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Genetic characterization and fine mapping of S25, a hybrid male sterility gene, on rice chromosome 12

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Hybrid male sterility genes are important factors in creating postzygotic reproductive isolation barriers in plants. One such gene, *S25*, is known to cause severe transmission ratio distortion in inter-subspecific progeny of cultivated rice *Oryza sativa* ssp. *indica* and *japonica*. To further characterize the *S25* gene, we fine-mapped and genetically characterized the *S25* gene using near-isogenic lines with reciprocal genetic backgrounds. We mapped the *S25* locus within the 0.67–1.02 Mb region on rice chromosome 12. Further genetic analyses revealed that *S25* substantially reduced male fertility in the *japonica* background, but not in the *indica* background. In first-generation hybrid progeny, *S25* had a milder effect than it had in the *japonica* background. These results suggest that the expression of *S25* is epistatically regulated by at least one partially dominant gene present in the *indica* genome. This finding supports our previous studies showing that hybrid male sterility due to pollen killer genes results from epistatic interaction with other genes that are hidden in the genetic background.

Key words: epistasis, fine mapping, hybrid male sterility, *Oryza sativa*, reproductive isolation

INTRODUCTION

Hybridization between genetically divergent populations and species often produces hybrid offspring with reproductive abnormalities such as hybrid inviability or hybrid sterility. These phenomena play pivotal roles in speciation by acting as postzygotic reproductive isolation barriers. Two major genetic elements, duplicated genes and gamete killers (including egg killers and pollen killers), are known to be involved in hybrid sterility. Reciprocal loss of duplicated genes between two species (including gene transposition as a similar mechanism) has been reported to be the cause of hybrid sterility in *Drosophila* (Masly et al., 2006), *Arabidopsis* (Bikard et al., 2009) and rice (Mizuta et al., 2010; Yamagata et al., 2010). Duplication of genes essential for reproductive development allows subsequent loss of one copy without any reduction in fitness, and reciprocal gene loss in both species leads to hybrid sterility. This is a logical and simple mechanism that can lead to hybrid sterility. On

the other hand, the genetic basis of gamete killers is not so simple. Gamete killers are selfish genetic elements that kill non-carrier gametes in heterozygous hybrid progeny. Initial examination of the genetics of gamete killers suggested that this phenomenon was caused by a simple allelic interaction at a single heterozygous locus; however, the molecular mechanism is far more complex than expected. Some examples include the *t*-complex in mice (Lyon, 2003), *Segregation distorter* (*Sd*) in *Drosophila* (Larracuente and Presgraves, 2012) and egg killer in rice (Yang et al., 2012). All three of these examples involve a complex interaction between multiple genes located within the same chromosomal region.

Since rice improvement is largely dependent on cross breeding, hybrid sterility is considered a critical issue for breeding programs using remote relatives. Seven species are known in the *Oryza sativa* complex (also known as AA genome species): two cultivated rice species (Asian rice *O. sativa* and African rice *O. glaberrima*) and five wild relatives (*O. rufipogon*, *O. barthii*, *O. glumaepatula*, *O. longistaminata* and *O. meridionalis*). *Oryza rufipogon*, a close wild relative of Asian cultivated rice *O. sativa*, is divided into two subgroups, an annual type (also referred to as a new species, *O. nivara*) and a perennial type (*O. rufipogon*) (Sharma and Shastry, 1965). All of the *Oryza sativa* complex species are diploid ($2n = 24$) and are distributed broadly in tropical areas including Asia, Africa

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and South America. More than 40 hybrid sterility loci have been reported in intraspecific crosses of *O. sativa* and in interspecific crosses between *O. sativa* and other AA genome species (reviewed in Koide et al., 2008 and Kubo, 2013). Of the more than 40 reported loci, only seven loci have been isolated and the causal gene characterized (Kubo, 2013; Kubo et al., 2016a). Most of the other gene loci, excluding these seven, were only mapped on the chromosome, and some are potentially identical and alleles of the same locus. A pollen killer gene on chromosome 5, *S24/Sb/f5*, provides a good example of this issue. Three genes *S24*, *Sb* and *f5*, were initially found in different varietal crosses between *O. sativa* ssp. *indica* and *japonica*. These three genes were eventually found to be alleles of the same locus because fine-mapping localized them within the same narrow interval (Zhao et al., 2011). Information about the phenotypic effects and the precise position of each gene (also of tightly linked DNA markers) is particularly important for promoting the development and adoption of hybrid rice technology. Sequenced genomes, varietal series of mutant libraries, and genome engineering tools such as TALEN and CRISPR/Cas9 are now available for rice, but map-based cloning using a hybrid population derived from a cross between two divergent species is still the required initial step toward identifying and cloning causal genetic factors for hybrid sterility. Additionally, a comprehensive understanding of the genetic components and their interaction with each other and the environment is necessary for elucidating the molecular mechanism of hybrid sterility.

Previously, we found three pollen killer genes (*S24*, *S25* and *S35*) responsible for hybrid male sterility in a single cross between the *indica* cultivar IR24 and the *japonica* cultivar Asominori (Kubo, 2013). In our earlier work, the *S24* and *S35* loci were finely mapped on chromosomes 5 and 1, respectively, and the genetic mechanisms with their interacting partner genes (*EFS* and *INK*) were characterized (Kubo et al., 2011, 2016b). In contrast, *S25*, which is located on rice chromosome 12, remains only roughly mapped and mostly uncharacterized (Win et al., 2009). The aim of this study was to characterize more completely the *S25* gene toward the goal of elucidating the genetic mechanism of hybrid male sterility caused by *S25*. Here, we have extended our earlier work by using a large segregating population and reciprocal near-isogenic lines (NILs) for *S25*. We found that hybrid male sterility due to *S25* is epistatically regulated by other unlinked gene(s) hidden in the genetic background. This study provides additional evidence that epistasis plays an essential role in pollen killer systems.

MATERIALS AND METHODS

Plant materials Reciprocal chromosome segment sub-

stitution lines (CSSLs) carrying *S25* segments derived from a cross between Asominori (*Oryza sativa* ssp. *japonica*) and IR24 (ssp. *indica*) (Kubo et al., 2002) were used for genetic analysis of *S25*. Consequently, we used AIS84 (BC₃F_n) and IAS73 (BC₂F_n), CSSLs carrying substitution segments of chromosome 12 with the Asominori and IR24 genetic backgrounds, respectively. These two lines were backcrossed with their recurrent parents, and *S25* heterozygous plants were phenotypically evaluated and genetically analyzed. Fine mapping of *S25* was performed using F₂ and F₃ selfed progeny of Asominori//AIS84/Asominori (N = 3,334). Genotype frequency at the *S25* locus was assessed using seedling leaves from the self-pollinated progeny of each plant heterozygous for *S25*. All plant materials in this study were grown under paddy field conditions in 2009–2015 in Mishima, Japan, where both parents produced fertile pollen (> 90%).

DNA analysis For DNA marker genotyping, crude DNA extracts from individual leaves were prepared using 0.25 M NaOH followed by neutralization with 0.1 M Tris-HCl (pH 8.0). These DNA extracts (1 µl) were used in PCR reactions (10-µl final volume) performed using GoTaq polymerase (Promega) with the following cycling profile: 94 °C for 2 min; followed by 30 cycles of 94 °C for 20 s, 56–60 °C for 20 s, and 72 °C for 30 s. The PCR amplicons were separated in 2% agarose gels, and visualized by ethidium bromide staining. Insertion/deletion (InDel) markers were developed for mapping the *S25* locus based on DNA sequence polymorphisms between Nipponbare and 93-11 (MSU6, <http://rice.plantbiology.msu.edu/>; BGI, <http://rice.genomics.org.cn/rice/index2.jsp>). The primer sequences for InDel markers were as follows:

12c046, 5'-TTGGGGAGTTGTTGACTGGT-3', 5'-GCGTT-CACAAGCAATGGTC-3'; *12c066*, 5'-TCCTCTCTATG-GTGCGTTGA-3', 5'-CGCTCTTTGTTAGGGCAATC-3'; *12c070*, 5'-TGGATAAGGCTGAGGTCAAA-3', 5'-GGCT-GCATAGGACAATTGAA-3'; *12c084*, 5'-AACAACGAC-GAATCCTGGTG-3', 5'-ATGTAGTGGGCCGAGATCA-3'; *12c093*, 5'-GACTGGCGTGTTTCATACGA-3', 5'-TGTTT-TCCTGGTTTAATGGAA-3'; *12c102*, 5'-CTTTTTATGTTTG-AATCACCATTCT-3', 5'-ATTGATCAGTTGGGGATTGG-3'; *12c104*, 5'-GGGACAAACATCGGTACGAG-3', 5'-ACGAG-CATTGAGGGGGTAAT-3'; *12c107*, 5'-GGGAGCTAGGGGAGT-GTGTT-3', 5'-TCCAAAGCAAAGCAAAAAGG-3'. The whole-genome genotyping data of AIS84 and IAS73 were obtained from Kubo et al. (2002). Gene annotations and positions of marker loci are based on the Nipponbare genome sequence (MSU7).

Scoring fertility Pre-flowering spikelets were collected from each individual in the population and were fixed and stored in 50% ethanol solution to evaluate pollen fertility. Pollen extracted from anthers was stained with 1% iodine-potassium iodide (I₂-KI) on glass slides, and the

pollen fertility of more than 400 pollen grains from each individual was evaluated by light microscopy. Stained pollen grains with a normal size were counted as fertile, and unstained small pollen grains were counted as sterile. In addition, ethanol-fixed pollen grains were stained with hematoxylin solution as described by Kindiger and Beckett (1985) to investigate the morphology of mature pollen grains. To assess seed fertility, we collected three panicles with fully ripened grains from each plant and counted the numbers of filled and unfilled spikelets. Seed set was defined as the percentage of filled grains relative to the total number of filled and unfilled grains.

RESULTS

Fine mapping of *S25* Heterozygous introgression of *S25* from IR24 in the Asominori genome induces pollen semi-sterility and selective abortion of the male gametes bearing Asominori alleles for *S25* (*S25-j*), but homozygotes for either allele do not show any notable phenotype in pollen development (Fig. 1A and Table 1, Win et al., 2009). Such preferential transmission of the IR24 allele for *S25* (*S25-i*) in hybrid progeny suggests that *S25* acts as a pollen killer. Cytological observation revealed that *S25* causes postmeiotic abortion of microspores (Fig. 1A). The *S25* gene has been roughly mapped to the short arm of chromosome 12 (Win et al., 2009). To determine a more precise position for the *S25* locus, we fine-mapped *S25* using F_2 and F_3 populations (a total of 3,334 plants)

derived from a backcross of AIS84 with Asominori. In the segregating population, we found an individual plant (#13-19) that carried a small heterozygous segment near the *S25* region (Fig. 2). Pollen of heterozygous progeny of the #13-19 plant was semi-sterile, indicating that the small segment harbored the *S25* locus. Together with the results from the other recombinants including #2-89 and 83-6, the *S25* locus was mapped within a 359-kb region between marker loci *12c066*–*12c102* (665–1024 kb of chromosome 12) (Fig. 2A). No recombination was observed between the *12c070*–*12c093* loci in the population we used. The candidate region for *S25* contains 55

Table 1. Segregation of the *S25* gene in reciprocal genetic backgrounds

Genetic background	Line#	No. of plants ^a				χ^2 (1:2:1)	% of <i>S25-j</i> ^b
		i/i	i/j	j/j	Total		
Asominori	A83	97	94	1	191	96.08***	0.5
	A84	429	435	0	864	426.06***	0.0
	A13-19	23	33	0	56	20.68***	0.0
IR24	I24	31	51	26	108	0.8 ^{ns}	24.1
	I19	29	47	14	90	5.18 ^{ns}	15.6

ns: not significant; ***: $P < 0.001$.

^a i/i: IR24 homozygote; i/j: heterozygote; j/j: Asominori homozygote.

DNA markers (*12c066*, *12c084* and *12c093*) tightly linked to *S25* were used for genotyping the individuals.

^b The expected percentage of the *S25-j* homozygote is 25%.

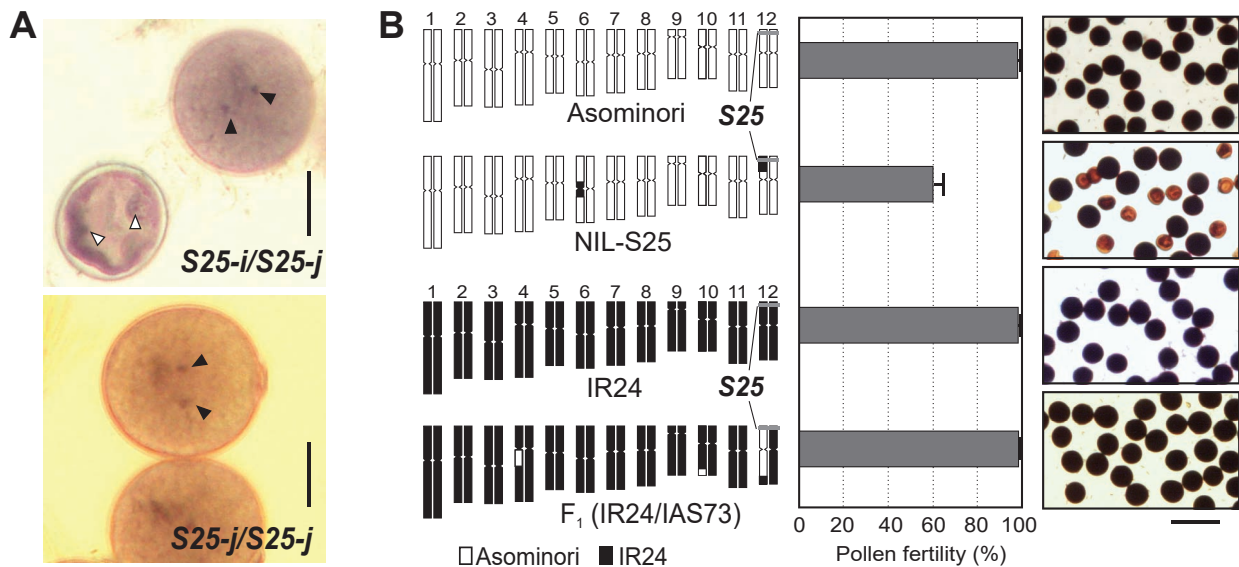


Fig. 1. Phenotype of *S25* in reciprocal genetic backgrounds. (A) Hematoxylin-stained pollen grains of the *S25* heterozygote and *S25* homozygote. Sterile pollen arrested at the bicellular microspore stage in *S25* heterozygotes (upper panel), and normal pollen in *S25* homozygotes (lower panel). Two normal sperm cells in mature pollen are indicated with black arrowheads, whereas nuclei in the arrested cells are shown with white arrowheads. Scale bars = 20 μ m. (B) Pollen phenotype of the NIL for *S25* in the Asominori and IR24 genetic backgrounds. Left panel: graphical representation of the NIL genotypes. Middle panel: pollen fertility of the NILs compared with the parents Asominori and IR24. Bars show the mean (%) with SD ($N = 5-6$). Right panel: photomicrograph of pollen grains stained with an I_2 -KI solution. Scale bar = 100 μ m.

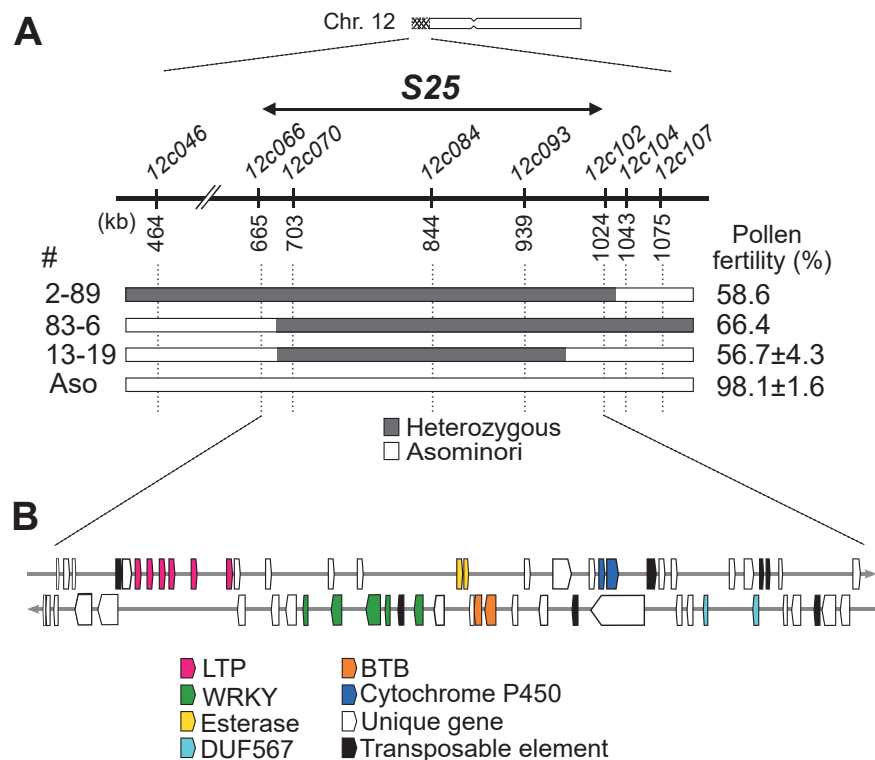


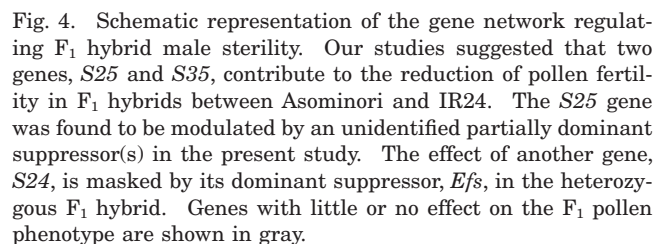
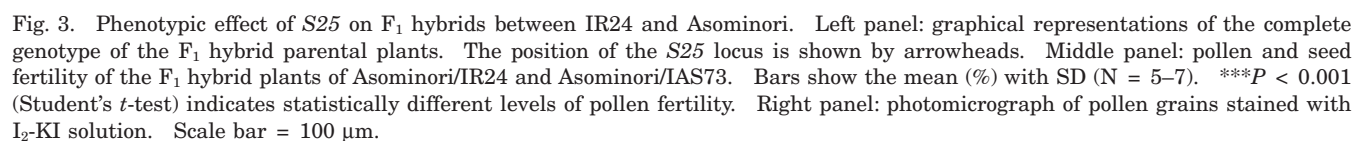
Fig. 2. Chromosome localization of the *S25* locus. (A) Physical map of chromosome 12 (upper) and the genotype of the three most informative recombinant plants selected from a BC₅F₃ population (lower) are shown. The pollen fertility phenotype of the #13-19 plant, which had the smallest IR24 segment, was evaluated in the selfed progeny (BC₅F₄) (N = 5). The others (#2-89 and 83-6) were phenotyped as a single plant. (B) Predicted genes in the *S25* candidate region based on the MSU Rice Genome Annotation Project (release 7). Tandem gene copies are shown in colored boxes on the forward (upper) and the reverse strands (lower). LTP: lipid transfer protein; BTB: broad-complex tramtrack and bab; DUF567: domain of unknown function 567.

putative protein-coding genes and seven transposable elements (Fig. 2B). Remarkably, there are five tandem copies of genes in the WRKY family, six tandem copies of LTP genes, and two tandem copies of other genes in the candidate region. These genes may be good candidates for the *S25* gene because gene duplication is a potential source of sequence diversification leading to functional differences.

Influence of genetic background on the *S25* phenotype For further characterization of *S25*, we investigated the effect of genetic background on pollen sterility due to *S25*. To evaluate the pollen phenotype of *S25* in the *indica* background, IAS73, a CSSL carrying a homozygous *japonica* segment around *S25* in the IR24 background (Kubo et al., 2002), was backcrossed with IR24, and the obtained F₁ plants heterozygous for *S25* were evaluated for pollen and seed fertility. Unlike the pollen fertility in the *japonica* background, pollen from the heterozygous *S25* had good fertility in the IR24 genetic background (Fig. 1B). Segregation of selfed progeny of the F₁ (IR24/IAS73) was not significantly different from a Mendelian segregation ratio (1:2:1) for the *S25* locus. In

this IR24 background population, the frequency of the *S25 japonica* homozygote recovered to 15.6–24.1% (Table 1), indicating that the *S25-j* allele can be transmitted through the male gamete. This finding demonstrates that pollen killing by *S25* occurs exclusively in the *japonica* genetic background and not in the *indica* genetic background. This result also suggests the presence of a suppressor gene(s) for *S25* in the IR24 genome.

Evaluation of phenotypic effects of *S25* on heterozygous F₁ hybrids As mentioned above, we found contrasting effects of *S25* on pollen fertility by using NILs with reciprocal genetic backgrounds, i.e., a noticeable phenotypic effect of *S25* in the *japonica* background but little or no effect in the *indica* background. We next assessed the phenotypic effects of *S25* on the first generation of the Asominori/IR24 hybrid. To do that, we compared the pollen fertility of the F₁ hybrid of Asominori/IR24 with that of other F₁ hybrid plants homozygous for the *S25* locus (Asominori/IAS73). The pollen fertility of the Asominori/IR24 hybrid was 66%, whereas that of the *S25*-homozygous F₁ hybrids was partially but significantly



partially dominant and a recessive suppressor gene(s) for *S25* should be present in the IR24 genome. No significant differences in seed fertility between Asominori/IR24 F₁ and the *S25*-homozygous F₁ (Asominori/IAS73) were observed (Fig. 3), indicating that *S25* has little or no effect on seed setting in the first generation of hybrid progeny. Importantly, Tsunematsu et al. (1996) reported a considerable reduction in the number of *japonica* alleles around the *S25* locus in recombinant inbred lines (RILs) between Asominori and IR24. The observed segregation ratio of IR24:Asominori was 0.85:0.15 in the RILs, whereas the expected ratio was 0.5:0.5. This significant distortion in the segregation of RILs supports our proposal that *S25* has a relatively reduced but substantial activity in the first generation of hybrids between Asominori and IR24.

DISCUSSION

Using a large segregating population in the *japonica* background, we mapped the *S25* locus within the 665–1024 kb region on chromosome 12. Earlier work

by Zhang et al. (2011) identified *qS12*, a hybrid male sterility gene, in the same position (622–1024 kb) using a cross between Nipponbare (*japonica*) and 93-11 (*indica*). Because *S25* and *qS12* were localized within the same narrow region (< 402 kb in size), these two genes are considered alleles within a single locus. Zhang et al. (2011) and our present study tried to determine the precise position of *S25/qS12* by fine mapping, but no recombination within the candidate region on chromosome 12 was observed. The lower recombination rate around the *S25/qS12* region possibly results from an inversion or other chromosomal rearrangement, as previously pointed out by Zhang et al. (2011). Although still only mapped roughly, two additional F_1 pollen sterility genes, *S-e* and *S36*, have been localized near the *S25/qS12* locus (Zhu et al., 2008; Win et al., 2009). *S-e* and *S36* were found in an inter-subspecific *japonica/indica* cross (Zhu et al., 2008) and an inter-specific *O. sativa/O. nivara* cross (Win et al., 2009), respectively. These findings suggest the possibility that these four loci (*S25/qS12/S-e/S36*) are the same and are polymorphic alleles that arose from the ancient gene pool of *O. sativa*, *O. rufipogon* and *O. nivara* before rice domestication. If the *S25* and *S36* loci are the same genes, the candidate region would be further narrowed to within a 118-kb region (906–1024 kb). In this region, two tandem gene copies encoding cytochrome P450 proteins were found (Fig. 2). Members of the cytochrome P450 protein family are required for pollen exine formation in *Brassica* (Yi et al., 2010) and function as male sterility genes in maize (Djukanovic et al., 2013). On the other hand, it is possible that more than one gene causing male sterility resides in the *S25* candidate region, because such a recombination cold spot tends to accumulate genetic mutations and form adaptive gene complexes (Lowry and Willis, 2010). In plants, three sets of hybrid male sterility genes (*Sa*, *S27/S28* and *DPL1/2*) have been isolated (Long et al., 2008; Mizuta et al., 2010; Yamagata et al., 2010). As the *S25* candidate region did not contain homologs or members of the same protein families as these characterized hybrid male sterility genes, isolation of the *S25* gene should provide new insights into mechanisms for hybrid male sterility in plants.

In our previous study, no difference was observed in pollen fertility between the reciprocal F_1 hybrids of Asominori and IR24 (Kubo et al., 2008). This result suggested that nuclear but not cytoplasmic genes are responsible for their F_1 pollen sterility. In this study, we found that the *S25* phenotype was modulated by the genetic background. *S25* was inactivated in the *indica* genetic background, whereas the *S25* activity was retained but slightly weakened in heterozygous F_1 hybrids (Figs. 1 and 3). Consequently, we concluded that there could be at least one partially dominant suppressor gene in the IR24 genome. Although we have tried to find the chromosome position of the putative suppressor locus, the

precise location remains elusive. The partial dominance of this locus should make it difficult to identify and map the suppressor on the chromosome because of the obscure pollen phenotype of the segregants and the low frequency of individuals homozygous for the suppressor and heterozygous for *S25* in the segregating population (e.g., 12.5% frequency in the F_2 population). Interestingly, all of our investigations have demonstrated that pollen killer genes do not have a consistent effect on pollen sterility in reciprocal genetic backgrounds (Kubo et al., 2016b). Two other pollen killers, *S24* and *S35*, have asymmetrical effects on pollen sterility in the reciprocal background of Asominori and IR24 (Kubo et al., 2016b). Our study identifying a dominant *S24*-suppressor gene (named *Efs*) on the *indica* chromosome provided clear evidence of pollen fertility restoration in the *indica* genetic background (Kubo et al., 2011). The presence of an unidentified suppressor(s) for *S24* other than *Efs* has also been hypothesized because restoration of pollen fertility occurs even in the recessive *efs/efs* homozygote with an IR24 genetic background (Kubo et al., 2016b). It is noteworthy that the *indica* genome has an activator for pollen killer (a dominant activator gene, *Ink*, has been found for *S35*) (Kubo et al., 2016b). These findings indicate that the pollen killer system is regulated by epistatic networks involving multiple factors including suppressors, activators and modifiers that may have either major or minor effects. Thus, our recent studies of pollen killer genes represent a different aspect of the gamete killer system in plants from the traditional model of “allelic interaction at a single locus”; here, “locus” includes not simply a single gene but rather a small chromosomal region such as a supergene. Previous gene cloning studies of *Sa* and *S5* highlighted the importance of interactions between tightly linked multiple genes at a single locus (Long et al., 2008; Yang et al., 2012). Since gamete killer genes often have a complex genetic architecture and are found in recombination cold spots, identifying the interacting partner genes will assist in the identification and characterization of molecular pathways for gamete killer systems.

Numerous hybrid rice cultivars have been developed and grown successfully in China and other Asian countries for many years. Exploitation of cytoplasmic male sterile lines and photoperiod/thermo-sensitive genic male lines resulted in successful large-scale hybrid rice commercialization, but this success is limited largely to certain compatible rice cultivars. We must unveil the molecular mechanisms for hybrid sterility as a next step toward expanding the genetic variation of hybrid rice parents. Despite the large number of hybrid sterility genes reported in rice, little is known about the individual genes and how each gene contributes to fertility in the first generation of hybrid offspring. Taking the *S24* locus as an example, a heterozygous *S24* is expected to have no

effect on pollen fertility in the F_1 hybrid due to inactivation by its dominant suppressor *Efs* (Fig. 4) (Kubo et al., 2011). Such a dominant suppressor gene is useful for a cross breeding program and for hybrid rice breeding to overcome hybrid sterility. Through a comparison of the F_1 hybrid phenotypes of Asominori/IAS73 and Asominori/IR24, we estimated the phenotypic effect of *S25* in the heterozygous F_1 hybrid. The results from this experiment showed that *S25* has a moderate but significant effect (~18%) on pollen sterility in the first generation of the Asominori/IR24 hybrids. This result was substantiated by the segregation distortion at the *S25* locus in RILs of Asominori/IR24 (Tsunematsu et al., 1996), and in the F_2 and RILs of Nipponbare/93-11 (Huang et al., 2009; Zhang et al., 2011). In the RILs of Asominori/IR24, however, the *japonica* homozygotes segregated at a frequency of 14.7% (expected 50%) (Tsunematsu et al., 1996), a level higher than the 0–0.5% frequency in the *japonica* background population in this study (Table 1). Some recovery in the frequency of the *japonica* alleles for *S25* offers supportive evidence for the presence of a partially dominant suppressor. Another locus, *S35*, has been shown to partially contribute to pollen sterility in F_1 hybrids between Asominori and IR24 (Kubo et al., 2016b). Taking these observations together, the semi-sterility of F_1 pollen seen in Asominori and IR24 seems most likely to result from the cumulative effect of two genes, *S25* and *S35* (Fig. 4).

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