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# Molecular Mapping of 2 Environmentally Sensitive Male-Sterile Mutants in Soybean

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## Abstract

In soybean [*Glycine max* (L.) Merr.], manual cross-pollination to produce large quantities of hybrid seed is difficult and time consuming. Identification of an environmentally stable male-sterility system could make hybrid seed production commercially valuable. In soybean, 2 environmentally sensitive male-sterile, female-fertile mutants (*ms8* and *msp*) have been identified. Inheritance studies showed that sterility in both mutants is inherited as a single gene. The objectives of this study were to 1) confirm that *msp* and *ms8* are independent genes; 2) identify the soybean chromosomes that contain the *msp* and the *ms8* genes using bulked segregant analyses (BSAs); and 3) make a genetic linkage map of the regions containing these genes. Mapping populations consisting of 176 $F_2$ plants for *ms8* and 134 $F_2$ plants for *msp* were generated. BSA revealed that Sat_389 and Satt172 are closely associated markers with *ms8* and *msp*, respectively. Map location of Sat_389 suggested that the *ms8* gene is located on chromosome 7; molecular linkage group (MLG) M. Map location of Satt172 indicated that the *msp* gene is located on chromosome 2 (MLG Dlb). Genetic linkage maps developed using $F_2$ populations revealed that *ms8* is flanked by a telomere and Sat_389 and *msp* is flanked by Sat_069 and GMES4176. The region between the telomere and Sat_389 is physically 160 Kb. Soybean sequence information revealed that there are 13 genes present in that region. Protein BLASTP analyses revealed that homologs of 3 of the 13 genes are known to a play role in cell division, suggesting putative candidates for *ms8*.

**Key words:** *environment,* glycine max*, male-sterility,* ms8 *mutant,* msp *mutant, soybean*

Male and female reproductive structures play an important role in seed development in plants. Abnormalities in male or female reproductive structures can lead to sterile plants (Kaul 1988b). Two main types of mutants have been characterized in soybeans. One type, in which both male and female gametophytic functions are lost or impaired, is referred to as male-sterile, female-sterile mutant. The second type, in which male gametophytic function is impaired but the female gametophyte is intact and functional, is known as male-sterile, female-fertile (MSFF) mutant (Kaul 1988a). MSFF mutants are important for the study of development and biology of reproductive systems in plants and may have applications in making soybean [*Glycine max* (L.) Merr.] hybrids commercially viable (Palmer et al. 2001; Pandini et al. 2002; Burton and Brownie 2006). Several MSFF mutants have been reported in soybean, and some of those have been mapped. The MSFF genes *ms1* and *ms6* map on chromosome 13 (molecular linkage group [MLG] F) (Palmer et al. 2004). A female-partial sterile 1 (*Fsp1*) mutant, the Midwest Oilseed male-sterile (*msMOS*), and the *ms3* genes were mapped to chromosome 2 (MLG D1b) in a small chromosomal region (Jin et al. 1998; Kato and Palmer 2003; Cervantes-Martinez et al. 2009). The *ms2* gene was mapped to chromosome 10 (MLG O), and *ms9* was mapped to chromosome 3 (MLG N) (Cervantes-Martinez et al. 2007).

Sterility systems are well characterized in some plant species such as rice and maize and are utilized for hybrid seed production (Coors and Pandey 1999). A sterility system provides convenience in hybrid seed production and is also instrumental in decreasing the cost of hybrid seeds by reducing the resource requirements (Palmer et al. 2001; Burton and Brownie 2006). With a viable sterility system in soybean, insects can be used for pollination, which can play a key role in the establishment of a viable hybrid seed industry (Palmer et al. 2009). Characterizing sterility genes is

the first step to develop a sterility system for possible commercial application. Identification of markers linked to genetic male-sterility genes can play an important role in early identification and removal of male-fertile plants before flowering (Horner and Palmer 1995). In addition, molecular markers can be used to select male-sterile lines in a backcrossing program. Development of genetic linkage maps for male-sterility genes may facilitate identification of morphological markers linked to the genes that can be utilized for quick and easier selection of male-sterile lines. Close linkage between flower color locus *W1* and *ms6* is commonly used for early selection of male-sterile lines in hybrid seed production (Lewers et al. 1996).

The environment in which a plant is grown can affect the way it develops. This is true for 2 MSFF soybean mutants, *ms8* and *msp* (Stelly and Palmer 1980a, 1980b; Carlson and Williams 1985; Perez PT, personal communication). The *ms8* mutant was 1 of 4 MSFF mutants identified from an experiment studying inheritance of *k2* (tan saddle), *Mdh1-n* (mitochondrial malate dehydrogenase one null), and the *y20* (yellow foliage) loci and was assigned genetic type collection number T358 (Palmer 2000). The $F_1$ seed produced on the *ms8ms8* female with 6 different male parents near Santiago, Chile from November 2004–March 2005 with alfalfa leaf-cutting bees, was not hybrid seed but 99% self-seed. This environment resulted in self-pollination not insect-mediated cross-pollination (Perez PT, personal communication). The *msp* gene was identified as a spontaneous mutant in the progeny of soybean germplasm population AP6(S1)C1 (Fehr and Ortiz 1975) in the field plots near Ames and Kanawha, IA, and was assigned the genetic type collection number T271. The recessive *mspmsp* plants had only a few pods. The anthers of the partially male-sterile *mspmsp* plants vary in appearance. In case of extreme sterility, anthers are small and often brown, even in newly opened flowers. Anther fertility sometimes varies not only among plants and flowers but also between whorls of anthers within flowers and anthers within whorls. Degenerating microspores or pollen often remain clumped. Anthers dehisce normally and clumping does not occur when anthers or flowers are fertile. The environment alters the degree of sterility in both of these monogenically inherited mutants (Stelly and Palmer 1980a, 1980b; Carlson and Williams 1985).

The objectives of our investigation were to 1) confirm that *msp* and *ms8* are independent genes; 2) identify the soybean chromosomes that contain the *msp* and the *ms8* genes using bulked segregant analyses (BSAs); and 3) make a genetic linkage map of the regions containing these genes.

## Materials and Methods

### Development of Genetic Materials

Three populations, A07-438, A07-448, and A07-455, consisting of 66, 50, and 60 $F_2$ plants, segregating for the male-fertility/sterility gene *Ms8/ms8* were developed by crossing Minsoy (*Ms8 Ms8*) as the female parent with the *ms8* mutant, T358H (*Ms8 ms8*) as the male parent.

Minsoy (PI27890) is a maturity group 0 plant introduction from France with no known pedigree. The *ms8* mutant is a maturity group II. Population A07-510, which segregated for the *Msp/msp* gene, consisted of 134 $F_2$ plants and was developed by crossing the *msp* mutant, T271 (*msp msp*) as female parent and Minsoy (*Msp Msp*) as male parent. The *msp* mutant is a maturity group II.

### Evaluation of Genetic Materials for Male Fertility/Male Sterility

Given the environmental sensitivity of the *ms8* and *msp* mutants; parental lines, $F_2$, and $F_{2:3}$ populations were evaluated under controlled environmental conditions (growth chambers and greenhouse) and in the field. Plants were either grown under 28/25 °C day/night temperature or 35/25 °C day/night temperature both in growth chamber and greenhouse. The plants were grown in medium containing 2 parts of soil, 1 part sand and 1 part peat moss. Soil was taken from the Bruner Farm near Ames, IA. In the field, lines were grown in 1.5 m rows with 2 biological replications at the Bruner Farm. No irrigation, fertilizers, or insecticides were used. To determine $F_2$ genotypes, $F_{2:3}$ families were also grown in 2 replications. Fertility was tested by pollen staining with iodine potassium iodine solution (Jensen 1962) and by seed set at harvest. Sterile pollen grain appeared to be light colored and translucent, whereas fertile pollen stained dark red–brown. Sterile plants had no or few pods. Generally, the field conditions in Ames, IA were fertility inducing. Both in the field and the greenhouse, mutants performed as expected.

### DNA Isolation and Pooling for BSAs

Genomic DNA was isolated using the cetyl trimethyl ammonium bromide extraction method described earlier (Sandhu et al. 2004). BSAs were used to identify the locus of interest for each gene (Michelmore et al. 1991). The fertile and sterile bulks were developed by mixing DNA from 10 homozygous fertile and 10 homozygous sterile $F_2$ plants, respectively. DNA bulks were prepared by pooling 1 μg DNA from each $F_2$ plant. Each bulk was diluted to a final concentration of 50 ng DNA/μl (Michelmore et al. 1991). Bulks were tested with simple sequence repeat (SSR) markers covering the entire soybean genome.

### Molecular Marker Analysis

Sequence information for developing SSR markers was obtained from http://soybase.org/resources/ssr.php (Song et al. 2004). For SSR analysis, 30 ng DNA was used as the template in a 10 μl reaction containing 1× reaction buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 2.0 mM $MgCl_2$; 0.25 μM of each primer; 200 μM of each dNTP; and 0.25 units of *Biolase* DNA polymerase (Bioline, USA Inc.). The polymerase chain reaction conditions were as follows:

**Table 1** Segregation patterns, Chi-square, and *P* values for populations of mutant lines of soybean *ms8* and *msp*

| | | No. $F_2$ plants[a] | | | | No. $F_2$ families[b,c] | | | |
|---|---|---|---|---|---|---|---|---|---|
| Population | Gene | Fertile | Sterile | $\chi^2$ | *P* | Homozygous fertile | Heterozygous fertile and sterile | $\chi^2$ | *P* |
| A07-438 | *ms8* | 52 | 12 | 1.3 | 0.25 | 19 | 33 | 0.24 | 0.62 |
| A07-448 | *ms8* | 33 | 12 | 0.1 | 0.80 | 14 | 19 | 1.23 | 0.27 |
| A07-455 | *ms8* | 41 | 14 | 0 | 0.94 | 16 | 25 | 0.60 | 0.44 |
| A07-510 | *msp* | 102 | 32 | 0.1 | 0.76 | 40 | 62 | 1.59 | 0.21 |

[a] $F_2$ plants were grown in the greenhouse.

[b] $F_{2:3}$ families were grown in the field. For some families, confirmation was done on remnant seed in growth chambers in sterility-inducing conditions.

[c] Homozygous $F_2$ families consisted of all male-fertile and female-fertile plants. Heterozygous $F_2$ families consisted of ~3 fertile plants: 1 sterile plant, that is, they were segregating $F_2$ families.

2 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 1 min at 72 °C, followed by 8 min at 72 °C. The amplification products were separated on a 4% agarose gel.

The Mapmaker 2.0 program was used to determine genetic linkages and genetic distances (Lander et al. 1987). Marker order was determined at a logarithm of odds threshold of 3.0. Linkage calculations were done using the Kosambi mapping function (Kosambi 1944).

## Results

Segregation for the 3 $F_2$ populations for *ms8* and 1 $F_2$ population for *msp* showed 3:1 ratios for fertile and sterile plants (Table 1). Progeny testing of male-fertile, female-fertile $F_2$ plants showed a 1:2 ratio of homozygous fertile:heterozygous fertile $F_2$ plants confirming monogenic inheritance of both of these genes (Table 1).

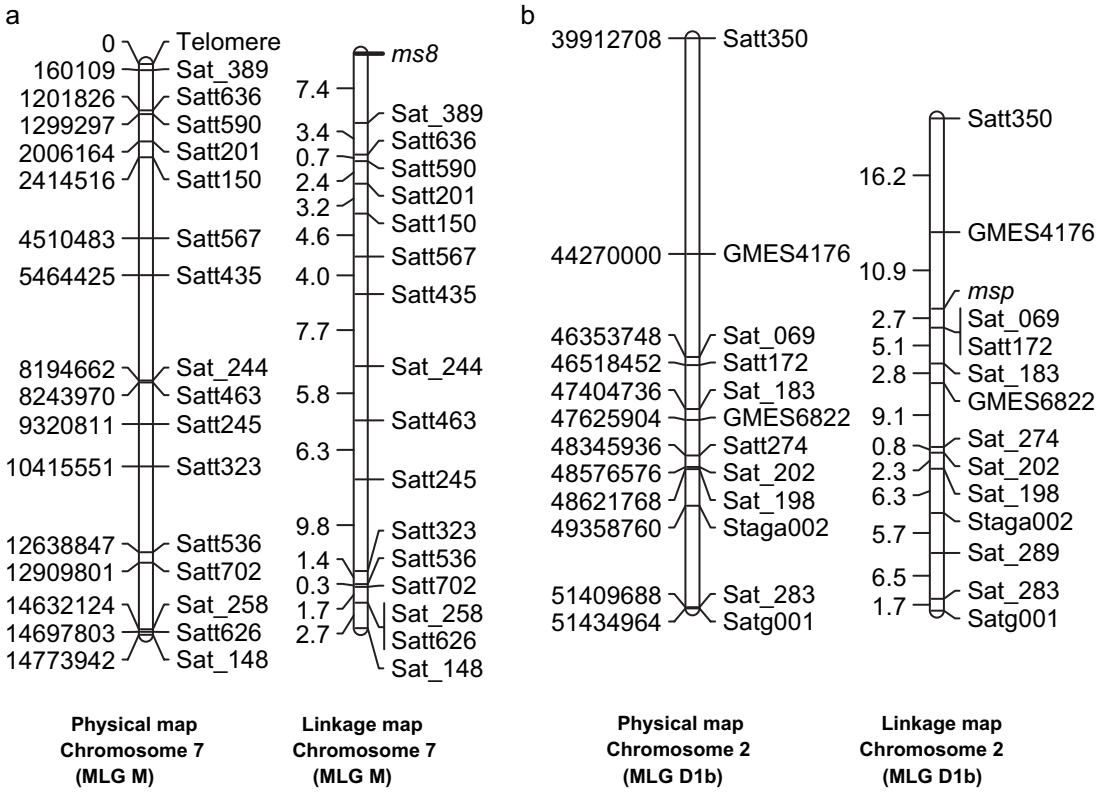


**Figure 1.** Genetic linkage maps and sequence-based physical maps of soybean chromosome 7 (MLG M) and chromosome 2 (MLG D1b) showing locations of SSR markers close to the *ms8* and the *msp* genes. (**a**) Male-sterile *ms8* locus, cross Minsoy (*Ms8 Ms8*) × *Ms8 ms8*; (**b**) Male-sterile *msp* locus, cross *msp msp* × Minsoy (*Msp Msp*). Physical distances are shown in base pairs (bp), and genetics distances are shown in centiMorgans (cM).

We used the bulked segregant analysis (BSA) method to find the locations of the *ms8* and the *msp* genes, using 560 SSR markers from the entire soybean genome on fertile and sterile bulks, with an average of 28 markers per chromosome. In the *ms8* populations, the SSR marker Sat_389 showed polymorphism between the bulks, suggesting that *ms8* was present in close proximity to Sat_389. Sat_389 was located on chromosome 7 (MLG M) (Song et al. 2004). We used 24 SSR markers close to Sat_389 from chromosome 7, 16 (Sat_389, Satt636, Satt590, Satt201, Satt150, Satt567, Satt435, Sat_244, Satt463, Satt245, Satt323, Satt536, Satt702, Sat_258, Satt626, and Sat_148) displayed polymorphism when tested on the parents. Polymorphic markers were then tested on all 3 $F_2$ mapping populations for *ms8*. Data from all 3 populations were pooled to develop a genetic linkage map of the chromosome. The gene was present on the telomeric end of chromosome 7 and showed 7.4 cM distance from the first marker, Sat_389, on the chromosome (Figure 1a).

In the *msp* population, the SSR marker Satt172 located on chromosome 2 (MLG D1b) (Song et al. 2004) was closely associated with the gene. We used 30 SSR markers from chromosome 2 that were in close proximity to the gene, 13 (Satt350, GMES4176, Sat_069, Satt172, Sat_183, GMES6822, Sat_274, Sat_202, Sat_198, Staga002, Sat_289, Sat_283, Satg001) of these displayed polymorphism when tested on the parents. Polymorphic markers were then tested on the entire $F_2$ population. The *msp* gene was flanked by markers Sat_069 and GMES4176 and was 6.5 cM from Sat_069 (Figure 1b). Markers Sat_069 and Satt172 were completely linked in our population.

## Discussion

In this study, we were able to confirm that 2 environmentally sensitive male-sterile genes, *ms8* and *msp*, showed monogenic inheritance. BSA and genetic linkage mapping demonstrated that the *ms8* gene and the *msp* gene were located on chromosome 7 and chromosome 2, respectively, confirming that they are independent genes. The *msp* gene was flanked by Sat_069 and GMES4176, whereas *ms8* was located near the telomere and was 7.4 cM from Sat_389, the most distal marker known on chromosome 7 (Figure 1a). Environmentally sensitive genic male-sterile lines have been identified in several other crop plants. For example, in rice, temperature was shown to play a significant role in fertility expression (Chen et al. 2003). Some of these genes have been characterized and genetically mapped to chromosomes (Wang et al. 2003; Lee et al. 2005).

The soybean genome has been sequenced and this sequence information can be accessed at the Phytozome website (Schmutz et al. 2010; http://www.phytozome.net/). We used the sequence information for all the SSR primers present on the genetic linkage maps to physically locate them on to the chromosomes (Figure 1a,b). The *msp* gene is flanked by Sat_069 and GMES4176 that encompass 13.6-cM region on chromosome 2. From these data, the

**Table 2** Genes present in the *ms8* region

| Gene | Putative protein | Homolog gene ID | Identities | *E* value | Predicted function |
|---|---|---|---|---|---|
| *Glyma07g00200.1* | Microtubule-associated protein | 814722 | 57% | 2e-89 | Cytokinesis regulation |
| *Glyma07g00220.1* | Condensation domain–containing protein | 824080 | 52% | 3e-146 | Unknown function |
| *Glyma07g00230.1* | P300/CBP-related protein | 5938778 | 62% | 0 | Cytoskeleton organization and transcriptional regulation |
| *Glyma07g00240.1* | Dihydroflavonal-4-reductase | 8281016 | 87% | 0 | Anthocyanin biosynthesis |
| *Glyma07g00260* | Salutaridinol 7-O-acetyltransferase | 8278440 | 57% | 7e-133 | Alkaloid biosynthesis |
| *Glyma07g00270.1* | Major facilitator superfamily | 8259933 | 64% | 1e-159 | Transport solutes in response to chemiosmotic ion gradients |
| *Glyma07g00280.1* | yt521-B-like family | 4334103 | 40% | 6e-88 | Silencing of genes involved in gametogenesis |
| *Glyma07g00290.1* | Phosphatidylcholine transfer protein | 8259936 | 63% | 1e-112 | Transport of lipids through cell membrane |
| *Glyma07g00300.1* | Strubbelig-receptor family 5 | 844238 | 40% | 3e-124 | Transmembrane receptor protein, protein phosphorylation |
| *Glyma07g00330.1* | Beta-amyrin synthase | 547702 | 96% | 0 | Synthesis of beta-amyrin from oxidosqualene |
| *Glyma07g00340.1* | Serine–threonine protein kinase | 821564 | 52% | 0 | Transfer of γ phosphate from ATP to amino acids |
| *Glyma07g00350.1* | Inorganic $H^+$ pyrophosphatase | 838249 | 86% | 0 | Involved in establishing the $H^+$ electrochemical potential difference between the vacuole lumen and the cell cytosol |
| *Glmya07g00360.1* | Ligand | 844230 | 61% | 7e-130 | Receptor binding |

Name and predicted functions of the putative proteins encoded by 13 genes that are flanked by telomere and Sat_389 on Gm07 (MLG M) are shown.

chromosome 2 region of the genome was examined to locate all the genes present between Sat_069 and GMES4176. Physically, the region was about 2.08 Mb (Figure 1b). Although the region was quite large, we were able to use soybean genome sequence flanked by these markers to locate putative genes present in this region. There were 182 genes identified in this region, which made it impossible to identify putative candidates (Schmutz et al. 2010); http://www.phytozome.net/. Fine mapping of the region is required using other types of marker systems to narrow down the region of interest.

The physical region between Sat_389 and the telomere that flanked the *ms8* gene was about 160 kb (Figure 1a), and there were 13 genes located in this region (Schmutz et al. 2010; http://www.phytozome.net/). We utilized the sequence information from these genes and used the National Center of Biotechnology Information (NCBI) basic local alignment search tool (BLAST) to find similarity with other know sequences. We performed protein BLAST (BLASTP) to compare protein sequences against nonredundant protein databases to find homologous sequences and to determine putative functions of these genes (Table 2). Function was not known for 1 of the 13 genes. Five genes were putatively involved in catalytic activity (metabolism), 2 had structural roles, 2 were involved in signal transduction, and 3 genes were involved in the cell cycle. The genes that have putative function in cell cycle were of particular interest as a mutation in a protein involved in the cell cycle can lead to problems in gamete formation, which in turn could result in sterility. The *Glyma07g00280.1* gene, codes for yt521-B-like family of proteins, which are known to play a role in gene regulation and have been shown to remove meiosis-specific genes prevalent in mitotic cells (Harigaya et al. 2006). *Glmya07g00230.1* codes for a protein that is involved in zinc ion interactions and affects cytoskeleton organization and transcription (Ponting et al. 1996). The cytoskeleton plays an important role in chromosome separation. *Glyma07g00200.1* codes for a microtubule-associated protein, affecting the cell during meiosis and mitosis (Pellman et al. 1995). Any defect of this gene during meiosis will render the gametes irregular, and as a result, sterile. Alterations in any of these 3 functions could lead to problems during meiosis and microsporogenesis, potentially leading to impaired pollen grains and male-sterile plants.

In soybean *st* lines, male and female sterility was caused by mutations that affected chromosome synapsis, whereas in the *ms* lines male sterility usually was caused by mutations that affect postmeiotic events or events leading to degeneration of the pollen grains later in microgametogenesis (Smith et al. 2002). In rice, detailed characterization of one thermosensitive male-sterile mutant revealed that premature programmed cell death of tapetum led to the collapse of pollen grains resulting in sterility (Ku et al. 2003). UDP-glucose pyrophophorylases that are normally associated with glycogenesis are also involved in callose deposition in pollen grain and silencing of these genes can result in temperature-sensitive male sterility in rice (Chen et al. 2007). Therefore, we cannot exclude the possibility of involvement of other predicted genes. Further investigations focusing on the genes involved in cell cycle and postmeiotic events are warranted.

Cloning and characterization of genes involved in male sterility may help us recognize molecular mechanisms controlling sterility and help us understand the biology of the male reproductive system in soybean. In addition, advancement of knowledge in this direction may assist in development of a stable sterility system in soybean that can be utilized for hybrid seed development.

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