

# Mapping Eight Male-Sterile, Female-Sterile Soybean Mutants

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## ABSTRACT

In soybean [*Glycine max* (L.) Merr.], mutations in genes involved in meiosis can lead to altered chromosome pairing and result in nonfunctional gametes. Mutability of the  $w_4$  flower color locus in soybean is due to an unstable allele designated  $w_4-m$  (mutable). Several germinal revertant studies using the  $w_4-m$  system resulted in generation of mutants for necrotic roots, chlorophyll-deficiency, and sterility. In the present study, six male-sterile, female-sterile mutant lines were identified from an independent mutational event that involved T366H (female-partial sterile mutant), which was a germinal revertant of  $w_4-m$ . In addition, two spontaneous mutations were identified that resulted in male-sterile, female-sterile mutants. The objectives of this study were to investigate if the newly identified six male-sterile, female-sterile germinal revertant mutants were allelic to previous germinal revertant steriles or are novel mutants and to molecularly map the locations of these six mutants along with two spontaneous male-sterile, female-sterile mutants. Three of the six mutants identified in the germinal revertant study mapped to the *st8* region on chromosome Gm16 (molecular linkage group [MLG] J). The other three mutants mapped to a novel location on chromosome Gm14 (MLG B2). Of the two spontaneous mutants, one mapped to chromosome Gm02 (MLG D1b) and the second one mapped to Gm18 (MLG G).

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**Abbreviations:** BSA, bulked segregant analysis; LOD, logarithm of odds; MLG, molecular linkage group; PCR, polymerase chain reaction; SSR, simple sequence repeat.

**I**N MEIOSIS, synapsis is an important process for ensuring normal chromosome segregation and development of gametes. Two important classes of mutants either leading to aberrant chromosomal pairing or abnormal maintenance of chromosomal pairing have been identified in soybean [*Glycine max* (L.) Merr.] (Gottschalk and Kaul, 1980a, b; Koduru and Rao, 1981). Mutations in genes involved in synapsis can result in either male-sterile, female-sterile plants, male-sterile, female-fertile plants, or male-fertile, female-sterile plants. In soybeans, several sterility mutants have been identified and studied (Cervantes-Martinez et al., 2007, 2009; Jin et al., 1998; Kato and Palmer, 2003a, b, 2004a; Palmer et al., 2004, 2008a)

An active transposable element has been characterized at the  $W_4$  locus in soybean (Xu et al., 2010). The  $W_4$  locus is involved in anthocyanin pigmentation and is mapped on the molecular linkage group (MLG) D2 (Xu and Palmer, 2005a). The unstable mutable  $w_4-m$  allele is the result of the transposon insertion in the  $W_4$  locus. When the transposon excises, the unstable allele reverts to  $W_4$ , and the flower regains pigmentation. Approximately 1% of the progenies derived from self-pollination of germinal revertant plants contain mutations at other loci (Palmer et al., 1989). The new mutants include chlorophyll deficiency, necrotic roots, and complete or

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partially sterile plants as the excised transposon reinserts into the corresponding locus (Kato and Palmer, 2003b, 2004b; Palmer et al., 1989, 2008a, b; Xu and Palmer, 2005b).

The first germinal revertant study yielded the completely sterile mutant *st8st8* and was mapped to Gm16 (MLG J) (Kato and Palmer, 2003b). In the second study, 36 individual reversion events led to 36 different male-sterile, female-sterile lines (Palmer et al., 2008a). Of these, 35 mapped to the *st8* region on Gm16 and one mutant mapped to Gm18 (MLG G) (Palmer et al., 2008a). In the third study, an independent mutational event(s) eventually resulted in the selection of six male-sterile, female-sterile mutants. The initial cross (allelism test) was Clark- $k_2$  ([L67-3483], Gm02 [MLG D1b])  $\times$  (T366H, Gm13 [MLG F]). Clark- $k_2$  is tan-saddle seed coat, and in the heterozygous condition expresses about 41% ovule sterility but has fertile pollen (Ilarslan et al., 2003). Clark- $k_2$  was identified as a mutation, tan saddle seed, in X-rayed cultivar Clark at the University of Missouri in Columbia, Missouri. Genetic Type T366 (*fsp3 fsp3*) is female-partial sterile that was found as a germinal revertant in T322 in 1997, and the genetic trait is not transmitted through the female parent (Pereira et al., 1997). The progeny row was segregating for plants producing normal and reduced numbers of seed per pod. One F<sub>2</sub> family descended from A96-1927-2 (F<sub>1</sub> plant; Clark- $k_2$   $\times$  T366H) segregated 42:3 (male-fertile and female-fertile):(male-sterile and female-sterile) plants. Fertile plants were threshed individually and progeny tested. After several field seasons of self-pollination, six families were selected and crossed to cv. Minsoy (PI 27890) for molecular mapping of the male-sterile, female-sterile trait(s). These six populations (A03-) are listed in Table 1.

In breeding populations, spontaneous mutations do occur at a low frequency. Some mutations with obvious phenotype such as sterility are very easy to notice. Two spontaneous male-sterile, female-sterile mutants were identified in separate breeding populations at Iowa State University in Ames, IA. A05-133, our designation, was given to fertile plants threshed individually from A01-406045 (IA 2058  $\times$  [A97-875044  $\times$  NK 528-T3]) from Dr. W.R. Fehr's soybean breeding project at Iowa State University in Ames, IA. A06-204, our designation, was given to fertile plants threshed individually from Ax18373 (IA 1006  $\times$  S21-A1) from Dr. S.R. Cianzio's soybean breeding project at Iowa State University in Ames, IA.

The objectives of this study were to investigate if six newly identified male-sterile, female-sterile germinal revertant mutants are allelic to *st8* or are novel mutants and to molecularly map the locations of these six mutants along with two spontaneous male-sterile, female-sterile mutants.

## MATERIALS AND METHODS

### Plant Materials

Six mapping populations (A03-2137, A03-2138, A03-2139, A03-2154, A03-2159, and A03-2160) were generated by crossing

plants (*St?* *St?* or *St?* *st?*) from each of the six independent sterility mutant lines from the second germinal revertant study with cultivar Minsoy (PI 27890) (*St St*) using standard soybean crossing techniques (Fehr, 1980) at the Bruner Farm near Ames, IA (Table 1). Two additional populations (A05-133 and A06-204) were generated by crossing plants (*St?* *St?* or *St?* *st?*) from the two breeding lines from Dr. W.R. Fehr's and Dr. S.R. Cianzio's projects, which gave rise to spontaneous sterility mutants with cultivar Minsoy (*St St*). Segregating F<sub>2</sub> populations were selected for each of the eight crosses by classification of fertile and sterile plants. The fertile F<sub>2</sub> plants were threshed separately. Each fertile F<sub>2</sub> plant was progeny tested by planting 50 F<sub>2,3</sub> descendents. Segregation of fertile and sterile plants, or all fertile plants, in each F<sub>2,3</sub> line was recorded at maturity to determine each F<sub>2</sub>-plant genotype.

For the six germinal revertant populations segregating for male-fertile, female-fertile and male-sterile, female-sterile phenotypes, between 32 and 48 F<sub>2</sub> plants were used for initial mapping, because previously 36 of 37 male-fertile, female-fertile and male-sterile, female-sterile mutants, from the second germinal revertant study, mapped to the same chromosome region (Palmer et al., 2008a). A larger size population consisting of 125 additional F<sub>2</sub> plants was generated from the remnant seed of A03-2137 for precise mapping.

### Determination of Association of New Male-Sterile, Female-Sterile Mutants with *St8*

Genomic DNA for parents and populations was isolated as described (Sandhu et al., 2004). To confirm if any of the six newly identified mutants in the *w<sub>4</sub>*-mutable study were different from the previously mapped *St8* mutant, simple sequence repeat (SSR) markers from the *St8* region Gm16 (MLG J) (Kato and Palmer, 2003b) were used to test for polymorphism between the parents. Polymorphic markers were screened on all six F<sub>2</sub> populations. Genetic linkage maps were constructed for individual populations. 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 (LOD) threshold of 3.0.

### Bulked Segregant Analyses

Of the six germinal revertant mutant genes, three mutant genes from entries A03-2137, A03-2138, and A03-2139 did not map to Gm16 (MLG J). To find the location of these three novel genes and two spontaneous mutant genes (from the previously mentioned breeding populations), bulked segregant analyses (BSAs) were used (Michelmore et al., 1991). For these five mapping populations, fertile and sterile bulks for the BSA were prepared from randomly selected DNA samples of either ten homozygous fertile (fertile bulk) or ten sterile (sterile bulk) F<sub>2,3</sub> families. DNA bulks were prepared by pooling 1  $\mu$ g DNA from each selected family. Each bulk was diluted to a final concentration of 50 ng DNA  $\mu$ L<sup>-1</sup>.

### Molecular Marker Analysis

For SSR analysis, 30 ng DNA was used as the template in a 10  $\mu$ L reaction containing 1X reaction buffer (10 mM Tris-HCl and 50 mM KCl with pH 8.3), 2.0 mM MgCl<sub>2</sub>, 0.25  $\mu$ M of each primer; 200  $\mu$ M of each dNTP, and 0.25 units of Biolase DNA polymerase (Biolone, USA Inc., Taunton, MA). The polymerase chain reaction (PCR) conditions consisted of: 94°C for 3 min,

**Table 1. Segregation patterns, chi-squares, and *p*-values for populations of male-sterile, female-sterile mutant lines of soybean.**

Population	No. F <sub>2</sub> plants		$\chi^2$ (3:1)	<i>p</i> -value	No. F <sub>2</sub> families		$\chi^2$ (1:2)	<i>p</i> -value
	Fertile	Sterile			Homozygous	Heterozygous		
A03-2154	37	11	0.11	0.7	8	29	2.28	0.13
A03-2159	26	6	0.67	0.4	9	17	0.02	0.89
A03-2160	35	12	0.01	0.9	13	22	0.23	0.63
Total	98	29	0.32	0.6	30	68	0.33	0.57
A03-2137	128	43	0.002	1	43	84	0.02	0.9
A03-2138	34	11	0.01	0.9	10	24	0.24	0.63
A03-2139	35	9	0.49	0.5	17	18	3.65	0.06
Total	197	63	0.08	0.77	70	126	0.51	0.47
A05-133	98	24	1.85	0.2	24	74	3.45	0.06
A06-204	89	29	0.1	0.9	26	63	0.68	0.41

11 cycles of 94°C for 30 s, 58°C for 30 s with an increment of -1°C per cycle and 72°C for one min, 35 cycles of 94°C for 30 s, 46°C for 30 s, and 72°C for one min, and a final 10 min at 72°C. The PCR products were separated on a 4% agarose gel at 150 v for 1 to 3 h. The Mapmaker 2.0 program was used to determine genetic linkages and genetic distances (Kosambi, 1944; Lander et al., 1987). Marker order was determined at a LOD threshold of 3.0. Sequence information for developing SSR markers was obtained from the soybean genome database at <http://soybase.org/resources/ssr.php> (verified 12 Oct. 2010) (Song et al., 2004).

## RESULTS

### Segregation in Populations

All F<sub>1</sub> plants were fertile for all of the crosses. Self-pollination of heterozygous F<sub>1</sub> plants from the crosses of Minsoy (*St St*) × *St?* *St?* or *St?* *st?* resulted in segregation of 3:1 male-fertile: male-sterile plant in the F<sub>2</sub> generation, suggesting monogenic inheritance. This was true for each of the eight different populations (Table 1). Each fertile F<sub>2</sub> plant of the eight mapping populations was single-plant threshed and progeny tested. The F<sub>2,3</sub> family segregation for each of the eight populations was the expected 1:2 nonsegregating:segregating ratio (Table 1).

### Molecular Mapping to Determine Association of Six Germinal Revertant Genes with *St8*

From each of the six different cross combinations, one segregating F<sub>2</sub> population was sampled for molecular mapping. Previous studies showed that 36 of 37 male-sterile, female-sterile mutants identified from the *w<sub>4</sub>-m* revertant study mapped to the same chromosomal region on Gm16 (MLG J) (Kato and Palmer, 2003b; Palmer et al., 2008a). Initially, we investigated if any of the six mutants from the revertant study mapped to the *St8* locus. We tested nine SSR markers (Satt132, Satt183, Satt215, Satt280, Satt285, Satt380, Satt406, Satt414, and Satt596) from *St8* region on the parents of the six F<sub>2</sub> populations for polymorphism. Of the nine SSR markers used, four (Satt132, Satt285, Satt414, and Satt693) showed polymorphism between the parents. These four markers were used on all six F<sub>2</sub> populations and genetic linkage maps were developed. In three (A03-2154,

A03-2159, and A03-2160) of the six populations, the male-sterile, female-sterile mutant gene mapped to the *St8* region. Genetic linkage map of male-sterile, female-sterile mutant gene from entry A03-2154 is shown as an example in Fig. 1. In the other three populations, male-sterility, female-sterility genes did not show association with markers from Gm16.

### Bulked Segregant Analysis for Mapping the Novel Male-Sterile, Female-Sterile Mutant Genes

In three of the populations (A03-2137, A03-2138, and A03-2139), the male-sterile, female-sterile mutant genes did not map to the *St8* region. To determine the map location of these three unknown male-sterile, female sterile mutant genes,

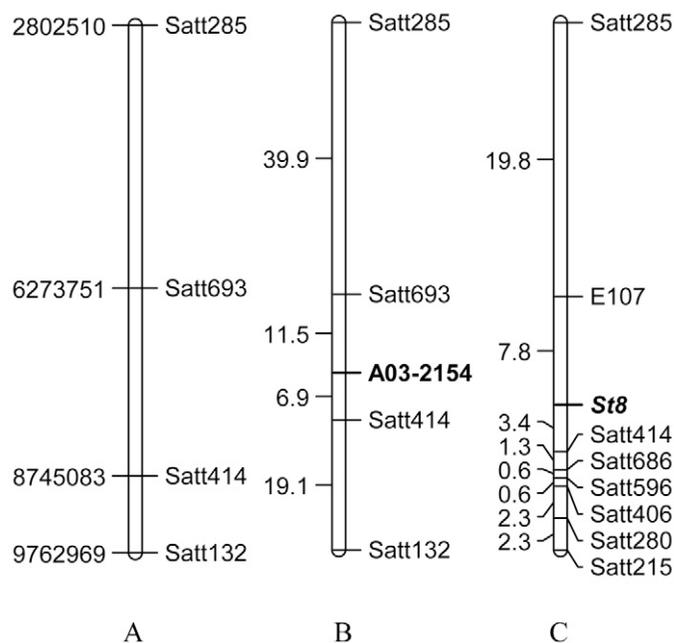


Figure 1. Physical and genetic linkage maps of Gm16 (molecular linkage group J) showing position of male-sterile, female-sterile mutant gene in comparison to *St8*. A. Sequence-based physical map of Gm16 (Schmutz et al., 2010). B. Genetic map position of the male-sterile, female-sterile mutant gene from entry A03-2154. C. Genetic linkage map of the male-sterile, female-sterile mutant gene, *St8*, from a cross of Minsoy × *St8st8* (T352H) (Kato and Palmer, 2003b). Distances are represented in centiMorgans (cM).

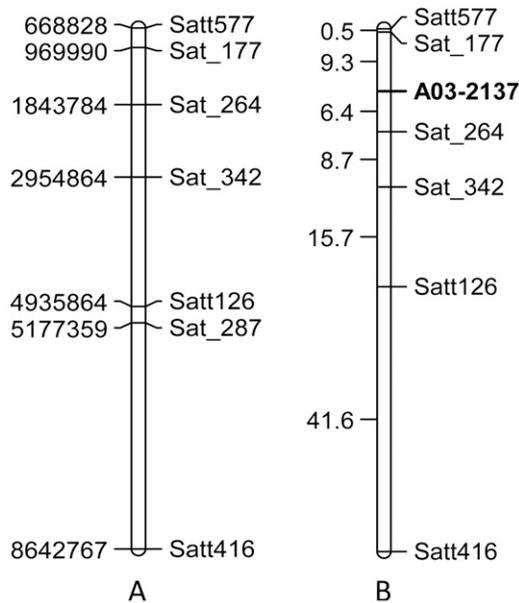


Figure 2. Physical and genetic linkage maps of soybean chromosome Gm14 molecular linkage group (MLG) B2 showing locations of simple sequence repeat (SSR) markers close to a male-sterile, female-sterile mutant generated in the germinal revertant study. A. Sequence-based physical map of Gm14 (Schmutz et al., 2010). B. Genetic map position of the male-sterile, female-sterile mutant gene from entry A03-2137. Physical distances are shown in base pairs (bp) and genetic distances are shown in centiMorgans (cM).

and two spontaneous male-sterile, female-sterile mutants, we applied BSA (Michelmore et al., 1991). We used 500 SSR markers representing all 20 soybean MLGs using fertile and sterile bulks developed from the  $F_2$  progeny for each of the mutants. In BSA, detection of polymorphism between the bulks suggested that the marker was present close to the gene of interest. Most of the markers tested did not detect polymorphisms between the contrasting bulks for any of the mutant populations. For all of the three novel mutants from the revertant study, Sat\_264 detected polymorphism between the bulks. Sat\_264 is located on Gm14 (MLG B2). Seven SSR markers located close to Sat\_264 were used to test polymorphism between the parents. Three markers (Sat\_177, Sat\_264, and Satt416) detected polymorphism and were used on the  $F_2$  populations. In all three populations, genes mapped between Sat\_177 and Sat\_264, indicating that these three mutants are allelic to each other or are very closely associated with each other (data not shown). For precise mapping of this gene, a large  $F_2$  population (from remnant  $F_2$  seed of A03-2137) consisting of 125 plants was used. Six polymorphic markers from Gm14 were used to develop a linkage map of the region (Fig. 2). The gene was flanked by Sat\_177 and Sat\_264 and was 6.4 cM from Sat\_264 and 9.3 cM from Sat\_177.

For one of the spontaneous mutants (A05-133), Satt271 showed polymorphism between the bulks, and for the second spontaneous mutant (A06-204), Satt472 showed polymorphism. Satt271 and Satt472 are located on Gm02 (MLG

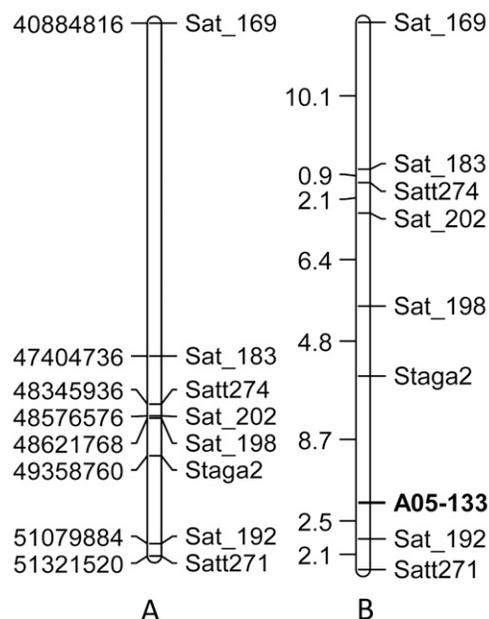


Figure 3. Physical and genetic linkage maps of soybean chromosome Gm02 molecular linkage group (MLG) D1b showing locations of simple sequence repeat (SSR) markers close to A05-133, one of the two spontaneous male-sterile, female-sterile mutants. A. Sequence-based physical map of Gm02 (Schmutz et al., 2010). B. Genetic map position of the male-sterile, female-sterile mutant gene from entry A05-133. Physical distances are shown in base pairs (bp) and genetic distances are shown in centiMorgans (cM).

D1b) and Gm18 (MLG G), respectively.  $F_2$  populations were used for the genetic linkage mapping of these mutants. For the mutant A05-133, the male-sterile, female-sterile gene was flanked by the markers Staga2 and Sat\_192 with Sat\_192 located 2.5 cM from the gene (Fig. 3). For the mutant A06-204, the sterility gene was flanked by AF162283 and Satt472 with AF162283 located 4.1 cM from the gene (Fig. 4).

## DISCUSSION

An active transposable element was cloned from soybean (Xu et al., 2010). The unstable nature of  $w_4-m$  can be explained by the presence of a transposable element in the line and has been utilized in genetic characterizations of sterility, chlorophyll deficiency, and necrotic root mutants found among self-pollinated progeny of germinal revertants (Kato and Palmer, 2003b, 2004b; Palmer et al., 1989, 2008a, b; Xu and Palmer, 2005b). In this study, six male-sterile, female-sterile mutants generated in the progeny of the  $w_4-m$  line were molecularly mapped. Three mapped to a genomic region on Gm16 (MLG J), where 36 male-sterile, female-sterile mutants had been mapped in previous studies (Kato and Palmer, 2003b; Palmer et al., 2008a). The other three male-sterile, female-sterile mutants mapped to a unique location on Gm14 (MLG B2). In these three populations, the genes mapped to the same location, suggesting that these three mutants are allelic to each other or are very closely associated. Of the previously known male-sterile,

female-sterile mutants in soybean, none were mapped on Gm14, suggesting that this is a new gene. Comparison of the genetic linkage map with the soybean genome sequence database (<http://www.phytozome.net/soybean> [verified 30 Sept. 2010]) suggested that the physical distance between Sat\_177 and Sat\_264 was about 874 kbp, and there are 148 putative genes located in this region (Schmutz et al., 2010).

Appearance of the complete male and female sterility in the progeny of two female-partial sterile lines (Clark- $k_2$  × T366) is very intriguing. More studies need to be conducted to decipher the mechanism involved. However, it is possible that T366, which has been completely stable for more than five selfed generations, contains a transposable element, and this transposon was activated by Clark- $k_2$  and produced the completely sterile mutant. The female semi-sterility in the F<sub>1</sub> plants with Clark- $k_2$  could be an interaction between protein subunits. Mutant protein from Clark- $k_2$  and normal protein from the other parent form a dimer or higher order interaction that impairs female function. When the mutant protein subunits are present in Clark- $k_2$ , in a dimer or higher order interaction, functionality approaches wild type (normal) and only a very low level of female sterility is seen phenotypically.

Mutations often lead to disease or dysfunction but also increase variability and provide key entrance points to the study of genetics. Per sexual generation of eukaryotes, mutation rates of the effective genome range from 0.0036 to 1.6, and the estimated rate for the entire genome is around 1 (Drake et al., 1998; Klekowski, 1988, 1992). In this study two spontaneous male-sterile, female-sterile soybean mutants, A05-133 and A06-204, were located on Gm02 (MLG D1b) and Gm18 (MLG G), respectively. Comparison of the genetic linkage map with the soybean genome sequence database (<http://www.phytozome.net/soybean> [verified 30 Sept. 2010]) suggested that the flanking markers around A05-133 encompass about 1.7 Mbp, and there are 270 putative genes in this region (Schmutz et al., 2010). However, markers flanking A06-204 are about 700 kbp apart, and there are only 72 predicted genes in this region.

The actual points during development at which the mutations occurred within the genome are still unknown. Since plants lack separation of the soma and germ line, mutations in the soma could potentially be passed onto the gametes. Additionally, mutations affecting reproduction-related genes in meristematic cells would not be expressed until after differentiation (Sutherland and Watkinson, 1986). However, mutations may occur within the reproductive tissues after differentiation as well. Further studies are needed to determine exactly when the mutations occurred, which processes the mutation directly affected in causing sterility, and whether the affected processes differ from previously characterized sterility mutants.

For all five novel mutants mapped in this study, future studies focusing on fine mapping of these regions

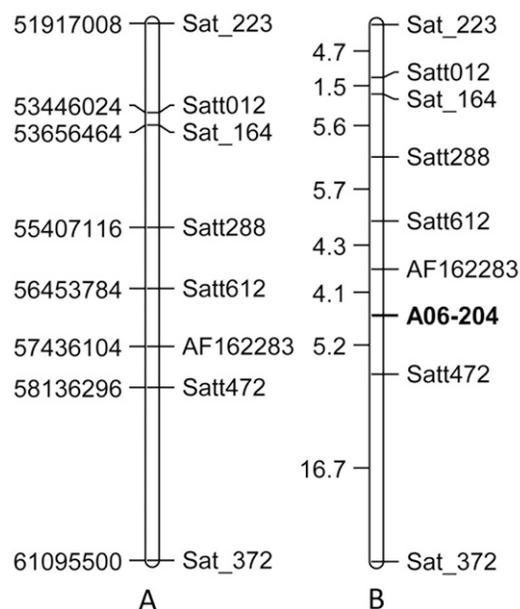


Figure 4. Genetic linkage maps and sequence based physical maps of soybean chromosome Gm18 molecular linkage group (MLG) G showing locations of simple sequence repeat (SSR) markers close to A06-204, the second spontaneous male-sterile, female-sterile mutant. A. Sequence-based physical map of Gm18 (Schmutz et al., 2010). B. Genetic map position of the male-sterile, female-sterile mutant gene from entry A06-204. Physical distances are shown in base pairs (bp) and genetic distances are shown in centiMorgans (cM).

are warranted. Their eventual cloning and sequencing will lead to further studies of the gene products and their functions in causing sterility in soybean. The collective results will yield a more complete picture of the pathways involved in reproduction processes of higher plants.

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