Hybrid male sterility in rice controlled by interaction between divergent alleles of two adjacent genes

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Sterility is common in hybrids between divergent populations, such as the *indica* and *japonica* subspecies of Asian cultivated rice (*Oryza sativa*). Although multiple loci for plant hybrid sterility have been identified, it remains unknown how alleles of the loci interact at the molecular level. Here we show that a locus for *indica-japonica* hybrid male sterility, *Sa*, comprises two adjacent genes, *SaM* and *SaF*, encoding a small ubiquitin-like modifier E3 ligase-like protein and an F-box protein, respectively. Most *indica* cultivars contain a haplotype *SaM*$^{+}$*SaF*$^{+}$, whereas all *japonica* cultivars have *SaM*$^{-}$*SaF*$^{-}$ that diverged by nucleotide variations in wild rice. Male semi-sterility in this heterozygous complex locus is caused by abortion of pollen carrying *SaM*$^{-}$. This allele-specific gamete elimination results from a selective interaction of SaF^{+} with SaM^{-}, a truncated protein, but not with SaM^{+} because of the presence of an inhibitory domain, although *SaM*$^{+}$ is required for this male sterility. Lack of any one of the three alleles in recombinant plants does not produce male sterility. We propose a two-gene/three-component interaction model for this hybrid male sterility system. The findings have implications for overcoming male sterility in inter-subspecific hybrid rice breeding.

allelic interaction | gamete selection | hybrid sterility | reproductive barrier | two-gene/three-component model

Hybrid sterility is the most common form of postzygotic isolation mechanisms between species or subspecies, provides an initial force driving genetic differentiation and speciation, and plays an important role in maintaining species identity (1, 2). Hybrid sterility in plants has been known for a long time (3–11). Cultivated rice comprises two species, *Oryza sativa* L. (Asian rice) and *O. glaberrima* Steud (African rice); *O. sativa* is classified further into two major types or subspecies, *indica* and *japonica*, which have been referred to as "Hsien" and "Keng" since the Han dynasty in China (12), based largely on the low affinity of fertility in their hybrids and other morphological features (3, 13, 14). Hybrids between the rice species or subspecies have significant hybrid vigor, or heterosis, that provides great potential for further productivity increases in rice. However, the partial or complete sterility of the hybrids forms a reproductive barrier hindering the utilization of heterosis in inter-(sub)specific hybrid rice breeding.

A number of loci conferring hybrid male or female sterility (or, in a few cases, both) have been identified in rice (8–10, 15–18); however, only a gene encoding an aspartic protease at the locus *S5* (9) conditioning embryo-sac sterility in *indica-japonica* hybrids has been cloned recently (19). Plant hybrid sterility is thought to be caused by interactions between alleles (called "pollen killer," "egg killer," or "gamete eliminator") at certain heterozygous loci derived from divergent populations (5–10). Several genetic models have been proposed for plant hybrid sterility, including the one-locus allelic interaction (one-locus sporogametophytic interaction) model (7–10), and the duplicate gametophytic lethal model (7). The one-locus allelic interaction model, which can explain the genetic behavior of most hybrid sterility loci, proposes that an allelic interaction of a hybrid sterility gene in a heterozygote causes selective abortion of gametes carrying a given allele, in most cases a *japonica* allele in the *indica-japonica* or *O. glaberrima-japonica* hybrids, thus resulting in poor transmission of this allele into the progeny. However, the molecular genetic mechanism for controlling the allele-specific gamete killing remains unknown.

In this study we cloned an *indica-japonica* hybrid male sterility locus, *Sa*, finding that it comprises two adjacently located genes, *SaM* and *SaF*, encoding a small ubiquitin-like modifier (SUMO) E3 ligase-like protein and an F-box protein, respectively. We show that allele-specific hybrid male sterility is controlled by direct and indirect interactions among three divergent alleles of the genes, suggesting that intercellular protein transport may occur during the early microspore development.

Results

Genetic Effect of *Sa* on Hybrid Male Sterility. To study rice hybrid sterility, several near-isogenic lines (NILs) were developed that contained chromosomal segments from *indica* cultivars in the genetic background of a *japonica* variety, Taichung 65 (T65) (7). Using T65 and NIL E4, a major locus conferring hybrid male sterility, *Sa*, was identified (15, 16). T65 with a locus-genotype Sa^j/Sa^j and E4 with Sa^i/Sa^i were fully (>90%) male fertile (pollen fertile, MFF), but their F_1 hybrid (Sa^i/Sa^j) exhibited male semi-sterility (MSS) in which ≈ 50% of pollen was sterile (Fig. 1 *A–C*). The male developmental defect caused by heterozygous *Sa* appeared in microspores of the early uni-nucleate stage (20), but female fertility was unaffected (15, 16). Of 666 analyzed F_2 plants, only three (0.45%) were of Sa^j/Sa^j; the others were either Sa^i/Sa^j or Sa^i/Sa^i, with distribution being equal (Fig. 1 *D–F*). This result showed that the heterozygous *Sa* locus has a strong effect, impairing the development of almost all male gametophytes carrying Sa^j. In typical *indica-japonica* hybrids the accumulative effect of multiple hybrid sterility loci usually leads to serious decreases in both male fertility and seed-setting rate (16).

Identification of Two Adjacent Genes at the *Sa* Locus. *Sa* was mapped primarily on chromosome 1 (21). We further located this locus to a 370-kb region with 200 F_2 plants of the T65 x E4 cross (Fig. 2*A*). We then used the flanking markers A10-2 and G02-148 to screen ~ 10,500 F_2 plants and obtained 322 recombinants. The

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EU337974–EU337977) .

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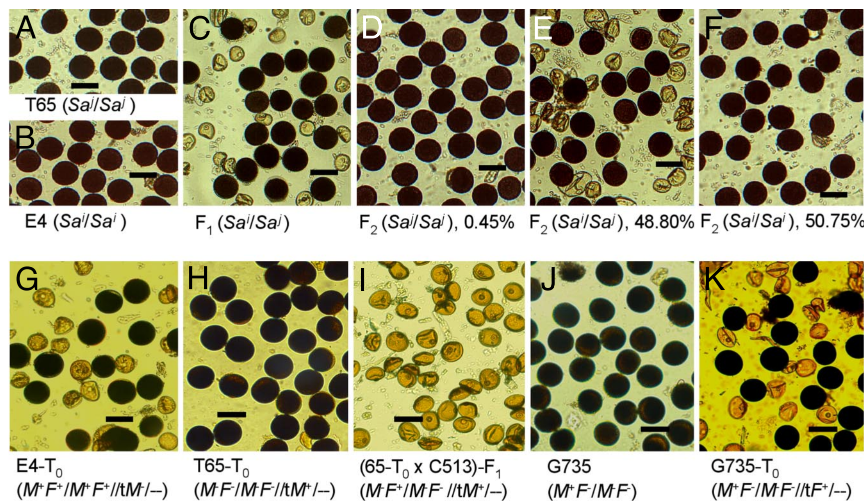

Fig. 1. Hybrid male sterility controlled by the *Sa* locus. (*A–F*) Pollen phenotypes and genotypes of *Sa* in T65 and E4, and their F_1 and F_2 plants. The genotypic frequencies (%) were determined using an *Sa*-cosegregation marker G02–76.3 (see Fig. 2*A*). (*G–L*) Pollen phenotype and genotype of the genes of a recombinant and transgenic plants. M^+, M^-, F^+, and F^- denote *SaM*$^+$, *SaM*$^-$, *SaF*$^+$, and *SaF*$^-$, respectively. tM^-/−, tM^+/−, and tF^+/− indicate hemizygotes of the transgenes. (Space bars, 50 μm.)

high-resolution mapping was accomplished with 11 polymorphic markers developed in this region (supporting information (SI) Table S1). Three crosses between certain recombinants and T65 (Fig. 2*A*) were conducted to obtain new recombinants for the genetic dissection.

Based on the two recombinants G392 and H122, *Sa* was delimited to an ∼ 10-kb region containing two predicted genes, which we named *SaF* and *SaM* (Fig. 2*A*). However, analysis of other recombinants showed that the induction of male sterility by this locus could not be explained by the single-gene hypothesis. Hereafter, the alleles of *SaF*-E4, *SaF*-T65, *SaM*-E4, and *SaM*-T65 are designated *SaF*$^+$, *SaF*$^-$, *SaM*$^+$, and *SaM*$^-$, respectively. Four recombinants (A408, H70, C513, and LS38) with heterozygous *SaF* and homozygous *SaM*$^+$ or *SaM*$^-$ were fully male fertile; three other recombinants (G735, H70T65, and A408T65) with homozygous *SaF*$^-$ and heterozygous *SaM* also showed full fertility, not semi-sterility. On the other hand, G392 and H122 containing heterozygous *SaF* and *SaM* and an individual H71 with homozygous *SaF*$^+$ and heterozygous *SaM* were consistently semi-sterile. A genotype (C513H70) with heterozygous *SaF* and *SaM* in a recombined arrangement between them (repulsion phase) exhibited male semi-sterility, with the *SaF*$^+$*SaM*$^-$ allele set to be eliminated in the male. This genetic dissection demonstrated that the male semi-sterility occurred only when both the *SaM* alleles (*SaM*$^+$/*SaM*$^-$) and at least one *SaF*$^+$ allele (*SaF*$^+$/*SaF*$^-$ or *SaF*$^+$/*SaF*$^+$) were present, and the pollen grains carrying *SaM*$^-$ were aborted. *SaF*$^-$ had no role in male sterility, as shown in G735, H70T65, and A408T65.

SaM Encodes a SUMO E3 Ligase-like Protein, and SaF Encodes an F-Box Protein. The *SaM*$^+$ allele encodes a protein of 257 aa with similarity to the C-terminal region including the SP-RING-like domain of Mms21 (Fig. 2 *B* and *C*), a SUMO E3 ligase in yeast (22). An SNP, the marker G02–74.6, is present in the *SaM*$^-$ allele. This G-to-T mutation changes the 3′ splicing site of the corresponding fifth intron of *SaM*$^+$, which results in a truncated 217-aa protein, by removing the entire sixth exon (80 bp) and creating a new stop codon in the seventh exon (Fig. 2 *A–E*). A database search showed that *SaM* is unique in rice; its nearest homolog in *Arabidopsis thaliana* encodes an unknown protein with a 95-aa region similar to the Mms21 domain in SaM$^+$ (Fig. 2*C*).

SaF encodes a 476-aa protein with an F-box and a plant-specific F-box protein domain (FBD). F-box proteins mediate protein–protein interactions, but the function of the FBD is unclear. The SaF protein is homologous to a ribosomal RNA apurinic site-specific lyase (RALyase) in wheat (23) with 33% identity in the overall sequences and 64% and 44% identities in the F-box and FBD, respectively (Fig. 2*C*). About 20 members of the F-box/FBD subfamily are predicted in rice, and they have up to 75% identity to RALyase and up to 40% identity to SaF. In the coding region of *SaF*, only one SNP was found between *SaF*$^+$ and *SaF*$^-$, which results in a Phe-to-Ser substitution in position 287 (Fig. 2 *A* and *B*).

SaM was expressed in all tissues tested as examined by RT-PCR, albeit at different levels (Fig. 3*A*). This analysis showed that only the cDNA of *SaM*$^+$ was detectable in the purified pollen of the F_1 plants, providing molecular evidence for the selective abortion of *SaM*$^-$-carrying pollen. *SaF* also was expressed constitutively (Fig. 3*A*). Immunoblotting confirmed that *SaM*$^-$ encoded a truncated protein (Fig. 3*B*).

SaF Interacts with SaM$^-$ but not with SaM$^+$. A bacterial two-hybrid (B2H) interaction assay demonstrated that both SaF$^+$ and SaF$^-$ interacted physically with SaM$^-$ but not with SaM$^+$ (Fig. 4*A*). Therefore, the amino acid substitution in SaF$^-$ did not affect its physical interaction with SaM$^-$ but impaired the biological function of SaF$^-$ for male sterility. The interactions were confirmed by a bimolecular fluorescence complementation (BiFC) assay (24), which also showed that the interacting proteins targeted to the cytoplasm and nuclei (Fig. 4*C*). Because both *SaM*$^+$ and *SaM*$^-$ are required for male sterility (see below), we tested whether their protein products interact directly. The results showed that no physical interactions occurred between them or between the homozygous protein molecules (Fig. 4*A*). To find out whether the interaction between SaM$^+$ and SaF is suppressed by an extra domain in SaM$^+$, we prepared four deletion-constructs of *SaM*$^+$ for the B2H assay. Only one construct with deletion to an amino acid position 202, near the divergent point of SaM$^-$, was able to interact with SaF (Fig. 4*B*). Therefore, we determined that a self-inhibitory domain within the 203–218 region of SaM$^+$ (see Fig. 2*C*) blocks the interaction, probably by affecting the protein's structure.

Both *SaM* Alleles Are Required, and *SaM*$^-$ Is a Gametophytic Determinant for the Selective Pollen Killing. Genetic analysis showed that microspores containing *SaM*$^+$ developed normally in F_1 or

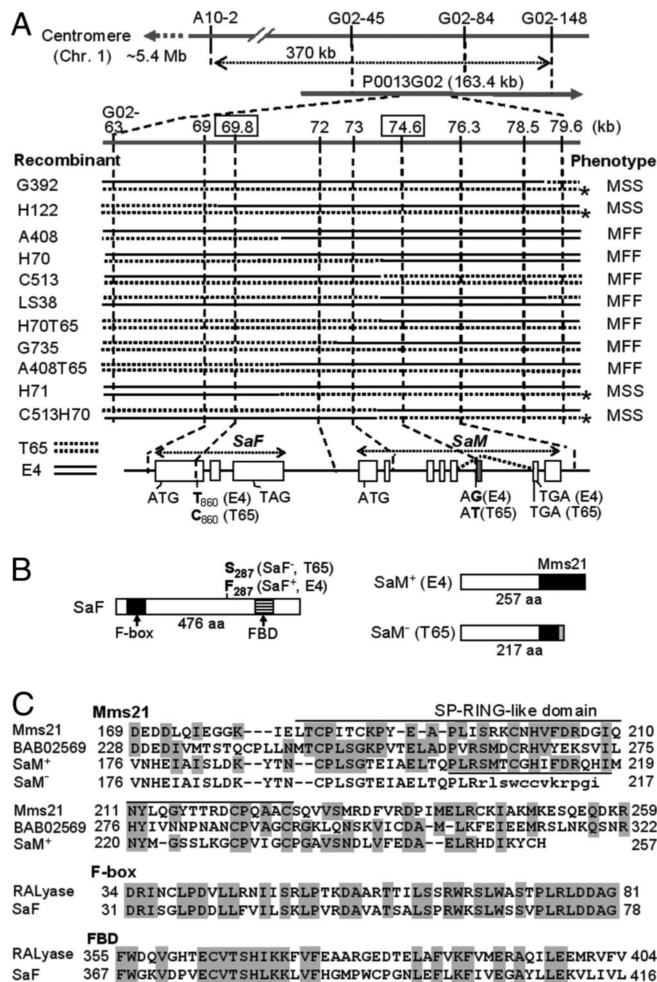

Fig. 2. Identification of two genes in the *Sa* locus. (*A*) *Top*: Primary mapping of *Sa*. The numbers of the markers following G02- indicate their positions (kb) in a P1 artificial chromosome clone P0013G02. *Middle*: Fine mapping with key recombinants obtained from 10,500 F_2 plants and crosses of H70 x T65 (H70T65), A408 x T65 (A408T65), and C513 x H70 (C513H70). The *SaF* and *SaM* alleles of E4 and T65 were determined by the functional SNPs G02-69.8 and G02-74.6, respectively, and G02-76.3 was used for segregation analysis of the recombinant's progenies. MFF, male full fertility; MSS, male semi-sterility; asterisk indicates the chromosome eliminated in male sterility. *Bottom*: Structures of *SaF* and *SaM* alleles. The fifth intron of the *SaM*-T65 allele is shown by a dotted line. ATG, start codon; TAG/TGA, stop codons. (*B*) Sequence and structural variations of the proteins. (*C*) Alignment of conserved domains among SaM, yeast Mms21, and BAB02569 of *Arabidopsis thaliana.* The underlined region in SaM^+ contains the self-inhibitory domain.

recombinants with the genotype SaM^+SaF^+/SaM^-SaF^- or SaM^+SaF^+/SaM^-SaF^+, whereas those carrying SaM^- aborted. To test the function of the alleles and identify the molecular mechanism for this allele-specific gamete selection, we transferred SaM^- (denoted tSaM^-) and SaM^+ (tSaM^+) into E4 and T65, respectively. Three E4 transformants (T_0 generation) with a single tSaM^- insertion showed typical male semi-sterility (see Fig. 1*G*). Furthermore, the transgene segregated in the presence: absence ratio 1:1 (Table 1) and co-segregated with male sterility in the T_1 generation (Table S2). This test also confirmed that SaF^- is not necessary for male sterility.

All 19 T65-T_0 plants with tSaM^+ were fully male fertile (see Table 1 and Fig. 1*H*). We then crossed them with a recombinant (C513) (see Fig. 2*A*) carrying SaM^-SaF^+/SaM^-SaF^-. As expected, $F_1(T_1)$ plants without tSaM^+ and/or SaF^+ were all fully

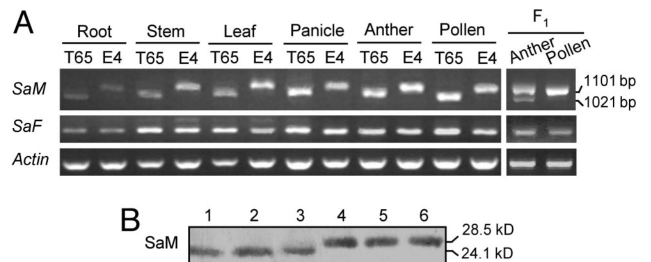

Fig. 3. Expression of *SaM* and *SaF.* (*A*) RT-PCR assay of the genes in T65, E4, and its F_1. Full-length cDNAs of the *SaM* alleles and a cDNA segment (480 bp) of the *SaF* alleles are shown. The anthers included pollen, and pure pollen grains were separated from the anther wall tissue. (*B*) Immunoblot detection of SaM^- of T65 (lanes 1–3) and SaM^+ of E4 (lanes 4–6) prepared from leaf (lanes 1 and 4), anther (lanes 2 and 5), and young panicle (lanes 3 and 6), as probed with an antibody to the N-terminal region.

male fertile (see Table 1). However, segregants with SaM^-SaF^+/SaM^-SaF^-//tSaM^+ were highly male sterile without or with very little viable pollen, rather than exhibiting semi-sterility (see Fig. 1*I*), because all microspores carried SaM^-. These results confirmed that SaM^- (tSaM^-) determined which microspores were aborted, whereas SaM^+(tSaM^+), like SaF^+ (see below), functioned in a sporophytic manner to induce the sterility of the SaM^--carrying microspores.

SaF^+ Is a Component of the *Sa* Male Sterility System. To test the function of SaF^+ for the male sterility, we transferred tSaF^+ into the progeny (three genotypes) of G735 (SaM^+SaF^-/SaM^-SaF^-), a full-fertile recombinant (see Fig. 1*J*). Eighteen T_0 plants were obtained, but only the four plants that had heterozygous *SaM* (and tSaF^+) showed male semi-sterility (see Table 1 and Fig. 1*K*). Furthermore, the segregation for *SaM* in their T_1 progeny was distorted from the Mendelian ratio (see Table 1), similar to the distortion observed in the F_2 of T65 x E4. Together with the genetic analysis and protein interaction assays, this evidence indicates that SaF^+ is a component of this hybrid male sterility system. Several genetic mechanisms, such as hybrid sterility, pollen tube competition (certation), specific pollen–pistil interactions, and hybrid

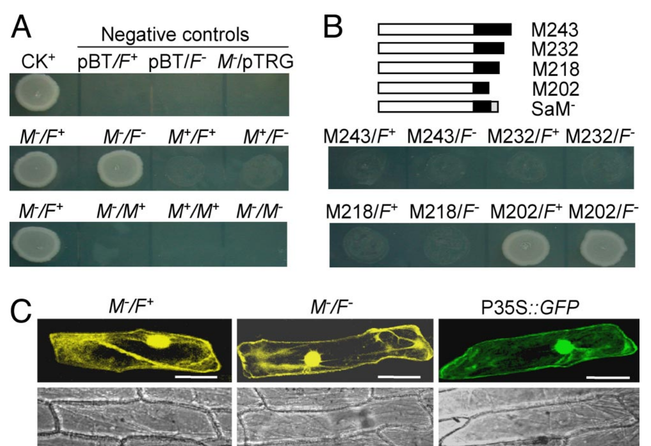

Fig. 4. Interaction between SaF and SaM proteins. (*A*) Bacterial two-hybrid assay. SaM^+(M^+) and SaM^- (M^-) were expressed in a bait vector, pBT, and SaF^+(F^+) and SaF^- (F^-) were expressed in a prey vector, pTRG. CK+, positive control. (*B*) Mapping of a self-inhibitory domain in SaM^+. Numbers of the deletion constructs indicate the lengths of the truncated proteins. (*C*) Interaction between SaM^- and SaF^+(or SaF^-) and the targeting to cytoplasm and nuclei in onion epidermal cells, as assayed by BiFC. *Top*: YFP signals on the basis of protein interaction. *Bottom*: Bright field of the same cells. (Scale bars, 100 μm.)

Table 1. Functional analysis of *SaM*⁺, *SaM*⁻, and *SaF*⁺ in rice by transformation

Plant (line)	Genotype (no. plants)	Phenotype	Segregation in Progenies
F_1	M^+F^+/M^-F^-	MSS	$M^-/M^-:M^+/M^-:M^+/M^+$ = 3:325:338 [χ^2(1:2:1) = 337.4***]
#1-1	$M^+F^+/M^+F^+//tM^-/-$	MSS	$tM^-:-$ = 61:48 [χ^2(1:1) = 1.6, χ^2(3:1) = 21.1***]
#1-2	$M^+F^+/M^+F^+//tM^-/-$	MSS	$tM^-:-$ = 72:58 [χ^2(1:1) = 1.5, χ^2(3:1) = 26.7***]
#1-3	$M^+F^+/M^+F^+//tM^-/-$	MSS	$tM^-:-$ = 111:138 [χ^2(1:1) = 2.9, χ^2(3:1) = 122.9***]
#2	$M^-F^-/M^-F^-//tM^+/-$ (19)	MFF	
#2-1	M^-F^-/M^-F^- (4)	MFF	
#2-2	M^-F^+/M^-F^- (7)	MFF	
#2-3	$M^-F^-/M^-F^-//tM^+/-$ (3)	MFF	
#2-4	$M^-F^+/M^-F^-//tM^+/-$ (8)	MCS	
#3	M^+F^-/M^-F^-	MFF	$M^-/M^-:M^+/M^-:M^+/M^+$ = 90:195:97 [χ^2(1:2:1) = 0.44]
#3-1	$M^+F^-/M^-F^-//tF^+/-$	MSS	$M^-/M^-:M^+/M^-:M^+/M^+$ = 2:96:100 [χ^2(1:2:1) = 93.2***]
#3-2	$M^+F^-/M^-F^-//tF^+/-$	MSS	$M^-/M^-:M^+/M^-:M^+/M^+$ = 3:62:60 [χ^2(1:2:1) = 52.0***]
#3-3	$M^+F^-/M^-F^-//tF^+/-$	MSS	$M^-/M^-:M^+/M^-:M^+/M^+$ = 20:83:65 [χ^2(1:2:1) = 24.1***]
#3-4	$M^+F^-/M^-F^-//tF^+/-$	MSS	$M^-/M^-:M^+/M^-:M^+/M^+$ = 7:45:27 [χ^2(1:2:1) = 11.6**]

Note: #1-1 to #1-3, t*SaM*⁻ T_0 plants of E4; #2, t*SaM*⁺ T_0 plants of T65; #2-1 to #2-4, $F_1(T_1)$ plants from a cross between #2 and a recombinant C513 (M^-F^+/M^-F^-); #3, the recombinant G735; #3-1 to #3-4, selected t*SaF*⁺ T_0 plants of G735 line with heterozygous *SaM*. MCS, male complete sterility. tM^- (tM^+, tF^+) and − indicate the presence and absence of the transgene, respectively. **, $P < 0.005$; ***, $P < 0.0001$.

breakdown or weakness, have been proposed as genetic mechanisms for the segregation distortion reported in plant hybrids (25, 26). Our results indicate that the segregation distortion of the *Sa* region is a consequence of hybrid male sterility caused by the allele-specific gamete selection.

Divergence of *SaM* and *SaF* Arose in Wild Rice Species. To trace the origins of the variation in *SaF* and *SaM*, we investigated the SNPs G02-69.8 and G02-74.6 (see Fig. 2*A*) in 13 wild species and in cultivated rice. The nucleotides "T" and "C" at G02-69.8 were variably present in populations of nine wild species (including the common wild rice, *O. rufipogon* Griff) and in *indica* cultivars (Table S3). For G02-74.6, only the nucleotide "G" was detected in all tested wild species and *indica*, with the exception of *O. rufipogon*, which was "G"-only in accessions distributing in South and Southeast Asia but contained both "G" and "T" (54 of 110 accessions carried "T") in the accessions from southern China. In summary, three haplotypes of the linked orthologs, *SaM*⁺*SaF*⁺, *SaM*⁺*SaF*⁻, and *SaM*⁻*SaF*⁻, were present in the *O. rufipogon* populations (Fig. 5*A*). Most *indica* cultivars (95 of 106 accessions) contained *SaM*⁺*SaF*⁺, but 11 accessions carried *SaM*⁺*SaF*⁻. All the tested 108 *japonica* cultivars possessed *SaM*⁻*SaF*⁻. These results indicate that the variation in *SaF* occurred before the split of most, if not all, of the *Oryza* species, whereas the mutation in *SaM* most likely arose in an *O. rufipogon* population with *SaM*⁺*SaF*⁻ in southern China and generated the haplotype *SaM*⁻*SaF*⁻ (Fig. 5*A*).

Discussion

The genetic behavior of most identified hybrid sterility loci in plants, including *Sa*, fits the one-locus allelic interaction model at the general genetic level (7–10). However, the molecular mechanism, that is, how an allele (in many cases from *indica* or *O. glaberrima*) of a heterozygous locus acts at the molecular level as a killer to eliminate the gametophytes carrying another allele (e.g., from *japonica*), is a longstanding question. This study shows that *Sa* is a complex locus comprising two adjacent genes, *SaM* and *SaF*, and that hybrid male sterility is controlled by a special interaction between these two genes.

A Molecular Genetic Model for Rice Hybrid Male Sterility. We propose a two-gene/three-component interaction model for this hybrid male sterility system (Fig. 5*B*). The absence of any one of the three alleles, *SaM*⁺, *SaM*⁻, or *SaF*⁺, fails to produce male sterility (see Fig. 2*A* and Table 1). In an F_1 plant, the linked allele sets (*SaM*⁺*SaF*⁺, *SaM*⁻*SaF*⁻) are separated from each other in the haploid microspores. Therefore, the SaF⁺ and SaM⁺ proteins may need to be transported from their own microspores to those carrying *SaM*⁻ for interaction. The SaF⁺-SaM⁻ complex may interact further with SaM⁺ indirectly to trigger a specific sterility process (Fig. 5*B*). Because the male developmental defect appears at the early uni-nucleate microspore stage (20), the protein transport may occur at the tetrad stage, through cytoplasmic channels existing between tetrad cells (27). However, SaM⁻ should be unable to move to the microspores carrying *SaM*⁺ to cause sterility, probably because of the loss of a necessary domain in the truncated region. The selective protein transport and the specific SaF⁺-SaM⁻ interaction restrict the sterility process in the *SaM*⁻-containing microspores, thereby resulting in allele-specific pollen killing. This model also can explain the induction of male sterility in other recombinants and in the transgenic plants (Fig. 5*B*). In some of these plants, *SaF*⁺ (t*SaF*⁺) and/or *SaM*⁺ (t*SaM*⁺) co-exist with *SaM*⁻ (t*SaM*⁻) in microspores, and the transgenes can function by ectopic expression to cause male sterility. Therefore, the molecular effect of the "allelic interaction" of gene sets does not necessarily require genetic allelism (i.e., location at the same position of the chromosomes). In conclusion, *SaM*⁻ acts as a gametophytic factor in the male sterility system, whereas *SaM*⁺ and *SaF*⁺ play their roles in any microspores in which they are located. On the other hand, the blocking of the SaF⁺–SaM⁺ interaction by the self-inhibitory domain in SaM⁺ may be an important mechanism to prevent triggering the sterility process in *SaM*⁺-carrying microspores, thus facilitating its transmission to hybrid progenies and avoiding male sterility in *indica* cultivars.

Intercellular protein trafficking through plasmodesmata is an important direct-signaling process for the development of organs such as shoot apical meristem and endodermis; often, specific domains are required for the targeted protein transports (28, 29), although this process has not been reported in developmental microspores. Therefore, our studies provide an ideal system for studying cell-to-cell interaction by protein trafficking in the early microspore development. Plants seem to have complex SUMOylation systems, and SUMO modification of proteins regulates a number of biological processes in plants (30). Although the activity of SaM as a SUMO E3 ligase needs to be tested, it is possible that a SUMOylation signaling pathway regulates the sterility process in microspore development of *indica-japonica* hybrids.

Fig. 5. Hybrid male sterility/fertility expression in rice hybrids. (*A*) Evolution of the *SaM* and *SaF* orthologs in wild and cultivated rice species. Asterisk indicates the occurrence of the SNPs at G02-69.8 and G02-74.6. Other wild species and other possible sequence variations are not shown. SA/SEA and SC indicate accessions from south Asia/Southeast Asia and south China, respectively. (*B*) A two-gene/three-component interaction model. It is assumed that the selective transport of the SaF^{+} and SaM^{+} proteins, shown by the same marks for the alleles without the horizontal or vertical lines, occurs at the tetrad stage (showing two cells only). Arrows of broken lines shown in H71 and E4-T0 indicate the transport of protein is not necessary for the male sterility.

Sa Hybrid Male Sterility and the *indica-japonica* Differentiation. Dobzhansky-Muller-type hybrid sterility in animals has been shown to result from the divergence of underlying genes that have primary functions (31, 32). The longstanding polymorphisms of the *SaF* and *SaM* orthologs among and within the *Oryza* species suggest that the varied alleles might have primary functions. We show that the incompatible haplotype $SaM^{-}SaF^{-}$ was generated by a two-step mutation process in different wild rice species (Fig. 5*A*). The intermediate haplotype $SaM^{+}SaF^{-}$ might have acted as a buffer to avoid the elimination of $SaM^{-}SaF^{-}$ from the wild rice populations when SaM^{-} arose. This process is similar to but distinct from the stepwise mutation model for the generation and maintenance of incompatible alleles of hybrid sterility genes in animals (33). The absence of SaM^{-} in *indica* rice suggests that this sterility system eliminated this allele during evolution; the fixation of $SaM^{-}SaF^{-}$ in *japonica* rice could be attributed to random genetic drift accompanied by its geographical isolation from *indica* or to an adaptive advantage of this haplotype for *japonica*. Therefore, this male sterility system might have contributed to the reproductive barrier and the genetic differentiation between the subspecies.

Early reports suggested that *indica* originally was domesticated from *O. rufipogon* and that *japonica* was further generated from *indica* (12, 34). Recent studies propose that *indica* and *japonica* originated independently from different populations of *O. rufipogon* (35, 36). Our results seem to corroborate the hypothesis of independent domestication (Fig. 5*A*). Furthermore, the data suggest that *indica* also evolved from different pre-differentiated populations of *O. rufipogon* and provide molecular evidence that *indica* and *japonica* originated from *O. rufipogon* in south or Southeast Asia and south China, respectively (36).

Compatible Haplotype Useful for Hybrid Rice Breeding. The haplotype $SaM^{+}SaF^{-}$ found in some *indica* cultivars (Table S3) is expected to be compatible with *japonica* cultivars as well as with the *indica* ones with $SaM^{+}SaF^{+}$ because of the absence of SaF^{+} or SaM^{-} in their hybrids (Fig. 5*A*) and thus can be defined as a neutral or compatible allele-complex, Sa^{n}. Therefore, this complex and the incompatible ones Sa^{i} ($SaM^{+}SaF^{+}$) and Sa^{j} ($SaM^{-}SaF^{-}$) form a tri-haplotypic system controlling the hybrid male sterility and fertility in *O. sativa*. Rice cultivars with Sa^{n} and other compatible alleles of hybrid sterility loci, such as $S5^{n}$ (9, 19), are valuable germplasm for overcoming the reproductive barrier in the breeding of inter-subspecific hybrid rice. Therefore, our findings have important implications for genetic improvements in rice, providing molecular markers for screening *Sa*-compatible germplasm and possibly creating transgenic *Sa*-compatible lines by suppressing the expression of the *Sa* gene(s).

Materials and Methods

Rice Materials. Rice materials for the gene cloning and functional analysis included the T65 and E4 (6) and their hybrid progeny prepared in this study. The wild and cultivated rice species used for haplotype survey (Table S3) were provided by the International Rice Research Institute and other institutions in China.

Map-Based Cloning. The pollen phenotypes (MFF, MSS) of rice were observed in a 1% I_2-KI (potassium iodide) solution under a microscope (Leica DMLB). F_2 plants of T65 x E4 were used for the mapping. Hybrid plants producing fully fertile pollen (> 95% pollen fertile) and semi-fertile pollen (≈ 50% of pollen grains sterile) represent the homozygote and heterozygote for *Sa*, respectively. Key recombinants (see Fig. 2*A*) were confirmed further by investigating the phenotypic and genotypic segregations in their progenies. Genotyping with SNP markers was done as described (37).

Genomic sequences of the *SaM* alleles and SaF^{+} (including their promoters and downstream sequences) were PCR-amplified with the primers SaM-p1/SaM-p2 for *SaM* and SaF-p1/SaF-p2 for SaF^{+} (all primers are listed in Table S4) and were cloned into a binary vector pCAMBIA1300 (Cambia). The constructs were transferred into suitable rice lines (see Table 1) by *Agrobacterium*-mediated transformation (38).

B2H and BiFC Assays. The coding regions of SaM^{+} and SaM^{-} cDNAs were amplified with SaM-p5/SaM-p6 and SaM-p5/SaM-p7, respectively, and were cloned into a bait vector pBT of the BacterioMatch two-hybrid system (Stratagene). The coding regions of SaF^{+} and SaF^{-} cDNAs were amplified with

GENETICS

SaF-p3/SaF-p4 and cloned into a prey vector pTRG. To check for possible interactions between the heterozygous or homozygous SaM^+ and SaM^-, the genes amplified with SaM-p8/SaM-p9; SaM-p10/SaM-p11 were cloned into pTRG. The deletion constructs of SaM^+ were prepared by inverse PCR of the gene's construct in pBT with an anchor primer (SaM-pa12) in combination with four inverse-directed primers (SaM-pr13–SaM-pr16), respectively, followed by digestion with a restriction endonuclease XhoI and self-ligation. The normal expression of the SaM fusion proteins in *Escherichia coli* was confirmed by immunoblot (Fig. S1).

For the BiFC assay, the *SaM*$^-$-coding cDNA amplified with SaM-p17/SaM-p18 was cloned into the vector pUC-SPYCE (24); the *SaF*$^+$- and *SaF*$^-$-coding cDNAs amplified with SaF-p5/SaF-p6 were cloned into the vector pUC-SPYNE (24). The combined constructs were co-transferred into onion epidermal cells by a helium-driven accelerator (BioRad). After culture for 10 to 15 h, the BiFC-based YFP expression was viewed with a confocal laser scanning microscope system TCS SP2 (Leica).

RT-PCR. Mature pollen grains were separated from anther wall tissue as described (39). Total RNAs were extracted from rice tissues and pollen with TRIZOL (Invitrogen). Reverse transcription reactions for *SaM* and *SaF* were performed with SuperScript III kit (Invitrogen), and expression of the target genes was assayed by RT-PCR with SaM-p19/SaM-p20 for *SaM* and SaF-p7/SaF-p8 for *SaF*.

Immunoblot. A peptide antigen corresponding to residues 1–25 of SaM was synthesized (Invitrogen) and used for immunizing rabbits. Detection of SaM^+ and SaM^- with the antiserum was carried out as described (37).

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1. Sano Y (1986) Sterility barriers between *Oryza sativa* and *O. glaberrima. Rice Genetics* (International Rice Research Institute, Manila) pp. 109–118.
2. Orr HA, Presgraves DC (2000) Speciation by postzygotic isolation: Forces, genes and molecules. *BioRssays* 22:1085–1094.
3. Kato S, Kosaka H, Hara S (1928) On the affinity of rice varieties as shown by fertility of hybrid plants. *Bulletin of Sciences of Faculty of Agriculture, Kyushu University* 3:132–147.
4. Watkins AE (1932) Hybrid sterility and incompatibility. *Journal of Genetics* 25:125–162.
5. Cameron DR, Moav R (1957) Inheritance in *Nicotiana tabacum* XXVII. Pollen killer, an alien genetic locus inducing abortion of microspores not carrying it. *Genetics* 42:326–335.
6. Rick CM (1966) Abortion of male and female gametes in the tomato determined by allelic interaction. *Genetics* 53:85–96.
7. Oka H-I (1974) Analysis of genes controlling FB_1 sterility in rice by the use of isogenic lines. *Genetics* 77:521–534.
8. Sano Y, Chu Y-E, Oka H-I (1979) Genetic studies of speciation in cultivated rice, I. Genic analysis for the F_1 sterility between *O. sativa* L. and *O. glaberrima* steud. *Japanese Journal of Genetics* 54:121–132.
9. Ikehashi H, Araki H (1986) Genetics of F_1 sterility in remote crosses of rice. *Rice Genetics* (International Rice Research Institute, Manila), pp 119–130.
10. Sano Y (1990) The genic nature of gamete eliminator in rice. *Genetics* 125:183–191.
11. Kitamura E (1962) Genetic studies on sterility observed in hybrids between distantly related varieties of rice, *Oryza sativa* L. *Bulletin of Chugoku Agricultural Experimental Station Series A* 141–205.
12. Ting Y (1957) The origin and evolution of cultivated rice in China. *Acta Agronomica Sinica* 8:243–260.
13. Oka H-I (1974) Experimental studies on the origin of cultivated rice. *Genetics* 78:475–486.
14. Oka H-I (1988) *Indica-japonica* differentiation of rice cultivars. *Origin of Cultivated Rice* (Japan Science Society Press/Elsevier, Tokyo/Amsterdam) pp. 141–179.
15. Zhang G, Lu Y, Zhang H, Yang J, Liu G (1994) Genetic studies on the hybrid sterility in cultivated rice (*Oryza sativa* L.) IV. Genotypes for F_1 pollen sterility. *Acta Genetics Sinica* 21:34–41.
16. Zhang G, Lu Y (1996) Genetics of F_1 pollen sterility in *Oryza sativa. Rice Genetics* (International Rice Research Institute, Manila) pp. 418–422.
17. Wan J, Yamaguchi Y, Kato H, Ikehashi H (1996) Two new loci for hybrid sterility in cultivated rice (*Oryza sativa* L.). *Theor Appl Genet* 92:183–190.
18. Li DT, *et al.* (2007) Fine mapping of *S32(t)*, a new gene causing hybrid embryo sac sterility in a Chinese landrace rice (*Oryza sativa* L.). *Theor Appl Genet* 114:515–524.
19. Chen J, *et al.* (2008) A triallelic system of *S5* is a major regulator of the reproductive barrier and compatibility of *indica-japonica* hybrids in rice. *Pro Natl Acad Sci USA* 105:11436–11441.
20. Zhang Z, Lu Y, Liu X, Feng J, Zhang G (2006) Cytological mechanism of pollen abortion resulting from allelic interaction of F_1 pollen sterility locus in rice (*Oryza sativa* L.). *Genetica (The Hague)* 127:295–302.
21. Zhuang C, Zhang G., Mei M, Lu Y (1999) Molecular mapping of the *Sa* locus for F_1 pollen sterility in cultivated rice (*Oryza sativa* L.). *Acta Genetics Sinica* 26:213–218.
22. Zhao X, Blobel G (2005) A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization. *Proc Natl Acad Sci USA*, 102:4777–4782.
23. Ogasawara T, *et al.* (1999) A new class of enzyme acting on damaged ribosomes: Ribosomal RNA apurinic site specific lyase found in wheat germ. *EMBO J* 18:6522–6531.
24. Walter M, *et al.* (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J* 40:428–438.
25. Harushima Y, Nakagahra M, Yano M, Sasaki T, Kurata N (2001) A genome-wide survey of reproductive barriers in an intraspecific hybrid. *Genetics* 159:883–892.
26. Hall MC, Willis JH, (2005) Transmission ration distortion in intraspecific hybrid of *Mimulus guttatus*: Implications for genomic divergence. *Genetics* 170:375–386.
27. Kenrick J, Knox RB (1979) Pollen development and cytochemistry in some Australian species of. *Acacia. Australian Journal of Botany* 27:413–427.
28. Lucas WJ, *et al.* (1995) Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. *Science* 270:1980–1983.
29. Gallagher KL, Benfey PN (2005) Not just another hole in the wall: Understanding intercellular protein trafficking. *Genes Dev* 19:189–195.
30. Colby T, Matthäi A, Boeckelmann A, Stuible H-P (2006) SUMO-conjugating and SUMO-deconjugating enzymes from Arabidopsis. *Plant Physiol* 142:318–332.
31. Wu C-I (2001) The genic view of the process of speciation. *J Evol Biol* 14:851–865.
32. Sun S, Ting C-T, Wu C-I (2004) The normal function of a speciation gene, *Odysseus*, and its hybrid sterility effect. *Science* 305:81–83.
33. Nei M, Maruyama T, Wu C-I (1983) Models of evolution of reproductive isolation. *Genetics* 103:557–579.
34. Chang TT (1976) Origin, evolution, cultivation, dissemination, and diversification of Asian and African rice. *Euphytica* 25:425–441.
35. Zhu Q, Ge S (2005) Phylogenetic relationships among A-genome species of the genus *Oryza* revealed by intron sequences of four nuclear genes. *New Phytol* 167:249–265.
36. Londo JP, Chiang Y-C, Hung K-H, Chiang T-Y, Schaal BA (2006) From the cover: Phylogeography of Asian wild rice, *Oryza rufipogon*, reveals multiple independent domestications of cultivated rice, *Oryza sativa. Proc Natl Acad Sci USA* 103:9578–9583.
37. Wang Z, *et al.* (2006) Cytoplasmic male sterility of rice with Boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. *Plant Cell* 18:676–687.
38. Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* 6:271–282.
39. Honys D, Twell D (2003) Comparative analysis of the Arabidopsis pollen transcriptome. *Plant Physiol* 132:640–652.