Hybrid Male Sterility in Rice Is Due to Epistatic Interactions with a Pollen Killer Locus

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ABSTRACT In intraspecific crosses between cultivated rice (*Oryza sativa*) subspecies *indica* and *japonica*, the hybrid male sterility gene *S24* causes the selective abortion of male gametes carrying the *japonica* allele (*S24-j*) via an allelic interaction in the heterozygous hybrids. In this study, we first examined whether male sterility is due solely to the single locus *S24*. An analysis of near-isogenic lines (NIL-F₁) showed different phenotypes for *S24* in different genetic backgrounds. The *S24* heterozygote with the *japonica* genetic background showed male semisterility, but no sterility was found in heterozygotes with the *indica* background. This result indicates that *S24* is regulated epistatically. A QTL analysis of a BC₂F₁ population revealed a novel sterility locus that interacts with *S24* and is found on rice chromosome 2. The locus was named **Epistatic Factor for *S24* (*EFS*)**. Further genetic analyses revealed that *S24* causes male sterility when in combination with the homozygous *japonica* *EFS* allele (*efs-j*). The results suggest that *efs-j* is a recessive sporophytic allele, while the *indica* allele (*EFS-i*) can dominantly counteract the pollen sterility caused by *S24* heterozygosity. In summary, our results demonstrate that an additional epistatic locus is an essential element in the hybrid sterility caused by allelic interaction at a single locus in rice. This finding provides a significant contribution to our understanding of the complex molecular mechanisms underlying hybrid sterility and microsporogenesis.

HYBRIDS between genetically divergent species often show abnormal phenotypes that reduce fitness, such as sterility, weakness, and inviability. Such hybrid characteristics, collectively called hybrid incompatibility, are assumed to play important roles in speciation by acting as postzygotic reproductive barriers. The genetic mechanisms of reproductive barriers and their implications in evolution have been studied using a variety of plant species including *Helianthus* (Rieseberg *et al.* 1996), *Mimulus* (Sweigart *et al.* 2006), *Iris* (Taylor *et al.* 2009), and rice (Sano 1990; Koide *et al.* 2008). These efforts have demonstrated that diverse mechanisms of hybrid incompatibility exist and have shed some light on the roles of these mechanisms in plant evolution. However, aside from a few cases, the molecules and networks that control hybrid incompatibility remain largely unknown. It is widely accepted that interactions between genetic loci

(called epistasis) contribute to reproductive isolation mechanisms. Such genetic interactions most likely reflect the existence of molecular networks that control reproductive isolation. For example, an immunity system was found to be involved in hybrid incompatibilities resulting from intraspecific crosses in *Arabidopsis thaliana* (Bomblies *et al.* 2007; Alcazar *et al.* 2010).

Because of its wide genetic diversity and well-characterized genetic base, cultivated rice (*Oryza sativa* L. $2n = 24$) is a useful model for the study of hybrid sterility in plants. A number of hybrid sterility genes/QTL have been reported in hybrids between *O. sativa* ssp. *japonica* and ssp. *indica*. So far, two major genetic models for F₁ hybrid sterility have been proposed, one involving interlocus epistasis and the other involving allelic interactions at a single locus. The interlocus epistasis model is also called the Bateson–Dobzhansky–Muller (BDM) model (Bateson 1909; Dobzhansky 1937; Muller 1942). Recent reports by Yamagata *et al.* (2010) and Mizuta *et al.* (2010) have provided experimental evidence for the BDM model, by demonstrating the reciprocal loss-of-function of duplicated genes that were involved in male gamete development. On the other hand, the interaction of alleles at a single locus model is illustrated by the hybrid

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doi: 10.1534/genetics.111.132035

Manuscript received June 24, 2011; accepted for publication August 18, 2011

Supporting information is available online at <http://www.genetics.org/content/suppl/2011/08/25/genetics.111.132035.DC1>.

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sterility gene (*S/S^a*), which causes the selective abortion of *S^a* gametes in *F*₁ heterozygotes, resulting in the predominant transmission of *S* alleles to their progeny. This model was first proposed for tomato by Rick (1966) and has been supported by numerous studies of gamete eliminator, pollen, and egg killer genes (Kitamura 1962; Ikehashi and Araki 1986; Sano 1990). In this model, it is assumed that stepwise mutations at a single locus underlie the development of reproductive barriers without a reduction in fitness, and consequently multiple alleles, including a neutral allele, are generated at the causal locus (Nei *et al.* 1983). The triallelic system of the rice *S5* locus is an example of this model. *S5-i* (the *indica* allele) and *S5-j* (the *japonica* allele), cause sterility when present as a heterozygous pair, while *S5-n* is a neutral allele that confers fertility when combined with either *S5-j* or *S5-i*. Positional cloning revealed that the *S5* gene encodes an aspartic protease (Chen *et al.* 2008). In another case, *SaM* and *SaF* are adjacent rice genes that encode a small ubiquitin-like modifier E3 ligase-like protein and an F-box protein, respectively. These two loci were originally thought to comprise one multiallelic locus, but it is now clear that they interact epistatically to cause hybrid male sterility (Long *et al.* 2008). Despite the identification of some of the gene products involved in hybrid sterility, the molecular mechanisms by which these proteins cause the sterility phenotype remain obscure. Furthermore, there have been very few investigations to discover whether the genetic background or epistasis have any effects on hybrid sterility involving allelic interactions at single loci.

The hybrid male sterility gene *S24*, which acts as a pollen killer gene, has a strong effect on male sterility and segregation distortion in hybrid progeny between *indica* and *japonica* (Kubo *et al.* 2008). Male gametes carrying the *japonica* allele for *S24* (*S24-j*) are selectively aborted during the mitotic stage after meiosis in *S24-i/S24-j* heterozygous plants, and the *indica* allele for *S24* (*S24-i*) is transmitted to about 90–100% of progeny through pollen (Kubo *et al.* 2008). Therefore selfing of the *S24* heterozygotes produce *S24-j/S24-j* (pollen fertile), *S24-i/S24-j* (semisterile) and *S24-i/S24-i* (fertile) plants in an ~0.1:1:1 ratio distorted from the expected 1:2:1 ratio. The parental homozygotes (*S24-i/S24-i* and *S24-j/S24-j*) exhibit no phenotypic abnormalities in either the pollen or other tissues. Thus, it was thought that *S24* specifically affects the male gamete via an allelic interaction in heterozygous hybrids. In addition it has been found that another locus, *S35* on chromosome 1, enhances pollen sterility through an interaction with *S24* (Kubo *et al.* 2008). Using different cross combinations of *indica* and *japonica* varieties, other researchers have reported on hybrid male sterility genes named *f5-Du* (Wang *et al.* 2006) and *Sb* (Li *et al.* 2006), which map to the *S24* region on chromosome 5. *S24*, *f5-Du*, and *Sb* occur within a single 90-kb region and are therefore likely to be the same gene (Zhao *et al.* 2010). Zhao *et al.* (2010) used a fine mapping strategy to identify two candidate genes for *S24* (*f5-Du* and *Sb*), one encoding an Ankyrin domain protein

and the other encoding an uncharacterized protein. Thus, *S24* (*f5-Du* and *Sb*) appears to be an important factor controlling hybrid male sterility in the *indica/japonica* hybrids. This locus should be a useful resource for exploring the following important questions: (1) What is the molecular mechanism of the allelic interaction at the hybrid male sterility locus? (2) How does the hybrid sterility gene effect rice evolution? (3) Would it be possible to overcome reduced fitness in the rice breeding program by using a neutral allele or an epistatic restorer gene for hybrid sterility?

Our study aims to reveal the genetic and molecular bases of hybrid male sterility in rice. Our previous genetic study of *S24* was performed using backcross progeny with the *japonica* background (Kubo *et al.* 2008). On the basis of its mode of inheritance, it appears that negative allelic interactions occur at the *S24* locus, as in other pollen killer systems. However, there has been no previous study to determine whether the hybrid male sterility caused by allelic interaction at *S24* is really controlled by a single genetic locus or whether epistasis is also involved. In this study, we addressed this question in a genetic analysis of *S24* using reciprocal near-isogenic lines (NILs). By this approach we unveiled a complex genetic mechanism in which interlocus epistasis is a necessary component of the hybrid male sterility system involving negative allelic interactions at *S24*. On the basis of these results we propose that epistasis plays a large and essential role in most cases of postzygotic reproductive barriers in rice.

Materials and Methods

Plant materials

Reciprocal chromosome segment substitution lines carrying *S24* segments were derived from a cross between the *japonica* variety Asominori and the *indica* variety IR24 (Kubo *et al.* 2002). These were backcrossed (as males) with their respective parents, and NILs for *S24* were obtained by marker-assisted selection (Figure 1). All the reciprocal NILs had the Asominori cytoplasm. The populations used for further genetic analyses of hybrid male sterility were developed from a cross between the *japonica* variety Nipponbare and the *indica* variety 93-11. The backcross populations BC₂F₁ (*N* = 47), BC₂F₂ (*N* = 153), BC₃F₂ (*N* = 101), BC₄F₁ (*N* = 44), and BC₄F₂ (*N* = 189) were derived from crosses with 93-11 as the donor parent and Nipponbare as the recurrent male parent (Supporting Information, Figure S1). These populations were cultivated during 2008–2010 and plants uniformly headed by early September.

Phenotypic evaluation

To examine the pollen phenotypes, preflowering panicles from each individual in the population were collected and fixed in a formalin/acetic acid/alcohol solution. The fixed samples were stored in a 70% ethanol solution. Three to 6 anthers collected 1 day before anthesis were stained with

Figure 1 Pollen fertilities of near-isogenic lines for *S24* in reciprocal genetic backgrounds. The NIL- F_1 populations carry heterozygous segments harboring the *S24* locus in either the Asominori (*japonica*) background (upper, AI-NIL- F_1), or the IR24 (*indica*) background (lower, IA-NIL- F_1). The genotypes of the reciprocal NIL- F_1 populations are represented on the left, their pollen fertility data ($N = 3$ for each NIL- F_1 line) are shown in the middle, and micrographs of their pollen grains are shown on the right.

1% iodine-potassium iodide and 1% acetocarmine solutions. More than 300 pollen grains were scored for each individual. Stained pollen grains with a normal size were considered to be fertile. Faintly stained small pollen grains and empty pollen grains were considered to be sterile. In this study, plants showing higher than 90% pollen fertility were classified as pollen fertile, and two classes of pollen sterility were identified: partial sterility (with 70–90% fertile pollen) and semisterility (30–70% fertile pollen).

DNA analysis and linkage map

DNA samples used for PCR analyses were crudely extracted using a 0.25 M NaOH solution, followed by neutralization with 0.1 M Tris-HCl. The crude extracts were diluted fourfold and 4.0- μ l aliquots of the diluted extracts were used as DNA templates in the PCR reactions. TIGR and BLAST analyses of the genomic DNA sequences of Nipponbare and 93-11 were used to design the indel and SSR markers used in this study. The primer sequences for all markers are listed in Table S1. The PCR reactions were performed using the GO-Taq Green Master mix (Promega) in 10- μ l final volumes according to the manufacturer's instructions. The amplification reactions were generally carried out as follows: 30 cycles of denaturation at 94° for 20 s, annealing at 56° for 20 s, and elongation at 72° for 30–60 s. The PCR products were resolved on 2.0% agarose gels and visualized by ethidium bromide staining. Linkage maps of marker loci were constructed with Map Manager QTX version 0.30 (Manly *et al.* 2001). The recombination frequencies (%) were converted into genetic distances (in cM) using the Kosambi function (Kosambi 1944).

QTL analysis and genetic dissection of the *EFS* locus

The QTL and epistatic interaction analyses were carried out using a marker regression analysis with Map Manager QTX version 0.30 (Manly *et al.* 2001). A likelihood ratio statistic (LRS) score of 12.0 was used as the threshold to declare the presence of a putative QTL. This corresponds to an LOD score of 2.6 (LOD = LRS/4.6). To detect epistatic interac-

tions between QTL, a genome-wide scan for all pairs of marker loci located on substituted segments was performed with Map Manager QTX, with the assumption that a QTL is right on a marker locus. The interaction effect and the magnitude were calculated using a two-way ANOVA. The *EFS* locus was mapped using the BC₂F₂, BC₃F₂, and BC₄F₁ populations derived from the cross between Nipponbare and 93-11. The fertile homozygotes for *S24* did not provide any recombination information for *EFS* mapping. Therefore, 140 *S24*-heterozygous plants, identified by marker-assisted selection with the *mS1* and *mS2* markers, were used for mapping *EFS* (Figure 3D).

Results

The *S24* sterility is dependent on genetic background

S24 was first identified in backcross populations for introduction of an *indica* chromosome segment into a *japonica* cultivar of rice (Kubo *et al.* 2008). The *S24* locus causes pollen semisterility owing to selective abortion of male gametes carrying *S24-j* (*japonica* allele for *S24*) in the heterozygote, leading to severe segregation distortion at the *S24* locus in favor of the *indica* alleles in the segregating population (Kubo *et al.* 2008). This pollen sterility appeared to be mainly caused by an allelic interaction at the *S24* locus. If this allelic interaction is sufficient to cause the phenotype, the same phenotype should be seen in any genetic background. To address the question we developed NILs carrying a single heterozygous segment harboring the *S24* locus in reciprocal backgrounds and examined their pollen phenotypes. A heterozygous NIL with Asominori (*japonica*) genetic background (AI-NIL- F_1), showed pollen semisterility (41.8% fertility) (Figure 1), as was demonstrated previously (Kubo *et al.* 2008). Its selfed progeny (AI-NIL- F_2) exhibited significant segregation distortion representing preferential transmission of the *indica* allele at *S24* (*jp/jp*: *in/jp*: *in/in* = 6:69:67, $\chi^2 = 52.52$, $P < 0.001$) (Table 1). However, a heterozygous NIL with the IR24 (*indica*) background (IA-NIL- F_1), produced fertile pollen grains (92.9% fertility)

Table 1 Genotype frequencies of a marker locus linked to *S24* in backcross populations derived from *indica/japonica* crosses

Population ^a	Parental genotype at the <i>EFS</i> locus ^b	No. of plants with each genotype ^c			Total	% of <i>jp/jp</i> ^d	χ^2 for 1:2:1
		<i>jp/jp</i>	<i>in/jp</i>	<i>in/in</i>			
AI-NIL-F ₂	<i>efs-j/efs-j</i>	6	69	67	142	4.2	52.52***
IA-NIL-F ₂	<i>EFS-i/EFS-i</i>	23	21	25	69	33.3	10.68**
BC ₂ F ₂ -1	<i>EFS-i/efs-j</i>	24	68	61	153	15.7	19.78***
BC ₃ F ₂ -8-4	<i>EFS-i/efs-j</i>	18	48	35	101	17.8	5.97 NS
BC ₄ F ₂ -8-4-1	<i>EFS-i/efs-j</i>	41	89	59	189	21.7	4.07 NS
BC ₃ F ₂ -8-6 ^e	<i>efs-j/efs-j</i>	7	65	50	122	5.7	30.83***

NS, not significant; ** $P < 0.01$, *** $P < 0.001$.

^a AI-NIL represents Asominori carrying the IR24 allele for *S24*, and IA-NIL represents IR24 carrying the Asominori allele for *S24*. See Figure 1. The BC₂₋₄ populations were derived from a cross between Nipponbare and 93-11.

^b All parents were heterozygous for *S24* (*S24-i/S24-j*). The parental genotypes at the *EFS* locus were determined on the basis of the marker locus *mE4*. Those that were homozygous for the *EFS* locus are shown in boldface type. *EFS-i* and *efs-j* denote *indica* and *japonica* alleles for the *EFS* locus, respectively.

^c DNA marker *mS2* linked to *S24* was used for genotyping, with the exception of the BC₂F₂ population, for which *mS3* was used. *jp/jp*, *japonica* homozygous; *in/jp*, heterozygous; *in/in*, *indica* homozygous.

^d The expected proportion (%) of the *jp/jp* genotype in each population is 25%. Note that a reduced proportion of the *jp/jp* genotype at *mS2* (linked to *S24*) (underlined) was observed in populations that were homozygous for the *japonica* *EFS* allele (*efs-j/efs-j*).

^e BC₃F₂-8-6, a sister line of BC₃F₂-8-4, was homozygous for the *japonica* *EFS* allele.

(Figure 1). Selfed progeny of the IA-NIL-F₁ (IA-NIL-F₂) did not show reduced frequency of *japonica* allele at *S24* (the frequency of *japonica* homozygote was 33.3%), even though the segregation ratios among the IA-NIL-F₂ also deviated from 1:2:1 (*jp/jp*: *in/jp*: *in/in* = 23:21:25, $\chi^2 = 10.68$, $P = 0.004$) (Table 1). This result indicated that the *S24-j* male gametes in the *S24* heterozygotes with the *indica* background were fertile and transmitted normally to their progeny. Therefore, we concluded that the *S24* heterozygous alleles can induce male sterility only in the *japonica* background, and that epistasis must be involved in the genetic mechanisms of the pollen sterility caused by *S24*.

Detection of the epistatic factor for pollen sterility

Since the Nipponbare (*japonica*) and 93-11 (*indica*) genomes have been fully sequenced, we decided to carry out further analyses with these varieties. First we needed to confirm that the same *S24* locus occurred in these lines and then identify the epistatic gene hidden in the *japonica* genetic background. To these ends we developed another set of backcross populations using Nipponbare as the recurrent parent and 93-11 as the donor parent (Figure S1). Both parents have >90.0% pollen fertility, while their reciprocal F₁ hybrids, Nipponbare/93-11 and 93-11/Nipponbare, showed $37.8 \pm 2.8\%$ ($N = 3$) and $42.9 \pm 7.6\%$ ($N = 3$) pollen fertility, respectively. Among eight plants from the BC₁F₁ generation (Nipponbare/93-11//Nipponbare), we identified a fertile segregant (90.8% pollen fertility) that carried heterozygous segments harboring the *S24* locus. To identify the other genetic factors that affect pollen sterility, the fertile BC₁F₁ plant was backcrossed with Nipponbare, and the segregating BC₂F₁ population ($N = 47$) was analyzed. The BC₂F₁ population showed a wide distribution for pollen fertility (37.7–100%) with two classes of sterility phenotypes: semisterility (37.7–70.0%) and partial sterility (70.0–90.0%) (Figure 2A). We performed a QTL analysis using the BC₂F₁ population with 76 PCR markers that were evenly distributed throughout the 12 rice chromosomes (Ta-

ble S1). A marker regression analysis detected two putative QTL for pollen sterility, designated *qPS2* and *qPS5*, which were located on chromosomes 2 and 5, respectively (Table 2). The *qPS5* QTL was linked to markers *chr05-109* and *mS3* and reduced pollen fertility in plants with the heterozygous genotype. On the other hand, the *qPS2* QTL at the marker locus *mE4* increased pollen fertility in the heterozygous plants. Since the position and phenotypic effect of the *qPS5* QTL coincided with those of the *S24* locus (Kubo *et al.* 2008), *qPS5* was considered to be *S24*. Next we performed a genome-wide interaction analysis to identify marker pairs that interacted epistatically to significantly affect pollen fertility. As a result, we identified two pairs of interactions, one involving the pair *qPS5* (*S24*) and *qPS2* and the other involving loci on chromosomes 4 (*chr4-3173*) and 9 (*chr9-0755*) ($P < 0.001$). A two-way ANOVA revealed a significant interaction effect between the marker loci *mS3* (linked to *S24*) and *mE4* (linked to *qPS2*) (Figure 2B, Table S2). The combination of heterozygous alleles for *mS3* (*S24*) and homozygous Nipponbare (*japonica*) alleles for *mE4* (*qPS2*) significantly reduced pollen fertility (63.7%) compared with the other genotype combinations (>95.0% fertility). This result suggests that *qPS2* may be associated with *S24* in its effects on pollen fertility. The *qPS2* QTL has not previously been characterized and no gene/QTL for pollen sterility has previously been reported for this region of chromosome 2. Therefore we named this new locus *EFS* (Epistatic Factor for *S24*). The other pair of interacting loci, *chr4-3173* and *chr9-0755*, significantly reduced pollen fertility (65.5%) (Table S2). However, further analyses did not show any interactive effects on sterility between these two loci (data not shown).

Genetic dissection of the *EFS* locus

To verify the effect and position of the *EFS* locus, we developed the advanced backcross populations BC₃F₂ and BC₄F₁ by making additional backcrosses between a fertile BC₂F₁ segregant and Nipponbare. Specific *S24* and *EFS*

Figure 2 Analysis of genetic interactions relating to pollen sterility in a BC₂F₁ population derived from the cross Nipponbare/93-11/Nipponbare//Nipponbare. (A) Histogram of pollen fertility in the BC₂F₁ population. Plants in the distribution were genotyped using the *mS3* marker, which is linked to the *S24* locus on rice chromosome 5. The *in/jp* plants were heterozygous for *mS3*, while *jp/jp* plants were homozygous for the Nipponbare (*japonica*) *mS3* allele. Note that semisterile plants were exclusively heterozygous at the *mS3* locus. (B) Interaction effects of marker loci *mS3* and *mE4* on pollen sterility in the BC₂F₁ population. The mean pollen fertility (%) of plants with each pair of genotypes at the *mS3* and *mE4* loci are shown. The *mE4* marker is linked to *qPS2* (*EFS*), a putative QTL located on chromosome 2. See also Table S2 where details of the statistics are shown.

alleles were identified by marker-assisted selection. The BC₃F₁-8-4 plant, which was used as the progenitor of the BC₃F₂ and BC₄F₁ populations, carried only small segments of chromosomes 2, 4, and 5 from 93-11 in an otherwise uniform Nipponbare background (Figure 3A). We evaluated the phenotypes of all nine genotype classes generated by different combinations of *S24*-linked and *EFS*-linked marker alleles, in both the BC₂F₂ and BC₃F₂ populations (Table S3, Figure S2). We found that the *S24-i/S24-j* heterozygotes showed pollen sterility (average 70.1% in the BC₃F₂ population) only when they were combined with the homozygous Nipponbare *EFS* alleles (*efs-j/efs-j*). All other genotype combinations showed no abnormalities in their pollen phenotypes (Table S3). This result provided conclusive evidence for the existence of *EFS* and its effect of inducing male sterility in *S24* heterozygotes when the recessive *efs-j* allele is present in the homozygous condition. The BC₄F₁ population was also analyzed to confirm the *EFS* effect on phenotype. Measurements of mean pollen fertilities, along with microscopic examinations of pollen grains, indicated that pollen sterility occurred only in plants that were heterozygous at the *S24* locus and homozygous for

the Nipponbare allele at the *EFS* locus (Figure 3, B and C). As in the BC₂F₁ and BC₃F₂ populations, the *S24-i/S24-j efs-j/efs-j* genotype in the BC₄F₁ generation showed two types of pollen sterility: semisterility (30.3–59.1% fertile) and partial sterility (74.0–82.3% fertile) (Figure S2).

We next performed a genetic dissection of the *EFS* locus to determine its position using the BC₂F₂, BC₃F₂, and BC₄F₁ populations. Since the fertile *S24* homozygotes did not provide any recombination information for *EFS* mapping, a total of 140 plants with the *S24* heterozygous genotype were examined. Of the 140 informative plants, we found 2 with recombinations between *EFS* and the marker loci. One recombinant, BC₄F₁-8-4-39, had a recombination breakpoint between *mE3* and *mE4*. The other, BC₃F₂-8-4-37, was recombined between *mE4* and *mE5*. Both recombinants showed the fertile phenotype, and we therefore concluded that the fertile *EFS* allele (*EFS-i*) resided on the substituted regions that overlapped in the two recombinants. Therefore, the *EFS* gene was mapped to a 817-kb region between the marker loci *mE3* and *mE5* on chromosome 2 (Figure 3D).

Selective transmission of the *S24-j* gamete is counteracted by *EFS-i* in the sporophyte

The transmission rates of individual *S24* alleles in male gametes were examined to determine whether the *EFS* effect on *S24* occurs in the gametophyte or the sporophyte. The transmission rates were evaluated using reciprocal crosses between the double heterozygote for *S24* and *EFS* (NIL^{*S24+EFS*}) and Nipponbare. If *EFS* acts gametophytically, pollen with the *S24-j efs-j* genotype would be selectively eliminated. In contrast, if *EFS* acts sporophytically then no biased transmission would be observed. The results revealed that the transmission ratio of each genotype fitted a theoretical ratio of 1:1:1:1. Male gametes carrying the *S24-j efs-j* alleles were transmitted with a frequency of 14.3% when the double heterozygote was used as the male parent (Table 3). This was slightly lower than the expected frequency of 25.0% but higher than that observed when a plant that was heterozygous at the *S24* locus and homozygous for the *efs-j* allele was used as the male parent [0% in our previous study (Kubo *et al.* 2008)]. In two different generations, the double heterozygotes for *S24* and *EFS* produced *S24-j/S24-j* progeny at frequencies of 17.8% (BC₃F₂-8-4) and 21.7% (BC₄F₂-8-4-1), on the basis of the transmission frequencies of a linked marker (Table 1). These frequencies were distinctly higher than the 5.7% observed in the BC₃F₂-8-6 population,

Table 2 Marker loci showing significant associations with pollen fertility in the BC₂F₁ population

QTL	Chromosome	Marker locus	Position (kb)	LRS	VAR (%)	P	Add
qPS5	5	<i>chr05-109</i>	1,112	13.2	24	0.00028	−16.65
	5	<i>mS3</i>	1,477	13.2	24	0.00028	−16.65
qPS2	2	<i>mE4</i>	20,300	14.3	26	0.00016	+17.20

Marker loci showing significant associations ($P < 0.01$) on the basis of a marker regression analysis are listed. Position, physical position on rice chromosome (MSU 6.0); LRS, likelihood ratio statistic; VAR, percentage of total variance attributable to the marker locus; Add, additive effect of the *indica* (93-11) allele compared to the *japonica* (Nipponbare) allele.

Figure 3 Genetic dissection of the *EFS* locus. (A) Diagram showing the genotype of the BC₃F₁ plant that was used as a parent of the BC₄F₁ population. White and black regions are derived from Nipponbare and 93-11, respectively. The long arm region of chromosome 2 containing the *chr2-2162* locus was homozygous for the Nipponbare allele (see also Figure 3D). (B) Mean pollen fertilities (%) of BC₄F₁ plants, classified according to their marker genotypes for *mS2* (linked to *S24* on chromosome 5) and *mE4* (linked to *EFS* on chromosome 2). The genotypes are shown by chromosome diagrams above each micrograph in Figure 3C. Error bars indicate standard deviations. (C) Micrographs of pollen grains collected from BC₄F₁ plants representing each of the four genotypes. (D) Physical maps of the regions surrounding the *S24* gene on chromosome 5 (Chr5) and the *EFS* gene on chromosome 2 (Chr2). Marker loci are shown above the lines representing each region, along with the genetic distances between them (in cM) obtained using the BC₃F₂ population. The physical positions of the markers (based on the International Rice Genome Sequencing Project build 4 sequence assembly) are shown below the lines. Below the maps are diagrams showing the genotypes of individual plants or lines as indicated on the left. Shaded bars represent heterozygous regions and open bars represent regions that are homozygous for Nipponbare alleles. NIL^{*S24+EFS*} and NIL^{*S24*} are BC₄F₁ segregants with and without recombinations between *EFS* and nearby marker loci, respectively. The mean fertilities (%) and standard deviations (*N* = 3) are shown on the right.

which was a sister line of BC₃F₂-8-4 that was homozygous for the Nipponbare *EFS* allele (genotype: *S24-i/S24-j efs-j/ efs-j*). These results indicate that the *S24-j* male gametes were able to develop normally in the *EFS-i/ efs-j* heterozygotes and be transmitted to the progeny. The results also suggest that the *EFS indica* allele (*EFS-i*) acts dominantly to counteract the sterility of the *S24-j* male gametes in the *S24* heterozygotes. Thus, our results consistently indicate that the allelic interaction at the *S24* locus requires the presence of the *efs-j* homozygous genotype to induce the antagonistic allelic interactions causing male sterility.

Discussion

Multiple epistatic interactions affect the *S24* gene

Here we present evidence that allelic interactions at the *S24* locus induce the selective abortion of male gametes carrying the *S24-japonica* allele (*S24-j*) via genetic interactions with the unlinked locus *EFS*, which is located on chromosome 2.

The recessive *japonica* allele for *EFS* (*efs-j*) causes male sterility in collaboration with *S24*. Conversely, the dominant *indica* allele for *EFS* (*EFS-i*) counteracts the pollen sterility by *S24*. Generally, pollen killer or gamete eliminator systems induce sterility only when the causal loci are heterozygous. Therefore it has been widely documented that allelic interactions are the major causes of these phenomena. Few epistatic factors regulating pollen/egg killer or gamete eliminator systems have been reported in any plant species, despite a long history of studies for nearly half a century. The reason why epistasis in such hybrid sterility systems stubbornly remains obscure might be because the allelic interaction model appears to sufficiently explain these phenomena. In fact, the allelic interaction at the *S24* heterozygous locus remains an important causal factor in this male sterility system. Our study demonstrated that the allelic interaction at *S24* becomes active only in the sporophyte homozygous for recessive allele of *EFS* (*efs-j*). The *S24-EFS* interaction can be interpreted as a sporogametophytic interaction by two independent loci as shown by

Table 3 Transmission frequencies of male gametes in reciprocal test crosses between NIL^{EFS+S24} and Nipponbare

Cross combination ♀/ ♂	Numbers of male gametes, classified using marker genotypes				Total	χ ² for 1:1:1:1
	<i>mS2-i</i> <i>mE4-i</i>	<i>mS2-i</i> <i>mE4-j</i>	<i>mS2-j</i> <i>mE4-i</i>	<i>mS2-j</i> <i>mE4-j</i>		
Nipponbare / NIL ^{EFS+S24}	14 (28.6)	17 (34.7)	11 (22.4)	7 (14.3)	49	4.47 NS
NIL ^{EFS+S24} / Nipponbare	12 (26.7)	8 (17.8)	15 (33.3)	9 (20.0)	44	2.72 NS

NS, not significant. The male gamete genotypes were determined by genotyping the F₁ progeny using the *mS2* marker (linked to the *S24* locus) and the *mE4* marker (linked to the *EFS* locus). The numbers in parentheses are the transmission frequencies (%).

model III in Figure 4A. This genetic model fits into neither of the previously recognized genetic models for hybrid sterility systems (models I and II in Figure 4A).

Another interactor, *S35*, has been shown to associate with *S24* and enhance pollen sterility in *S24* heterozygotes (Kubo *et al.* 2008). The *S35* gene requires the presence of the *S24-i* allele to cause pollen sterility, but *S24* can induce semisterility independently of the *S35* genotype, and thus this interaction is unidirectional (Figure 4B). The relationship between *EFS* and *S35* remains to be elucidated. However, since both *S35* and *EFS* interact with *S24* and affect its activity, we expect that *S35* participates in the *S24*–*EFS* network in some way. In another case, female sterility due to epistasis among three unlinked loci has been found in one cross combination in rice. In this case, interactions among one sporophytic and two gametophytic genes induced the selective abortion of female gametes carrying a specific pair of alleles (Kubo and Yoshimura 2005). These previous findings and those presented here emphasize a large contribution of epistasis to the genetic mechanisms of hybrid sterility.

They further suggest that hybrid sterility may be controlled by complicated networks composed of a variety of epistatic genes.

Molecular and functional aspects of *S24* and *EFS*

Our studies demonstrated that hybrid sterility is strictly controlled by the *S24* and *EFS* genotypes. In further studies we will identify and characterize the products of the *S24* and *EFS* genes, in an effort to understand how they cause the selective abortion of the *S24-j* male gametes. Since the genome sequence data for both the *S24* and *EFS* regions did not show any major structural differences such as large inversions, insertions, or deletions between Nipponbare and 93-11 (MSU Rice Genome Annotation Project ver. 6.0), the hybrid male sterility phenotype appears to be caused by negative interactions between proteins generated from polymorphic alleles at each locus. Zhao *et al.* (2010) identified the Ankyrin-3 (ANK3) protein as a primary candidate for the *S24* product, on the basis of a map-based cloning approach. We also performed fine mapping of *S24*, and

Figure 4 Genetic mechanisms of hybrid male sterility in rice. (A) Three genetic models for hybrid male sterility in plants. Model I assumes that sterility is controlled strictly by allelic interactions at a single locus and is currently the most widely accepted explanation for pollen killer/gamete eliminator systems. Model II is explained by a reciprocal loss of function at duplicated genes. In this model, only the gamete carrying the pair of loss-of-function alleles aborts, leading to 75% fertility in the F₁ hybrid. Model III, predicted by the present study, proposes that allelic interactions at *S24* cause the selective abortion of the *S24-j* male gamete via an epistatic interaction with *efs-j*. (B) Diagram showing the epistatic interactions among the *S24*, *S35*, and *EFS* loci for hybrid male sterility and their effects on pollen phenotype. The *efs-j* allele is required for the *S24*-induced male semisterility. In addition, a unidirectional interaction between *S35-i* and *S24-i* enhances the male sterility phenotype. Therefore, the double heterozygous genotype for *S24* and *S35* (*S24-i/S24-j S35-i/S35-j efs-j/efs-j*) shows high male sterility.

the candidate region of *S24* was narrowed down to the region between *mS1* and *mS2* containing ANK3 (122-kb region) (Figure 3D). ANK is an adapter protein that exclusively mediates protein–protein interactions and is involved in various biological activities including signal transduction and the maintenance of cytoskeleton integrity. Therefore, it is possible that *EFS* may encode a protein capable of binding with ANK3. Because the *EFS* candidate region contains ~80 predicted genes excluding transposable elements, it remains to be determined whether the *EFS* effects on pollen sterility involve protein interactions with ANK3. Since neither Nipponbare nor 93-11 have gene encoding ANK-domain protein at the *EFS* candidate region, the genetic interaction between *S24* and *EFS* should not be due to duplication of ANK3 at these loci. Meanwhile tandem array of ANK3 (three copies) were found at the 122-kb region of Nipponbare and 93-11 alleles, suggesting that allelic diversity at these ANK3 loci may be a cause of the pollen sterility. Histological analyses revealed that the *S24* genotype did not affect pollen development at the male meiotic stage, but that mitotic cell cycle arrest was observed at the mononuclear or bicellular pollen stage in affected genotypes (Kubo *et al.* 2008; Zhao *et al.* 2010). The (sporophytic) tapetal cells degenerate at this developmental stage, and this degeneration is essential for providing a supply of nutrients needed for normal pollen development. Since the *EFS* and *S24* genes function in a sporophytic manner, it seems likely that they function in diploid tissues of reproductive organs, such as tapetal cells, which have a strong influence on pollen development. A number of mutant analyses relating to microsporogenesis have been reported in plants (Borg *et al.* 2009). However, the molecular networks that control microsporogenesis remain largely unknown. Further studies of the *S24*–*EFS* network should aid our understanding of the molecular mechanisms involved in hybrid male sterility as well as the molecular networks underlying microsporogenesis.

Putative supergene complex

A few issues remain unresolved by this study. One is the large variation in the pollen sterile phenotypes due to *S24* (see Figure S2). Another is the incomplete elimination of the skewed transmission frequencies of male gametes in progeny of the double heterozygous plants (Tables 1 and 3). Previous studies showed that *S24* (*f5-Du*) consistently and drastically reduced pollen fertility without the partial sterility (i.e., 70.0–90.0% pollen fertility) found in this study, even in different cross-combinations and under different environmental conditions (Wang *et al.* 2006; Kubo *et al.* 2008). In the present study, we developed a BC₄F₁ population carrying only a few chromosomal segments spanning the *S24* and *EFS* loci, to evaluate the exact phenotype of the *EFS* gene (see Figure S1 and Figure 3). However, segregants carrying the *S24-i/S24j efs-j/efs-j* genotype showed large phenotypic variations (ranging from 30.3 to 82.3% pollen fertility), which were carried over from the BC₂F₁ and BC₃F₁

generations (Figure S2). Presumably, the causes of this variation are undetermined genetic factors that may or may not be linked with *S24*.

The incomplete elimination of the skewed transmission frequencies (mentioned above) may be due in part to an unidentified segregation distorter linked to *S24*. The lengths of the substituted segments in the populations analyzed in this study were different from those used in our previous study (Kubo *et al.* 2008). This allowed for different allele combinations via recombination within the linked loci. There are many cases in which the chromosomal region causing hybrid sterility contains two linked genetic loci (Sano 1990; Long *et al.* 2008). Intriguingly, a QTL analysis by Li *et al.* (1997) identified putative “supergenes” relating to hybrid sterility on rice chromosomes 5 and 2, in regions adjacent to the *S24* and *EFS* loci, respectively. Supergenes are defined as groups of tightly linked genes within which recombination will cause reduced fitness (Darlington and Mather 1949). In light of these observations, the genetic and genomic features of *S24* may closely resemble the segregation distorter (SD) system of *Drosophila*. SD is a multi-gene selfish gene complex (supergene cluster), in which different allele combinations regulate segregation distortion and determine phenotypic degrees (Presgraves 2007). *Sd*, one of the gene loci in the SD complex, encodes a truncated RanGAP that possesses enzyme activity but lacks intracellular localization domains, leading to mislocalization to the nucleus and causing segregation distortion. Unlinked suppressors have also been characterized in the SD system, suggesting that complex interactions between *cis* and *trans* components play roles in this reproductive isolation mechanism. If *S24* is part of an SD-like system in rice, there may be numerous epistatic factors at both linked and unlinked loci that have yet to be identified, and these may form a complicated mechanism regulating the hybrid male sterility.

Evolutionary aspects of *S24* and *EFS*

On the basis of the present results, the dominant *EFS-i* allele allows the abortive *S24-j* allele to be transmitted to the progeny by counteracting the pollen sterility by *S24*. Therefore, the *S24-i* allele has no strong advantage in early segregating generations of hybrid progeny between *indica* and *japonica*. Actually, no segregation distortion in the chromosomal region harboring *S24* has been detected in an F₇ population of recombinant inbred lines of Asominori/IR24 (Tsunematsu *et al.* 1996), and the same is true for the F₁₁ generation of a set of Nipponbare/93-11 recombinant inbred lines (Huang *et al.* 2009). The segregation distortion observed in this study was elicited by backcrossing with *japonica* parents. Once the *efs-j* allele becomes fixed as homozygous in a population, the abundance of the *S24-i* allele should drastically increase due to the selective elimination of the *S24-j* allele. To understand the role of the *EFS* locus in the evolution of the allelic *S24*-sterility system, it would be useful to determine the timing of the appearance of mutations at both the *S24* and *EFS* loci. It is not yet clear whether

the sterility-associated *S24* mutations arose before the epistatic *EFS* mutations in the ancestral species. For example, if the *S24-j* allele arose before the *efs-j* allele, *EFS* might have played an important role in allowing the deleterious *S24-j* mutation to spread and become fixed in the initial population without a reduction in fitness. More importantly, a sterility-neutral allele of *S24* (*S24-n*), conferring wide compatibility with both the *S24-i* and *S24-j* alleles, has been found in an analysis of the *aus* variety “Dular” (Wang *et al.* 1998; Zhao *et al.* 2010). This multiple allelism could also account for the development of a sterility barrier without a reduction of fitness during the process of evolution (Nei *et al.* 1983). On the basis of our findings and those of others, it is also possible that the sterility barrier became established through the gradual accumulation of mutations at multiple loci showing both epistatic and allelic interactions, without any fatal phenotypes developing during population divergence. Further molecular studies to elucidate gene structures and functions, along with phylogenetic and evolutionary analyses, will provide insights into the evolutionary history of the hybrid sterility system controlled by *S24* and *EFS*.

Acknowledgments

We thank T. Makino and Y. Gonohe (National Institute of Genetics, Mishima, Japan) for technical assistance and Y. Harushima for providing indel marker information and BC₁F₁ seeds derived from the cross between Nipponbare and 93-11. We thank Y. Yamagata and M. Shenton for a critical review of this manuscript. We also thank two anonymous reviewers for useful comments. This study was partly supported by a grant-in-aid for special research on priority areas from the Japanese Ministry of Education, Science, Culture, and Sports (no. 18075009 to N.K.) and partly by a grant-in-aid for young scientists (B) from the Japanese Society for the Promotion of Science and the Japanese Ministry of Education, Culture, Sports, Science, and Technology (no. 21780008 to T.K.).

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Communicating editor: A. H. Paterson

GENETICS

Supporting Information

<http://www.genetics.org/content/suppl/2011/08/25/genetics.111.132035.DC1>

Hybrid Male Sterility in Rice Is Due to Epistatic Interactions With a Pollen Killer Locus

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Figure S1 Crossing scheme used for the development of backcross populations.

Figure S2 Histograms of pollen fertility in the BC₂F₂, BC₃F₂, and BC₄F₁ populations. Plants were classified according to their marker genotypes for *mS2* or *mS3* (linked to *S24*) and *mE4* (linked to *EFS*). The marker *mS2* was used for genotyping in the BC₃F₂ and BC₄F₁ populations, while *mS3* was used for the BC₂F₂ population. Black: plants that were heterozygous for *mS2* (or *mS3*) and homozygous for the Nipponbare *mE4* allele. Gray: all other genotypes.

Table S1 Primer sequences used in this study

Marker ^a	Primer sequence(5'-3')		Position on chromosome ^b		Predicted size ^b	Purpose
	Forward	Reverse	bp	Chr.	(base pair)	
* chr01.0176	CTCCACGTAAATACGTCCTG	GTTGAACCACGCAACATCAG	1761487	1	372	Whole-genome genotyping
chr01.0724	AGAAGGGCAAAAGAGGAAGC	AACATCAAAATGCGGTGTTG	7242741	1	201	Whole-genome genotyping
* chr01.1172	CATGGATGGGATAAGCCAAC	TGGATGGAAGATGTTGCTCG	11720537	1	667	Whole-genome genotyping
* chr01.2217	AAGAGATGAACCAAGCCCAAC	CCTCATTGGCAACAAGATGG	22168461	1	458	Whole-genome genotyping
* chr01.2397	TGTCGGTTGACACCTTCTAG	TTATGATGAGGCCTGTGTGG	23965804	1	780	Whole-genome genotyping
* chr01.2584	GCCTAAAGTCAGGCGACTAG	GTTTGGACTGTTCCAACGTC	25843803	1	274	Whole-genome genotyping
* chr01.3340	GGTCAGCAAAGCAAGTCTAC	CATCGCAGCTGAATAGCATG	33404444	1	727	Whole-genome genotyping
* chr01.389	ATAGTCACTCACGATGCGTG	CATGGACTTTTGACACCAGG	40683184	1	547	Whole-genome genotyping
* chr01.450	TGTAGAGATAGACAGGGC	ACAGAGAGTGACATGCAGTG	45016779	1	610	Whole-genome genotyping
* chr02.0021	TGTGTTAGAAGCTAGCGTCG	CGCATCTGTTAATTGCTGGC	214200	2	789	Whole-genome genotyping
* chr02.0438	CAGAATGCCACTTTGGAAGC	TGATAGTGCAAGTCAGGAG	4386301	2	358	Whole-genome genotyping
* chr02.1037	CGTAGAATAGGAGGTATGCC	CTTCCAGATGAGTGTGACAC	10355285	2	380	Whole-genome genotyping
* chr02.190	TAGAGAGAGGCTGCATGAAG	CCTGGATTTTTGTGTGGTCG	19017401	2	601	Whole-genome genotyping
* chr02.201	AGTTAAGGGATTCTTCGGG	GGATTTCAATAGCACCTCCG	20114053	2	349	Whole-genome genotyping
* chr02.2162	TATCGGCCGAGTTAAAATC	CGATCTACTTCGATCGTTTGG	21619136	2	239	Whole-genome genotyping
* chr02.2966	TATTGCGCTACATGACCTCC	TTTGCTCGGTGTACAAGAG	29661769	2	484	Whole-genome genotyping
* chr02.3622	AGTCCTAATGCCAATCCCTC	CGCCCAATTCATTCTACC	36218157	2	318	Whole-genome genotyping
* chr03.026	ACTGCTACTCCGTACTGTAC	GCAAGGTTTCTCTGGTAAG	2639392	3	431	Whole-genome genotyping
* chr03.049	GGCTATAGATGAATTGGAGC	AAACTGTGTCGTCGGTTCAC	4899521	3	760	Whole-genome genotyping
* chr03.120	TAAGAAGTGAGGGTAGGTGG	CGTGTCCAATTCTCAAGGG	11997506	3	354	Whole-genome genotyping
* 76B11rpoly	AGCCGAAGTCAGGTAAAACC	CCTTTCAATGCCATACGTCC	13381447	3	303	Whole-genome genotyping
* chr03.1628	GAGGGAGTACTTACTTCGTC	CTCACACACTGGATCTTGAG	16279869	3	311	Whole-genome genotyping
* chr03.178	ATGAGCTGCCTTTTGCCAG	GGATCTGTACTAGTTGTGCG	17751662	3	182	Whole-genome genotyping
* chr03.2909	TGTTATCCTCTCTTGCTCTC	CAGCTTCAAATGCAAGCTGC	29089913	3	179	Whole-genome genotyping
* chr03.3716	ACAGTTCCTCCAAAGTCTGC	TGACCTATGAAAGCTGCCAG	37159059	3	351	Whole-genome genotyping
* chr04.036	TCTCTACGGATTGATGAGCC	TGTTCCGGCTCCGTTAGAAAC	3565475	4	427	Whole-genome genotyping
* chr04.192	AGTATCGGTTGCTTCGGTAC	CCACCTGTGTTTGTAGCC	19232094	4	294	Whole-genome genotyping
* chr04.269	TTGCGAATCTGAGACTCTGC	GGGAGTACTAGACACTACTC	26930216	4	587	Whole-genome genotyping
* chr04.3173	TATGTATAGCGTCTCACC	CCGTGAACGTACATATAGGG	31731831	4	565	Whole-genome genotyping
* chr04.358	ACAGACTCTAATCACCTCGC	GGATTAGCTATAAGGTTGGC	35821532	4	714	Whole-genome genotyping
* chr05.0109-2	TGCCAGGATCAGAACTTCAC	CCTGTAGGATTGAGCTAACC	1089969	5	414	Whole-genome genotyping
* chr05.0287	TTGTGAAGTTAGCCGAACCG	CCGTATGGAATAGCACAACC	2868553	5	645	Whole-genome genotyping

*	chr05.0858	GGACTCTGAATCCAGTAACC	CCTGCGGTTCAATCGAATAG	8583505	5	321	Whole-genome genotyping
*	chr05.2229	AGTTGCTGATTACGGTAGCG	TCTCTTCTCCACTCGAAAC	22290043	5	723	Whole-genome genotyping
*	chr05.2729	AGCTGATGGCGAAGAAGGAT	ATCCACTGCAGCTTACAGAC	27290011	5	597	Whole-genome genotyping
*	chr06.0021	GAATTTGACAGGTTCCGCTC	AGAGGTTGCCTACACTGTAG	213961	6	274	Whole-genome genotyping
*	i2121	AATTGATGCAGTGAGCTGCC	CAACCAACGAAAATCACGCC	2273355	6	578	Whole-genome genotyping
*	chr06.0488	GGCTGTAAAGAGTTTCGCAC	TAACGGTTAACGCCGATGAC	4880710	6	548	Whole-genome genotyping
*	i4790	ATACGGCTGATTAGACGGAG	TGTTTCGTGGGCTATAGCTC	6052885	6	828	Whole-genome genotyping
*	chr06.0793	ATCCTGCAGTAACAGTAGGC	ATCTGCTATGATGCTCCACG	7925041	6	510	Whole-genome genotyping
*	chr06.1660	TAGTTCGGTTTACCTGCCTC	TTTGTCTATGCCAGCAGCTC	16597091	6	277	Whole-genome genotyping
*	chr06.2301	GTGGCAGATCTCTGTCTATG	TCAGAGGCTATTCACTCTG	23013731	6	397	Whole-genome genotyping
	chr06.28077-2	TTTGGTACCAAAAGGCGTGG	ACGACATGCACTTATGACCG	28076771	6	265	Whole-genome genotyping
*	chr07.0001-3	ATGTGGAGGAGAGAGAAGCTC	TTCACCAAGCATGTGTGCTC	10088	7	459	Whole-genome genotyping
*	chr07.0317	CCATGAAAAGTCGCTATGGG	ACAGTTGCTGCAGTGTCTTG	3169581	7	281	Whole-genome genotyping
*	chr07.2058	TTTAGGTGGACTGTAGCACG	TCTTTCAGCAGCACAAAGACC	20577737	7	422	Whole-genome genotyping
*	chr07.2290	GGACCGAGTTATGGAGTATG	GCTTGTGTTCTTCTCTTGG	22903860	7	424	Whole-genome genotyping
	chr07.2525	GGCTAGAGAAGCAGCGAGAA	TTCACCGCAGATATGTCACC	25972792	7	488	Whole-genome genotyping
*	chr07.3032	TCACATTAGCTAGCCTAAGC	TGTATATTGCGAGCAGTGGC	30314554	7	543	Whole-genome genotyping
*	chr08.0002	ATCTTCTGGGATCTCAGCG	AGGTATTCTGCAAGGTCTCTG	25340	8	313	Whole-genome genotyping
*	chr08.0373	GCGCATTACCGCTTAGATTG	CACCTTGTTTTGCGCATCTG	3729067	8	315	Whole-genome genotyping
*	chr08.0792	TACTTGACAGACAACCTCAC	AGGAGGATTCTAAGCTCAC	7916010	8	597	Whole-genome genotyping
*	chr08.2372-2	CGGTACACGCTTAAGTATG	TGACTTCAAACCCAGCAAGC	23718926	8	571	Whole-genome genotyping
*	chr08.2701	TGATGATCTGTGGCACTACG	ACTGTCTGGATGACACAAGG	27009274	8	520	Whole-genome genotyping
*	chr08.2823	GGAGTAAGAGGCAACTTAGG	TCGGCATCCAACCTCAAATG	28381207	8	367	Whole-genome genotyping
*	chr09.0755	ATTGGCCTCCAATTCTCTC	CTAGCAAATCCACGGCAAG	7548073	9	430	Whole-genome genotyping
*	chr09.0963	AACACAATGTGCAGCGGATG	GCCATAGATCCTAGGAACAC	9629151	9	430	Whole-genome genotyping
*	chr09.1239	CCATTCCATACAATGCAGGG	AACTGATGCACTTTCACGCC	12385707	9	504	Whole-genome genotyping
*	chr09.1932	TGATGCAATATTGCCGTGCC	TTGTGGCTGAGCATTTGAGC	19323279	9	747	Whole-genome genotyping
*	chr09.2181	TGATGCAATATTGCCGTGCC	TTGTGGCTGAGCATTTGAGC	21814063	9	318	Whole-genome genotyping
*	chr10.0007	TTCTCTGAGCGTATGCTGTC	ACAATGCTGCAGGAAGCTAG	72746	10	515	Whole-genome genotyping
*	chr10.1354	CAACTAGCTAGACTGCAGAG	GACAACTGTCGTGCATAGAG	13544111	10	536	Whole-genome genotyping
*	chr10.1836	TTCTCGTATGGTGGCGTTTC	TAGTTCGGTACATCGATGGG	18359889	10	508	Whole-genome genotyping
*	chr10.2205	GTACTACTGCACGATTCCAC	GTCTAATCGAGTGAATATGC	22052887	10	541	Whole-genome genotyping
*	chr11.0016-1	AAGAGCAAAGCCTGAGGTAG	ACCTTCAAACAATTGTGGCC	158838	11	212	Whole-genome genotyping
*	chr11.0294	TGACTGGAATCTCGTAGTTAC	CCATATCATCAGTGTGGCAC	2942360	11	145	Whole-genome genotyping
*	chr11.0959	ACGAACCGAGACTACAAACG	CCATGACTGGAGAACAGATC	9593512	11	306	Whole-genome genotyping
*	chr11.2115	CATATCGACTCTGCCACTAC	CAAACCTCGAAAGCCTCGAG	21149987	11	707	Whole-genome genotyping

*	i25283_2	CCTGATTAATCCAGCCATGTG	GCTGCTCATTTACCCATTT	25135300	11	218	Whole-genome genotyping
*	chr11.2704	ATGCGTCCAGTTTGTGACCTG	GGTGAGTTGGTGACATAACC	27038247	11	337	Whole-genome genotyping
	chr12.0154	CATATCGATCATGCCTCTTGG	CTTAGTACATACCTGCCTGTC	1544585	12	418	Whole-genome genotyping
	chr12.3016	TCAAGAACAGTGCCAACTCG	GACATGGGCAAGTGGAAGAT	3284698	12	677	Whole-genome genotyping
	chr12.1577	ATTGGCAGCAAAGCTGAGAT	CGAGCTCCACCTTCTCTTTG	15982961	12	537	Whole-genome genotyping
*	i23966	TTGGTTGAGAGCCACACTTG	TTGAAAGGCACCAGGATTTC	21354881	12	670	Whole-genome genotyping
	AL5216	GTGGAACAATGGTTGCCTTT	GAACCCTGGAAACGAACGTA	24806040	12	195	Whole-genome genotyping
*	chr12.2535	AGGAGGTGATTTCCCATGGA	TATCGGGAAGCAGTTTGACC	25353649	12	216	Whole-genome genotyping
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	mE1	AATCAAGAGTGCAGCCAACA	TCTTTAACCGGTCTCGCTGT	19709334	2	218	EFS mapping
	mE2	GAATTCATGGGCATCCACTT	TGCCCATTTTGTGAGTTGAC	20297467	2	261	EFS mapping
	mE3	GCCTCCATTCCCTTCTTCTT	GTCACGCATGAGCCGTTG	20606778	2	273	EFS mapping
	mE4	TTTGACCATGCTTTGACTGG	CTTCCCACACCTGCTACTC	21163432	2	236	EFS mapping
	mE5	GCAAAGCCAAATAAAGCACA	GCTCAATTCATTTACATGACCA	21424538	2	297	EFS mapping
	mS1	AAACAGTAAGGCTCACCAATTT	GAAGCAACTCTGGGGTCAAT	1274926	5	217	MAS for S24
	mS2	GCATGGCATTTTCCAATTT	ATGGCAGATGCATCAGAATG	1397404	5	251	MAS for S24
	mS3	TCTTGAAATCTCCTGCCTTC	TCAGACAATTCAGCCTCTCA	1455302	5	225	MAS for S24

^a Markers with asterisks (*) were designed for a previous study (Mizuta *et al.* 2010).

^b The positions and the product sizes were estimated on the basis of the Nipponbare genome sequence (IRGSP build 4 version).

Table S2 Interaction effects of marker locus pairs on pollen fertility (%) in the BC₂F₁ population

Marker pair	<i>F</i>	<i>P</i>	Pollen fertility (%) in the digenic genotype ^a			
			N x N	N x H	H x N	H x H
<i>mS3 x mE4</i>	38.71	<10 ⁻⁶	98.7±1.9 (10)	99.5±0.6 (11)	63.7±16.7 (11)	96.2±5.3 (15)
<i>Chr4-3173 x Chr9-0755</i>	13.79	0.0006	96.4±6.3 (10)	93.1±11.8 (14)	65.5±26.2 (7)	93.7±11.3 (16)

^aN and H represent genotypes that are homozygous and heterozygous, respectively, for the Nipponbare alleles at the marker loci. The numbers in parentheses indicate number of plants.

Table S3 Pollen fertilities (%) of plants with different combinations of *mS2* and *mE4* alleles in the BC₂F₂ and BC₃F₂ populations

		<i>mS2</i> or <i>mS3</i> genotype ^{a,b}			χ^2
	<i>mE4</i> genotype	<i>jp/jp</i>	<i>in/jp</i>	<i>in/in</i>	
BC ₂ F ₂ -1 (N=153)	<i>jp/jp</i>	98.2±1.3	77.0±18.3	99.7±0.2	19.26*
		(5.2)	(11.1)	(7.2)	
	<i>in/jp</i>	98.0±2.6	98.7±1.1	98.4±1.3	
		(5.9)	(22.9)	(22.2)	
	<i>in/in</i>	98.2±3.3	97.6±1.7	97.2±4.4	
		(4.6)	(13.1)	(7.8)	
BC ₃ F ₂ -8-4 (N=101)	<i>jp/jp</i>	99.2±0.5	70.1±12.7	98.9±0.7	10.72 ^{ns}
		(5.9)	(17.8)	(7.9)	
	<i>in/jp</i>	98.2±1.1	97.5±1.8	98.9±0.9	
		(7.9)	(18.8)	(18.8)	
	<i>in/in</i>	99.1±0.2	97.7±1.7	94.2±12.6	
		(4.0)	(10.9)	(7.9)	

ns: not significant, *: $P < 0.05$

^aThe genotypes of individual plants were determined using *mS3* for BC₂F₂, and *mS2* for BC₃F₂. *jp*: *japonica* allele, *in*: *indica* allele

^bThe numbers in parentheses are genotypic frequencies (%).