Mutation in *CSA* creates a new photoperiod-sensitive genic male sterile line applicable for hybrid rice seed production

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Rice is a major staple food worldwide. Making hybrid rice has proved to be an effective strategy to significantly increase grain yield. Current hybrid rice technologies rely on male sterile lines and have been used predominantly in *indica* cultivars. However, intrinsic problems exist in the implementation of these technologies, such as limited germplasms and unpredictable conversions from sterility to fertility in the field. Here, we describe a photoperiod-controlled male sterile line, *carbon starved anther* (*csa*), which contains a mutation in an R2R3 MYB transcription regulator of pollen development. This mutation was introduced into *indica* and *japonica* rice, and it rendered male sterility under short-day conditions and male fertility under long-day conditions in both lines. Furthermore, F₁ plants of *csa* and a restorer line JP69 exhibited heterosis (hybrid vigor), suggesting the feasibility of using this mutation to create hybrid rice. The *csa*-based photoperiod-sensitive male sterile line allows the establishment of a stable two-line hybrid system, which promises to have a significant impact on agriculture.

PGMS line | hybrid breeding | sugar partitioning

Rice (*Oryza sativa* L.) is a major staple food that feeds more than one-half of the human population (1). The three- and two-line hybrid rice breeding technologies take advantage of heterosis (hybrid vigor) and have been successfully applied in many countries, leading to a more than 20% yield increase over inbred varieties (2). The three-line hybrid rice breeding system was developed in the 1970s after the revolutionary discovery of the wild abortive cytoplasmic male sterile cytoplasm (CMS-WA) for the breeding of CMS lines, which solved the problem of self-pollination in the varieties used for hybrid rice production (3). In this technology, a restorer line that contains the restorer gene(s) is used as the pollen donor to recover male fertility in F₁ plants (hybrid rice) after its cross with the CMS line (Fig. S1A). To maintain CMS, a maintainer line, which has viable pollen grains and normal seed sets, is used as the pollinator to cross with the CMS line (Fig. S1A). Although this system has been widely used for hybrid rice seed production, the requirement of a maintainer line with normal cytoplasm and a restorer line with nuclear-encoded fertility restorer genes restricts breeders from exploiting more rice germplasms and complicates the procedures for generating hybrid seeds (4).

As a replacement of the three-line technology, a two-line system has been developed and applied for hybrid rice seed production after the discovery of the environmentally sensitive genic male sterile lines controlled by nuclear recessive gene(s). A *japonica* cultivar, Nongken 58S (NK58S), was the first spontaneous photoperiod-sensitive genic male sterile (PGMS) mutant identified (5). Because *indica* varieties are predominant in most rice planting regions, the PGMS loci/locus in NK58S were/was introduced into *indica* to generate breeding cultivars, such as Peiai 64S (PA64S) and Xin'an S. These *indica* lines frequently exhibit thermosensitive genic male sterility (TGMS) with a

threshold of ~24 °C (6). PGMS/TGMS lines can be used as the male sterile line or the maintainer line depending on the light and temperature conditions in the environment, and most rice cultivars can be used as the restorer line to restore the fertility in F₁ hybrid plants (Fig. S1B). *UDP-glucose pyrophosphorylase 1* cosuppressed rice exhibits a TGMS phenotype (7). Recently, a locus conferring PGMS/TGMS in NK58S and its derived lines was identified as a noncoding RNA gene, but the underlying molecular mechanisms for its function remain unclear (8, 9).

The two-line hybrid technology has obvious advantages, including a simplified breeding and seed production procedure. In addition, a wider range of restorers can be used (i.e., basically, all rice cultivars are capable of restoring the fertility in F₁ hybrids) (10). However, the two-line hybrid technology for *indica* hybrid seed production frequently suffers from unpredictable conversions from sterility to fertility in the male sterile lines because of temperature changes in the field, which is problematic to hybrid seed production (10).

Given the limitation of currently used male sterile lines, exploring new male sterile lines is critical to improving hybrid rice technologies. Recent advances in rice functional genomic research have provided great opportunities to reveal additional beneficial alleles for breeding (11). Previously, we reported the identification of Carbon Starved Anther (CSA), an R2R3 MYB transcription factor that regulates sugar partitioning from photosynthetic tissues (sources) to anthers (sinks) to promote pollen maturation (12). The *csa* mutant, which contains a substitution of guanine with cytosine and one deletion of guanine in the first exon of *CSA* and displays male sterility under short-day (SD) conditions, was identified from a *japonica* cultivar, 9522 (12). Here, we report the finding that this *csa* mutation renders male fertility under long-day (LD) conditions in both *japonica* and *indica* rice backgrounds, and it can be used to create new and more stable PGMS lines for two-line hybrid rice seed production.

Result

***csa* Is a Photoperiod-Sensitive Genic Male Sterile Mutant.** Consistent with our previous report (12), when the *csa* mutant initiated panicle development during late August to early September in a

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microsporogenesis takes place (6, 13). To check the critical stage for male fertility conversion in *csa*, correlation analysis between seed setting and the time of LD treatment (Tables S1, S2, and S3) was performed using the SPSS statistical analysis software. Our results showed that the recovery of male fertility in *csa* correlated well with the LD treatment that took place 22–17 d before the heading stage; during this period, the secondary branch primordia are formed, and stamen and pistil are initiated (Fig. S3 and Tables S1, S2, and S3).

To further confirm the PGMS nature of *csa*, we grew the mutants in growth chambers under various photoperiods and a daily average temperature of 24 °C or 28 °C (Fig. S4 and Table S4), conditions frequently used for PGMS mutant characterizations (14). Irrespective of the ambient temperature, the *csa* mutant was completely male-sterile under the 11.5- or 12.5-h photoperiod conditions (Fig. S4A–D and Table S4) and exhibited recovery of pollen fertility under 13.5- and 14.5-h photoperiod conditions (Fig. S4E–H and Table S4).

These results together suggest that the fertility of *csa* can be rescued by longer photoperiod and that *csa* is a PGMS line, which seems to be capable of overcoming the problems of instable male sterility caused by unpredictable temperature changes in current TGMS lines.

Defect in Sugar Partitioning in *csa* Was Recovered Under LD Conditions. Starch, as an energy reserve for pollen germination, accumulates during rice pollen maturation and thus, serves as a marker for pollen maturity (15). We showed previously that, under SD conditions, *csa* has defective assimilate allocation (i.e., sugar and starch contents are increased in leaves and reduced in anthers during pollen maturation) (Fig. 2A and B and Table S5) (12). Under LD conditions, however, this defect was partially rescued in *csa*. At stage 13 of anther development, when mature pollen grains form, starch levels increased ~120% in the anther and decreased 42% in leaves compared with *csa* under SD conditions (Fig. 2A and B and Table S5). To measure the amount of sugars in the anther sink tissue, we used a ^{14}C -sucrose feeding assay on excised stems that contained leaves and panicles on which the anthers were at developmental stages 11 and 13. The *csa* mutant had increased accumulation of ^{14}C -labeled sugars in anthers under LD conditions compared with the same tissue under SD conditions (Fig. 2C). In agreement with this result, the expression of *OsMST8*, a monosaccharide transporter encoding gene that is critical for rice pollen starch accumulation and regulated by CSA (12), displayed a twofold increase in *csa* anthers under LD conditions compared with the SD conditions (Fig. 3A). These data collectively suggest that the deficiencies in sugar partitioning in *csa* can be rescued by LD conditions.

Differential Expression of CSA in SD and LD Conditions. To begin to understand the mechanism underlying the photoperiod-controlled conversion of male fertility in *csa*, we analyzed the expression patterns of CSA in the WT under SD and LD conditions. Quantitative RT-PCR (qRT-PCR) was performed with RNA collected from anthers at stage 9, when microspores are released from the tetrad. Under SD conditions, CSA is highly expressed in anthers, where its transcript level is ~6.7-fold higher than the level under LD conditions (Fig. 3B), suggesting that CSA may play a key role in male fertility under SD conditions and have a less important function in pollen development under LD conditions.

We next performed microarray analysis of RNA from stage 9 anthers of *csa* to determine the level of rescue by LD at the global gene expression level and explore whether there are genes that may function preferentially in LD to replace the function of CSA. In SD, 880 genes were up-regulated and 455 genes were down-regulated in *csa* compared with the WT (Fig. 3C and Dataset S1). Consistent with the recovery of male fertility in LD in *csa*, the expression of 908 of 1,335 genes was completely or partially restored to WT levels under LD conditions in the mutant (Fig. 3C and Dataset S1). Notably, two CSA homologs, *OsMYB5* (*Os05g41166*) and *OsMYB8* (*Os01g45090*), showed two- to threefold expression in LD vs. SD conditions in WT anthers (Fig. 3D and E). In addition, in *csa* anthers, the expression level of 392 genes (including *OsMYB5*) is higher in LD than SD conditions (Fig. 3C and Dataset S1). It remains to be elucidated whether these two CSA homologs or other proteins can function as the replacement of CSA in male reproductive development under LD conditions.

Applicability of *csa* in Conferring PGMS in *indica* and Breeding. Strains used for hybrid rice production are mainly *indica* cultivars. The original NK58S line is a *japonica* line displaying PGMS. However, *indica* cultivars such as PA64S, into which the loci from NK58S were introgressed, predominantly exhibit TGMS rather than PGMS (16, 17). To determine whether introducing the *csa* mutation into *indica* cultivars can reproduce the phenotype that it confers in *japonica*, we crossed *csa* with the *indica* rice Zhenshan 97, a variety widely used in rice breeding. In the progeny, we selected male sterile lines homozygous for the *csa* mutation and displaying typical Zhenshan 97 morphological traits, such as longer leaf and purple spikelet awn and stigma (Fig. 4A–C and Fig. S5A–G). After two backcrosses with *indica* and two self-pollinations, BC₂F₃ lines homozygous for *csa* also exhibited male sterility in SD conditions and recovered male fertility in LD conditions (Fig. 4A–G). These phenotypes are identical to those phenotypes of *csa* in the *japonica* background, suggesting that *csa* can produce new PGMS lines suitable for two-line hybrid rice breeding in both *indica* and *japonica*. These results confirmed that CSA is a PGMS gene.

Fig. 2. Sugar and starch levels and ^{14}C -signal accumulation in the flag leaf and anthers in the WT and *csa*. (A and B) Sucrose and starch levels at stages 11 and 13 in anthers (A) and the flag leaf (B). (C) ^{14}C -signal accumulation in stage 11 anthers of the WT, *csa* under SD, and *csa* under LD. The data are given as means $\pm$ SE ($n = 3$). cpm, unit for radioactivity; FW, fresh weight; S, starch; Suc, sucrose.

Fig. 3. Expression of *OsMST8*, *CSA*, *OsMYB5*, and *OsMYB8* and analysis of genes with expression changes in *csa*. (A) Expression levels of *OsMST8* in stage 9 anthers in WT (9522) and *csa* under SD and LD conditions. (B, D, and E) Expression levels of *CSA*, *OsMYB5*, and *OsMYB8* in WT (9522) stage 9 anthers under SD and LD conditions. (C) Clustering analysis of genes showing significant differential expressions in the anthers of WT (9522) under SD conditions and *csa* under SD and LD conditions. The candidate genes were categorized into six groups according to their different expression levels in stage 9 anthers of WT (SD), *csa* (SD), and *csa* (LD). Numbers on the graph indicate the number of hybridizing probes for each gene on the chip. Detailed information of the genes is listed in Dataset S1. GO analysis showed that these genes are mainly related to photosynthesis, light harvesting (GO: 0009765), lipid metabolic process (GO:0006629), response to abiotic stimulus (GO:0009628), and reproduction (GO:0000003) (Dataset S2). In A, B, D, and E, error bars indicate SD. Each reaction has three qPCR biological replicates.

The feasibility of *csa* in breeding was tested using *csa* as a female parent to cross with JP69, a *japonica* restorer line. F₁ plants displayed normal male fertility (Fig. 4 H and I and Fig. S5 H–J) and a strong hybrid vigor over JP69, such as significant increases in tiller number (77.2%, $n = 20$), grain number (~52.9%, $n = 10$), and 1,000-grain weight (12.9%, $n = 5$) (Fig. 4 J–L). Our data confirmed the applicability of *csa* in hybrid rice production.

Discussion

PGMS/TGMS lines are valuable germplasm resources for crop hybrid breeding. Since the discovery of the first spontaneous photoperiod-sensitive genic male sterile mutant NK58S, a series of TGMS has been developed and applied for two-line hybrid rice production in countries such as China, India, Philippines, and the United States (18). One of these TGMS lines, PA64S, predominantly displays a temperature-controlled conversion from male sterility to fertility. It is male-sterile when anther development occurs at temperatures higher than 23.5 °C and male-fertile when the temperature is in the range of ~21–23 °C (16, 17). Because of unpredictable environmental temperature changes, failure to produce hybrid rice seeds frequently occurs in TGMS in the field (10). In contrast to the fluctuating environmental temperatures, photoperiod is relatively stable in the field within a season; thus, PGMS is more preferred in hybrid rice breeding.

In this study, we showed that *csa* and its derived lines can be used as PGMS lines in the two-line hybrid rice breeding system. The *csa* mutation confers stable photoperiod-controlled male fertility in both *indica* and *japonica*, which is distinct from the currently used PGMS/TGMS lines. Until now, two types of rice PGMS lines have been reported. One type, such as NK58S (*japonica*), displays male sterility under LD and male fertility

under SD (5). The other type, such as YiD1S and D52S, shows male sterility under SD and male fertility under LD, and it is called reverse PGMS (19, 20). The genetic loci governing the photoperiod-sensitive male fertility of YiD1S and D52S remain to be determined at the molecular level (19, 20). In our work, introduction of the *csa* mutation into *indica* rice Zhenshan 97 rendered male sterility under SD conditions and male fertility under LD conditions, suggesting that *csa* belongs to the reverse PGMS category and can produce new PGMS lines suitable for two-line hybrid rice breeding in both *indica* and *japonica*. Thus, the *csa* mutation has a great potential in two-line hybrid rice breeding, overcoming some of the problems in the current hybrid rice systems, such as limited germplasm use and unstable male sterility, and it may greatly benefit large-scale crop breeding systems.

Plant male reproductive development is controlled by the interaction between genetic components and environmental signals such as photoperiod and temperature. This study suggests that photoperiod regulates the expression of *CSA*, which encodes a key regulator of sugar partitioning during anther development. Previously, we showed that, in late anther development, *CSA* plays a key role in promoting sugar partitioning from the source tissue leaf to the sink tissue anther by directly regulating the expression of *OsMST8*, which encodes a monosaccharide transporter (12). Monosaccharide transporters are capable of transporting various monosaccharides across membrane barriers, and rice *OsMST8* is a key component of the specialized anther apoplastic sugar transport pathway (21–23). The mutation in *CSA* caused a dramatic reduction of *OsMST8* expression in anthers and reduced amount of sugar accumulation in the anther under SD conditions, leading to male sterility (Figs. 2 and 3A) (12). In contrast, *OsMST8* expression and the sugar level in *csa*

Fig. 4. Phenotypes of Zhenshan 97 with or without *csa*, JP69, and hybrid F₁ lines. (A–C) Spikelet after the palea and lemma are removed. (D–F) Pollen grains stained with I₂-KI. (G) Strategy for screening *csa* in the *indica* background. (H) Morphologies of whole plant. (I) Panicles. (J–L) Comparison of the number of tillers (J) and grains (K) per plant with 1,000-grain weight (L).

anthers was recovered nearly to the normal level under LD conditions (Figs. 2 and 3A). We hypothesize that CSA plays a less important role in pollen development under LD conditions. In support of this hypothesis, *CSA* expression in SD conditions is about 6.7-fold higher than in LD conditions (Fig. 3B).

Our genome-wide analysis indicated that, in *csa* under LD conditions, the expression of a large number of genes with expression that is changed under SD conditions was completely or partially restored to WT levels. *CSA* is completely dysfunctional in the *csa* mutant (12); therefore, other genes may be mainly responsible for regulating anther development under LD conditions. Candidates for these regulators include the two CSA homologs, *OsMYB5* and *OsMYB8*, which exhibit higher expression levels in LD conditions (Fig. 3D and E and Dataset S1). *OsMYB5* is closely related to another *Arabidopsis* R2R3 MYB gene, *MYB33*, and double mutant between *myb33* and another *myb* mutant (*myb65*) displays conditional male sterility with reduced male fertility under low light intensity or higher temperatures (24). *OsMYB8* is closely related to *Arabidopsis* *MYB21*, which encodes a flower-specific transcription factor that affects anther development (25). Other genes with higher expression in LD than SD could also act in a CSA-independent regulatory pathway of anther development in the LD.

In summary, this work identified *csa* as a causal mutant displaying a PGMS trait. The *csa* mutant and derived *csa*-containing lines overcome some of the problems in the current hybrid rice systems and will potentially be greatly beneficial to large-scale crop breeding programs.

Methods

Materials and Growth Conditions. The varieties 9522 (*O. sativa* L. ssp. *japonica*), Zhenshan 97 (*O. sativa* L. ssp. *indica*), *csa*, Zhenshan 97 (*csa*), and JP69 (*O. sativa* L. ssp. *japonica*) were used for this study. *csa* was generated by treating rice seeds 9522 with ⁶⁰Co γ-ray radiation (26). Zhenshan 97 (*csa*) has the *csa* mutation in the Zhenshan 97 background, which was generated by backcrossing *csa* with the *indica* variety Zhenshan 97. The hybrid rice was bred by crossing JP69 (male) and *csa* (female) in Sanya (18_N, 109_E) and later grown in Shanghai (30_N, 121_E).

Experiments in the paddy fields were carried out at two sites, Sanya and Shanghai, China, from 2006 to 2010 in normal rice growing seasons. To

confirm the PGMS nature of *csa*, photoperiods were extended by illuminating rice plants (after panicle initiation) with artificial light with a 200-W incandescent source (lamp) that is ~50 cm above the rice panicles. We measured the natural photoperiod length according to the website record: www.time.ac.cn/serve/sun_time/sh.htm.

Experiments in the growth chambers were carried out in China National Rice Research Institute, with the mean temperature of 24 °C or 28 °C, photoperiod of 11.5, 12.5, 13.5, or 14.5 h, and 70% relative humidity. When each *csa* seedling had five leaves on the stem, SD (10 h light/14 h dark) treatment was conducted to promote the transition from vegetative growth to reproductive growth. After 10 d of SD treatments for flowering induction, the rice plants were moved into growth chambers for photoperiod treatments (Table S4). Rice anther staging was described in the work by Zhang et al. (27).

Phenotype Characterization and Fertility Examination. Plants were photographed with a Nikon E995 digital camera and a Leica S8 APO stereo microscope (Leica Microsystems). For transverse section analysis of anther locules, anthers were collected and fixed as described (28). Semithin (4 μm) sections were made using a Leica RM 2265 programmable rotary microtome (Leica Microsystems) and stained with 0.05% Toluidine Blue (28). Transverse sections were photographed using a Leica DM2500 microscope (Leica Microsystems). To analyze pollen fertility, anthers of five mature flowers from each plant at heading time were collected. Pollen grains were then stained with 1% potassium iodide solution (I₂-KI) and photographed using the Leica DM2500 microscope.

Sugar and Starch Measurement and ¹⁴C-Signal Accumulation. WT and *csa* (SD and LD) anthers and flag leaves were obtained at stages 11 and 13 of anther development for sucrose and starch measurement and analysis using previously published methods (12). To measure ¹⁴C-signal accumulation, WT and *csa* panicles from about 100 flowers at stage 11 of anther development were excised and treated with radiolabeled [fructose-U-¹⁴C]sucrose. Radioactivity (cpm) was measured as previously described (12).

qPCR Assay. Total RNA was isolated from rice anthers at stage 9 with the TRIzol Reagent Kit (Invitrogen) treated with DNase and then reverse-transcribed as previously described (12). qRT-PCR was performed with a Bio-Rad CFX96 Real-Time PCR Detection System using SYBR Green I. Samples were normalized using *ACTIN1* expression; the relative expression levels were measured using the 2^(-ΔC_T) analysis method. Primer sequences used are listed in Table S6.

Microarray Analysis. Total RNA was isolated from stage 9 anthers in *csa* under LD and SD conditions and stage 9 WT anthers under SD using TRIzol (Invitrogen). The microarray experiment was performed with Affymetrix rice

genome array by Gene Tech Biotechnology Company using the standard Affymetrix protocol (Affymetrix). Significance analysis of microarray was used to identify differentially expressed genes. Genes with at least a threefold change in expression, P values < 0.001 , and false discovery rate < 0.05 were chosen for additional analysis.

The microarray data were also verified by qRT-PCR using independently generated stage 9 samples. Eight genes, which have more than threefold changes in expression (Fig. S6), were selected for qRT-PCR. The fold changes observed from qRT-PCR closely mirrored those changes obtained from the microarray analysis, confirming the reliability of the microarray data.

Gene Ontology Analysis. The Gene Ontology (GO) annotations of microarray genes were downloaded from National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), UniProt (www.uniprot.org), and GO (www.geneontology.org). The elim Fisher algorithm was used to do the GO enrichment

test, because it can iteratively remove the genes mapped to significant GO terms from more general (higher-level) GO terms; therefore, the significant GO terms will not be overshadowed by the general GO terms (29). Gene ontology categories with a P value < 0.01 were selected (Dataset S2).

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