Construction of a male sterility system for hybrid rice breeding and seed production using a nuclear male sterility gene

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Contributed by Xing Wang Deng, August 23, 2016 (sent for review July 13, 2016; reviewed by Yaoguang Liu and Chuanqing Sun)

The breeding and large-scale adoption of hybrid seeds is an important achievement in agriculture. Rice hybrid seed production uses cytoplasmic male sterile lines or photoperiod/thermo-sensitive genic male sterile lines (PTGMS) as female parent. Cytoplasmic male sterile lines are propagated via cross-pollination by corresponding maintainer lines, whereas PTGMS lines are propagated via self-pollination under environmental conditions restoring male fertility. Despite huge successes, both systems have their intrinsic drawbacks. Here, we constructed a rice male sterility system using a nuclear gene named *Oryza sativa No Pollen 1* (*OsNP1*). *OsNP1* encodes a putative glucose–methanol–choline oxidoreductase regulating tapetum degeneration and pollen exine formation; it is specifically expressed in the tapetum and miscrospores. The *osnp1* mutant plant displays normal vegetative growth but complete male sterility insensitive to environmental conditions. *OsNP1* was coupled with an α-amylase gene to devitalize transgenic pollen and the red fluorescence protein (*DsRed*) gene to mark transgenic seed and transformed into the *osnp1* mutant. Self-pollination of the transgenic plant carrying a single hemizygous transgene produced nontransgenic male sterile and transgenic fertile seeds in 1:1 ratio that can be sorted out based on the red fluorescence coded by *DsRed*. Cross-pollination of the fertile transgenic plants to the nontransgenic male sterile plants propagated the male sterile seeds of high purity. The male sterile line was crossed with ∼1,200 individual rice germplasms available. Approximately 85% of the F1s outperformed their parents in per plant yield, and 10% out-yielded the best local cultivars, indicating that the technology is promising in hybrid rice breeding and production.

hybrid rice | male sterility | breeding | hybrid seed production | OsNP1

The breeding and large-scale adoption of hybrid rice contributes significantly to the food supply worldwide. Currently, commercial hybrid rice production includes a cytoplasmic male sterile (CMS) line-based three-line system and a photoperiod/thermo-sensitive genic male sterile (PTGMS) line-based two-line system (1). The CMS line, the maintainer line, and the restorer line are required for the three-line system (1, 2). CMS is correlated to aberrant, often chimeric, mitochondrial genes absent in maintainer lines, and the male sterility can be suppressed by *Rf* genes in restorer lines (2). CMS hybrid varieties have been deployed for commercial production since the 1970s and covered ∼40% of rice growing areas in China in 2012 (3). Despite the wide application, CMS systems suffer from several intrinsic problems, including the narrow germplasm resources of restorer lines, the poor genetic diversity between the CMS lines and restorer lines, the instability of male sterility under certain weather conditions, the negative impact of aberrant mitochondrial genes on hybrid performance, and the difficulty to breed new traits into the parental lines (1, 3, 4). These problems limit further improvement in CMS hybrid breeding, which is believed to be the reason that CMS varieties reached a yield plateau in the last 20 y (1, 3).

Photoperiod-sensitive and thermo-sensitive lines are the two major types of rice PTGMS germplasm resources (5–8). Two widely used PTGMS genes have been cloned: one is a noncoding RNA gene (6, 7), and the other codes for RNase Z^{S1} (8). The male fertility of PTGMS lines is reversible in response to environmental conditions, which enables the PTGMS lines to propagate via self-pollination under environmental conditions restoring the male fertility and to outcross with restorer lines for hybrid seed production under conditions suppressing male fertility (5–8). Because PTGMS traits are controlled by nuclear recessive genes and male fertility can be restored by any normal rice cultivars (5–8), broader genetic resources can be explored for strong heterosis (1, 5). PTGMS rice was first adopted for farming in 1995, and its planting area quickly increased and almost covered 20% of rice fields in China by 2012 (1, 3). In fact, the highest yielding varieties currently cultivated in China are mostly PTGMS hybrids (1, 3). Nonetheless, the PTGMS system also has intrinsic problems primarily in that its fertility is regulated by environmental conditions (5). Thus, propagation of PTGMS seeds and production of hybrid seeds both require strict environmental conditions, and both are vulnerable to unpredictable environmental changes. In addition, the critical temperature for fertility transformation (CTFT) in PTGMS lines often shifts up after a few generations of propagation, and PTGMS individuals of suitable

Significance

Nuclear male sterility is common in flowering plants, but its application in hybrid breeding and seed production is limited because of the inability to propagate a pure male sterile line for commercial hybrid seed production. Here, we characterized a rice nuclear gene essential for sporophytic male fertility and constructed a male sterility system that can propagate the pure male sterile seeds on a large scale. This system is fundamentally advantageous over the current cytoplasmic male sterile and photoperiod/thermo-sensitive genic male sterile systems. Application of this technology will greatly enhance the effectiveness and efficiency in hybrid rice breeding and production.

Author contributions: X.T. and X.W.D. designed research; Z. Chang, Z. Chen, N.W., G.X., J.L., W.Y., and J.Z. performed research; and X.T. and X.W.D. wrote the paper.

Reviewers: Y.L., South China Agricultural University; and C.S., China Agricultural University.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the NCBI Sequence Read Archive, https://www.ncbi.nlm.nih.gov/sra (accession nos. SRP073226 and SRP058039 for genome resequencing data and KX066198 and KX066199 for OsNP1 nucleotide sequences).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1613792113/-/DCSupplemental.

CTFT have to be reisolated repeatedly during production (5). Furthermore, the CTFT trait is influenced by genetic backgrounds, which significantly increases the difficulty and uncertainty to breed new practical PTGMS lines (5).

Plant male reproductive development involves a series of events, from stamen meristem specification to pollen grain formation and pollination. Defects in any of these events can lead to male sterility. More than 40 nuclear genes required for male fertility have been identified in rice (3, 9, 10), but these genes have not been tapped for hybrid production because of the inability to propagate pure male sterile seeds on a production scale. Here we isolated a nuclear gene, *Oryza sativa No Pollen 1* (*OsNP1*), essential for pollen development and male fertility in rice. By transforming the *osnp1* mutant with *OsNP1* coupled with a gene to deactivate the transgenic pollen and a gene to mark the transgenic seed, we constructed a male sterility system that can overcome the intrinsic problems of both CMS and PTGMS systems. The male sterile line generated by this system was tested for hybrid breeding, and the results show great practical potential of the system in hybrid rice breeding and production.

Results

***osnp1*-1 Mutant Exhibits Male Sterile Phenotype.** To identify new rice genes regulating male fertility, we generated an ethyl methanesulfonate-induced mutant library in Huanghuazhan (HHZ), an elite *indica* cultivar in China, and screened for male sterile mutants. One mutant, named *osnp1*-1, displayed complete male sterility but normal vegetative growth, inflorescence, and flower morphology (Fig. 1*A*). The mutant anther was small and whitish (Fig. 1*B*), lacking pollen grains (Fig. 1*C*), and this phenotype was stable under variable day length (10–16 h) and temperature range (20–38 °C). Approximately 87–92% of spikelets displayed extrusion of one or both stigmas (Fig. 1*D*), and cross-pollination by wild-type (WT) HHZ under natural field conditions resulted in 40% or higher seed set. All of these traits indicated that *osnp1*-1 could be a promising male sterile line for hybrid rice technology. When back-crossed with the WT HHZ, all F1 progeny were fertile, and the F2 population displayed 3:1 segregation of fertile to sterile plants (216:68), indicating that *osnp1*-1 is a single recessive mutation.

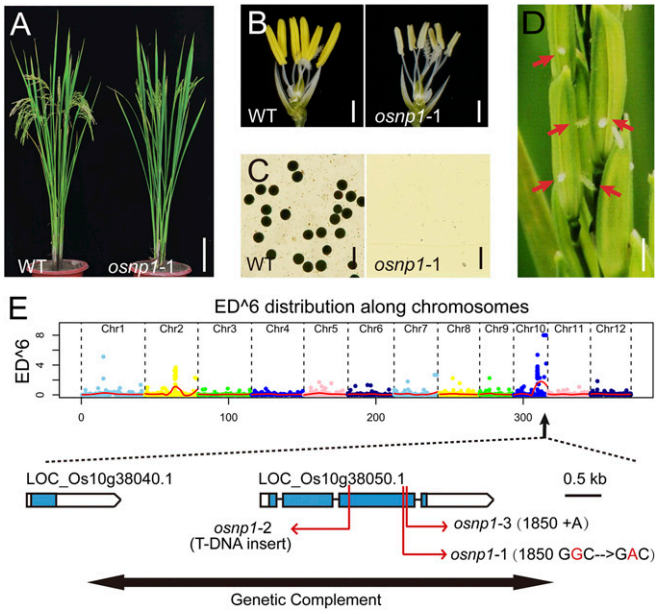

Fig. 1. Phenotypes of *osnp1*-1 and molecular identification of *OsNP1*. (*A*) A WT plant and *osnp1*-1 mutant plant after bolting. (Scale bar, 10 cm.) (*B*) Spikelets of WT and *osnp1*-1 with the palea and lemma removed. (Scale bar, 1 mm.) (*C*) I_2-KI staining of the WT and *osnp1*-1 pollen grains. (Scale bar, 100 μm.) (*D*) Inflorescence of *osnp1*-1, showing stigma extrusion. (Scale bar, 2 mm.) (*E*) The identification of *osnp1* alleles. The *Top* section showed distributions of Euclidean distance (ED) scores of SNP sites along chromosomes in *osnp1*-1. *OsNP1* gene structure and mutation sites of *osnp1*-1, *osnp1*-2, and *osnp1*-3 are shown in the *Bottom* section. The genomic fragment for gene complementation is shown by the double-headed black arrow. Empty boxes represent 5′- and 3′-UTRs, blue boxes represent exons, and the lines between boxes represent introns.

***OsNP1* Encodes a Putative Glucose–Methanol–Choline Oxidoreductase.** The *osnp1*-1 mutant gene was cloned via a modified MutMap method (11). The analyses identified a candidate region at the end of chromosome 10 (from 21.27 to 22.05 Mbp) harboring four candidate SNPs: two of which are in the intergenic regions, one in the intron of *LOC_Os10g39044*, and one (Chr10: 20,379,153) in the third exon of *LOC_Os10g38050* (Fig. 1*E*) that causes amino acid substitution from Gly_{561} (GGC) to Asp (GAC) (Fig. S1). Phenotype association assay showed that all 68 F2 male sterile plants carried this mutation, whereas the 216 F2 fertile plants showed 2:1 ratio of heterozygous and homozygous WT genotypes (149:67), suggesting that *LOC_Os10g38050* is *OsNP1*.

To confirm the prediction, we analyzed two other mutant alleles of *LOC_Os10g38050*. *osnp1*-2 was derived from *japonica* Zhonghua11, with a transfer DNA (T-DNA) insertion in the third exon, whereas *osnp1*-3 was created by CRISPR/Cas with a 1-bp insertion in the third exon (Fig. 1*E*). Both *osnp1*-2 and *osnp1*-3 exhibited normal plant growth but small whitish anthers lacking pollen grains and were completely male sterile (Fig. S2 *A–H*). When a genomic fragment covering *LOC_Os10g38050* with 2.5-kb upstream and 1.4-kb downstream regions was introduced into the *osnp1*-1 mutant, the transgenic plants developed normal anthers and fertile pollens (Fig. S2 *I–L*).

LOC_Os10g38050 proteins in Nipponbare and HHZ are almost identical (Fig. S1). Pfam analysis showed two conserved domains of the glucose–methanol–choline oxidoreductase superfamily. This superfamily catalyzes the oxidation of an alcohol moiety to the corresponding aldehyde in diverse substrates (12).

Both monocot and dicot species have genes highly homologous to OsNP1 (Fig. S3). There are six homologs in Nipponbare with >40% amino acid identity to OsNP1, of which ONI3 (Mini1, LOC_Os09g19930) has a role in preventing organ fusion during shoot development (13, 14). A phylogenetic tree constructed from alignment of OsNP1 and the highly similar sequences in rice and *Arabidopsis* defined four groups (Fig. S4). OsNP1 belongs to group A, represented by the *Arabidopsis* protein At1g72970, which was proposed to act as a long-chain fatty acid (LCFA) ω-alcohol dehydrogenase catalyzing the biosynthesis of long-chain α-,ω-dicarboxylic FAs (15). α-,ω-dicarboxylic FAs were proposed to participate in the cross-linking of surface cutin (15).

***OsNP1* Is an Anther-Specific Gene.** Quantitative real-time PCR (qPCR) revealed that *OsNP1* was specifically expressed in anthers during meiosis (stages 7 and 8), but not in the root, stem, leaf, glume, palea, lemma, pistil, and anthers at other developmental stages (Fig. 2*A*). Accordingly, transgenic plants expressing the promoter reporter gene $OsNP1_{pro}$*::GUS* showed β-glucuronidase (GUS) activity specifically in anthers at stages 7 and 8 (Fig. 2*B*), but not in other tissues. More precisely, *OsNP1* transcription was detected in the tapetum and microspores by in situ hybridization (Fig. 2*C*). When a Ubi_{pro}*::OsNP1* construct was introduced into the *osnp1*-1 mutant, the transgenic plants, which ectopically expressed *OsNP1* (Fig. S2*M*), showed normal vegetative and reproductive growth (Fig. S2*N*). It is noteworthy that pollen fertility was restored to 60–90% (Fig. S2 *O* and *P*). Together, these results indicated that *OsNP1* plays a specific role for the development of anther and microspore.

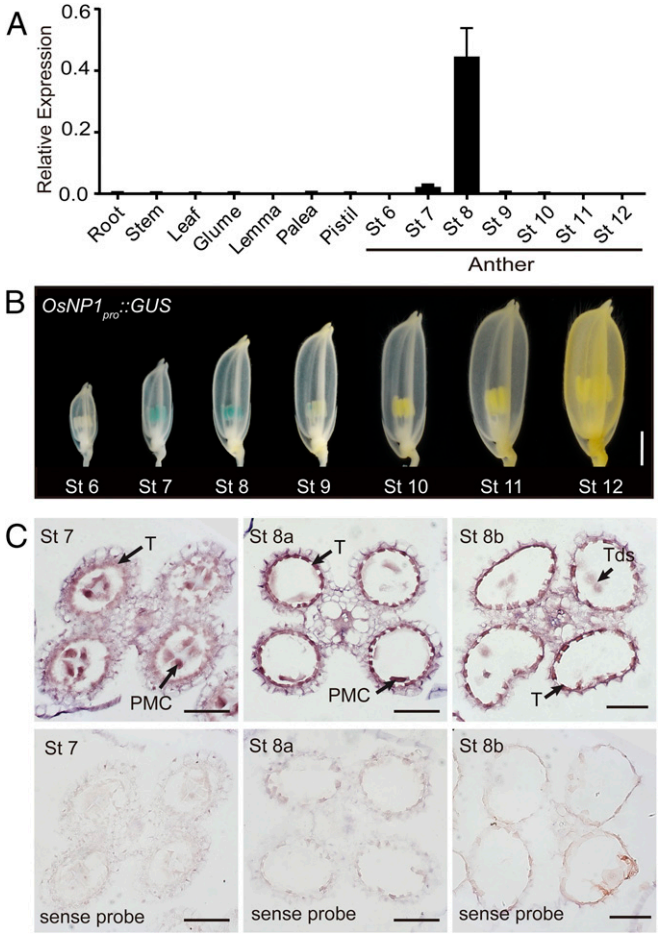

Fig. 2. Expression pattern of *OsNP1*. (*A*) qPCR analysis of *OsNP1* expression in different organs of WT. *OsACTIN1* served as a control. Data are shown as means ± SD (n = 3). (*B*) GUS expression (blue staining) patterns of developmental spikelets on the $OsNP1_{pro}$::*GUS* transgenic line. (Scale bar, 2 mm.) (*C*) In situ hybridization of *OsNP1* in WT anthers from stages 7–8b. Anthers hybridized with *OsNP1* sense probe served as controls. PMC, pollen mother cell; T, tapetal layer; Tds, tetrads. (Scale bars, 50 μm.)

OsNP1 Regulates the Tapetum Degeneration and Pollen Exine Formation. Microscopic analyses divide the rice anther development into 14 stages, from the formation of stamen primordium to the release of mature pollen during anther dehiscence (16). By stage 6, the WT anther primordia differentiate into a concentric structure, with pollen mother cells (PMCs) in the locule surrounded by a four-layered anther wall, from surface to interior, the epidermis, endothecium, middle layer, and tapetum. The PMC subsequently undergoes meiosis and generates a tetrad by the end of stage 8b. Meanwhile, tapetal cells initiate programmed cell death (PCD), and the middle layer becomes nearly invisible (16). No visible defect was observed in *osnp1*-1 anthers before tetrad formation (Fig. 3 *A*–*D*). However, both the anther wall and microspores displayed abnormal development after the microspores were released from the tetrads (stage 9). The WT tapetal cells gradually degenerate by PCD, becoming condensed and deeply stained (Fig. 3 *E* and *G*). The mutant tapetal cells were swollen and lightly stained (Fig. 3 *F* and *H*), indicating abnormal PCD. Different from the WT middle layer that vanishes after stage 9 (Fig. 3 *E*, *G*, and *I*), the mutant middle layer expanded at stage 10 (Fig. 3*H*). Unlike the WT microspores that complete pollen exine deposition and starch accumulation by stage 12 (Fig. 3*K*), the mutant microspores did not form exine, and they aborted by stage 11 (Fig. 3*J*), leaving debris in the locule (Fig. 3*L*).

Transmission and scanning electron microscopic analyses show that, in the WT anthers at stage 9, the tapetum starts to produce Ubisch bodies that gradually grow into electron-dense orbicules facing the locule (Fig. 4 *A*, *C*, *G*, *I*, *M*, and *O* and Fig. S5). Ubisch bodies are believed to secrete tapetum-produced sporopollenin precursors for pollen exine formation (17). The sporopollenin is made up of complex biopolymers derived mainly from LCFAs, long aliphatic chains, and phenolic compounds (17). Accumulation of sporopollenin on the microspore cell wall in the WT was visible at stage 9 (Fig. 4*E*), which eventually formed thick exine with distinctive layers of tectum, bacula, and nexine (Fig. 4 *G*, *K*, *M*, and *O*). Although the mutant tapetum appeared to form pre-Ubisch bodies at stage 9, these structures did not grow into mature Ubisch bodies (Fig. 4 *B*, *D*, *H*, *J*, *N*, and *P* and Fig. S5). Accumulation of electron-dense materials occurred on the mutant microspore cell wall at stage 9 (Fig. 4 *B* and *F*), but it stayed thin and failed to form an exine structure (Fig. 4 *H*, *L*, *N*, and *P*). Unlike the WT microspores that eventually grew into spherical pollen grains, the mutant microspores aborted at stage 11, leaving remnants in the locule (Fig. 4 *M*–*P* and Fig. S5).

In addition, defects were observed on the exterior of *osnp1*-1 anther. The WT anther epidermis was covered with a smooth cuticle layer at early developmental stages, which gradually thickened and formed grid-like structure at stages 11 and 12 (Fig. 4*Q* and Fig. S5). However, the grid-like structure was not formed on the surface of *osnp1*-1 anther (Fig. 4*R* and Fig. S5).

The anther surface cuticle consists of cutin and intracuticular and epicuticular waxes. Cutin is a polymer of ω-and midchain hydroxy and epoxy C16–C18 FAs, whereas cuticular waxes mainly comprise very LCFAs (18, 19). In rice, the lipid precursors for anther surface cuticle and sporopollenin are presumably produced in the tapetum, and then secreted via Ubisch bodies and

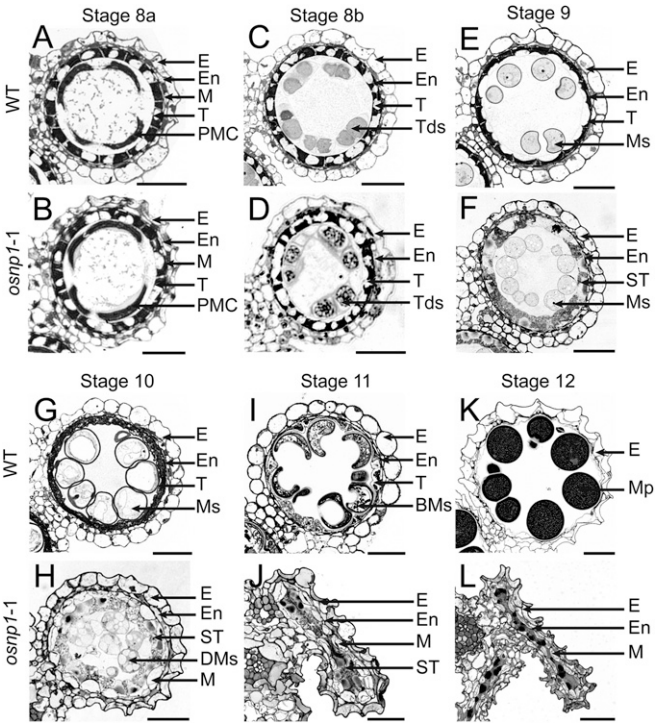

Fig. 3. Histological features of anther development in the WT and *osnp1*-1. Anther sections of WT (*A*, *C*, *E*, *G*, *I*, and *K*) and *osnp1*-1 (*B*, *D*, *F*, *H*, *J*, and *L*) plants from developmental stages 8a to 12 are shown. BMs, binuclear microspores; DMs, degenerated microspores; E, epidermis; En, endothecium; M, middle layer; Mp, mature pollen; Ms, microspores; PMC, pollen mother cell; ST, swollen tapetal layer; T, tapetal layer; Tds, tetrads. (Scale bars, 20 μm.)

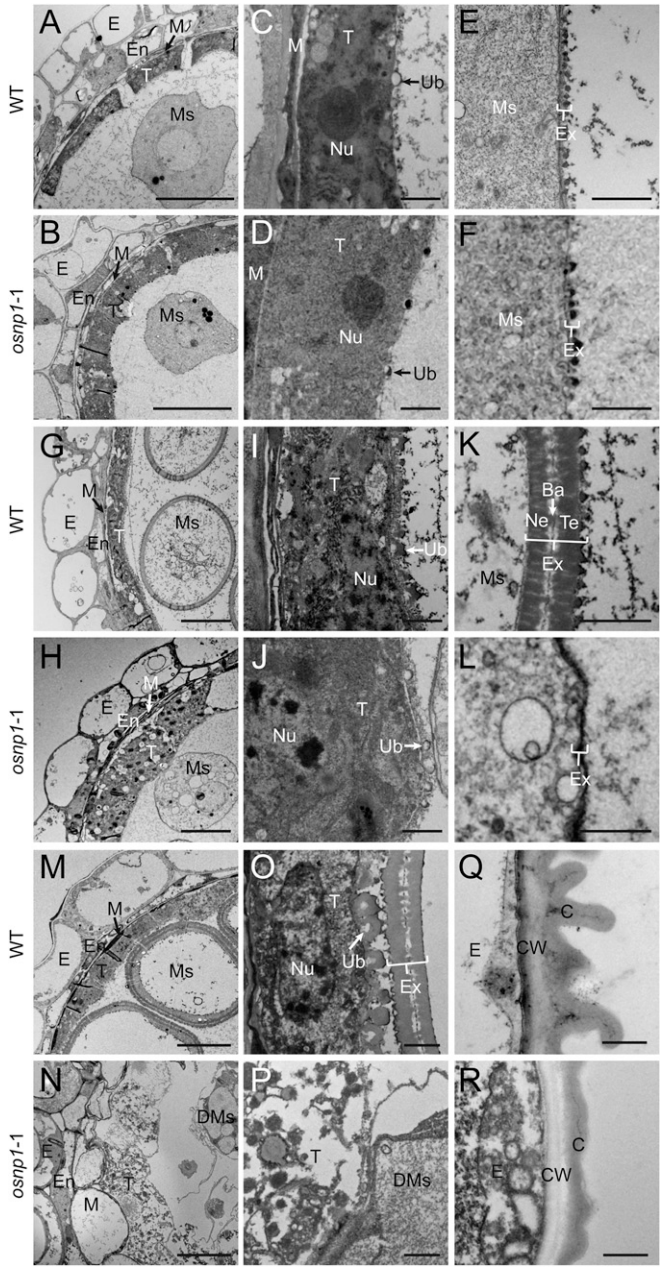

Fig. 4. TEM analyses of the WT and *osnp1*-1 anthers from stages 9–12. The transverse sections of the WT (*A*, *C*, *E*, *G*, *I*, *K*, *M*, *O*, and *Q*) and *osnp1*-1 (*B*, *D*, *F*, *H*, *J*, *L*, *N*, *P*, and *R*) anthers at stage 9 (*A*–*F*), early stage 10 (*G*–*L*), late stage 10 (*M*–*P*), and stage 12 (*Q* and *R*) are compared. (*C*, *D*, *I*, and *J*) Higher magnification of the tapetum showing Ubisch body. (*E*, *F*, *K*, and *L*) Higher magnification showing deposition of sporopollenin in pollen exine. (*O* and *P*) Higher magnification of the tapetum and pollen exine. (*Q* and *R*) The outer anther epidermis. Ba, bacula; C, cuticle; CW, cell wall; DMs, degenerated microspores; E, epidermis; En, endothecium; Ex, exine; M, middle layer; Ms, microspores; Nu, nucleus; Ne, nexine; T, tapetum; Te, tectum; Ub, Ubisch body. (Scale bars: *A*, *B*, *G*, *H*, *M*, and *N*, 10 μm; *C*, *D*, *I*–*L*, *O*, and *P*, 1 μm; *E*, *F*, *Q*, and *R*, 500 nm.)

transported to the anther and microspore surfaces (9, 17). Several rice male sterile mutants with defects in tapetum lipid metabolism or transportation also have defects in the development of Ubisch bodies, pollen exine, and anther surface cuticle (20–22). Moreover, the expression of several genes regulating tapetum PCD and synthesis/transportation of sporopollenin precursors displayed abnormal expression patterns in *osnp1*-1 (Fig. S6). Together, these results suggest that *osnp1*-1 has defects in the synthesis of lipophilic molecules for anther surface cuticle and sporopollenin formation, which is essential for pollen development.

Development of a Male Sterility System Using *OsNP1*. Because the *osnp1*-1 mutant exhibited traits desirable to the male sterile line, we sought to develop a nuclear male sterility system using the mutant plant and *OsNP1* gene. The strategy is shown in Fig. S7*A*. We transformed *osnp1*-1 with a double T-DNA binary vector *pZhen18B* (Fig. S7*B*). The first T-DNA contained *NPTII* under the CaMV 35S promoter for transformation selection. The second T-DNA contained three functional modules: *OsNP1* under its native promoter for restoration of male fertility, the maize α-amylase gene *ZM-AA1* under the pollen-specific *PG47* promoter to devitalize the transgenic pollen (23, 24), and the red fluorescence protein gene from *Discosoma sp*. (*DsRed*) (25) under the aleurone-specific *LTP2* promoter (26) to mark the transgenic seed. Because *OsNP1* is a sporophytic male fertility gene, a hemizygous *OsNP1* transgene in the *osnp1* mutant plant can fully restore the male fertility. Because *ZM-AA1* driven by a *PG47* promoter is a gametophytic factor that disrupts starch accumulation only in the transgenic pollen (23), only the transgenic pollen grains produced by the hemizygous transgenic plant are deactivated. The T0 transgenic plants were allowed to self-pollinate, and the T1 progeny was screened for the plant lacking the first T-DNA but carrying a single copy of the second T-DNA. The selected T1 plant was homozygous of the *osnp1*-1 locus but hemizygous of the second T-DNA, because pollen grains carrying the transgene were all defective, and the transgene was inherited only by the female gamete.

One T1 transgenic plant named Zhen18B was chosen as a representative for further study and presentation here. The plant exhibited normal vegetative and reproductive growth (Fig. 5*A*), normal male organ development (Fig. 5*B*), 1:1 ratio of fertile and defective pollen grains (Fig. 5*C*), and 1:1 (109:111) ratio of fluorescent seeds (with a hemizygous transgene) and nonfluorescent seeds (no transgene) on the panicle (Fig. 5*D*). The seeds were sorted out manually based on the fluorescence and cultivated for the next generation. All plants from the fluorescent seeds were fertile and genetically identical to the parent Zhen18B, and all plants from the nonfluorescent seeds (named Zhen18A) were male sterile. The fluorescence screening and fertility examination experiments were repeated for five generations, with a total of more than 15 million seeds, and all showed the same results, indicating the transgene was stable.

To determine the potential of Zhen18B as a maintainer, Zhen18A plants were cross-pollinated by Zhen18B plants. The Zhen18A plants exhibited 40% or higher seed setting, and one to three fluorescent seeds were found in 1×10^4 outcross seeds in each of five generations of the experiment, indicating the high stability and very low transgene transmission through pollen. Therefore, this system can efficiently propagate the male sterile seeds either by selfing or outcrossing the transgenics, and the maintainer and male sterile seeds can be sorted based on the fluorescence.

Zhen18A Is Promising for Commercial Hybrid Rice Breeding and Production. Propagation of pure Zhen18B seeds was achieved by Zhen18B self-pollination and mechanical sorting, whereas propagation of pure Zhen18A seeds was achieved by cross-pollination of Zhen18B to Zhen18A. With these prerequisites for commercial application fulfilled, we set to test the potential of Zhen18A in breeding of unique hybrid varieties. Zhen18A plants were pollinated with ∼1,200 individual rice germplasms available. Approximately 85% of the F1s outperformed their parents in per-plant yield, and 10% outyielded the local control cultivars (i.e., the best-yielding local varieties). A few representative F1s are shown in Fig. 6. These results indicated that our system is promising for hybrid breeding and production.

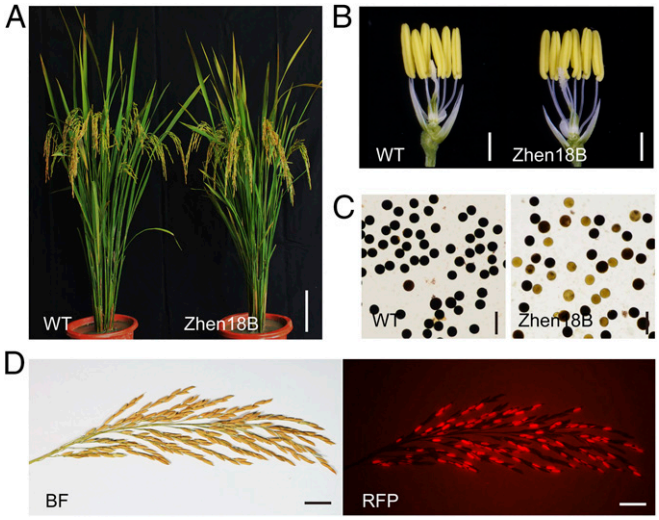

Fig. 5. Phenotype of the nuclear male sterility system. (*A*) A WT plant and Zhen18B plant after bolting. (Scale bar, 10 cm.) (*B*) Spikelets of WT and Zhen18B with the palea and lemma removed. (Scale bars, 1 mm.) (*C*) I_2-KI staining of the pollen grains in WT and Zhen18B. (Scale bars, 100 μm.) (*D*) Zhen18B panicle under bright field (BF) and a red fluorescence filter (RFP), respectively. (Scale bars, 2 cm.)

Zhen18B Provides a Platform for Breeding of Various Male Sterile Lines. To test whether the *osnp1*-1 and transgene loci can be transferred into other germplasms to breed male sterile lines, Zhen18B (as maternal parent) was crossed with 10 different rice germplasms as recurrent male parent. Progeny was analyzed for the presence of the *osnp1*-1 locus and transgene and their associated phenotypes in two consecutive generations of back-cross and self-pollination that have been obtained so far. Both *osnp1*-1 and the transgene were accurately associated with their respective phenotypes in the segregated progeny. The results of F1 and F2 progeny from Zhen18B crossed with *indica* rice 9311 are shown in Table S1 as representative. These results indicated that both *osnp1*-1 and the transgene from Zhen18B maintain their functions when transferred into different genetic backgrounds, making it possible to breed male sterile lines via traditional crossing and selection.

Discussion

Nuclear male sterility caused by non–PTGMS genes is common in flowering plants. However, commercial application of these mutants is limited because of the difficulty to propagate a large quantity of pure male sterile lines. In 1993, a strategy was proposed by Williams and Leemans to obtain a transgenic maintainer by transforming recessive male sterile plant with a fertility-restoration gene linked with a pollen-lethality gene and a marker gene (27). Self-pollination of the maintainer would propagate the maintainer line and the male sterile line as well. Later in 2002, Perez-Prat and van Lookeren Campagne (28) proposed two strategies to obtain maintainer lines: one to transform the fertility-restoration gene linked with a seed-color gene into the male sterile plant, and the other to transform the fertility-restoration gene linked with a pollen-lethality gene into the male sterile plant. Cross-pollination of the color-maintainer to the male sterile line would generate 50% of male sterile seeds and 50% of color-maintainer seeds that can be separated based on the seed color. Cross-pollination of the pollen-lethality maintainer to the male sterile line would generate a pure male sterile line. Based on these ideas, DuPont-Pioneer devised seed production technology in maize by transformation of a male sterile mutant with a fertility-restoration gene linked with the α-amylase gene to disrupt the transgenic pollen and the *DsRed* gene to mark the transgenic seed (24, 29). Self-pollination of the resulting maintainer propagates 50% of maintainer seeds and 50% of male sterile seeds that can be color sorted. Cross-pollination of the maintainer to the male sterile line produces 100% male sterile seeds. Application of seed production technology in maize would save the costs for mechanical detasseling, which is the predominant method for commercial maize hybrid seed production. However, nuclear male sterility maintainers are fundamentally more useful for crops that have bisexual flowers not amenable to manual emasculation, including rice, wheat, and sorghum.

Construction of the nuclear male sterility maintainer in rice was made possible by the isolation of *osnp1*-1 mutant and the cloning of *OsNP1*, an anther-specific gene. *osnp1*-1 is derived from HHZ, an elite *indica* cultivar that is semidwarf, super high yield, good eating quality, and widely cultivated in diverse geographical regions in China (30). As the mutant displays numerous traits highly desirable to the male sterile line, we sought to transform it into a practical male sterile system. In this work, we deployed the maize α-amylase as the pollen-lethality gene and *DsRed* as the seed-marker gene. We are also testing whether cytotoxin Barnase (31) can be used to deactivate the transgenic pollen and whether herbicide resistance genes can replace *DsRed* for seed-sorting during the maintainer propagation.

A transgenic line, Zhen18B, with a hemizygous single insertion of the transgene was identified. As expected, self-pollination of Zhen18B propagated itself and the male sterile Zhen18A in 1:1 ratio, whereas cross-pollination of Zhen18B to Zhen18A propagated pure Zhen18A seeds to a large quantity. Thus far, the transgene in Zhen18B is highly stable in five generations of ~10,000 plants tested. These results indicated that Zhen18B fulfills the role as a maintainer. The availability of a sorting machine makes it possible to separate the transgenic maintainer seeds from

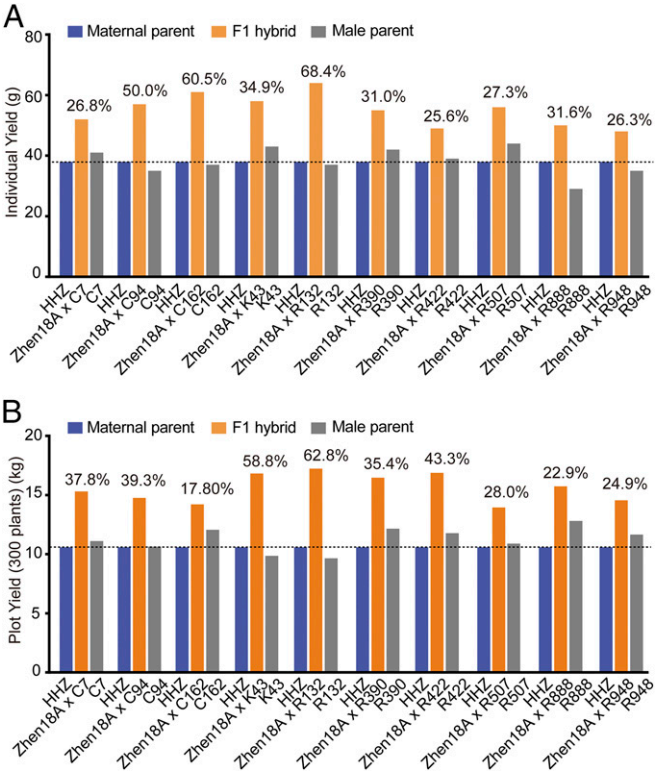

Fig. 6. Heterosis of Zhen18A-derived F1 hybrids. Representative F1s from Zhen18A cross with various germplasms out-performed their parents in individual yield (*A*) and plot yield (300 plants) (*B*). The number above the column represents the yield increase of the F1 over the better yielding parent.

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the nontransgenic male sterile seeds based on the presence of red fluorescence, which ensures high purity of both lines at a commercial scale. Hybridization of Zhen18A with other germplasms generated many hybrids outperforming the best local rice cultivars, indicating the system is promising in application.

This system is advantageous over CMS and PTGMS systems in several aspects. First, the male sterility is controlled by a single recessive nuclear gene; thus, any rice germplasms with the WT *OsNP1* gene can complement the mutation. This approach provides broader choices of germplasms as paternal lines to breed hybrids of superior heterosis. Second, the male sterility is insensitive to photoperiod and environmental temperature; thus, both the male sterile seeds and the hybrid seeds can be propagated under regular farming conditions, which significantly lowers the demand on specific environmental conditions for seed production and reduces the risk induced by weather changes. Furthermore, the male sterility and fertility restoration are each controlled only by a single genetic locus and thus can be easily bred into other cultivars to generate new sets of maintainer and male sterile lines. Our initial breeding experiments showed that both loci can be stably transferred from Zhen18B into different genetic backgrounds, without altering the corresponding phenotypes. Finally, although the technology involves transgenics, only the maintainer line carries the transgenes. Both the male sterile seeds and hybrid seeds are nontransgenic. Thus, transgenic oversight is applicable only to the maintainer line cultivation, which requires only a small acreage, and production of hybrid seeds and hybrid cultivation do not require transgenic oversight. Application of this technology will greatly enhance hybrid rice breeding and production.

Materials and Methods

All of the plants (*Oryza sativa*) were grown in the paddy field in Shenzhen during the natural growing season and maintained regularly. The *osnp1*-1 mutant was derived from HHZ by ethyl methanesulfonate mutagenesis, as described previously (11). Details of experimental procedures, such as cloning of *OsNP1*, qPCR, GUS staining, in situ hybridization, microscopic analyses, and field test, are described in *SI Materials and Methods*. See Table S2 for the primers used in this study.

ACKNOWLEDGMENTS. We thank Roger Thilmony for critical reading of the manuscript. This work was supported by Guangdong Innovative Research Team Program 201001S0104725509; Ministry of Agriculture Transgenic Program 2012ZX08001001; and Shenzhen Commission on Innovation and Technology Programs JCYJ20130402154520292, KQF201109160004A, and JSGG20150508105340526.

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