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Segregation distortion in a region containing a male-sterility, female-sterility locus in soybean☆

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ABSTRACT

In diploid segregation, each alternative allele has a 50% chance of being passed on to the offspring. Mutations in genes involved in the process of meiotic division or early stages of reproductive cell development can affect allele frequency in the gametes. In addition, competition among gametes and differential survival rates of gametes can lead to segregation distortion. In a recent transformation study, a male-sterile, female-sterile (MSFS) mutant was identified in the soybean cultivar, Williams. The mutant in heterozygous condition segregated 3 fertile:1 sterile in the progeny confirming monogenic inheritance. To map the lesion, we generated an F_2 mapping population by crossing the mutant (in heterozygous condition) with Minsoy (PI 27890). The F_2 progeny showed strong segregation distortion against the MSFS phenotype. The objectives of our study were to molecularly map the gene responsible for sterility in the soybean genome, to determine if the MSFS gene is a result of T-DNA insertion during *Agrobacterium*-mediated transformation, and to map the region that showed distorted segregation. The fertility/sterility locus was mapped to molecular linkage group (MLG) D1a (chromosome Gm01) using bulked segregant analysis. The closest marker, Satt531, mapped 9.4 cM from the gene. Cloning of insertion sites for T-DNA in the mutant plants revealed that there are two copies of T-DNA in the genome. Physical locations of these insertion sites do not correlate with the map location of the MSFS gene, suggesting that MSFS mutation may not be associated with T-DNA insertions. Segregation distortion was most extreme at or around the *st_A06-2/6* locus suggesting that sterility and segregation distortion are tightly linked attributes. Our results cue that the distorted segregation may be due to a gamete elimination system.

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1. Introduction

Meiosis is an important process in sexual reproduction. During the synapsis phase of meiosis homologous chromosomes come together, exchange chromosomal segments and later separate, allowing recombination between genes of a linkage group. Mutations in the genes responsible for proper chromosomal pairing and gamete formation can lead to sterile plants [1]. In soybean {*Glycine max* (L.) Merr.}, numerous mutations involved in male fertility and female fertility have been identified and many of those have been mapped [2–10].

Segregation distortion is a phenomenon observed for particular chromosomal regions in both plants and animals. Segregation distorters are genetic elements that alter the expected Mendelian ratio [11]. In fact, Mendelian segregation ratios are observed only if each gamete has the same probability of being fertilized by each other. Segregation distorters cause preferential success, or failure, of specific gamete types. Chromosomal structures such as those involved with movement on spindle fibers during meiosis can lead to distorted segregation ratios [11]. Segregation distortion can also be a result of differential gamete performance, and/or survival. Interactions between gametes can result in differential success [12]. In a transformation study in soybean, we found a male-sterile,

Abbreviations: SSR, simple sequence repeat; PCR, polymerase chain reaction; BSA, bulked segregant analysis; MSFS, male-sterile female-sterile; MFFF, male-fertile female-fertile; Cm, centiMorgan; MLG, molecular linkage group; T-DNA, transferred DNA; BLAST, basic local alignment search tool.

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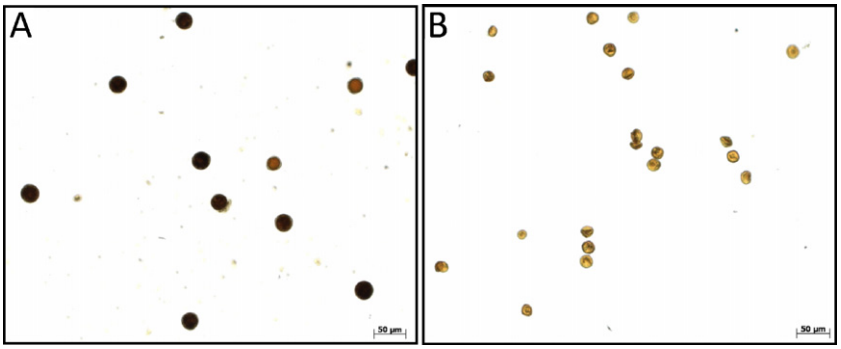

Fig. 1. Comparison of pollen grains of fertile and sterile plants: A, I_2KI-stained pollen from a fertile plant; B, I_2KI-stained pollen from a sterile plant.

female-sterile (MSFS) mutant plant. The heterozygous T_1 line showed a 3:1 segregation ratio of fertile:sterile plants in the T_2 generation suggesting monogenic inheritance. In an attempt to map the MSFS mutant, we crossed this mutant in heterozygous form to cultivar Minsoy (PI 27890). Segregating F_2 progeny showed segregation distortion in favor of the fertile phenotype. Investigation of segregation in the $F_{2:3}$ generation suggested that the distortion was caused most likely by one or more genetic factors.

The objectives of this investigation were to: (i) map the location of the MSFS gene, (ii) determine if the mutation in the MSFS gene was caused by T-DNA insertion, and (iii) map the region that showed distorted segregation.

2. Materials and methods

2.1. Plant materials

In a transformation experiment conducted at the Plant Transformation Facility, Iowa State University, the cultivar Williams was transformed with a candidate *Rps1*-k gene that confers **R**esistance to ***P**hytophthora **s**ojae* (N.N. Narayanan, M.K. Bhattacharyya, unpublished). Fourteen seeds were harvested from the T_2 generation of a transformation event, and were grown in the field at the Bruner Farm near Ames, IA. Three of the plants were MSFS as determined by I_2KI staining [13] of pollen grains, and lack of pod setting following cross pollinations with fertile pollen. The remaining plants were tested for segregation: three were found to be homozygous male-fertile, female-fertile (MFFF) and eight segregated for sterility. Heterozygous plants obtained from the remnant seed of two plants that segregated 18 fertile:7 sterile (A06-2), and 27 fertile: 11 sterile (A06-6) were crossed to Minsoy (PI 27890) as a female parent at the Bruner Farm, Iowa State University. We tentatively named the MSFS gene as *st_A06-2/6*. Two mapping populations were developed from these crosses and were designated A07-1132 and A07-1152, respectively. The F_2 populations were investigated for segregation to fertile and sterile plants at flowering by staining pollen grains with I_2KI. Light microscopic and cytological observations of microsporogenesis were carried out in the Microscopy and NanoImaging Facility at Iowa State University. In fertile plants, pollen grains were densely stained and spherical in shape as compared to sterile plants where pollen grains were lightly stained and irregular in shape (Fig. 1). The non-stained, somewhat misshapen pollen grains of the mutant had similar morphology to aborted pollen grains of soybean synaptic mutants *st4 st4* [14] and *st5 st5* [15]. To test for female fertility, cross-pollinations were made with sterile plants as female parent and cultivar 'BSR 101' (PI 548519) [16], as male parent. Ten cross-pollinations per day for 8 days were made. All 80 cross-pollinations aborted, suggesting that the mutant is female sterile. Each of the fertile F_2 plants was progeny tested by planting $F_{2:3}$ seeds. Segregation was determined in the $F_{2:3}$ families at maturity and used to genotype the F_2 individuals.

2.2. Bulked segregant analysis (BSA)

Genomic DNA for parents and the F_2 populations was isolated from leaf tissues following a method described elsewhere [17]. Fertile and sterile bulks were created by pooling DNA from 10 homozygous fertile or homozygous sterile F_2 plants, respectively [18]. DNA from each of the bulks was diluted to a final concentration of 50 ng/μl.

2.3. Molecular marker analysis

SSR markers were developed using information from http://soybase.org/resources/ssr.php [19]. For SSR analysis, 30 ng of DNA was used for 10 μl reaction with 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., Tauton, MA). PCR cycle was run with a temperature of 94 °C for 3 min, followed by 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 1 min, 35 cycles of 94 °C for 30 s, 46 °C for 30 s, and 72 °C for 1 min, with a final temperature of 72 °C for 10 min. The resulting PCR products were separated on a 4% agarose gel at 150 V for 1–3 h. Genetic linkages and distances were determined using Mapmaker 2.0 [20,21]. The order of markers was determined at LOD threshold of 3.0.

2.4. Genome walking

Four restriction enzymes, *Swa*I (eight cutter), *Dra*I, *Stu*I, and *Eco*RV (six cutters), were used to digest pooled genomic DNA of 10 homozygous MSFS plants. We used the GenomeWalker Universal kit (Clontech, Mountain View, CA), and followed the manufacturer's instruction with a few modifications. Digested products were purified using phenol: chloroform and were ligated to GenomeWalker Adaptors to generate four libraries. Each library was amplified with the outer adaptor primer (AP1) and an outer T-DNA primer (T-DNA R1) (Table 1). Two microliters of this reaction was then diluted 100× and used as a template for a second or "nested" PCR utilizing the nested adaptor primer (AP2) and a nested T-DNA primer (T-DNA R2) (Table 1). PCR products were size separated on 1.5% agarose gels. DNA bands were cut out of the gels and purified using a gel extraction kit (Qiagen, Valencia, CA). All the DNA fragments were sequenced and the sequences were used in BLAST analysis against the soybean genome sequence (Table 2).

Table 1
Primer sequences used for the genome walking experiment.

Primer name	Primer description	Primer sequence (5′ to 3′)
Adaptor primer 1 (AP1)	Outer adaptor primer	GTAATACGACTCACTATAGGGC
Adaptor primer 2 (AP2)	Nested adaptor primer	ACTATAGGGCACGCGTGGT
T-DNA R1	Outer T-DNA primer	TGG CGT TAC CCA ACT TAA TCG CCT
T-DNA R2	Nested T-DNA primer	ACT TAA TCG CCT TGC AGC ACA TCC

Table 2
Sequence comparison of the fragments identified in the genome walking experiment with the soybean genome.

Sequence name	Fragment sequence	Matching sequence
*Swa*1-1/*Dra*1-1/*Stu*I-2	1–58	Soybean chromosome Gm18: 60,057,477–60,057,534
	64–248	Binary cloning vector pPZP202 for plant transformation
*Eco*RV-1	53–167	Soybean chromosome Gm18: 60,057,420–60,057,534
	173–306	Binary cloning vector pPZP202 for plant transformation
*Stu*I-1	33–175	Soybean Chromosome Gm06: 37,592,039–37,592,181
	228–370	Binary cloning vector pPZP202 for plant transformation
*Stu*I-3	1–133	Binary cloning vector pPZP202 for plant transformation

3. Results

3.1. Genetic linkage mapping of the MSFS mutant

To determine the genetic location of *st_A06-2/6*, we used 700 SSR markers covering all 20 soybean molecular linkage groups (MLG) on the fertile and sterile bulks. Satt531 showed polymorphism between the fertile and the sterile bulks. Satt531 is located on chromosome Gm01 (MLG D1a). Thirty-one SSR markers from Gm01 were used on the parents to identify polymorphic markers. Of these, 12 (Sat_413, Satt184, Satt531, Satt320, Satt342, Satt532, Satt502, Sat_346, Satt603, Satt515, Sat_201, and Satt402) showed polymorphism. Polymorphic markers were run on the A07-1132 and A07-1152 F_2 populations. Linkage data revealed that *st_A06-2/6* is flanked by Satt320 and Satt531, with the closest marker, Satt531 located 9.4 cM away from the gene (Fig. 2). The soybean genome has been sequenced and can be accessed at the Phytozome website (http://www.phytozome.net/) [22]. We used the sequence information for all the SSR primers present on the genetic linkage map to physically locate them on the chromosome (Fig. 2). The *st_A06-2/6* region flanked by Satt320 and Satt531 markers on the physical map is 18.4 Mbp (Fig. 2).

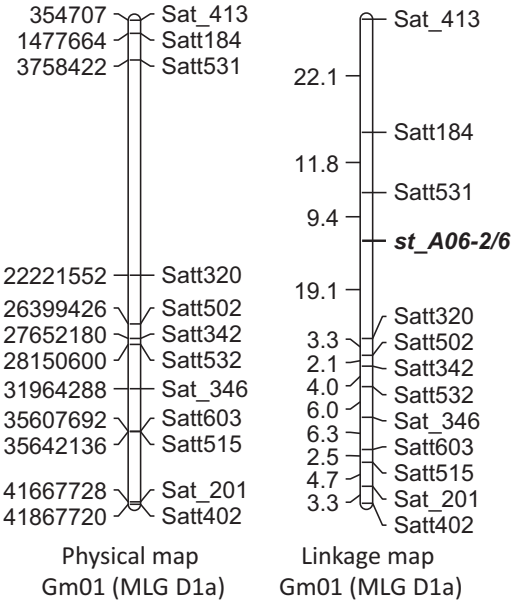

Fig. 2. Genetic linkage map and physical map of the Gm01 chromosome containing the *st_A06-2/6* locus. Physical distances are shown in base pairs (bp) and genetic distances are shown in centiMorgans (cM).

3.2. Association between T-DNA and sterility phenotype

The MSFS mutant was identified in the progeny of a line transformed with the candidate *Rps1*-k gene using *Agrobacterium*-mediated transformation. If the sterility was a result of T-DNA insertion, it would provide a means of cloning the gene responsible for the sterility trait. To investigate if T-DNA insertion led to a loss of function mutation, we used the genome walking technique to locate the insertion sites of T-DNA molecules in the soybean genome. Six fragments detected two loci in the soybean genome (Table 2; Fig. 3). As expected, one part of each of the fragments *Swa*I-1, *Stu*I-2, *Dra*I-1, and *Eco*RV-1 showed identity with soybean chromosome Gm18 and the second part showed identity with the plant transformation cloning vector (Table 2). Fragments *Swa*I-1, *Stu*I-2, and *Dra*I-1 are identical in sequence, however, *Stu*I-2 sequence is 17 bp longer than *Swa*I-1 and *Dra*I-1 sequences. Sequence for the fragment *Stu*I-3 is 163 bp long, 133 of which showed identity to the transformed vector sequence.

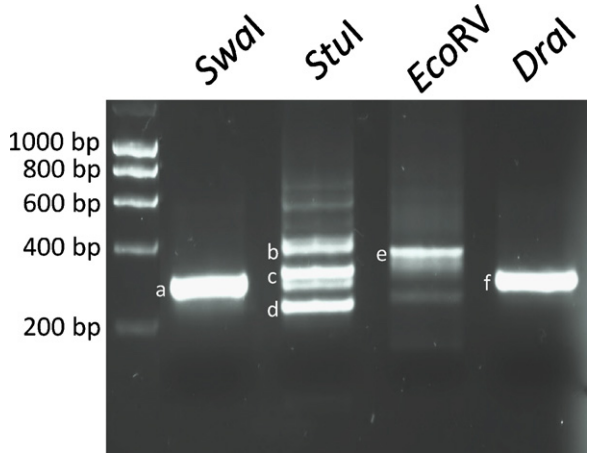

Fig. 3. PCR amplification of four libraries produced with the GenomeWalker Universal kit using primers AP2 and T-DNA R2. Libraries were generated by digesting pooled DNA from 10 sterile plants and ligating the products with Genome Walker adaptors. Fragment bands are marked using small letters: a, *Swa*I-1; b, *Stu*I-1; c, *Stu*I-2; d, *Stu*I-3; e, *Eco*RV-1; f, *Dra*I-1.

Table 3
χ^2 test showing segregation of the *st_A06-2/6* gene in F_2 and $F_{2:3}$ generations in crosses with Minsoy (PI 27890). St St, homozygous fertile; St st, heterozygous; st st, homozygous sterile.

Sample	No. of F_2 plants		χ^2(3:1)	*p*-Value	No. of $F_{2:3}$ families		χ^2(1:2)	*p*-Value
	St_	st st			St St[a]	St st		
A07-1132	47	3	9.63	0.0019	28	17	16.9	<0.0001
A07-1152	62	7	8.12	0.0044	30	24	12.00	0.0005
Total	109	10	17.48	<0.0001	58	41	28.41	<0.0001

[a] At least 47 plants per progeny were scored (misscoring $p \leq 0.05$).

Remaining 30 bp sequence did not show any homology to the soybean genome, maybe due to the small size of the fragment. A part of fragment *Stu*I-1 showed identity with chromosome Gm06 (Table 2). This suggests that there are two T-DNA insertion sites in the transformed plants. Detailed analysis of insertion sites revealed that in either location T-DNA did not land in any predicted gene (http://www.phytozome.net/). On Gm18, T-DNA landed 981 bp away from nearest predicted gene GDP-fucose protein O-fucosyltransferase. Insertion site on Gm06 is 15,829 bp away from nearest predicted gene GAG-POL-related retrotransposon. Although the T-DNA insertions are not in genes, the possibility that they affect promoter elements cannot be ruled out.

3.3. Segregation distortion

Severe deviation from the expected 3 fertile:1 sterile ratio for a monogenic trait was observed in the two mapping populations (Table 3). χ^2 test showed that the collective probability (*p*) value for both populations was <0.0001. The observed ratios appeared to follow expectations for a two-gene model, with linkage between the two gene copies. For two-gene model, however, 25% $F_{2:3}$ families are expected to segregate in a 3:1 ratio. We failed to observe a single $F_{2:3}$ family that segregated in a 3:1 ratio. The $F_{2:3}$ generation showed preference of homozygous fertile over both heterozygous fertile and homozygous sterile plants, with a collective *p* value for both populations <0.0001 (Table 3). To map the distorted region, markers that mapped close to the *st_A06-2/6* gene were tested for segregation distortion. Ten of the markers near the gene showed distorted segregation ratios covering a genetic length of 69.2 cM, which is equivalent to a physical length of about 40 Mbp (Table 4; Fig. 4). Markers from the opposite arm of Gm01 and from other chromosomes were tested to see if the segregation distortion was restricted to the chromosomal region near the *st_A06-2/6* gene. Markers distant from the *st_A06-2/6* region showed normal segregation ratios of 1:2:1 (Table 4; Fig. 4). Two markers from MLG K were tested for possible segregation distortion. Both markers showed normal segregation ratios in the $F_{2:3}$. These results suggested that segregation distortion is associated with the *st_A06-2/6* locus. Whether *st_A06-2/6* itself or one or more distinct factors are involved in this distorted segregation is not known.

Table 4
Mapping of the segregation distortion region containing *st_A06-2/6*. Markers are selected from the *st_A06-2/6* region and MLG K. Markers showing segregation distortion are underlined.

Marker	MLG	St St	St st	st st	χ^2(1:2:1)	*p*-Value
Sat_413	D1a	37	50	25	3.86	0.1454
Satt184	D1a	37	51	15	9.41	0.0091
Satt531	D1a	40	57	9	18.74	<0.0001
st_A06-2/6	D1a	58	41	10	48.96	<0.0001
Satt320	D1a	43	57	14	14.75	0.0006
Satt342	D1a	42	54	17	11.28	0.0035
Satt532	D1a	42	56	14	14.00	0.009
Satt502	D1a	41	56	15	12.07	0.0024
Sat_346	D1a	39	51	22	6.05	0.0485
Satt603	D1a	36	51	16	7.78	0.0205
Satt515	D1a	37	58	17	7.29	0.0262
Sat_201	D1a	40	55	18	8.65	0.0133
Satt402	D1a	33	51	23	2.10	0.3494
Satt588	K	36	62	19	5.28	0.0714
Satt178	K	24	49	39	5.77	0.0559

4. Discussion

In this study, we investigated if the MSFS mutant is due to T-DNA insertion during *Agrobacterium*-mediated soybean transformation. By cloning the insertion sites using the genome walking technique, we were able to show that T-DNA insertions are on chromosome Gm18 and Gm06. The MSFS locus was mapped to Gm01, suggesting no direct association between the mutant phenotype and insertion of a T-DNA molecule. Most likely, appearance of the MSFS mutant plant was due to a spontaneous mutation event or resulted from failed integration of a T-DNA molecule in the *st_A06-2/6* gene [23].

The MSFS mutant was originally identified in a transformation study in soybean. In the progeny of the original transgenic plant, the sterility gene segregated in a 3:1 ratio in the Williams background. Fourteen plants obtained in the T_2 generation showed segregation of 3 homozygous fertile:8 heterozygotes:3 sterile. To validate our results, we studied segregation in large number of heterozygous individuals. Heterozygotes segregated into 138 fertile to 49 sterile plants, a ratio close to 3:1. This analysis suggested monogenic inheritance of this sterility gene in Williams background. However, when the MSFS mutant was crossed to Minsoy to develop F_2 mapping populations, a fertile to sterile ratio of 10.9:1 was observed

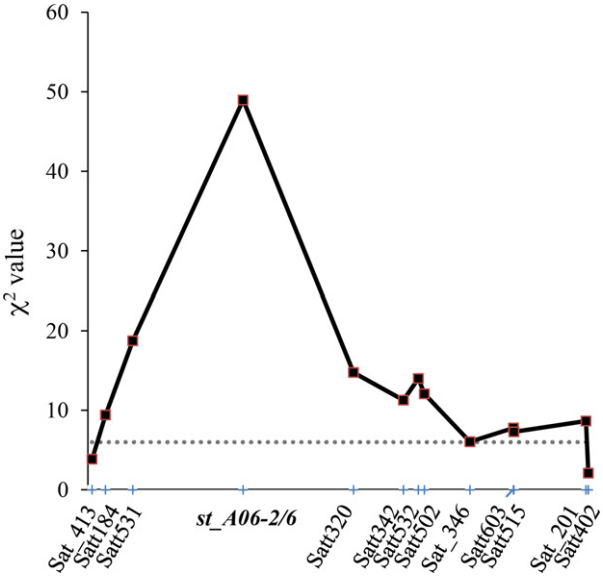

Fig. 4. Genomic region that showed distorted segregation with respect to the expected 1:2:1 F_2 ratio. The dotted line represents the critical χ^2 value at 2 degrees of freedom and $p = 0.05$. Physical locations of the markers were determined using the soybean genome sequence [19]. Physical location of *st_A06-2/6* is not absolute. It is placed between two flanking markers based on the genetic linkage map (Fig. 2).

Table 5
Expected number of F_2 plants in all fertile, segregating fertile and sterile plants, and sterile classes, based on different recombination fraction (θ) values between two redundant fertility genes. Bottom row of the table represents observed number of F_2 plants in different classes.

Recombination fraction (θ)	Expected number of F_2 plants		
	All fertile	Segregating	Sterile
0	27.3	54.5	27.3
0.1	32.4	54.5	22.1
0.2	37.1	54.5	17.4
0.3	41.1	54.5	13.4
0.4	44.7	54.5	9.8
0.5	47.7	54.5	6.8
Observed	58	41	10

(Table 3). One possible explanation of this segregation distortion may be the presence of a duplicated chromosome segment containing the fertility gene in the Minsoy background. In Drosophila, tandem duplication of a chromosomal segment containing the *Sd* locus was shown to be responsible for segregation distortion [24]. The observed F_2 ratio 10.9:1 is close to the 15:1 ratio for two functional genes in Minsoy and only one functional gene in Williams. However, the χ^2 test on the $F_{2:3}$ generation, did not conform to the expected 7 non-segregating fertile:8 segregating fertile (4 segregating in a 15:1 ratio for both genes and 4 segregating in a 3:1 ratio for one of the two genes):1 sterile ratio ($p = 0.0041$) for unlinked duplicated genes (Table 5). If indeed there were linkage between the two genes, the proportion of the heterozygous plants would only have increased. In addition, among the 41 heterozygous F_2 plants, none showed 3:1 ratio in the $F_{2:3}$ generation (Table S1). If two independent genes were controlling fertility, at least 1/4 of the 41 heterozygous F_2 plants should have segregated in a 3:1 ratio. Thus, it is most unlikely that two functional copies of the *st_A06-2/6* gene can explain the observed distorted segregation.

Recombination values ranging from 0% to 50% were used to calculate expected genotypic frequencies of the F_2 population (Table 5) [25]. Expected frequency of homozygous fertile was never more than that of the heterozygotes for any recombination value. In our study, homozygous fertile plants were about 1.41 times more frequent than the heterozygotes (Table 5). This further suggests that the altered segregation of the sterility phenotype was unlikely to have arisen from segregation of two functional linked genes from Minsoy. Segregation distortion was most extreme at or around the *st_A06-2/6* locus suggesting that sterility and segregation distortion are linked attributes (Fig. 4). In *Drosophila*, male sterility and segregation distortion are known to be controlled by a single gene [26]. It is possible that one or more Minsoy-specific factors may have contributed toward segregation distortion in a region that spans 69.2 cM. Whether Minsoy-specific functional *st_A06-2/6* gene or one or more *st_A06-2/6*-linked genes control this regional distorted segregation is yet to be determined. Mapping of addition markers in the marker-poor *st_A06-2/6* region will facilitate determining if the two traits are tightly linked.

There are several different mechanisms known to cause segregation distortion in plants. Two of the most common are 'gamete eliminator' and 'pollen killer' systems [27,28]. In the gamete eliminator system, one type of gamete is preferentially eliminated leading to a bias toward the other gamete. Both male and female gametes are affected by gamete eliminator [27]. In pollen killer, preferential elimination of one type of gamete only happens in male gametes [28]. Even if there are no affected gametes passed from the pollen side, unaffected homozygous to heterozygous ratio will be 1:1 in F_2 because of normal transmission of female gametes. With increasing frequencies of the affected gametes, the ratio will change in the favor of the heterozygotes. In the gamete eliminator system, if a type of gamete is preferentially selected in both male and female gametes, the proportion of homozygous fertile progenies may be higher than that of heterozygous progenies in F_2. For example, if instead of 50% participation of each type of gamete, the proportion of preferred male and female gametes is 72% and non-preferred male and female gametes is 28%, the expected proportion of homozygous fertiles:heterozygotes:steriles will be 0.52:0.40:0.08. These expectations very closely match with our observations (Table 5). Thus, one can expect to have more homozygous plants than the heterozygous plants as we have observed in our study. Our results are compatible with a gamete eliminator system in which gametes carrying the mutant allele have reduced viability or fertility. Other mechanisms of sterility acting post-fertilization may also play a role. Further studies are necessary to identify the specific mechanism involved in the segregation distortion in the region containing the *st_A06-2/6* locus in soybean.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2012.07.003

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