MALE STERILITY 3 encodes a plant homeodomain-finger protein for male fertility in soybean

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ABSTRACT

Male-sterile plants are used in hybrid breeding to improve yield in soybean (Glycine max (L.) Merr.). Developing the capability to alter fertility under different environmental conditions could broaden germplasm resources and simplify hybrid production. However, molecular mechanisms potentially underlying such a system in soybean were unclear. Here, using positional cloning, we identified a gene, MALE STERILITY 3 (MS3), which encodes a nuclear-localized protein containing a plant homeodomain (PHD)-finger domain. A spontaneous mutation in ms3 causing premature termination of MS3 translation and partial loss of the PHD-finger. Transgenetic analysis indicated that MS3 knockout resulted in nonfunctional pollen and no self-pollinated pods, and RNA-seq analysis revealed that MS3 affects the expression of genes associated with carbohydrate metabolism. Strikingly, the fertility of mutant ms3 can restore under long conditions. The mutant could thus be used to create a new, more stable photoperiod-sensitive genic male sterility line for two-line hybrid seed production, with significant impact on hybrid breeding and production.

Keywords: fertility restoration, male sterility, PHD-finger, photoperiod, soybean

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INTRODUCTION

Soybean (Glycine max (L.) Merr.) is a crop legume which globally constitutes one of the most important sources of protein and oil (Adak and Kibritci, 2016). Because of domestic imbalance in various countries between soybean supply and demand, the main goal of modern soybean breeding is to increase yield. Hybrid breeding technologies take advantage of heterosis (hybrid vigor), which has been applied successfully in major crops to substantially increase global food supplies and could potentially improve soybean yields as well (Kim and Zhang, 2018). Male-sterile plants are considered ideal female lines for breeding because they do not require removal of functional pollen grains for hybrid seed production, which eliminates the complicated process of artificial emasculation in the strictly self-pollinated soybean.
Male sterility can be classified as either cytoplasmic male sterility (CMS), caused by interactions of factors encoded by mitochondrial and nuclear genes, or genetic male sterility (GMS), caused by nuclear genes alone (Chen and Liu, 2014). CMS-based hybrid seed technology is used in the three-line hybrid breeding system to circumvent the problem of self-pollination in various cultivated plants. Although this system has been widely used for hybrid seed production, the requirement for a maintainer line and a restorer line prevents breeders from exploiting more germplasms and complicates the procedure for generating hybrid seeds. GMS often occurs in natural conditions, and GMS plants are easily distinguishable from non-male-sterile plants; scientists have thus collected and preserved such materials during traditional breeding and used them in creating hybrid seeds. In soybean, an ms series of 13 GMS mutants (ms1–ms9, msMOS, msp, mst-M, and msnH) have been identified and mapped to seven different chromosomes (Nie et al., 2019; Zhao et al., 2019). With the explosion of genomic resources and the rapid development of the third generation hybrid rice technology, more effort has been invested in identifying GMS genes for hybrid breeding in crops. Recently, a few GMS genes have been cloned, namely ms4 (Thu et al., 2019), ms1 (Fang et al., 2021; Jiang et al., 2021; Nadeem et al., 2021), and ms6 (Yu et al., 2021).

Environmentally sensitive genetic male-sterile mutants have enabled the use of some GMS traits for hybrid crop breeding. In the two-line hybrid breeding system, which uses photoperiod- and temperature-sensitive genetic male-sterile (PGMS and TGMS, respectively) mutants, reversion to fertility occurs through conditional self-pollination under different growth conditions. The reversibility of male fertility in PG/TGMS mutants enables researchers to simplify hybrid seed production and reduce costs with a two-line hybrid system, for which a broader range of germplasm resources are available (Chang et al., 2016). However, only a few PG/TGMS materials, such as the TGMS strain SS71-26 (Caviness and Fagala, 1973), PT/GMS line 88-4288BY (Wei, 1991), and TGMS mutant msp and ms8 (Yang et al., 2014), have been identified in soybean; gene exploration of PT/GMS loci is still under way in soybean.

In 1977, a single recessive nuclear gene was reported for the first time in the male-sterile, female-fertile mutant ms3 (Chaudhari and Davis, 1977). In this case, male sterility was possibly due to the abortion of microspores initiated by the failure of callose dissolution at the tetrad stage (Jin et al., 1997). A candidate region for this mutation flanked by two simple sequence repeat markers (Satt 157 and Satt 542) on molecular group D1b (Gm02) was reported (Cervantes-Martinez et al., 2009). In contrast to the other GMS mutants in the ms series, ms3 suffers from intrinsic problems: the 3/16 F2 populations deviate significantly from the expected 3:1 ratio, and at least 50% of the male-sterile plants in each population produce some pods, a variation that is probably due to environmental factors (Chaudhari and Davis, 1977). Until now, the identity of the ms3 gene had not been confirmed, and the reason for the abnormalities caused by environmental factors remained unclear.

In this study, based on a systematic analysis of phenotypes, we isolated the ms3 gene, which encodes a plant homeodomain (PHD)-finger domain, via a map-based cloning approach. Clustered regularly interspaced palindromic repeats (CRISPR) /CRISPR-associated protein 9 (Cas9)-mediated mutagenesis led to dysfunction of MS3, resulting in the male sterility phenotype. Transcriptome analyses showed that ms3 affects the expression of genes related to sugar metabolism. The fertility of ms3 can be rescued under long-d conditions. This work provides new insights into a molecular mechanism of male sterility in soybean and may enhance the efficiency of soybean hybrid breeding.

## RESULTS

### Identification of recessive genetic male-sterile material ms3

To investigate the cause of male infertility of the ms3 mutant, we carried out a comprehensive phenotypic analysis of ms3 mutants grown in different locations. In Changchun (43°50’ N), the average numbers of pods of MS3 and ms3 plants at the mature stage were 56.20 ± 14.54 and 18.00 ± 7.12, respectively (Figure 1A–C). In Sanya (18°25’ N), the pod numbers for MS3 and ms3 were 25.64 ± 4.43 and 0.00 ± 0.00, respectively (Figure S1A–C). Observation of other agronomic traits showed no clearly observable differences between MS3 and ms3 from the two locations (Figure S2A–H). The results indicate that the ms3 locus stably affects fertility phenotype under different conditions.

To investigate male gamete development, we compared mature pollen grains of MS3 and ms3 plants. Relative to MS3, ms3 had substantially fewer pollen grains, which were larger and more irregular in shape when observed by staining with 1% (w/v) iodine potassium iodide (I2-KI) solution (Figure 1D–E). Scanning electron microscopy (SEM) observation showed that ms3 anthers were nearly empty and the only remaining pollen grains were aborted (Figure 1F, G). Further observation by semithin section light microscopy revealed that the ms3 morphology of pollen grains were abnormal and aggregated into groups; the tapetum was clearly abnormally enlarged and vacuolated, resulting in tapetal extrusions into the anther locule (Figure 1H, I). These observations indicate that the ms3 mutant is unable to form normal tapetum and pollen, which leads to male sterility.

### Map-based cloning and functional analysis of ms3

To identify the intriguing gene ms3, we adopted a map-based cloning strategy. The mutant ms3 plants were crossed with the Jinlin NO.3 (Figure S3). All the ms3BC1F1 progenies were fertile, and the ms3BC1F2 plants displayed a genetic segregation ratio of 3:1 (fertility/sterility = 201/60, χ² (3:1) = 0.56, P = 0.7–0.8) under the conditions of Changchun (43°50’ N), indicating a recessive monofactorial inheritance for the ms3 mutation. Using the bulked segregant analysis (BSA) method (Michelmore et al., 1991) and data from a previous report
(Cervantes-Martinez et al., 2009), we located the ms3 gene on chromosome Gm02 (D1b) (Figure 2A). Genetic linkage mapping using 2,498 ms3BC4F2 plants revealed that ms3 was flanked by the M1 and M7 markers, which have a physical distance of 160 kb (Figure 2B). By analyzing the genotypes and phenotypes of 6,000 homozygous recombinant ms3BC4F3 plants, we mapped ms3 between two markers, M9 and M10. Based on the soybean reference genome annotation project (https://www.soybase.org/), this 22.07-kb region between M9 and M10 was predicted to contain two genes, designated open reading frames 1 and 2 (ORF1 and ORF2) (Figure 2C). ORF2 (Glyma.02g107600), from Integrating Genetics and Genomics to Advance Soybean Research, encodes a protein that contains a PHD-finger domain. Sequence analysis of 3′ random amplification of complementary DNA (cDNA) ends (3′-RACE) cDNA products indicated that the ORF2 cDNA was 2,126 bp long, with an ORF of 1,971 bp and a 155-bp 3′-untranslated region (3′-UTR), while the orf2 cDNA was 1,938 bp long, with an ORF of 1,887 bp and a 151-bp 3′-UTR. The sequence of the orf2 cDNA revealed three nucleotide substitutions, GTT+1882 to +1884TGG, in the third putative exon of orf2 caused a substitution of tryptophan (W) for valine (V) in the 628th amino acid, and a further three nucleotide substitutions, CGA+1885 to +1887TAA, led to premature termination of protein translation and, therefore, the partial loss of the PHD-finger domain (Figure 2D). Basic Local Alignment Search Tool analysis showed that the sequence of a 57-bp insertion fragment in natural mutant ms3 cDNA belongs to a part of a long terminal repeat retrotransposon. These results suggest that dysfunction of ORF2 (Glyma.02g107600) causes the male sterility phenotype of the ms3 mutant.

To further test whether the male sterility phenotype was caused by this mutation, we prepared a CRISPR/Cas9-based knockout construct, MS3-KNOCKOUT (MS3-KO), with two selected target sites 55-74 and 64-83 bp upstream of the MS3 PHD-finger domain, respectively, that have no homologous, potential off-target sites in the soybean genome; we then introduced this into the Williams 82 soybean cultivar (Figure S4A). Sequence analysis of the MS3-KO transgenic T3 plants identified

Figure 1. Identification of recessive genic male sterile material ms3

(A, B) Comparison of the plant phenotype between MS3 (A) and ms3 (B) in Changchun (43°50′ N); bars = 20 cm. (C) The pod numbers of 10 MS3 and ms3 plants from Changchun (43°50′ N) were measured. The double asterisks represent a significant difference determined by Student’s t-test at P < 0.01. (D, E) Mature pollen grains of MS3 (D) and ms3 (E) stained with iodine potassium iodide (I2-KI); bars = 50 μm. (F, G) Scanning electron microscope observation on the mature pollen grains of MS3 (F) and ms3 (G); bars = 25 μm. (H, I) Semithin sections observation on MS3 (H) and ms3 (I) at maturity stage; bars = 50 μm.
five knockout plants with different mutations, resulting in frame-shifts and loss of function of the MS3 gene (Figure S4B–D). Analysis of two independent MS3-KO transgenic T1 lines showed they produced almost no pollen grains or selfing (self-pollination) pods (Figure 2E–N), and that they showed no significant difference in any other measured agronomic traits, except for a small significant difference in plant height for one line (Figure S5A–H). These characteristics are similar to those of the ms3 mutant, which indicated that Glyma.02g107600 is essential for the development of male gametophytes in soybean.

**MS3 encodes a transcription factor with a PHD-finger**

The full-length cDNA of MS3 is 2,975 bp and comprises three exons (Figure S6). MS3 encodes a protein of 657 amino acid residues, forming a PHD-finger domain between residues 600 and 645 (Figure S7). Transient expression experiments indicated that an MS3-green fluorescent protein (GFP) fusion protein was specifically localized to the nucleus in tobacco (*Nicotiana tabacum*) leaf epidermal cells (Figure 3A). A transcriptional activation assay showed that a fusion of MS3 and a DNA binding domain (DB) in yeast activated the expression of reporter genes, implying that MS3 is a transcription factor with strong transcriptional activity. A truncation analysis revealed that residues 600–645 of MS3 are required for its transcriptional activity (Figure 3B). A phylogenetic analysis showed that MS3 is a soybean ortholog of ZmMs7 in maize (*Zea mays*) (Zhang et al., 2018), PTC1 in rice (*Oryza sativa*) (Li et al., 2011), AtMs1 and MMD1/DUET in *Arabidopsis thaliana* (Ito et al., 2007; Yang et al., 2003, 2007), and Ms4 in soybean (Thu et al., 2019) (Figure 3C), indicating that the PHD-finger domains have conserved functions in regulating the development of male gametophytes in monocots and dicots.

**MS3 affects the carbohydrate metabolism**

The main morphological defect of ms3 is male sterility, with no obvious vegetative phenotype suggesting anter-associated expression. Quantitative gene expression analysis in different tissues, including developing floral buds, full flowers, leaf and culms, showed that MS3 was specifically expressed in the early stage of anther formation in both ms3 and wild-type plants grown in Beijing (39°56′N), Changchun (43°50′N) and Jiamusi (46°82′N). Strikingly, the abundance of the ms3 mutant transcript was higher than that of the functional MS3 transcript in wild-type plants at the early stage of anther formation, consistent with the comparison in Changchun (43°50′N) and Jiamusi (46°82′N), respectively (Figure 4A, B).
Most PHD-finger proteins characterized to date regulate gene transcription (Viola and Gonzalez, 2016). It has been reported that PTC1 (OsMS1) and AtMS1 regulate tapetum development and pollen exine formation in rice and Arabidopsis, respectively (Yang et al., 2007; Li et al., 2011), in accord with the cytology we observed for ms3 plants. To identify the potential function of ms3 in regulating the expression of genes involved in tapetum formation during pollen development, we identified the differentially expressed genes (DEGs, |log2 (fold change)| > 0.585, padj < 0.05) between ms3 and MS3 plants at the early stage of B_Ms3_E/B_MS3_E, CL_MS3_E/CL_ms3_E and J_MS3_E/J_ms3_E via high-throughput whole-transcriptome sequencing (RNA-seq). In ms3 as compared to MS3, a total of 11,080, 8,776 and 6,235 up-regulated and 9,796, 8,466 and 6,551 down-regulated DEGs were identified in the three conditions, respectively (Figure S8A–C). We used the Gene Ontology (GO) database to categorize these DEGs, among which were a significant enrichment of genes involved in carbohydrate metabolic process and glycosyltransferase activity (Figure 4C, D; Table S1). A set of Arabidopsis and rice homolog genes associated with sugar metabolism and putatively related to tapetum and pollen exine formation showed altered expression patterns in mutant ms3 anthers (Figure S9; Table S2). These results suggest that the ms3 mutation affects the expression level of genes involved in carbohydrate metabolism.

Long-day photoperiod restores the fertility of ms3 plants

The fertility phenotype of ms3 suggests that the mutant ms3 is capable of fertility restoration. Previous phenotypic observations showed that mutant ms3 produces some selfing pods (3–12) under natural light conditions in Changchun (43°50′ N) late June to late July, which has a day–night time range of 14:50 h/9:10 h–15:25 h/8:35 h (Figure 5C, E). To determine if the fertility recovery ability of mutant ms3 is sensitive to environmental changes, the number of selfing pods identified by cleaved amplified polymorphism sequence (CAPS) markers in different conditions were investigated. The mature stage of ms3 from Jiamusi (46°82′N; diurnal temperature range (DTR), 1°C–14°C), with a day–night time range of 14:25 h/9:35 h–15:45 h/8:15 h produced 8–27 selfing pods
Figure 4. MS3 affects carbohydrate metabolism

(A) The expression level of the MS3 and ms3 genes in different tissues and different stages in Beijing. An, anther; Pi, pistil; Pe, petal; Se, sepals; Cu, culm; Le, leaf; Fl, flowers. The double asterisks represent a significant difference determined by Student’s t-test at $P < 0.01$. (B) The expression level of MS3 and ms3 anthers in different stages in Changchun (43°50′N) and Jiamusi (46°82′N), respectively. CCLLD, Changchun (43°50′N) long-d; JMS, Jiamusi (46°82′N). (C) Venn diagram of differentially expressed genes (DEGs) ($|\log_2 \text{fold change}| > 0.585, \text{padj} < 0.05$) in B_ms3_E vs. B_MS3_E, CL_ms3_E vs. CL_MS3_E, and J_ms3_E vs. J_MS3_E. B, Beijing (39°56′N); CL, Changchun (43°50′N) long-d; J, Jiamusi (46°82′N); E, early stage. (D) Gene Ontology (GO) term of 5,291 overlapping DEGs involved in molecular function, cellular component, and biological process. Red triangle, significantly enriched GO term.
We also conducted a survey of ms3 from Beijing (39°56′ N; DTR, 4℃−11℃), with a day–night time range of 14:15 h/9:15 h–14:55 h/9:05 h, which produced 2–6 selfing pods (Figure 5B, E). In addition, there was no significant difference in fertility phenotypes of MS3 under different latitudes (Figure S10A–E).

To further verify whether the phenotype was photoperiod-sensitive or temperature-sensitive, we grew the mutant ms3 plants under 13:30 h/10:30 h day/night time conditions in the growth chamber, similar to the natural temperature conditions (DTR, 4℃–16℃) in Changchun (43°50′ N), a region in northern China that is suitable for growing the Jilin No. 3 line. The mutant ms3 was completely sterile (Figure 5A, E). We also grew the ms3 mutant plants in an intelligent incubator (RGC-500D, Hefei, China) under various daily temperatures (25°C, 27°C, and 30°C) and a 14:30 h/9:30 h photoperiod.
The mutant again was completely male-sterile, irrespective of the ambient temperature (Figure S11A–C). We also assessed other agronomic traits of the plants under different conditions (Figure S12A–L). Together, these results suggest that the fertility of ms3 can be rescued by long-d photoperiod: although these plants are completely sterile under ≤13:30 h short-d conditions, their fertility can be rescued at ≥15:45 h long-d conditions.

DISCUSSION

Applicability of ms3 in conferring PGMS in two-line system

Compared with the three-line system, the two-line system, that is, P/TGMS, has many advantages for hybrid seed production, as it can be used both to produce hybrid seeds for a male-sterile line and as maintainer lines for self-propagation. A series of P/TGMS systems have been developed and applied to two-line hybrid breeding in crops such as rice and maize (Chen and Liu, 2014). However, no P/TGMS genes have been reported in soybean. Because fluctuating environmental temperatures lead to frequent failure in producing hybrid seeds in TGMS lines in the field, PGMS lines are preferred in hybrid breeding systems due to the relatively stable photoperiod during the period of pollen development within a season (Zhang et al., 1994, 2013). In our study, the degree of ms3 fertility recovery was different under different conditions. Mutant ms3 can be used as maintainer lines under long-d conditions in isolated field conditions (i.e., without other exogenous soybean pollen). Strikingly, based on the selfing pod number of ms3 (ranging 8–27) in Jiamusi (46°82′ N), the yield range was estimated to be 800–2,700 kg/ha with a planting density of 250,000 plants/ha, two-seeded pods and 100-seed weight of 20 g. According to the threshold for fertility recovery, suitable varieties, such as cultivars with a semi-determinate growth habit, the florescence of which can fall within the critical d-length range can be selected to produce more pods. In addition, based on the cross-pollination pod number of ms3 (ranging 11–41) in Jiamusi (46°82′ N) (Figure 5E), the yield range was estimated to be 1,100–4,100 kg/ha. This indicates that mutant ms3 also could be used to produce hybrid seeds, depending on the presence of bees or other insects as pollinators in a net house under short-d conditions. These results suggest that ms3 has great potential for use in the two-line hybrid breeding system.

PHD-finger domain is required for environmental response

The PHD-finger domain containing a conserved zinc-coordinating Cys2-His-Cys1 motif affects growth and development processes (Viola and Gonzalez, 2016). In a prior study, scientists found that ms3 F2 populations display segregation distortion and that male-sterile plants in populations produced some pods, presumably due to environmental factors (Chaudhari and Davis, 1977), but the molecular mechanism and environmental factors underlying these discrepancies remain unknown. In our study, the mutant ms3 from Changchun (43°50′ N) produced a few selfing pods, whereas the mutant ms3 from Jiamusi (46°82′ N), a more northerly latitude, produced a larger number of selfing pods. The transgenic experiment showed that no selfing pods were produced in MS3-KO transgenic plants from Changchun (43°50′ N), in which the PHD-finger domain in the MS3 protein is completely deleted. The spontaneous mutation of ms3, which contains a 17-amino acid deletion in the PHD-finger domain (whereby Cys3-His-Cys3 is replaced by Cys4-His-Cys), can restore the fertility of male-sterile plant lines under long-d conditions. Likewise, analysis of rice TMS9-1 proteins showed that the conversion of Leu to Phe in the region encoding the PHD-finger resulted in thermo-sensitive GMS in the traditional TGMS line HengnongS-1 (Li et al., 2011; Qi et al., 2014). These results indicate that the PHD-finger domain is required for the environmental response.

Environmental regulation of fertility conversion during pollen wall development is conserved in monocotyledons and dicotyledons

Previous studies have demonstrated that the transcription factors MS188 and MS1 participate in a genetic pathway that is important for tapetal development and pollen formation in rice and Arabidopsis (Shi et al., 2015). MS188 directly regulates the expression of RES2/QT3, which restores the fertility of the typical P/TGMS line rvms-2 (Shi et al., 2021). Similarly, other Arabidopsis P/TGMS mutants, such as acos5-2, cyp703a2, rnu-2, rpg1 and cals5-2, which contain mutations in genes required for pollen wall development, exhibit fertility restoration under low temperature or short-day photoperiod conditions (Zhang et al., 2020; Zhu et al., 2020). The rice thermo-sensitive GMS gene tms9-1 (PTC1), a homolog of MS1 in Arabidopsis and MS3 in soybean, is a candidate gene in the traditional TGMS line HengnongS-1. In our study, we prove that ms3 is an rpgms (reverse-photoperiod-sensitive GMS) mutant. These studies and the evidence provided here indicate that environmental regulation of fertility conversion during pollen wall development is conserved in monocotyledons and dicotyledons.

MATERIALS AND METHODS

Plant materials

The mutant ms3 (Glycine max) was used as a female parent in a cross with Jilin No. 3 as a male parent, and then the sterile F1 lines were selected to backcross with Jilin No. 3 four times successively and self-pollinated to generate the ms3BCF2 and ms3BCF3 genetic segregated populations. All materials were grown in experiment stations in Sanya (18°25′ N), Beijing (39°56′ N), Changchun (43°50′ N), and Jiamusi (46°82′ N). Plants were photographed by a Canon EOS 700D digital camera (Canon, Japan).

Histological analysis

Ten individual MS3 plants and 10 individual ms3 mutant plants were examined to determine pollen fertility. Five
MS3 involved in male fertility in soybean

Mature buds of each plant were collected 1-2 d before flowering. The pollen from one mature bud from each plant was mixed and stained by 1% (w/v) I2-KI solution, and three views were photographed by a charge-coupled device microscope (Olympus, Japan) with at least 50 pollen grains in each view. For the pollen scanning electron microscopy (SEM) and semithin section observation, the fresh anthers of MS3 and ms3 mutants at mature stage were immersed in 2.5% glutaraldehyde solution (5:1:4 (v/v/v) mixture of 0.2 mol/L phosphate buffer/25% glutaraldehyde/ddH2O) overnight.

DNA extraction, BSA and restriction endonuclease digestion
Genomic DNA was extracted from fresh leaves of each plant of the mapping populations, as well as from transgenic plants, using the cetyltrimethylammonium bromide method described by Doyle and Doyle (1990).

In the F2 population, DNAs of 12 individuals each from the sterile and fertile populations, selected randomly, were mixed in equal amounts to form the sterile and fertile bulk, respectively. Single nucleotide polymorphism and insertion/deletion (InDel) markers from the 20 soybean chromosomes were used to test the polymorphism of the parents and bulks. The MS Hiper Plus Taq HiFi Polymerase Chain Reaction (PCR) Mix (without dye) (Mei5 Biotechnology Co., Ltd) was used for PCR of marker MS3-CAPS according to the manufacturer’s instructions. The PCR was performed under the following conditions: (1) 94°C for 3 min; (2) 35 cycles of 94°C for 250 s, 55°C for 25 s and 72°C 10 s; and finally (3) 72°C for 5 min. The PCR products were digested with the restriction enzymes Ava I (New England Biolabs, Beijing, China) following the manufacturer’s instructions and the digests were examined via 5% agarose gel electrophoresis and stained with nucleic acid dye, respectively.

Vector construction and plant transformation
A CRISPR/Cas9 plasmid was constructed by introducing two knockout targets with 19-bp fragments in the MS3 coding region (5′-GGGTGAGGCTGGAAGTTGGCA-3′ and 5′-GACCCGCCCTCTCTGGTATG-3′) into pYLCRISPR/Cas9 (Ma et al., 2015), and was designated MS3-KNOCKOUT. The construct was introduced into Agrobacterium tumefaciens strain EHA105 and subsequently MS3-KNOCKOUT transferred into the Williams 82 soybean cultivar. Phenotypic measurements were conducted in transgenic plants from the T1 to T2 generations.

Reverse-transcription quantitative PCR (RT-qPCR) and 3′-RACE
The soybean tissues, including anthers, pistils, petals and sepals from the early, middle and late stages of buds, as well as flowers, leaves and culms, were harvested for RNA isolation by using RNA extraction and purification kits (GBTbiotech, Beijing, China), and the RT reaction was performed using an RT system (GenStar, China) following the manufacturer’s protocol. Additionally, RT-qPCR was performed using GenStar Green Fast Mixture with ROX II (GenStar, China) according to the manufacturer’s instructions on the ABI Prism 7500 Software (ABI, USA) in triplicate. The amplification of ACTIN was used as the internal control. Three replicates were performed for each experiment, the data was analyzed by the (2-ΔΔCT) method, and quantitative results were given as means ± SD (Livak and Schmittgen, 2001). 3′-RACE was performed using a 3′-Full RACE Core Set (Takara, Japan) following the manufacturer’s instructions.

Subcellular localization
The full-length cDNA of the MS3 and ms3 alleles was amplified and fused in-frame with the GFP coding sequence and then cloned into the vector via combinational joining. The resulting construct was used to transform tobacco (Nicotiana tabacum) leaves. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The GFP and DAPI fluorescent signals from tobacco leaf tissue were recorded with a Zeiss confocal laser scanning microscope (Zeiss, Germany).

Phylogenetic analysis of MS3
A neighbor-joining tree was reconstructed using the MEGA6 program (Tamura et al., 2013). For phylogenetic analysis, orthologs of MS3 were identified in the Arabidopsis, rice, maize and soybean reference genomes.

Identification of DEGs by RNA-seq
Total RNA was isolated from the anther of MS3 and mutant ms3, with three biological replicates each containing five plants. Paired-end libraries were constructed and sequenced using an Illumina sequencing kit (Illumina, USA). The raw reads were mapped to the reference genome using TopHat2 with the default parameters (Kim et al., 2013). Cuffdiff was used to calculate the fragments per kilobase of exon per million mapped reads of each gene and to identify the DEGs between MS3 and mutant ms3 (Trapnell et al., 2010). Heat map and GO terms were generated by TBtools (Chen et al., 2020).

Primers
The primers used in this study are listed in Table S3.

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CONFLICTS OF INTEREST
The authors declare they have no conflicts of interest associated with this work.
J.H. performed the experiments, analyzed the data, and wrote the paper. W.F., R.M., B.L., Z.Y., W.H., Y.W., Q.H., X.Z., C.L., and B.P. helped perform the experiments. L.Z. and C.Z. provided the plant strains. C.Z. identified the phenotypes and revised the paper. L.S. designed the research and revised the paper. All authors read and approved of this manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.13242/suppinfo

Figure S1. Comparison of plant phenotype and pod number between MS3 and ms3 in Sanya (18°25′N)

Figure S2. Comparison of agronomic traits between MS3 and ms3 in Changchun (43°50′N) under long-d conditions and Sanya (18°25′N)

Figure S3. Development of the ms3 locus-containing backcross segregation population

Figure S4. Genotyping between two independent transgenic lines and the corresponding controls

Figure S5. Comparison of other agronomic traits between two independent transgenic lines and the corresponding controls

Figure S6. Coding sequences of MS3 and ms3

Figure S7. Amino acid variations between MALE STERILITY 3 (MS3) and ms3

Figure S8. Transcriptome analysis of MS3 and ms3 in three conditions

Figure S9. The expression of genes related to sugar metabolism in anthers in different conditions

Figure S10. The fertility phenotypes of MS3 under different photoperiod conditions

Figure S11. The mature pollen grains of MS3 and ms3 stained with iodine potassium iodide at different temperatures

Figure S12. Comparison of agronomic traits between MS3 and ms3 in Changchun (43°50′N) under short-d conditions, Beijing (39°56′N) and Jiamusi (46°82′N)

Table S1. Gene Ontology (GO) term analysis of 5,291 overlapping differentially expressed genes (DEGs) in B_ms3_E vs. B_MS3_E, CL_ms3_E vs. CL_MS3_E and J_ms3_E vs. J_MS3_E

Table S2. The expression of differentially expressed genes (DEGs) related to sugar metabolism in B_ms3_E vs. B_MS3_E, CL_ms3_E vs. CL_MS3_E and J_ms3_E vs. J_MS3_E, respectively

Table S3. Primers used in this study