Hybrid sterility in rice (*Oryza sativa* L.) involves the tetratricopeptide repeat domain containing protein

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ABSTRACT

Inter-subspecific hybrid sterility is a common form of reproductive isolation in rice (*Oryza sativa* L.), which significantly hampers the utilization of heterosis between *indica* and *japonica* varieties. Here, we elucidated the mechanism of *S7*, which specially causes Aus-*japonica/indica* hybrid female sterility, through cytological and genetic analysis, map-based cloning and transformation experiments. Abnormal positioning of polar nuclei and smaller embryo sac were observed in *F1* compared with male and female parents. Female gametes carrying *S7cp* and *S7i* were aborted in *S7ai/S7cp* and *S7ai/S7* respectively, whereas normal in both N22 and Dular possessing a neutral allele, *S7n*. *S7* was fine-mapped to a 139-kb region in centromere region on chromosome 7, where the recombination was remarkably suppressed due to aggregation of retrotransposons. Among 16 putative open reading frames (ORFs) localized in the mapping region, *ORF3* encoding a tetratricopeptide repeat (TPR) domain containing protein was highly expressed in pistil. Transformation experiments demonstrated that *ORF3* is the candidate gene: down-regulated expression of *ORF3* restored spikelet fertility and eliminated absolutely preferential transmission of *S7ai* in heterozygote *S7ai/S7cp*; sterility occurred in the transformants Cpslo17-*S7ai*. Our results may provide implications for overcoming hybrid embryo sac sterility in inter-subspecific hybrid rice and utilization of hybrid heterosis for cultivated rice improvement.
INTRODUCTION

Hybridization between two different species can lead to a distinct phenotype, which can also be fitter than the parental lineage. However, reproductive isolation maintains the integrity of a species over time, reducing or directly impeding gene flow between individuals of different species (Mayr 1942; Grant 1981; Coyne and Orr 2004; Widmer et al. 2009; Baack et al. 2015). The mechanisms of reproductive isolation were classified into two broad categories: pre-zygotic and post-zygotic isolation mechanisms (Mayr 1963; Levin 1978; Sweigart and Willis 2012; Chen et al. 2014). According to the classical Dobzhansky-Muller model, post-zygotic isolation results from a deleterious interaction between functionally diverged genes from the hybridizing species (Dobzhansky 1937; Ting et al. 1998; Barbash et al. 2003; Presgraves et al. 2003; Brideau et al. 2006; Bayes and Malik 2009; Ferree and Barbash 2009; Phadnis and Orr 2009; Tang and Presgraves 2009; White et al. 2011). Genes for hybrid sterility, a common pattern of post-zygotic isolation, have been reported in several organisms, including fungi, animals, and plants (Brideau et al. 2006; Lee et al. 2008; Bikard et al. 2009; De Vienne et al. 2009).

Major progress has been made in rice since the inter-specific and inter-subspecific hybrid sterilities are perhaps the best known examples (Chen et al. 2008; Long et al. 2008; Mizuta et al. 2010; Yamagata et al. 2010; Yang et al. 2012). The evolutionary history of rice is complex, but recent work has shed light on the genetics of the transition from wild rice (Oryza rufipogon and Oryza nivara) to domesticated rice (Oryza sativa) which comprises two species, Asian rice (Oryza sativa L.) and African rice (Oryza glaberrima Steud) (Sweeney and McCouch 2007). The Asian cultivated rice consists of two major types or subspecies, indica and japonica, which are also referred to as ‘hsien’ and ‘keng’ respectively since the Han Dynasty (around 2000 years ago) in China (Ting 1957). Recently, a large numbers of
loci causing inter-specific or inter-subspecific hybrid sterility have been identified, including embryo sac abortion (Wan et al. 1993, 1996; Wan and Ikehashi 1995; Zhu et al. 2005; Li et al. 2007; Zhao et al. 2007; Chen et al. 2008; Chen et al. 2012; Yang et al. 2012), pollen sterility (Chen et al. 2006; Jing et al. 2007; Kubo et al. 2008, 2011; Long et al. 2008; Zhang et al. 2011; Zhao et al. 2011), and both in a few cases (Koide et al. 2008, 2012). The $S7$ locus was firstly identified causing hybrid sterility between an Aus variety ‘Ingra’ and some javanica varieties by Ikehashi and Araki (1987). Thereafter, $S7$ was located between $Rc$ (brown pericarp and seed coat) and $Est-9$ on Chromosome 7 (Yanagihara et al. 1992). So far, information is still limited about the molecular mechanism of controlling hybrid embryo sac sterility in rice with $S7$ locus.

The tetratricopeptide repeat (TPR) motif is a 34 amino acid consensus sequence, commonly found in multiple copies in the same protein. TPR-containing proteins from bacteria to humans have been reported participating in diverse processes such as cell cycle, protein folding, protein kinase inhibition, and hormone regulation (Wang et al. 2004; She et al. 2010; Zeytuni and Zarivach 2012; Li et al. 2015; Masuda et al. 2015). Some TPR-containing proteins, such as FKBP52, TRD-1, and SITPR1, play important roles in regulation of reproductive development (Tranguch et al. 2005; Yang et al. 2006; Lin et al. 2008; Hughes et al. 2014). In rice, a mutant related with TPR-containing protein ($OsAPC6$), showed abnormal central cell development during mega-gametogenesis, leading to failure of endosperm development and reduced seed set (Kumar et al. 2010; Awasthi et al. 2012). However, whether the TPR-containing proteins are involved in hybrid sterility has not been reported.

In this study, we showed that $S7$ encodes a TPR-containing protein and controls Aus-$japonica$/indica hybrid female sterility by sporo-gametophyte allelic interaction. These results are helpful for understanding on reproductive isolation and heterosis utilization in rice breeding.
MATERIALS AND METHODS

Plant materials and growth condition
Ingra with red pericarp belongs to Aus variety, whereas Cpslo17 possessing neutral allele at S5 locus is a javanica variety (Ji et al. 2010), and IR36 is a typical indica variety (Wan and Ikehashi, 1996). All plants and populations used for cytological analysis, genetic analysis, fine mapping, and neutral allele identification were planted at the Food Crop Institute, Jiangsu Academy of Agricultural Sciences. Each material was planted with spacing of 16.5 × 16.5 cm. A wide row spacing of 23.5 cm was set between the plots. The plants were managed following normal commercial practices.

Fertility evaluation of pollen, embryo sac and spikelet
Ten individuals from each parent and F₁ hybrid were examined to determine the pollen fertility. Six florets from three panicles of each plant were collected 1-2 days before flowering. One anther per floret per plant was mixed and stained with 1% iodine potassium iodide (I₂-KI) solution, and four views were observed by light microscope. The affinity between pollen and stigma was examined by observing the behavior of the pollen grains on the stigma after pollination. Twenty florets were collected at more than 30 min after flowering to examine the adherence of pollen on stigma, pollen germination, and elongation of the pollen tube by confocal laser scanning microscopy (Leica TCS SP5). The in vitro pollen germination was tested according to the method of Schreiber and Dresselhaus (2003).

To observe the embryo sac development of the parents and F₁ hybrids, spikelet at various development stages were excised and fixed in FAA fluid [containing an 18:1:1 (by vol.) mixture of formalin, 70% ethanol and acetic acid]. Before staining, the samples were transferred to 70% ethanol, removing lemma and palea to expose ovary. The tissue was then processed through an ethanol series (50, 30 and 15%) and finally transferred into distilled water (20 min each). The whole ovary was incubated in 1 mol
L⁻¹ Hydrochloric acid for 15 min, then held in Eosin Y water solution for 8 h, and in Citric acid-disodium hydrogen phosphate buffer (0.1 mol L⁻¹, pH 5.0) for another 8 h after washing with distilled water. Dyeing with 20 μg mL⁻¹ H33342 (Hoechest stain) for 24 h at 25° in the dark, washed with distilled water 3 or 4 times then dehydrated by passing through an ethanol series (15, 30, 50, 70, 85, 95%) (20 min each) and absolute ethyl alcohol for 3 times (2 h each), transited in mixture of absolute ethyl alcohol and methyl salicylate (1:1) for 1h, cleared in methyl salicylate 3 times (2 h each in former two steps and >15 h finally) (Dai et al. 2006). Fertility of embryo sacs was examined by confocal laser scanning microscopy (Leica TCS SP5).

Spikelet fertility of each plant was determined by counting fertile and sterile spikelet on the upper half of three panicles after maturation as described by Wan et al. (1996).

**Transmission ratio distortion (TRD) and recombination rate**

The TRD system was measured by the method of Koide et al. (2008). Based on this system, the degree of TRD was calculated in terms of a k value, varying from 0.5 (Mendelian segregation) to 1.0 (complete elimination of its allelic alternative). The $k_m$ and $k_f$ stood for the proportion of progeny that received the allele exhibiting the preferential transmission through male and female gametes, which were estimated from $N_s / (N_f + N_s)$ backcrossing data using heterozygote as male and female parent, respectively. While $N_s$ represented the number of semi-sterility (heterozygotes Ingra/Cpso17, $S^7a1/S^7cp$ or Ingra/IR36, $S^7a1/S^7i$) plants, $N_f$ stood for that of fertile (homozygotes $S^7cp/S^7cp$ or $S^7i/S^7i$).

Recombination frequency in units of physical distance was defined as the recombination rate for each interval, and estimated as (recombination frequency) / (total length of region) where recombination frequency=100 × (total number of recombinants) / (number of plants) (Lien et al. 2000).
**Vectors construction and assay of transgenic plants**

The construct pCUbi1390-ΔFAD2 (inserting ubiquitin promoter and a FAD2 intron into pCAMBIA1390) was used as an RNAi vector (Stoutjesdijk *et al.* 2002; Li *et al.* 2013). Both anti-sense and sense versions of a specific 468 bp fragment from coding region of *open reading frame 3 (ORF3)* were amplified (primer pairs TPR-RNAi-A and TPR-RNAi-S, Table S1), and successively inserted into pCUbi1390-ΔFAD2, to form the RNAi construct pUbi-dsRNAiTPR, which was then transformed into parents and heterozygote S7al/S7cp. The transformation was conducted according to a published method (Hiei *et al.* 1994).

A fragment containing the whole specific 468 bp fragment and part vector sequence were amplified by using primers RNAi-A (607 bp) and RNAi-S (851 bp) for assay of transgenic positive plants. For background analysis, the transgenic plants were tested with PCR aiming to amplify the S7-containing region with primers TI15 and TI53 (Table S1).

A 2,856 bp cDNA fragment containing the entire ORF3 coding region and a 1,719 bp upstream genomic region were amplified (primer pairs TPRai and TPRai-P) from Ingra, meanwhile a 2,859 bp cDNA fragment and a 1,610 bp upstream genomic region were amplified (primer pairs TPRcp and TPRcp-P) from Cpslo17. The PCR products from Ingra and Cpslo17 were inserted into the binary vector pCAMBIA1305 respectively, and then transformed into Cpslo17 and Ingra accordingly. Two primer pairs (P1-F & P2, P3 & P1-R) were used for assay of transgenic positive plants (Table S1).

All transgenic plants were grown either in a nursery in summer growing season in Beijing, China, or in a greenhouse in winter.

**RT-PCR analysis**

Total RNA was extracted from the leaf, stem and root at the seedling stage, or from
the pistil, lemma, palea, stamen, panicle at mature stage using an RNA Prep Pure Plant kit (Tiangen Co., Beijing, China), and then reverse transcribed using a SuperScript II kit (TaKaRa). Real-time PCR was performed using a SYBR® Premix Ex Taq™ kit (TaKaRa) on an ABI prism 7900 Real-Time PCR System. The $2^{-\Delta\Delta CT}$ method was used to analyze relative changes in gene expression (Livak and Schmittgen 2001). Primers for ORF3 and other two candidate genes (ORF15 and ORF16) were named as 27180-3, 27310-3, and 27320-1. The rice ubiquitin gene (Os03g0234200) was used as a reference in the experiment (primer pair Ubq) (Table S1).

**Bioinformatics analysis**

Gene prediction was performed using the Rice Genome Annotation Project database (RGAP, http://rice.plantbiology.msu.edu/). Homologous sequences of ORF3 were identified using the Blastp search program of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Molecular phylogenetic analysis was constructed by maximum likelihood method using MEGA6 (Tamura et al. 2013). Multiple sequence alignments were conducted with BioEdit software. Prediction of the 3-D structure was carried out using phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/).

**Transient expression analysis in *Nicotiana benthamiana***

The coding sequence of ORF3 from Ingra was amplified and fused to N-terminus of GFP under control of the CaMV 35S promoter in the transient expression vector pCAMBIA1305-GFP, referred to as ORF3-GFP. The complementary DNA (cDNA) fragment was PCR-amplified by the corresponding primer pair (TPRGFP) (Table S1). Transient expression construct was introduced into the *Agrobacterium* strain EHA105 and then used to infiltrate *N. benthamiana* leaves as described previously (Waadt and Kudla 2008). *N. benthamiana* protoplasts were isolated using the same method as *Arabidopsis* (Park et al. 2005). Confocal imaging analysis was performed using a Leica TCS SP5 laser scanning confocal microscope.
RESULTS

Cytological observation of pollen and embryo sacs in parents and F\textsubscript{1} hybrids

Pollen grains of all the three parents and their F\textsubscript{1}s were plump and stainable by I\textsubscript{2}-KI, suggesting pollen fertility of all genotypes was normal (Figure 1A and B and Table 1). However, spikelet fertility of hybrid F\textsubscript{1}s (Ingra/IR36 and Ingra/Cpslo17) showed typically semi-sterility, viz. 49.2±0.1 and 59.6±1.1% of seed setting rates, respectively, against with normal spikelet fertility of their parents (≥80% of seed setting rates, Figure 1C and Table 1).

To deeply investigate cytological mechanism of hybrid sterility in F\textsubscript{1} hybrids, we examined the in vitro germination of pollen grains from parents and their F\textsubscript{1}, which showed that the pollen grains from all plants could germinate efficiently (Figure 1D, E and Table 2). Furthermore, no distinction was observed between F\textsubscript{1}s and their parents when examining the number of pollen grains adhered to stigmas and pollen tube elongation (Figure 1F-I). However, the fertilization rate of ovaries measured at 2 days after flowering was lower in F\textsubscript{1} hybrids (Table 2). In addition, the spikelet fertility of F\textsubscript{1} hybrids was not restored to normal levels after hand pollination with pollen from each parent (Table 2). It suggested that the sterility of F\textsubscript{1} hybrids might be mainly due to defects in the female reproductive organs rather than pollen sterility or the frustration of fertilization.

Observation of the embryo sacs from mono-nucleate stage to mature embryo sac stage showed that abnormal embryo sacs occurred at eight-nucleate embryo sac developing-stage, of which two polar nuclei located in the central cavity with horizontal arrangement instead of vertical one (Figure 2A-J). There were mainly two different types of abnormalities observed in the mature embryo sacs of the F\textsubscript{1} hybrids, including embryo sacs with abnormal polar nuclei positioning and smaller embryo sacs. In the first type, the polar nuclei located either above the egg apparatus with
vertical arrangement or near the wall of embryo sac (Figure 2K-M). In the second type, embryo sacs were smaller in which abnormal positioning of polar nuclei was found occasionally (Figure 2N). Analysis of histological sections of mature embryo sacs revealed that frequency of abnormal embryo sacs in F\textsubscript{1} hybrids was significantly higher than that in parental controls (Figure 2O). Additionally, embryo sac fertility of Ingra/Cpslo17 and Ingra/IR36 F\textsubscript{1} corresponded to their spikelet fertilities separately (Table 2).

Taken together, these results suggested that the partial abortive embryo sac caused hybrid sterility. Meanwhile, the failure of normal fertilization in hybrids was mainly caused by abnormal development from eight-nucleate embryo sac developing-stage, which totally induced the occurring of smaller size embryo sac and embryo sac with abnormal positioning of polar nuclei consequently.

**Genetic mechanism and neutral allele identification of S7**

An framework linkage map was constructed based on a population of 272 F\textsubscript{1} plants derived from three-way cross Ingra/IR36//Cpslo17, where the average pollen fertility was 90.4±11.9% but the spikelet fertility showed significantly bimodal distribution with an apparent valley between 70~80% (Figure S1 and File S1). The locus showed a tightly linkage with Rc (Sweeney *et al.* 2006) on chromosome 7 (Figure S2 and File S1), which was demonstrated by the fact that nearly all hybrids F\textsubscript{2} of both Ingra/Cpslo17 and Ingra/IR36 showed red pericarp inherited from their female parent Ingra (Figure S3). These results confirmed that the locus found in our study is the S7 reported by Yanagihara *et al.* (1992).

Reciprocal crosses (Ingra/Cpslo17 and Cpslo17, Ingra/IR36 and IR36) were conducted separately to reveal the genetic mechanism of S7. The segregation of genotypes in progeny was not fit well to Mendel's law when pollinating Ingra/Cpslo17 and Ingra/IR36 with Cpslo17 and IR36 pollen grains respectively, of which the number
of individual with homozygote genotype decreased obviously. Since the male and female gametes could be affected differently, two parameters $k_m$ and $k_f$ were estimated for TRD. The parameters $k_m$ of $S7^{cp}$ and $S7'$ allele transmitted efficiently through male gametes were 0.49 and 0.48, respectively. Nevertheless, female gametes were significantly blocked, and the parameters $k_f$ of $S7^{cp}$ and $S7'$ allele were 0.97 and 0.94, respectively. These results indicated the $S7^{ai}$ allele exhibited absolutely preferential transmission by promoting the elimination of $S7^{cp}$ and $S7'$ to their progeny, mostly through female gamete (Table 3).

Based on the model of allelic interaction (Ikehashi and Araki 1984), for three given varieties, A, B and N, if a hybrid A/B shows gamete abortion, but N/A and N/B do not, the variety N possesses a neutral allele at this locus. To identify neutral allele of $S7$, thirteen crosses were constructed between the three parents and cultivars including indica and japonica. While the typical sterility was shown in both Ingra/IR36 and Ingra/Cpslo17 F$_1$ hybrids, spikelet fertilities were normal when Dular and N22 were crossed with Ingra, IR36 and Cpslo17, which suggested that Dular and N22 probably possess the neutral allele, $S7^n$, at the $S7$ locus (Table 1).

**Fine-mapping of $S7$**

A single, significant QTL was identified on chromosome 7 from three-way cross Ingra/IR36/Cpslo17 F$_1$ population, suggesting hybrid embryo sac sterility in Ingra/Cpslo17 and Ingra/IR36 was mainly controlled by $S7$ (Figure 3A). The peak of QTL appeared at molecular marker RM5543 where the LOD score was 80.0. $S7$ was flanked by Rc and RM445 including the centromere.

Two populations were used to narrow down the genomic region containing the $S7$ locus. We initially chose plants carrying $S7^{ai}/S7^{cp}$ between markers Rc and RM445 with seed setting rate less than 55% from Ingra/IR36/Cpslo17 F$_1$ and Ingra/Cpslo17 F$_2$ to generate high resolution mapping populations. Additional markers including
In-Del (Insertion-Deletion) and dCAPS (derived cleaved amplified polymorphic sequences) were developed in the Rc-RM445 interval. By analyzing recombinants from 12,000 Ingra/Cpslo17 F_{2:3} plants, the $S_7$ locus was delimited to the interval flanked by Y6 and TI53 with two recombinant events between Y6 and $S_7$ and five between TI53 and $S_7$. Meanwhile, the result from 6,999 Ingra/IR36//Cpslo17 F$_2$ population showed that $S_7$ was delimited to the interval between TH15 and TI44 with three recombinant events either end (Figure 3B and File S1). The overlapping fragment of 139-kb in the two mapping results included eight ORFs except retrotransposons (RTs) (Figure 3C and Table S2).

**Recombination rate**

A significant repression of recombination was observed across the primary mapping region of 754-kb (TI21-TI26, Figure 3B) in the centromeric region. The evaluation of 12,000 plants from Ingra/Cpslo17 F$_{2:3}$ for recombination allowed us to identify recombination frequency in units of physical distance across this region. The most recombinogenic interval was a 66-kb region on the left-hand defined by markers TI21 and TI24. Within this interval, the recombination rate was 12.63 cM Mb$^{-1}$, which was significantly higher than that of the 241-kb interval just downstream (TI24 to Y16) and the following 50-kb (Y16 to Y6). A similar case occurred on the right-hand but in lesser degree, which the interval between Y26 and TI53 showed a lower recombination rate compared with its downstream 1.01 cM Mb$^{-1}$. Besides, the 184-kb interval from Y6 to Y26 had no detectable recombination (Figure 4 and File S1).

While the whole region is rich in RTs, we analyzed the number of RTs in each of the divided region according to the calculation of recombination rate. Interestingly, the regions where recombination rate drops sharply were also found a surge of RTs (Figure 4). Considering the location in centromere region, it was likely that less occurrence of recombination events in Ingra/Cpslo17 F$_{2:3}$ populations were mainly
caused by aggregation of RTs in the target region.

**Analysis of candidate genes**

To define candidate gene for S7, we firstly sequenced the genome of five ORFs (ORF1, 3, 14, 15 and 16) which had functional annotation from varieties including parents, Dular (N22), and Ketan Nangka (K.N., possessing S7<sup>kn</sup> allele, Yanagihara et al. 1992). Since amino acid sequences of ORF1 and ORF14 had shown no obvious difference among those varieties, we excluded ORF1 and ORF14 as candidate gene (Figure S4 and File S2). Besides, in contrast to ORF15 and ORF16 which were predominantly expressed in mature panicle, ORF3 was mostly expressed in the pistil (Figure 5A and Figure S5).

The genomic sequence of ORF3 (LOC_Os07g27180) spans 17,504 bp, and contains 19 introns and 20 exons. It encodes a protein of 898 amino acids with a conserved TPR domain. Compared with others, Ingra showed more specific amino acid sites. Moreover, a variant amino acid was detected at amino acid 119 in Dular and N22 that has a Met (M) instead of a Leu (L) in Ingra, Cpslo17 and K.N. (Figure S6). Based on higher expression in mature pistil and unique differences in amino acid sequence, ORF3 was selected as the candidate gene for S7 tentatively.

**S7 encodes a TPR-containing protein**

To investigate function of ORF3, we knocked down ORF3 in parents and heterozygote S7<sup>al</sup>/S7<sup>cp</sup> by RNAi. 26 Ingra-ORF3 RNAi, 33 Cpslo17-ORF3 RNAi, and 53 Ingra/Cpslo17-ORF3 RNAi transgenic positive T<sub>0</sub> plants were obtained by background detection and transgenic PCR assay (Figure S7 and Figure S8). The pollen grains in all ORF3 RNAi plants showed no obvious abortion compared with those in transgenic negative plants, while embryo sacs with abnormal positioning of polar nuclei and small embryo sacs were significantly reduced in Ingra/Cpslo17-ORF3 RNAi plants (Figure 5B-E). ORF3 RNAi transgenic had no effect on spikelet fertility of both Ingra and
Cpslo17. However, significant restored spikelet fertility was observed in Ingra/Cpslo17-ORF3 RNAi positive plants (Table 4).

In order to verify the correlation between relative expression of ORF3 and spikelet fertility, eight families with different spikelet fertility level from Ingra-ORF3 RNAi, Cpslo17-ORF3 RNAi and Ingra/Cpslo17-ORF3 RNAi plants were randomly selected, respectively. Gene expression in mature pistil was examined by RT-RCR. In parental transgenic plants, correlation between relative expression of ORF3 and spikelet fertility was not obvious. However, the extent of down-regulation was consistent with the restored level of spikelet fertility in Ingra/Cpslo17-ORF3 RNAi positive plants. Strong interference plants (such as 27-6, 18-5 and 13-3) exhibited a high fertility rate, whereas weak interference plants (such as 21-1 and 14-4) were associated with a low fertility rate (Figure 5F).

To test the hypothesis that the restored spikelet fertility of the ORF3 RNAi plants might be mainly due to normal development of the embryo sac, we selected three strong interference plants (27-6, 18-5 and 13-3) as representative ORF3 RNAi plants for further analysis. The rate of embryo sac abortion in representative plants ranged from 6.13% to 15.04%, which was significantly lower than 51.46% detected in the negative plants. Correspondingly, the frequency of normal embryo sacs in the representative plants increased substantially (Figure 5G). These data were in accordance with the spikelet fertility in the RNAi positive and negative plants. The offspring of all the eight RNAi transgenic positive plants showed a similar phenotype of restored spikelet fertility from the T₁ to T₂ generation (Figure 6 and Table S3). Additionally, segregation ratio in T₂ generation of those three representative ORF3 RNAi families conformed to Mendel's law (Table 3). These results demonstrated that ORF3 RNAi could restore spikelet fertility of heterozygote S7\textsuperscript{ai}/S7\textsuperscript{cp} and eliminate absolutely preferential transmission of S7\textsuperscript{ai}.
In addition, complementation tests were performed to confirming the function of ORF3. The strategy that we adopted was transforming $S_7^{ai}$ allele into Cpslo17 and $S_7^{cp}$ allele into Ingra, respectively. Through PCR assay, a series of transgenic positive T₀ plants were obtained (Figure S9). Examination of the spikelet fertility showed that there was no statistically significant difference between the transgenic positive and negative plants of Ingra-$S_7^{cp}$. By contrast, transgenic positive and negative plants of the Cpslo17-$S_7^{ai}$ showed a highly significant difference in spikelet fertility; the average spikelet fertility of the positive plants (49.00%) was much lower than that of the negative plants (82.19%) (Table 4). Considering $S_7^{ai}$ allele exhibited absolutely preferential transmission by promoting the elimination of $S_7^{cp}$, we thus concluded that ORF3 is the candidate gene.

**Expression and subcellular location of ORF3**

Phylogenetic tree analysis showed that ORF3 exists in not only Asian rice (O. sativa L.) but also wild rice. Cpslo17 are closely related to indica type and distanced from Ingra, confirming the similarity with IR36 that could not transmit their female gamete to their progeny in hybrid $F_1$ s crossed with Ingra. Homologous proteins of ORF3 were identified in other plants, all of which belong to grass family (Figure S10). Further, protein sequence alignment suggested that ORF3 exhibited 76.0%-79.7% identity with its homologous proteins, indicating that the ORF3 is specific to monocots (Figure S11).

To determine experimentally subcellular localization, a fusion protein, ORF3-GFP, was generated and expressed transiently under control of the 35S promoter in tobacco (Nicotiana benthamiana) mesophyll protoplasts. As shown in Figure 7, ORF3-GFP was detected in the endoplasmic reticulum (ER) and nuclei.
DISCUSSION

Rice is one of the main crops providing the staple food for more than half the globe population. Utilization of strong heterosis in inter-subspecific crosses of rice has long been difficult due to semi-sterility of panicles in the hybrids between *indica* and *japonica* cultivars. One of the genes involved in this phenomenon is *S7*, which have been isolated here through a positional cloning approach. We demonstrate that a TPR-containing protein plays significant role in hybrid female sterility causing by *S7* locus, which would be particularly for understanding molecular mechanism of inter-subspecific hybrid in rice.

Evidence from the current study suggests that the function of *S7* is centered on functional female gametophyte formation. Abnormal female gametophyte of hybrid F₁ would appear at eight-nucleate embryo sac developing-stage when polar nuclei started their migration to the upward side of egg apparatus and embryo sac began to enlarge (Figure 2). In addition, abnormal embryo sac caused by *S7* led to the semi-sterility of spikelet directly (Table 2), which was consistent to a previous report that most of the smaller embryo sacs and abnormal polar nuclei positioning embryo sacs could not succeed in fertilization, even with normal female germ unit (Zeng *et al.* 2009). It was reported that orientation of polar nuclei always occurred not only during development of female gametophyte but also after fertilization, when one sperm cell nuclear moved toward central cell (Ding *et al.* 2009). Observation on the distribution and structural organization of the microtubule during mega-gametogenesis suggested that some new organizational patterns of microtubules surrounding the central cell might be associated with the probable movement and positioning of the polar nuclei (Xu *et al.* 2001). Furthermore, an involvement of F-actin in gamete nuclear migration had been suggested that F-actin disorganization in the central cell could disrupt the polarized nuclear location and the presence of intact F-actin cables in the central cell...
was correlated with successful karyogamy regardless of the central cell nuclear position (Kawashima et al. 2014; Ohnishi et al. 2014; Kawashima and Berger 2015). Therefore, we speculate that some cytoskeleton or signal molecules, involved in the migration of polar nuclei, were disorganized during the development of embryo sac, and led to incorrect guidance of polar nuclei fertilization eventually in both Ingra/IR36 and Ingra/Cpslo17 hybrids.

Analysis of recombination rate revealed that a large number of retrotransposons (RTs), especially Ty3-gypsy, were aggregated in S7-containing region near the centromere (Figure 4). In general, transposon elements (TEs) were found to be enriched in the pericentromeric regions of many plant genomes (Arabidopsis Genome Initiative 2000; International Rice Genome Sequencing Project 2005; Paterson et al. 2009; Schnable et al. 2009; Schmutz et al. 2010; Luo et al. 2012). Genes close to the centromere or retrotransposon clusters are less recombinogenic than other genes in a gene-rich region (Copenhaver et al. 1999; Fu et al. 2001). Significant recombinational repressions were reported in mapping region of Rc/rg7.1 (Sweeney et al. 2006) and Ghd7 (Xue et al. 2008). Both of the genes located close to S7, demonstrating that the low frequency of recombination in S7-containing region may be due to its particular location on centromeric region on chromosome 7. In plants, self-incompatibility genes were found to be recombinationally suppressed due to their subcentromeric location in Petunia (Coleman and Kao 1992; Entani et al. 1999), Antirrhinum (Ma et al. 2003; Yang et al. 2007), and the presence of repetitive DNA in Nicotiana (Matton et al. 1995). Additionally, the hybrid incompatibility genes Lhr, Zhr, and OdsH in Drosophila, were all mapped to recombinationally suppressed pericentric and heterochromatic regions with reduced or undetectable levels of recombination (Sawamura et al. 1993; Brideau et al. 2006; Bayes and Malik 2009). Consequently, it is still necessary to validate whether the suppression of recombination plays a role in species conservation.
TPR domain is one of the most frequently observed amino acid motifs in nature, which can be found in numerous proteins. TPR-containing proteins always serve as interaction modules and multi-protein complex mediators (Blatch and Lassle 1999; Zeytuni et al. 2011; Zeytuni and Zarivach 2012; Shin et al. 2014), and regulate diverse biological processes including reproductive development. In rice, mutation in OsAPC6, one of the TPR-containing APC/C (anaphase-promoting complex/cyclosome) subunits, caused a reduced number or complete absent of polar nuclei (Kumar et al. 2010; Awasthi et al. 2012). Therefore, protein-protein interaction may provide important clues to reveal the molecular mechanism of S7. In our study, down-regulated expression of ORF3 could restore spikelet fertility in the hybrid F1. However, there was no effect in either Ingra-ORF3 RNAi or Cpslo17-ORF3 RNAi plants (Figure 5 and Table 4). These results suggest that S7 may not be essential for growth, development, or reproduction, and hybrid sterility of Ingra/Cpslo17 is probably controlled by allelic interaction between $S_7^{ai}$ and $S_7^{cp}$. Therefore, weak allelic interaction in Ingra/Cpslo17-ORF3 RNAi plants could overcome the hybrid sterility. Although transformants Cpslo17-$S_7^{ai}$ and Ingra-$S_7^{cp}$ have $S_7^{ai}$ and $S_7^{cp}$ alleles simultaneously, the sterility occurs only in $S_7^{ai}/S_7^{ai}$-$S_7^{cp}$ (Table 4). Combined with the results of TRD analysis (Table 3), $S_7^{ai}$ allele exhibits stronger function during female gametes transferring in heterozygote ($S_7^{ai}/S_7^{cp}$) plants. Due to the fact that the F1 hybrids between three parents (Ingra, IR36 and Cpslo17) and wide-compatibility varieties (WCVs, Dular and N22) showed normal spikelet fertility (Table 1), Dular and N22 were assumed to carry the neutral allele $S_7^o$ at S7 locus. To confirm the function of $S_7^o$, we sequenced S7 region in three parents and WCVs, and found that a SNP (single nucleotide polymorphism) (C/A) caused an amino acid substitution (Leu-119 in parents, Met-119 in Dular and N22) (Figure S6 and Table S4). Additionally, a total of forty-seven varieties including indica, japonica,
and wild rice were compared with the genomic sequence of the \(S7\) region, of which the result indicated that the nucleotide C exists in most varieties (Table S4). However, Leu-119 is conserved not only in various rice species, but also in other plants (Figure S10). Moreover, predicted 3-D structures of ORF3 from Ingra, Cpslo17 and Dular indicated that amino acid sequence differences may induce their changes in spatial structure directly (Figure S12). Therefore, we speculate that during the formation of female gametophyte in heterozygotes \(S7^{ai}/S7^{cp}\) and \(S7^{ai}/S7^{i}\), gametes carrying \(S7^{cp}\) and \(S7^{i}\) could not resist the effect of \(S7^{ai}\) from sporophyte, thus leads to the embryo sac abortion. Whereas the \(S7^{ai}\) gametes could resolve such function of \(S7^{cp}\) and \(S7^{i}\), exhibiting preferential transmission. Additionally, substitution of Met-119 in \(S7^{n}\) may cause loss-function of protein-protein interactions in Dular and N22, which results in the failure to produce sterile offspring when crossed with either indica or japonica (Figure S13). Although how such likely nonfunctional is related to the hybrid fertility remains to be characterized, the discovery and molecular analysis of the \(S7^{n}\) in this study probably can provide functional markers for WCG germplasm screening to solve, at least partially, hybrid embryo sac sterility in rice breeding.

ACKNOWLEDGMENTS

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Figure 1 Phenotype of Ingra and Ingra/Cpslo17 F₁. Pollen I₂-KI staining of Ingra (A) and F₁ (B). Spikelet of Ingra (left) and F₁ (right), the arrow indicates the shriveled grain (C). In vitro pollen germination (D, E), germination of pollen on stigma (F, G) and pollen tube elongation in ovary (H, I) showed no difference between Ingra (D, F, H) and F₁ (E, G, I). Bars: (A, B, D, E) 50 μm; (C) 1 cm; (F-I) 500 μm.
Figure 2 Observation of embryo sac development. (A, F) Mono-nucleate embryo sac stage. (B, G) Bi-nucleate embryo sac stage. (C, H) Tetra-nucleate embryo sac stage. (D, I) Eight-nucleate embryo sac stage. (E, J) Eight-nucleate embryo sac developing-stage. (A-E) Ingra; (F-J) Ingra/Cpslo17 F₁. (K-N) Embryo sac matured-stage, (K) normal embryo sac; (L, M) abnormal positioning of polar nuclei; (N) small embryo sac. The arrow indicates the polar nuclei. (O) Statistics of different types of embryo sac in mature embryo sac. NES, normal embryo sac; APPN, abnormal positioning of polar nuclei; SES, small embryo sac; Other, other abnormal embryo sacs. Bars: (A-J) 50 μm; (K-N) 100 μm.
Figure 3 Fine mapping of S7. (A) Quantitative trait locus (QTL) analysis of S7. (B) The physical map of S7. S7-containing region determined by Ingra/Cpslo17 F_{2:3} (partial key recombinants were shown from 10-38 to 10-163) and Ingra/IR36//Cpslo17 F_{2} (partial key recombinants were shown from 08-N12 to 08-N1) population. (C) The overlapped 139-kb included sixteen ORFs, of which eight encode retrotransposon proteins (black boxes), three encode expressed or hypothetical proteins (blue boxes) and other five have functional annotation (green boxes).
Figure 4 Evaluation of recombination rate across the primary mapping region (754-kb). The x axis is positioned along the markers used in fine mapping, and the y axis shows the number of recombination rate, that is the recombination frequency in units of physical distance (cM Mb\(^{-1}\)). The number of retrotransposons is shown above the markers.
**Figure 5 Functional analysis of ORF3.** (A) Expression pattern of ORF3. YR, young root; YS, young stem; YL, young leaf; MP, mature panicle; Le, lemma; Pa, palea; St, stamen; Pi, pistil. (B-E) Cytological observation of RNAi transgenic plants: negative control (B, D); positive (C, E). (F) Regression analysis of relative expression of ORF3 and spikelet fertility in parent and Ingra/Cpslo17 F₁ RNAi transgenic plants. (G) Percentage of different type observed in mature embryo sacs of Ingra/Cpslo17-ORF3 RNAi representative and negative plants. NES, normal embryo sac; APPN, abnormal positioning of polar nuclei; SES, small embryo sac; Other, other abnormal embryo sacs. Bars, 50 μm.
Figure 6 Distribution of spikelet fertility of Ingra/Cpslo17-ORF3 RNAi from T\textsubscript{1} to T\textsubscript{2} generation.
Figure 7 Subcellular localization of ORF3 protein in tobacco mesophyll protoplasts. Bars, 20 μm.
Table 1 The pollen and spikelet fertility of three parents and their F<sub>1</sub> hybrids.

<table>
<thead>
<tr>
<th>Parents and crosses</th>
<th>Fertility of pollen (%)</th>
<th>Fertility of spikelet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingra</td>
<td>94.2±2.0</td>
<td>95.7±0.8</td>
</tr>
<tr>
<td>IR36</td>
<td>94.9±2.0</td>
<td>84.8±5.2</td>
</tr>
<tr>
<td>Cpslo17</td>
<td>94.8±1.4</td>
<td>86.1±6.2</td>
</tr>
<tr>
<td>Ingra/IR36</td>
<td>99.1±0.8</td>
<td>49.2±0.1***</td>
</tr>
<tr>
<td>IR36/Ingra</td>
<td>88.2±3.3</td>
<td>46.8±0.0***</td>
</tr>
<tr>
<td>Ingra/Cpslo17</td>
<td>89.2±3.5</td>
<td>59.6±1.1***</td>
</tr>
<tr>
<td>Cpslo17/Ingra</td>
<td>97.4±0.6</td>
<td>54.3±0.4***</td>
</tr>
<tr>
<td>Ingra/Nipponbare</td>
<td>80.8±0.8</td>
<td>74.4±0.6</td>
</tr>
<tr>
<td>Nipponbare/IR36</td>
<td>66.8±1.6</td>
<td>50.9±6.7</td>
</tr>
<tr>
<td>Ingra/9311</td>
<td>99.7±0.3</td>
<td>50.2±2.3</td>
</tr>
<tr>
<td>9311/Cpslo17</td>
<td>97.5±1.7</td>
<td>84.1±6.6</td>
</tr>
<tr>
<td>Ingra/Dular</td>
<td>98.4±1.6</td>
<td>87.1±2.0</td>
</tr>
<tr>
<td>Cpslo17/Dular</td>
<td>97.6±0.8</td>
<td>86.7±7.6</td>
</tr>
<tr>
<td>IR36/Dular</td>
<td>97.9±0.5</td>
<td>92.3±2.4</td>
</tr>
<tr>
<td>Ingra/IR24</td>
<td>97.6±1.2</td>
<td>44.4±4.2</td>
</tr>
<tr>
<td>Cpslo17/IR24</td>
<td>96.4±1.4</td>
<td>92.4±2.7</td>
</tr>
<tr>
<td>IR36/IR24</td>
<td>95.1±1.3</td>
<td>89.9±2.7</td>
</tr>
<tr>
<td>Ingra/N22</td>
<td>99.0±0.7</td>
<td>91.9±1.2</td>
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<tr>
<td>Cpslo17/N22</td>
<td>99.1±0.3</td>
<td>87.8±6.8</td>
</tr>
<tr>
<td>N22/IR36</td>
<td>73.4±3.5</td>
<td>83.1±3.3</td>
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</tbody>
</table>

*** Statistically significant difference with respect to their parents ($P < 0.001$).
Table 2 The fertility-related traits of three parents and their F₁ hybrids.

<table>
<thead>
<tr>
<th>Fertility-related traits (%)</th>
<th>Ingra</th>
<th>Cpslo17</th>
<th>IR36</th>
<th>Ingra/IR36</th>
<th>Ingra/Cpslo17</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro pollen germination rate</td>
<td>77.5±2.1</td>
<td>77.8±1.8</td>
<td>79.6±0.1</td>
<td>77.5±1.7</td>
<td>88.1±1.1</td>
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<tr>
<td>Normal embryo sac</td>
<td>95.1±1.9</td>
<td>85.3±2.4</td>
<td>81.4±0.7</td>
<td>47.8±5.1</td>
<td>50.8±4.4</td>
</tr>
<tr>
<td>Fertilized ovaries</td>
<td>89.4±4.1</td>
<td>82.6±1.6</td>
<td>92.4±1.6</td>
<td>48.2±2.5</td>
<td>55.6±4.3</td>
</tr>
<tr>
<td>Open seed-setting rate</td>
<td>95.7±0.8</td>
<td>86.1±6.2</td>
<td>86.6±4.7</td>
<td>49.2±2.2</td>
<td>59.6±8.6</td>
</tr>
<tr>
<td>Supplementary pollination with parents</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>44.3±7.5</td>
<td>49.6±4.1</td>
</tr>
</tbody>
</table>
Table 3 Genetic analysis of S7.

<table>
<thead>
<tr>
<th>Reciprocal crosses</th>
<th>Female genotype</th>
<th>Male genotype</th>
<th>Progeny genotypes</th>
<th>P</th>
<th>TRD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S7ai/S7ai</td>
<td>S7ai/S7cp</td>
<td>S7ai/S7i</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>10</td>
<td>309</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.6e-63</td>
<td>k=0.97</td>
</tr>
<tr>
<td></td>
<td>S7ai/S7cp</td>
<td>S7ai/S7i</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>209</td>
<td>198</td>
<td>-</td>
<td>0.59 k=m=0.49</td>
</tr>
<tr>
<td></td>
<td>S7ai/S7i</td>
<td>S7ai/S7i</td>
<td>-</td>
<td>19</td>
<td>293 2.9e-54 k=0.94</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td>202</td>
<td>187 0.45 k=m=0.48</td>
</tr>
<tr>
<td></td>
<td>27-6</td>
<td>30</td>
<td>50</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Ingra/Cpslo17-</td>
<td>18-5</td>
<td>9</td>
<td>20</td>
<td>5    0.37</td>
</tr>
<tr>
<td></td>
<td>ORF3 RNAi</td>
<td>13-3</td>
<td>33</td>
<td>56</td>
<td>22   0.33</td>
</tr>
</tbody>
</table>

P value obtained using the chi-squared test under the hypothesis of Mendelian segregation.
Table 4 Spikelet fertility of transgenic $T_0$ plants.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Types</th>
<th>No. of plants</th>
<th>Spikelet fertility (%)</th>
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</thead>
<tbody>
<tr>
<td>Ingra-ORF3 RNAi</td>
<td>Positive</td>
<td>26</td>
<td>92.03</td>
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<tr>
<td></td>
<td>Negative</td>
<td>12</td>
<td>92.37</td>
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<tr>
<td></td>
<td>$t$</td>
<td></td>
<td>2.03</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td></td>
<td>0.84</td>
</tr>
<tr>
<td>Cpslo17-ORF3 RNAi</td>
<td>Positive</td>
<td>33</td>
<td>93.54</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>13</td>
<td>92.17</td>
</tr>
<tr>
<td></td>
<td>$t$</td>
<td></td>
<td>2.12</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td></td>
<td>0.15</td>
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<tr>
<td>Ingra/Cpslo17-ORF3 RNAi</td>
<td>Positive</td>
<td>53</td>
<td>77.00</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>19</td>
<td>48.04</td>
</tr>
<tr>
<td></td>
<td>$t$</td>
<td></td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>Cpslo17-S$^{7al}$</td>
<td>Positive</td>
<td>11</td>
<td>49.00</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>6</td>
<td>82.19</td>
</tr>
<tr>
<td></td>
<td>$t$</td>
<td></td>
<td>2.18</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>Ingra-S$^{7sp}$</td>
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<td>7</td>
<td>90.03</td>
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<tr>
<td></td>
<td>Negative</td>
<td>12</td>
<td>93.56</td>
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<td></td>
<td>$t$</td>
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<td></td>
<td>$P$</td>
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