES, BM, S-NA, and GE contributed to the development of CSSL populations. NS, SH, GE, and HK phenotyped the plants. NS, KA, and SH genotyped the plants. YS and GD developed data management tools. VG and DW developed the CSSL Identifier R Shiny App. NS, DW, SH, GE, and SM analyzed the data and wrote the manuscript. SM conceptualized the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.564824/full#supplementary-material

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Gene Pyramiding for Achieving Enhanced Resistance to Bacterial Blight, Blast, and Sheath Blight Diseases in Rice

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Bacterial blight, blast, and sheath blight are the commonest diseases causing substantial yield loss in rice around the world. Stacking of broad-spectrum resistance genes/QTLs into popular cultivars is becoming a major objective of any disease resistance breeding program. The varieties ASD 16 and ADT 43 are the two popular, high yielding, and widely grown rice cultivars of South India, which are susceptible to bacterial blight (BB), blast, and sheath blight diseases. The present study was carried out to improve the cultivars (ASD 16 and ADT 43) through introgression of bacterial blight (*xa5*, *xa13*, and *Xa21*), blast (*Pi54*), and sheath blight (*qSBR7-1*, *qSBR11-1*, and *qSBR11-2*) resistance genes/QTLs by MABB (marker-assisted backcross breeding). IRBB60 (*xa5*, *xa13*, and *Xa21*) and Tetep (*Pi54*; *qSBR7-1*, *qSBR11-1*, and *qSBR11-2*) were used as donors to introgress BB, blast, and sheath blight resistance into the recurrent parents (ASD 16 and ADT 43). Homozygous (BC_3F_3 generation), three-gene bacterial blight pyramided (*xa5* + *xa13* + *Xa21*) lines were developed, and these lines were crossed with Tetep to combine blast (*Pi54*) and sheath blight (*qSBR7-1*, *qSBR11-1*, and *qSBR11-2*) resistance. In BC_3F_3 generation, the improved pyramided lines carrying a total of seven genes/QTLs (*xa5* + *xa13* + *Xa21* + *Pi54* + *qSBR7-1* + *qSBR11-1* + *qSBR11-2*) were selected through molecular and phenotypic assay, and these were evaluated for resistance against bacterial blight, blast, and sheath blight pathogens under greenhouse conditions. We have selected nine lines in ASD 16 background and 15 lines in ADT 43 background, exhibiting a high degree of resistance to BB, blast, and sheath blight diseases and also possessing phenotypes of recurrent parents. The improved pyramided lines are expected to be used as improved varieties or used as a potential donor in breeding programs. The present study successfully

introgressed *Pi54*, and *qSBR* QTLs (*qSBR7-1*, *qSBR11-1*, and *qSBR11-2*) from Tetep and major effective BB-resistant genes (*xa5*, *xa13*, and *Xa21*) from IRBB60 into the commercial varieties for durable resistance to multiple diseases.

Keywords: rice, multiple disease resistance, marker-assisted backcross breeding, gene pyramiding, phenotyping

INTRODUCTION

Rice (*Oryza sativa* L.) is considered a major staple food crop for billions of population across the globe, and it provides 23% of calories shared by different food crops (Sharma et al., 2012). Exponential growth of the world population demands an increase in rice production by 26% to fulfill calorie requirements (Khush, 2013). According to the Food and Agricultural Organization, the global rice production would have to increase by 42% over the present-day production to meet the growing population by 2050 (Ray et al., 2013). However, the yield potential is frequently threatened by various biotic stresses, mostly fungi, and bacteria. To address these problems and to increase production, developing cultivars with durable resistance is a prerequisite. The host-plant resistance can be ideally improved through pyramiding of major *R*-genes/QTLs for multiple diseases and biotic stress factors.

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a major destructive disease of rice, causing a yield loss of up to 80% depending on the severity (Kumar et al., 2012). Improving the host-plant resistance is the most efficient and eco-friendly approach, as chemical control of BB is not effective (Lee et al., 2003). The infection chain starts by entering into the plant through the hydathodes, and it reaches to xylem vessels, where the infection became systemic. Till date, 46 resistance genes have been identified from the different sources of rice (Chen et al., 2020). Of these, the *Xa4*, *xa5*, *Xa7*, *xa13*, *Xa21*, *Xa33*, and *Xa38* genes are most frequently utilized in hybridization programs for developing BB-resistant cultivars (Hsu et al., 2020). Natural allelic variations in *Xoo* challenges the resistance levels conferred by a single gene; hence, pyramiding of two or more effective resistance genes is highly essential for broad-spectrum and durable resistance to *Xoo* at field conditions. *Xa21*, a major dominant resistant gene, originated from African wild species, *Oryza longistaminata*, was observed to confer the resistance to many *Xoo* isolates (Nguyen et al., 2018). The encoding proteins of *Xa21* gene carries both leucine-rich repeats (LRR) and serine-threonine kinases, and these complexes perceives the presence of pathogen ligand on the cell surface and activates the subsequent intracellular defense response *R*-proteins (Song et al., 1995). The *Xa21* gene was physically mapped on the long arm of chromosome 11, and a highly efficient PCR-based co-dominant molecular marker (pTA 248) was developed for marker-assisted selection of *Xa21* (Ronald et al., 1992). A unique, fully recessive gene, *xa13*, was first identified in cultivar BJ1 and physically mapped on the long arm of chromosome 8 (Zhang et al., 1996). Mutations in the promoter region of dominant allele (*Xa13*) resulted in a recessive gene, *xa13*, which does not encode for a modulator for pathogen (Chu et al., 2006). Another broad-spectrum recessive resistant gene, *xa5*, was identified and mapped on the subtelomeric region of chromosome number 5 (Blair et al., 2003). Unlike other *R*-genes, the *xa5* gene encodes for a gamma transcription factor-like protein (TFIIAγ). Pyramiding of *xa5* gene with other dominant genes gives durable resistance to *Xoo* than the plants with single BB-resistant gene (Huang et al., 1997).

Rice blast, caused by *Magnaporthe oryzae* (Teleomorph: *Pyricularia oryzae*), is one of the devastating diseases of rice growing areas across the world. Yield loss is estimated to be more than 50% when it occurs in epidemic proportions (Babujee and Gnanamanickam, 2000). Similar to BB, developing of host-plant resistance is the most effective strategy for management of blast disease (Sharma et al., 2012). So far, about 100 resistance genes have been identified, and 37 of them were cloned (Zhang et al., 2019). Although several blast resistance genes have been identified, only a few of them were used in breeding programs for blast disease management in India (Singh et al., 2011). Among them, the *Pi54* gene located on chromosome 11 provides stable and durable resistance to diverse strains of *M. oryzae* collected across India (Thakur et al., 2015). The predicted proteins of the *Pi54* gene contains NBS-LRR proteins along with unique zinc finger domain (Sharma et al., 2005). During the host–pathogen interaction, the *Pi54* gene induces the synthesis of callose (β-1,3-glucan), which acts as a physical barrier by blocking the penetration of fungal hyphae (Gupta et al., 2012). A functional marker has been developed for the *Pi54* gene and used in maker-assisted selection for developing blast-resistant cultivars (Ramkumar et al., 2011).

Rhizoctonia solani Kühn, the causative agent of rice sheath blight disease (ShB), poses a significant impact on both yield and quality (Singh et al., 2019). Introduction of high yielding varieties and application of high doses of nitrogenous fertilizers resulted in a steep rise in incidence of sheath blight disease (Savary et al., 1997). *R. solani* Kühn is a soil-borne facultative parasite, survives as sclerotia or mycelium, or rarely as basidiospores. No varieties resistant to sheath blight were reported till date (Channamallikarjuna et al., 2010). Breeding for resistance to ShB is quite unsuccessful owing to inability to identify effective resistance sources from the available germplasm, wide-ranging host compatibility, high genetic variability, and capability of the pathogen to survive from season to season in the form of dormant sclerotia, makes additional complications in controlling the disease (Molla et al., 2020). Though qualitative resistance to ShB was not found, quantitative resistance was reported in some landraces *viz*., Tetep, Teqing, Jasmine 85, etc., (Channamallikarjuna et al., 2010; Wang et al., 2012; Yadav et al., 2015). Up to now, 50 QTLs conferring moderate resistance to rice sheath blight have been identified from the different sources of rice (Zhang et al., 2019). Among these, *qSBR7-1*, *qSBR11-1*, and *qSBR11-2* were identified in the background of Tetep (Channamallikarjuna et al., 2010) and pyramided in

Pusa 6B (Singh et al., 2015). We have used these three QTLs (*qSBR7-1*, *qSBR11-1*, and *qSBR11-2*) for pyramiding in our recurrent parents to improve sheath blight resistance.

In cognizance of the above reports, the present study was formulated with the following objectives: (i) introgression of BB (*xa5*, *xa13*, and *Xa21*), blast (*Pi54*), and sheath blight (*qSBR7*-1, *qSBR11-1*, and *qSBR11-2*) resistance genes/QTLs in the backgrounds of ASD 16 and ADT 43; (ii) analysis of recurrent parent genome recovery (RPG) with a set of polymorphic SSR (simple sequence repeats) markers; (iii) evaluation of improved pyramided lines for physical resistance against BB, blast, and sheath blight diseases; and (iv) evaluation of agro-morphological and quality traits of improved pyramided lines in comparison with parents.

MATERIALS AND METHODS

Plant Materials

The two recurrent parents, ASD 16 (ADT 31 × CO 39) and ADT 43 (IR 50 × Improved White Ponni) are popular, high yielding, and widely grown rice cultivars of South India. ASD 16 has short bold grains, while ADT 43 has medium slender fine grains. Though both cultivars are high yielding, they are highly susceptible to BB, blast, and sheath blight diseases. A bacterial blight-resistant genotype, IRBB60 harboring *xa5* + *xa13* + *Xa21* was used as donor for BB resistance genes. Tetep, a Vietnamese *indica* land race possessing blast resistance (*Pi54*) (Ramkumar et al., 2011) and moderately resistant to sheath blight, harboring qSBR QTLs (*qSBR7-1, qSBR11-1*, and *qSBR11-2*) (Channamallikarjuna et al., 2010) was also used as one of the donors for targeted transfer of blast and sheath blight resistance into the background of recurrent parents (ASD 16 and ADT 43).

Marker-Assisted Backcross Breeding for Targeted Gene/QTL Pyramiding of Bacterial Blight, Blast, and Sheath Blight in the Backgrounds of ASD 16, and ADT 43

Two independent crosses (ASD 16 × IRBB60 and ADT 43 × IRBB60) were made between recipient parents and IRBB60 for targeted gene transfer of *xa5*, *xa13*, and *Xa21*. The hybridity of the F_1 plants were confirmed by functional/linked PCR-based co-dominant molecular markers of *xa5*, *xa13*, and *Xa21* (**Table 1**). The heterozygous plants for *xa5*, *xa13*, and *Xa21* were backcrossed with respective recurrent parents (ASD 16 and ADT 43) to generate BC_1F_1. The BC_1F_1 hybrids were screened for targeted genes (*xa5*, *xa13*, and *Xa21*), and the confirmed plants were assessed with a set of polymorphic SSR markers to recover the maximum percentage of RPG (Sundaram et al., 2008). The solitary plant with targeted genes (*xa5*, *xa13*, and *Xa21*) and maximum recovery of RPG was selected and backcrossed to generate BC_2F_1, and this procedure was repeated till BC_3F_1. At every backcross, foreground and background selections were carried out to forward the plant with targeted genes and maximum recovery of RPG. Pedigree-based breeding strategy with marker-assisted selection was followed after BC_3F_1 to generate BC_3F_2 and BC_3F_3 populations. In BC_3F_2 population, the homozygous plants for targeted genes were identified through foreground selection and self-pollinated to generate BC_3F_3 population. The homozygous plants at BC_3F_3 were evaluated for resistance against BB as well as for key agronomic traits, and the best lines were pooled to generate three-gene bacterial blight (*xa5*, *xa13*, and *Xa21*) pyramided lines of ASD 16, and ADT 43. The three-gene bacterial blight pyramided lines were crossed with Tetep to introgress blast and sheath blight resistance. The plants showing heterozygous allele for all the targeted genes/QTLs were backcrossed with respective recurrent parent (three-gene bacterial blight pyramided lines of ASD 16 and ADT 43) to generate BC_1F_1. The BC_1F_1 hybrids were screened with molecular markers for all the targeted traits in the study (**Table 1**) followed by background selection with polymorphic SSR markers. The "positive" plants with maximum recovery of RPG were again backcrossed with recurrent parents to produce BC_2F_1 and BC_3F_1. In the BC_3F_2 population, foreground selection was carried out to identify the plants carrying all the targeted genes (*xa5* + *xa13* + *Xa21* + *Pi54*) and targeted QTLs (*qSBR7-1* + *qSBR11-1* + *qSBR11-2*) in homozygous condition, and the identified plants were self-pollinated to generate BC_3F_3 population. The plants carrying all the targeted traits in homozygous condition at BC_3F_3 generation were evaluated for resistance against bacterial blight, blast, and sheath blight diseases under greenhouse conditions and also assessed for key agronomic as well as grain quality traits. The detailed plan of program for marker-assisted gene pyramiding of bacterial blight, blast, and sheath blight resistance genes/QTLs is depicted in **Figure 1**.

DNA Extraction and PCR Amplification

The DNA extraction for PCR amplification was carried out by the CTAB method (Varghese et al., 1997). The PCR protocol for marker-assisted selection of targeted genes/QTLs was followed according to the earlier reports (Chu et al., 2006; Iyer-Pascuzzi and McCouch, 2007; Sundaram et al., 2008; Channamallikarjuna et al., 2010; Ramkumar et al., 2011; **Table 1**). Ten microliters of PCR reaction mixture contains 4 μl of DreamTaq green 2× PCR master mix (Thermo scientific, United States), 4 μl of water, 50 ng of template DNA, and 30 ng each of forward and reverse primers. To find the specific allelic pattern of *xa*5 allele, 5–10 μl of PCR product is digested with *Bsr*I (5 units of enzyme) at 65°C for 4 h with 2 μl of 10× PCR buffer (Iyer-Pascuzzi and McCouch, 2007). The amplified PCR products were separated by 2.5% agarose gel stained with ethidium bromide and visualized on UV light in gel documentation system (Bio-Rad Laboratories., United States). Background selection was carried out with a set of 463 SSR markers[1]; 69 and 68 markers were found to be polymorphic for ADT 43 and ASD 16 cross combinations, respectively (**Supplementary Table S1**), with a wide coverage of all the 12 chromosomes (five to six polymorphic

[1]https://archive.gramene.org/

TABLE 1 | Details of molecular markers used for foreground selection.

Gene/QTL	Marker	Sequence (5′–3′)	AT (°C)	Chr	Resistant allele size (bp)	Reference
Xa21	pTA248	F-AGACGCGGAAGGGTGGTTTCCCGGA R-AGACGCGGTAATCGAAAGATGAAA	65	11	925	Ronald et al. (1992)
xa13	xa13-prom	F-GAGCTCCAGCTCTCCAAATG R-GGCCATGGCTCAGTGTTTAT	59	8	500	Chu et al. (2006)
xa5	xa5-1	F-CTCTACCGGAGGTCCACCATTG R-AGGAACAGCAACATTGCAAC	53	5	299	Iyer-Pascuzzi and McCouch (2007)
Pi54	Pi54-MAS	F-CAATCTCCAAAGTTTTCAGG R-GCTTCAATCACTGCTAGACC	56	11	216	Ramkumar et al. (2011)
qSBR7-1	RM336	F-CTTACAGAGAAACGGCATCG R-GCTGGTTTGTTTCAGGTTCG	55	7	190	Channamallikarjuna et al. (2010)
qSBR11-1	RM224	F-ATCGATCGATCTTCACGAGG R-TGCTATAAAAGGCATTCGGG	55	11	130	
qSBR11-2	RM209	F-ATATGAGTTGCTGTCGTGCG R-CAACTTGCATCCTCCCCTCC	55	11	150	

markers per chromosome). Additional polymorphic markers, i.e., 10 polymorphic markers were employed on chromosome number 11, which carries *Xa21*, *Pi54*, *qSBR11-1*, and *qSBR11-2* to minimize the linkage drag.

Bioassays Against Bacterial Blight, Blast, and Sheath Blight Diseases

Bacterial Blight

The selected IPLs (improved pyramided lines) and parents (IRBB60 as resistant check and ASD 16 and ADT 43 as susceptible checks) were tested for resistance against the DX-027 of *Xoo* isolate under greenhouse conditions. In addition to the selected IPLs, single- and two-gene BB pyramided lines were also evaluated to check the effectiveness of three-gene BB pyramided lines. Three replications were maintained with 30 plants per replication. The top leaves were clipped off, and bacterial suspension was inoculated with a density of 10^9 cells/ml by clip inoculation method at maximum tillering stage (Kauffman et al., 1973). Eight leaves per plant were inoculated, and mean lesion length was taken on six leaves to measure the accurate disease reaction. Symptoms were measured 21 days post-inoculation, and observations were recorded based on visual score and lesion length (LL). The plants with an average lesion length of <5 cm were considered as resistant, and those with >5 cm were considered as susceptible (International Rice Research Institute [IRRI], 2002).

Blast

The IPLs and parents (Tetep as resistant check; ASD 16 and ADT 43 as susceptible checks) were evaluated for leaf blast resistance against IS (KUL)-6, a virulent local isolate of *M. oryzae*. Five-week-old seedlings were individually transplanted into mud pots (19 cm × 22 cm × 22 cm) with three replications and inoculated with *M. oryzae* IS (KUL)-6 isolate at a spore density of 5×10^5 spores/ml. Disease reaction was recorded 9 days post-inoculation and evaluated based on blast lesion type (BLT) according to the 0–9 scale of SES (Standard Evaluation System) (International Rice Research Institute [IRRI], 2002). The plants with a score of 0–3 were rated as resistant and those with more than a score of 4 were rated as susceptible.

Sheath Blight

A pure culture of *R. solani* collected from the Tamil Nadu Rice Research Institute (TRRI), Aduthurai, Tamil Nadu, India, was used for testing the resistance of IPLs and parents against sheath blight. The pathogen was multiplied on sterilized shoot bits of water sedge, *Typha angustata*. Infected *Typha* shoot bits with mycelium and sclerotia were used as source of inoculum and placed carefully between the tillers of rice hills with the help of forceps at 80 days after sowing. The inoculated portion was covered with wet cotton and aluminum foil to avoid the moisture loss in the inoculated portion. The observations were recorded 25 days post-inoculation on randomly selected plants consisting of three infected tillers in each of three replications. Disease reaction was measured based on RLH% (relative lesion height), and scoring (0–9) was given as per SES (International Rice Research Institute [IRRI], 2002) [0 (immune), 1–20% (resistant), 21–30% (moderately resistant), 31–45% (moderately susceptible), 46–65% (susceptible), and >65% (highly susceptible)].

Characterization of Agro-Morphological and Quality Traits

The 30-day old seedlings of recurrent parents and selected IPLs were transplanted to an experimental plot at the Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai, India, with a spacing of 15 cm × 20 cm. The experimental plot was arranged in a randomized block design (RBD) with four blocks, and three replications were maintained in each block. Standard agronomic practices were followed as prescribed by TNAU, Coimbatore, India[2], to raise the healthy crop. Observations were recorded on five plants in each line for key agronomic traits *viz*., days to 50% flowering (DFF), plant height (PH) (cm), number of productive tillers per plant (NPT), panicle length (PL) (cm), number of grains per panicle (NGP),

[2]http://agritech.tnau.ac.in/

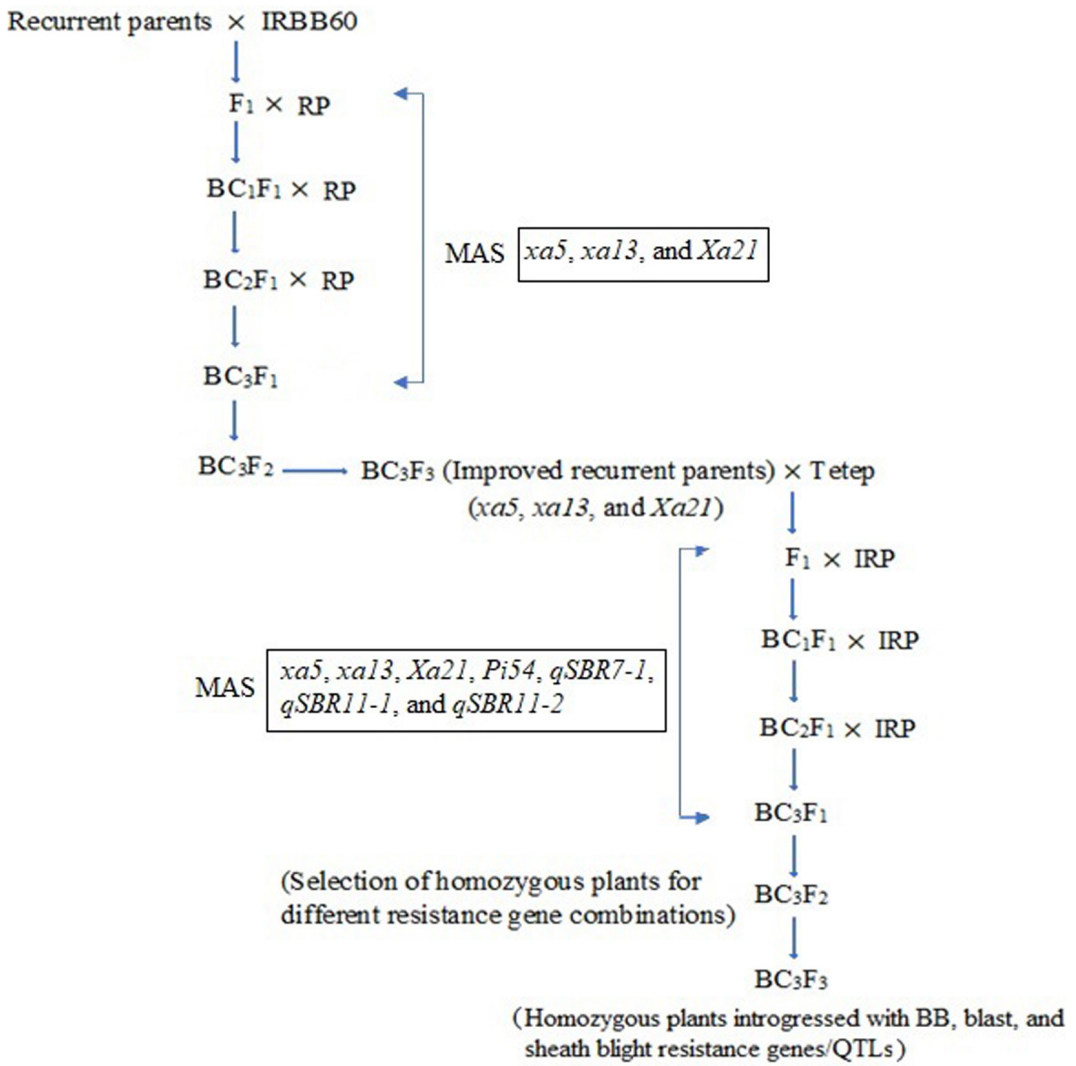

FIGURE 1 | Schematic illustration of marker-assisted gene pyramiding of bacterial blight, blast, and sheath blight resistance genes/QTLs in the backgrounds of ASD 16 and ADT 43. RP, recurrent parents (ASD 16 and ADT 43), IRP, improved recurrent parents (ASD 16 and ADT 43 introgressed with *xa5*, *xa13*, and *Xa21* genes); MAS, marker-assisted selection.

flag leaf length (FL) (cm), flag leaf width (FW) (cm), 1,000-grain weight (1,000-GW) (g), grain-L/B ratio (cm), and single plant yield (SPY) (g). In addition, the quality traits *viz.*, hulling percentage (HP%), milling percentage (MP%), head rice recovery (HRR%), kernel length (KL) (mm), kernel breadth (KB) (mm), kernel length breadth ratio (KLBR), milled rice length (MRL) (mm), milled rice breadth (MRB) (mm), kernel length after cooking (KLAC) (mm), kernel breadth after cooking (KBAC) (mm), and linear elongation ratio (LER) were analyzed in the homozygous improved pyramided lines.

Statistical Analysis

Statistical analysis was done with SPSS software to complement the ANOVA (analysis of variance) to determine the significant variation among the improved pyramided lines. The coefficient of genetic distance among the selected pyramided lines and parents was calculated based on 10 morphological characters and used for generating the dendrogram using the "R" software (R Core Team, 2013).

RESULTS

Marker-Aided Pyramiding of *Xa21*, *xa13*, and *xa5* in the Backgrounds of ASD 16 and ADT 43

The hybrids (F_1) from the crosses of ASD 16 × IRBB60 and ADT 43 × IRBB60 were analyzed with functional/linked molecular markers of *xa5*, *xa13*, and *Xa21*, i.e., *xa5*-1, *xa*-13 prom and pTA 248, respectively. A total of 12/138 plants in ASD 16 × IRBB60 cross combination and 15/157 plants in ADT

43 × IRBB60 cross combination were found to be "positive" for all the targeted genes (*xa5*, *xa13*, and *Xa21*). These positive plants were backcrossed with respective recurrent parents (ASD 16 and ADT 43) to generate the BC_1F_1 population. We have found that 9/134 plants in ASD 16 background and 13/150 plants in ADT 43 background were heterozygous for *xa5*, *xa13*, and *Xa21*, and these plants were screened with polymorphic SSR markers to assess the recovery of RPG. Background assay revealed that three plants in ASD 16 combination *viz*., IL-1-2-2, IL-1-5-6, and IL-1-8-18 with an RPG% recovery of 75.60, 76.82, and 76.56, respectively, and two plants in ADT 43 combination *viz*., IL-2-1-33 and IL-2-2-53 with a recovery of 77.08% and 76.04% were identified (**Supplementary Table S2**). These plants were backcrossed alone with respective recurrent parents to produce BC_2F_1. In BC_2F_1, 10/154 plants in ASD 16 background and 12/166 plants in ADT 43 background were found to be heterozygous for the targeted genes. In these BC_2F_1 populations, we have identified two plants in ASD 16 combination and three plants in ADT 43 combination with background genome recovery ranging from 87.5 to 89.02% (**Supplementary Table S2**), and these plants were backcrossed to produce BC_3F_1. In BC_3F_1, we have found that 11/92 in ASD 16 background and 17/110 in ADT 43 background were shown to be triple heterozygous, and background assay indicates that three plants with RPG recovery ranged from 93.85 to 94.96% in ASD 16 combination, and two plants with a recovery of 94.26% and 94.04% were observed in ADT 43 combination (**Supplementary Table S2**). Self-pollination was carried out in the selected plants to generate BC_3F_2 followed by BC_3F_3. Homozygous plants for *xa5*, *xa13*, and *Xa21* with key agro-morphological traits were identified through genotypic and phenotypic assays, and the best lines (six lines in ASD 16 combination and nine lines in the ADT 43 combination) were constituted to generate triple-gene (*xa5*, *xa13*, and *Xa21*) pyramided lines of ASD 16 and ADT 43 for bacterial blight resistance.

Combining Multiple Disease (BB, Blast, and Sheath Blight) Resistant Genes/QTLs in the Backgrounds of ASD 16, and ADT 43

The three-gene bacterial blight (*xa5*, *xa13*, and *Xa21*) pyramided lines of ASD 16 and ADT 43 were used as recipient parents for the targeted introgression of blast (*Pi54*) and sheath blight QTLs (*qSBR7-1*, *qSBR11-1*, and *qSBR11-2*) from the Tetep. The bacterial blight pyramided lines of ASD 16 and ADT 43 harboring *xa5*, *xa13*, and *Xa21* were crossed with Tetep, and the F_1 plants were analyzed for all the targeted traits (**Table 1**). The hybrids with *xa5* + *xa13* + *Xa21* + *Pi54* + *qSBR7-1* + *qSBR11-1* + *qSBR11-2* in heterozygous condition were selected and backcrossed with ASD 16 and ADT 43 introgressions with the three-gene bacterial blight genes (*xa5*, *xa13*, and *Xa21*) to recover the original RPG. Up to BC_3F_1, all the foreground and background selections were made similar to pyramiding of bacterial blight-resistant genes, as explained above. Foreground selection of BC_3F_1 hybrids revealed five plants in ASD 16 background and seven plants in ADT 43 background possessing all the targeted genes and QTLs (*xa5* + *xa13* + *Xa21* + *Pi54* + *qSBR7-1* + *qSBR11-1* + *qSBR11-2*) in heterozygous condition. Background selection of these positive plants revealed two plants with RPG recovery ranging from 93.88 to 94.92% in both combinations (ASD 16, and ADT 43) (**Supplementary Table S2**). Selfing was done in "positive" BC_3F_1 hybrids with high RPG recovery to generate BC_3F_2. In BC_3F_2 generation, 11 plants in the ASD 16 background and 14 plants in the ADT 43 background were homozygous for targeted genes (*xa5* + *xa13* + *Xa21* + *Pi54*) and targeted QTLs (*qSBR7-1* + *qSBR11-1* + *qSBR11-2*), and these were selfed to produce BC_3F_3. These homozygous-improved gene pyramided lines at BC_3F_3 generation were evaluated for physical expression of resistance against bacterial blight, blast, and sheath blight pathogens under greenhouse conditions. Out of 1,500 plants screened in both the crosses at field conditions, we found nine lines in ASD 16 background and 15 lines in ADT 43 background performing on par with the recurrent parents and, moreover, exhibiting a high level of resistance to all the three major diseases (**Tables 2, 3** and **Figures 2–5**).

TABLE 2 | Phenotypic reaction and disease score of improved pyramided lines against bacterial blight (BB), blast, and sheath blight diseases.

Genotype	Bacterial blight		Blast	Sheath blight	
	LL (cm)	Score	Score	RLH (%)	Score
ACM 18243	3.15 ± 0.56	0	2	44	5
ACM 18244	3.28 ± 0.67	0	1	28	3
ACM 18242	3.17 ± 1.43	1	1	39	5
ACM 18245	3.35 ± 1.02	1	3	30	3
ACM 18249	3.67 ± 0.62	0	1	37	5
ACM 18012	4.03 ± 0.34	1	2	43	5
ACM 18014	3.87 ± 0.34	0	1	38	5
ACM 18015	3.36 ± 0.70	0	0	29	3
ACM 18023	3.33 ± 0.78	1	1	35	5
ACM 18020	4.21 ± 0.49	1	2	30	3
IRBB60	3.10 ± 0.45	0	–	–	–
Tetep	–	–	0	28	3
ASD 16	15.12 ± 1.46	9	9	78	9
ADT 43	16.88 ± 0.56	9	9	72	9

LL, lesion length;
RLH (%), relative lesion height expressed in percentage;
BB – LL of <5 cm is considered as resistant, and LL > 5 cm is considered as susceptible.
Blast – 0–1 (highly resistant), 2–3 (resistant), 4 (moderately resistant), 5–6 (moderately susceptible), 7 (susceptible), and 8–9 (highly susceptible).
Sheath blight – 0 (Immune), 1 (resistant), 3 (moderately resistant), 5 (moderately susceptible), 7 (susceptible), and 9 (highly susceptible).

Evaluation of Improved Pyramided Lines for Resistance to Bacterial Blight, Blast, and Sheath Blight Diseases

Bioassay for BB Resistance

The resistant parent, IRBB60, possessing *xa5*, *xa13*, and *Xa21* exhibited a mean lesion length of 3.10 ± 0.45 with a disease reaction score of 0–1 (highly resistant) against the isolate of

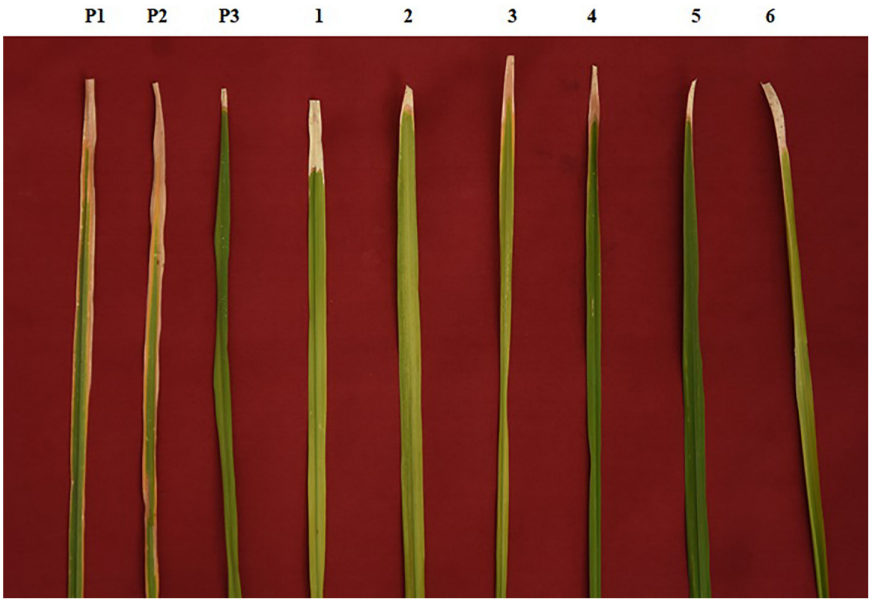

FIGURE 2 | Phenotypic screening of improved pyramided lines against DX-027 isolate of bacterial blight (BB). P1 – ASD 16, P2 – ADT 43, P3 – IRBB60; serially numbered 1, 2, 3, 4, 5, and 6 lines indicate improved pyramided lines of recurrent parents harboring *xa5*, *xa13*, and *Xa21* genes.

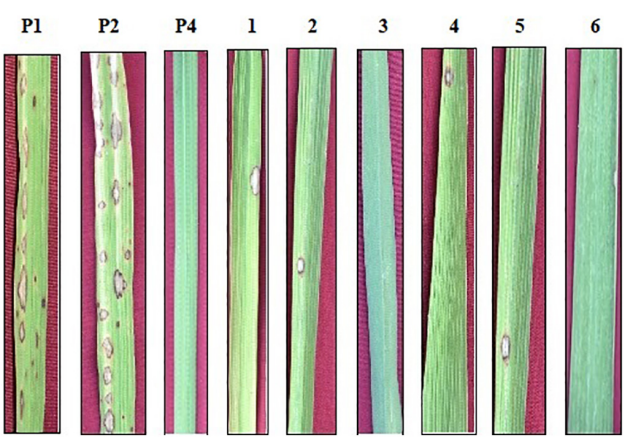

FIGURE 3 | Phenotypic screening of improved pyramided lines against IS (KUL)-6 isolate of *Magnaporthe oryzae* for leaf blast. P1 – ASD 16, P2 – ADT 43, P4 – Tetep; serially numbered 1, 2, 3, 4, 5, and 6 lines indicate improved pyramided lines of recurrent parents harboring the *Pi54* gene.

DX-027. Similarly, the identified homozygous IPLs were also expressed, and the similar mean lesion length ranged from 3.15 ± 0.56 to 4.21 ± 0.49 with the same disease reaction score as the resistant parent. The two-gene pyramided lines (*xa5* + *xa13*, *xa5* + *Xa21*, and *xa13* + *Xa21*) were shown a mean lesion length of 4.58 ± 1.15 with a score of 1–2 (resistant). Evaluation of single-gene pyramided lines showed that *Xa21* pyramided lines expressed a mean lesion length of 4.93 ± 0.22, *xa13* lines with 6.45 ± 0.79, and *xa5* lines with 6.85 ± 0.64 lesion lengths. This indicates that the lines that harbor the *Xa21* component exhibited a high level of resistance than the other components in the disease reaction (data not shown), while the recurrent parents (ASD 16 and ADT 43) have shown an average lesion length of more than 15 cm with a disease reaction score of 9 (highly susceptible) for the same isolate of BB (**Table 2** and **Figure 2**).

Bioassay for Blast and Sheath Blight Resistance

The resistant check, Tetep, possessing the *Pi54* gene, exhibited a high level of resistance to leaf blast with no lesions observed on the leaf with a disease score of 0–1 (highly resistant) against the isolate of IS (KUL)-6. Similarly, the selected IPLs harboring the *Pi54* gene displayed small pin-point size brown specks to slightly elongated necrotic patches with a sporulating center with a disease score of 1–3 (highly resistant to resistant). While, the recurrent parents (ASD 16 and ADT 43) displayed spindle-shaped lesions with brown margin with a disease reaction score of 9 (highly susceptible) (**Table 2** and **Figure 3**). With regard to the sheath blight, Tetep possessing *qSBR* QTLs (*qSBR7-1*, *qSBR11-1*, and *qSBR11-2*) exhibited an RLH up to 28% with a disease score of 3 (moderately resistant). The susceptible checks, both ASD 16 and ADT 43, expressed an RLH of 78% and 71%, respectively, with disease score of 9 (highly susceptible). While the selected IPLs possessing *qSBR7-1*, *qSBR11-1*, and *qSBR11-2* have expressed an RLH ranging from 28 to 45% with a disease score of 3 to 5 (moderately resistant to moderately susceptible) (**Table 2** and **Figure 4**).

Evaluation of Improved Pyramided Lines for Agro-Morphological and Quality Traits

All the selected improved pyramided lines (nine lines in ASD 16 background and 15 lines in ADT 43 background) have similar

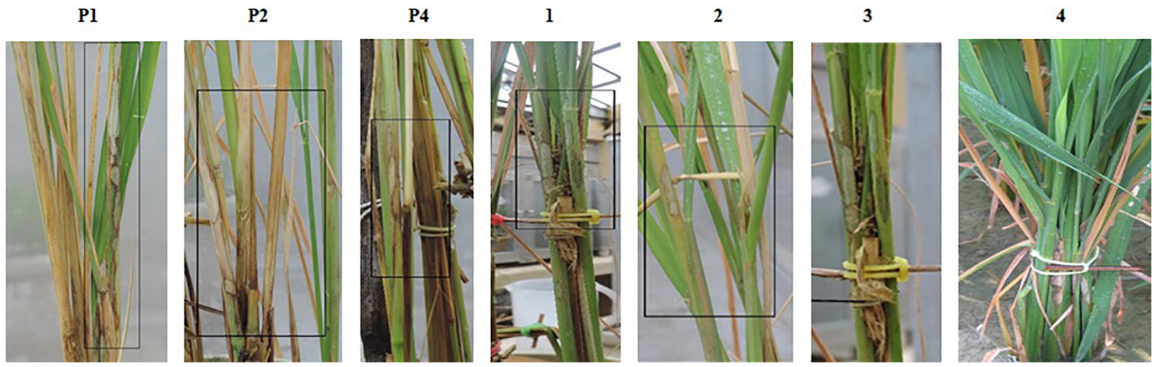

FIGURE 4 | Phenotypic screening of improved pyramided lines against sheath blight resistance. P1 – ASD 16, P2 – ADT 43, P4 – Tetep; serially numbered 1, 2, 3, and 4 lines indicate improved pyramided lines harboring *qSBR7-1*, *qSBR11-1*, and *qSBR11-2* QTLs.

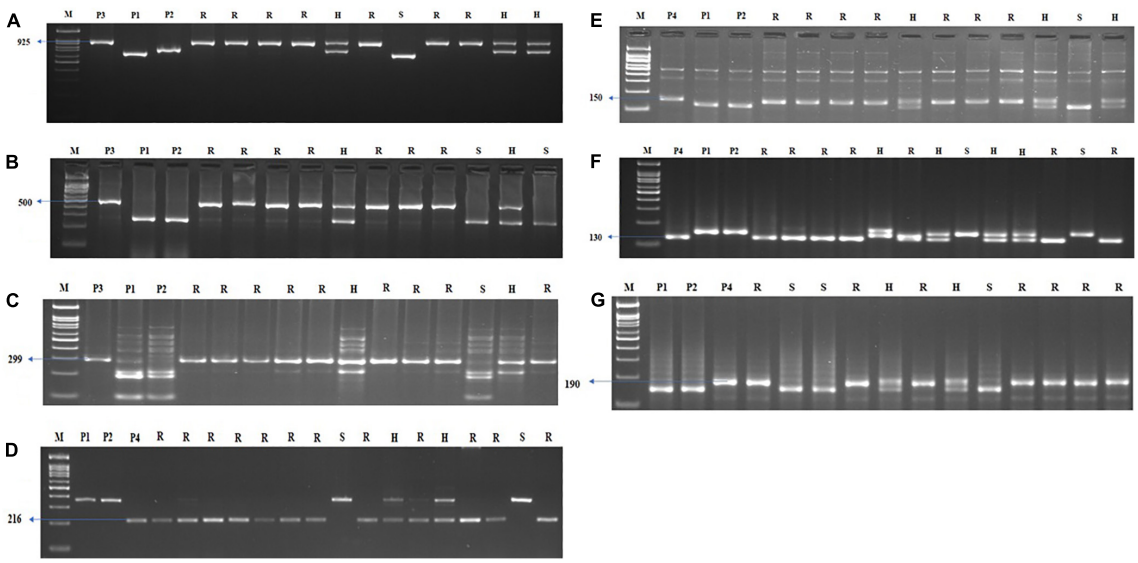

FIGURE 5 | Agarose gel electrophoresis images illustrating the presence of **(A)** *Xa21*, **(B)** *xa13*, **(C)** *xa5*, **(D)** *Pi54*, **(E)** *qSBR11-2*, **(F)** *qSBR11-I*, and **(G)** *qSBR7-1* alleles. P1 – ASD 16, P2 – ADT 43, P3 – IRBB60, P4 – Tetep, M – 100-bp ladder, R, resistant; H, heterozygote; S, susceptible.

agro-morphological and quality traits as recurrent parents. Some promising lines have shown superior agro-morphological and quality traits and, moreover harboring BB, blast, and sheath blight resistance. Significant differences for plant height were observed among the few improved pyramided lines (BC_3F_3 generation), which were shown as taller than recurrent parents *viz.*, ACM 18012, ACM 18245, ACM 18242, ACM 18243, ACM 18244, ACM 18013, and ACM 18014 (**Table 3**). The DFF for recurrent parents (ASD 16 and ADT 43) and donor parents (IRBB60 and Tetep) were 85 and 97 days, respectively. The selected improved pyramided lines at BC_3F_3 generation harboring *xa5* + *xa13* + *Xa21* + *Pi54* + *qSBR7-1* + *qSBR11-1* + *qSBR11-2* also showed a similar range for DFF, i.e., 78–95 days. Two lines in the background of ASD 16 *viz.*, ACM 18242 and ACM 18246 were significantly flowered ∼13–18 days earlier than its respective recurrent parent, ASD 16 (**Table 3**). The mean values of recurrent parents for the number of grains per panicle ranged from 191 ± 14.3 to 201 ± 11.2. Several improved pyramided lines *viz.*, ACM 18012, ACM 18245, ACM 18242, ACM 18243, ACM 18017, ACM 18016, ACM 18247, and ACM 18015 have shown significantly higher number of grains per panicle than both recurrent parents (**Table 3**). The mean 1,000-grain weight (g) of recurrent parents ranged from 15.6 ± 0.4 to 23.2 ± 0.5 for ADT 43 and ASD 16, respectively. The selected improved pyramided lines, ACM 18245, ACM 18244, ACM 18013, ACM 18016, ACM 18017, and ACM 18050 have shown significantly higher 1,000-grain weight than both the recurrent parents ASD 16 and ADT 43 (**Table 3**). The HRR% of recurrent parents ranged from 59.87 to 62.14 for ADT 43 and ASD 16, respectively, while the selected homozygous lines HRR% are also on par with the recurrent parents and some of the lines have recovered more HRR% than both the recurrent parents (**Supplementary Table S3**). Some of the promising lines, ACM 18012, ACM 18014, and ACM 18015 in the background of ADT 43 and ACM 18244, ACM

TABLE 3 | Agro-morphological characters of selected improved pyramided lines at BC_3F_3 generation.

Genotype	Days to 50% flowering (DFF)	Plant height (PH) (cm)	Flag leaf length (FL) (cm)	Flag leaf width (FW) (cm)	Number of productive tillers per plant (NPT)	Panicle length (PL) (cm)	Number of grains per panicle (NGP)	1,000-Grain weight (1,000-GW) (g)	L/B ratio	Single plant yield (SPY) (g)
ACM 18012	85	88.3 ± 1.2	32.5 ± 0.6	1.0 ± 0.01	18 ± 2.1	24.2 ± 0.4	199 ± 4.5	15.21 ± 0.2	3.71 ± 0.02	27.11 ± 0.4
ACM 18242	64	87.4 ± 1.4	29.3 ± 0.4	1.3 ± 0.02	16 ± 1.4	26.5 ± 0.3	205 ± 9.7	22.9 ± 0.3	2.32 ± 0.04	30.13 ± 0.3
ACM 18243	82	89.1 ± 2.1	28.4 ± 0.4	1.2 ± 0.01	20 ± 3.2	23.4 ± 0.6	207 ± 8.1	22.89 ± 0.5	2.43 ± 0.01	30.19 ± 0.4
ACM 18244	89	86.4 ± 1.1	28.8 ± 0.5	1.2 ± 0.01	17 ± 2.1	20.6 ± 0.2	202 ± 9.4	24.15 ± 0.1	2.59 ± 0.02	30.24 ± 0.4
ACM 18013	78	87.6 ± 1.2	33.1 ± 0.4	1.0 ± 0.02	14 ± 3.2	19.3 ± 1.8	194 ± 12.6	16.32 ± 0.6	3.68 ± 0.05	28.06 ± 0.7
ACM 18014	81	86.5 ± 1.8	32.5 ± 0.3	1.0 ± 0.03	16 ± 1.4	20.5 ± 1.2	188 ± 10.3	15.94 ± 0.4	3.61 ± 0.03	27.62 ± 0.8
ACM 18245	79	88.3 ± 1.7	29.4 ± 0.4	1.3 ± 0.01	14 ± 2.4	27.8 ± 0.3	212 ± 11.2	23.61 ± 0.4	2.62 ± 0.02	29.65 ± 0.4
ACM 18246	65	83.8 ± 2.3	28.7 ± 0.1	1.2 ± 0.02	16 ± 2.1	24.3 ± 1.1	201 ± 6.8	22.78 ± 0.5	2.32 ± 0.01	27.19 ± 1.1
ACM 18247	88	78.8 ± 3.4	29.3 ± 0.4	1.2 ± 0.03	14 ± 3.2	21.2 ± 0.6	213 ± 14.1	22.19 ± 0.1	2.18 ± 0.01	29.89 ± 0.3
ACM 18015	83	79.7 ± 3.8	33.2 ± 0.3	1.0 ± 0.01	15 ± 1.5	20.8 ± 1.4	198 ± 12.2	15.29 ± 0.4	3.78 ± 0.05	28.18 ± 0.2
ACM 18016	84	85.4 ± 2.4	32.8 ± 0.6	0.9 ± 0.01	15 ± 2.1	21.5 ± 0.5	198 ± 13.4	15.23 ± 0.6	3.91 ± 0.03	27.95 ± 1.2
ACM 18017	81	88.3 ± 1.3	33.1 ± 0.2	0.9 ± 0.02	16 ± 2.3	23.8 ± 0.8	192 ± 11.9	16.65 ± 1.2	3.89 ± 0.08	28.24 ± 0.5
ACM 18248	85	86.2 ± 1.7	28.6 ± 0.5	1.2 ± 0.02	14 ± 2.8	23.7 ± 0.5	188 ± 16.6	24.08 ± 0.6	2.32 ± 0.05	29.85 ± 0.8
ACM 18018	85	81.5 ± 2.5	33.2 ± 0.2	0.9 ± 0.01	18 ± 1.2	18.3 ± 1.8	185 ± 6.5	15.27 ± 0.4	3.81 ± 0.04	27.49 ± 1.1
ACM 18249	85	81.6 ± 2.6	29.1 ± 0.2	1.1 ± 0.06	16 ± 1.4	24.1 ± 1.1	194 ± 8.5	22.34 ± 0.1	2.24 ± 0.04	29.71 ± 0.4
ACM 18250	79	79.7 ± 3.9	29.2 ± 0.5	1.2 ± 0.03	16 ± 2.1	23.4 ± 0.7	197 ± 8.1	22.56 ± 0.8	2.14 ± 0.06	27.15 ± 1.1
ACM 18019	83	89.4 ± 0.8	32.8 ± 0.3	0.9 ± 0.02	14 ± 2.4	23.8 ± 0.4	195 ± 12.9	15.15 ± 1.1	3.68 ± 0.04	28.23 ± 0.7
ACM 18020	81	81.3 ± 3.1	33.1 ± 0.1	1.0 ± 0.01	17 ± 3.3	25.9 ± 0.3	196 ± 4.8	15.87 ± 1.3	3.72 ± 0.06	27.48 ± 0.5
ACM 18021	82	85.3 ± 1.1	32.4 ± 0.2	1.0 ± 0.01	17 ± 1.2	24.2 ± 0.4	195 ± 3.4	15.52 ± 1.1	3.69 ± 0.02	26.2 ± 1.1
ACM 18022	83	84.7 ± 1.9	33.2 ± 0.1	0.9 ± 0.01	16 ± 1.6	24.6 ± 0.2	182 ± 4.7	15.23 ± 0.4	3.69 ± 0.05	25.8 ± 0.4
ACM 18023	78	82.5 ± 2.3	32.7 ± 0.3	0.9 ± 0.03	17 ± 1.2	25.1 ± 0.3	189 ± 2.1	15.29 ± 0.8	3.70 ± 0.02	26.4 ± 0.2
ASD 16	86	84.4 ± 3.1	29.3 ± 0.4	1.2 ± 0.05	19 ± 2.6	22.5 ± 0.6	201 ± 11.2	23.2 ± 0.5	2.08 ± 0.01	29.2 ± 0.2
ADT 43	85	85.2 ± 2.4	32.7 ± 0.4	1.0 ± 0.03	18 ± 1.3	24.3 ± 0.8	191 ± 14.3	15.6 ± 0.4	3.69 ± 0.07	27.6 ± 0.4
IRBB60	91	87.7 ± 4.8	31.1 ± 0.4	1.1 ± 0.02	15 ± 2.5	22.83 ± 2.5	148 ± 7.8	18.76 ± 0.6	3.34 ± 0.04	25.32 ± 0.3
Tetep	97	123.2 ± 5.8	35.5 ± 0.3	0.85 ± 0.05	17 ± 2.2	27.13 ± 3.1	153 ± 19.7	19.15 ± 1.2	3.43 ± 0.05	26.15 ± 0.9
SE	1.199	0.646	0.392	0.0282	0.312	0.471	1.594	0.762	0.142	0.296
CD (5%)	1.74	1.62	0.6	0.05	1.34	0.75	3.81	0.54	0.02	0.36

18245, and ACM 18249 in the background of ASD 16 have shown superior quality traits than both the recurrent parents (**Supplementary Table S3**).

Cluster Analysis

The coefficient of genetic distance on 10 morphological traits of 24 pyramided lines and four parents revealed that all the pyramided lines were very similar with their respective recurrent parent. It was observed that two solitary clusters were formed, one with Tetep alone and another with improved pyramided lines, IRBB60 and recurrent parents (ASD 16, and ADT 43). Cluster II was subdivided into two clusters, i.e., one subcluster with ASD 16 and its respective pyramided lines, another subcluster with IRBB60, ADT 43 parent, and its respective pyramided lines. Obviously, all the improved pyramided lines were being clubbed into their respective recurrent parent cluster (**Figure 6**).

DISCUSSION

More than half of the population of the globe is consuming rice to meet their dietary requirements. The demand for rice production is increasing, and many studies have reported that global rice production need to be doubled to meet the demands of the growing population (Ray et al., 2013). Besides the growing population, many biotic and abiotic stresses are affecting both yield and quality of rice crop. To address these constraints and to increase rice production, development of high-yielding cultivars enriched with resistant genes will enhance the yield as well as host-plant resistance. Conventional backcross method is the primary approach to develop resistant cultivars for single gene resistance, but phenotypic selection is a difficult and time-consuming process when multiple genes are involved in disease resistance (Crossa et al., 2017). The use of marker-assisted selection with stringent phenotypic selection enhances the efficiency and precision of breeding program for developing multiple disease-resistant varieties.

Marker-assisted backcross breeding (MABB) is a fastidious method for introgression of two or more targeted genes in the elite cultivars for improving the deficient trait. The prime aim of MABB is to transfer the targeted genes into the background of elite cultivars and to recover the RP genome as quickly as possible with a limited number of backcrosses. MABB also focuses on gradual reduction of donor parent genome as much as possible to avoid the undesirable effects on agronomical, yield, and quality

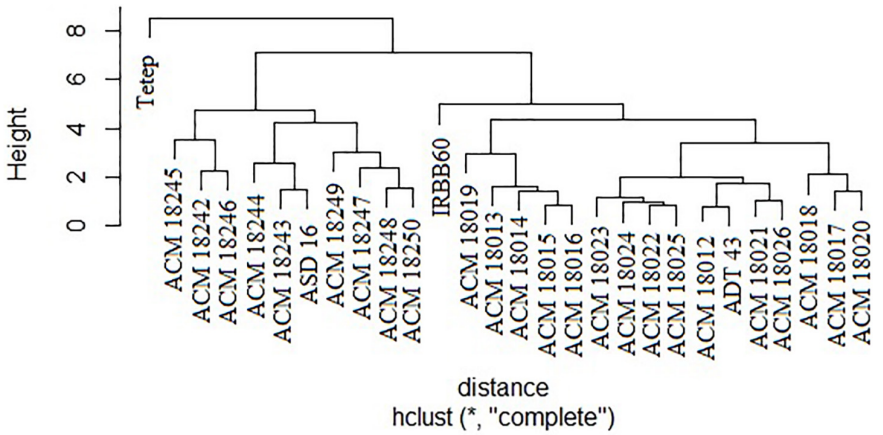

FIGURE 6 | Clustering of 24 selected improved pyramided lines based on 10 morphological characters. *Euclidean distance.

traits. The recurrent parents (ASD 16 and ADT 43) in this study are the popular and high-yielding cultivars of South India. Bacterial blight, blast, and sheath blight diseases are the major diseases of South India causing huge losses to the crop.

Many studies have focused on developing resistance to one or two diseases, as developing resistance to more than two diseases is a complex and time-consuming process. There are a few reports on developing multiple disease-resistant cultivars. Singh et al. (2012) introgressed blast and sheath blight resistance genes/QTLs in the background of Improved Pusa Basmati 1, harboring *xa13*, and *Xa21* genes. Das and Rao (2015) introgressed blast (*Pi2* and *Pi9*), submergence (*Sub1*), gall midge (*Gm1*, *Gm4*), and salinity (*Saltol1*) genes/QTLs in the background of Improved Lalat harboring *Xa4*, *xa5*, *xa13*, and *Xa21*. Arunakumari et al. (2016) has introgressed bacterial blight and blast resistance genes into the popular cultivar MTU 1010. As a part of sustainable management, we have planned and executed the introgression of BB (*xa5*, *xa13*, and *Xa21*), blast (*Pi54*), and sheath blight (*qSBR7-1*, *qSBR11-1*, and *qSBR11-2*) resistance genes/QTLs in the backgrounds of ASD 16 and ADT 43 for achieving multiple disease resistance and to offer IPL to farmers.

In this study, background screening with polymorphic SSR markers was used to recover the maximum percentage of RPG in early generations. At BC_3F_1, we have achieved RPG recovery in both ASD 16 and ADT 43 backgrounds. The background recovery rate in this study was at par with the theoretical recovery rate. We have observed inheritance of some unfavorable characters (plant height and grain qualities) along with favorable resistant traits while introgressing genes/QTLs from Tetep. Nevertheless, we have identified superior segregates with minimal residual effect from the Tetep genome by assessing greater population size (1,500 plants in both crosses). Sundaram et al. (2008), reported that introgression of *Xa21*, *xa13*, and *xa5* genes from SS1113 exercises a "pull" through inheritance of undesirable loci from a donor segment. Singh et al. (2015) introgressed *qSBR7-1*, *qSBR11-1*, and *qSBR11-2* into Pusa 6B, but they have not observed the inheritance of undesirable loci from Tetep. The observed undesirable effects of Tetep on the agronomic characters might be due to the linkage drag or environmental influence on agronomic characters.

The agronomic performance of 24 selected improved pyramided lines of both ASD 16 and ADT 43 backgrounds at BC_3F_3 generation revealed that most of the agro-morphological traits were on par with their recurrent parents (ASD 16 and ADT 43) and also showed durable resistance to BB, blast, and sheath blight diseases. The higher yield and superior quality traits of improved pyramided lines were probably due to the inheritance of yield contributing traits from the recurrent parents. The yield, agro-morphological, and quality traits were normally controlled by polygenes, and these are distributed throughout the genome. Employment of a greater number of background markers accelerated the recovery of RPG in the early generations. Cluster analysis of selected pyramided lines and parents based on 10 morphological characters revealed that all the selected pyramided lines were clustered in their recurrent parent's cluster. This is due to the similar morphological characters of pyramided lines with their recurrent parent's. Similar results were also obtained by Pradhan et al. (2015) and Hsu et al. (2020), while introgressing BB resistance genes, which supports the present study results.

The homozygous improved pyramided lines (BC_3F_3 generation) harboring *xa5* + *xa13* + *Xa21* + *Pi54* + *qSBR7-1* + *qSBR11-1* + *qSBR11-2* were assessed for physical resistance under greenhouse conditions. The results of bioassays suggest that pyramiding three BB-resistant genes exhibited higher resistance levels than the lines with one or two genes. In addition, the present study results also reveal that the gene combination with *Xa21* component expressed a shorter lesion length than the remaining gene combinations. These results were consistent with the results of previous studies (Peng et al., 2015; Pradhan et al., 2015; Ramalingam et al., 2017; Yugander et al., 2018; Hsu et al., 2020). The improved pyramided lines harboring the *Pi54* gene and ShB-resistant QTLs have expressed a similar disease reaction as donor parent, Tetep. This is due to the transfer of resistant alleles from the donor parent, which was confirmed by

functional/linked molecular markers as well as phenotypic screening methods.

The field evaluation of improved pyramided lines at BC_3F_3 generation demonstrated that the candidate lines of both recurrent parents had equivalent expression of yield, agro-morphological, and quality traits and, more importantly, with pyramided genes for BB, blast, and sheath blight. The higher levels of resistance to multiple diseases, without any yield penalty, is an integrated approach of genotypic and phenotypic selection methods. Developing broad-spectrum resistance to multiple diseases is a challenging task due to rich diversity of agro-climatic conditions in India along with the existence of genetically distinct virulent strains of different plant pathogens. Pyramiding of multiple or effective resistant genes/QTLs for different biotic stresses can contribute broad-spectrum and durable resistance to multiple diseases to the rice regions in India. The present study results prove that MABB is an effective tool for pyramiding major genes/QTLs to obtain improved plant lines in a quick time frame.

Introgression of BB resistance genes from IRBB60, blast and sheath blight resistance genes/QTLs from Tetep into the commercial cultivars is a significant achievement for obtaining durable resistance to multiple diseases. Introgression of identified effective resistance genes from the wild relatives or landraces into the commercial cultivars gradually improves the host-plant resistance to different biotic and abiotic stresses for attaining food and nutritional security. In conclusion, we have introgressed *xa5*, *xa13*, *Xa21*, *Pi54*, and *qSBR* QTLs (*qSBR7-1*, *qSBR11-1*, and *qSBR11-2*) in the backgrounds of ASD 16 and ADT 43 to improve host-plant resistance to BB, blast, and sheath blight diseases. The improved pyramided lines can be further tested in multilocational trails and could be released as improved variety or used as a potential donor in hybridization programs for developing multiple disease-resistant cultivars in rice.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JR designed the experiment. ChR, PS, RS, VV, and TC were involved in the experiments. JR, ChR, RS, and PS developed the improved pyramided lines. SV and MA were involved in a part of the experiment. JR, ChR, VV, and AR were involved in the screening of improved pyramided lines. JR, SA, and CV were involved in the revision of the final version of the manuscript. JR and all the authors prepared and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.591457/full#supplementary-material

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Domain Unknown Function DUF1668-Containing Genes in Multiple Lineages Are Responsible for F_1 Pollen Sterility in Rice

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Postzygotic reproductive isolation maintains species integrity and uniformity and contributes to speciation by restricting the free gene flow between divergent species. In this study we identify causal genes of two Mendelian factors *S22A* and *S22B* on rice chromosome 2 inducing F_1 pollen sterility in hybrids between *Oryza sativa* japonica-type cultivar Taichung 65 (T65) and a wild relative of rice species *Oryza glumaepatula*. The causal gene of *S22B* in T65 encodes a protein containing DUF1668 and gametophytically expressed in the anthers, designated *S22B_j*. The *O. glumaepatula* allele *S22B-g*, allelic to *S22B_j*, possesses three non-synonymous substitutions and a 2-bp deletion, leading to a frameshifted translation at the S22B C-terminal region. Transcription level of *S22B-j* and/or *S22B_g* did not solely determine the fertility of pollen grains by genotypes at *S22B*. Western blotting of S22B found that one major band with approximately 46 kDa appeared only at the mature stage and was reduced on semi-sterile heterozygotes at *S22B*, implying that the 46 kDa band may associated in hybrid sterility. In addition, causal genes of *S22A* in T65 were found to be *S22A_j1* and *S22A_j3* encoding DUF1668-containing protein. The allele of a wild rice species *Oryza meridionalis* Ng at *S22B*, designated *S22B_m*, is a loss-of-function allele probably due to large deletion of the gene lacking DUF1668 domain and evolved from the different lineage of *O. glumaepatula*. Phylogenetic analysis of DUF1668 suggested that many gene duplications occurred before the divergence of current crops in Poaceae, and loss-of-function mutations of DUF1668-containing genes represent the candidate causal genetic events contributing to hybrid incompatibilities. The duplicated DUF1668-domain gene may provide genetic potential to induce hybrid incompatibility by consequent mutations after divergence.

Keywords: hybrid incompatibility, reproductive isolation, rice, *Oryza*, pollen sterility, DUF1668, domain unknown function

INTRODUCTION

Hybrid incompatibilities (HIs), with respect to both intra- and interspecific hybridizations, are a widespread mechanism of postzygotic reproductive isolation, which restricts free gene flow between divergent species (Coyne and Orr, 2004). In plants, post-zygotic reproductive isolation occurs throughout the various life cycle stages of hybrids, from fertilization to sexual reproduction (inviability), gametogenesis and fertilization of gametes (sterility) in F_1 hybrids, and in the subsequent generation (hybrid breakdown; Stebbins, 1950). In cross breeding for genetic improvement in crop species, HI frequently hinders unflagging efforts of hybridization between cultivated and wild species to exploit useful genes and quantitative trait loci (QTLs) from wild genetic resources. Therefore, understanding genetic and molecular basis of HI play an important role in broadening a gene pool for crop improvement and in understanding evolutionary pathway of postzygotic reproductive isolation in crop species. Chromosomal or genic changes that occur during species divergence from common ancestral species are considered to be the main causal events in the evolution of HI (Maheshwari and Barbash, 2011). With respect to genic incompatibility, incompatible combinations of genes, each of which is generally an ancestral and variant allele derived from two reproductively isolated species, combine in the sporophytes or gametophytes of hybrids, resulting in maladaptive phenotypes, leading to inviability, sterility, and/or hybrid breakdown. These incompatible zygotes or gametes with reduced fitness are subsequently eliminated in hybrid populations. However, nucleotide variants causing HI are heterozygous only at birth; thus, a simple genetic model assuming a single locus is insufficient to explain the evolution of HI (Coyne and Orr, 2004). Therefore, it is currently central question of evolutionary genetics of HI that how alleles causing HI can evolve and be maintained in a population without falling into fitness valleys.

Bateson-Dobzhansky-Muller (BDM) incompatibilities are a classical genetic model (Dobzhansky, 1937; Muller, 1942), which proposes that reciprocal genetic changes in divergent species at two or multiple loci allow for the maintenance of new alleles causing HI without negative selection in the intermediate step (Coyne and Orr, 2004; Noor and Feder, 2006). The BDM incompatibilities model emerged based on observations of genetic interactions among multiple loci using a forward genetics approach with hybrid populations derived from inter- or intraspecific crosses. The HI system is controlled by the interaction of multiple genes located on different chromosomes, and epistatic complementarity between two genetic loci is exhibited in many plant species such as lettuce (Jeuken et al., 2009), *Arabidopsis* (Bomblies et al., 2007), cotton (Deng et al., 2019), monkey flower (Zuellig and Sweigart, 2018), and wheat (Matsuda et al., 2012). Gene cloning studies have also supported the molecular basis of BDM incompatibilities in plants (Rieseberg and Blackman, 2010; Chen et al., 2016). Gene duplication *via* whole-genome duplication or segmental genome duplication is a primordial event for gene diversification by neofunctionalization and subfunctionalization, as well as for species diversification due to nonfunctionalization (Lynch and Conery, 2000). Reciprocal losses of duplicated genes in divergent plant species were shown to cause F_1 pollen sterility at the *S27*/*S28* (Yamagata et al., 2010), *DPL1*/*DPL2* (Mizuta et al., 2010), and *DGS1*/*DGS2* (Nguyen et al., 2017) loci, and could lead to hybrid breakdown (Bikard et al., 2009).

When incompatible genes from two divergent populations are closely linked and cause BDM incompatibilities in the hybrid, inheritance of HI appears as a genetic interaction of the gene complex (or haplotype) at a single Mendelian locus in heterozygotes in plants. For example, in rice, intra-subspecific hybrids of cultivated rice carry two adjacent genes encoding F-Box protein and SUMO E3 ligase-like protein at the *Sa* locus to induce pollen sterility (Long et al., 2008), and endoplasmic reticulum stress due to incompatible interactions among heat shock protein Hsp70, an unknown protein with a transmembrane region, and eukaryotic aspartic proteases resulted in embryo sac sterility governed by *S5* (Yang et al., 2012). Recently, the *S1* locus identified in hybrids between *Oryza sativa* and *Oryza glaberrima* that induces F_1 pollen and embryo sac sterility was found to have originated from a gene complex consisting of *S1A6* and *S1A4* derived from an *O. sativa* allele (Xie et al., 2019) and *S1TPR/SSP* encoding a peptidase-like protein derived from an *O. glaberrima* allele at *S1* (Xie et al., 2017; Koide et al., 2018). As another mechanism, multiple gene copies derived from tandem duplications can acquire new promoter sequences and suppress the expression of genes essential for pollen formation of alternative alleles (Shen et al., 2017).

It has been controversial whether specific protein families or domains are likely to induce HI, although dozens of HI genes have been isolated in plants to date (Rieseberg and Blackman, 2010). HI, including necrosis or weakness, is frequently caused by deleterious interactions of pathogen and insect resistance (*R*) genes in plants (Bomblies and Weigel, 2007), affecting autoimmune responses to ultimately reduce growth, deregulate cell death, and cause sterility (Bomblies et al., 2007; Alcázar et al., 2009; Jeuken et al., 2009; Yamamoto et al., 2010; Chen et al., 2014; Atanasov et al., 2018). In rice, the protease genes involved in hybrid pollen sterility are located at *S1* (Xie et al., 2017; Koide et al., 2018) and involved in embryo sac sterility are located at *S5* (Yang et al., 2012). Genes encoding domain unknown function DUF1618-containing protein at the *Sc* allele on chromosome 3 (Shen et al., 2017) and at *HSA1A* on chromosome 12 in an inter-subspecific hybrid of *O. sativa* (Kubo et al., 2016) were recognized as causal genes of male hybrid sterility. If a specific pattern of functional change or loss of function in a particular domain such as DUF1618 has the potential to induce HI, sporadic independent origins of HI might have occurred across species or genera, which would allow for prediction of the evolution of HI and speciation in geographically isolated species. However, these domains responsible for speciation have been little reported to date.

In the genus *Oryza*, six wild species with an AA genome show an allopatric or sympatric distribution in several continents. *Oryza rufipogon* Griff. and *Oryza nivara* Sharma et Shastry are wild species in South and Southeast Asia, *Oryza longistaminata* A. Chev. & Roehr. and *Oryza barthii* A. Chev. are wild species

in Africa, *Oryza glumaepatula* Steud. is a wild species in South America, and *Oryza meridionalis* Ng is a wild species in Australia (Vaughan et al., 2005). Two cultivated species, *O. sativa* L. and *O. glaberrima* Steud., are considered to have been domesticated from *O. rufipogon* and *O. barthii*, respectively. More than 50 loci/QLTs for hybrid sterility have been reported in inter- and intraspecific hybrids of rice. Incompatible genotypes of the sporophyte or gametophyte determine sterility, and causal genes at 11 loci have been characterized based on molecular evidence (Chen et al., 2008; Long et al., 2008; Mizuta et al., 2010; Yamagata et al., 2010; Yang et al., 2012; Kubo et al., 2016; Yu et al., 2016; Nguyen et al., 2017; Shen et al., 2017; Koide et al., 2018; Xie et al., 2019).

In backcrossed hybrid progenies derived from a cross between *O. sativa* japonica-type cultivar Taichung 65 (T65) and *O. glumaepatula* accession IRGC105668 in the genetic background of T65, the F_1 pollen sterility gene *S22* was identified as a Mendelian genetic factor on the short-arm end of chromosome 2 (Sobrizal et al., 2000). The genomic regions of *S22* responsible for pollen sterility were dissected into the two independent genetic loci: *S22A* and *S22B* (Sakata et al., 2014). In the T65 genetic background, plants with the *S22A-T65*$^+$/*S22A-glum*^{s}|*S22B-T65*$^+$/*S22B-T65*$^+$ genotype (*S22A*_SS plants) showed approximately 50% pollen fertility (semi-sterility) due to sterility of pollen grains carrying the "sterile allele" *S22A-glum*^{s}. Similarly, plants carrying the *S22A-T65*$^+$/*S22A-T65*$^+$|*S22B-T65*$^+$/*S22B-glum*^{s} genotype (*S22B*_SS plants) showed pollen semi-sterility because of sterility of pollen grains carrying the "sterile allele" *S22B-glum*^{s}. The coupling phase linkage of *S22A-glum*^{s} and *S22B-glum*^{s} on *O. glumaepatula*-derived chromosomal segments could explain the initial identification of *S22* as a single Mendelian factor (Sakata et al., 2014).

In this study, to elucidate molecular players determining postzygotic reproductive isolation between *O. sativa* and *O. glumaepatula*, causal genes of *S22A* and *S22B* for F_1 pollen sterility between these divergent species were identified by map-based cloning approach. The allelism of another allele in *O. meridionalis* possibly at *S22B* also investigated. Since causal genes of HI both at *S22A* and *S22B* were found to encode DUF1668-containing proteins, which were found to be diversified in Poaceae, phylogenetic analysis of DUF1668 domain in Poaceae was conducted to know evolutionary timing of their occurrence of duplicated copies during divergence of Poaceae.

MATERIALS AND METHODS

Plant Materials and Phenotyping

The backcrossed progenies carrying chromosomal segments derived from *O. glumaepatula* accession IRGC105668 at *S22A* or *S22B* in the *O. sativa* L. cultivar T65 genetic background were developed in a previous study (Sakata et al., 2014). In this study, plants with the genotypes *S22A-T65*$^+$/*S22A-glum*^{s}|*S22B-T65*$^+$/*S22B-T65*$^+$, *S22A-T65*$^+$/*S22A-T65*$^+$|*S22B-T65*$^+$/*S22B-glum*^{s}, or *S22A-T65*$^+$/*S22A-glum*^{s}|*S22B-T65*$^+$/*S22B-glum*^{s} were designated *S22A*_SS, *S22B*_SS, or *S22A+B*_SS plants, respectively. These lines were maintained by marker-assisted selection using the simple sequence repeat (SSR) markers *RM12317* and *RM7451* for *S22A*_SS plants, *RM7033* and *RM279* for *S22B*_SS plants, and *RM12317* and *RM279* for *S22A+B*_SS plants. Phenotypes of *S22A*_SS, *S22A+B*_SS, and *S22B*_SS plants were discriminated according to the morphology of the sterile pollen grains stained with a 1% iodine-potassium iodide solution. Semi-sterile plants, in which almost all of the sterile pollen grains show no staining, stain slightly, and stain dark brown in color and are smaller than normal grains, were classified as *S22A+B*_SS, *S22A*_SS, and *S22B*_SS (**Supplementary Figure S1**). Transmission frequency of allele was estimated by maximum likelihood method (**Supplementary Methods**). The homozygous plants for *S22B-glum*^{s} and *S22A-T65*$^+$ obtained in the previous study (Sakata et al., 2014) were used in this study.

Map-Based Cloning

Total genomic DNA was extracted according to the method described by Dellaporta et al. (1983), with minor modifications. Primer sequences of the polymerase chain reaction (PCR)-based DNA markers used in this study are listed in **Supplementary Table S1**. Each 15-μl reaction mixture consisted of 50 mM KCl, 10 mM Tris (pH 9.0), 1.5 mM $MgCl_2$, 200 mM dNTPs, 0.2 mM primers, 0.75 units of *Taq* polymerase (Takara, Otsu, Japan), and 10 ng genomic DNA template. PCR was performed in a GeneAmp PCR System 9,700 (Applied Biosystems, Foster City, CA, United States). The cycling profile was an initial denaturation step at 95°C for 5 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 40 s; and a final elongation step at 72°C for 7 min. Amplified products were electrophoresed on a 4% agarose gel in 0.5× TBE buffer. *Agrobacterium*-mediated transformation was conducted as described by Yamagata et al. (2010). In brief, genomic fragments digested by restriction enzymes were cloned into the Ti-plasmid binary vector pPZP2H-lac (Fuse et al., 2001), and were then transformed into *S22A*_SS and *S22B*_SS plants. The copy numbers of transgenes were analyzed by quantitative PCR (qPCR) in an MX3000P QPCR system (Agilent Technologies, Santa Clara, CA, United States) using QuantiTect SYBR Green PCR Kits (Qiagen, Venlo, The Netherlands) according to our previous analysis (Nguyen et al., 2017). The gene models in the mapping region were obtained from MSU Rice Genome Annotation Project Database Release 7 (MSU7; http://rice.plantbiology.msu.edu/) in *O. sativa* cv. Nipponbare Os-Nipponbare-Reference-IRGSP-1.0 and *O. glumaepatula* W1183 accession ALNU02000000 (Jacquemin et al., 2013). The homologous sequences were searched in bl2seq program with cut off score at 1e-50 (Altschul et al., 1990).

Expression Analysis

For temporal expression analysis of *S22B*, the developmental stages of male gametophytes in T65, *S22B-glum*^{s} heterozygotes, and *S22B-glum*^{s} homozygotes were observed under a light microscope without staining. Fifty anthers were sampled from the unicellular, bicellular, and mature stages, respectively, which were placed into 1.5-ml tubes and then frozen in liquid nitrogen. Total RNA was extracted using Trizol reagent

(Life Technologies Japan, Tokyo, Japan) from the ground anther using a Multibeads shocker (Yasui Kikai, Osaka, Japan). Protein fractions of the Trizol extract were reserved for subsequent western blotting analysis. Extracted RNA was treated with DNase I (Takara, Otsu, Japan) to degrade contaminated genomic DNA. The first-strand cDNA was synthesized from approximately 100 ng of the extracted RNA and then reverse-transcribed using Revertra Ace (Toyobo, Otsu, Japan). Reverse transcription-quantitative PCR (RT-qPCR) was conducted in a MX3000P QPCR system (Agilent Technologies) using QuantiTect SYBR Green PCR Kits (Qiagen). For RT-qPCR of *S22B*, a pair of primers, 5'-CTC TGC CAA CTT CTG CAT CGC CAG G-3'/5'-GCT GAT AAG CTT GTA CAT CTC CGA C-3' and 5'-TGG AGG ATC CAT CTT GGC ATC AT-3'/5'-ACA GCT CCT CTT GGC TTA GCA-3' were used for amplification of *S22B* and the *actin 1* gene (*Os03g0718100*) as an internal control, respectively.

For the promoter-β-glucuronidase (*GUS*) assay, genomic sequences of the 1,572-bp region upstream of the initiation codon (ATG) of *S22B-T65*$^+$ were cloned into the Gateway-entry vector pENTR/TOPO using pENTR Directional TOPO Cloning Kits (Life Technologies Japan). The cloned insert was transferred into the destination vector pGWB3 (Nakagawa et al., 2007) using LR clonase (Life Technologies Japan) to fuse the promoter and *GUS* gene derived from pGWB3. The construct was transformed to T65 by *Agrobacterium*-mediated transformation.

Subcellular Localization

The coding sequence at *S22B* was amplified from the Nipponbare full-length cDNA clone J023058D10 (AK070727) provided by the National Agriculture and Food Research Organization (NARO), Japan, and was cloned into the Gateway-entry vector pENTR/TOPO (Life Technologies Japan). The *GFP*-fused gene at N-terminal under a control of 35S CaMV promoter was constructed using LR clonase (Life Technologies Japan) into the destination binary vector pGWB6 (Nakagawa et al., 2007). The construct was transformed into T65 by *Agrobacterium*-mediated transformation. The root of the obtained T_0 plants was observed in a fluorescence microscope (Biozero, BZ-8000, Keyence, Osaka, Japan) after 500 nM of mitochondrial fluorescent dye MitoTracker Red CMXRos staining.

Western Blotting

For western blotting of the *S22B* product, we prepared a rabbit polyclonal antibody against two synthesized 14-amino acid peptide sequences: N-KLATPLDAGAHDG-C and N-ISGGRKPEQHSLLP-C (Eurofins Genomics, Tokyo, Japan). The antibody specificity was confirmed by western blotting of a 6×His + S22B-T65 fused recombinant protein expressed in *Escherichia coli* strain BL21. The protein fractions extracted by Trizol were mixed with 2× Laemmli sample buffer and 2-mercaptoethanol, and then run on a 12% TGX gel (Biorad, Hercules, CA, United States). The proteins were transferred onto a polyvinylidene difluoride membrane with a 0.2-µm pore size (ATTO, Tokyo, Japan). Goat anti-rabbit IgG (H + L) antibody and horseradish peroxidase-conjugated antibody (Bio-Rad) were used for the secondary antibody reactions, and detection was performed using chemiluminescence detection with Western BLoT Hyper HRP Substrate (Takara Bio, Shiga, Japan).

Phylogenetic Analysis of DUF1668-Containing Sequences

All protein sequence data deduced in *Setaria viridis*, *Setaria italica*, *Panicum virgatum*, *Botryococcus distachyon*, *Sorghum bicolor*, *Zea mays*, *Botryococcus stacei*, and *Oryza sativa* were downloaded from the Phytozome 12 database (Goodstein et al., 2012). The protein sequences harboring the DUF1668 domain were detected by hidden Markov model searches using hmmsearch software[1] with a cutoff score of 1e−8. After the amino acid sequences of the DUF1668 domains were aligned using MUSCLE software with default parameters (Edgar, 2004), a phylogenetic tree based on maximum-likelihood inference was constructed in RAxML v. 8.2.8 software (Stamatakis, 2014) and drawn in FigTree v. 1.4.2 software.[2]

RESULTS

Map-Based Cloning of *S22B*

To narrow down the candidate region of *S22B*, we conducted high-resolution mapping of *S22B* in the BC_4F_6 population in which both *S22A* and *S22B* segregated (n = 7,424; **Figure 1**). Since *S22B* was previously mapped between the SSR markers *RM12329* and *RM279* (Sakata et al., 2014), 308 recombinants obtained between the SSR markers *RM12329* and *RM279* were screened by genotyping at the seedling stage. Linkage analysis of the 308 recombinants demonstrated that five recombinants (9-7, 23-2, 28-1, 38-3, and 39-7) were the most informative plants to map *S22B* within 18.4 kb of the genomic region in the reference sequence Nipponbare between DNA markers *M48* and *M46* (**Supplementary Figure S2**; **Figure 1B**). For the complementation test of *S22B*, 13.1-kb *Eco*72I, 13.5-kb *Xmn*I (*Xmn*I_a), 13.7-kb *Sal*I, and 10.7-kb *Xmn*I (*Xmn*I_b) fragments of the Nipponbare genomic fragment derived from BAC clone OSJNBa008C13 were introduced into *S22B*_SS plants by *Agrobacterium*-mediated transformation (**Figure 1B**). In the non-transgenic progeny of *S22B*_SS, a reduced transmission efficiency (k) of *S22-glum*^{s} alleles (k = 0.11) *via* pollen from the theoretical transmission (k = 0.5) was observed, which was attributed to the sterility of pollen grains carrying *S22-glum*^{s} (**Table 1**). Similarly, the T_1 generation derived from the T_0 plants transformed by the *Eco*72I, *Xmn*I_a, and *Xmn*I_b fragments showed segregation distortion of genotypes at *RM7033* linking to *S22B* because of reduced transmission of *S22-glum*^{s} (**Table 1**). Meanwhile, transformation of the *Sal*I fragment significantly altered the segregation of the genotype at *RM7033* in the T_1 generation (**Table 1**), suggesting that pollen grains harboring

[1]http://hmmer.org/
[2]http://tree.bio.ed.ac.uk/software/figtree/

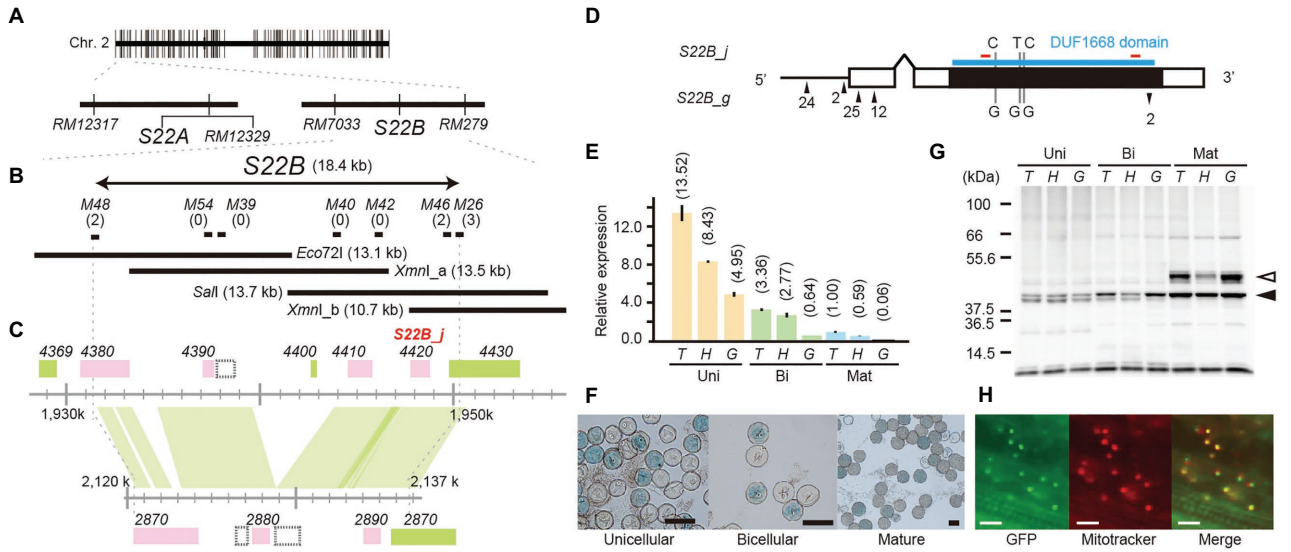

FIGURE 1 | Map-based cloning of *S22B*. **(A)** Two tightly linked genes, *S22A* and *S22B*, independently induce F_1 pollen sterility. **(B)** High-resolution linkage mapping of *S22B*. Numbers of recombinants between DNA markers and phenotypes are shown in parentheses. The restriction fragments *Eco*72I, *Xmn*I_a, *Sal*I, and *Xmn*I_b were used for transformation experiments. **(C)** Genomic structures on the reference genome assembly of *Oryza sativa* cv. Nipponbare Os-Nipponbare-Reference-IRGSP-1.0 (Kawahara et al., 2013) and *Oryza glumaepatula* W1183 accession ALNU02000000 (Jacquemin et al., 2013). Green and pink rectangles represent annotated genes encoded in the plus and minus chain of the reference sequences, respectively. The hatched rectangles represent predicted transposable elements. **(D)** Nucleotide variations on the 1.5-kb promoter (black horizontal bar) and coding sequence (black rectangles) between *S22B_j* and *S22B_g*. Up and down arrowheads represent nucleotide insertions and deletions, respectively, in *S22B_g* against *S22B_j*. The DUF1668 domain is shown as a blue line. Red bars show the location of peptide sequences to develop S22B antibody. **(E)** Reverse transcription-quantitative PCR (RT-qPCR) of *S22B* in the anthers during male gametogenesis for each genotype at *S22B*. Relative expression compared to the transcript in homozygotes for T65 on mature stage is represented in parentheses. **(F)** Promoter-glucuronidase (*GUS*) assay using the *S22B* promoter. T, H, and G represent genotypes homozygous for the T65 allele, heterozygous, and homozygous for IRGC105668, respectively. Scale bar: 50 μm. **(G)** Western blotting of S22B protein in the anthers. Total protein levels among samples were adjusted according to the numbers of collected anthers from 50 spikelets. White and black arrowheads represent the two major bands mentioned in text with the estimated mass of 37 and 46 kDa, respectively. **(H)** Subcellular localization of N-GFP-S22B in root cells of the stable transformant (T_0). Scale bar: 20 μm.

TABLE 1 | Frequency of genotypes at *RM7033* in the T_1 generation for the complementation test of *S22B*.

Construct	Recipient	Frequency of genotypes at *RM7033* at T_1[1]					
		TT	*TG*	*GG*	Total	*p*	*k*
*Eco*72I	*S22B*_SS	13	20	0	33	0.32	0
*Xmn*I_a	*S22B*_SS	26	21	5	52	0.52	0.16
*Sal*I	*S22B*_SS	18	41	19	78	0.00017***	0.51
*Xmn*I_b	*S22B*_SS	15	12	2	29	0.74	0.12
Non-transgenic	*S22B*_SS	42	43	5	90	-	0.11

[1]*TT, TG, and GG are homozygous for S22B-T65[+], heterozygous, and homozygous for S22B-glum[s], respectively. Values of p for independence of the segregation ratio in the T_1 generation were tested using Fisher's exact test at empirical segregation ratios of 42:43:5 for TT, TG, and GG plants derived from self-pollination of non-transgenic S22B_SS plants. k represents the transmission efficiency of S22B-glum[s] via pollen from maximum-likelihood estimation. ***represents significance at 0.1% significance level.*

S22-glum[s] recovered pollen fertility by the transformation of the *Sal*I fragment. Pollen fertility of the T_1 plants derived from the T_0 plant with the *Sal*I fragment or *Xmn*I_a fragment was observed (**Supplementary Figure S3**). The two T_1 lines, 6 and 7, were derived from the two independent T_0 plants carrying a single copy and more than two copies of the *Sal*I fragment, respectively. The heterozygotes at *S22B* harboring *Sal*I fragment showed recovered pollen fertility as compared with null segregants of heterozygotes or the heterozygotes harboring *Xmn*I_a fragment (**Supplementary Figure S3**). In the T_1 line 6, transgene segregated at one locus and the heterozygous plants at *S22B* carrying two, one, and zero copies of the *Sal*I fragment showed more than 90%, approximately 75, and 50% of pollen fertility, respectively, whereas the transformation of the *Xmn*I_a fragment did not recover pollen fertility in T_1 as a negative control (**Supplementary Figure S4**). *LOC_Os02g04420* was predicted to be located on the *Sal*I fragment but not on the other genomic fragments in the MSU7. These data demonstrated that the causal gene of *S22B* is *LOC_Os02g04420*, which encodes a protein containing DUF1668, designated *S22B_j* (**Figures 1C,D**). The genomic sequences of *S22B* including 2,500 bp of upstream region from the transcription initiation site are identical to those of Nipponbare. Sequencing of the *O. glumaepatula* (Acc. IRGC105668) BAC clone GL47D11 showed that the *O. glumaepatula* allele *S22B-g*, allelic to *S22B_j*, possesses three non-synonymous substitutions CCG(P) > GCG(A), TTC(F) > GTC (V), and CCC(P) > GCC(A) and a 2-bp deletion, leading to a frameshifted translation at the S22B C-terminal region (**Supplementary Data 1**). Many nucleotide substitutions at the promoter and first exon regions

were also observed (**Figures 1C,D**; **Supplementary Data 1**). The *S22B* sequences of T65 and IRGC105668 were deposited to DNA databank of Japan (DDBJ; LC596092 and LC596094).

Molecular Characterization of *S22B*

Expression of *S22B* from the anthers at the unicellular, bicellular, and mature stages was investigated by RT-qPCR among *S22B-T65*$^+$ homozygotes, *S22B-glum*^{s} heterozygotes, and *S22B-glum*^{s} homozygotes. Transcript levels gradually decreased during the progression of post-meiotic male gametogenesis (**Figure 1E**). At the unicellular, bicellular, and mature stages, the *S22B* expression level was elevated in the order *S22B-T65*$^+$ homozygotes, heterozygotes, and *S22B-glum*^{s} homozygotes, suggesting that transcription of *S22B-glum*^{s} was not as active as that of *S22B-T65*$^+$. The promoter-*GUS* assay was conducted in T_0 plants with 1.5 kb of endogenous genomic sequences upstream from the initial codon of *S22B-T65* in the unicellular, bicellular, and mature pollen stages (**Figure 1F**). Half of the pollen grains displayed GUS signals in T_0 transgenic plants carrying a single copy of the transgene, but no GUS signals were detected in the anther tissues, demonstrating that *S22B* is gametophytically expressed in the haploid generation. Together, the results from these expression analyses suggested that the level of gametophytic transcripts of *S22B-T65*$^+$ or *S22B-glum*^{s} did not solely determine the fertility of pollen grains carrying *S22B-T65*$^+$ or *S22B-glum*^{s} alleles.

Expression of S22B protein was investigated by western blotting using anti-S22B antibody in the anthers at the unicellular, bicellular, and mature stages (**Figure 1G**). The deduced molecular mass of S22B-T65$^+$ and S22B-glum^s proteins was expected to be approximately 39 kDa based on the coding nucleotide sequences. The density of approximately 37 kDa band estimated in western blotting (37 kDa) gradually increased during the pollen stage. Another major band at a molecular mass probably 46 kDa (46 kDa band) appeared only at the mature stage, and was reduced in the *S22B-glum*^{s} heterozygotes as compared with those of homozygotes for *S22B-T65*$^+$ or *S22B-glum*^{s} alleles. Although the levels of *S22B* transcripts were reduced in homozygotes for the *S22B-glum*^{s} allele (**Figure 1E**), protein levels between homozygotes for the *S22B-T65*$^+$ or *S22B-glum*^{s} allele were comparable (**Figure 1G**), implying that the transcript level was sufficient for expression of S22B protein and could be adjusted *via* feedback regulation.

The intracellular localization of S22B was investigated on root cells of the stable transformant (T_0) transformed by the construct of 35SCaMV prom::N-GFP-S22B protein (**Figure 1H**). Green fluorescent protein (GFP) signals were colocalized with the mitochondrial fluorescent dye MitoTracker Red CMXRos on root cells. The program for subcellular localization prediction in plant cells, TargetP-2.0 (Almagro Armenteros et al., 2019), MitoFate (Fukusawa et al., 2015), and Localizer (Sperschneider et al., 2017) did not find apparent prediction of localization to the mitochondria. WolfPSORT programs (Horton et al., 2007) weakly suggested that S22B possesses mitochondrial-targeting peptide sequences.

Map-Based Cloning of Gametophytic Factors at *S22A*

High-resolution mapping of *S22A* was conducted using the BC_4F_7 population (n = 3,072) derived from the BC_4F_6 plants heterozygous at the *S22A* genomic region between the SSR markers *RM12317* and *RM12350*, and homozygous for T65 at *S22B* in the genetic background of T65 (*S22A*_SS plants; **Figure 2**). Our previous study revealed that *S22A*_SS plants showed approximately 50% pollen sterility due to the sterility of pollen grains harboring *S22A-glum*^{s}; consequently, homozygotes for *S22A-T65*$^+$, heterozygotes, and homozygotes for *S22A-glum*^{s} segregated at a 1:1:0 ratio (Sakata et al., 2014). The genomic region responsible for *S22A* was delimited within a 151.4-kb region between the DNA markers *M24* and *SSR33* (**Figure 2A**). Homozygous plants for *S22A-glum*^{s} were not obtained in the high-resolution mapping population, suggesting that male gametophytes with *S22A-glum*^{s} in heterozygotes are completely sterile. Within the candidate genomic region, three gene models, *LOC_Os02g01790* designated *S22A_j1*, *LOC_Os02g01870* designated *S22A_j2*, and *LOC_Os02g01900* designated *S22A_j3*, were found to harbor DUF1668 in the reference genomic sequence of Nipponbare based on a Pfam search (**Figure 2B**). We speculated that the *S22A-T65*$^+$ allele has a function to gametophytically provide fertility to pollen grains carrying the *S22A-T65*$^+$ allele in spite of the haploid genotype, as in the case of *S22B*. Three *Acc*65I-digested genomic fragments *Acc*65I_a, *Acc*65I_b, and *Acc*65I_c, containing *S22A_j1*, *S22A_j2*, or *S22A_j3*, respectively, were subcloned from the Nipponbare BAC clone OSNBb0096M07 and transformed into *S22A*_SS plants. The T_1 population for *S22A_j1* showed segregation of homozygous genotypes for *S22A-glum*^{s}, demonstrating that pollen grains carrying the *S22A-glum*^{s} allele restored pollen fertility due to the effect of the *S22A_j1* transgene (**Table 2; Supplementary Table S2**). The transgene in the T_1 population for *S22A_j3* resulted in segregation of two homozygotes for *S22A-glum*^{s}, the frequency of which was lower than that in the T_1 population in *S22A_j1*. Moreover, the homozygous plants for *S22A-glum*^{s} were not observed in the T_1 population derived from the T_0 plants transformed by *S22A_j2*. If *S22A_j2* has equivalent function to *S22A_j1*, non-segregation of the homozygous plants for *S22A-glum*^{s} means *S22A_j2* does not function to restore pollen fertility with *S22A-glum*^{s}. Meanwhile, if *S22A_j2* has a partial function to restore the sterility as shown in transformation of *S22A_j3*, the number of T_1 individuals in this population might not have been sufficient to detect complementation of gene function. Therefore, further study is necessary to elucidate the function of this gene. Eventually, it was concluded that transgenes encoding protein with the DUF1668 domain derived from *S22A-T65*$^+$, at least *S22A_j1* and *S22A_j3*, gametophytically restored the fertility of pollen grains carrying the transgene. The amino acid sequences of *S22A_j1*, *S22A_j2*, and *S22A_j3* deduced from *O. glumaepatula* genomic sequences lacked DUF1668, suggesting that these three alleles at *S22A-glum*^{s} lost their function as a DUF1668-harboring protein (**Figure 2C**).

Independent Origin of Sterile Alleles in *O. meridionalis* at *S22B*

During the process of development of introgression lines of *O. meridionalis* accession W1625 (MER-ILs) in the genetic background of T65 (Yoshimura et al., 2010), pollen semi-sterile

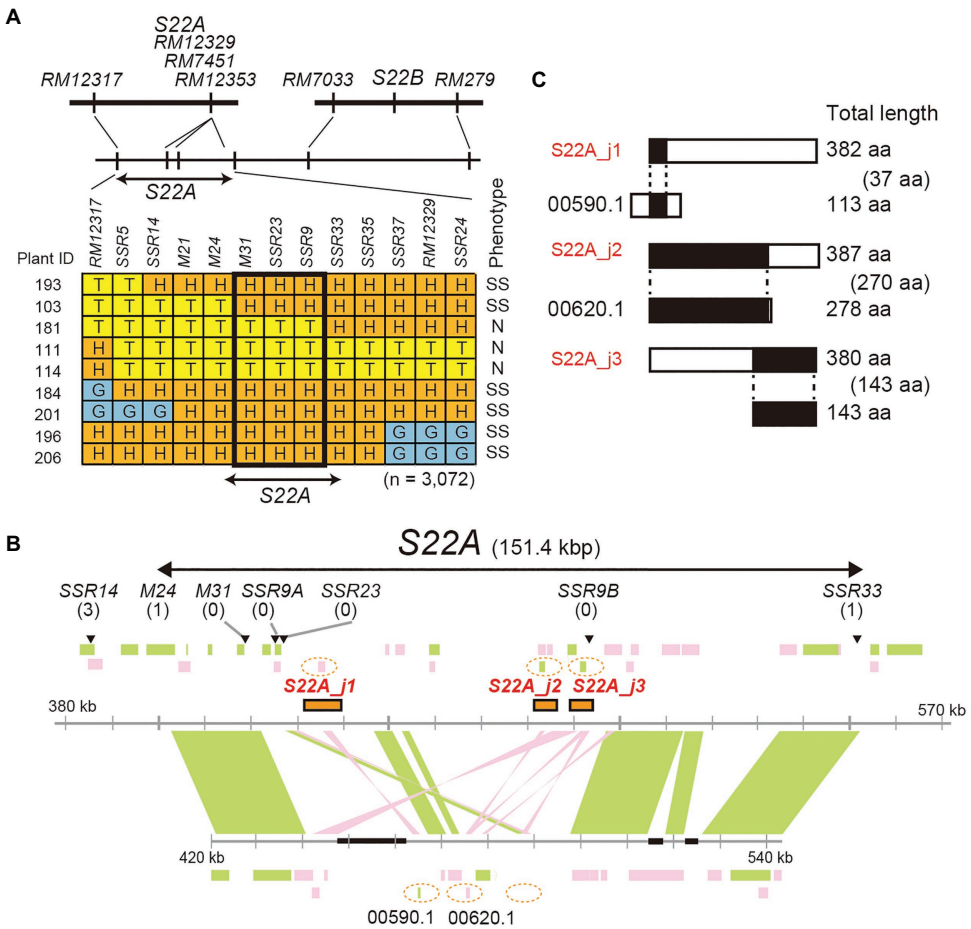

FIGURE 2 | Map-based cloning of *S22A*. **(A)** High-resolution linkage mapping of *S22A*. T, H, and G represent genotypes homozygous for the T65 allele, heterozygous, and homozygous for IRGC105668, respectively. N and SS represent a pollen phenotype of normal fertility and semi-sterility, respectively. **(B)** Genomic structures on the reference genome assembly of *O. sativa* cv. Nipponbare Os-Nipponbare-Reference-IRGSP-1.0 and *O. glumaepatula* W1183 accession ALNU02000000. Three restriction fragments, *Acc*65I_a, *Acc*65I_b, and *Acc*65I_c, including *S22A_j1*, *S22A_j2*, and *S22A_j3*, respectively, were used for transformation experiments. These fragments are shown as orange rectangles. Black boxes represent a sequence gap in the reference sequence. Numbers of recombinants between DNA markers and phenotypes are shown in parentheses. Green and pink rectangles represent annotated genes encoded in the plus and minus chain of the reference sequences, respectively. **(C)** Putative pseudogenes for DUF1668-containing protein in the *O. glumaepatula* genome at *S22A*. A similarity search using the BLASTn program revealed that only parts of DUF1668-containing protein S22A_j1, S22A_j2, and S22A_j3 showed sequence similarity in the *O. glumaepatula* genome. *S22A_j1* and *S22A_j2* appear to be allelic to *OGLUM02G00590.1* and *OGLUM02G00620.1*, respectively. However, DUF1668 domains were lost in the *O. glumaepatula* genome. Alignment lengths between proteins are shown in parentheses.

TABLE 2 | Complementation test of *S22A* by transmission analysis at *SSR23*.

Transgene	Genotype at *SSR23*[1]				
	TT	***TG***	***GG***	**Total**	***k***
S22A_j1	54	57	17	128	0.239
S22A_j2	19	20	0	39	0.000
S22A_j3	46	55	2	103	0.042
Empty vector	35	41	0	76	0.000

[1]*TT, TG, and GG are homozygous for S22A-T65$^+$, heterozygous, and homozygous for S22B-glum^s, respectively. k represents the transmission efficiency of S22A-glum^s via pollen from maximum-likelihood estimation. Segregation of the homozygous plants for S22B-glum^s (underlined) represent transmission of S22B-glum^s due to recovery of pollen fertility of pollen grains carrying S22B-glum^s.*

plants carrying W1625 chromosomal segments on the short arm of chromosome 2 with T65 cytoplasm were observed in the BC_4F_2 population (**Supplementary Figure S5**). Linkage mapping using the BC_4F_3 progeny demonstrated that semi-sterile and normal fertile pollen completely co-segregated to *RM7033*, and one single Mendelian factor close to *RM7033* located in the genomic region between SSR markers *RM7451* and *RM5984* controls pollen sterility in this population. This genetic factor was designated as *S22-mer*. Further genetic dissection of the Mendelian factor *S22-mer* using 753 plants of the mapping population indicated that *S22-mer* is located between *SSR24* and *RM5984* containing the *S22B* locus. The *O. meridionalis* allele at *LOC_Os02g04420* corresponding to *S22B_j* in *O. sativa* was designated *S22B_m*. Sequencing of

the *O. meridionalis* BAC clone OMERIa-82O19 containing *S22B_j* allelic region showed that *O. meridionalis* has lost the upstream gene region, including the promoter, transcription initiation site, and N-terminal region of the DUF1668-containing protein (**Supplementary Data 1**). However, *S22B_m* does not possess the non-synonymous substitutions and a 2-bp deletion found in *O. glumaepatula* (**Supplementary Data 1**) except the CCG (P) > GCG (A) mutation. These results suggest that *S22B_m* is a loss-of-function allele and evolved from the different lineage of *O. glumaepatula*. The *S22B* sequence of W1625 was deposited to DDBJ (LC596093).

Diversity Analysis of DUF1668

A BLASTP search using the amino acid sequences of *S22B_j* as a query in the proteome of angiosperm species in the Phytozome 12 database (Goodstein et al., 2012) found homologous sequences of proteins only in Poaceae species, including the PACMAD clade species *S. viridis*, *S. italica*, *P. virgatum*, *S. bicolor*, and *Z. mays*, and the BOP clade species *B. distachyon*, *B. stacei*, and *O. sativa* (**Supplementary Figure S6**). Phylogenetic relationships based on DUF1668-containing sequences in Poaceae species revealed that each genome showed more than 40 copies of DUF1668-containing genes except for *Z. mays* (**Supplementary Figure S6**). The phylogenetic tree of DUF1668 domain sequences did not exhibit apparent monophyletic branches by species (**Figure 3A**), suggesting that active gene duplication and diversification of multiple copies of DUF1668-containing genes likely started before the diversification of Poaceae. However, *S22B_j*, *S22A_j1*, *S22A_j2*, and *S22A_j3* were included in a single clade (Clade 1) harboring other members from *Setaria*, *Panicum*, *Botryococcus*, and *Oryza* species (**Figure 3**, yellow-shaded area). Reconstruction of the phylogenetic tree of DUF1668 members in Clade 1 revealed that DUF1668 sequences deduced from *S22A_j1*, *S22A_j2*, and *S22A_j3* formed a monophyletic clade, but *S22B_j* was found to have an independent origin from the clade containing *S22A_j1*, *S22A_j2*, and *S22A_j3* (**Figure 3B**).

DISCUSSION

Here, we revealed that the genes *S22B_j*, *S22A_j1*, and *S22A_j3* encoding DUF1668-containing protein are causal genes of F_1 pollen sterility controlled by *S22A* or *S22B* in hybrids derived from a cross between *O. sativa* cultivar T65 and *O. glumaepatula* accession IRGC105668. Our previous genetic analysis demonstrated that *S22A* and *S22B* independently induced pollen semi-sterility on heterozygotes, and that pollen grains carrying the sterility allele *S22A-glum*^{s} and *S22B-glum*^{s} in *S22A*_SS and *S22B*_SS plants are, respectively, sterile (Sakata et al., 2014). The transformation of *S22B_j* to *S22B*_SS plants recovered the fertility of pollen grains harboring sterile alleles (**Supplementary Table S3**) and transmission of the sterile allele *via* pollen increased (**Table 1**). Although the sterile allele *S22A-glum*^{s} had never transmitted *via* pollen in the high-resolution linkage mapping, the transformation of *S22A_j1* or *S22A_j3* to *S22A*_SS plants also archived transmission of the sterile allele *via* pollen (**Table 2**), demonstrating recovery of the fertility of pollen grains harboring sterile alleles by the transgene. Since *S22B_j*, *S22A_j1*, and *S22A_j3* are linked in the coupling phase, *S22A* and *S22B* were previously considered to represent the single Mendelian locus *S22* based on genetic mapping (Sobrizal et al., 2000). Although *S22A* and *S22B* harbor the same domain, DUF1668, the DUF1668-containing proteins at *S22A* and *S22B* are not functionally redundant. Phylogenetic analysis of DUF1668-containing sequences in Poaceae revealed that gene duplication and diversification of DUF1668-containing genes occurred in ancestral species of Poaceae, and the sterility-causing genes *S22A* and *S22B* belong to a specific clade, Clade 1 (**Figure 3A**). Since Clade 1 contains both species from the BOP and PACMAD clades (including the genera *Setaria*, *Panicum*, *Botryococcus*, and *Oryza*), ancestral DUF1668-containing genes likely originated before divergence of the BOP and PACMAD clades and are thus shared among these species.

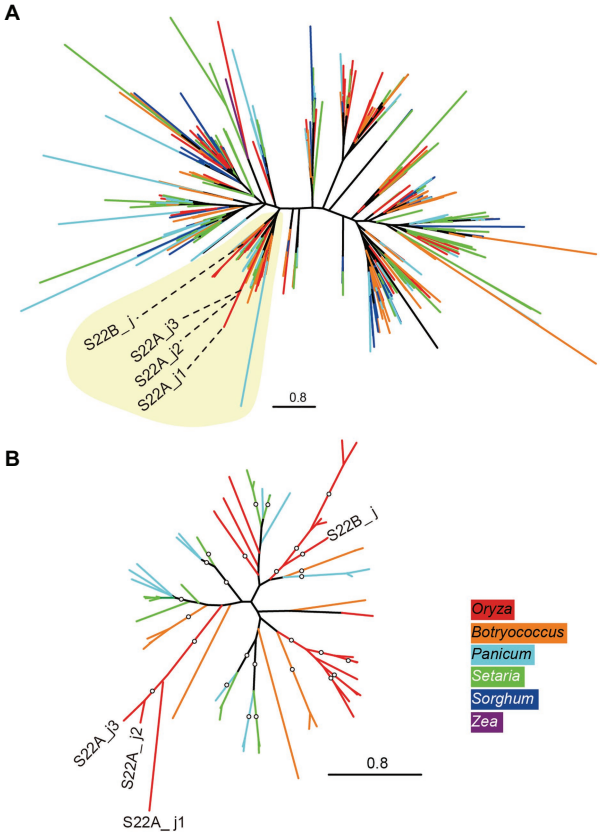

FIGURE 3 | Phylogenetic tree of DUF1668 domain in Poaceae species. **(A)** Maximum-likelihood trees showing the phylogenetic relationships of DUF1668 domain found in Poaceae species were constructed. The colors of external nodes (leaves) indicate the genus of DUF1668 domain members: red (*Oryza*), orange (*Botryococcus*), light blue (*Panicum*), green (*Setaria*), dark blue (*Sorghum*), and purple (*Zea*). Clade 1 (shaded in yellow) includes the DUF1668 members from the genera *Oryza*, *Botryococcus*, *Panicum*, and *Setaria*. **(B)** Reconstructed tree of DUF1668 members belonging to Clade 1. The branches supported by more than 75% bootstrap values are indicated by circles.

Since *S22A_j1*, *S22A_j2*, and *S22A_j3* also formed a monophyletic clade within Clade 1 (**Figure 3B**), they likely originated from gene duplications at *S22A* after divergence of the genus *Oryza*. Therefore, the genetic functions of these DUF1668-containing gene copies at *S22A* are likely to be redundant, and transformation of these copies restored the fertility of sterile pollen grains carrying *S22A-glum*^{s}. By contrast, gene duplication between *S22A* (*S22A_j1*, *S22A_j2*, and *S22A_j3*) and *S22B* may have at least an ancestral population of the divergence of the BOP and PACMAD clades. Although this study demonstrated that genetic variants at both *S22A* and *S22B* induce HI, it will be necessary to further examine whether the DUF1668 domain has evolved as an HI factor in other Poaceae species.

Molecular Behavior of S22B

S22B transcript levels were reduced in the order of homozygotes for *S22B-T65*$^+$, heterozygotes, and homozygotes for *S22B-glum*^{s} in the anthers at each of the unicellular, bicellular, and mature stages using forward and reverse primers targeting the same sequences at *S22B_j* and *S22B_g* (**Figure 1E**). These data suggest that the expression level of *S22B_g* is lower than that of *S22B_j*. The expression level of *S22B* also decreased as male gametogenesis progressed. The *S22B* transcripts are likely mainly contributed from male gametophytes rather than from sporophytic tissues (anthers) based on the results of the promoter-*GUS* assay (**Figure 1F**). On the other hand, *S22B* broadly expressed in vegetative tissues in RiceXpro expression database (**Supplementary Figure S6**; Sato et al., 2013). Comparison of the genomic regions of *S22B_j* and *S22B_g* revealed many nucleotide substitutions in the promoter region and the 5' untranslated region near the transcriptional start site. These substitutions may be involved in regulating interspecific differences in transcription between the two alleles.

Western blotting showed that the level of S22B protein increased with the progression of male gametogenesis, in contrast to the decrease observed at the transcription level (**Figure 1G**). No obvious difference in S22B protein accumulation was observed between the two homozygotes for *S22B-T65*$^+$ and *S22B-glum*^{s}. These data suggest that the level of S22B protein is under control by a post-translational regulation mechanism or that *S22B-glum*^{s} is sufficient for male gametogenesis. By contrast, the level of S22B protein was reduced in heterozygotes (**Figure 1G**). As one example of reduced expression only in heterozygotes in HI systems, genetic variants of a single gene between two diverged alleles at the *Sc* locus were reported to constitute the HI system (Shen et al., 2017). The japonica allele *Sc-j* contains a pollen-essential gene, and the indica allele *Sc-i* contains two or three tandem duplicates of an *Sc-j* homolog with a distinct promoter. In *Sc-j/Sc-i* hybrids, the high expression level of *Sc-i* in sporophytic cells causes suppression of *Sc-j* expression in pollen and selective abortion of *Sc-j*-pollen (Shen et al., 2017). Their study further revealed that feedback-mediated regulation of genes or proteins may result in the misregulation of gene expression between differentiated alleles in heterozygotes. Similar to the *Sc* system, the reduced S22B protein level in the anthers of heterozygotes observed in the present study could be due to allelic suppression of S22B protein *via* incompatible feedback modulation between the two alleles.

Our western blotting analysis of S22B also revealed that the 46 kDa band appeared specifically at the mature stage. In S22B_SS, development of pollen grains carrying the *S22B-glum*^{s} allele starts to delay from the late bicellular stages, and this delay is particularly apparent at the mature stage as compared with normal genotypes (Sakata et al., 2014). Almost all of the pollen grains carrying the *S22B-glum*^{s} allele could reach the tricellular stage but did not complete the formation of the male germ unit and failed to produce the pollen tube. We speculate that the reduction of the 46 kDa band in heterozygotes is involved in pollen semi-sterility. It is possible that the 46 kDa band resulted from alternative splicing of *S22B_j* and/or *S22B_g*, or from post-translational modification of the 37 kDa band, such as phosphorylation, lipidation, or glycosylation. The genetic effects of nucleotide substitutions and the 2-bp deletion on the *S22B* coding sequence for protein modification, and the mitochondrial localization of S22B have not yet been elucidated. Thus, these biochemical properties require further study.

In the linkage analysis of *S22A* (n = 3,072), homozygotes for *S22A-glum*^{s} were not obtained (**Supplementary Figure S2**). The total seed set of self-pollinated *S22A*_SS plants was fertile, and self-pollinated seeds of *S22A*_SS showed normal seed germination. These results demonstrate that the *S22A-glum*^{s} allele is insufficient for male gametogenesis in heterozygotes. Alternatively, the *S22B-glum*^{s} allele is transmitted *via* male gametophytes in heterozygotes and homozygotes, which segregates at low frequency. The *S22A-glum*^{s} allele encodes a truncated protein with loss of the complete DUF1668 domain (**Figure 2C**). In contrast, the *S22B-glum*^{s} allele has a few single nucleotide substitutions and a 2-bp frameshift mutation at the C-terminal, but the DUF1668 domain was predicted to exist in the Pfam search (Finn et al., 2014). *S22B_m* is likely a loss-of-function allele, and a homozygous plant for the *S22-mer* allele segregated and showed normal pollen fertility (**Supplementary Figure S4**). Therefore, an unidentified causative mutation in *O. glumaepatula* may inactivate gene function, although the DUF1668 domain was predicted *in silico*.

Hybrid Incompatibility at *S22A* and *S22B*

Diverged haplotypes, including multiple tightly linked genes, are known to induce HI in intra-specific and interspecific hybrids of rice, such as *Sa*, *S5*, and *S1*. The incompatible gene complex includes sporophytic genetic factors and gametophytic genetic factors that determine pollen fertility of its own gametophytes in heterozygotes. The BLAST similarity search using the cloned genes at the known HI as a query did not find homologous sequences within the *S22A* and *S22B* mapping regions. If HI systems conferred by *S22A* and *S22B* also evolve incompatible haplotypes to induce HI in hybrids, the genes cloned in this study only represent a portion of gametophytic members acting as a protector and were not sufficient to induce HI. To further identify other

possible genes including a killer factor involved in HI at *S22A* or *S22B*, it is necessary to conduct defective mutant experiments using genome-editing methods such as CRISPR/Cas9 in heterozygotes showing HI. As suggested in the BDM model, nucleotide variants causing HI are heterozygous only at birth, which is necessary to escape from negative selection due to their own maladaptive phenotypes owing to this incompatibility. When *S22A_j1*, *S22A_j2*, and *S22A_j3* are considered as the ancestral types and the truncated genes at *S22A-glum*^{s} are considered as the variant types, nucleotide variants of *S22A-glum*^{s} occurring only at birth need to escape from natural selection *via* BDM partners such as duplicated genes or interacting genes in other genomic regions of *O. glumaepatula* or ancestral species. Since near-isogenic lines of *S22A* and *S22B* in the T65 genetic background were used in this study, the genetic phenomena may appear as monogenic events, and other BDM partners have not yet been identified.

In contrast, a single gene can also cause an HI system. Allelic suppression of the japonica allele at the *Sc* locus results from the feedback-mediated regulation of gene expression between differentiated alleles in inter-subspecific hybrids between japonica and indica rice (Shen et al., 2017). If the allelic suppression occurred on the semi-sterile *S22B*_SS, transcription level on heterozygote would show half of the homozygotes for *S22B-T65*$^+$ because transcription of *S22B-glum*^{s} is suppressed on heterozygote. However, expression level on the *S22B*_SS was closed to average of homozygotes of *S22B-T65*$^+$ and *S22B-glum*^{s}, suggesting that allelic suppression has not occurred on transcription level. Instead, we suggest that alternative splicing of S22B_j and/or S22B_g or production of the 46 kDa band resulted from post-translational modification is suppressed in pollen grains harboring *S22B-glum*^{s} in allele-specific manner. The further studies may reveal this opinion. Another possibility may be that copy number variation of functional copies of DUF1668-containing genes in pollen grains results in competitive transmission efficiencies between fertile and sterile alleles. The allelic differences in development and starch absorption capacity may also result in the biased allocation of nutrient resources between gametophytes, leading to the distorted transmission efficiency of alleles.

In summary, HI at *S22A* and *S22B* could be caused by gene complexes within the candidate region, or by structural changes in a single gene. Further genetic analysis to identify other genetic loci interacting with *S22A* or *S22B*, and biochemical and genetic characterization of DUF1668-containing genes may reveal the HI mechanism *via* DUF1668.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MS: investigation, development of genetic materials, review, writing draft manuscript, and editing of the manuscript. NS and YM: investigation and development of plant materials. HK, JW, and TM: investigation and sequencing. HY and AY: project administration, funding acquisition, and supervision. YY: conceptualization, methodology, investigation, data curation, and writing draft manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.632420/full#supplementary-material

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REVIEW
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Hybrid Incompatibility of the Plant Immune System: An Opposite Force to Heterosis Equilibrating Hybrid Performances

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Hybridization is a core element in modern rice breeding as beneficial combinations of two parental genomes often result in the expression of heterosis. On the contrary, genetic incompatibility between parents can manifest as hybrid necrosis, which leads to tissue necrosis accompanied by compromised growth and/or reduced reproductive success. Genetic and molecular studies of hybrid necrosis in numerous plant species revealed that such self-destructing symptoms in most cases are attributed to autoimmunity: plant immune responses are inadvertently activated in the absence of pathogenic invasion. Autoimmunity in hybrids predominantly occurs due to a conflict involving a member of the major plant immune receptor family, the nucleotide-binding domain and leucine-rich repeat containing protein (NLR; formerly known as NBS-LRR). NLR genes are associated with disease resistance traits, and recent population datasets reveal tremendous diversity in this class of immune receptors. Cases of hybrid necrosis involving highly polymorphic NLRs as major causes suggest that diversified *R* gene repertoires found in different lineages would require a compatible immune match for hybridization, which is a prerequisite to ensure increased fitness in the resulting hybrids. In this review, we overview recent genetic and molecular findings on hybrid necrosis in multiple plant species to provide an insight on how the trade-off between growth and immunity is equilibrated to affect hybrid performances. We also revisit the cases of hybrid weakness in which immune system components are found or implicated to play a causative role. Based on our understanding on the trade-off, we propose that the immune system incompatibility in plants might play an opposite force to restrict the expression of heterosis in hybrids. The antagonism is illustrated under the plant fitness equilibrium, in which the two extremes lead to either hybrid necrosis or heterosis. Practical proposition from the equilibrium model is that breeding efforts for combining enhanced disease resistance and high yield shall be achieved by balancing the two forces. Reverse breeding toward utilizing genomic data centered on immune components is proposed as a strategy to generate elite hybrids with balanced immunity and growth.

Keywords: hybrid necrosis, autoimmunity, anti-hybrid necrosis, heterosis, immunity, trade-off, NLR, growth

INTRODUCTION

Hybridization between individuals of the same or different plant species leads to the formation of hybrids that contain a copy of each parental genome. This heterozygosity results in novel genetic combinations that were never found in the respective parental lines, and thus distinct phenotypes can be sometimes expressed in the hybrid (Mallet, 2007). Genetic interactions in the hybrid can result in beneficial phenotypes, of which the phenomenon is termed as heterosis or hybrid vigor (Shull, 1948; Schnable and Springer, 2013). On the contrary, such interactions can lead to detrimental phenotypes, as found in hybrid weakness or hybrid necrosis cases. Hybrid necrosis is a common phenomenon observed in plant hybrids featuring immune-related deleterious phenotypes (Bomblies and Weigel, 2007; Bomblies, 2009). The term reflects phenotypically recognizable damages in plant tissues with visible necrosis resulting from uncontrolled cell death and/or autoimmune-like symptoms including dwarfism, stunted growth, leaf crinkling, reduced fertility, and in severe instances death before transitioning to the reproductive phase (Bomblies and Weigel, 2007; Chae et al., 2014). The overall compromise in plant performances observed in hybrid necrosis cases is believed to reflect trade-offs between immunity and growth (Todesco et al., 2014; Chae et al., 2016; Świadek et al., 2017). Due to its drastic effect on survival and prevalence in F_1 generations, hybrid necrosis is postulated to provide plant populations with a conducive mechanism to create post-zygotic barriers toward eventual speciation (Bomblies and Weigel, 2007).

Hybrid Necrosis Under the Bateson-Dobzhansky-Muller Model and Negative Epistasis

Hybrid necrosis arises from novel genetic interactions in heterozygous backgrounds. This phenomenon often manifests in the F_1 generation, but cases in the F_2 have also been described. Numerous examples of hybrid necrosis in plants demonstrate that the dynamics of genetic incompatibilities found in F_1 and F_2 hybrids conform to the simple Bateson-Dobzhansky-Muller (BDM) model for two-locus interaction that had been formulated to explain the speciation mechanism (**Figure 1A**; Orr, 1996). The BDM model posits that independently evolved genetic variants are harmless in their respective parental lineages but the combination of a pair becomes incompatible in the hybrid (**Figure 1A**). Since inviable, inferior or infertile offspring are the outcome of such incompatibilities, such genetic mechanisms are considered to establish major postzygotic reproductive barriers between different lineages, hence promoting speciation (Orr, 1996).

A wider spectrum of phenotypes are found in hybrids as compared to the parents, including heterosis and hybrid necrosis, and the underlying causes can be generally explained by genetic interactions following the three schemes, namely (i) dominance, (ii) overdominance, and (iii) epistasis (**Figure 1B**). These three mechanisms can operate simultaneously, but independently, to allow the expression of different traits (Li et al., 2008; Huang et al., 2016). The dominance hypothesis is often favored to explain heterotic phenotypes, which attributes the expression of heterosis to the heterozygous allelic status at multiple loci in hybrids. The premise is that in parental lines phenotypic expression for a given trait is constrained by the sum of deleterious, recessive alleles. In heterotic hybrids, the introduction of beneficial or dominant alleles from both parents at these multiple loci breaks the recessiveness that had caused inferiority in the respective parental backgrounds, thus improving hybrid performance (**Figure 1B**, left; Hochholdinger and Hoecker, 2007). The overdominance hypothesis, on the other hand, attributes superior- or under-performance in the hybrid to the heterozygosity at a single locus as compared to the homozygous status in parents (**Figure 1B**, middle; Hochholdinger and Hoecker, 2007). Lastly, epistasis refers to non-additive genetic interactions deviating from the summation of allelic effects at the involved loci (**Figure 1B**, right; Fisher, 1919). It is necessary to point out, however, that other mechanisms such as epigenetics can control the expression of traits in hybrids (Fujimoto et al., 2018). For instance, experiments in *Arabidopsis thaliana* using epigenetic recombinant inbred lines (Epi-RILs) have shown that epialleles are inheritable and can explain phenotypic variation in certain developmental traits (Cortijo et al., 2014; Zhang et al., 2018). The BDM model for hybrid necrosis is certainly a well spelled-out case of negative epistasis involving two derived alleles from the involved genetic loci. In most hybrid necrosis cases, the derived alleles act as dominant or semi-dominant, in a dosage-dependent manner. There are hybrid necrosis cases only visible in the F_2 generation, of which genetic scheme can be similar to the classical F_2 hybrid breakdown, albeit with strong dose-dependent effects (**Figure 1A**). Genetic studies on hybrid necrosis cases have demonstrated that a pairwise epistatic relationship between alleles at two distinct loci inherited from each of the parents does condition autoimmunity in both F_1 and F_2 hybrid generations (**Figure 1A**, left; Bomblies et al., 2007; Alcázar et al., 2009; Chae et al., 2014). In addition, cases of hybrid necrosis involving different alleles at a single locus have also been identified. Such negative epistasis can be easily explained by a simple modification of the BDM model with a replacement of one unlinked locus with the different alleles of the same locus (**Figure 1A**, right; Alcázar et al., 2014; Chae et al., 2014). To conclude, the BDM model explains the majority of F_1 or F_2 hybrid necrosis cases as a result of negative epistasis. Due to the deleterious effect on hybrid performance, hybrid necrosis can serve as a conducive mechanism to create a reproductive barrier between two independently evolving parental lineages (**Figure 1A**).

Autoimmunity as an Underlying Mechanism for Hybrid Necrosis

Numerous hybrid necrosis cases have been identified in crops of economic relevance such as wheat (Hermsen, 1963), interspecific hybrids of *Triticum-Aegilops* (Mizuno et al., 2010), rye (Ren and Lelley, 1988), lettuce (Jeuken et al., 2009), rice

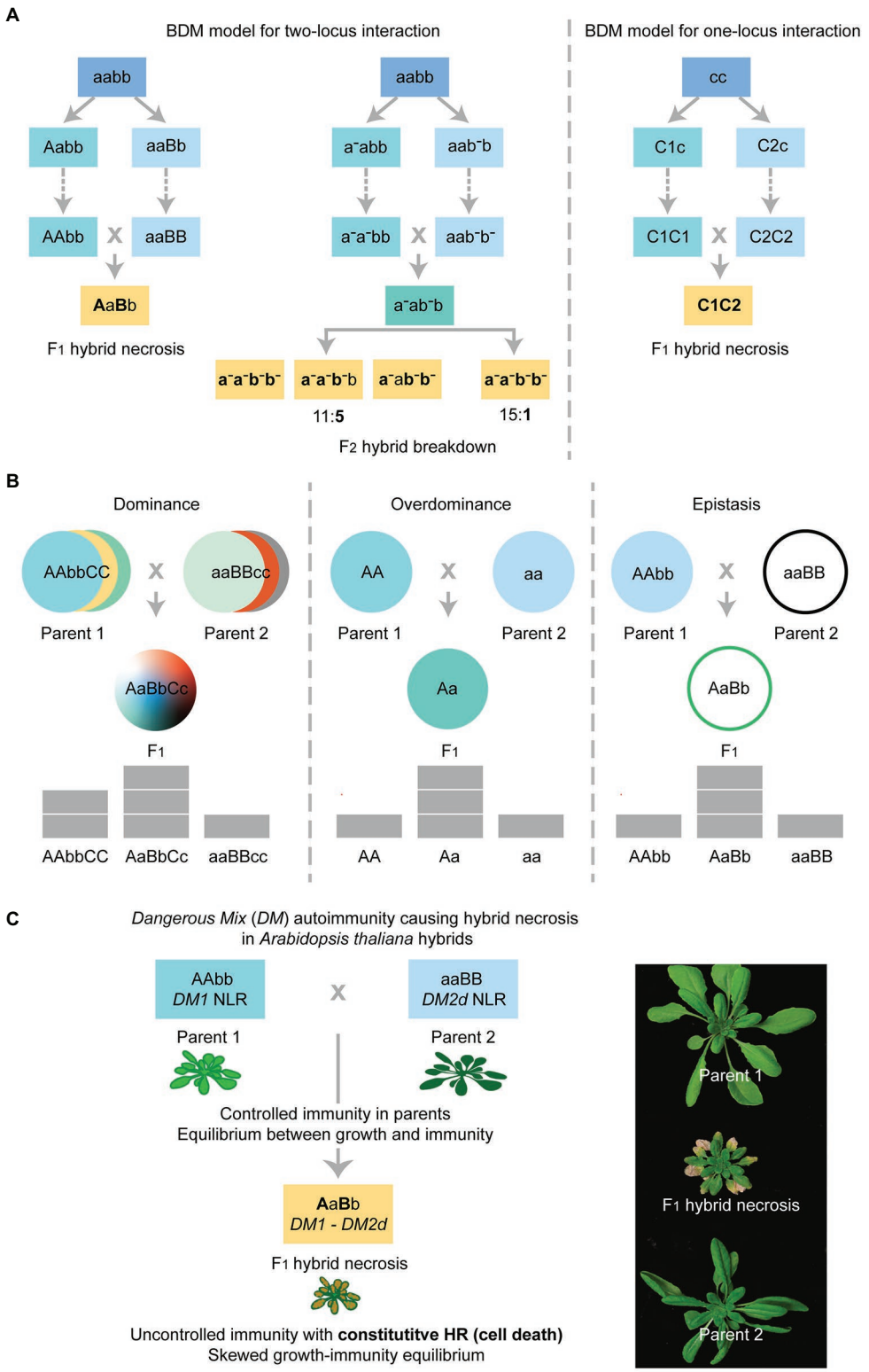


FIGURE 1 | The genetic mechanisms of hybrid necrosis caused by negative epistasis explained by the Bateson-Dobzhansky-Muller (BDM) model. **(A)** The BDM model for two-locus or one-locus interaction triggering F_1 hybrid necrosis and F_2 hybrid breakdown in hybrid necrosis cases (yellow text-boxes). Ancestral status of alleles at indicated loci is uncapitalized and derived allelic status is indicated as capitalized for hybrid necrosis cases. Boldface letters indicate alleles that are under

(Continued)

FIGURE 1 | deleterious epistasis for hybrid necrosis and hybrid breakdown cases. The dotted arrows in each scheme indicate that numerous generations are required before the different allelic combinations represented in the colored boxes are fixed in independent lineages. In the cases of hybrid necrosis locus/loci, derived alleles behave as dominant or semi-dominant (Bomblies et al., 2007; Alcázar et al., 2009; Chae et al., 2014). In F_2 hybrid breakdown, ancestral status and derived allelic status differ from each other by the presence of the upper dash for derived recessive alleles. Note that classical F_2 hybrid breakdown cases manifest when the homozygous recessive state at two loci is met with the derived alleles (a-a-;b-b-) or the homozygous recessive state of one locus is combined with the heterozygous status of the other locus (a-a-;b-b or aa-;b-b-) in 15:1 or 11:5 segregation (Oka, 1957; Fukuoka et al., 1998; Okuno, 1999; Matsubara et al., 2007, 2014; Ichitani et al., 2012). **(B)** Genetic mechanisms of dominance, overdominance, and epistasis leading to phenotypes that respond to both qualitative (color) or quantitative (pyramid) traits. The first two mechanisms attribute the observed phenotype in the hybrid to additive effects, arising from allelic interactions between dominant over recessive alleles at various loci (dominance) or at a single locus (overdominance). Epistasis attributes any phenotype that deviates from the expected additive effect of two alleles to non-allelic interactions (i.e., two different loci). Note that the BDM model for two-locus interaction shown in **(A)** corresponds to epistasis, while overdominance explains the BDM model for one-locus interaction shown in **(A)**. **(C)** Left: Autoimmunity triggered by the *DM1/DM2d* hybrid necrosis pair. The two loci, *DM1* and *DM2d*, are innocuous in their respective parental lineages. The combination of *DM1* and *DM2d* in the *Arabidopsis thaliana* hybrid triggers a constitutive activation of defense mechanisms in the absence of pathogen attack, which results in the hypersensitive response (HR) and hybrid necrosis (Bomblies et al., 2007; Tran et al., 2017). Right: F_1 hybrid displaying cell death in somatic tissues and the comparison to the respective parents.

(Yamamoto et al., 2010), cotton (Phillips, 1977; Deng et al., 2019), and in natural germplasms of *A. thaliana* and *Capsella* species (Bomblies et al., 2007; Chae et al., 2014; Sicard et al., 2015). Because hybrid necrosis has been observed in a broad range of species (see review Bomblies et al., 2007), it is of major interest to define the causal genetic components and investigate if there is a common underlying mechanism. Accumulating fine-mapping data obtained from various hybrid necrosis cases in different species point out that this phenomenon arises from the uncontrolled activation of immune responses in the absence of pathogens. Such links to autoimmunity had already been suggested by Kostoff in 1930, who indicated that hybrid necrosis phenotypes resemble those triggered by pathogens and therefore suggested autoimmunity as a major cause of hybrid necrosis (Kostoff, 1930). However, it has only been over a decade since the causality was identified to the R protein families by molecular cloning (Krüger et al., 2002; Rooney et al., 2005; Bomblies et al., 2007; Alcázar et al., 2009; Chae et al., 2014). Nucleotide-binding and leucine-rich repeat (NLR) immune receptors in the majority and in some cases receptor-like proteins (RLPs) or receptor-like kinases (RLKs) families contribute to trigger autoimmunity in hybrids. NLRs constitute the major class of resistance genes (hereafter *R* genes) characterized to date, and plant genomes often carry hundreds of *R* gene homologs, some of which are highly variable even within a species (Sarris et al., 2016; Kourelis and van der Hoorn, 2018; Van de Weyer et al., 2019). It is thought that such variability reflects the need for the gene-for-gene hypothesis, in which a specific R protein recognizes a matching avirulent effector that can be highly variable in pathogen populations (Flor, 1971; Chen and Ronald, 2011). Because pathogens evolve rapidly, plant NLRs in a given population are under the pressure of diversifying to provide sufficient recognition-specificity for effectors, of which coevolution is best explained under the concept of an arms race between host and parasites (Holub, 2001; Borrelli et al., 2018). Such diversity, however, can result in a collateral conflict involving NLRs; when divergently evolved NLRs are found in combination with other immune components, one can miss-recognize the other and trigger autoimmunity (Chae et al., 2014).

In the past years, thanks to fine-scale genetics and well-annotated genomes, it has been made possible to identify hybrid necrosis triggering epistatic loci down to individual genes. The first causal link between hybrid necrosis and autoimmunity has been established in *A. thaliana* with the epistatic interaction between *DANGEROUS MIX1* (*DM1*) and *DANGEROUS MIX2* (*DM2*; Bomblies et al., 2007). Both *DM* genes are located in the polymorphic, multi-gene clusters encoding multiple NLRs, with *DM2* homologs having known isolate-specific resistance function to the adapted pathogen *Hyaloperonospora arabidopsidis* (*Hpa*; **Figure 1C**). Later, a systematic study carried out with over 6000 F_1 hybrids generated from 80 *A. thaliana* parental accessions corroborated the notion that hybrid necrosis is commonly mediated by negative epistasis between highly polymorphic components of the immune system, such as NLRs (Chae et al., 2014). This large-scale investigation provides a species- and genome-wide insight on which immune components are more prone to generate predisposed alleles for autoimmunity. Interestingly, not all NLR loci turned out to contribute to the generation of autoimmunity-risk alleles but there exist particular genome loci recurrently spawning the risk alleles (Chae et al., 2014). Among such hybrid necrosis hot spots in the *A. thaliana* genome, the *DM2* cluster is the most prominent one producing multiple hybrid necrosis alleles interacting with a broad range of loci, including other NLR-containing clusters and the loci encoding enzymes (Alcázar et al., 2010; Chae et al., 2014; Tran et al., 2017). For instance, hybrids between L*er* and Kas-2 accessions of *A. thaliana* display mild-F_1 hybrid necrosis with obvious phenotypes found in F_2 and later generations, and the causal BDM pair is an NLR encoded by the *DM2* locus from L*er* and the SRF3 RLK from Kas-2 (Alcázar et al., 2009, 2010). There also seems to be a loyal partnership between BDM epistatic loci: multiple allelic pairs of *DM6* and *DM7* have been discovered to cause distinct classes of hybrid necrosis (Chae et al., 2014; Barragan et al., 2019). It has also been revealed that deleterious interactions can occur between different alleles at a single locus, such as *DM8* and *DM9*, as illustrated in **Figure 1A** (right; Chae et al., 2014). Recent analyses on the complex NLR encoding loci, of which dataset had been much improved by long-read sequences compared to Illumina short read-mapping based NLR assembly, revealed that the *DM* loci are definitely characterized with a high propensity of genomic rearrangements, including asymmetric cluster expansion and contraction in the *A. thaliana* natural accessions (Van de Weyer et al., 2019; Jiao and Schneeberger, 2020; Lee and Chae, 2020). Thus, a high correlation between the *DM*

locus predisposition and structural variation involving copy number variations (CNVs) emerges as an obvious feature. Given that the causality often maps to a single gene in the *DM* multi-gene clusters, it is likely that the cluster expansion and contraction events promote to generate such deleterious alleles. Importantly, the large-scale hybrid necrosis survey in *A. thaliana* has not only provided the incidence rate of hybrid necrosis at about 2% but also established high-throughput platforms for identifying causal allelic variants under epistatic interaction. Once an allele frequency contributing to the same type of hybrid necrosis is determined under a large-scale crossing scheme, such as diallelic crosses, genome-wide association studies (GWAS) can be utilized to identify single-nucleotide polymorphisms (SNPs) tagging the causal variants (Barragan et al., 2019, 2020). It has been also found that geographic distances between two parental lines do not necessarily correlate with the chances of running into immune system incompatibilities, as was demonstrated by *DM1* and *DM2d* carriers, Uk-3/Uk-6 and Uk-1 coming from a small town in Germany, Umkirch (Mouchel et al., 2004; Bomblies et al., 2007).

In crops, a systematic study focused on autoimmune hybrids has not been carried out obviously due to its seemingly low economic and agricultural potential, but there are certainly a massive number of examples reported from breeding panels. In an interspecific hybrid of lettuce, a deleterious epistatic interaction involving *RPM1 INTERACTING PROTEIN4* (*RIN4*) and potentially an *R* gene has been identified to lead to a dosage-dependent hybrid necrosis (Jeuken et al., 2009). Later, the causal interacting *R* locus, *DM39*, was mapped to an NLR gene cluster (Christopoulou et al., 2015; Parra et al., 2016). The protein RIN4 is one of the most well studied immune components (guardee, see section Diversity of NLRs in Sequence and Function for details) in plants, of which integrity is being monitored by multiple *R* proteins. Importantly, the most extreme case of hybrid necrosis has been reported in cotton in which an NLR gene, *Le4* leads to F_1 interspecific hybrid lethality in the cross between *Gossypium barbadense* and *Gossypium hirsutum* (Deng et al., 2019). The relevance of these findings in crops lies in the fact that a functional correlation could be made between hybrid necrosis alleles and disease resistance traits in the field. One of the most important unanswered questions is whether hybrid necrosis alleles can arise through the course of enhancing disease resistance either from selective breeding or through evolution in the wild. This connection is to be best addressed when hybrid necrosis research is carried out in crops with well-defined pathosystems and available breeding history such as in rice.

In this review article, we overview recent progress in hybrid necrosis research focusing on genetic and molecular details. Although initial works were favorably discussed under speciation mechanism, current research outcomes are heavily detailed in inbreeding species for obvious practicality in the pursuit of identifying causal genes. With a strong link to the plant immune system, we take an initiative to extend our view of hybrid necrosis to study plant performance in hybrids under the equilibrium of growth and immunity. We also attempt to revisit the cases of rice genetic incompatibilities responsible for various deleterious phenotypes found in F_1 and F_2 hybrids to examine if any of the reported criteria falls under the definition of immune system incompatibility. We propose that hybrid necrosis as a result of immune system incompatibility illustrates different levels of balancing act between immunity and growth. Thus, the characterization of hybrid necrosis in crops can guide breeding efforts to equilibrate resistance and yield to produce elite hybrids. This strategy will be highly informative in crops relying on hybridization as a major breeding method such as in rice. To this end, we explain in detail the molecular mechanism governing the autoimmunity in hybrids and suggest a way to mitigate autoimmunity to simultaneously enhance growth and resistance in breeding lines. Finally, we present the possible application of reverse breeding, a breeding technique that can rapidly deliver different types of mapping populations useful for the study of hybrid necrosis and heterosis.

HYBRID NECROSIS AND HETEROSIS: A MATTER OF EXTREMES

Trade-off Between Immunity and Growth

The trade-off between immunity and growth in plants is thought to occur as a result of prioritizing resource allocation to either of the two processes. An optimized equilibrium in response to external or internal factors would be achieved to ensure plant fitness and health (Huot et al., 2014). Optimal immune responses certainly promote the survival of plants in the time of needs, such as to fend off pathogen attack. For instance, under a certain avirulent bacterial pathogen load, it has been demonstrated that *A. thaliana* plants carrying the matching NLR will initialize an acute immune response, also known as effector-triggered immunity (ETI). Despite the fact that such a response disposes infection sites *via* activating local cell death, known as the hypersensitive response (HR), these plants display overall enhanced fitness as compared to those that do not carry the corresponding NLR. Indeed, regarding lifetime fitness, resistant lines can significantly outperform susceptible individuals when both are infected with pathogens. When total silique length was used as a proxy for lifetime fitness, it has been demonstrated that resistant *A. thaliana* isolines carrying an allele of *RPS5* (RPS5+) NLR that confers resistance to the avirulent *Pseudomonas syringae* strain show enhanced values by 9.6–32% in overall fitness measurement compared to susceptible lines (RPS5−) under the semi-controlled glasshouse condition (Gao et al., 2009). These findings support the idea that robust resistance conferred by an NLR for a matching pathogen enhances fitness if plants are under pathogenic attack.

The fact that energetic resources will be consumed once ETI is activated can logically explain a growth penalty. The complexity of this trade-off, however, largely resides in the fact that a fitness cost is associated with resistance even in the absence of obvious pathogen load. The pioneering work from Bergelson's group provided strong quantitative evidence for this (Tian et al., 2003). The *RPM1* NLR is an *R* gene in *A. thaliana* that confers resistance against the *P. syringae* strain DC3000 carrying matching avirulence (Avr) effectors

(Dangl et al., 1992; Innes et al., 1993; Bisgrove et al., 1994). To examine the cost that *RPM1* resistance would exact, susceptible host lines were transformed with the *RPM1* resistant allele to create a total of four isogenic lines (*RPM1+*). When *RPM1+* lines were compared against susceptible lines (*RPM1−*) in a field trial where the avirulent strain was absent, *RPM1+* showed lower shoot biomass and an average of 9% seed-set reduction as compared to *RPM1−*. Interestingly, resistant and susceptible alleles of *RPM1* coexist in the global populations of *A. thaliana*, indicating that balancing selection acts on to maintain both alleles. The signature of balancing selection endorses the premise that *RPM1* confers both a fitness advantage and a fitness cost depending on the presence and absence of the matching pathogens (Tian et al., 2003). A similar experimental setup has shown that carriers of an NLR, *RPS5* (*RPS5+*), that confers resistance to *P. syringae* carrying *AvrPphB* or its homologs, suffer from a yield penalty in comparison to lines carrying the susceptibility allele (*RPS5−*; Karasov et al., 2014). Field trials performed with the *RPS5+* and *RPS5−* that were not exposed to *P. syringae* showed a reduction in the seed-set between 5 and 10.2% in the *RPS5+* lines in comparison to *RPS5−* lines. Supporting the fitness cost associated with *RPS5* resistance, a balanced polymorphism for *RPS5* has also been observed (Tian et al., 2002). In both cases, fitness costs associated with the NLRs led to about 9–10% decrease in seed production in the absence of obvious pathogen infection, and such costs are not likely to be explained merely by the metabolic cost of RPM1 and RPS5 synthesis. There can be alternative reasons for the observed fitness costs in both experimental setups. First, although the particular pathogen that is thought to trigger the HR was not found during the field trials, it is possible that other, yet unknown, effectors from several pathogen species can interact with RPS5 and RPM1, triggering a constitutive defense response that would negatively impact growth (Karasov et al., 2014). RPM1 in particular has been shown to be a common target of multiple effectors in laboratory conditions (Grant et al., 1995; Kapos et al., 2019; Ray et al., 2019). Such diffused *R-Avr* interactions, as opposed to Flor's gene-for-gene hypothesis, would also explain relatively high frequencies of RPM1+ and RPS5+ natural accessions of *A. thaliana* despite the cost (Stahl et al., 1999; Rose et al., 2012; Karasov et al., 2014). Second, RPM1 and RPS5 may negatively interact with other *R* genes in the introduced background (Tian et al., 2003). Such type of interaction could very well correspond to negative epistasis following the BDM model, but further research is required to confirm whether there are partners for *RPM1* or *RPS5* exerting mild autoimmunity. Intriguingly, *RIN4*, an important host partner of *RPM1*, has been identified as a hybrid necrosis gene in lettuce interacting potentially with an NLR gene, indicating the BDM epistasis may occur through a host protein that such costly NLRs surveil. Overall, these findings imply that the composition of *R* gene repertoire in a given population determines the cost exerted to affect overall fitness because a fraction of costly *R* genes would bring down yield even under seemingly non-pathogenic conditions. In addition, these results highlight the importance of controlling such costly *R* genes in the host germplasm.

Since a well-managed trade-off can reward breeding efforts, there have been substantial research endeavors to uncover regulatory mechanisms that coordinate growth and immune responses (Karasov et al., 2017). Immune responses supposedly divert substantial amounts of resources that otherwise would be invested in growth, and multiple hormonal pathways have been under investigation to establish the link between growth and immunity (Purrington, 2000; Huot et al., 2014). Several studies have pointed out that crosstalks between different hormonal pathways are instrumental in shifting the balance between growth and defense (Glazebrook, 2005). Generally, but not exclusively, it is considered that growth-promoting hormones, including cytokinins, brassinosteroids (BR), auxins and gibberellins (GA), act against defense-promoting hormones such as ethylene (ET), salicylic acid (SA), and jasmonates (JA; Liu et al., 2008; Tsuda et al., 2009; Clouse, 2011; Emenecker and Strader, 2020). However, direct molecular links between an individual or a suit of hormonal pathways and immune responses still remain to be investigated in-depth (An and Mou, 2011; Chen and Ronald, 2011; De Vleesschauwer et al., 2014; Lozano-Durán and Zipfel, 2015). A mechanistic insight about the trade-off involving NLR-mediated immunity has been provided by the work in rice, demonstrating functional dependency between the fungal resistance NLR *Pik-H4* and its interacting protein OsBIHD1 that encodes a homeodomain transcription factor (Liu et al., 2017). Physical interaction between Pik-H4 and OsBIHD1 is necessary for ETI, supporting the importance of OsBIHD1-mediated control in immunity. During infection, OsBIHD1 launches a growth-inhibiting process by activating reactions that catabolize the growth-promoting BR. OsBIHD1, in addition, facilitates ET synthesis, which in turn enhances the NLR-mediated immunity. Interestingly, not only the overexpression but also the knockout of *OsBIHD1* leads to dwarfism, suggesting that OsBIHD1 is a critical factor coordinating growth potential through the regulation of other growth-promoting hormones or processes (Liu et al., 2017). This work elegantly describes how a single factor is able to modulate ET-BR crosstalk in response to NLR-mediated defense and how such modulation affects plant growth. Engagement of such an important growth regulator into the NLR action during infection appears to be a way to effectively reallocate resources.

On the other hand, untimely or uncontrolled activation of immunity often results in a decrease in plant fitness. Indeed, numerous lesion-mimic mutants identified in *A. thaliana* and rice illustrate a skewed balance toward constitutive activation of immune responses, resulting in growth compromise typically expressed with dwarfism, leaf curling, stunted roots, and death (Heil and Baldwin, 2002; Zhu et al., 2020). Some of these lesion-mimic mutants in *A. thaliana* were further characterized to reveal the contribution of NLR to such autoimmunity (van Wersch et al., 2016). For instance, a gain-of-function mutation in one of the *DM1* homolog is named *SSI4* (*SUPPRESSOR OF SALICYLIC ACID INSENSITIVITY OF NPR1-5, 4*) characterized with obvious autoimmune symptoms (Shirano et al., 2002). The most well-characterized autoimmune mutant would be *snc1* (*suppressor of npr1-1, constitutive 1*) in *A. thaliana*,

which had been identified with its dominant allele suppressing defects of *npr1-1* plants in SA pathway regulation (Li et al., 2001; Zhang et al., 2003). Both dominant mutations fall in the domain of the two NLRs important for regulating its activity. Another lesion mimic mutant in *A. thaliana*, *acd11* clearly shows autoimmunity-related compromise in growth due to a mutation in the gene involved in sphingolipid modification (Brodersen et al., 2002). However, later it has been demonstrated that the cell-death observed in *acd11* can be suppressed by mutations in *LAZ2*, a gene that encodes a histone-methyltransferase, which in turn controls the expression of an NLR named *LAZ5* (Palma et al., 2010). Likewise, *ACD6*-mediated growth compromise commonly observed in naturally occurring autoimmune accessions has recently been shown to require particular natural alleles of *SNC1* (Todesco et al., 2010; Zhu et al., 2018). Similar contributions of *SNC1* and other NLR alleles had been observed to regulate the class of *BONZAI* mutants characterized by dwarf phenotypes (Hua et al., 2001; Yang and Hua, 2004; Li et al., 2009). The involvement of NLRs in these lesion-mimic mutants strongly suggest that cell death execution in these mutants likely is mediated by NLRs, and such mutants indeed reveal regulatory mechanisms tightly controlling NLR activity to balance the equilibrium with growth promotion.

The above-described findings hint us to assess NLR function in lesion-mimic mutants in other species. For instance, a rice lesion-mimic mutant collection includes *Spl26* and *spl17* exhibiting chlorosis and cell death, as well as conferring resistance to both fungal blast and bacterial blight (Wu et al., 2008). The penalty on plant fitness is high in these mutants such that homozygosity of the dominant lesion-mimic mutations prevents them from reaching maturity and that the trans-heterozygosity between them makes plants lethal (Wu et al., 2008). Due to the enhanced resistance conferred by these rice mutants, one can easily hypothesize a link to NLR-mediated strong immune responses as the cause for the severe autoimmunity in rice. It would be a rewarding research agenda to further investigate the lesion-mimic mutants in rice with beneficial field resistance traits, which will allow us to examine how such enhanced resistance is achieved and examine the relative importance of causal factors in the plant immune system. In rice, constitutive activation of defense signaling and growth inhibition can also be simultaneously mediated by WRKY transcription factors. Overexpressing lines of *OsWRKY31* show constitutive activation of defense-related genes and enhanced resistance to rice blast, however, growth penalty is observed in lateral root formation and elongation (Zhang et al., 2008). The growth-deficient phenotype has been attempted for rescue by the administration of exogenous auxin, but due to a reduced auxin sensitivity in this line the trial failed. The result suggests that OsWRKY31 could act in the signal transduction of both the auxin and defense responses (Zhang et al., 2008), offering promising grounds to investigate the role OsWRKY31 in growth and immunity. WRKY transcription factors are known for its general function in defense in plants such as OsWRKY03 acting upstream of NPR1 (Liu et al., 2005), WRKY70 in *A. thaliana* mediating crosstalk between JA and SA (Li et al., 2004), and most importantly, decoy WRKY domains integrated to NLRs (Cesari et al., 2014a; see NLR-IDs in section Mechanisms of NLR-Mediated Autoimmunity Responsible for Hybrid Necrosis for detail). An additional functional link between WRKY and NLRs in rice has been established with five rice NLR proteins (Piz-t, Pib, Pi36, Pit, and Pita) interacting with WRKY45 and WRKY66 (Liu et al., 2016). Thus, it seems that growth-affecting defense pathways known in rice shall be revisited to further refine their molecular details to reveal regulatory mechanisms for the trade-off between growth and immunity.

Yield Penalty Accompanying Disease Resistance Traits Reflects the Trade-off

In breeding panels, the above-mentioned trade-off has been conceptualized under yield penalties associated with disease resistance traits. Low yield, due to reduced grain weight and/or a general reduction in plant growth, reflects a skewed balance favoring the maintenance of resistance traits that would protect crops from pathogenic infection at the expense of yield (Ning et al., 2017). Rice yield has steadily increased since the early 1960s in Asian countries like China, Vietnam, Indonesia, and India (Qian et al., 2016). The success of hybridization programs and the identification of major quantitative trait loci (QTLs) for heterosis along with their systematic incorporation to the existing elite lines are certainly responsible for major increases in rice production (Qian et al., 2016). Despite breeding success in yield traits *per se*, sustaining high yield in the deployed lines is evidently facing problems due to pest management. Indeed, rice production is being seriously affected by several pathogens such as the insect brown planthopper (*Nilaparvata lugens*), rice blast fungus (*Magnaporthe oryzae*), rice bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*), rice bacterial leaf strike (*X. oryzae* pv. *oryzicola*), and Tungro virus (Azzam and Chancellor, 2002; Bottrell et al., 2012; Liu et al., 2014). Climate change is predicted to escalate yield losses as both disease resistance and yield traits are optimized for certain environmental conditions (Qian et al., 2016; Muehe et al., 2019). Thus, engineering robust and durable disease resistance traits with a minimized yield penalty in elite rice hybrids is an urgent matter.

Unfortunately, the catalog of field-effective *R* genes in rice rather remains short-listed. Although hundreds of QTLs for resistance against different pathogens across taxa have been identified in experimental populations, only a handful of *R* genes have been used for hybrid breeding (Liu et al., 2014; Qian et al., 2016; Li et al., 2019). For instance, among ~30 genes cloned to date, most of which encoding NLRs (Wang et al., 2017), only a limited number of loci, for example, the locus encoding *Pi9/Pi2/PigmR*, have been under frequent use due to their ability to confer durable and/or broad-spectrum resistance for rice blast isolates (Cesari and Kroj, 2017; Deng et al., 2017; Tian et al., 2019). Although recent reports have successfully identified over a hundred potential new *R* genes against different isolates of rice blast out of hundreds of cloned rice NLRs present in Tetep and other blast-resistant cultivars (Zhang et al., 2015; Wang et al., 2019c), introgression of the newly discovered in elite lines have not yet been reported. Given that *Xa2*, a favorable blight *R* gene with no obvious

yield penalty is of Tetep origin (Ogawa et al., 1991), genome-assisted *R* gene discovery from a cultivar with broad-spectrum resistance offers a promising path. In addition, *Xa4* and *Xa21*, along with *Xa2*, are non-NLR R proteins and characterized by their durable nature and least yield penalty (Ogawa et al., 1991; He et al., 2006; Kottapalli et al., 2010; Ning et al., 2017), suggesting potential routes to broaden our search for functional R proteins other than NLRs. On the other hand, achieving durable resistance for pathogens like rice blast is still challenging as resistance conferred by single *R* genes would often break down within 3–5 years due to rapid cycling of pathogens that outpace the host immune system adaptation (Devi et al., 2015). To broaden the spectrum and durability of blast resistance, several *R* genes have been combined in the same background (Wang et al., 2019c). Although the current endeavor focuses on pyramiding *R* genes to promote both broad-spectrum and durable resistance in commercial lines (Hu et al., 2012; Miah et al., 2013; Xiao et al., 2017; Sabar et al., 2019), pyramiding resistance genes for any of the major rice pathogens in a novel genetic background is expected to encounter negative epistasis between newly assembled *R* gene combinations to a certain extent (**Figure 1C**). In addition, usually each set of *R* genes has been cloned based on the specific resistance to a single pathogen species or even to an isolate (Flor, 1971). Furthermore, it is technically tedious and costly to incorporate several *R* genes in new breeding lines every few years (Li et al., 2019). The introgression of *R* genes from donor lines does not always result in enhanced resistance in the recipient background due to an interference in immunity from the existing immune components (Hurni et al., 2014; Stirnweis et al., 2014; Kim et al., 2019). Finally, only several *R* genes are known not to affect yield after introgression in the commercial lines (Ning and Wang, 2018).

Negative effects on yield born by *R* gene pyramiding in crops are well documented (Ning and Wang, 2018). For this reason, introgression of new *R* genes in rice elite varieties is very often accompanied by a thorough evaluation of possible yield penalties (Xiao et al., 2017). Due to the correlation observed between *R* gene pyramiding with a decrease in yield, it is tempting to speculate that rice elite varieties that produce high-yielding hybrids have not only accumulated heterotic traits but also dispensed a suit of costly or unnecessary *R* genes during the breeding. Therefore, it is plausible that these lines have been selected for *R* gene loci that had accumulated mutations abrogating or compromising the resistance function. Supporting this proposal, an impressive large-scale GWAS on rice heterosis revealed that breeding strategies for increasing yield inadvertently selected for susceptibility alleles for blast and leaf-blight resistance genes in rice. Huang et al. (2015) analyzed 1,495 elite hybrid and their respective inbred parental lines of *Oryza sativa* spp. *indica* and *O. sativa* ssp. *japonica* to investigate the genetic architecture governing heterosis in rice hybrids mainly bred in 1990s and 2000s. This study has identified major loci contributing to heterosis with large effects on certain traits, including disease resistance and grain quality together with multiple small-effect loci for grain yield, thus presenting a comprehensive view on trait fixation during domestication. One of the most striking results from this work regarding the link between immunity and growth is that high yield in hybrids is associated with the accumulation of susceptibility alleles. In the evaluated hybrids, the frequency of the resistant alleles for the locus *Pi2/Pi9* for rice blast and a locus associated with resistance to leaf-blight in chromosome 6 was 3.7 and 2.6%, respectively, which implies that the accumulation of susceptibility alleles had resulted in positive effects on yield production (Huang et al., 2015).

Accumulating susceptibility alleles on known *R* genes for rice blast and leaf-blight during breeding suggests that there had been a selection pressure favoring susceptibility alleles at those loci. Potentially, the absence or low number of avirulent strains for these resistances as well as fitness cost innate in such alleles might have rendered the resistant carrier generating low performing hybrids than the ones carrying a susceptible allele when used as parents. Unlike natural populations where such resistant and susceptible alleles are being maintained by balancing selection due to stochastic selective pressure imposed by pathogens (Tian et al., 2003; Karasov et al., 2014), selective breeding would favor seemingly higher-yielding ones at the expense of resistant alleles. These examples suggest that the trade-off between growth and immunity had played a critical role in directing the *R* gene repertoire in crops to be streamlined only for an immediate purpose. The artificial selection against resistant alleles of no immediate benefits would make the elite germplasms susceptible to diseases that can be easily resisted by their wild relatives. Less diversity in *R* genes in a population would increase the vulnerability to new pathogens, while the introduction of a new immune component such as an *R* gene becomes cumbersome. Problems in readapting *R* genes from wild relatives in the introgressed cultivated lines are evident, which caught not only breeders attention but also researchers who investigated complexity in the plant immune system. The first scientific report for a clashing immune component with the introgressed *R* gene appeared with the identification of Rcr3, an important immune papain-like cysteine endoprotease in tomato, that conflicted with the *Cf-2 R* gene from wild tomato *Lycopersicon pimpinellifolium* (Krüger et al., 2002). Negative epistasis clearly explains the incompatibility between *Rcr3* and *Cf-2*, triggering uncontrolled cell death and compromise in growth. The fine genetic analysis revealing epistasis in tomato made a foundation for hybrid necrosis studies to be established under the BDM model.

Hybrid Necrosis Exemplifies a Trade-off Between Immunity and Growth

An obvious trend observed for hybrid necrosis cases in *A. thaliana* and other species is that uncontrolled immune responses negatively impacts on overall plant performance. The effects could range from mild compromise in growth to severe tissue necrosis and distortion in developmental programming (Alcázar et al., 2009; Jeuken et al., 2009; Yamamoto et al., 2010; Chae et al., 2014; Todesco et al., 2014; Sicard et al., 2015; Deng et al., 2019). In most hybrid necrosis cases, plants do not reach maturity to produce seeds. The characterization of overt hybrid necrosis in multiple plant species thus faces challenges in obtaining viable

seeds to generate mapping populations unless advanced crossing schemes are engaged. Nonetheless, genetic architecture and molecular mechanisms underlying hybrid necrosis have been discovered since the detrimental autoimmune symptoms are temperature sensitive; changes in temperature can drastically suppress the symptoms and F_2 progeny becomes available (Bomblies et al., 2007; Jeuken et al., 2009; Muralidharan et al., 2014). Our current understanding on the link between uncontrolled immunity and developmental defects and growth compromise still remains to be descriptive and further molecular characterization to define the connection is in need. Considering that the major mechanism underlying hybrid necrosis has been unequivocally attributed to autoimmunity only in recent years, it is very likely that the incidence of hybrid necrosis cases in rice and other crop species have been so far overlooked. The lettuce *RIN4* is an example of the discovery of epistasis upon revisiting the previous QTL mapping results for disease resistance traits under scrutiny (Jeuken et al., 2009). Therefore, to estimate the frequency of hybrid necrosis in crops, it would be necessary to reassess the data available from the literature and examine the cases of deleterious phenotypes observed in F_1 and F_2 hybrids to address how many of those could be contributed by immune genes and thus categorized under hybrid necrosis stemming from negative epistatic loci.

Deleterious phenotypes in rice hybrids have been reported as early as in 1957 by Oka, who described two different phenomena: hybrid inviability in the F_1 generation and hybrid breakdown in F_2. Oka established that dominant alleles cause plant death (Oka, 1957), which essentially conveys the concept of an allelic interaction following deleterious epistasis. In addition, he demonstrated that the genetic cause of the F_2 hybrid breakdown was due to a set of duplicated genes in which the combination of recessive alleles leads to poor growth, proving a classic Michael Lynch's prediction of hybrid incompatibilities arising from the combination of duplicate genes that had followed the independent fate (Lynch and Conery, 2000). Hybrid incompatibility due to negative epistasis between duplicated copies of the histidinol-phosphate aminotransferase gene which codes for HPA, a protein important in the biosynthesis pathway of histidine, has also been reported in *A. thaliana* (Bikard et al., 2009). The framework of hybrid weakness in rice prompted researchers in the following years to pursue more cases and characterize pairs of loci responsible for the range of hybrid weakness phenotypes (**Table 1**). Such research endeavors have identified some of the incompatibility loci to be mapped or linked to the *R* gene family (**Table 1**). For instance, *Hw4* was found to induce hybrid weakness when interacting with *Hw3*, which encodes for a putative calmodulin-binding protein (CaMBPs). We found it intriguing as CaMBPs are involved, among other processes, in plant defense mechanisms (Ali et al., 2003; Fu et al., 2013). Interestingly, further linkage-mapping analysis defined that *Hw3* was linked to *Pi1*, an allele of the *Pika* locus that confers resistance to blast, although it turned out that *Pi1* and *Hw3* was not the same gene (Hua et al., 2012; Fu et al., 2013). These findings show that *Hw3* and *Hw4* are both the causal genes of the hybrid weakness phenotype observed in rice, although the role played by components of the immune system in this genetic background and by the linkage between *Hw3* and the *R* gene is yet to be investigated. Moreover, the work of Yamamoto et al. (2007, 2010) has undoubtedly established the correlation between hybrid necrosis and autoimmunity in an *indica-japonica* F_2 rice hybrid. Indeed, this particular genetic incompatibility case was investigated under the BDM-epistasis model, revealing that the interaction between two recessive genes, *hbd2* that codes for a casein kinase I (CKI1) and *hbd3* that is mapped to an NLR gene cluster region, is responsible for the reduced height and number of tillers in the autoimmune lines (Yamamoto et al., 2010). Interestingly, the deleterious allele effect of *hbd2* has been attributed to one amino acid change, indicating that a reproductive barrier between varieties of rice can be easily established due to autoimmunity (Yamamoto et al., 2010).

The number of underperforming hybrids described in the literature (**Table 1**) indicates that this phenomenon has an appreciable incidence in rice, which requires further attention for various reasons. First, we cannot exclude the possibility that hybrid necrosis has played a role in the observed underperformance. During rice domestication, disease resistance traits have been primarily selected within breeding panels of a region. This suggests that an imminent local pathogen load may have imposed a strong pressure selecting for a robust *R* gene in the bred lines. As has been demonstrated in hybrid necrosis cases, a new combination of *R* repertoire could result in underperforming hybrids, which may have not yet been rigorously classified under the hybrid necrosis category. As genetic architecture is simple, cases under BDM epistasis would easily generate lists of causal loci. In addition, it has been shown that in permissive or close-to-permissive temperature ranges the inferior hybrid phenotypes are alleviated (Saito et al., 2007; Fu et al., 2013; Chen et al., 2014; Muralidharan et al., 2014). Therefore, the characterization of underperforming hybrids under different environmental conditions and genetic identification of causality are relevant for improving hybrid performance in general. Finally, hybrid necrosis, not only severe but also mild ones, would preclude the expression of heterotic phenotypes. We believe that cryptic heterosis can be easily released if deleterious effects from a degree of autoimmunity are managed. We speculate that the negative correlation between immunity and plant performance, in particular growth and yield, is an important bottleneck for achieving heterosis in hybrid rice, which shall be extensively investigated with the underperforming hybrids. Not all underperformance would be attributed to the trade-off between growth and immunity, but addressing the proportional contribution made by elevated immunity to a decrease in growth can be effectively carried out using such rice hybrid germplasms. For this reason, we propose that the heterotic expression of agricultural traits can be improved when a degree of autoimmunity triggered by immune system conflicts is properly defined and mitigated.

Fitness Equilibrium Model in Plants: Anti-hybrid Necrosis as Part of Heterosis?

The idea of mitigating autoimmunity in hybrids to increase overall fitness posits that the equilibrium between growth and immunity is an important factor determining plant performances.

TABLE 1 | Summary of hybrid weakness genetics in rice.

Hybrid	Phenotype	F	Genetics of the incompatibility	Causal gene	Reference
Intersubspecific *indica* × *japonica* hybrids	Sterility	F_1	Intra-allelic and intra-genic interaction at the *S5* locus, which encodes 3 tightly linked genes.	*S5* encodes an Aspartic protease, potentially involved in disease resistance signaling and programmed cell death in reproductive tissues.	Yanagihara et al., 1995; Chen et al., 2008; Yang et al., 2012
Teqing × *O. rufipogon Griff*. hybrids	Autoimmunity. Interrupted root formation and impaired growth.	F_1	Interaction *Hwi1* and *Hwi2*	*Hwi1* comprises 2 LRR-RLK genes, *25L1* and *25L2*. *Hwi2* encodes a putative subtilisin-like protease.	Chen et al., 2013, 2014
Intraspecific *indica* hybrids Taifeng A × V1134	Retarded growth, reduced panicle number, pale green leaves with chlorotic spots.	F_1 F_2	Two dominant loci: *Hw3* (From V113) and *Hw4* (from Taifeng)	*Hw3* encodes a putative calmodulin-binding protein.	Fu et al., 2013
Nipponbare × Jamaica hybrids	Compromised root growth (severe hybrid necrosis), difficulties maintaining root apical meristem (RAM), and shoot apical meristem (SAM).	F_1	Interaction between *Hwc2* and *Hwc1*, with *Hwc1* being semi-dominant and having a stronger dosage effect than *Hwc2*.	*Hwc1* (possible candidates are orthologs to *LEUNIG* or *STYLOSA Arabidopsis thaliana* genes)	Ichitani et al., 2007; Saito et al., 2007; Kuboyama et al., 2009
	Rolled leaves and a short stature. Arrested leave development in embryos. Dwarfism, delayed flowering.	F_2		Resistance genes linked to *Hwc2 locus: Pikahei-1(t), Pi39(t), Xa1, Xa2, Gm7*.	
Intersubspecific hybrids Sasanishiki (*japonica*) × Habataki (*indica*).	Reduction in the number of panicles per plant and in culm length	F_2	Set of complementary recessive genes, *hbd2* and *hbd3*	N/A	Matsubara et al., 2007
Intersubspecific hybrids Koshihikari (*japonica*) × Habataki (*indica*)	Reduced growth, reduced number of tillers.	F_2	Interaction between two recessive genes, *hbd2* and *hbd3*	*hbd2* (*deleterious CKI1*) and *hbd3* (mapped to NLR cluster region)	Yamamoto et al., 2007, 2010
Intraspecific *japonica* hybrids J-321 × J-147	Chlorosis and plant death	F_2 F_3	Two duplicated recessive loci: *HCA1* and *HCA2*	N/A	Sato and Morishima, 1988; Ichitani et al., 2012
Reciprocal hybrids of *Oryza perennis* (*barthii*) × related *Oryza* spp.	Embryo lethality, compromised growth of survivals.	F_1	Dominant lethal alleles at two loci	N/A	Chu and Oka, 1970

(Continued)

TABLE 1 | Continued

Hybrid	Phenotype	F	Genetics of the incompatibility	Causal gene	Reference
Intraspecific *Indica* hybrids	Chlorosis, arrested root growth and development.	F_2	Recessive alleles at one locus or two loci.	N/A	Oka, 1957; Ichitani et al., 2011; Shiragaki et al., 2019
	Cell death, chlorosis, growth termination.	F_1	Interaction between weakness-causing alleles of *HWA-1* and *HWA-2*.		
Interspecific hybrids Koshihikari (*japonica*) × wild species *Oryza nivara*	Compromised growth and die before heading.	F_2	Locus *hbd1(t)* from *O. nivara* interacting with other locus/loci in Koshikari background.	N/A	Miura et al., 2008
Intersubspecific hybrids Asominori (*indica*) × IR24 (*japonica*)	Sterility	F_2	Epistasis between three unlinked loci *Hsa1*, *Hsa2*, *Hsa3*. *Hsa1 indica* locus containing *Hsa1a* and *Hsa1b* in homozygosity causes female sterility in *japonica*.	N/A	Kubo and Yoshimura, 2005; Kubo et al., 2016
Intersubspecific hybrids IR24 (*japonica*) × Asominori (*indica*)	Poor growth, complete sterility	F_2	Duplicated recessive genes *Hwe1* and *Hwe2*. A rice blast resistance gene is located near *hwe1*.	N/A	Kubo and Yoshimura, 2002
Intraspecific hybrids Sanghaehyanghe olua (*japonica*) × Aranghyangchalb yeo (*japonica*)	Short, chlorotic plants with wilted leaves. Slow growth.	F_1	Possible interaction between *Hwc1*, *Hwc2*, and *Hwc3*. *Hwc3* is cloned from Aranghyangchalbyeo. Sanghaehyangheolua carried *Hwc1*.	*Hwc3* encodes a LRR-containing protein with sequence similarity to *Xa1*, an NLR gene.	Yoshimura et al., 1998; Ichitani et al., 2007; Nadir et al., 2019
Intersubspecific *indica-japonica* hybrids "Tachisugata" and "Hokuriku 193"	Reduced growth	F_2	Epistasis between two recessive alleles *hbd4*, carried by Tachisugata and *hbd5* carried by Hokuriku 193.	N/A	Matsubara et al., 2014
Intersubspecific *japonica-indica* reciprocal hybrids of Sasanishiki and other *japonica* cultivars and Col.no.15	Reduced growth and number of tillers	F_2	*hwd1* (*Col.no.15 indica*) and *hwd2* (Sasanishiki-*japonica*) as double-recessive homozygous or homozygous for one locus and heterozygous for the second locus.	N/A	Okuno, 1985; Fukuoka et al., 1998; Okuno and Fukuoka, 1999

How such an equilibrium can be regulated and how the overall immunity of an individual limit the manifestation of heterotic traits remains to be determined. One might easily question if dampening residual or basal immunity level by systematically knocking down prominent immune receptor genes would indeed increase plant performances and yield under no obvious pathogenic loads. Such a systematic approach has not been embarked on; however, it appears that nature has embraced the needs to evolve certain microRNAs that can target multiple NLRs at a time to reduce NLR activity by and large (Zhai et al., 2011; Shivaprasad et al., 2012; Zhang et al., 2016). In addition, the expression of a cohort of NLRs clustered in a multigene cluster can be coregulated through the action of small-RNAs produced within the cluster (Yi and Richards, 2007). The complexity of such regulatory mechanisms as well as the genome studies focused on NLR evolution in different species at least provides us with the breadth and intricacy of NLR diversity present in natural populations (Van de Weyer et al., 2019; Lee and Chae, 2020; Seong et al., 2020). Although cataloging NLRs present in a species would not pinpoint which NLRs and how many of them are cost-exacting or predisposed to trigger autoimmunity in hybrids, detailed analysis of the pan-NLRome might inform us which of them had undergone either positive or negative selection and are associated with other traits than disease resistance, such as yield. Due to the poor-mapping quality of short reads, GWAS studies or other SNP-based genome-wide scans certainly have missed information covering complex NLR loci, often tandemly repeated with a massive structural variation. An improved assembly by whole genome sequencing with longer reads as well as the targeted assembly of NLR genes will greatly improve the resolution of trait mapping and genotype-phenotype association. In this regard, rice hybrids on the other hand offer a great system to investigate the potential contribution of hybrid incompatibilities of the plant immune system to limit the expression of heterosis. The exceptional amount of field-collected phenotype data in rice are not comparable to those existing in any other species, which will become a crucial component in the attempts to associate the phenome to immune-centered genotype platforms in rice.

Here, we illustrate an equilibrium model that represents different manifestations of the trade-off between growth and immunity in natural populations, crop inbred lines, and elite hybrids on the basis of genetic trait distribution for disease resistance and heterosis (**Figure 2**). We propose that hybrid necrosis and heterosis lie in the extreme end of such equilibrium as the result of disproportionate phenotypic manifestations of uncontrolled immunity and exacerbated growth, respectively. As explained above, the genetic architecture regarding the *R* repertoire affects plant fitness both in the presence and absence of pathogens. In addition, the accumulation of heterotic alleles corresponds to an increase in growth and yield, as long as they contain an optimum *R* repertoire that does not cause yield penalty. In a given natural population (gray) where multiple types of immune alleles are being maintained, we envision that the fitness level of individuals in the population would show a wide distribution with a peak that reaches the point optimally balanced with the two opposing forces, immunity and growth (**Figure 2**). Under a pathogen attack, immune responses in the natural population are activated only among the individuals carrying the cognate immune receptors that can mount robust immunity. On the other hand, non-carriers would suffer serious disease symptoms which eventually decrease the allele frequency of the susceptibility alleles in the population. However, when the pathogenic load is low or absent, the discrepancy in allele frequencies between the carriers and non-carriers becomes rebalanced because the fitness cost in the carriers would render them as unfavorable ones over the non-carriers (Tian et al., 2003; Karasov et al., 2014). For instance, stable natural populations of *A. thaliana* would experience bouts of such fluctuating events given that pathogenic loads in nature are believed to be stochastic (Karasov et al., 2018). In our model, as the immunity and growth traits are simplified to manifest as antagonistic forces, the high genetic diversity in the immune system will result in high diversity in growth traits. Thus, compared to selectively bred materials, natural populations would show the widest genetic trait distribution (gray; **Figure 2**). On the other hand, in the inbreeding crop parents (yellow) that are developed to generate elite hybrids, the peak of equilibrium shifts toward favoring growth as selective breeding has accumulated heterosis alleles (**Figure 2**). Some inbred lines could even reach a point exceeding the fitness equilibrium, resulting in enhanced resistance for certain pathogens yet with a minimized yield penalty. This has been achieved through domestication efforts that had largely reconstructed *R* gene repertoires in the genomes (Stein et al., 2018) and through breeding strategies successfully pyramiding a few *R* genes with the least yield penalty (Xiao et al., 2017; Chen et al., 2020). Under the breeding goal of enhancing yield and resistance in hybrids, *R* genes in inbred lines predisposed to show extreme deleterious phenotypes in later generations (i.e., hybrid necrosis) should have been eliminated or largely reduced. Additionally, a common breeding practice had favorably used only a small number of *R* genes which show broad and/or durable resistance for dominant field pathogens (Li et al., 2019). Thus, inbred individuals would show a much narrower distribution for resistant and heterotic traits as compared to ones found in natural populations (**Figure 2**). Hybridization can easily disrupt the fitness equilibrium to a large extent due to the presence of novel allelic combinations and genetic interactions (**Figure 1**) that often leads to antagonistic events: hybrid necrosis (red) or heterosis (blue; **Figure 2**). Elite hybrids (purple) would occupy space in this equilibrium reaching toward far right with the expression of maximum heterotic traits and of least number of resistance traits only suited for a purpose. Maximization of heterotic traits has been shown to be achieved due to the concomitant accumulation of heterotic and/or susceptible alleles in rice breeding (Huang et al., 2015, 2016; Lin et al., 2020). Therefore, this group is skewed toward increased growth and yield reaching a much higher level for optimum fitness if we consider that those plants are grown under controlled environments in the absence of pathogens or in the presence of only a known pathogen that can be resisted (**Figure 2**).

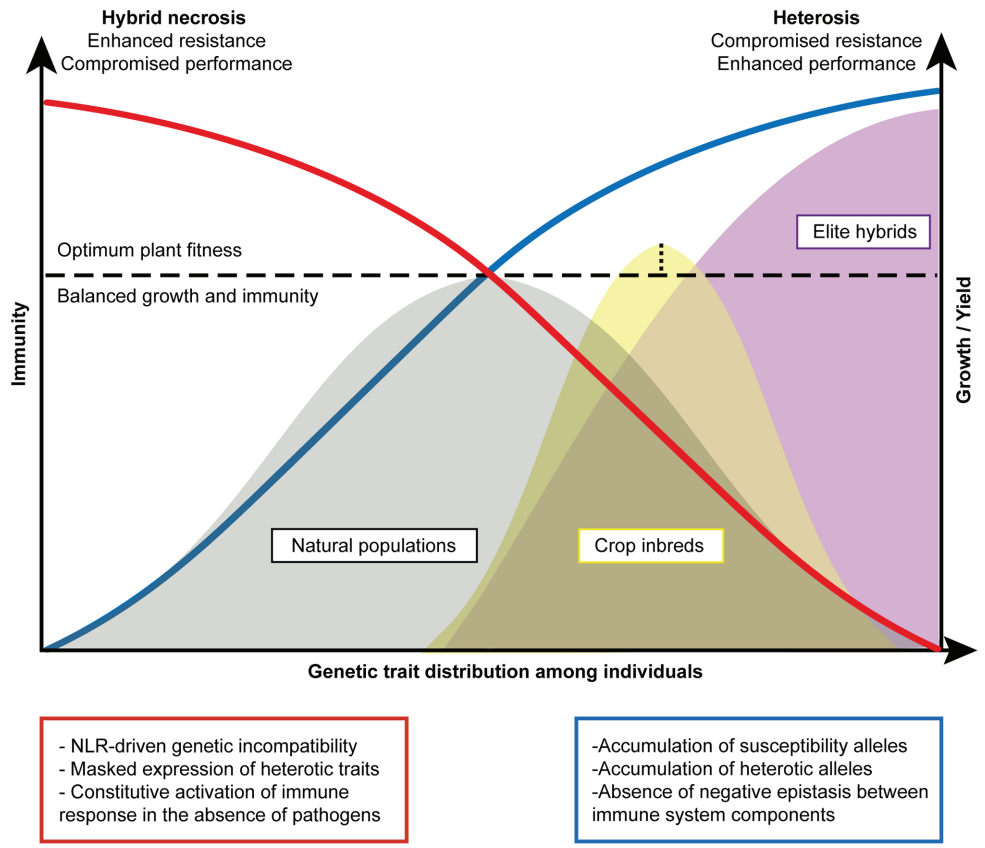


FIGURE 2 | Phenotypic expression of hybrid necrosis vs. heterosis. The model represents an equilibrium between immunity and growth and/or yield achieved essentially to ensure optimum plant fitness (horizontal dotted line). The expression of two extreme phenotypes, heterosis (blue) and hybrid necrosis (red), results from drastic disruption of the equilibrium. The optimum fitness equilibrium is predominantly reached by natural populations (gray area) with robust immune systems characterized by a broad and controlled immune response to biotic stresses. The total frequency of resistant alleles in the natural population potential limits overall growth and yield as compared to commercial lines. Crop inbred lines (yellow area) show a disproportioned accumulation of heterotic alleles. In optimal growth conditions, the fitness of these lines may be superior to the one reached by natural populations (vertical dotted line). Predominant accumulation of heterotic traits shifts the distribution of these lines toward the right, prioritizing the expression of heterosis over a robust immune system. Elite hybrids (purple area) show the highest accumulation of heterotic alleles accompanied by the accumulation of susceptibility alleles for blast rice and leaf-blight (Huang et al., 2015). The performance for elite hybrids can surpass the optimum fitness point under controlled conditions. Nonetheless, in three scenarios, the expression of heterosis may be compromised being: (i) hybrid necrosis, (ii) introduction of *R* genes to the cost of yield, and (iii) susceptibility to pathogen attack.

Looking at the bigger picture, however, losses in rice production worldwide are certainly caused by a generalized compromise in immunity in elite hybrids, as no one can predict the next wave of pathogenic attack. We then propose that elite hybrids display an underperforming *R* gene repertoire with insufficient diversity to face a wide range of pathogenic strains. Such underrepresentation of optimum *R* repertoires in elite hybrids can be explained by the trade-off favoring growth and selection against hybrid necrosis alleles that might have great resistance potential.

MOLECULAR MECHANISMS UNDERLYING HYBRID NECROSIS

One of the breeding goals in crops lies in broadening the spectrum of disease resistance. Understanding how plants have evolved the immune system to defend multiple pathogens is the first step to deploy different disease resistance traits into breeding panels. Although phytopathogens utilize diverse infection strategies, plants employ seemingly common yet complex immune pathways for defense (Mukhtar et al., 2011; Dou and Zhou, 2012; Weßling et al., 2014; Zhou and Zhang, 2020). The dedicated immune network is featured with prominent classes of immune receptors that perceive invasive events and transduce immune signaling. Coevolution between plants and pathogens drives the diversification of plant immune networks that immune receptors participate in; the system found in a given plant individual is supposedly optimized for its own, but not always function properly when combined in hybrids (Bomblies and Weigel, 2007; Chae et al., 2016). Hybrid necrosis is an extreme outcome of the immune system dysfunction resulting from newly combined immune components independently evolved from each parental lineage.

In this section, we summarize recent advances that revealed the mechanism of plant immune receptor action and explain how such mechanistic understanding rationalizes the action of NLR variants predisposed for hybrid necrosis. From the molecular framework, we propose how uncontrolled autoimmune signaling could be derived due to the combination of incompatible immune components involving NLRs. Our mechanistic view on the NLR-based autoimmunity provides a strategy to optimize the growth-immunity equilibrium toward breeding elite hybrids.

Diversity of NLRs in Sequence and Function

Faced with constant threats from pathogens, plants use a suit of membrane-bound and intracellular receptors to initiate defense signaling pathways to confer disease resistance (Kourelis and van der Hoorn, 2018). Most cell surface receptors are pattern-recognition receptors (PRRs), which include RLKs and RLPs. RLKs contain an extracellular LRR domain, a single-pass transmembrane (TM) domain, and an intracellular kinase domain. RLPs are structurally similar to RLKs except they do not contain a kinase domain (Boutrot and Zipfel, 2017). They could perceive the conserved non-self molecules, termed as pathogen-associated molecular patterns (PAMPs) or self-molecule damage-associated molecular patterns (DAMPs), and activate PAMP-triggered immunity (PTI; Boller and Felix, 2009; Boutrot and Zipfel, 2017). Some pathogens could yet evade from the PTI-based host immunity by secreting effectors that promote pathogenic virulence by suppressing immune perception or modifying host physiology (Toruño et al., 2016). NLRs are the family of immune receptors that sense the effectors or effector-driven modifications in the plant cell to initiate an acute immune response, ETI. Reflecting the effectiveness of ETI, NLRs constitute a major fraction of R proteins discovered to date across plant species (Cui et al., 2015; Kourelis and van der Hoorn, 2018). Depending on whether an effector and the matching NLR physically associate, the NLR recognition strategy could be divided into "direct" and "indirect" modes. The indirect recognition mode could be further refined to engage a third protein that can be classified either as "guard" or "decoy" in the plant cell (Van der Biezen and Jones, 1998; Dangl and Jones, 2001; van der Hoorn and Kamoun, 2008; Cesari, 2018). Homeostasis in the plant cell is gauged by the status of such host proteins, for which the matching NLRs monitor. Those host proteins become effector-targets upon infection, and NLRs sense such events to trigger ETI. Here, decoys are considered to have been generated to mimic guardees that have important immune functions. Under arms race between rapidly evolving pathogen effectors and host proteins, plants seem to have widened the net to catch the effectors by duplicating effector targets as decoys that in essence have no other roles affecting plant fitness (van der Hoorn and Kamoun, 2008; Ilyas et al., 2015).

NLRs are multidomain proteins with high diversity in structure and sequence. Based on the N-terminal structure, plant NLRs could be divided into three groups; substantial numbers of NLRs, in particular in eudicots, contain the Toll/Interleukin-1 receptor (TIR) domain at their N-terminus, termed as TIR-NLR or TNL in short; NLRs carrying predicted coiled-coil (CC) domains, albeit more variable than TIR in domain definition, are named CC-NLR or CNL; a less-abundant NLR group carries a non-canonical CC that bears similarity to RESISTANCE TO POWDERY MILDEW8 (RPW8) and named as RPW8-NLR or RNL (Shao et al., 2016). The small RPW8 proteins themselves serve as atypical non-NLR R proteins for conferring broad-spectrum resistance to powdery mildew, while some RPW8 domains experimentally cloned out from RNLs have been demonstrated to trigger cell death on its own when expressed in *Nicotiana benthamiana* (Xiao et al., 2001; Collier et al., 2011). Interestingly, one of the hybrid necrosis *DM* loci, *DM7*, encodes multiple members of RPW8s and HOMOLOG OF RPW8s (HRs), highlighting their connection to cell death activity (Barragan et al., 2019). The central part of NLRs is occupied by the nucleotide-binding and Apaf-1, R protein and CED4 homology (NB-ARC) domain followed by the C-terminal leucine-rich repeat (LRR) domain. The NB-ARC domain offers a conserved nucleotide-binding pocket, while LRR plays an essential role in effector recognition and features the highest sequence variability compared to other domains (Collier and Moffett, 2009; Takken and Goverse, 2012; Cesari, 2018; Wang et al., 2019a,b). The modular nature in domain structure, as well as high variability in sequence and copy number, are the key NLR features which presumably promote its versatility in the plant immune system.

Beyond a classical role of NLRs conferring a strain-specific disease resistance, recent findings highlight functional diversity of NLRs in the plant immune system. It is apparent that some NLRs form a pair, commonly encoded in head-to-head orientation in the genome, and require each other to fulfill resistance functions (Sinapidou et al., 2004; Narusaka et al., 2009; Okuyama et al., 2011; van Wersch and Li, 2019). Another group of NLRs assists immune signaling initiated from other NLRs, which are named as helpers. Well-characterized helpers in *N. benthamiana* and *A. thaliana* belong to RNLs (Peart et al., 2005; Bonardi et al., 2011; Collier et al., 2011; Castel et al., 2019), while helpers found in multiple Solanaceae species belong to a clade of CNL (Wu et al., 2016, 2017). Diversity in NLR function is likely to reflect the evolutionary processes followed by the massive expansion of NLRs in the plant lineage. Indeed, a recent pan-NLRome analysis carried out in *A. thaliana* has shown that distinct patterns of sequence polymorphisms exist for the different functional NLR groups (Van de Weyer et al., 2019). Paired NLRs evolve as one functional unit, while classical NLRs conferring resistance to adapted pathogens show the highest sequence diversity in populations, indicating diversifying forces imposed on such NLRs. On the other hand, helpers remain relatively conserved, meeting the needs to work with multiple sensors. A surprising versatility of NLR is also found in its modularity incorporating extra domains into its classical tripartite domains. In particular, domains found in decoys are integrated as part of NLRs as a single translation unit. This type of NLR is called NLR-ID with ID standing for the "integrated decoy" (Cesari et al., 2014a; Wu et al., 2015). Decoy domains of NLR-IDs are known to readily sense the effector presence through physical association, and thus NLR-IDs are considered as sensor NLRs

(Cesari, 2018; Adachi et al., 2019). The concept of the sensor has been elaborated through the identification of functionally dependent paired NLRs in rice. Rice NLRs, the RGA4/RGA5 and Pi5-1/Pi5-2 pairs require each other to confer resistance to rice blast fungus *M. oryzae* through one NLR of the pair, the sensor, which directly binds with the matching effectors through its ID (Lee et al., 2009; Okuyama et al., 2011; Cesari et al., 2013, 2014b). Functional dependency is explained by their ability of forming a heterodimer, in which effector sensing through the ID in the sensor NLR instigates the other to activate ETI to constrain rice blast growth (Cesari et al., 2014b; **Figure 3A**, middle). In this partnership, the matching NLR to the sensor is considered as an executor NLR due to its ability to transduce immune signaling leading to the HR, of which action appears to be under a negative control of the partnering sensor in the absence of pathogenic effectors. Up to date, a handful of NLR pairs are found under the same working mechanism: in *A. thaliana* RPS4/RRS1, CHS3/CSA1 NLRs are characterized as pairs with one NLR as a sensor with ID and the other as an executor (Cesari et al., 2014b; Huh et al., 2017; Ma et al., 2018). Although the initially characterized NLR-IDs haven been discovered by their head-to-head configuration in the genomic loci, later systematic search for NLR-IDs across plant genomes revealed that such configuration is not always the case, implying that a gained functional dependency might have promoted a genomic relocation of non-paralogous NLRs to be placed in tandem under linkage to facilitate coevolution of the two.

Sometimes, NLRs residing within a cluster can impede the signaling capacity of the paralogous NLRs. One example is the *Pigm* multigene cluster in rice that has been utilized for conferring broad-spectrum resistance to *M. oryzae* in many regions in China over the decades of breeding (Deng et al., 2017). The enhanced *R* trait is conferred by *PigmR* encoding a CNL, while one of the closely related paralogs *PigmS* in the same cluster opposes the *PigmR*'s function in a tissue-specific manner. As being a strong *R*, *PigmR* shows clear yield penalty as its uncontrolled function in the absence of antagonizing *PigmS* could cause a reduction in grain weight and size, potentially through autoimmunity. It has been shown that PigmS and PigmR could form a heterocomplex through their CC domains and thus it is plausible that the heterocomplex formation might inhibit the formation of PigmR homo-signaling complexes required for strong immunity. In the extant high-yielding rice lines, the *PigmS* promoter is highly methylated and the expression is suppressed in foliar tissues but not in the pollen. The tissue-specific expression would condition the trade-off to be balanced differently. In the vegetative tissue, PigmR homo-complexes would form to confer broad-spectrum resistance to *M. oryzae* when infected or to exert some degree of yield penalty when not infected. However, the net yield penalty is much alleviated as the epigenetic repression of the yield-saving *PigmS* expression is released in the pollen: supposedly PigmR/PigmS heterocomplex would reduce *PigmR*-mediated detrimental effects in the pollen, such as cell death or residual autoimmunity, and healthy pollen improve grain quality. This is a great example of how epigenetic regulation, potentially selected by rice breeding, contributes to achieve a balance between resistance and yield (Cesari and Kroj, 2017; Deng et al., 2017). While antagonistic paralogs could positively regulate the trade-off through tissue specificity, it can also hinder efforts to pyramid paralogous NLRs. When an introgressed paralogous NLR find its partner in the recipient germplasms to form a non-functional heteromeric NLR complex, the desired immunity from the introgressed *R* traits would be suppressed (**Figure 3A**, right), of which molecular examples are reported in wheat breeding (Hurni et al., 2014; Stirnweis et al., 2014).

Contrary to NLRs requiring or assisting others for full functionality, there are NLRs self-sufficient to complete its function without the need of other NLRs (Adachi et al., 2019). ZAR1 in *A. thaliana* is the most well-characterized NLR of which structure is recently resolved to reveal successive events accompanying NLR activation and signaling complex formation (Wang et al., 2019a,b). The ZAR1 structure revealed by cryo-EM explains how the previously postulated "on" and "off" status influence NLR activity and signaling complex assembly. The resolved complex includes not only ZAR1 but also resistance-related kinase 1 (RKS1) bound to the LRR domain of ZAR1. When ADP is bound to the NB-ARC of ZAR1, the ZAR1-RKS1 complex remains at resting state and inactive. Yet, when the effector-driven uridylation on PBL2 occurs, RKS1 tethers it to form a ZAR1-RKS1-PBL2^{UMP} complex and ADP/dATP exchange occurs, which is a key process ensuing conformational changes of ZAR1 and successive oligomerization. The resulting full signaling ZAR1 (or ZAR1-RKS1-PBL2) complex appears as a wheel-like pentamer, which is referred as ZAR1 resistosome that is sufficient to fully trigger the HR to restrict *Xanthomonas campestris* bacterial growth (Wang et al., 2019a,b; **Figure 3A**, middle). The mechanism underlying the NLR assembly offers an excellent template to envision NLR action and (auto)immunity, in general, to explain how plant performance is affected by controlled or uncontrolled immunity in hybrid plants, which we discuss in the next section.

Mechanisms of NLR-Mediated Autoimmunity Responsible for Hybrid Necrosis

Hybrid necrosis caused by immune system incompatibility appears to result from too much variability in the NLR sequences and potentially in function. Almost all NLRs predisposed to hybrid necrosis are encoded by tandem clusters (**Figure 3B**); a recently cloned *DM10* is encoded by a single locus, however, the locus had been generated through a recent chromosomal relocation event of a multigene TNL cluster in *A. thaliana* (Barragan et al., 2020). Moreover, the most prominent *DM2* cluster along with other large *DM* clusters show great expandability in cluster size with high CNV detected in the 64 natural accessions (Lee and Chae, 2020). An interesting finding from the pattern analysis of NLR cluster expansion is that not all NLRs in the cluster contribute to the expansion in the global *A. thaliana* population, suggesting that differentiated functionality within a given NLR cluster may contribute to

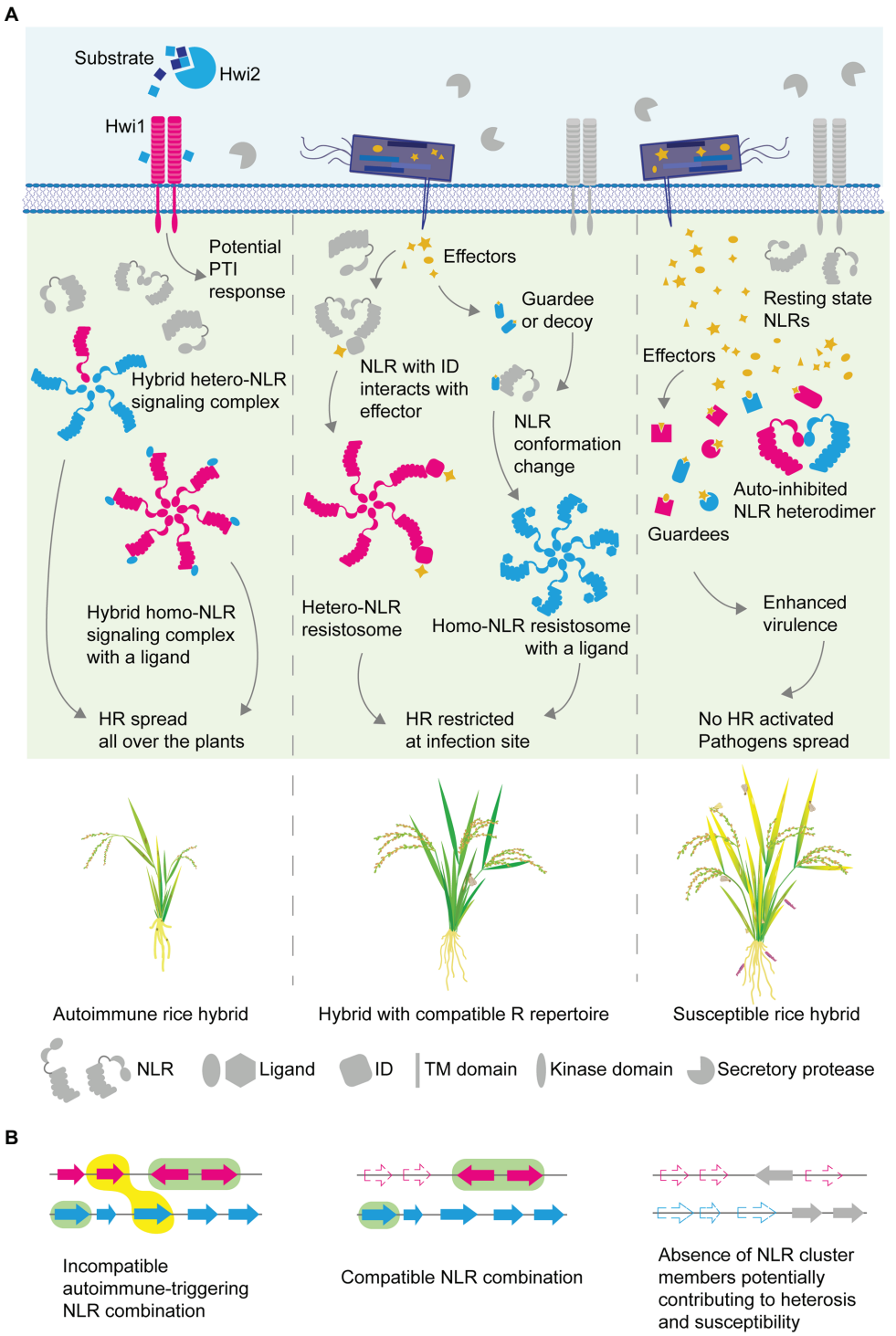


FIGURE 3 | Molecular mechanisms for disease resistance, hybrid necrosis and heterosis in the view of NLR receptor complex formation. **(A)** NLR complex assembly illustrated in the plant cell of autoimmune (left), normal (middle) or heterotic (right) hybrids. Immune components are color-coded as magenta or cyan to indicate parental origins. Different architecture in NLR illustration, such as a fusion of ID at the C-terminal end, was used to differentiate distinct NLR proteins in the hetero-complex. Domain illustrations constituting the membrane-bound receptor-like kinase (RLK)/receptor-like proteins (RLPs) and intracellular NLRs are indicated at the bottom. The resting state is indicated with gray fill while the active state is with cyan or magenta. Effectors are filled with orange. In a rice hybrid with compatible immune components (middle), effector-modified host proteins are perceived by NLRs, which induces the assembly of a signaling complex, so-called resistosome. NLR-IDs with a decoy domain are hypothesized to form a resistosome with their pair NLRs, forming a heteromeric complex

(Continued)

FIGURE 3 | (middle). Conformation changes occurring upon perception are indicated with the closed-form of NLRs and linear form of NLRs. In an autoimmune hybrid (left), signaling NLR complexes form constitutively without effector perception. The complex may consist of NLRs of different parental origins, or of NLR and other host proteins that function as ligands to activate the NLR to initiate and complete the assembly. RLKs of one parent could be constitutively activated by ligands from the other parent to trigger pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). Accumulation of susceptibility alleles or interference between NLRs in breeding lines (right) can lead to hybrids with compromised resistance. NLR underrepresentation may facilitate effector action toward disease. **(B)** Schematic diagram of NLR cluster composition assembled in hybrids. The incompatible NLR combination (left) is highlighted with a yellow-filled object. Compatible combinations are marked with green-filled objects (left, middle). Each filled arrow indicates an NLR, while pseudogenization or locus deletion is represented with dashed-lined arrows. Arrows are colored to indicate different parental origins.

shaping the NLR repertoire available in the population. Potentially deleterious hybrid necrosis NLR alleles may rise during cluster expansion and contraction, which in some clusters may not be easily purged but maintained due to the linkage to beneficial *R* alleles within the cluster.

The first cloned BDM pair for hybrid necrosis in *A. thaliana* is the two NLR-encoding genes, *DM1* and *DM2d*, and these NLRs have been shown to form a heteromeric oligomerized complex in plants through their TIR domains (Tran et al., 2017). A recent molecular investigation on DM6/DM7 hybrid necrosis also has shown that DM6/RPP7 NLR oligomeric status is augmented by the presence of the matching hybrid necrosis pair DM7, RPW8/HR protein through physical interaction between the two proteins (Li et al., 2020). Based on the ZAR1 resistosome structure, we postulate that in the autoimmune hybrids the causal NLR pairs are likely to formulate a hetero-signaling complex consisting of NLRs from different parents. If the BDM pair involves a non-NLR protein and an NLR, the NLR complex would include the host protein such as in RPP7/RPW8-mediated autoimmunity, which is also reflective of the ZAR1 NLR complex including decoys (**Figure 3A**, left and middle). The hybrid NLR signaling complex exerts immune responses in an uncontrolled manner since the completion of the signaling complex would not depend on an effector-trigger but on their own conformation of participating NLRs. Naturally evolved NLR variants with high sequence similarity have been experimentally demonstrated to potentiate different degrees of signaling (Bernoux et al., 2016). Based on the comparative analysis of closely related flax NLR L6 and L7, Bernoux et al. (2016) proposed that NLRs are present both in active vs. inactive status under equilibrium, which becomes readily shifted toward the active state when the effector is bound. We hypothesize that naturally occurring hybrid necrosis NLR variants adopt conformations close to the active status in a way that the equilibrium is already pulled close to fully active status. Independently evolved NLRs from the reciprocal parent would mistakenly recognize this form as an effector-bound conformation and assemble a fully functional NLR signaling complex. This proposition reminds a situation of paired NLR, where one is an effector-bound sensor and the other is a signaling executer (**Figure 3B**, middle). Although we proposed that DM1 and DM2d fit under this molecular category, with DM2d masquadering an effector-bound conformation and DM1 recognizing this form to help signaling, none of them carries IDs (Tran et al., 2017). It would be interesting to uncover molecular details of more hybrid necrosis cases, which will expand our knowledge on the functional diversity of NLRs in complex assembly and signaling. The new findings on RPW8/HR4 facilitating a higher-order NLR assembly also reminds of the ZAR1 complex assembly initiated by decoy binding (**Figure 3B**, left and middle). Whether or not RPW8 proteins are effector targets and how this class of proteins potentiate cell death remain as an active area of research. Our proposition of anti-hybrid necrosis potentially explaining heterosis could be illustrated with the molecular scenarios in the view of NLR complex formation. In the heterotic hybrids, NLR repertoires inherited from parents are supposedly much reduced as a result of selective breeding and the chance of formulating a hybrid NLR complex or decoy-assisted assembly of NLR complex is low (**Figures 3A,B**, right). Even worse scenario would be the auto-inhibiting NLR complex formation, which can suppress the particular disease resistance traits bred in the respective parents as was discussed in the above section with examples in wheat breeding (Hurni et al., 2014; Stirnweis et al., 2014).

In rice, most hybrid dysfunction has been categorized under hybrid weakness. The case involving *Hwi1/Hwi2* caught our attention as *Hwi1* encodes two tandemly repeated RLKs from wild rice and *Hwi2* encodes a secreted putative subtilisin-like protease in the *indica* rice variety Teqing (Chen et al., 2013, 2014). With the established contribution of RLKs to PTI, we could hypothesize that Hwi1 from wild origin mistakenly perceives Hwi2-generated products as PAMPs or DAMPs in the hybrid, presumably activating uncontrolled PTI at the cost of yield in the hybrid rice (**Figure 3A**, upper left). Another immune-related factor contributing to hybrid performance in rice is S5, a disease resistance-related aspartic protease (AP). Allelic conflict at S5 contributes to set up a reproductive barrier between *indica* and *japonica* by interfering with the functional embryo-sac production (Chen et al., 2008; Ji et al., 2012; Yang et al., 2012). Although the AP family has not yet been investigated in line with NLR activity, members of this family are involved in defense responses with activated SA in addition to pollen and ovule development in multiple plant species, including rice, grape and *A. thaliana* (Xia et al., 2004; Ge et al., 2005; Prasad et al., 2009; Huang et al., 2013; Guo et al., 2016; Gao et al., 2017). Given that immune incompatibility between the protease Rcr3 from domesticated tomatoes and R protein Cf-2 from wild tomatoes are known, it will be interesting to see if the rice AP important for setting up the reproductive barrier would engage an immune system incompatibility under BDM epistasis. Breeding history and already existing *indica-japonica* rice hybrids that overcame the AP-mediated barrier would provide a hint on

the involvement of immune components as well as the possibility of APs as host targets that an NLR monitors.

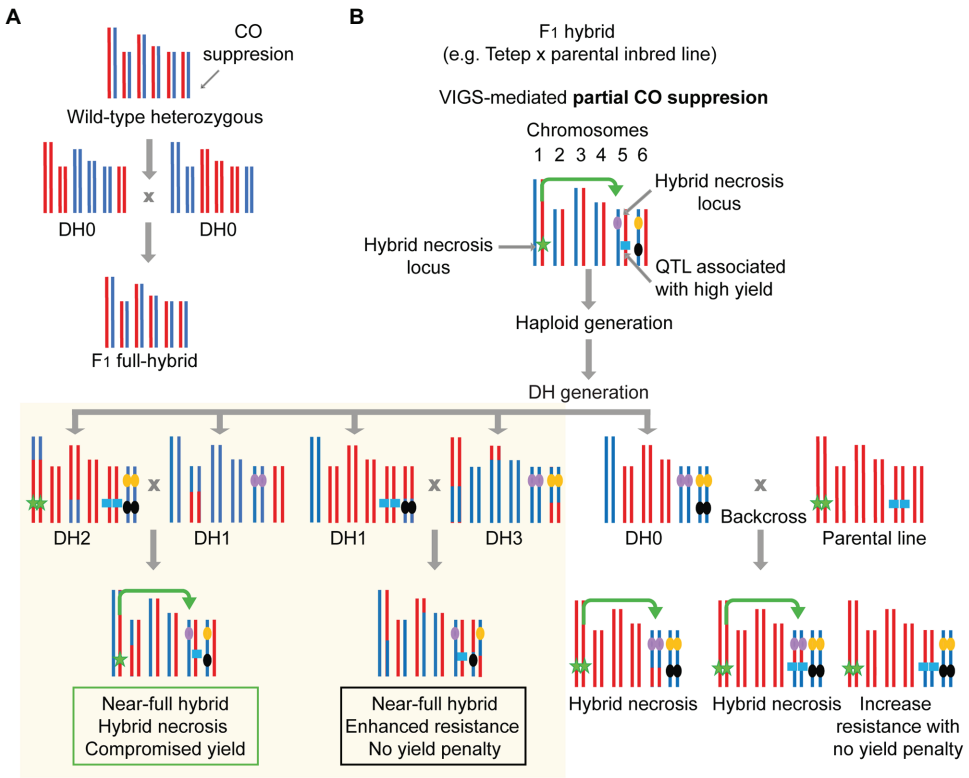
REVERSE BREEDING APPLICATION TO HYBRID NECROSIS AND HETEROSIS

To improve the combining ability of antagonistic traits such as growth and immunity, understanding genetic architectures underlying heterosis and hybrid necrosis is the first step. With the advances in crop genomics in recent years, it is becoming feasible to map responsible loci associated with such traits and retrieve relevant SNP sets, which will guide the design of a crop based on genomic information. However, challenges still remain to precisely and efficiently incorporate new traits into elite varieties. Given that fine-mapping resolution in crops still span a region including multiple loci and that immune genes particularly tend to form a cluster in the genome, substituting a responsible chromosome segment can be a useful genetic solution both to detect causal loci in bulk and to incorporate allele replacement when causal genes had not been cloned (Balakrishnan et al., 2019). Reverse breeding is an effective way to fix heterosis from hybrids expressing a suit of beneficial traits (Dirks et al., 2009). It first decomposes traits by generating numerous homozygous lines from a given heterozygous plant, from which desirable phenotypic traits can be selected in combination or in part. The crossing of complementing homozygous lines for the desired traits yields a hybrid which resembles fully or partially the genome of the starting heterozygous (Wijnker et al., 2012; Calvo-Baltanás et al., 2020; **Figure 4A**).

The technical application of reverse breeding demands two conditions: (1) the downregulation of meiotic recombination to preserve the two parental haplotypes that were combined in the starting hybrid and (2) the regeneration of the resulting gametes as a double-haploid (hereafter DH) population to obtain homozygous lines (Dirks et al., 2009; Wijnker et al., 2012; **Figure 4**). To date, reverse breeding has been shown in the model organism *A. thaliana* through complete and partial crossover (hereafter CO) suppression by silencing genes essential for meiotic recombination (Wijnker et al., 2012; Calvo-Baltanás et al., 2020). In the latest design, a meiotic gene required for about 83–87% of the total number of COs, *MSH5* (*MUT-S HOMOLOG 5*; Higgins et al., 2004, 2008; Lu et al., 2008), was silenced in a wild-type hybrid using virus-induced gene silencing (VIGS; Calvo-Baltanás et al., 2020). The silencing of *MSH5* resulted in non-recombinant and low-recombinant chromosomes segregating to gametes. These gametes are later grown into DH lines which show variable recombination rates, from zero COs (DH0) to three COs (thus generating DH1, DH2, or DH3; **Figure 4B**). If gametes carrying non-recombinant chromosomes are grown into DHs (DH0), chromosome-substitution lines (CSLs) will be obtained. The combination of two fully complementing CSLs will reconstitute the initial heterozygous genotype (**Figure 4A**). Gametes carrying low-recombinant chromosomes, however, can also be of use for reverse breeding. The combination of DH1 and DH2 lines, with one and two CO, respectively, will generate near-full hybrids (**Figure 4B**). Near-full hybrids largely retain the heterozygosity of the full hybrid with the exception of certain homozygous regions (**Figure 4B**). These hybrids, therefore, can be used to experimentally assess the effect that such homozygous regions have on hybrid performance (Calvo-Baltanás et al., 2020).

Pyramiding *R* genes in elite-hybrids is challenging, especially when it entails *R* gene cloning, which is particularly complicated due to massive structural variation often associated with the encoding loci (Jiao and Schneeberger, 2020). Alternatively, a backcrossing scheme between a donor and recipient line can facilitate the introduction of *R* genes in breeding lines. However, this is a time-consuming process and unknown deleterious combinations can lead to hybrid necrosis when parents containing newly assembled *R* genes sets are used to produce hybrids. To increase the efficiency of this process, one can use reverse breeding as an alternative technique to NLR cloning or to assist in backcrossing schemes (**Figure 4B**). In an ideal scenario, the genome of the initial (wild-type) heterozygote for a reverse breeding experiment will contain loci associated with hybrid necrosis, heterosis, and resistance. Reverse breeding based on partial CO suppression applied to this heterozygote will deliver DHs that will segregate for the desired number of *R* genes, heterotic loci and hybrid necrosis loci (**Figure 4B**). The combination of these lines will then produce hybrids with different genetic composition. Because DHs are grown from gametes that contain recombinant and non-recombinant chromosomes, depending on the combination of DHs used, some regions will become homozygous in the near-full hybrids (**Figure 4B**). In the event that such homozygous regions are not associated with yield, the expression of this trait should not be compromised in near-full hybrids. Some works already showed that the accumulation of several positive alleles was responsible for the heterotic traits in rice and for this reason, the reconstitution of the full hybrid genotype would not be essential to obtain elite heterozygous lines from a reverse breeding experiment (Huang et al., 2015, 2016). In addition, homozygous regions present in the near-full hybrids may be necessary to eliminate hybrid necrosis loci while retaining all the other desirable traits (i.e., *R* genes and heterotic loci; **Figure 4B**).

The use of reverse breeding offspring has further advantages. DH0s are also CSLs, a type of mapping population useful to dissect epistasis (Nadeau et al., 2000; Spiezio et al., 2012; Ziolkowski et al., 2017; Wijnen et al., 2018). CSLs can also be used as donor lines for single or entire sets of *R* genes; crossing CSLs to an existing breeding line allows the sequential introduction of desirable *R* genes into that background (**Figure 4B**). Because only specific chromosomes segregate, the associated genotyping effort to identify the lines with the desired introgression is largely reduced as compared to traditional backcrossing schemes. Moreover, if CSLs are crossed to an inbred parental line, in later generations, individuals segregating for specific loci will be obtained (**Figure 4B**). Such lines may contain a single chromosome segment from a donor parent introduced to a second, recurrent background. These genetic stocks are valuable tools for crop improvement, as they allow to map QTLs, causal genes, and gene interactions (Balakrishnan et al., 2019). Therefore, the rapid generation of CSLs through



C

NLRs in Tetep associated with blast resistance

Chr	Gene ID	Rice blast strain
Chr 6	Fgenesh.1195	TPXL / S30 / S2004 / ZB15 / S1 / B7 / B13 / B15 / LaiXian / Y10 / B3 / S21 / S32 / Y1
Chr 6	Fgenesh.378	S2007 / BEI1 / TPXL / S30 / ZB15 / S1 / B13 / S32 / S2 / S33
Chr 5	Fgenesh.1166	S2007 / TPXL / S30 / S1 / B7 / B13

FIGURE 4 | Reverse breeding through virus-induced gene silencing (VIGS)-mediated partial crossover suppression in a rice hybrid. **(A)** Reverse breeding allows the recreation of a heterozygous plant as an F_1 hybrid through the generation of genetic complementing parental lines. **(B)** A schematic reverse breeding strategy in rice. Only the first six chromosomes of a putative rice hybrid between Tetep (blue) and a hypothetical parental inbred line (red) are presented for simplicity. Three NLRs found in the Tetep genome are represented with purple, yellow, and black circles in chromosomes 5 and 6. The quantitative trait locus (QTL) associated with high yield is represented by the light blue square in chromosome 5. Hypothetical genetic interaction between two loci (green star and purple circle) results in hybrid necrosis, thus affecting yield. Reverse breeding based on partial suppression of meiotic recombination will yield plants producing gametes carrying chromosomes with 0, 1, 2, or 3 crossover (COs) which will be grown into DH0, DH1, DH2, and DH3, respectively. Different DHs can be crossed to produce near-full hybrids. The homozygous regions found in one near-full hybrid eliminate one of the hybrid necrosis loci (green star) while retaining the QTL for increased yield and three NLRs from Tetep, hence allowing the generation of a resistant and high-yielding hybrid. The DH0 lines [chromosome-substitution lines (CSLs)] can be used as parental donors to introduce *R* genes in a recipient background. Note that the lines obtained in this scheme can be instrumental to map hybrid necrosis loci **(C)**. List of NLRs in the Tetep represented in **(B)** and the list of pathogens they confer resistance against (Wang et al., 2019c). The font color corresponds to the NLR represented in each chromosome in **(B)**.

reverse breeding could be instrumental to facilitate the study of the genetic mechanisms of hybrid necrosis in rice.

Practical Application of Reverse Breeding in Rice

For the application of reverse breeding in rice, silencing of genes essential for CO formation shall be achieved in hybrids. To this end, different approaches such as microRNA, RNAi, or VIGS can be used. There are, however, benefits of using VIGS over other silencing methods that require stable transformation. First, for a gene to be silenced by RNAi or microRNAs in the hybrid, at least one of the parental lines needs to be previously transformed with dominantly acting transgenes. Consequently, inbred lines for that particular hybrid need to be available. VIGS, however, allows direct silencing of the target in the chosen hybrid, even if this is a heterozygote from outcrossing populations. Therefore, VIGS represents a convenient, broad, rapid method over the use of stable transgenes. In rice, the rice tungro bacilliform virus (RTBV)-VIGS system has been shown to reduce gene expression

by 80% (Kant and Dasgupta, 2017). The silencing efficiency of meiotic genes, however, is yet to be assessed using this system. The most obvious target for VIGS in a reverse breeding experiment would be *OsMSH5/OsMSH4* and/or other proteins involved in class I CO formation, which would lead to a comparable decrease in CO numbers as for the *A. thaliana* mutants defective in this CO pathway (Luo et al., 2013; Zhang et al., 2014, 2019; Wang et al., 2016; Calvo-Baltanás et al., 2020).

The effect of CO reduction on gamete viability can be calculated as $(1/2)^x$, where "x" represents the species' chromosome number. In rice, the average number of COs in wild-type meiosis has been estimated to be between 20.1 and 33.9 (Si et al., 2015; Zhang et al., 2019). The estimated 83% reduction in CO formation when class I CO formation is suppressed and considering 33.9 or 20.1 COs would result in about six or three residual COs per meiosis, respectively. While complete CO suppression would yield $(1/2)^{12} \times 100\% = 0.02\%$ of viable gametes, in the event that either six or three residual COs are present, and assuming the occurrence of one CO per chromosome pair this frequency will vary greatly, which will equal to $(1/2)^{12-6} \times 100\% = 1.6\%$ or $(1/2)^{12-3} \times 100\% = 0.2\%$. Therefore, partial COs suppression represents an 80 to 10-fold increase in gamete viability over complete CO suppression, depending on the residual number of COs present. In addition, it is important to consider the number of gametes produced by a single plant, as this will affect the number of viable gametes obtained from a reverse breeding experiment in rice. The number of pollen grains produced by rice plants is rather variable among varieties, and it is influenced by anther-length and environmental conditions such as temperature (Fukushima et al., 2017). Under optimal growth conditions, each anther may contain 1,000–2,000 pollen grains (Fukushima et al., 2017). If COs are reduced by 83% and six or three residual COs are present, each anther will produce between 16 and 32 or between 2 and 4 viable pollen grains, respectively. Such pollen viability rate *a priori* may seem low, but it would certainly enable the recovery of reverse breeding offspring if several silenced anthers from each plant are used for DH regeneration.

The last step requires haploid and/or DH technology to be established. Although haploid-induction efficiency and DH regeneration in rice has long remained largely genotype-dependent, these constraints have been greatly overcome in recent years (Bishnoi et al., 2000; Maharani et al., 2020). Indeed, different anther culture techniques allow DH-recovery frequency that ranges from 30 to 60% (Mishra and Rao, 2016; Ren et al., 2017; Sarao and Gosal, 2018). Interestingly, one of the latest protocols available for anther-culture using an elite *indica* rice hybrid even reports a regeneration of exclusively DHs from callus (Naik et al., 2017). Such frequencies integrated in a reverse breeding scheme implies that useful DHs and near-full hybrids can be obtained in a maximum of two generations from the starting hybrid (**Figure 4B**).

CONCLUSION

In this review, we put forward our proposition that hybrid necrosis resulting from immune incompatibility would function as a potential opposing force to the expression heterosis in hybrids. Our reasoning bases on recent molecular and genetic findings on hybrid necrosis in plant species covering both natural and selectively bred germplasms. Autoimmunity is a major mechanism underlying hybrid necrosis arising from a new combination of highly diversified immune components from different parental origins, which obligatorily results in compromise in growth and yield. Thus, hybrid necrosis illustrates an extreme degree of trade-off manifestation between growth and immunity. In the plant performance equilibrium model that we propose, the shifted equilibrium to the extremes is expressed as either hybrid necrosis or heterosis. Although the degree of contribution made from anti-hybrid necrosis to determining heterosis is yet to be examined, yield penalty associated with enhanced resistance observed throughout breeding history, including hybrid breeding in rice, clearly points to the link between heterosis and disease resistance traits. We encourage rice researchers to revisit the cases of underperforming hybrids in the breeding panel under the new concept of hybrid necrosis as an opposing force to heterosis. Despite the seemingly low breeding interests, such underperformance, when addressed under epistatic interactions and environmental variables affecting performances, might reveal an underestimated contribution of hybrid necrosis to restrict the full manifestation of heterosis. The findings will not only shed light on guided breeding strategies in the post-genome era but also greatly inform us of evolutionary processes shaping up an immune system including the valuable *R* gene repertoire. The more we understand the downside of hybrid performance, the better could we combine beneficial traits of yield and disease resistance. Valuable rice germplasms bred throughout history await a new wave of immune-centered breeding for heterosis.

AUTHOR CONTRIBUTIONS

VCB, JW, and EC formulated concepts and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Confirmation of a Gametophytic Self-Incompatibility in *Oryza longistaminata*

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Oryza longistaminata, a wild species of African origin, has been reported to exhibit self-incompatibility (SI). However, the genetic pattern of its SI remained unknown. In this study, we conducted self-pollination and reciprocal cross-pollination experiments to verify that *O. longistaminata* is a strictly self-incompatible species. The staining of pollen with aniline blue following self-pollination revealed that although pollen could germinate on the stigma, the pollen tube was unable to enter the style to complete pollination, thereby resulting in gametophytic self-incompatibility (GSI). *LpSDUF247*, a *S*-locus male determinant in the gametophytic SI system of perennial ryegrass, is predicted to encode a DUF247 protein. On the basic of chromosome alignment with *LpSDUF247*, we identified *OlSS1* and *OlSS2* as *Self-Incompatibility Stamen* candidate genes in *O. longistaminata*. Chromosome segment analysis revealed that the *Self-Incompatibility Pistil* candidate gene of *O. longistaminata* (*OlSP*) is a polymorphic gene located in a region flanking *OlSS1*. *OlSS1* was expressed mainly in the stamens, whereas *OlSS2* was expressed in both the stamens and pistils. *OlSP* was specifically highly expressed in the pistils, as revealed by RT-PCR and qRT-PCR analyses. Collectively, our observations indicate the occurrence of GSI in *O. longistaminata* and that this process is potentially controlled by *OlSS1*, *OlSS2*, and *OlSP*. These findings provide further insights into the genetic mechanisms underlying self-compatibility in plants.

Keywords: gametophytic, *OlSP*, *OlSS*, *Oryza longistaminata*, self-incompatibility

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INTRODUCTION

Self-incompatibility (SI) is a reproductive mechanism of higher plants for preventing inbreeding by inhibiting self-pollination, and has been widely observed in Brassicaceae, Solanaceae, Rosaceae, Plantaginaceae, Scrophulariaceae, Gramineae, and Rutaceae (Pandey, 1960; Goring et al., 1993; Vaughan, 1994; Dickinson, 1995; McCormick, 1998; Liu et al., 2014; Iwano et al., 2015; Li et al., 2017; Ye et al., 2018; Liang et al., 2020). SI is described as a mechanism whereby plants are able to produce functional male and female gametes, but are incapable of producing seeds subsequent to self-pollination or the cross-pollination of plants containing SI alleles (Pandey, 1960; Takayama and Isogai, 2005). SI was divided into gametophytic self-incompatibility (GSI) and sporophytic self-incompatibility (SSI), which is defined based on the genetic mode of control of the incompatibility phenotype in pollen (de Nettancourt, 1997; McCubbin and Kao, 2000; Silva and Goring, 2001). In GSI, incompatibility is determined by pollen genotype, where growth inhibition of incompatible

pollen occurs mainly in the style. In SSI, pollen grain incompatibility is determined by the diploid genome of the parent, in which mature pollen carry SI signal elements secreted by the tapetum, which lead to inhibiting of the germination of incompatible pollen on the stigma surface (de Nettancourt, 1997; Hiscock and Tabah, 2003; Hiscock and McInnis, 2004). The genetic mechanism of SI is dependent on signal transmission controlled by a single or multiple genetic loci between pollen and stigma (Franklin-Tong et al., 1988; Franklin-Tong and Franklin, 2003; Castric and Vekemans, 2004). The genetic loci associated with SI characteristically show high polymorphism, with respect to both the number of *S* alleles and differences in nucleotide sequences (Vekemans and Slatkin, 1994).

The SI system in Gramineae is GSI, which is thought to be controlled by two non-linked and polymorphic multi-allelic *S* and *Z* loci (Lundqvist, 1954; Cornish et al., 1979; Baumann et al., 2000; Thorogood et al., 2002). The *S* and *Z* loci in *Lolium perenne* L. have been found to be correspond to syntenic regions of chromosomes 5 and 4 in the genome of *O. sativa*, respectively (Yang et al., 2009). In total, 10 SI candidate genes have been identified in the SI cDNA libraries of *L. perenne*, and qRT-PCR analysis has revealed a rapid increase in the expression of these genes following pollen–stigma contact (Yang et al., 2009). Subsequently, nine candidate genes associated with SI were screened from *L. perenne* using molecular markers, and on the basis of expression profiling and nucleotide diversity assessment, two *Z*-linked genes, a *ubiquitin-specific protease* and a *LpDUF247* gene, have been identified as the most plausible candidates for the *S*-*Z* SI system (Shinozuka et al., 2010). In further studies, a total of 10,177 individuals of *L. perenne* from seven different mapping populations segregating for *S* locus showed a highly polymorphic gene encoding for a protein containing a DUF247 was fully predictive of known *S*-locus genotypes at the amino acid level (Manzanares et al., 2016). In addition, genome wide association studies and BLAST alignment with the *Brachypodium* physical map revealed highly significantly associated markers with peak associations from the chromosomal locations of candidate SI genes *S*- and *Z*-DUF247 (Thorogood et al., 2017). *LpSDUF247* is considered to be a *S*-locus male determinant in the gametophytic SI system that has five haplotypes with sequence polymorphism, which is a typical characteristic of SI. However, the molecular mechanisms underlying the regulation of SI in *L. perenne* have yet to be clarified (Thorogood et al., 2002; Shinozuka et al., 2010; Manzanares et al., 2016).

Oryza longistaminata is an AA-genome wild *Oryza* species of African origin that exhibits SI (Nayar, 1967). *O. longistaminata* has numerous desirable traits, such as long stigma and large anthers (Morishima, 1967), a rhizome (Ghesquiere, 2008), and high resistance to abiotic and biotic stresses (Khush et al., 1990; Song et al., 1995; Hu et al., 2003; Dong et al., 2017), and accordingly could represent a beneficial resource from the perspective of rice breeding. However, SI limits its free mating and hinders the exploitation of such desirable traits in rice cultivar improvement. In the present study, we identified a polymorphic *S*-locus associated with SI in this species, which will provide a basis for the determination of genetic patterns and molecular mechanisms of SI. Accordingly, we believe that the findings of this study could potentially facilitate exploitation of the desirable traits of *O. longistaminata*.

MATERIALS AND METHODS

Plant Materials

For the determining the SI of *O. longistaminata*, we used the germplasms OlMK23 and OlMK68, which were originally collected from Ethiopia and were kindly provided by Dr. Melaku Getachew of the Institute of Biotechnology, College of Natural and Computational Sciences, Addis Ababa University, Ethiopia. For reciprocal hybrid testing, OlMK68 and OlMK23 plants were cultivated at Jinghong, Yunnan Province, China (21°59′N, 87 100°44′E, 611 m a.s.l.). For hybridization experiments, stamens were removed manually from the pollen recipients and mature pollen was collected from the pollen donors.

Reciprocal Hybrids and Statistics

The *O. longistaminata* germplasms OlMK23 and OlMK68, and their reciprocal hybrids, each of which was represented by 10 samples, were cultivated for SI testing. For the reciprocal hybrids, the stamens were removed from a pollen recipient and mature pollen grains were collected from a pollen donor, after which plant were cross-pollinated and bagged. The seed-setting rate of OlMK23, OlMK68, and their reciprocal hybrids was determined after harvesting. Statistical analyses of seed-setting rate were based on the ratio between the number of harvested mature seeds and the total number of pollinated flowers in 10 independent pollination experiments.

Aniline Blue Staining

At 5 min, 30 min, 1 h, and 2 h following self-/cross-pollination, more than 10 pollinated *O. longistaminata* and *O. sativa* pistils were collected and subsequently fixed overnight in Carnoy's solution (90 mL of 50% ethanol, 5 mL of formaldehyde, and 5 mL of acetic acid). The pistils were then sequentially rehydrated with ethanol (50, 30, and 10%, each for 10 min) and then washed with deionized water. Thereafter, the pistils were softened in 1 M NaOH for 45 min at 55°C and stained with water-soluble aniline blue [0.1% (W/V) aniline blue and 0.1 M K_2HPO_4, pH 8.5] for 6 h in the dark at room temperature. The stained pistils were visualized by fluorescence microscopy.

Identified and Syntenic Analysis of *OlSSs* and *OlSP*

LpSDUF247 is a male component of the *S*-locus in *L. perenne* and is syntenic with *O. sativa* (Manzanares et al., 2016). *OsDUF247* (*Os05g0197900*), which corresponds to *LpSDUF247*, is located in the 6,030–6,090 kb region of *O. sativa* chromosome A05. Comparative genomics analyses were performed by comparing

the genome sequences of *O. longistaminata* with chromosome 5 of *O. sativa*. *OlSS1* on Contig10 of *O. longistaminata* corresponds to *OsDUF247* (*Os05g0197900*). Sequence analysis was performed on the sequence flanking *OlSS1* to identify candidate polymorphic sequences. Haplotype and sequence polymorphism analyses of *OlSS1*, *OlSS2*, and *OlSP* were based on published transcriptome sequencing data (Zhang et al., 2015), whereas analysis of the conserved domain of OlSS1, OlSS2, and OlSP was performed using SMART[1].

RNA Isolation

The roots, stems, leaves, stamens, and pistils of *O. longistaminata* were used for tissue-specific expression analysis. The stamens were collected to determine the expression of *OlSS1* and *OlSS2* from stages 7 to 14 and expression in the pistils was determined from stages 9 to 14 as described by Zhang and Zoe (2009) for *O. sativa*. Total RNA was isolated from *O. longistaminata* using an Eastep® Super Total RNA Extraction Kit (Promega, Shanghai, China), with first-strand cDNA being synthesized from 1 μg of the isolated RNA using a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa BIO, Shiga, Japan).

RT-PCR and qRT-PCR Analysis

The cDNAs of roots, stems, leaves, stamens, and pistils of *O. longistaminata* were used as templates for RT-PCR assays. RT-PCR and qRT-PCR amplifications were performed using the following primer pairs: *OlSS1*-1-F and *OlSS1*-1-R for *OlSS1*-1; *OlSS1*-2-F and *OlSS1*-2-R for *OlSS1*-2; *OlSS2*-1-F and *OlSS2*-1-R for *OlSS2*-1; *OlSS2*-2-F and *OlSS2*-2-R for *OlSS2*-2; *OlSP*-1-F and *OlSP*-1-R for *OlSP*-1; and *OlSP*-2-F and *OlSP*-2-R for *OlSP*-2. qRT-PCR analysis was performed using SYBR Premix Ex Taq Kit (TaKaRa BIO) in a Quant Studio 7 Flex real-time PCR system (Applied Biosystems, Foster City, CA, United States) with the following amplification program: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and primer annealing and extension at 58°C for 34 s. The results were normalized using the relative expression level of *Actin2* and analyzed using the $2^{-\Delta\ \Delta\ Ct}$ method (Livak and Schmittgen, 2001). Three biological replicates were performed for RT-PCR and qRT-PCR analysis. The sequences of the primers used in this study are presented in **Supplementary Table 1**.

Homologous Cloning of *OlSSs* and *OlSP*

Relevant genomic and transcriptomic data for homologous cloning obtained from a previous study on *O. longistaminata* (Zhang et al., 2015). The cDNA sequences of *OlSSs* and *OlSP* were cloned from the transcriptomic database of *O. longistaminata* by homologous cloning (Zhang et al., 2015). For cDNA cloning, we used the following primers pairs: *OlSS1-1-cF* and *OlSS1-1-cR* were used for *OlSS1-1*; *OlSS1-2-cF* and *OlSS1-2-cR* for *OlSS1-2*; *OlSS2-1-cF* and *OlSS2-1-cR* for *OlSS2-1*; *OlSS2-2-cF* and *OlSS2-2-cR* for *OlSS2-2*; *OlSP-1-cF* and *OlSP-1-cR* for *OlSP-1*; and *OlSP-2-cF* and *OlSP-2-cR* for *OlSP-2*. The products thus amplified were inserted in the *pMD19-T* vector, and monoclonal sequencing was performed for different haplotypes.

[1]http://smart.embl.de/

RESULTS

Oryza longistaminata Is a Strictly Self-Incompatible Species

The definitive characteristic of SI is that self-pollination of identical haplotypes results in sterility, whereas pollen is fertile when crossing different haplotypes (Franklin-Tong et al., 1988; Franklin-Tong and Franklin, 2003). In the present study, we grew plants of OlMK68 and OlMK23 in a field to evaluate the SI in *O. longistaminata* through self-pollination and reciprocal cross-pollination. Although the seed-setting rate of the hybrid (OlMK68/OlMK23) and its reciprocal hybrid was 20.5 ± 3.8 and 26.2 ± 4.7%, respectively (n = 10), in the case of both OlMK23 and OlMK68, self-pollination of plants failed to result in seed set, suggesting strict SI in this species (**Table 1** and **Supplementary Table 2**).

Oryza longistaminata Exhibits Gametophytic Self-Incompatibility

To determine pollen phenotype, we performed staining with aniline blue, which revealed that the pollen of *O. longistaminata* is able to germinate on the stigma after self-pollination (**Figures 1A–D**), with germination being observed within 5 min after self-pollination of OlMK68 (**Figure 1A**), a high rate of germination after 30 min, and the pollen tube reaching the top of the style within 1 h (**Figures 1B,C**). Subsequently, however, pollen tube growth was inhibited, with the pollen tube appearing to be incapable of passing through the style (**Figure 1D**). In contrast, pollen growth was consistent in self-pollinated of *O. sativa* after 5 and 30 min (**Figures 1E,F**), and the pollen tube was observed to have passed through the style within 1–2 h (**Figures 1G,H**). These results indicated that although the pollen of *O. longistaminata* is able to germinate on the stigma after self-pollination, it is incapable of subsequent passage through the style, there implying that this species exhibits GSI.

Oryza longistaminata Self-Incompatibility Determinants Are Linked *S*-Loci

The corresponding region of the *S*-locus of *L. perenne* was found on chromosome 5 of *O. sativa*, wherein a gene homolog of *LpSDUF247* is predicted to encode a DUF247 domain-containing protein (Manzanares et al., 2016). Comparative genomic analysis revealed that a gene located on contig10 of *O. longistaminata* is syntenic with *Os05g0197900* (*OsDUF247*),

TABLE 1 | Seed-setting rate after self-/cross-pollination in *O. longistaminata*.

Female	Male	Seed-setting rate (%)
OlMK68	OlMK68	0
OlMK68	OlMK23	20.5 ± 3.8
OlMK23	OlMK23	0
OlMK23	OlMK68	26.2 ± 4.7

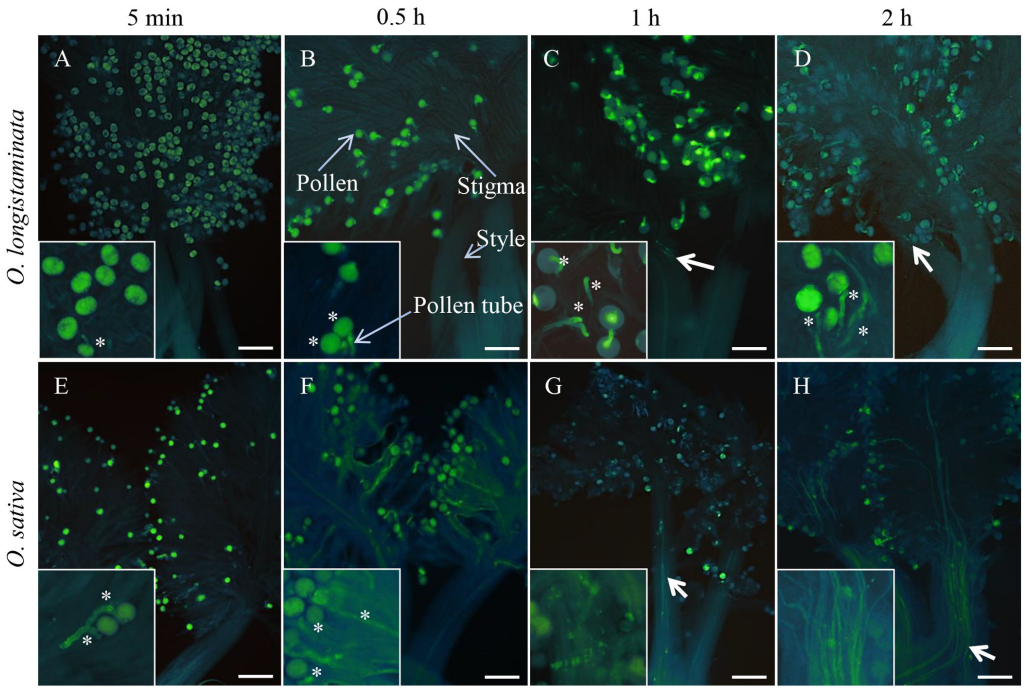

FIGURE 1 | Pollen germination of *Oryza longistaminata* and *Oryza sativa*. **(A–D)** Self-pollination of *O. longistaminata*: **(A)** The pistils of self-pollinated OlMK68 after 5 min, stained with aniline blue solution. **(B)** The majority of pollen grains had germinated at 0.5 h following self-pollination. **(C)** The pollen tube reached the top of the style at 1 h after self-pollination. **(D)** At 2 h after pollination, the pollen tube remained on top of the style and appeared incapable of passing through the style. **(E–H)** Self-pollination of *O. sativa*: **(E)** Pollen began to germinate 5 min after self-pollination. **(F)** The pollen tube began to elongate at 0.5 h after self-pollination and passed through the style within 1–2 h following cross-pollination **(G,H)**. Bar = 50 μm. The asterisk indicates the germination of pollen grains. Bold arrows indicate pollen tubes in the style.

named *Self-Incompatibility Stamen1* of *O. longistaminata* (*OlSS1*) (**Figure 2A**). *OlSS1* encode a DUF247 domain protein, which is similar to LpSDUF247 and considered to be involved in SI (**Figure 2B**). On the basis of homologous sequence alignment, a gene was predicted to encode a protein containing a DUF247 domain about 19 kb downstream of *OlSS1*, named *Self-Incompatibility Stamen 2* of *O. longistaminata* (*OlSS2*) (**Figures 2A,B**). Previous genomic and transcriptomic sequencing has revealed that both *OlSS1* and *OlSS2* have two alleles in *O. longistaminata* (Zhang et al., 2015). RT-PCR assays indicated that alleles *OlSS1-1* and *OlSS1-2* are expressed primarily in the stamens, whereas the *OlSS2-1* and *OlSS2-2* alleles are expressed in both stamens and pistils (**Figure 2C** and **Supplementary Figure 1**). A defining characteristic of SI is that there should be a close linkage between male and female determinants and expression in stamens and pistils (Franklin-Tong, 2008). Both *OlSS1* and *OlSS2* show sequence polymorphisms and are expressed in stamens, we speculated that there is a *Self-Incompatibility Pistil factor* on contig10 of *O. longistaminata*. Indeed, subsequent sequence polymorphism analysis of a region flanking *OlSSs* revealed a candidate gene for such a factor, named *Self-Incompatibility Pistil factor* (*OlSP*), which may be involved in the SI of *O. longistaminata* (**Figure 2A**), given *OlSP* has a pair of alleles, *OlSP-1* and *OlSP-2*, that are highly and specifically expressed in the pistil (**Figure 2C** and **Supplementary Figure 1**). Structural analysis revealed that *OlSP* encodes an N-terminal YfaZ domain of unknown function (**Figure 2B**). Consequently, given that *OlSSs* and *OlSP* are closely linked and highly expressed in the stamens or pistils of *O. longistaminata,* we believe that *OlSSs* and *OlSP* could be candidate genes controlling SI in this species.

The alignment of OlSS1-1 and OlSS1-2 amino acid sequences with that of OsDUF247 exhibited similarity level of 80.7 and 69.8%, respectively (**Figure 3**), whereas the sequences of OlSS2-1 and OlSS2-2 showed lower similarities of 38.9 and 36.9%, respectively (**Figure 3**). Both OlSS1 and OlSS2 are characterized by conserved regions at the N and C termini. Furthermore, we established that similarities between the amino acid sequences of OlSP1 and OlSP2 and Os05g0198000 are 51.8 and 63.2%, respectively (**Figure 4**). These findings thus indicate that the *OlSS1*, *OlSS2*, and *OlSP* exhibit sequence polymorphism and can be considered putative SI candidate genes in *O. longistaminata*.

OlSSs and *OlSP* Expression Patterns

The expression levels of *OlSS1*, *OlSS2*, and *OlSP* at different development stages of stamen and pistil were analyzed by qRT-PCR assay. The expression of *OlSS1-1* was down-regulated during all developmental stages of the stamens, whereas the expression of *OlSS1-2* was up-regulated in stages 7–9 and

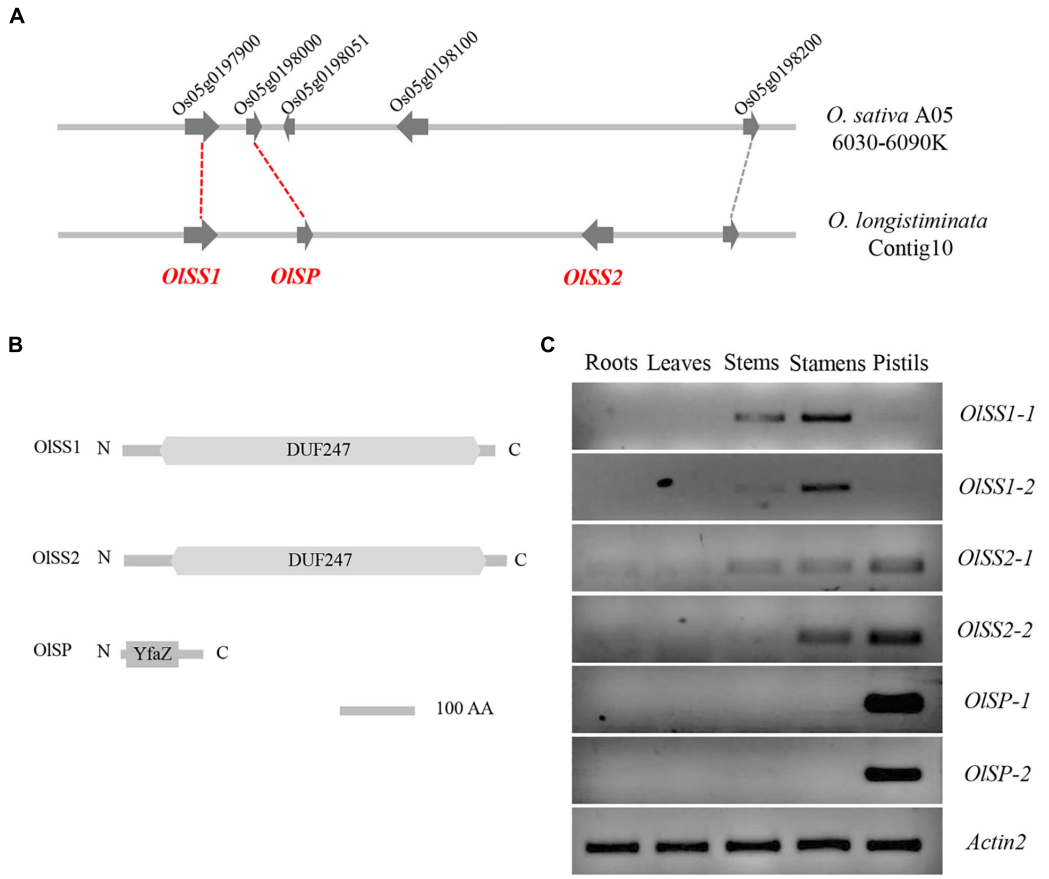

FIGURE 2 | Syntenic and tissue expression of *OlSSs* and *OlSP*. **(A)** Syntenic representation of *OlSSs* and *OlSP* in *Oryza sativa* and *Oryza longistaminata*. *Os05g0197900*, which encodes an unknown DUF247 domain protein in *O. sativa*, corresponds to *OlSS1*, whereas *Os05g0198000* and *Os05g0198100* correspond to *OlSP* and *OlSS2* respectively. **(B)** Analysis of OlSS1, OlSS2, and OlSP protein domains. Both OlSS1 and OlSS2 contain a conserved DUF247 domain, whereas OlSP contains an N-terminal YfaZ domain of unknown function. **(C)** RT-PCR determination of the tissue-specific expression pattern of *OlSSs* and *OlSP* in *O. longistaminata*. *OlSS1-1* and *OlSS1-2* are mainly expressed in stamens, whereas *OlSS2-1* and *OlSS2-2* are expressed in both stamens and pistils, and *OlSP-1* and *OlSP-2* are highly and specifically expressed in pistils. *OlSS1*: *Self-incompatibility stamen1* from *O. longistaminata*; *OlSS2*: *Self-incompatibility stamen2* from *O. longistaminata*; *OlSP*: *Self-Incompatibility Pistil factor* from *O. longistaminata*; *O. sativa* A05: *Oryza sativa* chromosome 5.

thereafter gradually down-regulated in stages 9–14 (**Figure 5A**). Interestingly, the expression *OlSS2* in stamens was found to show a pattern similar to that of *OlSS1*, with the down-regulated expression of *OlSS2-1* started commencing from stage 10 (**Figure 5B**). Of the two *OlSS2* alleles, whereas *OlSS2-2* was up-regulated during pistil development but the expression of *OlSS2-1* no significant change (**Figure 5C**). Moreover, both *OlSP-1* and *OlSP-2* were rapidly up-regulated during pistil development, with highest expression levels being detected at stage 13 (**Figure 5D**).

Analysis of the expression profiles of *OlSP-1*, *OlSP-2*, *OlSS2-1*, and *OlSS2-2* after self-/cross-pollination revealed that the expression of *OlSP-1* was highest 5 min after pollination and increased gradually within 1 h after cross-pollination (**Figure 5E**). Contrastingly, *OlSP-2* exhibited no significant change following self-pollination, although showed an expression pattern similar to that of *OlSP-1* following cross-pollination (**Figure 5F**). Expression of *OlSS2-1* was found to be highest at 30 min after self-pollination, whereas no significant change following cross-pollination (**Figure 5G**). *OlSS2-2* was up-regulated within 30 min and subsequently down-regulated after self-pollination, whereas following cross-pollination, highest expression levels were detected within 5 min, after which expression was down-regulated (**Figure 5H**). These observations indicate that whereas *OlSS1* and *OlSP* are expressed in the stamens and pistils, respectively, *OlSS2* is expressed in both stamens and pistils, thereby indicating that these three genes are associated with stamen and pistil development. In addition, the results revealed that *OlSS1*, *OlSS2*, and *OlSP* have different expression patterns after self-/cross-pollination.

DISCUSSION

Studies on the genetics of SI in Gramineae were mainly conducted on *Secale cereale* and *Phalaris coerulescens*, and have indicated that SI in grass species is under

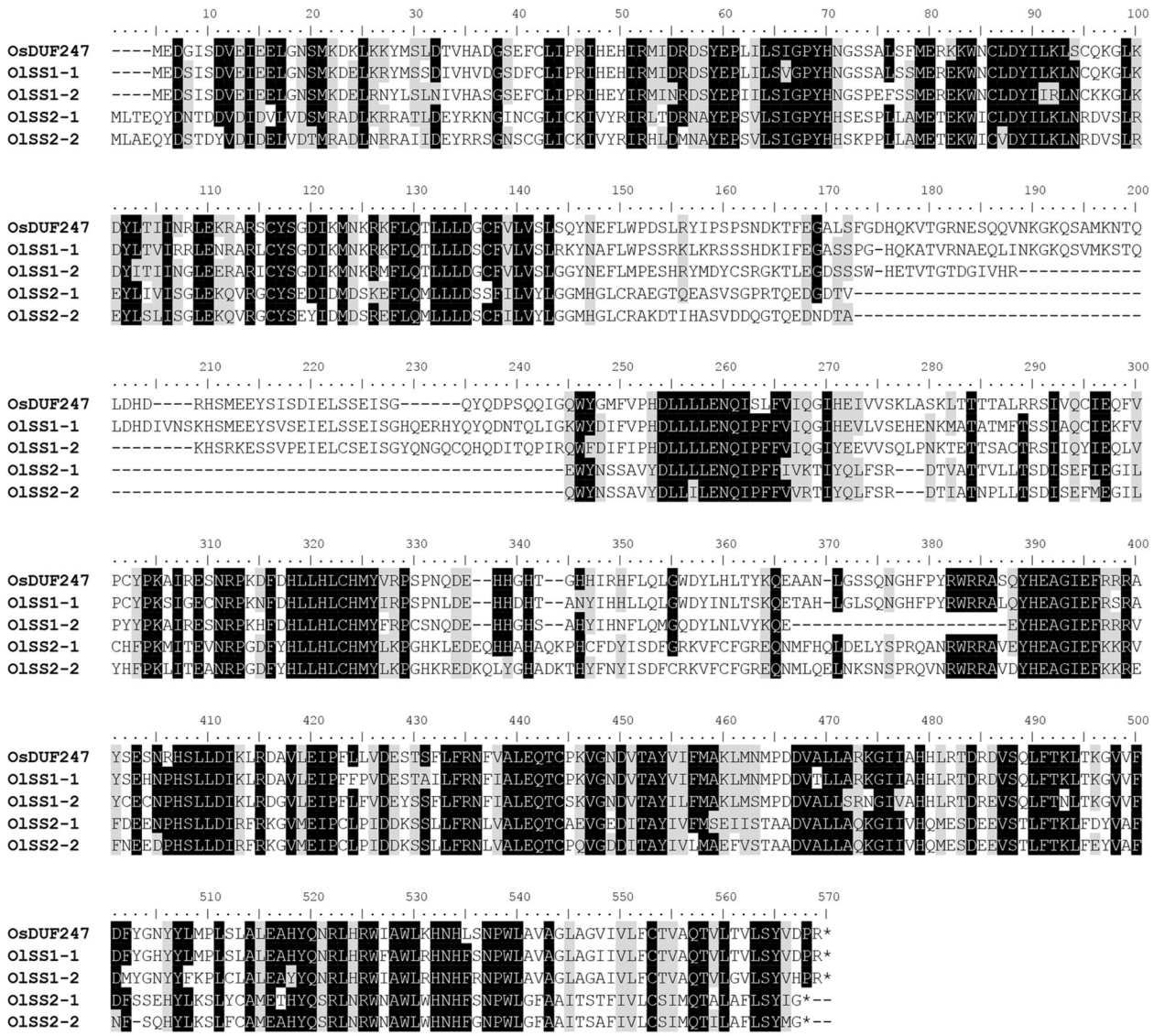

FIGURE 3 | Amino acid sequence polymorphism of OlSSs. Alignment of the amino acid sequences of OlSS1-1 and OlSS1-2 with that of OsDUF247 revealed similarities of 80.7 and 69.8%, respectively, whereas the corresponding similarities of OlSS2-1 and OlSS2-2 are 38.9 and 36.9%, respectively. Both OlSS1 and OlSS2 showed conserved regions at the N and C termini.

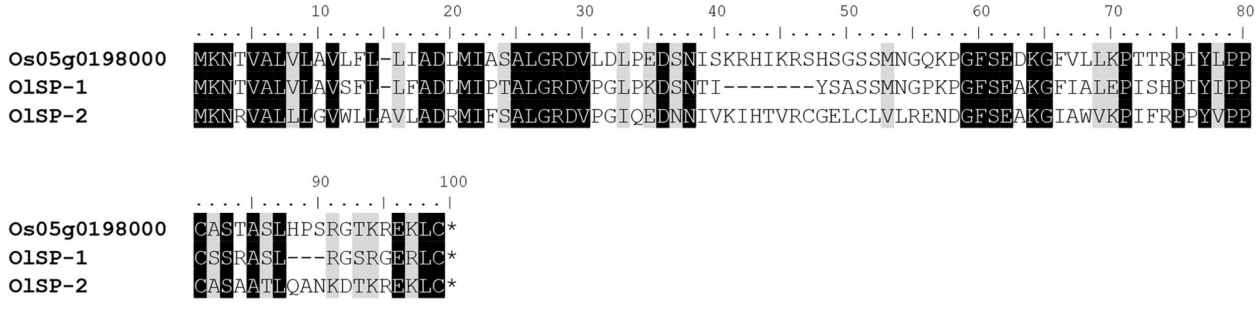

FIGURE 4 | Amino acid sequence polymorphism of OlSP. OlSP-1 and OlSP-2 are the amino acid sequences encoded by the two alleles of *OlSP* in *Oryza longistaminata*. The sequence similarity between OlSP-1 and OlSP-2 and Os05g0198000 is 51.8 and 63.2%, respectively.

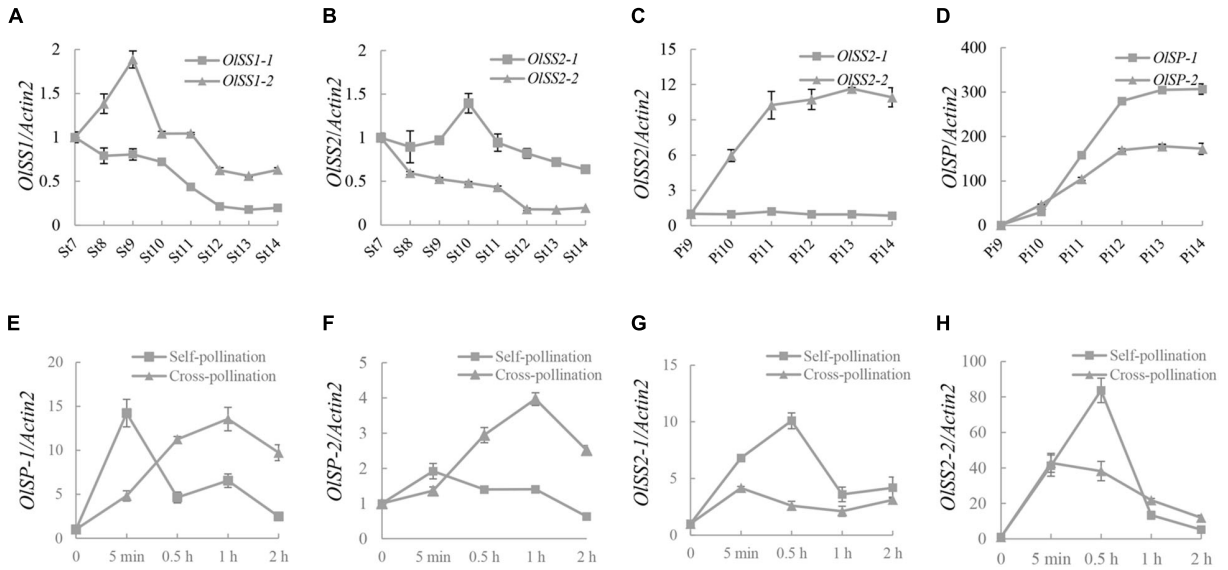

FIGURE 5 | The expression pattern of *OlSSs* and *OlSP*. **(A)** Expression profile of *OlSS1* from stage 7 to stage 14 in stamens. *OlSS1-1* was down-regulated at all stamen developmental stages, whereas *OlSS1-2* was up-regulated at stages 7–9, and gradually down-regulated from stages 9 to 14. **(B)** The expression pattern of *OlSS2* from stage 7 to 14 in stamens. The down-related expression of *OlSS2-1* commenced at stage 10. **(C)** The expression pattern of *OlSS2* in pistils. *OlSS2-2* up-regulated during pistil development, whereas we detected no significant changes in the expression of *OlSS2-1*. **(D)** The expression profile of *OlSP* in pistils. *OlSP-1* and *OlSP-2* were up-regulated in pistils. **(E)** The expression profile of *OlSP-1* in pistils after self-/cross-pollination. The down-regulated expression of *OlSP-1* commenced 5 min after self-pollination, and reached the highest level at 1 h after cross-pollination. **(F)** The expression profile of *OlSP-2* in pistils after self-/cross-pollination. *OlSP-2* showed significant changes in expression following self-pollination, but was up-regulated after cross-pollination, attaining the highest level after 1 h. **(G)** The expression profile of *OlSS2-1* in pistils after self-/cross-pollination. *OlSS2-1* was up-regulated within 0.5 h of self-pollination, after which the levels of expression began to decline. In contrast, no significant changes were detected in the expression of *OlSS2-1* following cross-pollination. **(H)** The expression profile of *OlSS2-2* in pistils after self-/cross-pollination. *OlSS2-2* was up-regulated within 0.5 h of self-pollination, and subsequently down-regulated. Following cross-pollination, the highest level of *OlSS2-2* expression was detected within 5 min, after which expression was down-regulated. St, stamen; Pi, pistil; St7 to St14, pollen development from stages 7 to 14; Pi9 to Pi14, pistil development from stages 9 to 14.

gametophytic control mediated by two unlinked multi-allelic loci referred to as *S* and *Z* (Lundqvist, 1954, 1956). Available data indicate that the two-locus SI system is common to all self-incompatible grass species of the subfamily Pooideae, and possibly to all members of the family Gramineae (Connor, 1979). The most significant differences with regards to SI are reciprocal differences in compatibility between two parents (Leach, 1988). In the present study, examination of the compatibility between the two investigated *O. longistaminata* germplasms (OlMK68 and OlMK23) revealed complete infertility following self-pollination and different seed-setting rates in reciprocal hybrids, indicating strict SI.

In SI, the transmission of signals between the stamens and pistils is dependent on the interaction of *S*-haplotypes with polymorphic sequences (Franklin-Tong et al., 1988; Franklin-Tong and Franklin, 2003). Therefore, putative specificity determinants should meet the following three criteria suggested by the biology and genetics of SI: linkage to the *S*-locus, polymorphism between different *S*-haplotypes, and expression in the pollen or pistil (Franklin-Tong, 2008). In the present study, the sequence polymorphisms and expression patterns of *OlSSs* and *OlSP* were in accordance with the characteristics of SI. Moreover, *OlSSs* and *OlSP* were found to be closely linked on the *O. longistaminata* genome, which is consistent with the signaling mechanism of SI.

The primer pairs used in this study were designed based on the allele sequences of *OlSSs* and *OlSP* from OlMK68, and on the basis of the tissue-specific expression and expression levels in stamens and pistils at different stages of developmental, we confirmed that *OlSSs* and *OlSP* are polymorphic loci. Moreover, qRT-PCR analysis revealed that the alleles of *OlSS1* and *OlSS2* show identical expression patterns in stamens, thereby indicating the comparable or synergistic functions of *OlSS1* and *OlSS2*. Given that the expression of *OlSS1* was gradually down-regulated during the latter stages of pollen maturation, further examination of the expression of this gene at the cellular level will be necessary to more precisely determine whether *OlSS1* is expressed in the pollen or in the tapetum. Besides, *OlSS2* was also expressed in the pistil, but only the *OlSS2-2* allele is up-regulated during pistil development. The reason for this phenomenon could be indictive of the complex mechanism of SI in *O. longistaminata*, which requires further study. A notable observation in the present study was that pollen on the stigma was unable to enter the style following self-pollination, indicating that either the pollen carries a male SI signal factor and/or that a pistil signal factor is secreted into the style. Although

OlSSs and *OlSP* are considered to be the respective candidate genes for these factors in the SI of *O. longistaminata*, the mechanisms whereby they contribute to the regulation SI need to be further studied.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FH and XL designed the experiments and wrote the manuscript. XL, SZ, and JZ performed the experiments. GH and LH analyzed the data. All authors have read and agreed to the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Substitution Mapping of a Locus Responsible for Hybrid Breakdown in Populations Derived From Interspecific Introgression Line

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Hybrid breakdown, a form of postzygotic reproductive barrier, has been reported to hinder gene flow in many crosses between wild and cultivated rice. Here, the phenomenon of hybrid breakdown was observed as low-tillering (i.e., low tiller number) in some progeny of an interspecific cross produced in an attempt to introduce *Oryza meridionalis* Ng (W1625) chromosomal segments into *Oryza sativa* L. ssp. *japonica* "Taichung 65" (T65). Low-tillering lines were obtained in BC_4-derived progeny from a cross between W1625 and "Taichung 65," but the locus for low-tillering could not be mapped in segregating populations. As a second approach to map the locus for low-tillering, we analyzed an F_2 population derived from a cross between the low-tillering lines and a high-yielding *indica* cultivar, "Takanari." A major QTL for low-tillering, *qLTN4*, was detected between PCR-based markers MS10 and RM307 on the long arm of chromosome 4, with a LOD score of 15.6. The low-tillering phenotype was associated with weak growth and pale yellow phenotype; however, low-tillering plant had less reduction of grain fertility. In an F_4 population (4896 plants), 563 recombinant plants were identified and the low-tillering locus was delimited to a 4.6-Mbp region between markers W1 and C5-indel3729. This region could not be further delimited because recombination is restricted in this region of *qLTN4*, which is near the centromere. Understanding the genetic basis of hybrid breakdown, including the low-tillering habit, will be important for improving varieties in rice breeding.

Keywords: rice, *Oryza meridionalis*, tiller number, substitution mapping, hybrid breakdown

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INTRODUCTION

Panicle number in rice (*Oryza sativa* L.) is a yield component that is directly influenced by tillering ability during the growth period. Tillers are additional culms (stems) that develop from the main culm and are similar to branches (Smith and Dilday, 2003). Tiller number is an important trait for improving rice varieties in breeding programs. In the late 1980s, new plant type (NPT) breeding was launched at the International Rice Research Institute (IRRI, Los Banos, Philippines) to increase rice yield potential. One of the target traits for the NPT was the low-tillering trait

(i.e., low number of tillers) because low-tillering varieties have fewer unproductive tillers (Janoria, 1989). Rice varieties with high tiller number are generally suitable under some conditions; however, excessive tillering can lead to yield reductions due to an increased number of unproductive tillers. Under wet direct-seeding conditions or in locations where drought is expected to occur episodically, desirable genotypes are expected to have few but vigorous tillers (Fujita et al., 2010). Therefore, the development of rice varieties with optimal tiller number for a specific environment could play an important role in increasing production (Kebrom et al., 2012).

Numerous studies have reported that tiller and panicle number (PN) is controlled by multiple genes (Zhu et al., 2011; Hussien et al., 2014). Across these studies, several major genes for tiller number, initially identified through mutant phenotypes, have been isolated and validated. The gene *TEOSINTE BRANCHED-1,* which negatively regulated lateral branching, was detected on chromosome 3 (Takeda et al., 2003). *MONOCULM 1*, which encodes a putative GRAS family nuclear protein, was located on chromosome 6 and promotes the formation of axillary buds (Li et al., 2003). Two genes for reduced culm number, *RCN8* and *RCN9*, have been mapped on the long arm of chromosome 1 and the long arm of chromosome 6, respectively (Jiang et al., 2006). The *Ltn* (low tiller number) gene from "Aikawa 1" was detected on the long arm of chromosome 8 (Fujita et al., 2010), and *ltn2* from NPT rice was identified on chromosome 7 at a distance of 2.1 cM from the SSR marker RM21950 (Uddin et al., 2016).

The use of exotic germplasm, including wild rice species, is expected to extend the available genetic variation in cultivated rice. To facilitate improvement of disease and insect resistance, resistance genes from wild rice species have been introduced into cultivated rice varieties (Khush, 1997). However, the identification of genes from wild rice species is difficult because of reproductive barriers between rice cultivars and wild rice species. Thus, there are few reports on exploitation of genes from wild rice species related to agronomic traits such as tiller number. To identify potentially useful genetic factors in wild rice species, Yoshimura et al. (2010) developed a set of introgression lines (ILs) carrying wild donor chromosomes segments in an uniform genetic background. The ILs are important materials for precisely evaluating the phenotype of each plant and fine-mapping genes as single Mendelian factors.

As mentioned above, the introgression of agronomically useful genes from wild rice into cultivated rice is often prevented by reproductive barriers. Hybrid breakdown, a type of postzygotic reproductive barrier, is defined as weakness or sterility in the F_2 or later generations (Fukuoka et al., 1998; Yamamoto et al., 2007, 2010; Miura et al., 2008; Kubo, 2013). In previous studies, genes inducing hybrid breakdown have been reported in both inter- and intraspecific crosses of AA genome species. The AA genome wild rice is considered to be the direct ancestor of cultivated rice. The *hybrid weakness f-1* gene from *O*ryza *glumaepatula* was located on the short arm of chromosome 4 and found to induce hybrid breakdown in the genetic background of *japonica* rice variety "Taichung 65" (T65) (Sobrizal and Yoshimura, 2009). The *hbd1(t)* gene from *O*ryza *nivara* was located on the short arm of chromosome 2 and produced a similar hybrid breakdown phenotype in the "Koshihikari" genetic background (Miura et al., 2008). To our knowledge there are no other reports of hybrid breakdown genes involving other AA-genome species.

To broaden the available rice genetic resources from wild species, *O. meridionalis* (AA genome), a wild rice that is endemic to Oceania, New Guinea, and Australia (Sotowa et al., 2013) was used to develop 36 ILs with introduced chromosome segments of *O. meridionalis* in the genetic background of T65 (Yoshimura et al., 2010). Among these ILs, we identified one line with yellow leaves and greatly reduced tiller number. Through analysis of a segregating population derived from a cross between the low-tillering IL and T65, we identified a locus related to low-tillering but could not map it in that population. By crossing the low-tillering line to another cultivar ("Takanari"), producing segregating populations, and performing substitution mapping, we delimited a major QTL for low-tillering.

MATERIALS AND METHODS

Plant Materials Containing Low-Tillering Trait in T65 Genetic Background

In a previous study *O. meridionalis* (W1625) was crossed with *O. sativa* ssp. *japonica* "Taichung 65", and the F_1 plants were repeatedly backcrossed to T65 as the recurrent parent (**Figure 1**; Yoshimura et al., 2010). A set of W1625 ILs (36 lines) in BC_4F_4 was selected by marker-assisted selection (MAS) (Yoshimura et al., 2010). One line among the W1625 ILs showed segregation of a particular phenotype with weaker growth (low-tillering and yellow leaves) compared with normal plants. To identify the locus controlling low tiller number, a BC_4F_5 segregating population (derived from a single self-pollinated BC_4F_4 plant) was used for genetic analysis. To confirm the genotype at the low-tillering locus for each BC_4F_5 plant, the corresponding BC_4F_6 lines were used for progeny tests. A set of 119 BC_4F_5-derived lines was used for analyzing the introgressed chromosomal segments with SSR markers. BC_5F_1 plants derived from a cross between T65 and low-tillering plant (BC_4F_5) were used for confirming the effect of the low-tillering trait on other agronomic traits. The BC_5F_2 plants were evaluated for tillering number and panicle structure.

Plant Materials for Mapping the QTL

To map the locus for low-tillering, we developed an F_2 population derived from a cross between the low-tillering plant (BC_4F_5) and a high-yielding *indica* cultivar, "Takanari." The F_2 plants were self-pollinated to generate F_3, F_4, and F_5 lines for substitution mapping of the low-tillering locus. $F_{2:3}$ and $F_{4:5}$ lines were used for progeny tests to confirm the genotypes at the low-tillering locus of the F_2 and F_4 plants, respectively.

Characterization of Agronomic Traits in Normal- and Low-Tillering Lines

All seeds were incubated at 25°C for 3 days to induce germination and then sown in seeding trays. The seedlings were transplanted

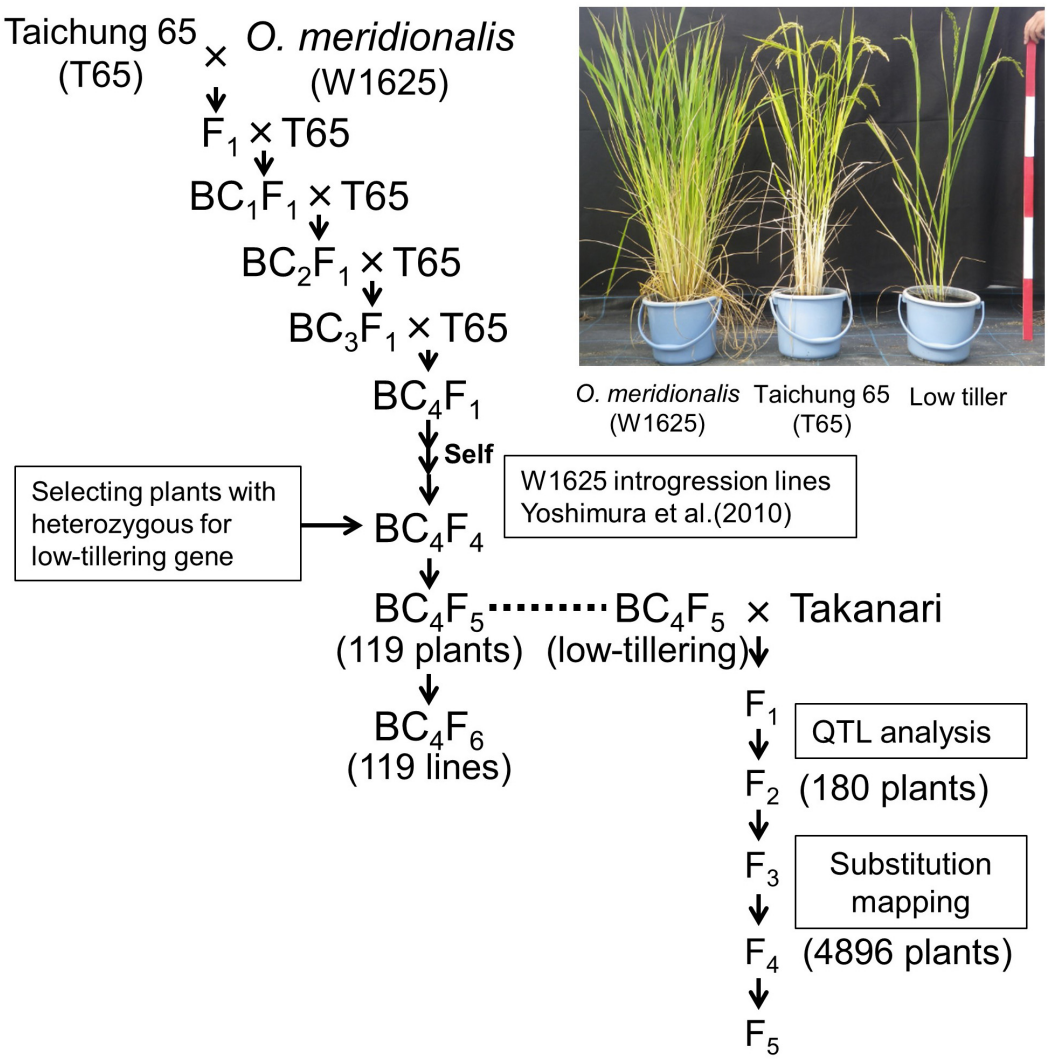

FIGURE 1 | Breeding scheme for developing lines with low-tillering. The BC_4F_5 and BC_4F_6 populations were derived from a cross between "Taichung 65" (T65) and *O. meridionalis*. The F_2 population was derived from a cross between low-tillering BC_4F_5 plants and "Takanari."

to the paddy field at Saga University (Saga, Japan) at 30 days after sowing and planted one plant per hill. The BC_4F_5 population was planted with 18 cm between plants within a row and 30 cm between rows in an experimental field at Kyushu University (Kasuya, Fukuoka, Japan). The other materials (BC_4F_6, BC_5F_1, BC_5F_2, F_2, F_3, F_4, and F_5) were planted with 20 cm between plants within a row and 25 cm between rows in an experimental field at Saga University (Saga, Japan). The PNs per plant were observed at the maturity stage and measured in all segregating populations. In the F_3, and F_5 generations, which were used for progeny tests (using 20 plants of each entry), plants with fewer than 5 panicles were considered as low-tillering. To examine tiller number after transplanting, 10 plants per entry were transplanted to 5-L pots and tiller numbers were counted every week. To understand the effects on other agronomic traits caused by low-tillering locus, the BC_4F_6 and BC_5F_1 plants (10 plants of each entry) were evaluated for culm length (CL), panicle length (PL), PN, leaf length (LL), leaf width (LW), number of primary branches (NPB), number of secondary branches (NSB), total spikelet number per panicle (TSN), grain fertility per panicle (GF), total number of spikelets on the primary branch (TSPB), and total number of spikelet on the secondary branch (TSSB).

DNA Extraction and Genotyping

The genomic DNA of parents and segregating populations was extracted from freeze-dried leaves by using the potassium acetate protocol (Dellaporta et al., 1983). In preparation for extraction, the leaves were collected in 96-well deep-well plates and crushed using a FastPrep 96 (MP Biomedicals, United States). DNA marker genotypes were determined by PCR with a Gene Atlas thermal cycler (ASTEC, Japan). PCR amplification conditions consisted of 96°C for 5 min; followed by 35 cycles of 96°C for 5 min, 55°C for 30 s, and 72°C for 30 s; followed by a final

extension of 25°C for 1 min. The PCR products were separated by electrophoresis in 4% agarose gel, stained with ethidium bromide, and viewed under ultraviolet light.

Construction of the Linkage Map and QTL Analysis

The DNA markers were first screened for polymorphism between the low-tillering line and "Takanari" and then used for genotyping the F_2 populations. The genetic map for the F_2 population was constructed using 113 markers that were polymorphic between the parents and distributed across all 12 chromosomes. In this map, genetic distances were determined using the Kosambi function. The QTL analysis was performed by Windows QTL Cartographer software version 2.5 and conducted using data for PN and DNA marker genotypes (Wang et al., 2012). On the basis of a 1000-permutation test, a LOD score greater than 3.6 was considered as the threshold at a 0.05 level of significance.

Substitution Mapping of the Low-Tillering Locus

To determine the precise location of the low-tillering locus, the tillering phenotypes and genotypes of DNA markers around the low-tillering locus in 180 F_3 lines were used to confirm the genotypes at that locus in the F_2 population. To further delimit the location of the low-tillering locus, a large segregating F_4 population (4896 plants) derived from F_3 lines heterozygous at the low-tillering locus were screened with markers RM8213 and RM307 to select recombinant plants. The genotypes of recombinant plants were analyzed using nine SSR markers (RM16459, RM16502, RM16535, RM16550, RM16605, RM16626, and RM307) and three Indel markers (RH7, W1, C5-indel3729, C5-indel3743, and C5-indel3757) around the low-tillering locus (**Supplementary Table 1**). Finally, an F_5 progeny test was performed to confirm the genotype of the low-tillering locus for each F_4 individual.

RESULTS

Identification of the Low-Tillering Locus

The tiller numbers per plant of W1625, T65, and a low-tillering line (BC_4F_6 generation which seems to have had homozygous low-tillering lines) were evaluated every week during the tillering stage (**Figure 2**). Throughout the tiller development stage, the low-tillering line continually showed few tillers. The PNs at maturity of W1625, T65, and the low-tillering line were 44, 13, and 4, respectively. The number of panicles per plant of W1625 was higher than that of T65 at the maturing and greatly reduced in the low-tillering line compared with both parents. These data suggested that the total number of panicles in the low-tillering line (with T65 genetic background) was affected by one or more introgressed segments from W1625.

To identify the locus for low-tillering, the BC_4F_5 segregating population was analyzed. The frequency distribution of PN in that population showed a bimodal distribution: 15 plants with low-tillering (one panicle per plant) and 104 plants with PN ranging from 4 to 10 (**Figure 3A**). To elucidate the genotypes at the low-tillering locus in the BC_4F_5 segregating population, the PNs in BC_4F_6 lines were observed as a progeny test. Among the 119 BC_4F_5 plants, 15 plants were homozygous for the W1625 allele at the low-tillering locus (progeny were all low-tillering), 60 plants were heterozygous (progeny segregated for low-tillering phenotype), and 44 plants were homozygous for the T65 allele (progeny were all normal tillering). The segregation ratio in the BC_4F_5 population (15:60:44) did not fit the 1:2:1 expected ratio ($\chi^2 = 14.1$, $P = 0.001$).

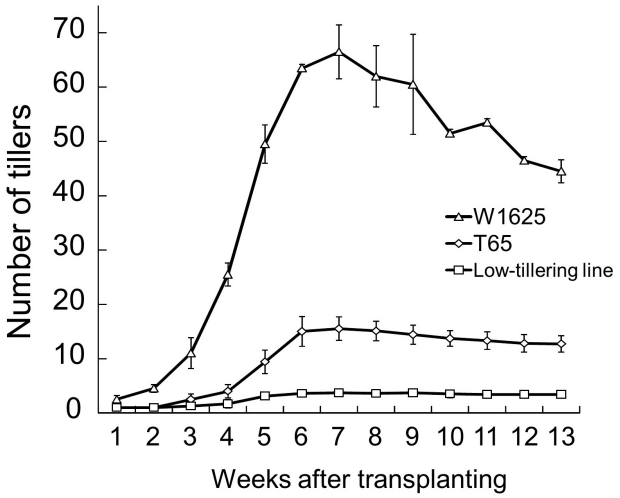

FIGURE 2 | Fluctuation in tiller number during plant development in W1625, T65, and the low-tillering line. Data are means ± SD (n = 10).

Identification of W1625 Chromosomal Segments Retained in the BC_4F_5 Population

The chromosomal constitution of the low-tillering line was assessed by using 492 SSR markers (**Figure 3B**). There were three introgressed segments from W1625 detected in the low-tillering line: one on the short arm of chromosome 1 and two on chromosome 3. Additionally, through genotyping by sequencing, three large introgressed segments from W1625 on the low-tillering line were identified at same regions using 5445 SNPs and there are three small regions introgressed segment from W1625 on chromosomes 4, 10, and 12 (**Supplementary Figure 1**). There was no association between tiller number and genotype of these three large W1625 segments in the BC_4F_5 segregating population. In BC_5F_2 segregating population, there are no association between agronomic traits (PN, TSN, and SNB) and genotype of these three large W1625 segments and the TSN and SNB of BC_5F_2 plants with low-tillering showed lower than that of T65 (**Supplementary Figure 2**). These results suggested that other chromosomes might harbor small introgressed segments from W1625 that are related to the low-tillering phenotype, TSN and SNB.

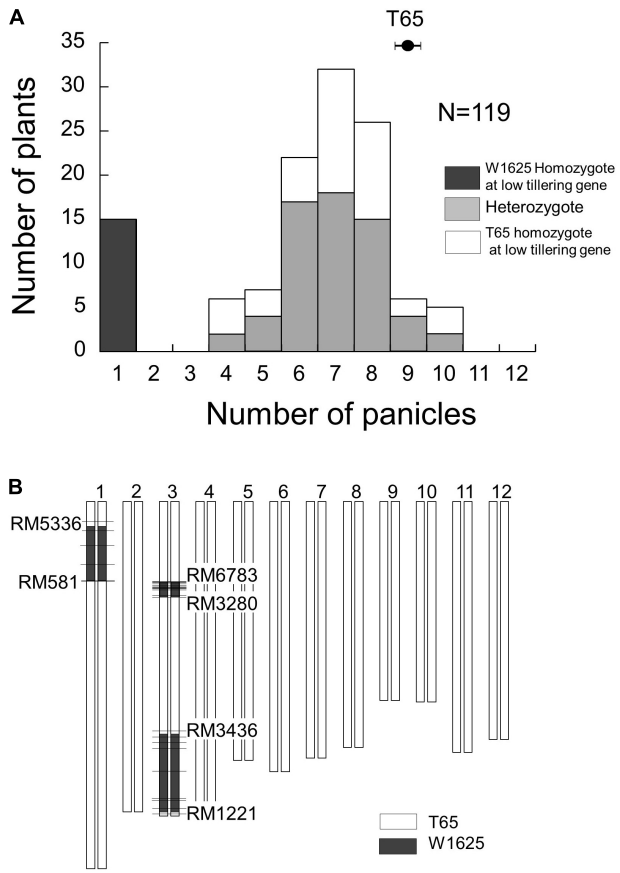

FIGURE 3 | Frequency distribution of panicle number **(A)** and graphical genotype of low-tillering line **(B)** in BC_4F_5 population derived from a cross between T65 and W1625. Black, gray, and white colors in **(A)** indicate W1625 homozygote, heterozygote, and T65 homozygote for low-tillering locus through scored based on segregation of phenotypes in the next generation. The horizontal bars in **(B)** show the location of polymorphic SSR markers.

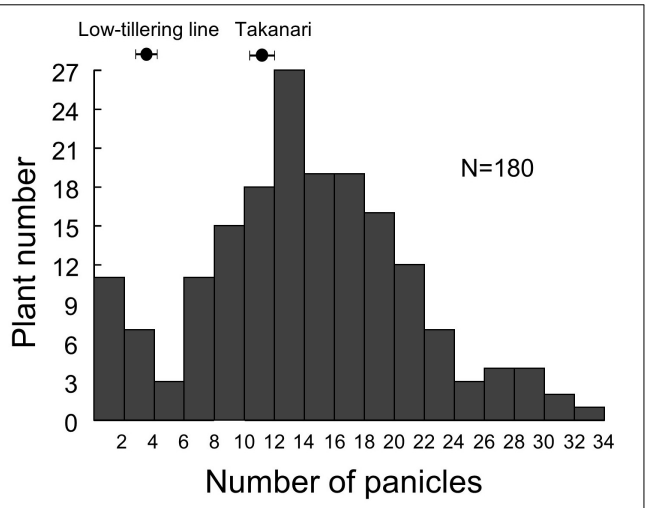

FIGURE 4 | Frequency distribution of panicle number in F_2 population derived from a cross between low-tillering plants and "Takanari."

Detection of a QTL for Panicle Number

Because we were unable to detect the location of the low-tillering locus in the BC_4F_5 population, we developed an F_2 population from a cross between the low-tillering line and "Takanari" to conduct QTL analysis. The PNs of the parents were 4 for the low-tillering line and 11 for "Takanari" (**Figure 4**). The frequency distribution for PN per plant in the F_2 population showed a continuous distribution with approximately 60% of the individuals having a higher PN than the parents. To estimate the location of the QTL for PN, a QTL analysis was conducted using this F_2 population. A QTL designated *qLTN4* (QTL for low tiller number on chromosome 4) was detected between MS10 and RM307 on the short arm of chromosome 4 (**Table 1**). This QTL had a LOD score of 15.6 and explained 30.4% of the phenotypic variation for PN. The allele from the low-tillering line at *qLTN4* was associated with decreased PN per plant. Additionally, five regions on chromosomes 5, 6, 8, 10, and 11 had LOD peaks and LOD score is less than threshold of LOD score at 5% level. The alleles from the low-tillering line at two QTLs, *qLTN5*, and *qLTN6*, showed increasing PN per plant, while the alleles from the low-tillering line at three QTLs, *qLTN8, qLTN10,* and *qLTN11*, showed decreasing PN per plant.

Substitution Mapping of *qLTN4*

To delimit the location of *qLTN4*, a progeny test was performed using F_3 lines. These lines, derived from F_2 plants with recombination between markers RM8213 and RM307, were classified into three phenotypes: low-tillering fixed, segregating, and normal-tillering fixed. By comparing F_2 genotypes and F_3 phenotypes, *qLTN4* was located within an 8.8-Mbp interval between markers RM8213 and RM307 (**Figure 5A**). To further refine the location of *qLTN4*, recombinant plants were selected from 13 F_4 families with heterozygosity at *qLTN4*. Among a large segregating population (4896 F_4 plants), 563 plants with a recombination event between markers RM8213 and RM307 were selected. These 563 plants were evaluated for PN and genotyped using PCR-based markers. Based on the phenotypes and genotypes at each marker for 13 recombinant plants, lines ISC11-3 and ISC8-18, with recombination that took place between markers W1 and C5-indel3729, delimited the low-tillering locus to a 4.6-Mbp region (**Figure 5B**).

Characterization of Agronomic Traits Affected by *qLTN4* on Low-Tillering Line

To determine the influence of *qLTN4* on agronomic traits, we characterized panicle architecture and other traits in the low-tillering line (BC_4F_6). CL and PN, of the low-tillering line were significantly lower than those of T65 despite their similar genetic background (**Table 2**). On the other hand, LW was significantly larger in the low-tillering line than in T65 or the BC_5F_1. BC_5F_1 plants derived from a cross between T65 and the low-tillering line had similar agronomic traits to T65 with the exception of PL, which was significantly larger in the BC_5F_1.

TABLE 1 | The QTL for tiller number identified in an F_2 population derived from a cross between "Takanari" and low-tillering line.

QTL	Chromosome	Marker interval	Peak LOD score	PVE (%)[a]	Additive effect[b]	Dominance effect[b]
qLTN4	4	MS10-RM307	15.6	30.4	7.4	5.5
qLTN5	5	RM249-RM430	2.9	5.4	−1.9	−1.8
qLTN6	6	RM6395-RM1370	1.6	2.8	−1.8	0.4
qLTN8	8	RM1345-RM25	2.4	6.2	2.5	−0.5
qLTN10	10	RM1873-RM496	1.8	3.2	1.3	−1.6
qLTN11	11	RM6091-RM229	2.5	4.4	0.7	2.8

The QTLs for panicle number has more than 1.5 of LOD score.
[a]*Proportion of the phenotypic variation explained by QTL.*
[b]*Values indicate the relative effect of the "Takanari" allele.*

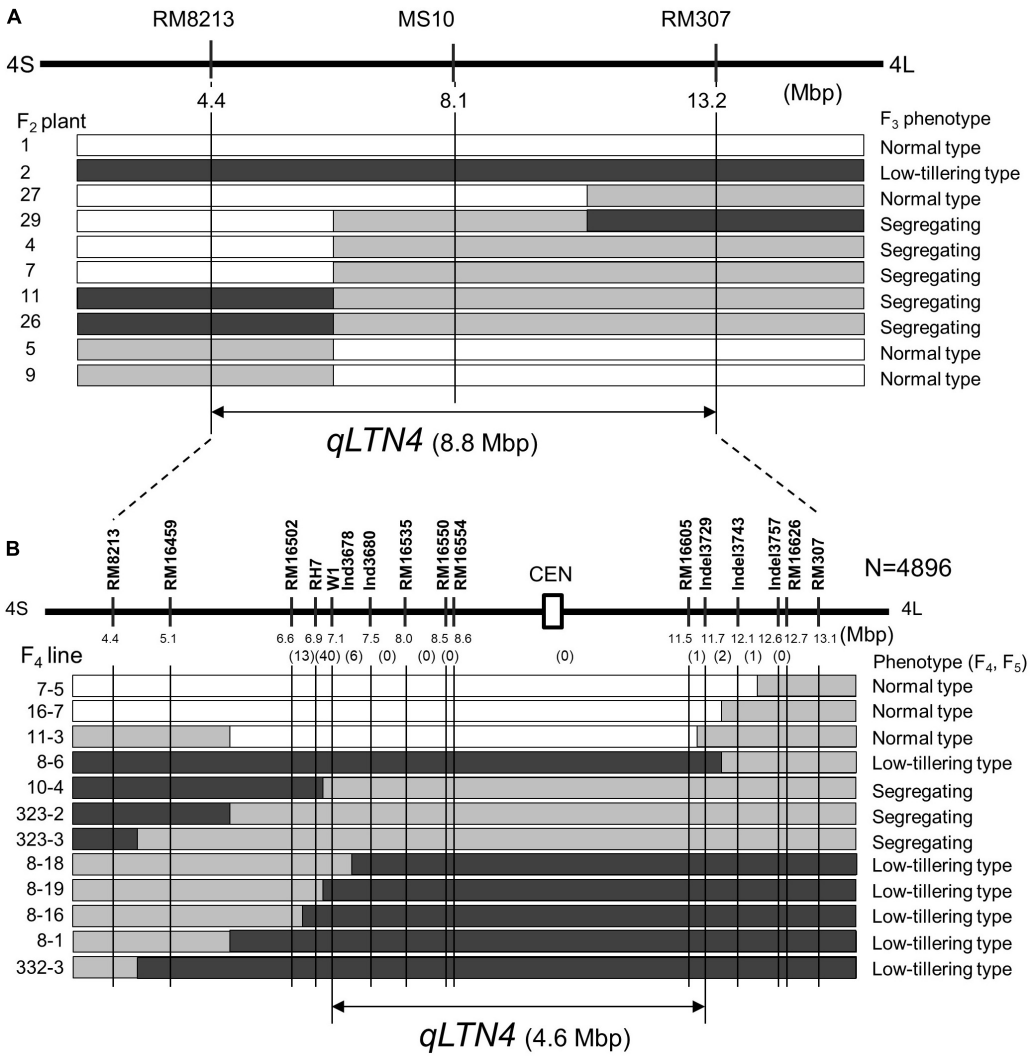

FIGURE 5 | Substitution map of locus for low tiller number. **(A)** Substitution mapping of *qLTN4* in F_2 plants and F_3 lines derived from a cross between "Takanari" and the low-tillering line. **(B)** Substitution mapping of *qLTN4* in F_4 and F_5 lines. Black, gray, and white indicate regions homozygous for marker allele from low-tillering line, heterozygous, and homozygous for marker allele from "Takanari." The white rectangle with CEN indicate centromere region of chromosome 4.

There were no significant differences for CL, PN, LL, and LW between T65 and BC_5F_1 plants. Several traits related to panicle architecture, i.e., TSPB, TSSB, and TSN, were significantly lower in the low-tillering line than in T65. The grain fertility of the low-tillering line was 75.4%, significantly lower than that of T65 (94.5%; **Table 3**). However, NPB, NSB, and TSPB of the BC_5F_1 were not significantly different from those of the low-tillering line.

TABLE 2 | Agronomic traits of "Taichung 65" (T65), BC_5F_1 (T65/low-tillering line), and low-tillering line.

Line	CL (cm)	PL (cm)	PN	LL (cm)	LW (cm)
Taichung 65 (T65)	99.6 ± 3.6a	19.8 ± 1.8a	13.0 ± 3.6a	42.8 ± 4.2a	1.41 ± 0.09a
BC_5F_1 (T65/Low-tillering line)	102.2 ± 4.3a	24.4 ± 0.6b	9.8 ± 1.9a	39.0 ± 3.5a	1.44 ± 0.09a
Low-tillering line	67.6 ± 4.4b	20.5 ± 1.3a	3.8 ± 1.7b	37.9 ± 5.3a	1.70 ± 0.09b

Data are means ± SD (n = 10). The different alphabetical letters indicate significant difference ($p < 0.05$, Tukey Kramer test).

TABLE 3 | Panicle structure of "Taichung 65" (T65), BC_5F_1 (T65/low-tillering line), and low-tillering line.

Line	NPB	NSB	TSPB	TSSB	TSN	GF (%)
Taichung 65 (T65)	12.0 ± 1.1a	34.5 ± 6.3a	68.7 ± 7.1a	102.9 ± 23.5a	171.5 ± 24.2a	94.5 ± 3.5
BC_5F_1 (T65/Low-tillering line)	10.7 ± 2.9a	34.1 ± 10.1a	58.1 ± 15.6b	109.3 ± 33.9a	167.3 ± 48.1a	ND[1]
Low-tillering line	10.1 ± 1.6a	22.0 ± 5.4a	55.6 ± 7.0b	63.4 ± 18.2b	118.9 ± 23.2b	75.4 ± 10.3

Data are means ± SD (n = 5).
[1]The BC_5F_1 plants were not observed grain fertility. The different alphabetical letters indicate significant difference ($p < 0.05$, Tukey Kramer test).

DISCUSSION

Tillering, i.e., the production of multiple stems, is an important agronomic trait that has been widely studied in cereals. In previous studies, genes and QTLs with large effects on tiller number were mapped in various regions of the rice genome. *MONOCULM 1* was located on chromosome 6 (Li et al., 2003), *Ltn* from "Aiwaka 1" on chromosome 8 (Fujita et al., 2010), and *ltn2* from an NPT variety on chromosome 7 (Uddin et al., 2016). Among the QTLs and genes for tiller number on chromosome 4, *tn4-1* was located between markers *RG190* and *RG908* and was detected by the conditional QTL method (Yan et al., 1998). The QTL detected in the present study, *qLTN4*, was detected on chromosome 4 and had a large contribution to the phenotypic variation (30.4%) and high LOD score (15.6). The chromosomal region of *qLTN4* overlapped with that of *tn4-1* (Yan et al., 1998), suggesting that *qLTN4* might be same QTL.

In a previous study, segregation distortion as a consequence of nuclear and cytoplasmic factors and zygotic selection was observed in two reciprocal F_2 crosses and a BC_1 population (Reflinur et al., 2014). Another report mentioned that segregation distortion was caused by other factors such as population size, genotyping errors, and some non-biological factors (Falconer and Mackay, 1996). Furthermore, among different cross combination, different factors may contribute to segregation distortion (Xu et al., 1997). In the present study, the distribution of PN in the BC_4F_5 segregating population showed a slightly bimodal distribution but did not fit the expected 3:1 ratio. In the F_2 population, significant segregation distortion was observed at MS10 (near *qLTN4*), reducing the frequency of low-tillering plants. Similarly, the low-tillering gene locus segregated in the BC_4F_5 population as 44 homozygous for the T65 allele, 60 heterozygous and 15 homozygous for the W1625 allele. This same tendency for segregation distortion around *qLTN4* in both F_2 and BC_4F_5 populations is evidence for segregation distortion in this region.

The low-tillering lines were characterized in different growth conditions. In BC_4F_5 population, the plants with 18 cm between plants within a row and 30 cm were grown in Fukuoka, JAPAN. In BC_4F_6, BC_5F_1, F_2, F_3, F_4, and F_5, the plants with 20 cm between plants within a row and 25 cm were grown in Saga, JAPAN. The PN of low-tillering plant is one in BC_4F_5 population (**Figure 3A**), while the PN of low-tillering line of BC_4F_6 is 3.8 (**Table 2**). The difference suggested that the PN of low-tillering line is influenced by environmental factors such as transplanting interval. Therefore, we defined plants with fewer than 5 panicles as low-tillering in progeny test (F_3 and F_5 those were grown in Saga, JAPAN)

The low-tillering line was analyzed for detecting introgression segments from W1625. Based on SSR markers, three segments were identified on chromosomes 1 and 3. However, the chromosome segment from W1625 around the *qLTN4* region on the low-tillering line was not detected. Therefore, through genotyping by sequencing, 5445 SNPs was used for detecting W1625 chromosomal segment on low-tillering line. Three large segments on chromosomes 1 and 3 and three additional small segment on chromosomes 4, 10, and 12 have been detected. However, the location of small chromosomal segment of W1625 on chromosome 4 is outside *qLTN4* region. Furthermore, we read whole genome sequence of low-tillering lines and several SNPs from W1625 detected around *qLTN4* region (**Supplementary Figure 3**). However, we cannot find exact evidence of association between low-tillering and W1625 chromosomal segment in this area. Therefore, these results suggested that there are possibilities for small introgression segment of W1625 locating on this region or natural mutation of low tiller line. The SNPs and NGS analysis were conducted based on Nipponbare genome sequence, therefore, we might not detect W1625 chromosomal segments that are absent in Nipponbare genome. In future study, *de novo* sequence of W1625 would be necessary for detecting W1625 chromosomal segments. Also, the detection of *qLTN4* on earlier generation of segregation population derived from cross between T65 and W1625 would be possible to confirm association between low-tillering and W1625 chromosomal segments.

Meiotic recombination, which is the exchange of DNA between homologous chromosomes, is a fundamental process in eukaryotic reproduction (Dreissig et al., 2019). Previous

studies have reported that recombination rates vary along chromosomes in most organisms with large genomes. High recombination rates were found near the distal ends of the chromosomes while low recombination rates were found in the region surrounding the centromere (Stapley et al., 2017; Haenel et al., 2018). Here, *qLTN4* was detected near the centromeric region of chromosome 4. In substitution mapping of *qLTN4*, we failed to narrow down the candidate region due to difficulty in finding recombinant plants in the $F_{3:4}$ generation. This difficulty might have been caused by recombination restriction around the centromeric region of chromosome 4. In 4896 plants those were segregated at *qLTN4*, there is region for recombination restriction between RM16535 (8.0-Mbp) and RM16605 (11.5-Mbp) and we cannot obtain any recombinant plants between RM16535 and RM16605. Additionally, the genotypes of *qLTN4* through progeny test were completely linked to those of RM16535 and RM16605. To further delimit the *qLTN4* genomic region and identify candidate genes, expanding segregating population will be essential in a future study.

Hybrid breakdown has been described as sterility and weakness in F_2 or later generations resulting from inter- or intraspecific crosses and can affect all aspects of plant development. Several studies have reported plants expressing various phenotypes such as sterility; severe reduction in plant height, tiller number, root number, and root length; or even plant death before heading time (Sato and Morishima, 1988; Kubo and Yoshimura, 2002; Miura et al., 2008). Yamamoto et al. (2010) observed two types of weak plants, severe and mild, in the F_2 progeny of a cross between "Koshihikari" and "Habataki." In the present study, the low-tillering plants (BC_4F_5) derived from a cross between T65 and *O. meridionalis* showed severe reduction in PN but only moderate reduction in other panicle traits, CL, and grain fertility compared with the recurrent parent. The phenotypes of the low-tillering line were considered to be caused by hybrid breakdown because they were observed in later generations derived from crosses between normal parental lines. We consider the type of hybrid breakdown that occurred in this study as the mild type described by Yamamoto et al. (2010). The low-tillering locus on the short arm of chromosome 4 was associated with reduction in CL, PL, PN, LW, NSB, and TSN. In a previous study by Sobrizal and Yoshimura (2009), the *hybrid weakness f-1* gene, associated with hybrid breakdown in a cross between T65 and *O. glumaepatula*, was located between G3006 and C933 (12.7-Mbp) on the short arm chromosome 4 and was completely linked to C708 (6.3-Mbp), C802 (6.9-Mbp), and R288 (8.2-Mbp). The *hybrid weakness f-1* gene showed a similar phenotype to *qLTN4* in the genetic background of T65. The *qLTN4* was located between W1 (7.1-Mbp) to Indel3729 (11.7-Mbp) and was overlapped with the regions of *hybrid weakness f-1* gene. Thus, we infer that the locus identified in our study might be the same locus that was previously identified by Sobrizal and Yoshimura (2009). Several studies have reported distant relationships between *O. meridionalis* and other AA-genome rice species (Second, 1986; Noredo et al., 1998). These results suggest that various AA-genome rice species may share a conserved region around *qLTN4* that is related to hybrid breakdown.

Several studies have proposed that interactions between complementary genes derived from each parent are the cause of hybrid weakness and hybrid breakdown (Fukuoka et al., 1998; Kubo and Yoshimura, 2002; Ichitani et al., 2007, 2011). In our study, only one locus involved in hybrid breakdown was detected. Assuming that hybrid breakdown in this case was also caused by the interaction of two complementary genes, the hybrid breakdown gene from T65 remains to be identified. Miura et al. (2008) were able to identify the gene responsible for hybrid breakdown in *O. nivara*; however, the complementary gene for hybrid breakdown from "Koshihikari" was not identified, suggesting a possible complex mechanism controlling this trait.

Hybrid breakdown has been reported as a hindrance to gene flow in many plant crosses. In this study, we observed a beneficial trait, low-tillering frequency, as a result of hybrid breakdown. Because the effects on other agronomic traits were mild, the allele with low-tillering on chromosome 4 might be useful for rice genetic improvement via production of low-tillering lines in breeding programs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

NM, SI, and DF designed the research. DF, NM, SI, and ZD performed the research. S-HZ, YY, and HY provided advice on the experiments. NM and DF wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.633247/full#supplementary-material

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