Cytoplasmic male sterility (CMS) determined by mitochondrial genes and restorer of fertility (Rf) controlled by nuclear-encoded genes provide the breeding systems of many hybrid crops for the utilization of heterosis. Although several CMS/Rf systems have been widely exploited in rice, hybrid breeding using these systems has encountered difficulties due to either fertility instability or complications of two-locus inheritance or both. In this work, we characterized a type of CMS, Fujian Abortive cytoplasmic male sterility (CMS-FA), with stable sporophytic male sterility and a nuclear restorer gene that completely restores hybrid fertility. CMS is caused by the chimeric open reading frame FA182 that specifically occurs in the mitochondrial genome of CMS-FA rice. The restorer gene OsRf19 encodes a pentatricopeptide repeat (PPR) protein targeted to mitochondria, where it mediates the cleavage of FA182 transcripts, thus restoring male fertility. Comparative sequence analysis revealed that OsRf19 originated through a recent duplication in wild rice relatives, sharing a common ancestor with OsRf1a/OsRf5, a fertility restorer gene for Boro II and Hong-Lian CMS. We developed six restorer lines by introgressing OsRf19 into parental lines of elite CMS-WA hybrids; hybrids produced from these lines showed equivalent or better agronomic performance relative to their counterparts based on the CMS-WA system. These results demonstrate that CMS-FA/OsRf19 provides a highly promising system for future hybrid rice breeding.

Cytoplasmic male sterility (CMS), a maternally inherited trait characterized by a lack of functional pollen, is a widespread phenomenon observed in more than 200 species of higher plants (1). CMS is usually determined by the mitochondrial genome but can be suppressed or counteracted by nuclear genes known as restorer of fertility (Rf). The CMS/Rf system is widely used in commercial production of hybrid seeds of many crops and also provides an excellent model for studying mitochondrial–nuclear interaction and coevolution in plants. CMS hybrid rice has been successfully developed with a three-line system containing a male sterile line, a maintainer line, and a restorer line, which has contributed greatly to food production worldwide since the 1970s.

Several types of CMS systems have been identified in rice, including CMS-BT (2, 3), CMS-HL (4), CMS-LD (5, 6), CMS-CW (7, 8), CMS-WA (9), CMS-RT102 (10), CMS-RT98 (11), CMS-D1 (12), and CMS-TA (13, 14). Each of the above CMS systems is associated with a chimeric open reading frame (ORF) that is thought to originate from mitochondrial genomic rearrangement (15). For example, orf79 for CMS-BT and orfH79 for CMS-HL share 98% identity at the nucleotide level and encode small proteins with an N terminus similar to COX1 (3, 4). The orf307 gene for CMS-CW contains the 5′ sequence of orf288 and a sequence of unknown origin (7, 8). The orf352 gene for CMS-RT102 is cotranscribed with the ribosomal protein gene rpL5 and the 2.8-kb orf532 transcripts are processed into 2.6-kb transcripts with a cleavage inside the orf352 coding region in the presence of the Rf gene (10). The orf113 for CMS-RT98 is cotranscribed with atp6 and cox3 encoding adenosine 5′-triphosphate (ATP) synthase F0 subunit 4 and Cyt c oxidase subunit 3, respectively, and their transcripts are distinctly processed in the presence of a fertility restorer gene (11). The CMS-WA gene W4352 comprises three segments derived from the putative mitochondrial ORFs orf284, orf224, and orf288 and a short sequence of unknown origin (9). W4314 is another CMS gene found in CMS-WA that causes partial male sterility in transgenic plants (16). The CMS-D1 gene orf182 is composed of three recombinant fragments, the largest of which has high similarity to the Sorghum bicolor mitochondrial sequence (12). The orf312 gene for CMS-TA is similar to the previously described orf288, a part of which is among the components comprising W4352, a chimeric CMS-associated gene of CMS-WA (13).

**Significance**

Although hybrid rice has been widely utilized for nearly half a century, tremendously improving rice productivity worldwide, the breeding of hybrids has been difficult because of genetic complications in male sterility and fertility-restoring systems currently available in rice. Here, we characterized Fujian Abortive cytoplasmic male sterility (CMS-FA) rice, which has shown stable male sterility controlled by the mitochondrial gene FA182; a single nuclear gene, OsRf19, completely restores fertility. This single-gene inheritance has greatly eased the breeding process. By converting CMS-WA hybrids with the CMS-FA system, we developed six hybrids that showed equivalent or better performance relative to their CMS-WA counterparts. CMS-FA/OsRf19 provides a promising system for future hybrid rice breeding.
Most of the cloned Rf genes belong to a large, well-defined family of genes that encode organelle-targeted pentatricopeptide repeat (PPR) proteins. To date, five Rf have been cloned and characterized as PPR proteins in rice: Rf1a and Rf1b in CMS-BT rice (2, 3), Rf5 and Rf6 in CMS-HL rice (17, 18), and Rf4 in CMS-WA rice (19, 20). Rf6 in Brassica napus Ogura-CMS (21), Rf6 in Brassica napus pol-CMS (22), Rf6 in Brassica napus hau-CMS (23), and Rf5 in maize CMS-S (24) also encode PPR proteins. In addition to PPR proteins, other types of Rf have been identified, including Rf2 in CMS-LD encoding a glycine-rich protein (5) and Rf17 encoding a mitochondrial-targeting protein containing a part of an acyl-carrier protein synthase-like domain in rice (25), as well as Rf2 in maize CMS-T encoding an aldehyde dehydrogenase (26).

PPR proteins are characterized by the presence of a degenerate 35-amino acid repeat, the PPR motif, which is arranged in tandem 2 to 50 times (27). Several PPR proteins exhibit RNA-binding activity and have been implicated in posttranscriptional regulation in organelle (mitochondria and chloroplasts) gene expression, including RNA editing, processing, intron splicing, translation, and messenger RNA (mRNA) stability (28–30). In organelles of flowering plants, RNA editing results in alteration of the coding sequences in many of the organelar transcripts and produces translatable mRNAs by creating AUG start sites or eliminating premature stop codons. RNA editing may also affect the RNA structure, influence splicing, or alter the stability of RNAs (31–33).

Fujian Abortive (FA) CMS rice was bred using the cytoplasm from a common wild rice (Oryza rufipogon L.), which was found in Fujian Province, China (34–36). Similar to CMS-WA, the most widely used system in hybrid rice breeding worldwide, the male sterility of CMS-FA is sporophytic, with fertility determined by the parental genotype (37), which is a preferred system compared to gametophytic male sterility, in which fertility is determined by the pollen genotype. An effort to search for restorers by crossing the CMS-FA line Jinnong1A with 220 diverse rice varieties, including many restorer lines of the CMS-WA system, failed to find any restorer line (35). Therefore, CMS-FA is genetically distinct from CMS-WA with respect to CMS and fertility restoration genes. Moreover, the sterility-restoration of the CMS-FA/RF progeny segregated as a single-locus inheritance (36, 37), which is also different from the two-locus inheritance of the CMS-WA system. Together, these two characteristics make CMS-FA more appealing for hybrid rice breeding than other currently widely used CMS systems such as CMS-WA and CMS-HL. A number of FA sterile lines and restorer lines have been bred and released for commercial use and have demonstrated superior performance in rice production, showing the promise for future hybrid rice breeding.

In the work reported here, we identified the chimeric ORF orf182 in the mitochondrial genome of CMS-FA as the cause of male sterility and a gene encoding a PPR protein as the restorer gene. Considering the current situation in which CMS and fertility restorer genes have been identified from many species and all are named orf and Rf irrespective of their molecular nature, for clarity we suggest adding a prefix of the species name to each Rf gene as well as a prefix to specify the cytoplasm source for each CMS gene. We thus named this restorer gene OsRf19 in sequence with other identified CMS-restorer genes in rice (https://archive.gramene.org/db/genes/search_gene?query=fertility+restoration&vocabulary=genes&search_box_id=gene_search_for&search_field=name&gene_type_id=&species=1&x=9&y=9) and the CMS gene FA182.

We showed that OsRf19 restores fertility by cleaving/degrading FA182 mRNA.

**Results**

**The Anther Morphology of the CMS-FA Lines.** Both the maintainer line Shen95B and the male sterile line Shen95(FA)A of CMS-FA grew normally during the vegetative stage (SI Appendix, Fig. S1A). Shen95B has normal anthers that are plump and yellow, but the anthers of Shen95(FA)A are small, shrunken, and transparent (SI Appendix, Fig. S1 B and C), producing no or very few pollen grains that could not be stained with I2-KI (SI Appendix, Fig. S1D). Transverse sectioning of Shen95(FA)A showed that the tapetum cells were vacuolated and expanded abnormally compared to those in the Shen95B at stage 9 of anther development. Most microspores of Shen95(FA)A gradually degenerated and disintegrated compared to those in Shen95B at stages 10 to 12 of anther development (SI Appendix, Fig. S1E).

**The Chimeric Gene FA182 Causes Male Sterility in CMS-FA.** To identify the gene that causes CMS traits in the CMS-FA line, we sequenced the Shen95(FA)A mitochondrial genome, which is 457,380 bp in total with a 43.83% G+C content (SI Appendix, Fig. S2). We compared the mitochondrial genome sequences of four rice varieties, the CMS-FA line Shen95(FA)A, the restorer line 9311, the CMS-RT98 line RT98C, and the maintainer line WA-N (SI Appendix, Fig. S3A). Two highly diverged regions, which contained four predicted ORFs in each of the regions (orf56, orf205, orf126, and orf182 in region 1; orf51, orf86, orf56, and orf55 in region 2), were specifically found in the Shen95(FA)A mitochondrial genome and were subjected to further investigation (SI Appendix, Fig. S3B). Using BLASTN, orf56, orf205, and orf126 were detected in the mitochondrial genome of Nipponbare, a japonica rice variety, whereas orf61 showed an unknown origin. Intriguingly, orf51 and orf55 showed high identity (91 to 99%) to mitochondrial genome sequences of the other grasses but not rice, while orf182 and orf86 were two chimeric genes generated by the fusion of two or more fragments with different origins. Orf86 was composed of a segment with unknown origin and a very short fragment having a homologous sequence to mitochondrial sequences of Zea mays, Sorghum bicolor, and Eleusine indica. Orf182 was formed by merging three components, a 421-bp fragment showing 79% nucleotide identity to a mitochondrial sequence in Sorghum bicolor, a 71-bp fragment identical to a mitochondrial sequence in rice, and a 57-bp fragment with unknown origin. Interestingly, orf182 is identical to the orf182 gene for CMS in CMS-D1 rice (12); we thus selected orf182 as the candidate gene for CMS-FA for subsequent investigation.

To determine whether orf182 is the direct cause of male sterility in CMS-FA, three chimeric expression cassettes were constructed with the orf182 sequence driven by the constitutive promoter CaMV35S or Pubi, and a mitochondrial transit peptide sequence (MTS) from Rf1b or ATTp was added to the N terminus of orf182 to enable its transport from the cytoplasm into mitochondria (Fig. 1A). The three plasmids were transformed into the variety Zhonghua11 (Oryza sativa ssp. japonica). A total of 15 independent positive transgenic plants with the 35S-Rf1bMTS-orf182 vector were obtained, of which nine plants had unstable pollen grains with no seed setting (Fig. 1A and SI Appendix, Table S1), six plants had 1 to 50.2% I2-KI-stainable pollen grains, and the seed setting rate was 0 to 12.8%. Nine independent positive transgenic plants with the
Plants had unstainable pollen grains and the other four plants derived from the Rf1b gene. (FA182

1% I2-KI carrying T0 plants from each transformation construct were randomly chosen to cross with wild-type plants. In the F1 segregation population consisting of 4,059 plants from the cross of Jinhui3:× Jinnong2(FA)A, we identified 25 recombinants and determined their genotypes at the OsRf19 locus by progeny testing. This analysis mapped OsRf19 between the markers Rf1D6 and Rf1D7 (SI Appendix, Fig. S5B). Subsequently, we constructed a BC2F2 population consisting of 4,059 plants from the cross of Jinhui3 (with FA cytoplasm) and Huazhan and detected seven recombinants between markers Rf1D6 and Rf1D7. Using information from these recombinants, we delimited OsRf19 between two markers, Rf1D3 and TMRf1M10, spanning a 16-kb region based on the Nipponbare genome.

We constructed a bacterial artificial chromosome (BAC) library of genomic DNA from Jinhui3, consisting of 41,472 clones with an average DNA insert size of 114 kb. This library
was screened with the markers TMRfl1M06, Rf1D3, and Rf1D7 using the DNA pooling method. We isolated two BAC clones (71-N-20 and 90-J-22) that might cover the corresponding region of the Jinhui3 genome and determined their nucleotide sequences. A 194.7-kb sequence was obtained based on the two BAC clones. The region between markers Rf1D3 and TMRfl1M10 in the Jinhui3 genome is 35 kb, 19 kb longer than the corresponding region in the Nipponbare genome.

Four ORFs, ORF1, ORF2, ORF3, and ORF4, were predicted in this region (SI Appendix, Fig. S5B). To identify OsRf19, we constructed subclone libraries of the two BAC clones using binary vectors. Three clones (71-N-20-06, 71-N-20-18, and 90-J-22-09) were identified to contain the genomic sequences, including promoters, ORFs, and 3' downstream regions of ORF1+ORF2, ORF2+ORF3, and ORF3+ORF4. Four PCR fragments containing the genomic sequences for ORF1, ORF2, ORF3, and ORF4, including the promoter ORF and 3' downstream region, were also individually cloned into binary vectors. These seven constructs were introduced into the CMS-FA line 9311(FA)A (SI Appendix, Fig. S6) by Agrobacterium-mediated transformation. The positive transgenic T0 plants with ORF1+ORF2 (three plants) and ORF1 (eight plants) showed restored male fertility, producing pollen grains stainable with I2-KI. Nontoxic T0 plants were obtained in transgenic plants of the other five constructs. T1 seeds from ORF1+ORF2 and ORF1-positive plants were obtained. In three T1 families of ORF1+ORF2, a total of 139 plants with the transgene produced nearly 100% I2-KI-stainable pollen grains showing 68.2 to 91.7% seed-setting rates, while 45 plants without the transgene were completely male sterile (SI Appendix, Fig. S7). In three T1 families of ORF1, the 172 transgenic plants with all stained pollen grains showed spikelet fertility of 64.4 to 92.0%, but the 56 plants without the transgene were completely male sterile (Fig. 1B). These results indicated that ORF1 restores the fertility of CMS-FA lines and is the OsRf19 gene.

We also generated an OsRf19 knockout mutant using CRISPR/Cas9 in the iso-cytoplasmic restorer line 9311(FA)R, which has OsRf19 in the 9311 background and CMS-FA cytoplasm. Seventeen independent transgenic T0 plants were obtained showing complete sterility (Fig. 1C). Three of these T0 plants were crossed with 9311 and 9311(FA)R. The F1 plants derived from the cross of the T0 plants with 9311 showed complete sterility, whereas plants derived from the cross with 9311(FA)R were fertile. Progeny plants resulting from self-fertilization of the fertile plants above showed a 3:1 segregation ratio between the fertile and sterile plants. This result further supported the conclusion that ORF1 is OsRf19.

OsRf19 encodes a PPR protein, which was predicted to localize to the mitochondria (iasb.biol.uoa.gr/PredSL). To verify this prediction, a transformation construct expressing an OsRf19–green fluorescent protein (GFP) fusion protein was made and introduced into rice protoplasts. Confocal laser-scanning microscopy showed that the green fluorescence of OsRf19-GFP exclusively colocalized with mCherry fluorescence in the mitochondria (SI Appendix, Fig. S8).

OsRf19 Reduces the Cytotoxicity to Bacteria Caused by FA182. Several CMS-associated genes encode peptides that are deadly to Escherichia coli (3, 39). To evaluate whether the expression of FA182 and the edited version FA182UU was also cytotoxic to bacteria, we cloned the coding sequences of FA182 and FA182UU into the first multiple cloning site of the pET-Duet1 vector. Expression of either FA182 or FA182UU in E. coli induced by Isopropyl-β-D-thiogalactopyranoside (IPTG) hindered E. coli growth (SI Appendix, Fig. S9A).

To investigate whether OsRf19 mitigates the toxicity caused by FA182, we cloned the coding sequence of OsRf19 into the second multiple cloning site of the pETDuet1 vector, which contained FA182 in the first multiple cloning site, to coexpress the two genes. The vector containing only the OsRf19 gene in the second multiple cloning site of pETDuet1 was used as the control; the expression of OsRf19 did not affect the growth of E. coli. Coexpression of the OsRf19 and FA182 genes in E. coli removed the cytotoxicity to bacteria caused by FA182 as the bacteria continued to grow (SI Appendix, Fig. S9B).

OsRf19 Mediates RNA Cleavage of FA182. To determine how OsRf19 affects the mRNA of FA182, we employed 5' RNA ligase–mediated rapid amplification of cDNA ends (RLM-RACE) to analyze the cleavage of FA182 using RNA samples prepared from the panicles of 9311(FA)A, 9311(FA)R, and OsRf19 transgenic T2 plants (Fig. 2A). Ligation was carried out by incubating RNA from the samples with a 45-base RNA adapter oligonucleotide mediated by T4 RNA ligase, which would ligate with cleaved RNA but not the CAP-protected uncleaved RNA. A PCR assay was employed to detect cDNA in the samples. Agarose gel electrophoresis detected amplified DNA from 9311(FA)R and T2 plants, but no product from 9311(FA)A (Fig. 2B). The PCR products from 9311(FA)R and T2 plants were cloned for DNA sequencing, which showed that the FA182 mRNA was cleaved between 414 and 415 nt. Thus, OsRf19 mediates RNA cleavage of FA182.

To quantify how much of the FA182 transcript was reduced by OsRf19, we first analyzed the expression patterns of OsRf19 and FA182 in the CMS-FA lines 9311(FA)R and 9311(FA)A, respectively, using qRT-PCR. The transcript level of OsRf19 was relatively low in the roots, stems, and leaves but high in anther, while FA182 was high in all the tissues assayed (SI Appendix, Fig. S10A).

A pair of primers (FA182-1F/1R) flanking both sides of the FA182 cleavage site was used to compare the transcripts of FA182 in 9311(FA)R, 9311(FA)R, and OsRf19 transgenic T2 plants by RT-PCR and qRT-PCR. The results showed that the transcript levels were much lower in 9311(FA)R and T2 plants than in 9311(FA)A in all tissues, as listed above (Fig. 2C and D). Compared with 9311(FA)R, the mRNA level of FA182 in 9311(FA)A was ~3.3 times higher in root, 11.5 times higher in stem, 8.2 times higher in leaf, 6.7 times higher in panicle, and 8.5 times higher in anther (Fig. 2D).

Another pair of primers (FA182-2F/2R) located upstream of the FA182 cleavage site was also used to detect the transcript of FA182 in 9311(FA)R, 9311(FA)R, and OsRf19 transgenic T2 plants. Similar to the above result, the transcript levels of FA182 were much lower in 9311(FA)R and T2 plants than in 9311(FA)A, suggesting that cleaved FA182 was mostly degraded (SI Appendix, Fig. S10B). These results confirmed that OsRf19 affects the transcript level of FA182, as much as ~90% of the FA182 transcripts were degraded in anthers in the restorer line. The results also suggest that reduction of the FA182 transcript also occurred in tissues other than the anther.

Origin of OsRf19 through Stepwise Duplications in the Oryza Genus. Five PPR genes, PPR830, OsRf19, ORF2, ORF3, and ORF4, are positionally clustered in a linear order on chromosome 10 in the Jinhui3 genome. (The GenBank accession no. of the Jinhui3 BAC clone sequence containing OsRf19 is ON855493.) To assess the relationship among members in this
cluster, we first compared the coding sequence of OsRf19 with each of the other four PPRs (SI Appendix, Fig. S11A and Dataset S1). PPR830 showed the highest identity in the predicted coding sequence with OsRf19 (95.1%), followed by ORF3 (94.6%), ORF4 (92.6%), and ORF2 (91.4%). Considering the high similarity in the coding region, we also analyzed the adjacent sequence of OsRf19 and found that the downstream regions of OsRf19 and PPR830 were extremely conserved, with sequence similarity up to 99.7% in the ~6-kb fragment, whereas the downstream regions of the other three PPRs were more divergent relative to those of OsRf19. These data suggested that OsRf19 was evolutionarily more closely related to PPR830. Likewise, high levels of sequence identity were found between ORF3 and ORF4 in the upstream region (~2 kb, 99.0%), coding sequence (2.3 kb, 96.3%), and downstream region (484 bp, 95.4%), suggesting a very close relationship between these two PPRs. ORF2 encodes a truncated protein as a result of a premature stop codon, and in the coding sequence along with adjacent regions, it showed higher sequence similarity to ORF3 (95.1%) and ORF4 (96.5%) than to PPR830 (92.6%) and OsRf19 (91.4%).

To trace the evolutionary processes stepwise for each of these PPR genes, we performed a comparative sequence analysis of the OsRf19 orthologous regions from the Oryza genus, including Oryza brachyantha (FF genome type, accession no. PRJNA70533), Oryza punctata (BB, accession number PRJNA13770), Oryza meridionalis (AA, accession no. PRJNA48433), Oryza barthii (AA, accession no. PRJNA30379), Oryza glaberrima (AA, accession no. PRJNA13765), Oryza rufipogon (AA), Oryza nivara (AA, accession no. PRJNA48107), and xian indica and georg japonica subspecies of O. sativa (AA), using reciprocal-best alignments across the phylogeny (Fig. 3 and SI Appendix, Fig. S11B and Table S2) (2, 40–42). We also included the closely related species Leersia perrieri (accession no. PRJNA163065) from a grass genus as an outgroup in the analysis. A single PPR copy was detected in L. perrieri in the syntenic region, suggesting that the origin of this PPR cluster can be traced back to an ancestor predating the occurrence of L. perrieri. The copy number of PPRs in this syntenic region differed greatly among the species in this writing based on the available data. The ancestral copies of PPR830 and ORF4 appeared initially in the most recent
common ancestor of the AA and BB lineages and were presumably generated by a local duplication event of a single progenitor gene.

Subsequently, independent duplication events occurred in these two ancestral PPR copies after the split of the AA lineage. The orthologs of ORF3 might originate from ORF4 by duplication inferred from their high similarity and adjacent genomic location, and they were preserved in O. meridionalis and other Asian species but were not detected in the African species possibly due to limited sample size (Fig. 3). Afterward, duplication of ORF3 in the most recent common ancestor of Asian rice species gave rise to ORF2 in O. rufipogon, PPR791N1 and PPR791N2 in O. nivara, and PPR883 in the xian/indica subspecies of O. sativa. Interestingly, the ortholog of ORF4 in this lineage gained restoration function to become the restorer gene OsRf1a/OsRf5 for Boro II and Hong-Lian CMS (3, 15).

In the other lineage, PPR883 gave rise to OsRf19 through a recent duplication in the wild relatives of Asian cultivated rice, as supported by the observed high sequence similarity and collinearity. Hence, two fertility restorer genes, OsRf1a/OsRf5 and OsRf19, were found to originate from a common ancestor preceding the split between L. perrieri. OsRf1a/OsRf5 represented an older PPR gene arising by an ancient duplication before the split of AA and BB lineages in the Oryza genus, while OsRf19 was a young PPR gene of a recent origination after the divergence of O. rufipogon and O. nivara. Consequently, OsRf1a and OsRf19 maintained 93.5% sequence identity after experiencing a range of evolutionary changes in their respective lineages, resulting in a distinct fertility restoration spectrum for different types of cytoplasmic male sterility.

We also noticed that the adjacent genomic region surrounding OsRf19 on chromosome 10 is a hotspot for fertility restorer genes. The restorer line MH63 contains the restorer gene OsRf1a/OsRf5 for Boro II and Hong-Lian cytoplasmic male sterility, the OsRf1b for Boro II cytoplasmic male sterility, and two copies of OsRf4 for WA CMS (SI Appendix, Fig. S11C). Another three restorer lines, R527, FH838, and R498, also carry OsRf1a/OsRf5 and OsRf1b with one copy of OsRf4. In addition, 9311 contains two restorer genes, OsRf1a/OsRf5 and OsRf1b.

Application of CMS-FA/OsRf19 in Hybrid Rice Breeding. We introduced the OsRf19 locus from Jinhui3 into six CMS-WA restorer lines, all carrying OsRf3 and OsRf4, HR2168, R498, α7–3, Zhonggeng57, Chenghui727, and Huazhan, through recurrent backcrossing and whole-genome selection using the RICE6K microarray and relevant markers (SI Appendix, Fig. S12 A–C). Six OsRf19-containing lines, HR2168-OsRf19, R498-OsRf19, α7–3-OsRf19, Zhonggeng57–OsRf19, Chenghui727–OsRf19, and Huazhan–OsRf19, were obtained with genetic background recovery rates of 66.2 to 99.9% (SI Appendix, Fig. S12B). Field evaluation showed that the OsRf19-containing lines had essentially the same agronomic performance as their corresponding parents (SI Appendix, Table S3).

These OsRf19 restorer lines plus 9311–OsRf19, also named 9311(FA)R, were crossed with the CMS-FA line Shen95(FA)A. For comparison, the original parental lines HR2168, R498, α7–3, Zhonggeng57, Chenghui727, and 9311 were also crossed with the CMS-WA line Shen95(WA)A. Field tests of the F1s based on CMS-FA/OsRf19 showed equivalent or better agronomic performance compared with the corresponding CMS-WA hybrids (Table 1). The yield per plant of the WA-hybrids ranged from 19.3 to 43.0 g, while that of the FA-hybrids ranged from 32.2 to 41.6 g.

These OsRf19-restorer lines were also crossed with the commercial CMS-FA line Jinnong3A. The hybrids showed excellent performance compared with the check Fengliangyou4, a popular high-yielding two-line hybrid (SI Appendix, Table S4). The grain yield per plant of hybrids from the cross of Jinnong3(FA)A with these restorer lines ranged from 32.4 to 38.6 g compared to 32.0 g for Fengliangyou4. In particular, in the field plot trial, grain yields of the three FA-combinations, Shen95(FA)A/Huazhan–OsRf19, Jinnong3(FA)A/Yuehui94–OsRf19, and Jinnong3(FA)A/Jinhui3–OsRf19, were 10.9, 11.8, and 12.3 ton/ha, respectively, and the yield of the check Fengliangyou4 was 10.8 ton/ha (SI Appendix, Table S5). These results demonstrate that the CMS-FA/OsRf19 system holds great promise for hybrid rice breeding.
Table 1. Agronomic performance of test-cross F1s in the FA system against WA counterparts

| Hybrid     | Days to heading (cm) | Plant height (cm) | No. of tillers per plant | No. of grains per panicle | Spikelet fertility (%) | 1,000-grain weight (g) | Yield per plant (g) |
|------------|----------------------|-------------------|--------------------------|---------------------------|------------------------|------------------------|----------------------|
| 1.1        | 85.1 ± 0.7           | 110.2 ± 5.2       | 10.4 ± 2.0               | 244.2 ± 33.8              | 82.9 ± 9.3             | 20.5 ± 0.8              | 43.0 ± 10.7          |
| 1.2        | 84.2 ± 0.8           | 107.2 ± 4.5       | 10.7 ± 2.7               | 220.4 ± 44.8              | 85.8 ± 3.7             | 20.7 ± 1.0              | 41.6 ± 12.5          |
| 2.1        | 79.2 ± 0.6           | 115.1 ± 5.7       | 9.1 ± 2.9                | 190.5 ± 20.8              | 79.3 ± 7.8             | 24.1 ± 0.9              | 34.3 ± 16.0          |
| 2.2        | 79.1 ± 0.9           | 115.5 ± 1.9       | 8.8 ± 2.4                | 190.5 ± 21.3              | 87.9 ± 6.2             | 24.8 ± 1.0              | 38.5 ± 15.1          |
| 3.1        | 86.1 ± 0.6           | 119.7 ± 4.5       | 8.5 ± 2.3                | 215.2 ± 36.1              | 63.0 ± 8.6             | 25.1 ± 1.1              | 29.0 ± 10.3          |
| 3.2        | 86.9 ± 0.6           | 115.3 ± 4.1       | 7.9 ± 1.5                | 211.6 ± 39.6              | 74.2 ± 7.0             | 26.0 ± 0.7              | 32.2 ± 9.9           |
| 4.1        | 81.6 ± 0.5           | 113.9 ± 3.7       | 10.1 ± 2.8               | 194.7 ± 36.5              | 77.1 ± 7.3             | 22.5 ± 0.7              | 35.8 ± 17.0          |
| 4.2        | 82.7 ± 0.7           | 116.1 ± 3.2       | 8.9 ± 2.5                | 203.4 ± 31.1              | 80.2 ± 9.5             | 23.4 ± 1.3              | 35.0 ± 13.8          |
| 5.1        | 83.4 ± 0.7           | 109.5 ± 4.2       | 9.2 ± 2.7                | 190.4 ± 23.0              | 87.4 ± 5.5             | 25.2 ± 0.7              | 39.3 ± 15.1          |
| 5.2        | 83.6 ± 0.5           | 106.7 ± 4.3       | 9.9 ± 3.1                | 181.3 ± 25.8              | 89.8 ± 3.3             | 25.2 ± 0.6              | 41.0 ± 14.3          |
| 6.1        | 81.4 ± 0.5           | 106.6 ± 4.3       | 9.1 ± 2.8                | 260.4 ± 38.3              | 55.5 ± 9.2             | 22.1 ± 0.7              | 29.1 ± 11.6          |
| 6.2        | 80.6 ± 0.5           | 107.0 ± 6.3       | 8.8 ± 3.3                | 242.7 ± 29.6              | 81.4 ± 7.1*            | 22.4 ± 1.2              | 38.3 ± 14.1*         |
| 7.1        | 90.3 ± 0.7           | 113.9 ± 4.9       | 10.2 ± 2.6               | 241.4 ± 35.5              | 31.1 ± 12.2            | 24.7 ± 1.3              | 19.3 ± 9.1           |
| 7.2        | 89.4 ± 0.5           | 118.4 ± 5.0       | 9.2 ± 2.4                | 233.6 ± 39.6              | 75.4 ± 6.4*            | 24.5 ± 0.9              | 39.8 ± 13.3*         |

1.1 Shen95(WA)/Huadan, 1.2 Shen95(WA)/Huadan-OSrift9; 2.1 Shen95(WA)/HR2168, 2.2 Shen95(WA)/HR2168-OSrift9; 3.1 Shen95(WA)/R498, 3.2 Shen95(WA)/R498-OSrift9; 4.1 Shen95(WA)/Os7-3, 4.2 Shen95(WA)/Ai7-3-OSrift9; 5.1 Shen95(WA)/Chenghu722, 5.2 Shen95(WA)/Chenghu727-OSrift9; 6.1 Shen95(WA)/Zhonggeng57, 6.2 Shen95(WA)/Zhonggeng57- OSrift9, 7.1 Shen95(WA)/9311, 7.2 Shen95(WA)/9311-OSrift9 (9311 is not a restorer of CMS-WA). Values are presented as the means ± SD.

*Significant difference from the performance of the F1s (FA) when compared with F1s (WA) at P < 0.01.

Discussion

Based on our results, we proposed a model to illustrate the mechanism of CMS and fertility restoration in CMS-FA rice (Fig. 4). Without the restorer protein, the mitochondria-encoded gene FA182 is transcribed, and the transcript, with or without editing, is translated into the FA182 protein, which causes male sterility in the CMS-FA line (Fig. 4). In the presence of the nuclear-encoded OsRf19 protein, the FA182 transcript is cleaved, to avoid causing male sterility in CMS-FA/OsRf19 hybrid rice.

RNA editing in plant mitochondria or chloroplasts is a posttranscriptional process that directs C to U changes in RNA (43, 44). RNA editing plays many important roles in gene expression by generating start codons at ACG sites, correcting codons to encode conserved amino acids, and generating required stop codons. Such editing may change the biochemical nature of the resulting proteins. An analysis of RNA editing in Arabidopsis mitochondria showed that 35% of the modifications of the codons altered the amino acids from hydrophilic to hydrophobic, while 35% were hydrophobic to hydrophobic (45). Thus, the general tendency of the effect of RNA editing in Arabidopsis mitochondria is to increase the proportion of hydrophobic amino acid codons. In addition, RNA editing may increase the stability and thus the quantity of the protein. For example, in maize, six C to U editing sites have been identified in the transcripts of the ribosomal protein S13 gene (rps13) in the mitochondria (46). Sequence analysis demonstrated that 73% of the transcripts of S13 were edited at all six sites and only 3% were completely unedited. Immunological analyses showed that only polypeptides from the edited RNA are detected and accumulate, whereas polypeptides from unedited RNA do not accumulate in maize mitochondria. This study indicates that translational products from unedited RNAs may be unstable and consequently fail to accumulate (46). In our study, the C to U editing in the 62 nt of FA182 led to an amino acid change of proline (hydrophobic) to leucine (hydrophobic) in the predicted protein, and the editing at 65 nt resulted in a change from serine (hydrophilic) to phenylalanine (hydrophobic). Therefore, RNA editing may have changed the stability and biochemical nature of the FA182 protein.

Numerous CMS germplasms have been found in various plant species, which in general can be characterized as two classes, gametophytic male sterility with pollen fertility specified by pollen genotype and sporophytic male sterility with pollen fertility decided by the maternal plant. Experience with hybrid rice breeding in the past decades clearly indicates that the gametophytic male sterility systems may suffer two disadvantages: sterility fluctuation of the male sterile lines to occasionally produce small amounts of fertile pollen and suboptimal seed setting of the F1 hybrids due to inadequate pollen fertility resulting in yield loss. This explains why the sporophytic CMS-WA/Rfs system is still dominant in hybrid rice breeding both in China and other countries. However, obvious difficulties have been encountered with the WA system in hybrid rice breeding. First, the need for simultaneous transfer of two restorer genes causes difficulties in the background improvement of the restorer lines due to linkage drag at two loci and also for phenotyping fertility of the two-locus genotypes. Second, such two-locus segregation also causes complications for breeding of maintainer lines. These difficulties have certainly hindered the development of hybrid rice breeding.

The CMS-FA/OsRf19 reported here may provide a promising alternative to improve the situation. We showed that the male sterile lines bred using this system can produce no pollen, or at least no stainable pollen, thus exhibiting complete male sterility and that a single restorer gene adequately restores male fertility of the hybrids with no negative effects on hybrid performance compared to WA counterparts. We also showed that this single-gene inheritance greatly eases the breeding process of both maintainer and restorer lines. Such progress has demonstrated a perspective that tremendously accelerates the development of hybrid rice breeding, especially when equipped with genomic selection technologies, although its usefulness remains to be tested by large-scale utilization in breeding programs.

A major remaining question is how the PPR protein OsRF19 mediates the cleavage of the FA182 transcripts to restore pollen fertility. Although we found that expression of FA182 in E. coli hindered cell growth and coexpression of OsRf19 and FA182 removed the cytotoxicity, we do not know how OsRf19 functions to remove the deadly effect of FA182 in bacterial cells. However, it can be reasoned that the action by

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which OsRf19 reduces the toxicity of FA182 in bacteria is likely different from its function in restoring male fertility in the rice plant. We attempted to assay whether OsRf19 protein binds to the FA182 transcripts without success. An alternative possibility is that OsRf19 may act on the FA182 transcript with the participation of other proteins, as in the Hong-Lian CMS line in which OsRF5 physically interacts with GRP162, which has an RNA recognition motif to bind atp6-orfH79 (17). Likewise, OsRF6 does not directly bind to the atp6-orfH79 transcript but physically interacts with OsHXK6 and promotes the processing of atp6-orfH79 (18). Further investigation is needed to answer this question.

**Materials and Methods**

Detailed descriptions for characterization of the phenotypes of Shen95(FA1), mitochondrial genomic sequencing and comparative analysis, vector construction and transformation of FA182, RNA editing analysis, mapping of OsRf19, construction of a BAC library and two subclone libraries, transformation materials and vector construction for OsRf19 candidate genes, subcellular localization analysis of OsRf19, expression of FA182 and OsRf19 in E. coli, RLM-RACE assay, expression analysis, genomic variation analysis of the OsRf19 locus, and breeding application and trait measurement are provided in SI Appendix.

**Data Availability.** All study data are included in the article and/or supporting information.

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