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A candidate male-fertility female-fertility gene tagged by the soybean endogenous transposon, Tgm9

Jaydeep Raval · Jordan Baumbach · Alexandrea R. Ollhoff · Ramesh N. Pudake · Reid G. Palmer · Madan K. Bhattacharyya · Devinder Sandhu

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Abstract In soybean, the *W4* gene encoding dihydroflavonol-4-reductase controls anthocyanin pigment biosynthesis in flowers. The mutant allele, *w4-m*, is characterized by variegated flowers and was evolved from the insertion of an endogenous transposable element, *Tgm9*, in intron II of the *W4* gene. In the *w4-m* mutant line, reversion of the unstable allele from variegated to normal purple flower in revertants would indicate *Tgm9*'s excision accompanied by its insertion into a second locus. We identified a male-sterile, female-sterile mutant from such germinal revertant bearing purple flowers. The objectives of our investigation were to map the sterility locus, identify candidate genes for the male-fertile, female-fertile phenotype, and then determine if sterility is associated with the insertion of *Tgm9* in the sterility locus. We used bulked segregant analysis to map the locus to molecular linkage group J (chromosome 16). Fine mapping enabled us to flank the locus to a 62-kb region that contains only five predicted genes. One of the genes in that region, *Glyma16g07850.1*, codes for a helicase. A rice homolog of this gene has been shown to control crossing over and fertility phenotype. Thus, *Glyma16g07850.1* is most likely the gene regulating the male and female fertility phenotype in soybean. DNA blot analysis of the segregating individuals for *Tgm9* showed perfect association between sterility and the presence of the transposon. Most likely, the sterility mutation was caused by the insertion of *Tgm9*. The transposable element should facilitate identification of the male- and female-fertility gene. Characterization of the fertility gene will provide vital molecular insight on the reproductive biology of soybean and other plants.

Keywords *Glycine max* · Sterility · Genetic linkage mapping · Transposon tagging

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J. Raval · J. Baumbach · A. R. Ollhoff · D. Sandhu (✉)
Department of Biology, University of Wisconsin-Stevens Point,
800 Reserve Street,
Stevens Point, WI 54481, USA
e-mail: dsandhu@uwsp.edu

R. N. Pudake · R. G. Palmer · M. K. Bhattacharyya
Department of Agronomy, Iowa State University, Ames,
IA 50011-1010, USA

Introduction

In flowering plants, normal development of male and female reproductive structures is crucial for the seed set. Germ cell formation is the result of a series of highly synchronized events controlled by a large number of genes. Genetic mutations that affect the processes of sporogenesis and/or gametogenesis can result in reproductive sterility. In soybean [*Glycine max* (L.) Merr.], several mutants affecting reproduction have been described cytologically and genetically (Palmer et al. 2004) and are available from the genetic type collection. There are several types of sterility mutations in soybean: male sterile female sterile (MSFS), male sterile female fertile, male partial sterile, and female partial sterile (Johns et al. 1981; Kaul 1988). The mutant plants that exhibit MSFS phenotype are usually classified as synaptic mutants and rarely produce seed (Kaul 1988).

An unstable mutation for flower color in soybean was conditioned by an allele at the W_4 locus named w_4-*m* (w_4-mutable; genetic type collection T322) (Palmer et al. 1990). The W_4 gene encodes dihydroflavonol-4-reductase (DFR2), and w_4-*m* arose through insertion of *Tgm9*, a 20,548-bp

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CACTA-like transposable element, into the second intron of the *DFR2* gene (Xu et al. 2010). The element excises at a high frequency from both somatic and germinal tissues (Groose et al. 1988, 1990; Palmer et al. 1989).

Study of germinal revertants was initiated using the w_4-*m* system (Palmer et al. 1989). If the reversion of the unstable allele was the result of an excision of the *Tgm9* element from the w_4-*m* allele, new mutations could arise from insertion of the excised copies of *Tgm9* into new genetic loci. Thus, new mutants could be identified just by searching progenies of germinal revertants bearing purple flowers and descended from the w_4-*m* mutable plants. For example, in a screen of a collection of 3,206 revertants, 24 independent necrotic root mutant lines and 36 independent male-sterile, female-sterile mutants were recovered (Palmer et al. 2008a, b). Mutation in all 24 necrotic mutants were allelic and mapped to molecular linkage group (MLG) G (Palmer et al. 2008b). Of the 36 MSFS mutants, 35 mapped to the *st8* region on MLG J (Palmer et al. 2008a), and one, to MLG G.

A novel MSFS mutant line (ASR-10-181) was identified from the high frequency, and late excision mutable category has been mapped in this study as follows. Four mapping populations consisting of a total 578 F_2 plants were generated by crossing Minsoy (PI 27890) × ASR-10-181. Of these, 145 MSFS plants produced no seed. One exceptional F_2 plant had two three-seeded pods and one one-seeded pod on a single node. The seven seeds from three pods were germinated, and plants were grown in greenhouse in spring 2007. Five plants were fertile and produced seed, and two plants were sterile and did not have seed, suggesting a possible reversion event in the gene for the fertility phenotype. The objectives of our investigation were to map the sterility locus, identify candidate genes for the male-fertile and female-fertile phenotype, and determine if the sterility is associated with the insertion of *Tgm9* in the sterility locus.

Material and methods

Genetic materials

Heterozygous fertile plants (*Stst*) in segregating entry (A05-221) were used as the male parent and crossed to Minsoy (PI 27890). The F_1 seeds were advanced to the F_2 generation at the University of Puerto Rico/Iowa State University soybean nursery near Isabela, Puerto Rico. The F_2 population, A06-321, that segregated for fertility/sterility was chosen as the mapping population (Table 1). We temporarily named the sterility mutant gene as *st_A06-321* based on the population name. The four $F_{2:3}$ rows were grown at the Bruner Farm near Ames, Iowa, in 2006, of which progenies of one F_2 plant were used to map the *st_A06-321* gene.

Evaluation and testing of revertants

The seven seed (revertants) on the MSFS plant from the A06-321 population were planted in the USDA greenhouse in January 2007. Seed from the five fertile plants were planted in the field in summer 2008. The fertile plants were single-plant threshed. Fifty seeds from the fertile plants were planted in the field in summer 2009, in a conventional planting, and ten seed from eight of the fertile plants from A08-88 were planted in the field in pollinator exclusion cages. Six of the eight progenies segregated for fertile and sterile plants.

DNA isolation and bulked segregant analysis

Genomic DNA was isolated following a method described earlier (Sandhu et al. 2004). Fertile and sterile bulks were created by pooling 1 μg DNA from ten homozygous fertile or ten homozygous sterile F_2 plants, respectively (Michelmore et al. 1991). Each bulk was diluted to a final DNA concentration of 50 ng/μl.

Molecular marker analysis

For simple sequence repeat (SSR) analysis, 50 ng of DNA was used for 10 μl reaction with 1× reaction buffer (10 mM Tris–HCl, 50 mM KCl, pH8.3), 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

Table 1 Chi-square test showing segregation of the *st_A06-321*gene in F_2 and $F_{2:3}$ generations in entry A06-321

Population	No. of F_2 plants		χ^2 (3:1)	*p* value	No. of $F_{2:3}$ families		χ^2 (1:2)	*p* value	No. plants within seg families		χ^2 (3:1)	*p* value
	F_	ff			FF	Ff			F_	ff		
A06-321	93	29	0.09	0.75	29	64	0.19	0.6	1,815	609	0.02	0.89

Chi-square and *p* values for FF, Ff, and ff are displayed

FF homozygous fertile, *Ff* heterozygous, *ff* homozygous sterile

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 to 3 h. The genetic linkages and distances were determined using Mapmaker 2.0 (Kosambi 1944; Lander et al. 1987). The order of markers was determined at LOD threshold of 3.0. Markers were developed using information from http://soybase.org/resources/ssr.php and http://www.phytozome.net/ (Song et al. 2004, 2010).

DNA gel blot analysis

For each sample, 10 μg of genomic DNA was digested with appropriate restriction enzyme (*Hin*dIII and/or *Eco*RI) and electrophoretically separated on 0.8 % agarose gel as previously described (Sandhu et al. 2004). DNA gel was blotted onto nylon membrane (GE Water & Process Technologies, Trevose, PA, USA) using capillary action of 0.4 M NaOH overnight at room temperature (Sandhu et al. 2004).

Probe preparation, hybridization, and autoradiography

The 5′ end of *Tgm9* was PCR amplified using primers 1F (5′ CGTAGTATTTAAGTCGGTTATC3′) and 1R (5′ GGCCAAAATAATAGGAAAGTAG3′) and then gel purified. About 80 ng DNA was labeled with 50 μCi of (α^{32}P)-dATP (Feinberg and Vogelstein 1983). Hybridization was performed in 10 ml of hybridization buffer (50 % formamide, 1 % sodium dodecyl sulfate (SDS), 1 M NaCl, 5× Denhardt's, 100 μg/ml Herring sperm DNA), incubated at 42 °C for 16–18 h in a hybridization rotisserie oven. Blots were washed with 2× saline sodium citrate (SSC) for 5 min at 42 °C, followed by 0.2× SSC, 0.1 % SDS solution at 65 °C in for 45 min, and then once more at 65 °C in 0.1 SSC×, 0.1 % SDS for 45 min. Blots were exposed to X-ray films for 3 to 7 days.

Results

Genetic mapping of the *st_A06-321* gene

To determine the genetic location of the *st_A06-321* gene, we used 600 SSR markers covering the entire soybean genome on the fertile and sterile bulks. Satt414 showed polymorphism between the fertile and sterile bulks. Satt414 maps on MLG J (Song et al. 2004). Thirty-four SSR markers in the vicinity of Satt414 were analyzed for polymorphisms between the parents. Of these, 12 markers showed polymorphism. Polymorphic markers were evaluated for the entire segregating $F_{2:3}$ population. Analysis of the marker data showed that *st_A06-321* is flanked by BARCSOYSSR_16_428 and BARCSOYSSR_16_430. The closet marker, BARCSOYSSR_16_428 was mapped 0.4 cM away from the gene (Fig. 1). Using the soybean genome sequence (www.phytozome.net) (Schmutz et al. 2010), we physically mapped the SSR primers of the *st_A06-321* region. The *st_A06-321* region between BARCSOYSSR_16_0428 and BARCSOYSSR_16_0430 markers is about 62 kb (Fig. 1). We identified only five

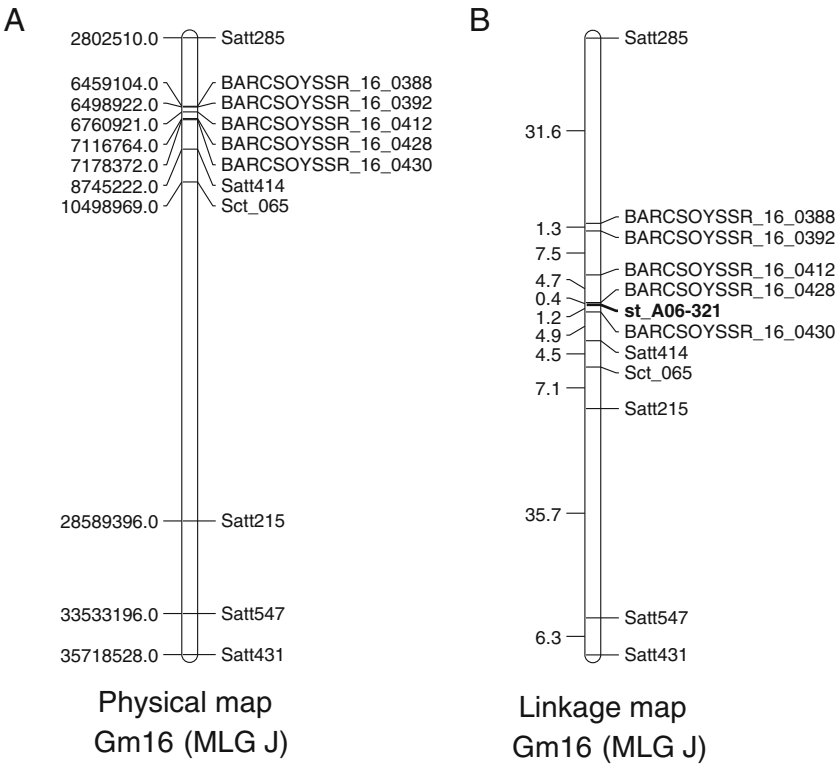

Fig. 1 Sequence-based physical and genetic linkage maps of the soybean chromosome Gm16 (MLG J) showing locations of SSR markers close to the male-sterile female-sterile locus *st_A06-321*. **a** Sequence-based physical map of Gm16 (Schmutz et al. 2010). **b** Genetic map position of the MSFS mutant gene from entry A06-321. Physical distances are shown in base pairs, and genetic distances are shown in centimorgans

Table 2 Genes located in the *st_A06-321* region

Gene	Putative protein	Homolog gene ID	Identities (%)	*E* value	Predicted function
Glyma16g07850.1	DNA/RNA helicase MER3/SLH1; DEAD-box superfamily	822395 RCK	75	0.0	Meiotic recombination and cytokinesis
Glyma16g07870.1	Gluteradoxin	834035 AT5G40370	72	$1e^{-57}$	Catalyzing disulfide bond reduction
Glyma16g07890.1	Unknown	5938778	43	$5e^{-92}$	Transcription related
Glyma16g07910.1	Polynucleotidyl transferase, ribonuclease H domain	8281016	23	$4e^{-28}$	Cleaves the RNA strand of an RNA/DNA complex
Glyma16g07920.1	Histone deacetylase	8284435 RCOM_1469870	48	$3e^{-129}$	Histone deacetylase

Putative proteins encoded by five genes that are flanked by BARCSOYSSR_16_0428 and BARCSOYSSR_16_0430 markers on soybean chromosome 16 (MLG J) are shown

predicted genes from the *st_A06-321* region, one of which may be the candidate gene controlling male-fertility and female-fertility phenotype in soybean (Table 2).

Transcriptomics analysis of these five candidate genes using Solexa/Illumina RNA-seq data (Joshi et al. 2012) revealed that *Glyma16g07850.1* expresses selectively in the apical meristem and root tip (Supp. Fig. S1). Due to unavailability of comprehensive RNA-seq data for different reproductive stages in soybean, it was not possible to present conclusive evidence for or against any of the five genes to be the putative gene of interest. However, expression in the apical meristem and root tip suggests the involvement of *Glyma16g07850.1* encoding a DNA/RNA helicase/DEAD-box protein in the cell cycle.

Evaluation of germinal revertants

The mutant MSFS plants do not set any seeds. Seven seeds, presumably developed from germinal reversion events, were harvested from three pods developed on a single branch of an MSFS plant from the mapping population A06-321 and grown in the greenhouse. Five of these seeds produced fertile plants (Table 3). The five fertile plants were progeny tested in the field in the summer of 2007. One plant was homozygous fertile, and the other four segregated in a 3:1 fertile/sterile ratio (Table 3).

The 2009 field study consisted of tractor- and hand-planted progeny rows of $F_{2:3}$ A08-88 grown in pollinator exclusion cages. The number of seeds (revertants) on the sterile plants was recorded for all progeny rows (Table 4). Different families varied in their reversion rates. The A08-88 family showed a very high rate of reversion (72.7 and 75 %) compared to the cumulative reversion rate of 11.8 % of all other families (Table 4).

Association between transposon and sterility phenotype

The MSFS mutant trait in the entry A06-321 was identified from screening of a collection of 3,206 germinal revertants with purple flowers. If the sterility phenotype developed from *Tgm9* insertion, it would provide a means of cloning the *st_A06-321* gene for fertility. To investigate if transposon insertion led to the loss of fertility function in the MSFS mutant, we conducted DNA gel blot analysis to study the association between the *Tgm9* insertion and sterility phenotype. We grew eight $F_{2:5}$ families (A09-1-26, A09-1-27, A09-70-1, A09-70-2, A09-115-5, A09-115-6, A09-133-4, and A09-133-5) descended from a fertile F_2 plants and tested these $F_{2:5}$ families for fertility. In this preliminary analysis, only 12 plants of each $F_{2:5}$ family were grown. Three of eight families, A09-115-5, A09-115-6, and A09-133-4, segregated for the trait; however, only A09-133-4 displayed a 3:1 fertile/sterile segregating ratio. Progeny testing of the fertile plants indicated a perfect 1:2 homozygous/heterozygous fertile segregating ratio. We selected this

Table 3 Investigation of the seeds developed in a single branch of an MSFS plant of the mapping population A06-321

Year	No. of plants	Phenotype		Progeny test	
		Fertile	Sterile	All fertile	Segregating for fertile and sterile phenotypes
2006 (greenhouse)	7[a]	5	2		
2007 (field)	5			1	4[b]

[a] Seeds harvested from three pods in a single branch of an MSFS plant of the $F_{2:3}$ mapping population, A06-321

[b] The combined segregation of the four families was 116:41 fertile/sterile plants. χ^2 (3:1)=0.10; p=0.74

Table 4 Extent of possible reversion event at the *st_A06-321* locus

	All families (except families derived from A08-88)	Families derived from A08-88	
		Field	Pollinator exclusion cages
Total no. of sterile plants	609	11	8
Without seed	537	3	2
With seed	72	8	6
Percent with seed	11.8	72.7	75
Total no. of seeds on sterile plants	277	99	96
No. of seeds per sterile plant	3.8	12.4	16

family and performed DNA gel blot analysis using a probe designed from *Tgm9* (Xu et al. 2010). Data showed a perfect association between the presence of the transposon with the sterility phenotype (Supp. Fig. S2).

DNA analysis of an additional 88 lines from the A09-133-4 family revealed that there is a close association between the transposon and the fertility and sterility phenotypes. We observed two, one, and no copies of *Tgm9* among the sterile, fertile heterozygous, and fertile homozygous plants, respectively (Fig. 2). These results are consistent with sterility phenotype being due to the insertion of *Tgm9*.

Discussion

In this study, we set out to fine map the *st_A06-321* gene in the soybean genome and to investigate if the sterility phenotype correlates with insertion of the endogenous transposable element *Tgm9* in the *st_A06-321* region. *st_A06-321* shows monogenic inheritance and mutation in the gene results in male sterility and female sterility. As both micro- and megagametogenesis are affected, action of the *st_A06-321* gene product, most likely, precedes the differentiation of male and female reproductive organs. In soybean, stamen and carpel differentiation take place 20–15 days before flowering (Kato et al. 1954). The first appearance of flower is in the form of knob-like primordium in the axil of a bract (Guard 1931; Miksche 1961). First, five calyx lobes develop and bases of the lobes broaden to form calyx tube (Guard 1931). The primordia of the petals are produced inwards and alternate with lobes of the calyx that is followed by the primordia of two alternating rings of stamens (Guard 1931). Followed by the development of stamens, a single primordium of the carpel appears. Development of microsporocyte precedes the development of megasprocyte (He et al. 1979).

We mapped the *st_A06-321* gene to the *st8* region on chromosome Gm16 (MLG J) (Kato and Palmer 2003; Palmer et al. 2008a; Slattery et al. 2011). The location of the *st_A06-321* in relation to the linked markers indicated that *st_A06-321* and *st8* are either allelic or are two tightly linked genes. Complementation tests will be required to confirm either of these possibilities.

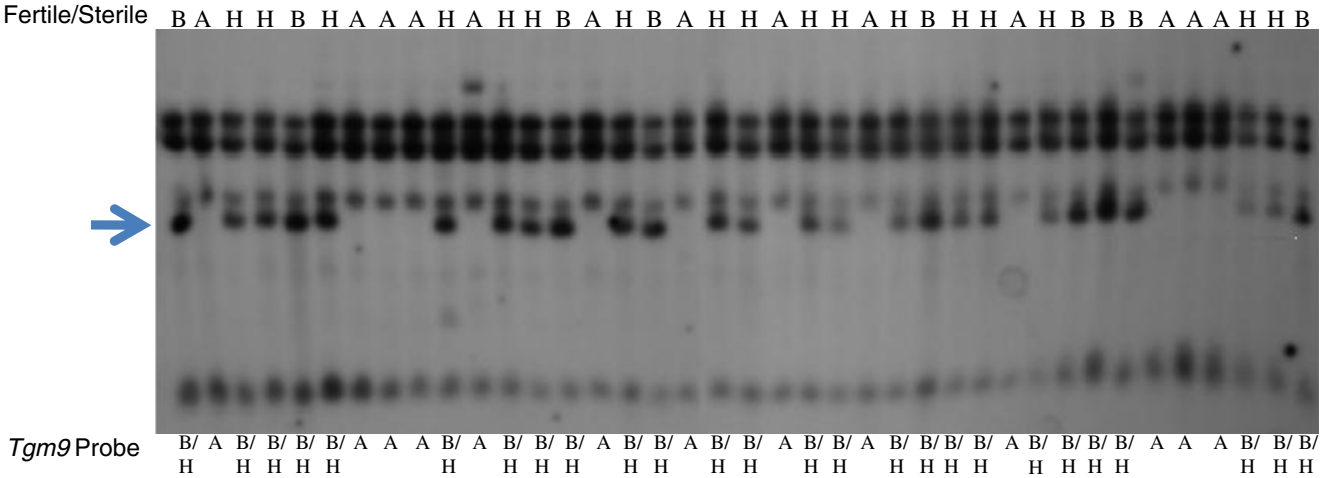

Fig. 2 DNA gel blot analysis of plants segregating for fertility and sterility phenotypes. The 5′ end of the *Tgm9* transposon was used as a probe. Plants were scored for fertility and sterility; and fertile plants were progeny tested to score homozygous fertile and heterozygous plants. Fertility/sterility phenotype is shown *on the top* of the gel. *A* homozygous fertile, *B* homozygous sterile, *H* heterozygous. The *arrow* represents a band that is missing in all the homozygous fertile plants and is always present in homozygous sterile plants. Scoring for that band is written *at the bottom of the gel*. *B*/*H* band present, *A* band missing

The *st_A06-321* gene is flanked by BARCSOYSSR_16_0428 and BARCSOYSSR_16_0430, which are about 62 kb apart. Only five predicted genes were found in this region (Fig. 1; Table 2; http://www.phytozome.net/). We annotated the five genes by searching their possible similarities to genes with known function using BLASTP program of the National Center of Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and results are presented in Table 2. The *Glyma16g07870.1* gene codes for glutaredoxins, which are small redox enzymes that use glutathione as a cofactor and are known to be involved in deoxyribonucleotide synthesis (Marmagne et al. 2007). *Glyma16g07890.1* did not show any functional annotation. Limited homology with a known protein, however, indicates its involvement in the transcription process (Matsumoto et al. 2011). The *Glyma16g07910.1* gene is predicted to code ribonuclease H that specifically degrades the RNA moiety of DNA/RNA hybrid duplexes. This enzyme may play a key role in DNA replication and repair (Town et al. 2004). The function of the predicted protein encoded by *Glyma16g07920.1* is histone deacetylation, which promotes high-affinity binding of histones to chromatin (Rabinowicz et al. 2009). The increased histone binding condenses DNA structure, preventing transcription. The *Glyma16g07850.1* gene codes for a DNA/RNA helicase/DEAD-box protein and is known to play a critical role during the cell cycle (Mercier et al. 2005). Transcriptomics analysis revealed that *Glyma16g07850.1* expresses selectively in the apical meristem and root tip, further suggesting its involvement in the cell cycle (Supp. Fig. S1).

The cell cycle is a complex process, which is involved in the development of gametes, and is mediated by numerous proteins. Deficiency in any one of these proteins may alter the further advancement of the cell cycle. This, in turn, can lead to sterile gametes. *Glyma16g07850.1* showed high identity to a gene that encodes for a Rock-N-Roller protein. The protein is a homolog of the yeast ATP-dependent DNA helicase MER3 that is required for normal meiotic crossover formation in plants and yeast (Borner et al. 2004; Mercier et al. 2005). Mutation in Rock-N-Roller, a MER3 homolog protein, exhibits defective meiosis and sterility in rice (Wang et al. 2009). Cytological studies indicated that the mutants were defective in synapsis and crossover formation during meiosis. These defects resulted in a reduction of bivalents formation at late prophase I (Wang et al. 2009). These observations suggest that loss of function of the *Glyma16g07850.1* gene due to the insertion of *Tgm9* could have led to sterility phenotype in soybean. Therefore, although involvement of the other four genes in fertility cannot be completely ruled out, *Glyma16g07850.1* is a strong candidate for the male-sterility, female-sterility *st_A06-321* gene.

In this study we have shown perfect association between the sterility phenotype and insertion of the transposon (Fig. 2). This is the first evidence to suggest that excised *Tgm9* most likely causes mutations in random soybean genes from all the mutants identified by screening revertants with purple flowers (Palmer et al. 1989). Several MSFS mutants, female partial sterile mutants, necrotic root mutants, flower color/distribution pattern mutants, chlorophyll deficient, tan-saddle seed coat, and malate dehydrogenase 1 mutants have been identified among the germinal revertants plants carrying purple flowers generated from the *w4-m* line (Chen and Palmer 1998; Kato and Palmer 2003, 2004; Palmer et al. 1989, 2008a, b; Xu and Palmer 2005a, b). This study suggests that *Tgm9* may facilitate cloning many soybean genes including the one under investigation.

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