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CANDIDATE GENE IDENTIFICATION FOR A FERTILITY LOCUS IN SOYBEAN

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ABSTRACT

Mutations in soybean genes involved in meiosis can lead to altered chromosome pairing and result in non-functional gametes causing sterility. There are several male-sterile, female-sterile mutants identified in soybean. Some of these mutants are mapped on soybean chromosomes, whereas locations of others are unknown. The objectives of this study were to find the genetic location of a male-sterile, female-sterile mutant gene, *st6*, in the soybean genome, to develop a molecular map of the region, and to identify putative candidate genes for *st6*. The *st6* gene was located on Molecular Linkage Group (MLG) B2 (chromosome Gm14) using Bulked Segregant Analysis. The gene was flanked by the markers Sat_177 and BARCSOYSSR_14_84 with a genetic distance of 8.5 cM and 1.7 cM, respectively. The region encompassed by the flanking markers is ~644 kb and there are 97 predicated genes in this region. Of these, three predicted genes, one coding for a stigma specific protein and two for microtubule associated proteins, are excellent candidates for the fertility gene. Future characterization of candidate genes should facilitate identification of the male- and female-fertility gene, which may provide vital insight on structure and function of genes involved in the reproductive pathway.

Keywords: Bulked segregant analysis, Genetic map, *Glycine max*, Molecular linkage Group (MLG), Sterility

In meiosis, synapsis is an important process for ensuring normal chromosome segregation and development of gametes. Two important classes of mutants either leading to aberrant chromosome pairing (asynaptic) or abnormal maintenance of chromosome pairing (desynaptic) have been identified in soybean (Gottschalk and Kaul, 1980a; Gottschalk and Kaul, 1980b; Koduru and Rao, 1981). Mutations in genes involved in synapsis can result in either male-sterile, female-sterile plants or male-sterile, female-fertile plants or male-fertile, female-sterile plants. In soybean, several male-sterile, female-sterile mutants have been identified and studied (Table 1) (Cervantes-Martinez *et al.,* 2009; Cervantes-Martinez *et al.,* 2007; Hadley and Starnes, 1964; Ilarslan *et al,* 1997; Jin *et al.,* 1998; Kato and Palmer, 2003a; Kato and Palmer, 2003b; Kato and Palmer, 2004; Palmer, 1974; Palmer and Kaul, 1983; Palmer *et al.,*2004; Palmer *et al.,* 2008; Slattery *et al.,* 2011). In a tissue culture study, two sterility mutants were identified in 89 families generated from cotyledonary node tissue culture (Graybosch *et al.,* 1987). One of these mutants appeared in cultivar Calland that showed male-sterile, female-sterile phenotype and was named as Calland TC (Ilarslan *et al.,* 1997). Inheritance studies revealed that two redundant recessive genes (*st6* and *st7*) control sterility in the mutant (Ilarslan *et al.,* 1997). Calland TC was designated *St6st6st7st7* and assigned the genetic type collection number T331H. The mutant plant showed abnormalities in chromosome segregation which resulted in non-viable pollen grains. The sterility in Calland TC was ascribed to desynapsis (Ilarslan *et al.,* 1997).

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The objectives of this study were to find the genetic location of the *st6* gene in the soybean genome, to develop a molecular map of the region, and to identify putative candidate genes for *st6*.

MATERIALS AND METHODS

Plant materials

A mapping population (A10-121), consisting of 63 F_2 plants, was generated by crossing cultivar Minsoy (PI 27890) (*St6St6*) with the sterility mutant line T331H (*St6st6*), using standard soybean crossing techniques at the Bruner Farm near Ames, Iowa (Fig. 1; Table 2). A segregating F_2 population was selected by classification of fertile and sterile plants. The fertile F_2 plants were threshed separately. Each fertile F_2 plant was progeny tested by planting 50 $F_{2:3}$ descendants. Segregation of fertile and sterile plants, or all fertile plants, in each $F_{2:3}$ line was recorded at maturity to determine each F_2-plant genotype (Fig. 1).

Bulked segregant analysis (BSA)

To find the location of the *st6* gene, BSA was used (Michelmore *et al.,* 1991). For the mapping population, fertile and sterile bulks for BSA were prepared from randomly selected DNA samples of either eight homozygous fertile (fertile bulk) or eight sterile (sterile bulk) F_2 plants (Fig. 1). DNA bulks were prepared by pooling 1 µg DNA from each selected F_2 plant. Each bulk was diluted to a final concentration of 50 ng DNA/µl.

Molecular marker analysis

For SSR analysis, 30 ng DNA was used as the template

Table 1. Male-sterile, female-sterile mutants in soybean with gene symbols and/or locations

Gene	*Phenotype*	*Molecular Linkage Group*	*Reference*
st2	Asynaptic male and female sterile	Not known	(Hadley and Starnes, 1964)
st3	Asynaptic male and female sterile	Not known	(Hadley and Starnes, 1964)
st4	Desynaptic male and female sterile	Not known	(Palmer, 1974)
st5	Desynaptic male and female sterile	F	(Palmer and Kaul, 1983)
st6st7	Desynaptic male and female sterile	Not known	(Ilarslan *et al.,*1997)
st8	Desynaptic male and female sterile	J	(Kato and Palmer, 2003b; Palmer *et al.,* 2008; Slattery *et al.,* 2011)

Table 2. Segregation patterns, Chi-square values and P-values for the A10-121 population

Population	No. F_2 plants		χ^2 (3:1)	P	No. F_2 families		χ^2 (1:2)	P
	Fertile	Sterile			Homozygous	Heterozygous		
A10-121	28	35	31.37	<0.01	8	18	0.08	0.78

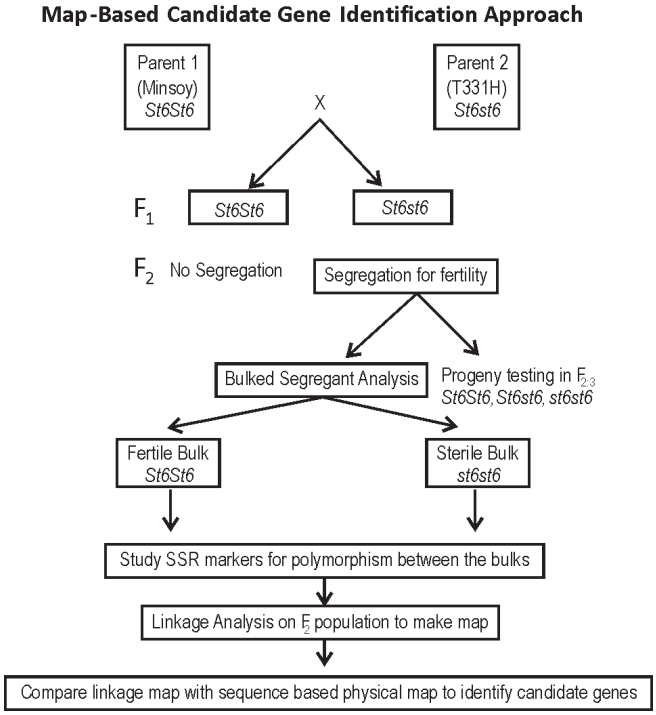

Fig. 1. Flowchart showing sequence of steps used in candidate gene identification approach for the st6 gene in soybean.

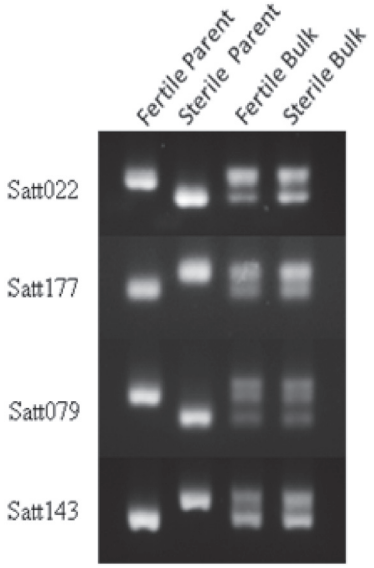

Fig. 2. Bulked segregant analysis to locate the st6 gene on a soybean chromosome. All the SSR markers show similar patterns in the fertile and sterile bulks suggesting no association between the sterility gene and the marker. Fertile parent, Minsoy (St6St6); sterile parent, T331H St6st6; fertile bulk, bulk of 8 homozygous fertile F¬2 plants; sterile bulk, bulk of 8 sterile F¬2 plants.

in a 10 µl reaction containing 1x 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., Taunton, MA). The PCR conditions consisted of: 94° C for three minutes, 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 ten minutes at 72°C. The PCR products were separated on a 4% agarose gel at 150 v for one to three hours. 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 http://soybase.org/resources/ssr.php (Song *et al.,* 2010; Song *et al*., 2004). Software "Map Chart" was used to create maps (Voorrips, 2002).

RESULTS AND DISCUSSION

Segregation in the population

All F_1 plants were fertile for the cross. Self-pollination of

Table 3. Genes present in the *st6* region

Predicted Gene	*Location (bp)*	*Predicted Protein/ Function*
Glyma14g01660	960490 - 969118	Cellulose synthase
Glyma14g01670	970081 - 974666	Cellulose synthase
Glyma14g01681	978123 - 979780	Cleavage site for pathogenic type III effector avirulence factor Avr
Glyma14g01691	982114 - 985375	NOL1/NOP2/sun family
Glyma14g01700	988926 - 990297	no function
Glyma14g01710	990990 - 992843	no function
Glyma14g01720	996228 - 999053	Protein kinase domain, Legume lectin domain
Glyma14g01730	999532 - 1004650	Cytidylyltransferase
Glyma14g01740	1007698 - 1012431	NUDIX domain
Glyma14g01750	1013308 - 1014336	no function
Glyma14g01760	1016459 - 1017549	no function
Glyma14g01765	1021713 - 1022498	no function
Glyma14g01770	1025668 - 1027469	no function
Glyma14g01780	1027893 - 1030449	Pyridoxal-phosphate dependent enzyme
Glyma14g01790	1031918 - 1035014	Ion channel
Glyma14g01800	1041075 - 1042155	no function
Glyma14g01810	1043203 - 1047425	NLI interacting factor-like phosphatase
Glyma14g01820	1048072 - 1051254	Pectinesterase
Glyma14g01830	1055906 - 1061089	Pectinesterase
Glyma14g01840	1066563 - 1068225	Serine acetyltransferase, N-terminal
Glyma14g01850	1069699 - 1073991	Proteasome subunit
Glyma14g01860	1075615 - 1078186	PPR repeat
Glyma14g01871	1079329 - 1081198	Cytochrome P450
Glyma14g01880	1083680 - 1086440	Cytochrome P450
Glyma14g01891	1089668 - 1092808	no function
Glyma14g01900	1098549 - 1105476	ABC transporter
Glyma14g01911	1108802 - 1111422	Leucine rich repeat
Glyma14g01920	1113614 - 1123499	Transporter associated domain
Glyma14g01930	1127860 - 1134992	Zinc finger C-x8-C-x5-C-x3-H type (and similar)
Glyma14g01940	1136434 - 1139527	mTERF
Glyma14g01950	1139586 - 1141565	A2L zinc ribbon domain
Glyma14g01960	1145373 - 1148815	GRAS family transcription factor
Glyma14g01970	1148816 - 1150115	no function
Glyma14g01980	1152651 - 1158381	WRKY DNA -binding domain
Glyma14g01990	1169082 - 1172360	Zinc finger C-x8-C-x5-C-x3-H type (and similar)
Glyma14g02000	1176248 - 1179309	Protein kinase domain
Glyma14g02011	1181809 - 1184171	Protein kinase domain
Glyma14g02020	1187060 - 1191355	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain)
Glyma14g02030	1194852 - 1198417	C2 domain
Glyma14g02040	1205094 - 1211218	Kinesin motor domain
Glyma14g02050	1212132 - 1214421	Tim17/Tim22/Tim23 family
Glyma14g02060	1216786 - 1220215	Pirin C-terminal cupin domain
Glyma14g02070	1227950 - 1235072	Glycerophosphoryl diester phosphodiesterase family
Glyma14g02080	1238519 - 1240240	Leucine Rich Repeat
Glyma14g02085	1241571 - 1243064	PPR repeat
Glyma14g02090	1244561 - 1250936	Calponin homology (CH) domain
Glyma14g02100	1252813 - 1254940	ATP synthase subunit H
Glyma14g02110	1256028 - 1259399	FtsZ family, C-terminal domain, Tubulin/FtsZ family, GTPase domain
Glyma14g02120	1260343 - 1262849	SNARE domain

Glyma14g02130	1267103 - 1272395	Calcineurin-like phosphoesterase
Glyma14g02140	1274036 - 1278986	LBP / BPI / CETP family, C-terminal domain
Glyma14g02150	1279846 - 1288205	PHD-finger
Glyma14g02160	1297714 - 1302384	SAM dependent carboxyl methyltransferase
Glyma14g02170	1306245 - 1309328	Vta1 like
Glyma14g02180	1311201 - 1315063	Microtubule associated protein (MAP65/ASE1 family)
Glyma14g02191	1324856 - 1325657	no function
Glyma14g02200	1332026 - 1335108	Microtubule associated protein (MAP65/ASE1 family)
Glyma14g02210	1338958 - 1344330	Cytidylyltransferase family
Glyma14g02220	1344313 - 1350644	FKBP-type peptidyl-prolyl cis-trans isomerase
Glyma14g02230	1352505 - 1353599	no function
Glyma14g02240	1359066 - 1359834	no function
Glyma14g02260	1368644 - 1373381	ThiF family, MoeZ/MoeB domain
Glyma14g02271	1380184 - 1386403	no function
Glyma14g02281	1388043 - 1388952	Cation efflux family
Glyma14g02290	1392361 - 1392861	K-box region
Glyma14g02300	1394304 - 1396287	Leucine Rich Repeat
Glyma14g02320	1398189 - 1400904	no function
Glyma14g02330	1402394 - 1402651	Hydroxymethylglutaryl-coenzyme A reductase
Glyma14g02340	1405542 - 1408806	Glycosyl hydrolase family 9
Glyma14g02350	1417342 - 1419893	Glycosyl hydrolases family 17
Glyma14g02360	1430045 - 1431949	AP2 domain
Glyma14g02380	1433503 - 1438427	Transketolase, pyrimidine binding domain
Glyma14g02385	1441025 - 1443235	Pectinesterase
Glyma14g02391	1444527 - 1445107	no function
Glyma14g02395	1446990 - 1448540	Plant invertase/pectin methylesterase inhibitor
Glyma14g02400	1455362 - 1457112	Bacterial trigger factor protein (TF)
Glyma14g02405	1458399 - 1458929	Plant invertase/pectin methylesterase inhibitor
Glyma14g02410	1461560 - 1465210	ThiF family
Glyma14g02416	1469202 - 1472441	Bacterial trigger factor protein (TF)
Glyma14g02423	1473701 - 1474252	Plant invertase/pectin methylesterase inhibitor
Glyma14g02430	1478867 - 1482330	Aspartate/ornithine carbamoyltransferase, carbamoyl-P binding domain
Glyma14g02440	1484339 - 1484922	Protein of unknown function DUF260
Glyma14g02450	1487558 - 1492612	Alternative splicing regulator
Glyma14g02461	1496359 - 1511294	Esophageal cancer associated protein
Glyma14g02470	1516171 - 1519245	Protein of unknown function (DUF568)
Glyma14g02480	1519878 - 1524657	Cell differentiation family, Rcd1-like
Glyma14g02490	1526617 - 1528705	Double-stranded RNA binding motif
Glyma14g02495	1535742 - 1536287	Cotton fibre expressed protein
Glyma14g02500	1546292 - 1549303	Subfamily not named
Glyma14g02510	**1550924 - 1551487**	**Stigma-specific protein, Stig1**
Glyma14g02520	1551828 - 1553347	B-cell receptor-associated protein 31-like
Glyma14g02530	1554050 - 1559697	Biotin-requiring enzyme
Glyma14g02540	1564680 - 1565860	no function
Glyma14g02550	1566279 - 1574854	no function
Glyma14g02561	1576628 - 1591862	MutS domain III
Glyma14g02570	1591893 - 1596493	GDSL-like Lipase/Acylhydrolase
Glyma14g02590	1602849 - 1610880	Bromodomain

Names and predicted functions of the putative proteins encoded by 97 genes that are flanked by Sat_177 and BARCSOYSSR_14_84 on Gm14 (MLG B2) are shown. Genes of interest are shown in bold font.

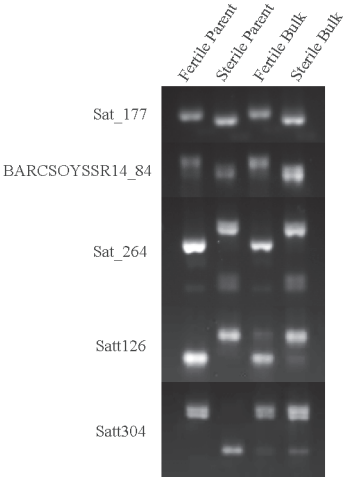

Fig. 3. Bulked segregant analysis showing identification of SSR markers linked to st6. SSR markers from Gm14 (MLG B2) show polymorphisms between the fertile and sterile bulks suggesting close association between the st6 gene and the marker. Satt304, although located on the same chromosome, does not show polymorphism between the bulks, suggesting distant location from st6. Fertile parent, Minsoy (St6St6); sterile parent, T331H St6st6; fertile bulk, bulk of 8 homozygous fertile F¬2 plants; sterile bulk, bulk of 8 sterile F¬2 plants.

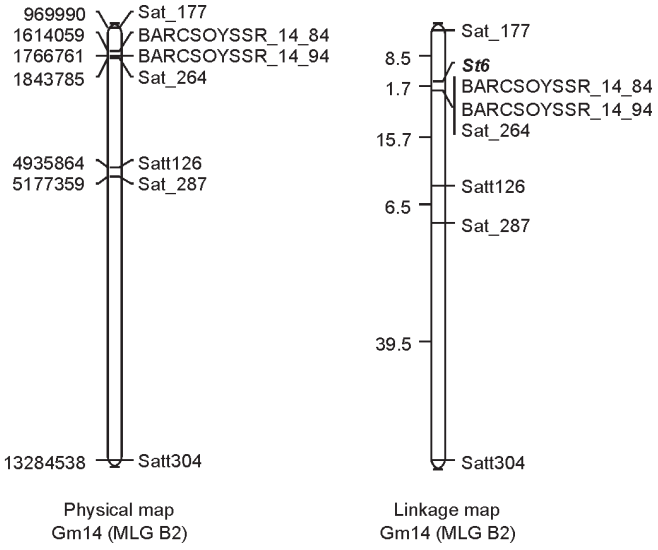

Fig. 4. Physical and genetic linkage maps of soybean chromosome Gm14 (MLG B2) showing locations of SSR markers close to the st6 gene. Physical distances are shown in base pairs (bp) and genetic distances are shown in centiMorgans (cM).

heterozygous F_1 plants from the crosses of Minsoy (*St6 St6*) x T331H (*St6st6*) resulted in deviation from segregation of 3 male-fertile :1 male-sterile plant in the F_2 generation (Table 2). Each fertile F_2 plant of the mapping population was single-plant threshed and progeny tested. The $F_{2:3}$ family segregation for the population was the expected 1 non-segregating: 2 segregating ratio (Table 2).

BSA for mapping the st6 gene

To determine the map location of the *st6* gene, we applied BSA (Fig. 1) (Michelmore *et al.,* 1991). We used over 800 SSR markers representing all twenty soybean MLGs using fertile and sterile bulks developed from the F_2 progeny. In BSA, detection of polymorphism between the bulks suggested that the marker was close to the gene of interest. Most of the markers tested did not detect polymorphisms between the contrasting bulks (Fig. 2). However, Sat_264 detected clear polymorphism between the bulks (Fig. 3). The fertile bulk displayed exactly the same fragment pattern as the fertile parent and the sterile bulk as the sterile parent (Fig. 3). Sat_264 is located on Gm14 (MLG B2). Eighteen SSR markers located close to Sat_264 were used to test polymorphism between the parents. Seven markers (Sat_177, BARCSOYSSR_14_84, BARCSOYSSR_14_94, Sat_264, Satt126, Sat_287, and Satt304) detected polymorphism. Most of these markers showed polymorphism between the bulks. However, Satt126 and Satt304 did not show clear differences between the bulks, suggesting that although these markers are present on the same chromosome, they are not closely linked with the *st6* gene (Fig. 3). For the linkage analysis, all polymorphic markers were used on the F_2 population. The *st6* gene mapped between Sat_177 and BARCSOYSSR_14_84 and was linked to each by 8.5 and 1.7 cM, respectively (Fig. 4).

In this study, the segregation pattern of the *st6* gene in F_2 did not follow 3 fertile : 1 sterile expectation. However, segregation in $F_{2:3}$ families was 1 homozygous : 2 segregating as expected for monogenic inheritance (Table 2). Distorted segregation in the F_2 may be the result of small sample size or an unknown factor involved in segregation distortion. We used BSA to locate *st6* on Gm14 (MLG B2), and molecularly mapped the gene to a 10.2 cM region.

The soybean genome has been sequenced and can be accessed at the Phytozome website http://www.phytozome.net/soybean (Schmutz *et al.,* 2010). We used sequence information for all SSR primers present on the genetic linkage map to physically locate them onto the chromosome (Fig. 4). The *st6* gene was flanked by Sat_177 and BARCOSYSSR_14_84. Physically, the region was ~644 kb (Fig. 4). We were able to use the soybean genome sequence flanked by these markers to locate putative genes present in the region. There were 97 genes identified in this region. Three genes involved in the cell cycle were particularly of interest (Table 3) (http://www.phytozome.net/soybean). The genes involved in the cell cycle could directly affect gamete formation and control fertility. One gene, *Glyma14g02510,* codes for a stigma specific protein and may affect female reproductive structures (Table 3) (Goldman *et al,* 1994). Any defect in these genes during meiosis could result in deformed

gametes, which may lead to sterility.

Future studies focusing on characterization of the candidate genes may result in cloning of the *st6* gene, which could shed light on the reproductive pathway in soybean and other plants.

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