Identification of the First Nuclear Male Sterility Gene (Male-sterile 9) in Sorghum

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ABSTRACT Nuclear male sterility (NMS) is important for understanding microspore development and could facilitate the development of new strategies to control male sterility. Several NMS lines and mutants have been reported in sorghum [Sorghum bicolor (L.) Moench] previously. However, no male-sterile gene has been identified, hampering the utility of NMS in sorghum breeding. In this study, we characterized a new NMS mutant, male sterile 9 (ms9), which is distinct from all other reported NMS loci. The ms9 mutant is stable under a variety of environmental conditions. Homozygous ms9 plants produced normal ovaries but small pale-colored anthers that contained no pollen grains. Microscopic analyses revealed abnormal microspore development of ms9 at the midmicrospore stage, causing degeneration of microspore inside the anther lobes and male sterility of ms9 plants. Using MutMap, we identified the Ms9 gene as a plant homeotic domain (PHD)-finger transcription factor similar to Ms1 in Arabidopsis thaliana (L.) Heynh. and Ptc1 in rice (Oryza sativa L.). Ms9 is the first NMS gene identified in sorghum. Thus, the Ms9 gene and ms9 mutant provide new genetic tools for studying pollen development and controlling male sterility in sorghum.

Abbreviations: CMS, cytoplasmic male sterility; LBK, USDA–ARS Cropping Systems Research Laboratory in Lubbock, TX; NMS, nuclear male sterility; PHD, plant homeotic domain; PR, winter nursery farm in Guayanilla, PR; SEM, scanning electron microscopy; WT, wild-type.

CORE IDEAS
- The male-sterile 9 (ms9) is a novel nuclear male-sterile mutant in sorghum.
- The Ms9 gene encodes a PHD-finger transcription factor critical for pollen development.
- The identification of the Ms9 gene provides a strategy to control male sterility in sorghum.

Sorghum is a major grain crop used for human consumption and animal feed as well as a promising bioenergy crop for sugar, biomass, and biofuel production (de Siqueira Ferreira et al., 2013; Rooney et al., 2007). Like maize (Zea mays L.), sorghum is a diploid C4 plant that, in contrast to maize, is well adapted to drought-prone and high-temperature environments and can thrive on marginal soils (Morris et al., 2013). Sorghum also has a much smaller genome than maize (~800 vs. 2500 Mb), and after the recent completion of a high-quality diploid genome sequence, sorghum has become an emerging model for highly productive C4 crops (McCormick et al., 2018; Paterson et al., 2009).

Heterosis, or hybrid vigor, the ability of hybrids to outperform the best inbred line parents is probably the most important strategy to increase grain yield in many crops including sorghum (Kim and Zhang, 2018). A critical requirement of using hybrids to increase yield is the ability to produce a pure male-sterile female parent that...
can be used for production large quantities of hybrid seeds by cross-pollination with a fertile male parent. At present, cytoplasmic male sterility (CMS) is the predominant strategy to produce male-sterile parents in most crops for which mass production of hybrid seed is possible. Most grain sorghum varieties currently used in agricultural production are hybrids. The A1 CMS reported in the 1950s (Stephens and Holland, 1954) remains the predominant CMS line used for producing hybrids in sorghum, although other types of CMS lines are available (Jordan et al., 2011). The CMS breeding system is a three-line system in which the CMS female parent A line is pollinated by a maintainer B line to maintain cytoplasmic male sterility of the A line, or by a fertility restorer R line to produce a fertile hybrid (Rooney, 2004). The three-line breeding system requires a perfect B line for each A–B pair to maintain the absolute male sterility of the A line. It also requires a perfect R line that can fully restore the fertility of the hybrid plants by complementing the cytoplasmic defect of the A line (Jordan et al., 2011). Simultaneous development of all three lines is time consuming, expensive, and complicated. Moreover, a significant amount of sorghum germplasm, especially bioenergy sorghums, is neither characteristic B line nor R line, and therefore, does not meet the strict requirements for the three-line sorghum hybrid breeding system. The limitation in the number of accessions that can be used in hybrid breeding prevents sorghum breeders from creating all possible hybrids to exploit heterosis. Furthermore, since almost all current commercial grain sorghum hybrids are produced using the A1 CMS, the homogeneity of A1 cytoplasm could predispose sorghum hybrids prone to severe diseases and other biotic and abiotic stresses. One example was the widespread use of the T-cytoplasm maize in 1960s and in early 1970s. The devastating epidemic of southern corn leaf blight disease caused by race T of Bipolaris maydis caused over 80% yield loss in maize production in 1970 (Ullstrup, 1972). To avoid catastrophic yield losses similar to those that occurred in T-cytoplasm maize, it is necessary to develop new sorghum hybrids by using a variety of sources of male sterility.

Recently, two-line hybrid breeding systems using NMS have been explored in rice (Chang et al., 2016). The two-line breeding system not only simplifies the hybrid seed production procedure but also dramatically expands the possibilities for making hybrids between accessions that cannot be generated with the current three-line breeding system (Dong et al., 2000; Zhang et al., 2010, 2013). Furthermore, hybrids made with the two-line breeding system have a 5 to 10% higher grain yield over similar hybrids produced using a CMS three-line breeding system (Chang et al., 2016; Kim and Zhang, 2018; Zhou et al., 2014). Therefore, the two-line breeding system for hybrid production of major crops holds great potential for increasing future agricultural production. The identification of nuclear genes involved in regulating male sterility in crops is the first and most essential step in the development of a two-line breeding system.

In sorghum, eight NMS lines, ms1 through ms8, and msal (antherless) have been reported (Andrews and Webster, 1971; Xin et al., 2017) previously. The ms4, ms5, and ms6 lines are no longer available, but the other lines have been preserved and introduced into different genetic backgrounds (Pedersen and Toy, 2001). These NMS lines could make important contributions to the elucidation of male gametophyte development and aid in the development of new breeding systems for making sorghum hybrids. However, to date, none of the genes that mediate male sterility in these lines has been identified, which greatly hinders the potential of using NMS in sorghum breeding.

In this study, we reported the characterization of a new NMS mutant isolated from an ethyl methane sulfonylate–mutagenized mutant population in the sorghum inbred line BTx623 (Jiao et al., 2016; Xin et al., 2009) and identification of the first NMS gene in sorghum. Since the result of our genetic study indicated that the ms mutant represents a new locus for NMS, we named it as male sterile 9 (ms9) and the casual gene as Ms9. Morphological analyses of ms9 flowers and spikelets revealed that the ovary of ms9 develops normally and sets seed after manual pollination with wild-type (WT) pollen. Detailed light and electron microscopic analyses revealed that the male-sterile phenotype in ms9 is caused by a defect in pollen development that occurs at microspore stage in the antith. The microspores inside the developing anthers begin to degenerate prematurely at the midvacuolated stage, leading to small, pale-colored anthers with no pollen grains at anthesis. The causal gene mutation for male sterility in ms9, identified using MutMap, encodes a PHD-finger transcription factor critical for tapetum degeneration and pollen formation. The Ms9 gene and its mutants represent new genetic tools for dissecting pollen development and controlling male sterility in sorghum.

MATERIALS AND METHODS

Growing Condition and Management

All plant materials used in this study were planted annually on the research farm of the USDA–ARS Cropping Systems Research Laboratory in Lubbock (LBK), TX (33° 35’ N, 101° 53’ W, 958 m asl), during the normal growing season (May–September) and in a winter nursery farm in Guayanilla, PR (PR) (18.0373° N, 66.7963° W, 49 m asl) during the winter months (December–April). Soil type was Amarillo (fine-loamy, mixed, superactive, thermic Aridic Paleustalfs) fine sandy loam at LBK and Caguabo (loamy, mixed, active, isohyperthermic, shallow Typic Eutrudepts) well-drained loamy soil at PR. The plot size at both locations was 4.6 m long. For genetic analysis, the mutant and F1 seeds were planted in a single plot, and the F2 seeds (regenerating population) were planted in eight plots. Approximately 60 sorghum seeds were planted per plot using a John Deere MaxEmerge planter at a planting depth of 3 cm. Plots were furrow irrigated 1 wk before planting to provide saturated soil moisture for optimal seed germination. Two weeks after germination, the field
was irrigated as needed by an automated subsurface drip system at LBK and surface drip lines at PR. Routine farm practices were applied with regard to fertilizer, pest control, and other field management.

The environmental conditions at the two field locations differed greatly. The weather during the summer growth season at LBK is typically hot and dry with sporadic occurrences of heat-wave events (Supplemental Fig. S1) and relative low humidity (~50% on average), whereas the tropical weathers in the winter nursery in PR are mild with high humidity (~75% on average) and much less temperature fluctuation (Supplemental Fig. S1). The photoperiod (from sun rise to sun set) in winter times in PR is short day with an average daylength of 12 h, while the photoperiod during the summer growth season at LBK is long day (13.5–14.3 h). The greenhouse test was conducted throughout of the year at LBK with temperatures set at 28°C during the day and 23°C at night and a 14 h light–10 h dark cycle with ambient light and supplemental light.

Isolation of ms9 Mutant

The ms9 mutant was identified in 2015 at LBK from an M4 mutant line, M2P0110 (ARS178). This mutant was one of the 256 sorghum mutant lines that were sequenced and annotated for gene mutations (Jiao et al., 2016). After identifying potential sterility phenotype of the main shoot at anthesis, flowered panicle of that particular mutant plant was bagged immediately, and a line below the flowering zone was marked on the sorghum bag to indicate the separation of the open-pollinated part of the panicle from the self-pollinated part. The seed set of the main panicle was examined 2 wk after bagging. Sterility on the main panicle was confirmed by having seed set in the open-pollinated zone and the absence of seed set in the part that flowered after being bagged. Approximately 10 d after the main panicle flowered, panicles from two tillers were bagged prior to anthesis. One tiller panicle was bagged for 45 d until harvest and set no seed. The other bagged tiller panicle was checked daily for flowering, pollinated with inbred line BTx623 WT pollen on the third day of anthesis (~50% of the sessile spikelets flowered), and continually bagged for 45 d after pollination. Additionally, another independent cross was made using a tiller panicle from a different sterile plant identified from the same plot as M2P0110. The seeds produced (F1) from the two independent ms plants crossed by the original WT inbred line BTx623 were harvested at maturation and used for genetic analysis.

Genetic Analysis of the Male Sterile Phenotype of the ms9 Mutant

The generations notations used for the genetic analysis of ms9 in this study was in accordance with the standard breeding terms. The F1 seeds refer to the seeds produced on the original ms mutant plants crossed by the WT BTx623 pollen. The F1 plants produced F2 seeds through self-fertilization. The BC1F1 seeds were those produced on male-sterile plant in the F2 segregating population pollinated with BTx623. Self-fertilization of BC1F1 plants produced BC1F2 seeds. BC2F1 and BC2F2 were produced by pollination of male-sterile BC1F2 by BTx623 pollen and self-fertilization of BC2F1, respectively.

For genetic analysis, both the F1 and open-pollinated seeds, along with the same M4 mutant seeds, from which the ms plant was identified, were planted in the PR winter nursery in 2015 to examine the nature of genetic control of male sterility in the F1 generation and to confirm the sterility phenotype in the same M4 line. The F1 plants were self-fertilized, the resultant F2 seeds were planted at LBK in the 2016 growing season. Segregation for fertile and sterile phenotypes was analyzed in two F2 populations. We used three approaches to further examine the nature of the sterility in the F1 generation. First, at anthesis, we marked a subset of F2 sterile plants and let them open-pollinate in the field for 1, 2, or 3 d before bagging the panicles. Second, we bagged another subset of F2 panicles prior to anthesis to produce completely self-pollinated panicles. Third, on the first day of anthesis, we bagged the ms panicle after removing the top, manually pollinated it with BTx623 pollen 4 d later, and then bagged it again until maturation to check the seed set. Seeds produced from two manually pollinated F2ms plants in the third approach (BC1F1 seeds) were harvested and planted in PR in the winter of 2016 to produce self-pollinated BC1F2 seeds. The BC1F2ms was backcrossed again with BTx623 pollen in the 2017 season at LBK, and the resultant BC2F2 were grown at PR in the winter and self-pollinated to produce the BC2F2 seeds. The field-grown BC2F2 fertile and sterile plants as well as the BTx623 WT plants were used for phenotypical and morphological characterization of the M2P0110ms mutant in 2018.

To determine the genetic relationship of ms9 to previous reported male-sterile lines in sorghum, we manually pollinated the male-sterile plants from other available NMS lines (ms1, ms2, ms3, ms7, msal, and ms8) with pollen collected from the heterozygous Ms9 fertile BC2F1 plants. The resulting complement-crossing F1 seeds (cpF1) were planted in the field and then subsequently examined for the male sterility phenotype of cpF1 plants during flowering time. If ms9 were allelic to any of these NMS lines, we would expect to observe a 1:1 fertile to sterile segregation ratio among the cpF1 progenies. For an NMS locus that is distinct from Ms9, we would expect all cpF1 plants to be fertile.

Examination of Female Fertility

The development and fertility of the female floral organs of the BC2F2 of ms9 plants were examined and compared with those of BTx623 WT plants. Specifically, on the day of flowering, ovaries from these plants were dissected from freshly flowered spikelets and compared morphologically. The top flowering parts of BTx623 and ms9 BC2F2ms panicles were removed, and the remaining nonflowering parts of the panicle were bagged. The next day, ~4 h into the photoperiod, the flowering zones of the bagged panicles were marked for both BTx623 and ms9 panicles and were
manually pollinated with BTx623 pollen. This process was repeated one more time on the following day. The development of ovaries from spikelets of the pollinated zones at 0 and 4 d after pollination was examined and photographed using a Leica MZ6 stereomicroscope equipped with a Leica Fire Cam program (Microsystems Inc.).

Examination of Anther and Pollen Development by Staining
Pollen viability was examined by alexander staining method as described previously (Huang et al., 2016; Zhao et al., 2002). Briefly, spikelets containing anthers were excised from the sorghum panicle prior to dehiscence and immediately submerged into Carnoy’s fixative solution in glass tubes (60 mL ethanol; 30 mL chloroform; 10 mL acetic acid). The tissues were vacuum-infiltrated for 15 min and fixed overnight at room temperature. Anthers from the fixed spikelet tissues were excised, rinsed in a series of increasing percentage ethanol solutions, placed in Alexander staining buffer (Xin et al., 2017), and incubated at 37°C for 2 h. The stained anthers were rinsed several times with staining solution without dye to remove the free dye and then mounted on a glass slide. The morphological characteristics of the stained anthers and pollen grains were examined and photographed using an Olympus BX60 microscope equipped with an Olympus DP 80 digital camera.

Light and Scanning Electron Microscopic Analyses of Anther Development
Cross-sections of flowers and individual anthers were obtained by paraffin embedding and CRYO-scanning electron microscopy (SEM) procedures. Whole-flower samples collected at early developmental stages (prior to booting) were fixed in FAA (contains 95% ethanol, water, 37% formaldehyde, and acetic acid at a ratio of 50:35:10:5) at room temperature for 24 h. The fixed samples were stored for 1 wk at 4°C, followed by dehydration at room temperature in an ethanol concentration series (from 30 to 100%), increasing concentration by 10% per hour, and then by 1 h incubation in 100% xylene. The total dehydration time was 24 to 30 h depending on the tissue stage. Dehydrated flower tissues were infiltrated (12–24 h) and embedded in 100% Paraplast Plus (Sigma-Aldrich) at 56 to 60°C. Wax blocks were cut into 5- to 10-μm sections using a Leica RM2125 RTS (Leica Biosystems) manual rotatory microtome. Slides containing sections were submerged three times in 100% xylene for 5 min followed by dehydration in a series of ethanol solutions (100% two times, and 95, 80, and 70% for 3 min each). Cross-sections were then stained with 0.1% toluidine blue or 0.01% Safranin O solution. Flower and pollen images were photographed and analyzed at 10 to 40× magnification on a Olympus BX60 microscope equipped with an Olympus DP 80 digital camera.

For SEM analysis, the fresh anthers dissected from sorghum spikelets were processed and analyzed according to the CRYO-SEM procedures described in Tsou et al. (2015). Surface and cross-section images of anther and pollen were analyzed and photographed at 100×, 400×, and 800× magnifications using a Hitachi S-4300 SEM (Hitachi America).

Whole Genome Sequencing and Identification of Ms9 Gene
MutMap, which is based on whole-genome sequencing data of bulked F₂ segregants, was used to identify the causal mutation in the ms9 mutant (Abe et al., 2012; Jiao et al., 2018). Leaf tissues from 40 confirmed back-crossed F₂ms mutants were sampled and used to isolate genomic DNA (gDNA) as described previously (Xin and Chen, 2012). Equal amounts of gDNA from each of the 40 samples were pooled, quality-checked, and subjected to 150-bp paired-end sequencing on an Illumina X-10 instrument (https://en.novogene.com). Whole-genome sequencing was performed using three bulked F₂pools: two BC1F2ms pools (A and B) for the two backcrosses made on two sterile plants in 2015 and one BC2F2ms pool containing equal amounts of gDNA of 20 BC2F2ms plants generated from each of the BC1F2ms A and B populations. For each of the F₂ pools, we obtained ~10 Gb of high-quality sequence data, corresponding to ~15× coverage of the whole genome. Sorghum reference genome version 2 was used for all sequencing data analyses. Standards, programs, database and processes used for bioinformatic analyses of the whole-genome sequencing data were essentially as described previously (Jiao et al., 2018). After identification of the candidate causal mutations for each of the BC1F2ms A and B pools, the candidate mutations from two F₂ populations were compared to determine the overlap of the mutations. The candidate mutations identified for the combined BC2F2ms pool were also compared with those identified from the BC1F2ms pools. The bulked segregant analysis sequencing data has been deposited to NCBI sequence read archive database under accession number of SRP183026 (https://www.ncbi.nlm.nih.gov/sra/?term=SRP183026).

The orthologs of the Ms9 gene were obtained from the Gramene database (http://ensembl.granome.org/Sorghum_bicolor/Gene/Compara_Ortholog?g=SORBI_3002G234700;r=2:62572601–62576927;t=KXG35825). The phylogenetic relationship of several selected homologous genes of Ms9 was constructed using Mega 7 (Kumar et al., 2016) with ClustalW alignment and the maximum likelihood method (Tello-Ruiz et al., 2016). Multiple alignments from ClustalW were visualized with MSView (Yachdav et al., 2016).

RESULTS

Morphological Characterization of the ms9 Mutant
In WT BTx623 plants, the first visible sign of anthesis is the protrusion of yellow anthers from the sessile spikelets on the top of panicles (Fig. 1A). The female stigmas of WT plants were only transiently and partially visible because they are overshadowed by the large fluffy yellow
and became withered following pollination (Fig. 1A,C). For *ms9* plants, however, anthers extruded from the sessile spikelets were pale in color, shed no pollen, and were much flatter and thinner than those of WT plants (Fig. 1B,D). Additionally, the white hairy stigmas of *ms9* that emerged from the spikelet were longer, fluffer, and much more visible (Fig. 1D,F) than those of WT plants (Fig. 1C,E). The small pale anthers and fluffy white hairy stigmas of *ms9* made it very easy to identify the sterile plants at a very early stage of anthesis (Fig. 1B). Overall, other than the sterile phenotype, the development and morphological structure of *ms9* sessile spikelets were similar to those of BTx623 (Fig. 1E,F). No noticeable morphological or developmental differences in any other parts of the sorghum floral tissues were observed between the *ms9* mutant and WT plants (Fig. 1).

**Female Fertility is Normal in the *ms9* Mutant**

To test for defects in female organs, we examined ovary development and seed set of *ms9* plants by manual pollination of *ms9* panicles with BTx623 pollen. Dissection of sorghum sessile spikelets just prior to anther extrusion revealed that female organs in *ms9* sessile spikelets had normal structures (Fig. 1G). On the day of flowering, we observed no differences between *ms9* and BTx623 in stigma, style, or ovary size and appearance (Fig. 1H,I), indicating that development of female organs was normal in mutant plants. The only difference observed was the absence of pollen grains on *ms9* stigmas (Fig. 1H). In addition, we examined the development of ovaries before and 4 d after pollination and found that the pollinated ovaries of *ms9* developed similarly to those of BTx623 plants (Fig. 1J,K). Moreover, the ovaries of *ms9* sessile spikelets were able to complete fertilization processes and produce normal seeds after pollination with WT pollen, providing further evidence of normal development. Examination of seed set, seed size, and seed morphology of manually pollinated *ms9* panicles revealed normal seed set on pollinated parts (Fig. 2A–D), no seed set on nonpollinated parts (Fig. 2A–C), and no seed set on the bagged, self-pollinated *ms9* panicles (Fig. 2F and 2G) compared with the normal seed set of the self-pollinated WT BTx623 (Fig. 2E). These results indicated that the development of female reproductive organs and female fertility were not affected by the *ms9* mutation. Therefore, we concluded that the *ms9* mutation only affected male sterility, the phenotypes shown in Fig. 1B, 1D, and 1F.

**The Sterile Phenotype of *ms9* Mutant is a Result of Male Sterility**

The normal seed set on manually pollinated and cross-pollinated (not-bagged) BC1F2 *ms9* panicles (Fig. 2A–D), in contrast to the lack of seed set on bagged pollinated parts (Fig. 2A–C), and no seed set on the bagged, self-pollinated *ms9* panicles (Fig. 2F and 2G) compared with the normal seed set of the self-pollinated WT BTx623 (Fig. 2E). These results indicated that the development of female reproductive organs and female fertility were not affected by the *ms9* mutation. Therefore, we concluded that the *ms9* mutation only affected male sterility, the phenotypes shown in Fig. 1B, 1D, and 1F.
temperature and humid PR field site, or under long-day and optimal temperature greenhouse conditions. These results indicated that the male sterility of ms9 is stable under variation in day length and temperature conditions, and that the mutation is fully penetrant.

The ms9 Anthers Lack Mature Pollen as a Result of Abnormal Microspore Development

Morphologically, the three extruding anthers of the sessile spikelets in ms9 mutant panicle were pale in color and much thinner than the plump yellow anthers on BTx623 panicles (Fig. 1). At anthesis, the ms9 anthers dispersed no pollen (Fig. 1D), and therefore the two hairy stigmas of ms9 were free of pollen grains (Fig. 1F, H) in contrast to the two pollen-receiving hairy stigmas observed on BTx623 sessile spikelets (Fig. 1E, I). To investigate the cause of male sterility in the ms9 mutant, we examined pollen production and pollen viability in mature anthers of sessile spikelets prior to their extrusion. The BTx623 anther lobes were full of round, reddish, mature pollen grains (Fig. 3A, C), whereas the anther lobes of ms9 mutants were empty (Fig. 3B). A microscopic close-up of the ms9 anther showed no sign of mature pollen being released (Fig. 3D). We conclude that the lack of mature pollen formation is the underlying cause for male sterility in ms9.

To determine the cause of failed pollen production in the ms9 mutant, we examined and compared

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**Fig. 2.** Seed set phenotypes of BTx623 and ms9 plants grown under field conditions. (A to D) Seed sets of the ms9 panicles manually pollinated with BTx623 pollens in the morning of the (A) first, (B) second, and (C) third day, and (D) at completion of anthesis showing normal seed set of ms9 panicles after manual pollination and (A–C) the absence of seed set after the panicles were bagged for self-pollination compared with (E) a self-pollinated BTx623 panicle and (F) the absence of seed set after the panicle was bagged for self-pollination. (F) An ms9 panicle bagged prior to anthesis. (G) An ms9 panicle bagged after removing the top open flowered spikelets during anthesis. The self-pollinated BTx623 wild-type panicle set normal seeds (E), whereas the self-pollinated ms9 panicles (F and G) produced no seeds.

**Fig. 3.** Morphological analyses of anther and pollen development in BTx623 wild-type and ms9 mutant plants. Alexander staining of anthers and pollen of (A, C) BTx623 and (B, D) ms9. The BTx623 anther contains plenty round pollen grains (A), and the mature pollens are viable (C, viable tissues are stained in reddish-pink in color), whereas the ms9 anther is visibly empty (B) and contains no pollen grains (D).
morphological features of the anthers at different developmental stages by histological and SEM analyses. We identified no morphological differences in the lobule anthers at or prior to the microspore midvacuolated stages (Fig. 4A,B,E,F). The four distinct layers of anther wall, epidermis, endothecium, middle layer, and tapetum formed normally in ms9 anthers (Fig. 4E,F). While the tapetum of WT anther started to degenerate during microspore stages (Fig. 4C,I,J), the tapetum of ms9 anther remained clearly noticeable (Fig. 4G,M,N). In addition, analyses revealed that the microspores of the ms9 plants started to degenerate prematurely at midmicrospore stage and the microspores became further shriveled (Fig. 4N) at the late-vacuolated stage, whereas the microspores of the WT BTx623 increased in size and became spherical (Fig. 4J). At this stage, the epidermal layers of both the WT and the mutant anthers were concentrically organized and the middle layer became imperceptible (Fig. 4C,G,J,N). In ms9, however, the tapetum layer was still largely noticeable (Fig. 4G,N) whereas the microspores shriveled (Fig. 4N). During the pollen maturation stages, WT pollen became round, and the tapetum and endothecium layers degenerated completely (Fig. 4D,L). By contrast, the microspores in ms9 anther lobes degenerated completely, resulting in little or no cell debris within the empty anthers at pollen maturation stage (Fig. 4H,P). These results indicate that mutation in ms9 hinders the normal degeneration process of tapetal cells of the anther wall layer and causes abnormal development of microspores at the midmicrospore developmental stage, resulting in degeneration of microspores inside the anther lobes.

**ms9 is a Novel Recessive Nuclear Male Sterility Mutant**

Several lines of genetic evidence revealed in this study indicated that the male sterility of ms9 was caused by a recessive mutation on a single nuclear gene. The F1 plants produced by manual pollination of the ms9 mutant with BTx623 pollen were all male-fertile and set seeds, indicating that the ms9 mutation is recessive (Table 1). Phenotyping of F1 plants derived from self-pollinated F1 progeny identified 161 fertile plants and 43 male-sterile plants, reflecting a segregation ratio of approximately 3:1. Phenotyping of the subsequent advanced backcrossing F2...
populations (BC1F2 and BC2F2) yielded similar ratios of fertile to sterile plants (Table 1). This segregation analysis indicated that the male sterility in ms9 is caused by a recessive mutation in a single nuclear gene.

We then performed complementation analyses to determine whether ms9 is allelic to any previously reported sorghum NMS mutants (Pedersen and Toy, 2001; Xin et al., 2018). Phenotyping results revealed that all cpF1 plants from the complementation crosses between male-sterile plants of known NMS lines and heterozygous BC2F1 ms9 plants were fertile (Table 1). These findings demonstrated that the ms9 is not allelic to any of the currently available sorghum NMS mutants and that Ms9 is most likely a new male-sterile locus.

**Ms9 Encodes a Plant Homeotic Domain–Finger Transcription Factor**

We used a modified MutMap approach (Jiao et al., 2018) to identify the causal gene mutation that leads to male sterility in ms9. Specifically, we performed whole-genome sequencing of two bulked F2 populations independently generated from two different original crosses, each containing 40 homozygous F2 ms9 mutants. Bioinformatic analysis of the whole-genome sequencing datasets using our in-house pipeline (Jiao et al., 2018) identified homozygous nonsynonymous mutations in nine genes in the first population and five genes in the second population, only two of which overlapped (Fig. 5A). Analysis of the two combined BC2F2 ms9 whole-genome sequencing datasets identified homozygous nonsynonymous mutations in six genes, including mutations in the same two overlapping genes (Fig. 5A). Therefore, the number of causal gene mutations for male sterility in ms9 was reduced to the two located on chromosome 2. One is a deleterious mutation located in Sobic.002G221000 gene encoding a PHD-finger transcription factor. A C-to-T transition at nucleotide 1207 in Sobic.002G221000 gene in ms9 causes an amino acid change from arginine to tryptophan at conserved position 218 (R218W, Fig. 5B). The other mutation located in Sobic.002G234700 gene encoding an ubiquitin carboxyl-terminal hydrolase family protein. A C-to-T transition in Sobic.002G234700 causes an amino acid changes from proline to leucine at position 98 (P98L). The M4 mutant line (ARS178) from which the ms9 mutant was isolated has already been sequenced and annotated for gene mutations (Jiao et al., 2016). Therefore, we searched the mutant database and confirmed that the ARS178 M4 mutant line contained the same nonsynonymous mutations (but in the heterozygous state) in the two candidate genes identified by MutMap.

We employed four approaches to determine which of the two gene mutations is responsible for the male-sterile phenotype in ms9. First, we mined the mutation database of our 256 sequenced mutant lines to identify mutant lines that contained independent mutation alleles in one of the two candidate genes to determine whether they segregated a male-sterile phenotype. One of the two M4 lines harboring heterozygous mutations in Sobic.002G221000 segregated a male sterility phenotype [ARS84 (25M2-1505)], whereas none of the five M4 mutant lines harboring heterozygous mutations in Sobic.002G234700 did. The ARS84 line harbored a C-to-T mutation at base 208, changing alanine to valine at conserved position 37 (A37V). The other line, ARS44, harbors a predicted amino acid change (G419S) in a nonconserved region in Sobic.002G221000 with a high SIFT score (0.74), implying that the predicted amino acid substitution (G419S) in ARS44 would have little effect on the function of the Sobic.002G221000 protein, and is therefore unlikely to alter the phenotype. Consistent with this prediction, no male-sterile plant was observed in the M4 plants. Together, these results suggest that the R218W mutation identified in the sorghum Sobic.002G221000 gene is the likely cause of male sterility in ms9.

Second, we performed complementation analysis between ARS178 and ARS84 by crossing a male-sterile plant in ARS84 with pollen collected from a heterozygous BC2F1 ms9 plant and then phenotyping segregation of male-sterile and fertile plants in the resulting cpF1 progenies. We obtained 21 fertile cpF1 plants and 20 male-sterile cpF1 plants, a segregation ratio of approximately 1:1 (Table 1). This result indicated that the male sterility phenotypes in ARS84 and ms9 were caused by mutations in the same gene, Sobic.002G221000. Therefore, we designated Sobic.002G221000 as Ms9. The ms mutation isolated

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<th>Crosses</th>
<th>Generation</th>
<th>Total no. of plants</th>
<th>Sterile plants</th>
<th>Fertile plants</th>
<th>Sterile/fertile ratios</th>
<th>c2 (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ms9*BTx623</td>
<td>F1</td>
<td>33</td>
<td>0</td>
<td>33</td>
<td>0:1</td>
<td>–</td>
</tr>
<tr>
<td>ms9*BTx623</td>
<td>F1</td>
<td>204</td>
<td>43</td>
<td>161</td>
<td>1:3</td>
<td>0.94</td>
</tr>
<tr>
<td>ms9*BTx623</td>
<td>BC1F2</td>
<td>461</td>
<td>117</td>
<td>344</td>
<td>1:3</td>
<td>0.87</td>
</tr>
<tr>
<td>ms9*BTx623</td>
<td>BC2F2</td>
<td>493</td>
<td>122</td>
<td>371</td>
<td>1:3</td>
<td>0.90</td>
</tr>
<tr>
<td>ms1<em>ms9BC2F1(Ms9</em>ms9)</td>
<td>cpF1</td>
<td>32</td>
<td>0</td>
<td>32</td>
<td>0:1</td>
<td>–</td>
</tr>
<tr>
<td>ms2*ms9BC2F1</td>
<td>cpF1</td>
<td>27</td>
<td>0</td>
<td>27</td>
<td>0:1</td>
<td>–</td>
</tr>
<tr>
<td>ms3*ms9BC2F1</td>
<td>cpF1</td>
<td>36</td>
<td>0</td>
<td>36</td>
<td>0:1</td>
<td>–</td>
</tr>
<tr>
<td>Ms7*ms9BC2F1</td>
<td>cpF1</td>
<td>33</td>
<td>0</td>
<td>33</td>
<td>0:1</td>
<td>–</td>
</tr>
<tr>
<td>ms9*ms9BC2F1</td>
<td>cpF1</td>
<td>18</td>
<td>0</td>
<td>18</td>
<td>0:1</td>
<td>–</td>
</tr>
<tr>
<td>ms8*ms9BC2F1</td>
<td>cpF1</td>
<td>39</td>
<td>0</td>
<td>39</td>
<td>0:1</td>
<td>–</td>
</tr>
<tr>
<td>ARS94ms*ms9BC2F1</td>
<td>cpF1</td>
<td>41</td>
<td>20</td>
<td>21</td>
<td>1:1</td>
<td>0.86</td>
</tr>
</tbody>
</table>
from ARS178 (M2P110) as ms9-1 and the ms mutation identified in ARS84 as ms9-2 (Fig. 5B). The nucleotide sequences of Ms9, Ms9-1, and Ms9-2 have been deposited into the GenBank with accession numbers of MK908415, MK908416, and MK908417, respectively.

Third, to obtain additional evidence that the mutations of Ms9 are the cause of male sterility in sorghum, we compared the functions of homologs of the two candidate genes. Ms9 homologous genes include male sterile 1 (Ms1, AT5G22260) in Arabidopsis (Wilson et al., 2001), persistent tapetal cell 1 (Ptc1, Os09g0449000) in rice (Li et al., 2011), and the male sterile 7 (zm0001d020680) in maize (Zhang et al., 2018), all of which cause male sterility in their respective plant species. The homologs of the other candidate gene identified have no reported roles in male sterility.

The sorghum Ms9 gene has three exons and two introns; the mature cDNA is 2444 bp in length (Fig. 5B). This gene contains a PHD-finger protein domain from the 612 to 658 amino acids according to the Pfam annotation (Fig. 6). This protein domain has been known to regulate the plant developmental transitions by recognizing the histone covalent modification and recruiting chromatin remodeling complexes and the transcriptional machinery (Mouriz et al., 2015). The structure of Ms9 in sorghum is similar to those of its homologs in Arabidopsis and cereal crops, and the gene encodes a protein of 668 amino acids (Fig. 6). Phylogenetic analysis of nine homologs selected from grass species, one from Arabidopsis, and one from tomato (Solanum lycopersicum L.) indicated that the closest homolog is the maize gene zm0001d020680 (Fig. 5C), which has recently been identified as male sterile 7 in maize (Zhang et al., 2018). Amino-acid sequence alignment revealed that the Ms9 in sorghum is highly similar to all nine genes used to construct the alignment (Supplemental Fig. S2). Furthermore, the amino acid sequences in conserved regions of Ms9 in sorghum are 100% identical to those of Ms7 in maize (Fig. 6) and almost identical to those of homologs in other cereal crops (Supplemental Fig. S2). Both R218W and A37V amino acid changes resulting from the nonsynonymous mutations in ms9-1 and ms9-2, respectively, occur within conserved regions of SbMS9 (Fig. 6). Together, these results provide strong evidence that Sobic.002G221000 is Ms9 in sorghum.

In the fourth approach, the expression pattern of the Ms9 gene was compared with its orthologs in Arabidopsis and rice, in which the expression pattern and its role in programed cell death are well characterized. We took advantage of the publicly available sorghum gene expression atlas (http://sorghum.riken.jp/morokoshi/Home.html) and showed that the Ms9 gene has similar expression pattern as Ms1 in Arabidopsis and Ptc1 in rice (Supplemental Fig. S3).

DISCUSSION

Pollen production is essential for pollination, which is the first step in setting seed. Although failure to pollinate (male sterility) can prevent seed set in self-pollinated plant species, male sterility is highly beneficial in hybrid breeding. The discovery of male sterility traits in plants has enabled breeders to produce hybrid seeds much more efficiently in a wide range of crops (Kim and Zhang, 2018). The direct benefit of hybrid production has been demonstrated by the significant yield advantage of maize hybrids over land races as well as improved yields and fitness in many other major crops and bioenergy species (Zhang et al., 2018). Control of pollen production is critical for hybrid breeding especially for hybrid seed production in self-pollinated species. Therefore, identifying male sterility in important crop species and improving their use in hybrid breeding systems could make important contributions to increasing future agricultural production and food security.

No gene that mediates NMS in sorghum has been cloned previously despite the discovery of several sorghum NMS lines and mutants (Andrews and Webster, 1971; Pedersen and Toy, 2001; Xin et al., 2017). Here, we report the isolation and characterization of a new male-sterile mutant and the identification of the first NMS gene in sorghum. This mutant, designated ms9, is distinct from all other sorghum NMS lines reported.
previously (Andrews and Webster, 1971; Pedersen and Toy, 2001; Xin et al., 2017). The male-sterile phenotype in ms9 mutants can be easily recognized at onset of anthesis because of its thin pale anthers and exaggerated stigmas. Other than male sterility, the ms9 mutants develop similarly to WT BTx623 plants. The characteristics of ms9 make it ideal for development of a two-line breeding system in sorghum based on NMS (Chang et al., 2016; Zhang et al., 2018).

The identification of Ms9 as the causal mutation for the male-sterile phenotype of the ms9 mutant is supported by bioinformatic analysis of two independent whole-genome sequencing data sets of pooled F2 mutants as well as by identification of another independent allele (Fig. 5) from the sequenced mutant library (Jiao et al., 2016). The cloned Ms9 gene encodes a PHD-finger transcription factor with a gene structure very similar to that of Ms1 in Arabidopsis, Ptc1 in rice, and Ms7 in maize (Li et al., 2011; Wilson et al., 2001; Zhang et al., 2018) as well as homologs in other cereals and plant species (Fig. 5C). The mutations identified in the two ms9 mutant alleles (R218W and A37V) cause amino-acid changes in the conserved domains of this protein (Fig. 6). The amino-acid sequence of SbMs9 is also very similar to its orthologous counterpart other crops (Supplemental Fig. S2). MS7, recently identified in maize, is the closest homolog to SbMs9, with 100% identity in the conserved region (Fig. 5C, 6). The phenotype of ms9 is similar to that of the male-sterility phenotypes of Arabidopsis ms1, the rice ptc1 and maize ms7 lines (Li et al., 2011; Wilson et al., 2001; Zhang et al., 2018), all of which have significant effects on anther morphology and pollen development but no effect on other aspects of floral development and morphology (Fig. 1, 3, 4).

The expression pattern of the Ms9 gene is also similar to its orthologs in other species. The expression data of Ms9 gene was extracted from the Morokoshi Sorghum Gene Expression Atlas (Makita et al., 2015). SbMs9 is highly expressed in young inflorescence tissues and anthers (Supplemental Fig. S3A). This tissue-specific gene expression pattern is similar to that of Ms1 in Arabidopsis and Ptc1 in rice (Li et al., 2011; Wilson et al., 2001), further supporting the role of Ms9 in the development of tapetum and pollen grains. As with Ms1 and Ptc1 in Arabidopsis and rice, Ms9 may serve as a critical regulator in tapetal cell degeneration and pollen development during anther development.

At present, the three-line hybrid breeding system relies exclusively on CMS for the male-sterile female parent in sorghum (Praveen et al., 2015; Rooney, 2004). Although several types of cytoplasmic male-sterile lines are available, commercial hybrid production uses mainly
the A1 cytoplasm (Jordan et al., 2011). A main advantage of the three-line breeding system in sorghum is that good A–B pairs can produce nearly 100% male-sterile line to serve as a female parent during production of hybrid seeds. Furthermore, this system has been used in breeding grain sorghum since 1940s, and many breeding materials in grain sorghum have been converted for use in this breeding system (Stephens and Holland, 1954). However, several aspects of the CMS breeding system need to be improved. For example, many sorghum accessions are neither perfect B nor R lines and cannot be used in breeding sorghum hybrids without lengthy period of conversion to B or R lines. In addition, many lines suitable for breeding biomass or sweet sorghum hybrids have not been converted and cannot be used directly to breed hybrids with the CMS-based breeding system. Furthermore, the A1 cytoplasmic homogeneity may predispose sorghum hybrids to devastating diseases, as in the T-cytoplasmic maize hybrids produced in the 1970s (Ullstrup, 1972).

A two-line breeding system that uses NMS can potentially overcome the disadvantage of the CMS-based three-line breeding system. For example, the male-sterility of a two-line breeding system based on a nuclear mutation can be restored by any line that does not carry the same mutation (Chang et al., 2016). This advantage is particularly useful for breeding biomass and sweet sorghum hybrids because accessions with the useful bioenergy traits can be directly used as parents for the hybrids. The two-line breeding system based on NMS also does not require male-sterile cytoplasm and, therefore, can avoid homogeneity of cytoplasm in hybrid. The main disadvantage of directly using NMS in hybrid breeding is that a fertile plant, heterozygous for an NMS mutation, only produces 25% of homozygous male-sterile plants. To remove the fertile plants from a breeder’s field is nearly impossible. Fortunately, through strategic manipulation of the NMS gene and its mutation, the two-line breeding system has been shown to produce pure male-sterile lines in rice (Chang et al., 2016; Huang et al., 2014; Zhou et al., 2014). In Maize, Ms7, the closest homolog of the sorghum Ms9 gene, was identified by map-based cloning and used, along with its WT gene, to engineer a controllable male-sterile line for a two-line breeding system (Zhang et al., 2018). A gene construct with the WT Ms7 gene and multiple control elements has been used to rescue the male sterility of the ms7 mutant and produce pure male-sterile seeds (Zhang et al., 2018). The identification of the Ms9 gene and its causal mutations provides critical tools to manipulate the production of male-sterile parent that is 100% male sterile.

In summary, we characterized a new sorghum NMS mutant, ms9, and identified the first NMS gene in sorghum. The identification of the SbMs9 provides an opportunity to engineer controllable male sterility for development of a two-line breeding system in sorghum.

**Supplemental Information Available**

Supplemental information is available with the online version of this manuscript.

**Conflict of Interest**

The authors declare that there is no conflict of interest.

**Author Contributions**

JC and ZX conceived the idea and designed experiment, YJ analyzed the data, HL performed histological and SEM analyses of anther features, all performed the experiment. JC and ZX drafted the manuscript with input from all authors. All authors agree with the final manuscript.

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**REFERENCES**


