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The Male Sterility Locus *ms3* Is Present in a Fertility Controlling Gene Cluster in Soybean

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Abstract

Soybean [*Glycine max* (L.) Merr.] is self-pollinated. To produce large quantities of hybrid seed, insect-mediated cross-pollination is necessary. An efficient nuclear male-sterile system for hybrid seed production would benefit from molecular and/or phenotypic markers linked to male fertility/sterility loci to facilitate early identification of phenotypes. Nuclear male-sterile, female-fertile *ms3* mutant is a single recessive gene and displays high outcrossed seed set with pollinators. Our objective was to map the *ms3* locus. A segregating population of 150 F_2 plants from Minsoy (PI 27890) × T284H, *Ms3ms3* (A00-68), was screened with 231 simple sequence repeat markers. The *ms3* locus mapped to molecular linkage group (MLG) D1b (Gm02) and is flanked by markers Satt157 and Satt542, with a distance of 3.7 and 12.3 cM, respectively. Female-partial sterile-1 (*Fsp1*) and the Midwest Oilseed male-sterile (*ms*MOS) mutants previously were located on MLG D1b. *ms*MOS and *Fsp1* are independent genes located very close to each other. All 3 genes are located in close proximity of Satt157. We believe that this is the first report of clustering of fertility-related genes in plants. Characterization of these closely linked genes may help in understanding the evolutionary relationship among them.

Key words: *Fsp1, gene cluster, Glycine max, male sterility, ms3 mutant, msMOS*

Nuclear male-sterility mutations in self-compatible plants, such as soybean, result in impaired pollen function, but such plants usually have normal female fertility (Chaudhury 1983). These nuclear mutations are classified as synaptic, structural, male-partial sterile or female-partial sterile, male sterile, female sterile, and complete male sterile, female fertile (Kaul 1988). Pollen development is a complex process involving network of pathways and is vital for male fertility and reproduction in plants. Numerous genes involved in anther development, tapetum development, pollen wall formation, tapetal programmed cell death, and exine formation have been cloned and characterized in various plant species (Wilson and Zhang 2009). At molecular level, any problems in any of these genes lead to a mutant or no protein, which may cause obstruction in the pathway, resulting in male-sterile plants.

The male-sterile, female-fertile soybean *ms3* mutant has normal meiosis I and II. Microspore degeneration precedes tapetal breakdown; therefore, microspore and tapetum degeneration prevent formation of pollen at anthesis (Graybosch and Palmer 1987). The *ms3* mutant is inherited as a single recessive nuclear gene (Chaudhari and Davis 1977) and has not been mapped by classical or molecular methods. The *ms3* mutant was an outcross found in the cultivar Wabash (Chaudhari and Davis 1977). Classical genetic studies revealed no linkage between *ms3* and flower color (*W1* is on chromosome Gm13) or between *ms3* and pubescence color (*T1* is on chromosome Gm06) (Palmer et al. 1980). For the assignment of soybean molecular groups to chromosomes, see http://soybase.org/LG2Xsome.php. The *ms3* mutant line has been used in insect-mediated cross-pollination studies to produce large quantities of hybrid soybean seed (Ortiz-Pérez et al. 2006), which have been used for agronomic performance (heterosis) studies (Ortiz-Pérez et al. 2007).

Even though many fertility/sterility genes have been reported in soybean (Palmer et al. 2004), there are few citations of DNA markers linked to fertility/sterility genes in soybean. A female-partial sterile-1 (*Fsp1*) mutant, found in Clark-k_2 (L67-3483), was positioned on molecular linkage group (MLG) D1b of the USDA-ARS-ISU map (Kato and Palmer 2003a). The Midwest Oilseed male sterile (*ms*MOS) also was positioned on MLG D1b of the USDA-ARS-ISU map (Jin et al. 1998). MLG D1b was assigned chromosome Gm02. The *ms*MOS trait segregated as a single Mendelian locus, was expressed in the recessive condition, and was not linked to either *W1* or *T1* (Jin et al. 1998). Male-sterile, female-fertile mutants *ms2* and *ms9* were positioned on MLGs O (chromosome Gm10) and N (chromosome Gm03), respectively (Cervantes-Martinez et al. 2007).

The objective of this study was to molecularly map the *ms3* locus to a chromosome location by using simple sequence repeat (SSR) markers. These results should facilitate the transfer of the *ms3* allele to suitable lines by using marker-assisted selection in soybean breeding programs.

Materials and Methods

Mapping Population

A segregating F_2 population derived from a single F_1 seed was developed from the cross of cultivar Minsoy (PI 27890) × T284H, *Ms3ms3* (A00-68) (Table 1). Fertile plants from segregating progeny of T284H were used as male parents. Minsoy (*MsMs*) was used as the female parent.

During summer 2004, F_2 seeds of the cross Minsoy × *Ms3ms3* were grown at the Bruner Farm near Ames, IA. About 220 seeds of each F_2 family were planted with 15 cm between seeds. At the beginning of flowering, stage R1 (Fehr and Caviness 1979), flowers of 10 plants per F_2 family were collected to identify segregation for the presence/absence of pollen. On that basis, 1 segregating F_2 family was selected as the mapping population, which consisted of 150 plants.

To determine the recombination rate between the *ms*MOS and *Fsp1* (Clark-k_2) loci, a cross was made between *ms*MOS*ms*MOS × Clark-k_2 at the Bruner Farm, IA, during summer 2003 (Table 1). The F_1 seeds were planted in the USDA greenhouse in winter 2004. The F_2 population was planted at the Bruner Farm during summer 2004, where 500 F_2 plants were selected at random, consisting of 100 plants from self-pollinated progeny of 5 F_1 plants and single-plant threshed. The plants were fertile (*Fsp1Fsp1*) or partial sterile (*Fsp1fsp1*). No attempt was made to distinguish between these 2 phenotypes. The male-sterile, female-sterile *ms*MOS*ms*MOS plants were not harvested.

The F_3 seeds from the 500 F_2 plants were planted in summer 2005 at the Bruner Farm as $F_{2:3}$ families. At maturity, each F_2-derived family was classified as follows: 1) all fertile F_3 plants, 2) segregating fertile and sterile F_3 plants (*ms*MOS*ms*MOS), 3) segregating fertile and female-partial sterile F_3 plants (*Fsp1fsp1*), or 4) segregating fertile plants, sterile plants, and female-partial sterile plants.

A closer placement of *ms*MOS to SSR markers on MLG D1b (chromosome Gm02) was deemed necessary to have a meaningful comparison with the *ms3* and the *Fsp1* genes. DNA from F_2 plants used in the mapping population of *ms*MOS × Minsoy (Jin et al. 1998) was not available. Remnant F_2 seeds of the original cross (Table 1) were planted in the USDA greenhouse at Iowa State University, winter/spring 2008. This mapping population (A08g-29 + 30) consisted of 110 F_2 plants.

DNA Extraction

In all, 2–3 g of young trifoliolate leaves were collected from each F_2 plant of the mapping populations, stored in individually labeled plastic bags, and freeze-dried for 48–72 h. Dry leaves were transferred to 15-ml sterile propylene tubes, ground to powder using glass beads, and kept at −80 °C until DNA extraction. The protocol for DNA extractions used in previous research was followed (Kato and Palmer 2003a, 2003b, 2004).

Also during summer 2004, seeds of Minsoy and T284H were planted in the USDA greenhouse at Iowa State University. During winter/spring 2008, seeds of 527-8 × 91133 (Table 1) were planted in the USDA greenhouse at Iowa State University. Young trifoliolates were collected in bulk and prepared for DNA extraction by the same procedure used for F_2 plants.

SSR Analysis

The DNA of the 150 F_2 plants of the Minsoy × *Ms3ms3* mating were assayed using SSR markers. A total of 231

Table 1. The F_2 soybean populations created to map the 3 male-sterile genes on Gm02 (MLG D1b)

Cross						
Female	Male	Source of sterility mutant	Grown	Population size	Mutant	Reference
Minsoy	*Ms3ms3*	T284H[a]	Summer 2004	150	*Ms3*	Present research
Minsoy	Clark-k_2	L67-3483[b]	Spring 2001	141	*Fsp1*	Kato and Palmer (2003a)
*ms*MOS	Minsoy	527-8 × 91133[c]	Summer 2008	110	*ms*MOS	Present research

[a] Genetic type collection number; USDA-ARS, Urbana, IL.

[b] Isoline collection; USDA-ARS, Urbana, IL.

[c] Midwest Oilseed Inc., Adel, IA.

markers were selected from the 20 soybean chromosomes (Song et al. 2004), using as the criterion for marker selection an intermarker genetic distance of 25 cM.

At maturity, each F_2 plant was phenotypically scored as either sterile or fertile. The initial SSR screening was done using the bulk segregant analysis method (Michelmore et al. 1991). The bulks consisted of DNA of 14 plants with the sterile phenotype and DNA of 14 plants with the fertile phenotype. The initial molecular screening was done using the 2 parents and the 2 contrasted bulks for each SSR marker. The markers showing polymorphisms between bulks were used to screen F_2 plants for linkage to the *ms3* locus. After this screening, possible linkage between the *ms3* locus and SSR markers Satt157 and Satt542 was identified, and the polymorphic markers Sat_227, Sat_351, Sat_373, Satt266, Satt005, and Satt141 were used to screen individual F_2 plants; all of them corresponding to chromosome Gm02.

Each polymerase chain reaction (PCR) mixture had a volume of 15 µl, including ×1 PCR buffer, 1.8 mM of $MgCl_2$, 0.17 mM of each of deoxynucleoside triphosphates, 0.25 units µl Biolase DNA polymerase (Bioline, Inc., Randolph, MA), 0.3 µM of forward and reverse primers, and 50 ng of template DNA. PCR was done with a PTC-100TM Programmable Thermal Controller (MJ Research, Inc., Waltham, MA), and PCR conditions were 45 s at 94 °C, 45 s at 47 °C, and 45 s at 68 °C for 32 cycles.

The PCR products were separated on 2–5% agarose gels (Agarose 3:1, Amresco Inc., Solon, OH), containing 0.50 mg/ml ethidium bromide, in ×1 TBE buffer (0.089 M Tris base, 0.089 M boric acid, and 0.002 M Ethylenediaminetetraacetic acid). The percentage of agarose used for preparing the gels depended on the difficulty of band separation. The DNA banding patterns were photographed using UV light.

For the *ms*MOS populations, 25 SSR markers close to the region, where *ms*MOS was previously placed, were used for polymorphism test. Of these, 11 markers detected polymorphism and were used for mapping.

Progeny Testing

The F_3 seeds from individual F_2 fertile plants were harvested and seeds planted in summer 2005 at the Bruner Farm. Segregation for fertility/sterility in the F_2-derived progeny rows was used to determine the genotype of each fertile F_2 plant. The expected phenotypic ratio in the F_2 generation was 3:1 (male-fertile:male-sterile plants), and the expected genotypic ratio of the $F_{2:3}$ population was 1:2:1 (*Ms3Ms3*:*Ms3ms3*:*ms3ms3*) or 1:2:1 (*Ms*MOS*Ms*MOS:*Ms*MOS *ms*MOS:*ms*MOS*ms*MOS).

Data Analysis

Each plant in the F_2 population was scored according to its SSR alleles at the locus, that is, a score of A was assigned if the plant was homozygous for the alleles from Minsoy, B if it was homozygous for the alleles from T284, and H if it was heterozygous. After scoring the population, recombination values were calculated to determine if each given SSR marker was linked to the gene of interest, using the LINKAGE-1 program (Suiter et al. 1983).

MAPMAKER V3.0 (Lander et al. 1987) program was used to make the final map that included all linked SSR markers. A minimum logarithm of the odd ratio score of 3 was used for accepting linkage between 2 markers. Recombination frequencies were converted to map distances in centimorgans using the Kosambi map function (Kosambi 1944). Percentage of recombination between the *Fsp1* and *ms*MOS loci was calculated from the classification data of $F_{2:3}$ families using the method reported by Mahama et al. (2002).

Results

The chi-square calculations for the *ms3*-segregating population showed a good fit to the genotypic ratio of 1 male-fertile, female-fertile homozygote:2 heterozygotes:1 male-sterile, female-fertile homozygote. The values were $\chi^2 = 1.52$ and $P = 0.47$ (Table 2).

Initial molecular screening of the 2 parents and of the 2 contrasted bulks indicated linkage between the *ms3* locus and SSR markers (Table 2). Markers on chromosome Gm02, Sat_227, Sat_351, Sat_373, Satt157, Satt542, Satt266, Satt005, and Satt141 were used to assay DNA of the individual F_2 plants (Table 2 and Figure 1A). The initial map, using MAPMAKER V3.0 (Lander et al. 1987), showed that the *ms3* locus was positioned between markers Satt157 and Satt542, with a distance of 3.7 and 12.3 cM, respectively (Figure 1A).

Previous results had shown that the *Fsp1* locus (Kato and Palmer 2003a) and male-sterile locus (*ms*MOS) (Jin et al. 1998) were located in the same region on chromosome Gm02. The original *Fsp1* locus data were used to construct the correspondent map (Figure 1B). Due to availability of

Table 2. F_2 genotypic segregation of the *ms3* locus and SSR markers linked to that locus

		Segregation[b]					
Marker	Map distance[a] (cM)	A	H	B	Number of plants	χ^2 (1:2:1)	*P*
Sat_227	37.3	38	78	34	150	0.45	0.80
Sat_351	28.5	40	75	35	150	0.33	0.85
Sat_373	10.1	34	81	33	148	1.33	0.51
Satt157	3.7	37	79	34	150	0.55	0.76
ms3	0.0	36	70	44	150	1.52	0.47
Satt542	12.3	37	80	33	150	0.88	0.64
Satt266	22.7	37	79	34	150	0.55	0.76
Satt005	45.2	36	77	36	149	0.17	0.92
Satt141	52.6	39	77	33	149	0.65	0.72

Markers on soybean chromosome Gm02 (MLG D1b) in the F_2 population derived from the cross Minsoy × *Ms3ms3*.

[a] Distance to *ms3* locus.

[b] Genotypes: A, homozygous for the alleles from Minsoy; H, heterozygous; B, homozygous for the alleles from T284.

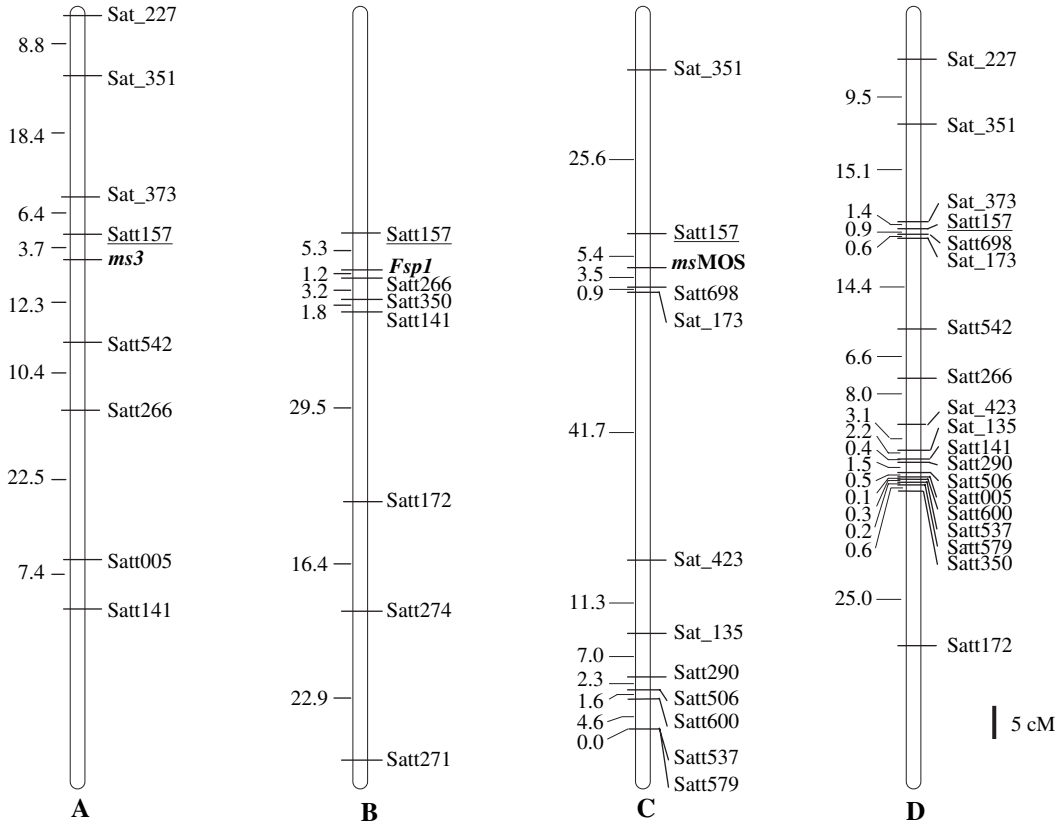

Figure 1. Genetic maps of soybean chromosome Gm02 (MLG D1b). (**A**) Male-sterile *ms3* locus, cross Minsoy × *Ms3ms3*; (**B**) *Fsp1* locus, cross Minsoy × Clark-k_2 (Kato and Palmer 2003a); (**C**) *ms*MOS, cross *ms*MOS × Minsoy. (**D**) Soybean composite map (Song et al. 2004). Note that Satt157 (underlined) segregated in all 3 populations, and each of the genes is present proximal to Satt157. Map distances are shown in centimorgans (cM).

a small number of markers at the time, the original map developed for *ms*MOS contained only a few markers (Jin et al. 1998). To have a meaningful comparison for the mapping location of all 3 genes, we decided to map more markers in the vicinity of the *ms*MOS gene. DNA from the F_2 plants used in the mapping population of *ms*MOS × Minsoy (Jin et al. 1998) was not available. Remnant F_2 seed of the original cross was used for mapping. Figure 1C shows the chromosomal position of *ms*MOS with the additional markers from the 2008 data (A08g-29 + 30 population). The percentage of recombination between the *Fsp1* and *ms*MOS loci was 9.04% (±0.01). The *ms3*, *Fsp1*, and *ms*MOS loci were localized to the same chromosomal region (Figure 1).

Discussion

The *ms3* linkage map showed that the order of the SSR markers, with the exception of Satt005, was the same as the soybean composite map based on Song et al. (2004). Map distances between markers were different, perhaps because the maps were constructed using crosses with different parents or because of vastly different population sizes. The soybean composite map was constructed by combining information from maps from 5 different populations including interspecific crosses (Song et al. 2004). The *ms3* population used in this study included 150 F_2 plants derived from the cross Minsoy (PI 27890) × T284H with both parents being *Glycine max*.

The *Fsp1* location in MLG D1b (Gm02) reported by Kato and Palmer (2003a) was derived from the cross of Minsoy × Clark-k_2, where both parents were *G. max*. Clark-k_2 (L67-3483) was a mutation in X-rayed Clark (Rode and Bernard 1975). Homozygous Clark-k_2 (k_2k_2) had 6.4% ovule abortion. However, in the F_1 hybrid of Clark × Clark-k_2, 41.4% of the ovules were aborted (Ilarslan et al. 2003). In the *Fsp1* map, the order of the SSR markers Satt350 and Satt141 is reversed as compared with the soybean composite map (Figure 1B). The data of Kato and Palmer (2003a) from the cross of Minsoy × Clark-k_2 support the hypothesis of a restriction of recombination around the heterozygous (*Fsp1fsp1*) locus in F_1 plants. However, the *Fsp1* map location was confirmed in the present study by reconstruction of the map.

In addition, the *ms*MOS mutation, mapped from the cross of Minsoy × *ms*MOS (527-8 × 91133), was positioned on MLG D1b (Gm02) between SSR markers Satt157 and Satt412 (Jin et al. 1998). Markers Satt157 and Satt412 were

18.7 cM apart on the map (Jin et al. 1998). In the map made from 2008 data, the *ms*MOS gene was flanked by Satt157 and Satt698, which are only 8.9 cM apart (Figure 1C). On soybean composite map, Satt157 and Satt698 are only 0.9 cM apart (Figure 1D), suggesting a precise localization for *ms*MOS. Close location of satt157 to all 3 genes makes it a suitable marker for marker-assisted selection of male-sterile, female-fertile plants in hybrid seed production.

Populations used to map the *ms3*, *Fsp1*, and *ms*MOS loci were derived from different crosses. Minsoy was a parent in each of the populations, and the plants were grown in different years. Nevertheless, all 3 genes *ms3*, *Fsp1*, and *ms*MOS are closely associated with Satt157 and are present proximal to Satt157 with genetic distance of 3.7, 5.3, and 5.4 cM, respectively (Figure 1). This is strong indication that the 3 mutations involved in fertility/sterility are clustered in the same chromosomal region of Gm02. This is the first report on clustering of fertility-related genes in a chromosomal region in plants. Although, clustering of genes with similar function has been reported for several groups of genes in prokaryotes and eukaryotes. Detailed genome analyses have revealed that disease-resistance genes are commonly found in clusters in plant genomes (Kanazin et al. 1996; Michelmore and Meyers 1998; Graham et al. 2002). For example, in lettuce, the *Dm* genes provide resistance against fungus *Bremia lactucae* that causes downy mildew. At least 15 *Dm* genes are located in 3 small gene clusters in lettuce genome (Chin et al. 2001). In soybean, at least 11 disease-resistance genes, including 2 genes controlling resistance to *Phytophthora sojae* (*Rps3* and *Rps8*), resistance to *Pseudomonas syringae* pv. *glycinea* (*Rpg1*), resistance against soybean mosaic virus (*Rsv1*), and resistance to peanut mottle virus (*Rpv1*), have been mapped to a small chromosomal region on chromosome Gm13 (MLG F) (Gore et al. 2002; Ashfield et al. 2004; Sandhu et al. 2004, 2005). Multiple gene copies or related genes present in a cluster may provide resistance against different races of a pathogen or against diverse pathogens.

In addition, comparative sequences analysis of 31 prokaryotic genomes revealed functional clustering of many other types of genes (Overbeek et al. 1999). A recent report extended this approach to eukaryotes and showed distinct functional coupling in cotton, where fiber development genes are present in gene-rich islands on 4 chromosomes (Xu et al. 2008). A gene cluster on an individual chromosome contained genes that corresponded with specific fiber developmental stage (Xu et al. 2008).

Recently, a soybean gene leading to a male-sterile phenotype was cloned using transposon-based mutagenesis (Mathieu et al. 2009). Mutant plants displayed defects in microspore development leading to small pods with no seeds (Mathieu et al. 2009). The gene codes for strictosidine synthase enzyme that is known to be involved in the condensation of tryptamine and secologanin into a vital indole alkaloid strictosidine (Bracher and Kutchan 1992). Mutation in the strictosidine synthase gene in corn also resulted in male sterility due to defects in pollen formation (Cigan et al. 2001). In *Silene latifolia* (white campion), 5 cDNA clones including strictosidine synthase were associated with fertility of anther and development of pollen (Ageez et al. 2005).

With the release 7.2× draft sequence by the soybean genome project, several studies are undertaken to establish a link between structure and function of soybean genome. Sequence comparison across species will help in characterization of fertility genes and will play a critical role in figuring out complex pathways involved in male and female fertility.

It will be interesting to clone and characterize clustered fertility genes and to understand the significance of functional clustering of fertility genes in soybean. This may provide important insights on the evolutionary relationship among these fertility-related genes.

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