Molecular Plant
Letter to the Editor

CellPress
PARTNER JOURNAL

Interspecific Hybrid Sterility in Rice Is Mediated by *OgTPR1* at the *S1* Locus Encoding a Peptidase-like Protein

Dear Editor,

The Asian cultivated rice (*Oryza sativa* L. ssp *japonica* and ssp *indica*) is a staple food crop, and current rice breeding for the utilization of hybrid vigor (heterosis) mainly uses crosses within and between *japonica* and *indica* varieties (Chen and Liu, 2016). However, another cultivated rice, African rice (*Oryza glaberrima* Steud), has many important traits, such as tolerance to heat, drought, aluminum toxicity, and disease (Brar and Khush, 1997). Hybrids from crosses between species, such as between African and Asian rice varieties, have stronger hybrid vigor and greater yield potential than those within subspecies; these hybrids can also incorporate many important traits from the parents. However, such hybrids often show severe hybrid sterility (HS), a postzygotic reproductive barrier that restricts gene flow between species and maintains species identity during speciation (Ouyang and Zhang, 2013). Thus, HS hampers the utilization of heterosis in crops (Linares, 2002). Therefore, identification of the causal genes for HS is important for molecular genetic and evolutionary studies and for crop breeding.

Genetic studies in rice have identified several loci for HS, but only a few of the causal genes have been cloned (Ouyang and Zhang, 2013). A "two gene/three component interaction" model is proposed for the *Sa* locus, in which protein–protein interactions among the diverged alleles of two adjacent genes cause allele-specific pollen abortion in *indica–japonica* hybrids (Long et al., 2008), while another "Killer-Protector" model is suggested for the *S5* locus, in which three closely located genes interact to cause hybrid female sterility in *indica–japonica* hybrids (Yang et al., 2012). The *S1* locus is the most important genetic factor affecting the incompatibility between Asian and African rice, as *S1* heterozygotes show both male and female sterilities, which eliminate transmission of majority of the *S1* allele of Asian rice in the hybrid progenies (Koide et al., 2008). The *S1* locus was mapped to chromosome 6 (Garavito et al., 2010), but the gene for *S1*-mediated HS has not yet been identified.

To clone *S1*, we developed a near-isogenic line (NIL-g, *S1-g/S1-g*) using an African rice line (IRGC102203) as the *S1-g* allele donor and a *japonica* rice line (IRAT216), carrying the *S1-s* allele as the recurrent parent (RP-s, *S1-s/S1-s*) (Supplemental Figure 1). The RP-s (*S1-s/S1-s*) and NIL-g (*S1-g/S1-g*) lines exhibited full fertility, but their hybrid plants (BC_9F_1, *S1-g/S1-s*) showed semi-sterility of pollen and spikelets due to selective abortion of most male and female gametes carrying *S1-s* (Supplemental Figure 2). Using the BC_9F_2 population (1335 plants) and molecular markers (Supplemental Table 1), we primarily mapped *S1* to a 90-kb region on chromosome 6 (Supplemental Table 2 and Supplemental Figure 3A). Further fine mapping with 11 000 BC_9F_3 individuals delimited the *S1* locus to a 29-kb region, based on the *japonica* (cv. Nipponbare) genomic sequence (Figure 1A and Supplemental Figure 3B), which overlapped mostly with the previously mapped *S1* region of 27.8 kb (Garavito et al., 2010). Based on the annotation of the *japonica* genome, this 29-kb region contains seven predicted open reading frames (ORFs), and we temporarily named them *OsORF1–OsORF7* (Figure 1A). According to our sequencing data and the previously reported genomic sequence (Guyot et al., 2011), this region of the *O. glaberrima* genome lacks *ORF2* and *ORF5*, but possesses five genomic insertions (IF1–5) that harbor six additional predicted ORFs, *OgORFa1–OgORFa6* (Figure 1A), indicating complex structural variation in this region between the two species. Semi-quantitative and quantitative RT-PCR analyses showed that, of the seven common ORFs, only *ORF6* and *ORF7* were expressed in anthers and young panicles of NIL-g and RP-s at different developmental stages (Supplemental Figure 4).

Previous studies on the *Sa* and *S5* loci indicated that HS is generally caused by genetic interaction between/among diverged alleles of the two parents. Therefore, the common transcription-active *ORF6* and *ORF7* in the two species, which have nucleotide variations, were selected as the candidate genes for the *S1*-mediated HS. Sequencing analysis found a single nucleotide polymorphism (SNP, C-A) in the fifth exon (+731 bp of the coding sequence) of the Asian rice *OsORF6* compared with *OgORF6*, resulting in a premature stop codon in *OsORF6* (Figure 1A). *OgORF6* encodes a 774-amino acid (aa) protein containing two trypsin-like peptidase domains and a ribosome biogenesis regulatory domain (RRS). However, *OsORF6* in *O. sativa* encodes a truncated protein of 243 aa, containing only one trypsin-like peptidase domain (Figure 1A and Supplemental Figures 5 and 6). To distinguish these two proteins, we designated that from *OgORF6* as OgTPR1, which harbors trypsin-like peptidase and RRS domains, and that from *OsORF6* as OsTP1, which contains only a trypsin-like peptidase domain (Figure 1A). *OsORF7* and *OgORF7* encode F-box-like proteins with 19 aa differences between them (Supplemental Figures 7 and 8).

To investigate the function of these candidate genes in HS, we used the CRISPR/Cas9 genome editing system (Ma et al., 2015a) to knock out *OgTPR1* (*OgORF6*) and *OgORF7* of NIL-g, with a selected unique target site in the coding region of

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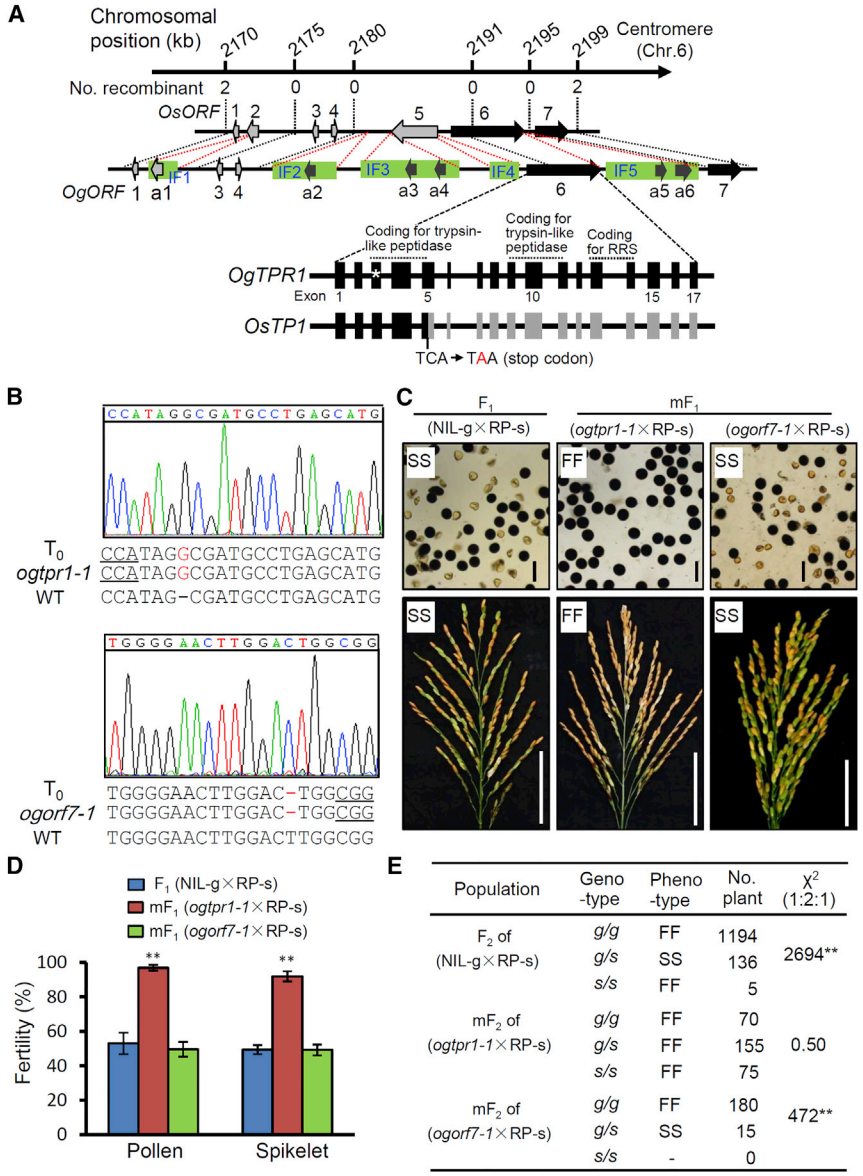

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Population	Geno -type	Pheno -type	No. plant	χ^2 (1:2:1)
F_2 of (NIL-g×RP-s)	*g/g*	FF	1194	2694**
	g/s	SS	136	
	s/s	FF	5	
mF_2 of (*ogtpr1-1*×RP-s)	*g/g*	FF	70	0.50
	g/s	FF	155	
	s/s	FF	75	
mF_2 of (*ogorf7-1*×RP-s)	*g/g*	FF	180	472**
	g/s	SS	15	
	s/s	-	0	

Figure 1. Cloning and Functional Analysis of the Genes at the *S1* Locus for Interspecific HS between Asian and African Rice.

(A) The *S1* locus was mapped to a 29-kb region (within a PAC clone P0535G04) using 1335 BC_9F_2 plants and 11 000 BC_9F_3 plants. The In/Del marker numbers also indicate their positions (kb) on chromosome 6 (see also Supplemental Figure 3 and Supplemental Tables 1, 2). In the mapped region, *japonica* rice possesses seven predicted ORFs (*OsORF1–7*). Only *ORF6* and *ORF7* were expressed in the young panicle and anthers (see also Figure S4). This region of African rice lacks *ORF2* and *ORF5*, but has five different inserted fragments (IF1–5, green box), which contain six additional ORFs, *OgORFa1–a6*. *OgORF6* of African rice encodes a protein with two trypsin-like peptidase domains and one ribosome biogenesis regulatory protein domain, thus we named it OgTPR1 (see also Supplemental Figures 5 and 6). *OsORF6* of Asian rice encodes a truncated protein OsTP1 with only one trypsin-like peptidase domain, due to a premature stop codon caused by the C-to-A mutation (in +731 bp position of the coding sequence) (see also Supplemental Figures 5 and 6). * indicates the target site in *OgTPR1* for CRISPR/Cas9 editing.

(B) Sequencing of the CRISPR/Cas9-targeted sites in *ogtpr1-1* (top) and *ogorf7-1* (bottom) (see also Supplemental Figure 9). The PCR fragments containing the targeted sites were directly sequenced. References are the wild-type (WT) sequences.

(C) Pollen fertility (top) and spikelet fertility (bottom) in F_1 derived from crossing NIL-g (carrying *S1-g* with intact *OgTPR1* and *OgORF7*) with the recurrent parent (RP-s, carrying *S1-s* with *OsTP1* and *OsORF7*), and mF_1 derived from crossing *ogtpr1-1* and *ogorf7-1* of NIL-g with RP-s. SS, semi-sterility of pollen or spikelet; FF, full fertility of pollen or spikelet (see also Supplemental Figure 11). Scale bars, 100 μm for pollen and 5 cm for panicles.

(D) Pollen and spikelet fertility rates in the F_1 and mF_1 plants. ** indicates significance between the F_1 and mF_1 plants at $P < 0.01$. Data are shown as means ± SD (n = 10 major panicles from different individuals).

(E) Segregation analysis of the F_2 and mF_2 families. The *S1* genotypes (*g/g*, *S1-g/S1-g*; *g/s*, *S1-g/S1-s*; *s/s*, *S1-s/S1-s*, including the natural and mutant alleles) were determined using marker 2170 (see also Supplemental Table 3). Test of χ^2 (1:2:1) assumed that no allele-specific abortion of male and female gametes occurred.

the genes, respectively, which has no any homologous, potential off-targeting sites in the rice genome (Figure 1A and Supplemental Figures 5 and 7). Sequence analysis of the *Cas9*-transgenic T_0 plants identified four and two plants that had homozygous or biallelic mutations in *OgTPR1* and *OgORF7*, respectively (Figure 1B and Supplemental Figure 9). These targeted mutations caused frame-shifts and thus plausible loss-of-function of the respective genes, but all these plants exhibited normal pollen and spikelet fertilities (Supplemental Figure 10), indicating that these genes are not essential for the development of male and female gametophytes in rice. To assess whether *OgTPR1* and/or *OgORF7* function in *S1*-mediated HS, we crossed the four *ogtpr1* mutants (*ogtpr1-1–ogtpr1-4*) and the two *ogorf7* mutants (*ogorf7-1* and *ogorf7-2*) with the parent RPs to produce mutant type F_1 hybrids (mF_1). All the mF_1 plants with *ogorf7-1* or *ogorf7-2* showed the semi-sterile phenotype of pollen and spikelets, and the distorted genotypic segregation, similar to the F_1 plants of the NIL-g × RP-s cross (Figure 1C–1E, Supplemental Figure 11, and Supplemental Table 3). However, all the mF_1 plants with the four mutant *ogtpr1* alleles exhibited normal fertilities of pollen and spikelets (Figure 1C and 1D and Supplemental Figure 11), and the segregation of the *S1* genotypes fitted the 1:2:1 ratio (Figure 1E and Supplemental Table 3). These results indicated that *OgTPR1* has a positive role in the *S1*-mediated interspecific HS; loss of mutation of this gene can rescue male and female fertility in hybrids.

There are more than 16 genes encoding the trypsin-like peptidase-domain-containing proteins in the genome of *japonica*

rice, but only five of them show over 35% amino acid sequence identity in the trypsin-like peptidase domain with that in OgTPR1 (Supplemental Figure 12). To trace the origin of the C-A variation that causes the genetic divergence between *OgTPR1* and *OsTP1*, we investigated the sequences of this gene in public databases. In *O. glaberrima* (101 accessions) and its wild progenitor species *O. bathi* (57 accessions), *ORF6* has the C nucleotide at this position, but in all *japonica* and *indica* cultivars (100 accessions for each) and the majority (109 of 114 accessions) of progenitors of the *O. sativa* (*O. rufipogon* and *O. nivara*), *ORF6* harbors the A nucleotide at this position (Supplemental Table 4). Furthermore, the wild species *O. longistaminata* (2 accessions) and *O. officinalis* (10 accessions) also have the C nucleotide at this gene position, but most of *O. meridionalis* (14 of 18 accessions) have the A nucleotide. These data suggest that this nucleotide variation of the gene occurred early in the ancestral *Oryza* species and the diverged alleles have co-existed in the primitive gene pool of the *Oryza* genus. The A allele (the truncated form) of this gene might have been inherited from *O. rufipogon* into *O. sativa*.

The "two gene/three component" and "killer-protector" models have been proposed to explain the selective abortion of gametes with a certain allele of a parent driven by the opposite allele (e.g., gamete killer) of another parent in hybrids with heterozygous HS loci (Long et al., 2008; Yang et al., 2012). This study shows that, although the functional defect of *OgTPR1* and *OgORF7* does not affect male and female gamete development, *OgTPR1*, but not *OgORF7*, of the *S1-g* allele is indeed necessary for *S1*-mediated HS (Figure 1C–1E, Supplemental Figures 10, 11, and Supplemental Table 3). In the interspecific hybrids, OgTPR1 may function as an *S1-s*-specific gamete killer during male and female gametogenesis in the African–Asian rice hybrids. The sterility process probably involves either in activation of certain precursor molecules via the peptidase domains or in the ribosome assembly through the RRS domain, as the proposed basic functions of peptidases and RRS proteins (Morita et al., 2002; Antalis et al., 2010). However, as previously shown at the *Sa* and *S5* complex loci that HS can involve multiple genetic factors, it is possible that other component(s) may also be required for *S1*-mediated HS. Therefore, it is necessary to investigate whether the putative gene(s) specifically present in the *S1-g* allele region (Figure 1A) function in this HS system. This study provides the basis for further study of the underlying molecular mechanism of *S1*-mediated HS and its effect on the speciation of the *Oryza* species.

Hybrid-compatible (neutral) alleles found at some loci do not produce HS in hybrids, and thus are important germplasm resources for hybrid breeding (Long et al., 2008; Yang et al., 2012). However, breeding of hybrid-compatible lines by successive backcrossing is time- consuming, or not feasible if no such neutral allele(s) are detected. In fact, at present, breeders lack a natural neutral allele of *S1*. In this study, we provide a novel approach to rapidly breed hybrid-compatible lines with an artificial neutral *S1* allele (*S1-n*) using the CRISPR/Cas9 system to knock out *OgTPR1* of *O. glaberrima* cultivars (Figure 1B–1E, Supplemental Figures 9 and 11 and Supplemental Table 3). Such *S1-n* alleles can be used to break down the interspecific reproductive barrier, enabling effective utilization of interspecific hybrid vigor and important traits across the two species in rice breeding programs.

ACCESSION NUMBERS

Sequence data in this letter have been deposited in GenBank under the following accession numbers: GenBank: KY457222, KY462781, KY486273, KY486274.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

FUNDING

This research was supported by grants from the National Basic Research Program of China (2013CB126904), the National Key Research and Development Program of China (2016YFD0100804), the China Postdoctoral Science Foundation (2016M592502) and the Key Research Program of Guangzhou Science Technology and Innovation Commission (201606201443109).

AUTHOR CONTRIBUTIONS

Y.X. performed most of the experiments; P.X. and D.T. developed the NIL-g; S.M. contributed to the primary mapping of *S1*; J.H. analyzed the segregation of knockout mutants and the mF_2 population; X.X. helped with analyzing the distribution of the key SNP; Y.-G.L. and L.C. conceived the idea, analyzed the data, and wrote the paper.

ACKNOWLEDGMENTS

We thank Rod A. Wing (the University of Arizona) for providing the BAC clones of *O. glaberrima*. No conflict of interest has been declared.

Received: February 3, 2017
Revised: May 11, 2017
Accepted: May 11, 2017
Published: May 19, 2017

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http://dx.doi.org/10.1016/j.molp.2017.05.005

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