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Molecular mapping of 36 soybean male-sterile, female-sterile mutants

R. G. Palmer · D. Sandhu · K. Curran · M. K. Bhattacharyya

Abstract Mutability of the w_4 flower color locus in soybean [*Glycine max* (L.) Merr.] is conditioned by an unstable allele designated w_4-*m*. Germinal revertants, purple-flower plants, recovered among self-pollinated progeny of mutable flower plants were associated with the generation of necrotic root, chlorophyll-deficiency, and sterility mutations. Thirty-seven male-sterile, female-sterile mutant lines were generated from 37 independent reversion events at the w_4-*m* locus. The first germinal revertant study had one male-sterile, female-sterile mutant (*st8*, T352), located on Molecular Linkage Group (MLG) J. The second study had 36 germinal-revertant derived sterility mutants descended from four mutable categories of w_4-*m*. The mutable categories were designated; (1) low frequency of early excisions, (2) low frequency of late excisions, (3) high frequency of early excisions, and (4) high frequency of late excisions. The objectives of the present study were to; (1) molecularly map the 36 male-sterile, female-sterile mutants, and to (2) compare map locations of these mutants with T352 (*st8*), identified from the first germinal revertant study. Thirty-three of 36 male-sterile, female-sterile mutations were derived from germinal reversions that were classified in the late excision categories. Thirty-five male-sterile mutants mapped to the *st8* region on MLG J. The only exception mapped to MLG G. Most likely mutants were generated through insertion of a putative transposon that was excised from the w_4 locus. The location of 36 of 37 mutations to a single chromosomal region suggests preference for sequence-dependent insertion.

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Introduction

Mutations that affect microsporogenesis and microgametogenesis causing male sterility have been described for many plant genera (Kaul 1988). In meiosis, synapsis of homologous chromosomes is a key event because it is essential for normal chromosome segregation and is critical in the regulation of chiasmata and crossover frequency. An important class of meiotic mutants, the synaptic mutants are usually subdivided into two groups; (1) asynaptic mutations, in which asynaptic mutants are defective in homolog synapsis, and (2) desynaptic mutations, in which desynaptic mutants are defective in the maintenance of synapsis after the normal synapsis process (Gottschalk and Kaul 1980a, b; Koduru and Rao 1981).

In soybean [*Glycine max* (L.) Merr.], meiotic mutants have been described genetically and cytologically (Palmer et al. 2004). There are 19 male-sterile, female-fertile lines and 6 male-sterile, female-sterile (synaptic) mutant lines in the soybean Genetic Type Collection. Several meiotic mutants have been mapped on the Classical Linkage Map (Palmer et al. 2004) and by inference to the Molecular Linkage Map (Song et al. 2004; http://soybase.org/resources/ssr.php).

An unstable mutation for anthocyanin pigmentation in soybean was conditioned by an allele at the w_4 locus named w_4-*m* (w_4-mutable). This mutant line was added to the Genetic Type Collection and assigned T322 (Palmer et al. 1990). Thus far, no molecular evidence is available to confirm that the w_4-mutable phenotype in soybean is produced by a transposable element(s). Collectively, however, the data strongly suggest that a transposable element system is producing the unstable flower phenotype (Xu and Palmer 2005b).

Two germinal revertant studies were initiated using the w_4-*m* system. The hypothesis was that the occurrence of new mutations would be maximized by searching among progenies of germinal revertant (wild-type; purple flower) plants descended from mutable plants. If the reversion of the unstable allele is the result of an excision of the element from the w_4-*m* locus, new mutations might be detected among the progenies of these revertants. Such mutants would be expected if the excised element were to insert at a second locus.

The first germinal revertant study generated mutants that included chlorophyll deficiency/malate dehydrogenase nulls, root necrosis, female partial sterility, complete male and female sterility, and flower color/distribution pattern plants (Palmer et al. 1989). Genetic studies have shown that the chromosomal region containing tan-saddle seed coat (k_2) locus, the mitochondrial localized, nuclearly encoded malate dehydrogenase 1 (*Mdh1*) locus, and the chlorophyll-deficient foliage (*y20*) locus, is very unstable (Chen and Palmer 1998a). A total of 31 independently derived mutants have been reported from this chromosomal region and were mapped to Molecular Linkage Group (MLG) H (Xu and Palmer 2005a). The three independently derived necrotic root mutants from the first germinal revertant study were allelic and were classified as disease lesion mimic mutants. These mutant lines showed an increased tolerance to root-borne infection of the fungal pathogen *Phytophthora sojae* (Kosslak et al. 1996). They were designated *rn1* (Ames 1), *rn1* (Ames 2), and *rn1* (Ames 3) and assigned Genetic Type Collection numbers T328, T329, and T330, respectively (Kosslak et al. 1997). The female partial-sterile 1 mutant (*Fsp2*, T364), female partial-sterile 2 mutant (*Fsp3*, T365), female partial-sterile 3 mutant (*Fsp4*, T366), and female partial-sterile 4 mutant (*Fsp5*, T367), were

located on MLGs C2, A2, F, and G, respectively (Kato and Palmer 2004). The complete sterility mutant was designated st_8 st_8 and assigned Genetic Type Collection number T352 (Palmer and Horner 2000), and mapped to MLG J (Kato and Palmer 2003). The two flower color/distribution pattern mutants were dilute purple flower (T321, w_4-*dp*) (Palmer and Groose 1993), and pale flower (T369, w_4-*p*) (Xu and Palmer 2005a).

The second germinal revertant study generated a few chlorophyll deficiency mutants. However, a total of 24 independent germinal reversion events generated 24 necrotic root mutant lines. These 24 mutant lines, plus three previously identified necrotic root mutants from the first germinal revertant study, and three additional necrotic root mutants, were allelic and were mapped to MLG G (Palmer et al. 2008). In addition, a total of 36 independent germinal reversion events generated 36 male-sterile, female-sterile mutant lines. The objectives of the present study were to; (1) molecularly map the 36 male-sterile, female-sterile mutants, and to (2) compare map locations of these mutants with T352 (*st8*), identified from the first germinal revertant study.

Materials and methods

Plant materials: generation of mutants

Six mutable lines representing four mutable categories were used in the second germinal revertant study. Two mutable categories did not contain male-sterile, female-sterile plants among self-pollinated progeny of the germinal revertant plants. The four mutable categories, number of germinal revertant entries, and the number and percentage of male-sterile, female-sterile phenotypes are given in Table 1. For comparison, the number and percentage of necrotic root phenotypes are given in Table 1.

Plant materials: cross-pollinations for mapping

Cultivar Minsoy (PI 27890; St_8 St_8) female parent, was crossed with fertile plants ($St_?$ $St_?$ or $St_?$ $st_?$) derived from self-pollination from each of the 36 unknown male-sterile, female-sterile mutant lines using standard soybean crossing techniques at the Bruner Farm near Ames, Iowa. Fertile progeny from self-pollination of $St_?$ $st_?$ plants were in the genotypic ratio of 1 $St_?$ $St_?$:2 $St_?$ $st_?$. 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. All F_1 plants were fertile as expected. The F_2 seeds were planted at the Bruner Farm. At flowering, segregation for fertile and sterile plants was recorded among F_2 families that were descended from self-pollination of heterozygous F_1 plants.

Table 1 Generation, from w_4-mutable, of germinal revertant (purple flower) male-sterile, female-sterile phenotypes, and necrotic root phenotypes by mutable category, and number and percentage of mutant phenotypes per mutable category

Mutable category[a]	Mutable line designation	No. of entries	No. male-sterile female-sterile phenotypes	% male-sterile female-sterile phenotypes	No. of entries	No. necrotic root phenotypes	% necrotic root phenotypes
Low frequency, late excision	ASR-3	961	14	1.46	961	0	0
High frequency, early excision	ASR-6	410	0	0	410	14	3.41
Low frequency, early excision	ASR-7	236	1	0.42	236	1	0.42
Low frequency, early excision	ASR-8	294	0	0	294	0	0
Low frequency, early excision	ASR-9	747	2	0.27	747	1	0.13
High frequency, late excision	ASR-10	558	19	3.41	558	8	1.43
Total		2,502[b]	36	1.44	1,951[c]	24	1.23

[a] Mutable category based upon flower phenotype. A total of 50 flowers from each segregating entry of the four mutable categories were classified. Five flowers from five plants were collected on 10 July and 17 July (1988) from each of the segregating entries

[b] Does not count entries ASR-6 and ASR-8 (male-sterile, female-sterile phenotype)

[c] Does not count entries ASR-3 and ASR-8 (necrotic root phenotype)

Between 40 and 48 F_2 plants were randomly sampled from each of the 36 F_2 populations segregating for the sterility phenotype. Each fertile F_2 plant in each of the 36 populations used for mapping was threshed individually. Fifty $F_{2:3}$ descendants from each fertile F_2 plant were planted the following summer at the Bruner Farm. 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.

Determination of association of new male-sterile, female-sterile mutants with *St8*

Genomic DNA of parents and 36 F_2 populations was isolated at the Iowa State University DNA facility. The F_2 populations varied in size from 40 to 48 plants. To confirm if any of the newly identified mutants were different from the previously mapped *St8* mutant, SSR markers from the *St8* region MLG J (Kato and Palmer 2003) were used to test polymorphism between the parents. Polymorphic markers were screened on all 36 F_2 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 LOD threshold of 3.0.

The mutant gene from entry ASR-7-206 was mapped to MLG G and was added to the Soybean Genetic Type Collection as T377H. A gene symbol has not been assigned because allelism tests with known male-sterile, female-sterile mutants have not been done.

DNA isolation and pooling for bulk segregant analyses (BSA)

Based upon our results, we used BSA (Michelmore et al. 1991) to map the mutated gene in mutable line ASR-7 (entry ASR-7-206) (Table 2). Fertile and sterile bulks for the BSA were prepared from randomly selected DNA samples of either (1) ten homozygous fertile (Fertile Bulk) or (2) ten sterile (Sterile Bulk) $F_{2:3}$ families. DNA bulks were prepared by pooling 1 μg DNA from each selected family. Each bulk was diluted to a final concentration of 50 ng DNA/μl (Michelmore et al. 1991).

Molecular marker analysis

Sequence information for developing SSR markers was obtained from Song et al. (2004) and http://soybase.org/resources/ssr.php. For SSR analysis, 30 ng DNA was used as the template 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.). The PCR conditions were as follows: 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 47°C, 1 min at 72°C; followed by 8 min at 72°C. The amplification products were separated on a 4% agarose gel.

The Mapmaker 2.0 program was used to determine genetic linkages and genetic distances (Lander et al. 1987). Marker order was determined at a LOD threshold of 3.0.

Results

Generation of mutants

In the second germinal revertant study, parents X1878 and X2717 of the w_4-mutable line (T322) had 1,227 and 996 entries, respectively, and no mutant phenotypes were observed. Cultivar Harosoy, also used as a control, had 1,000 entries and no mutant phenotypes were observed

Table 2 F_2, and $F_{2:3}$ generations for inheritance studies of Minsoy ($St_8\ St_8$) crossed to 36 unknown heterozygous male-sterile, female-sterile soybean ($St_?\ st_?$) mutants found among progeny of germinal revertants of *w4*-mutable

Parent: ASR entry no.	No. F_2 plants				No. $F_{2:3}$ families				No. F_3 plants in segregating $F_{2:3}$ families			
	Fertile	Sterile	χ^2 (3:1)	P	All fertile	Segregating	χ^2 (1:2)	P	Fertile	Sterile	χ^2 (3:1)	P
3-5	209	65	0.24	0.63	7	13	0.03	0.87	480	159	0.00	0.95
3-35	190	60	0.13	0.72	7	13	0.03	0.87	469	149	0.26	0.61
3-42	377	129	0.07	0.80	10	22	0.06	0.80	821	278	0.05	0.82
3-51	175	62	0.17	0.68	8	15	0.02	0.88	332	114	0.07	0.78
3-203	319	112	0.22	0.64	9	18	0.00	1.00	655	226	0.20	0.65
3–205	331	107	0.08	0.78	9	15	0.19	0.67	568	183	0.16	0.69
3–259	291	103	0.27	0.60	8	18	0.08	0.78	668	227	0.06	0.80
3-383	185	65	0.13	0.72	8	13	0.21	0.64	467	160	0.09	0.76
3-461	391	137	0.25	0.62	11	24	0.06	0.81	886	303	0.15	0.70
3-495	149	48	0.04	0.84	7	14	0.00	1.00	364	131	0.57	0.45
3-664	318	109	0.06	0.80	9	15	0.19	0.67	574	200	0.29	0.59
3-862	263	85	0.06	0.80	8	14	0.09	0.76	439	149	0.04	0.85
3-871	182	67	0.48	0.49	6	14	0.10	0.75	525	173	0.02	0.90
3-961	194	62	0.08	0.77	8	15	0.02	0.88	466	161	0.15	0.70
7-206	326	112	0.08	0.78	9	16	0.08	0.78	603	212	0.45	0.50
9-727	187	59	0.14	0.71	7	15	0.02	0.88	446	152	0.06	0.81
9-734	288	100	0.12	0.73	8	18	0.08	0.78	677	221	0.07	0.79
10-43	260	91	0.16	0.69	7	13	0.03	0.87	474	155	0.04	0.84
10-100	172	54	0.15	0.70	10	19	0.02	0.90	343	110	0.12	0.72
10-121	292	101	0.10	0.75	10	17	0.17	0.68	389	140	0.61	0.44
10-122	303	97	0.12	0.73	9	21	0.15	0.70	335	109	0.05	0.83
10-152	194	60	0.26	0.61	7	16	0.09	0.77	341	117	0.07	0.79
10-194	226	72	0.11	0.74	11	19	0.15	0.70	462	162	0.31	0.58
10-236	228	72	0.16	0.69	8	17	0.02	0.89	327	116	0.33	0.56
10-273	317	109	0.08	0.78	9	19	0.02	0.89	348	121	0.16	0.69
10-312	309	109	0.26	0.61	8	18	0.08	0.78	507	158	0.55	0.46
10-371	318	103	0.06	0.80	8	15	0.02	0.88	516	180	0.28	0.60
10-374	256	84	0.02	0.90	9	20	0.07	0.79	609	217	0.71	0.40
10-384	271	95	0.18	0.67	10	17	0.17	0.68	643	203	0.46	0.50
10-407	285	86	0.65	0.42	12	26	0.05	0.82	704	224	0.37	0.54
10-422	187	58	0.23	0.63	8	17	0.02	0.89	605	215	0.65	0.42
10-423	222	77	0.09	0.76	8	14	0.09	0.76	523	149	2.87	0.09
10-432	269	93	0.09	0.76	9	14	0.35	0.56	588	192	0.06	0.80
10-451	190	60	0.13	0.72	10	22	0.06	0.80	611	210	0.15	0.70
10-464	301	108	0.43	0.51	9	17	0.02	0.89	572	198	0.21	0.65
10-553	187	66	0.16	0.69	11	19	0.15	0.70	714	226	0.46	0.50

(data not shown). Two mutable categories, ASR-6, with high frequency, early revertant events with 410 entries, and ASR-8, with low frequency, early revertant events with 294 entries had no male-sterile, female-sterile phenotypes (Table 1).

Only the two late revertant ASR-3 and ASR-10 designations generated sterility phenotypes with a frequency greater than 1%. The low frequency, early germinal revertant events, ASR-7 and ASR-9 designations had frequencies less than 1% (Table 1).

Cross-pollinations for mapping

All F_1 plants were fertile. Self-pollination of heterozygous F_1 plants from the crosses of Minsoy x $St_?\ st_?$ segregated about 3 fertile:1 sterile plant in the F_2 generation (Table 2). This was true for each of the 36 different populations. Each fertile F_2 plant of the 36 mapping populations was single-plant threshed and progeny tested. The $F_{2:3}$ family segregation fit the expected 1 non-segregating:2 segregating ratio (Table 2). Within segregating $F_{2:3}$

Fig. 1 Genetic linkage map of Molecular Linkage Group J showing position of a male-sterile, female-sterile mutant gene in comparison to *St8*. **a** Consensus genetic linkage map (Song et al. 2004). **b** Genetic map position of one of the male-sterile, female-sterile mutant genes (from entry ASR-3-664) mapped to MLG J. **c** Genetic linkage map of the male-sterile, female-sterile mutant gene, *St8*, from a cross of Minsoy × *St8st8* (T352H) (Kato and Palmer 2003). Distances are represented in centiMorgans (cM)
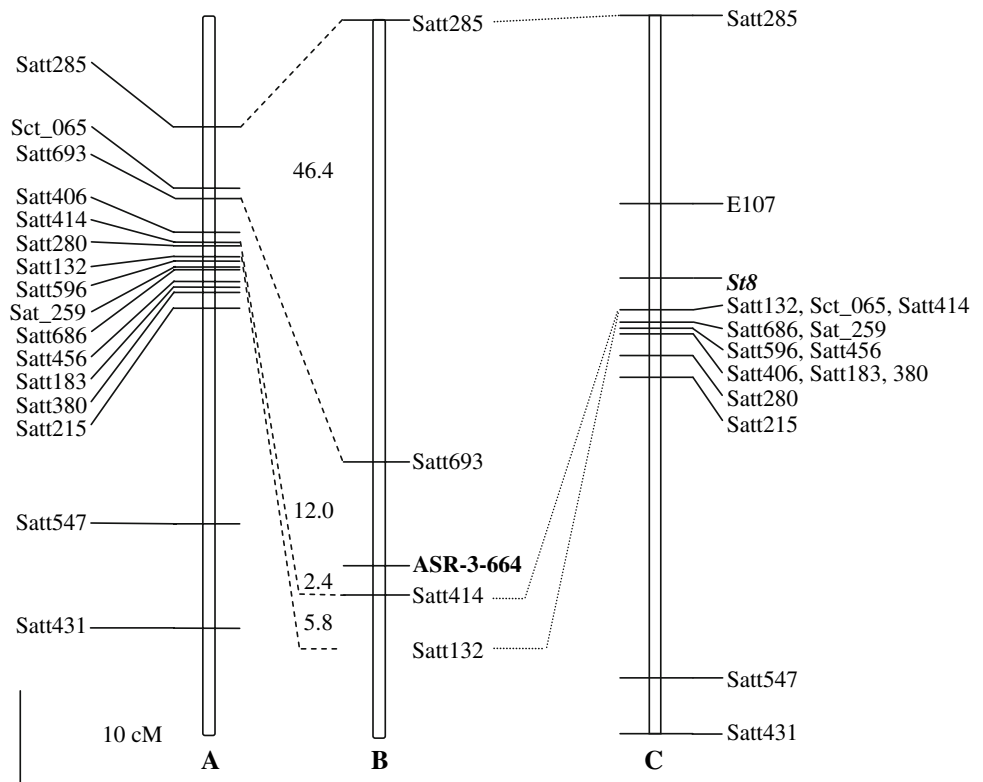
families, the expected 3 fertile:1 sterile plant ratio was observed (Table 2).

Molecular mapping

From each of the 36 different cross combinations, one segregating F_2 population for each of the male-sterile, female-sterile mutant lines was sampled for molecular mapping. Between 40 and 48 F_2 plants were randomly sampled from each F_2 population segregating for the sterility phenotype.

Initially, we investigated if any of the mutants mapped to the *St8* locus. A test for polymorphism was done using parents of the 36 F_2 populations. Of the 9 SSR markers (Satt132, Satt183, Satt215, Satt280, Satt285, Satt380, Satt406, Satt414, Satt596) mapped to the *St8* region (MLG J), four (Satt132, Satt285, Satt414, Satt693) showed clear polymorphism between the parents. These four markers were used on all 36 F_2 populations and genetic linkage maps were developed. In 35 of the 36 populations, the male-sterile, female-sterile mutant gene mapped to the *St8* region (Supplemental Table 1). An example, from entry ASR-3-664, of one of the mutant genes that mapped in the *St8* region is shown in Fig. 1. To show relative position of *St8* and other markers, the map is compared with consensus genetic linkage map for MLG J (Song et al. 2004), and map for the *St8* region (Kato and Palmer 2003) (Fig. 1). The male-sterile, female-sterile mutant gene maps near to Satt414.

Bulk Segregant Analysis (BSA) for mapping the new male-sterile, female-sterile mutant gene

In one of the populations (from entry ASR-7-206), the male-sterile, female-sterile mutant gene did not map to the *St8* region. To determine map location of the unknown male-sterile, female sterile mutant gene, we applied BSA (Michelmore et al. 1991).

We used 400 SSR markers representing all twenty soybean MLGs using fertile and sterile bulks developed from the $F_{2:3}$ families. Most of the markers tested did not detect polymorphisms between the contrasting bulks. An example of a polymorphic marker is shown in Fig. 2a. Satt309 showed polymorphism between the fertile and sterile bulks (Fig. 2b). Satt309 maps on MLG G (Song et al. 2004). Twenty-five SSR markers from the vicinity of Satt309 were analyzed for polymorphisms between the parents. Nine markers segregated in the mapping population. Analysis of the data showed that the new male-sterile, female-sterile gene is flanked by Satt163 and Satt275 (Fig. 3). Satt163 is the closest marker and is 6.1 cM away from the gene.

Discussion

To date, 31 mutants have been identified from the k_2 *Mdh1-n y20* chromosomal region in soybean (Xu and Palmer 2005a). A total of 25 *y20* mutants have been described; 18
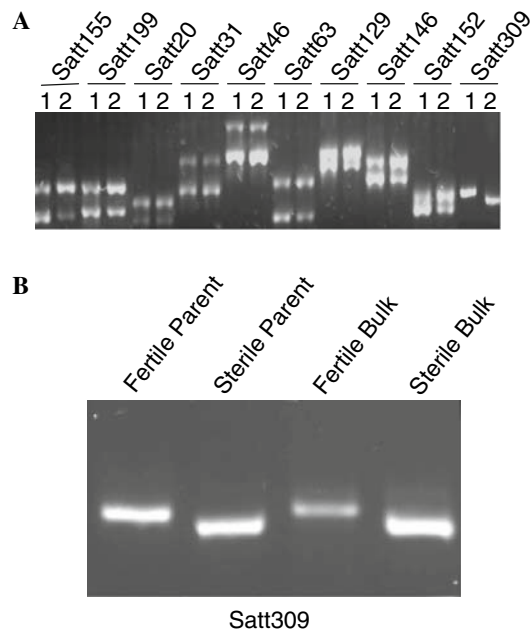
Fig. 2 Identification of SSR marker linked to a male-sterile, female-sterile mutant gene (ASR-7-206) using bulk segregant analysis. **a** SSR markers evaluated for Fertile Bulk (1) and Sterile Bulk (2). First nine markers detected no polymorphism between the two bulks, whereas Satt309 did. **b** Fertile parent, Minsoy; sterile parent, $St_?$ (from entry ASR-7-206); Fertile Bulk, bulk of 10 homozygous fertile $F_{2:3}$ families from the cross of Minsoy x $St_?$ $st_?$ (from entry ASR-7-206); sterile Bulk, bulk of 10 sterile $F_{2:3}$ families
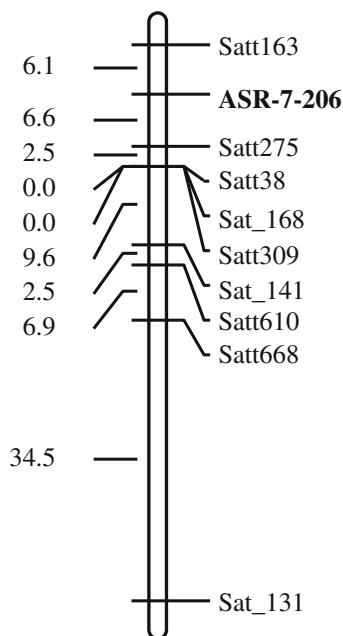
Fig. 3 Genetic linkage map of Molecular Linkage Group G showing location of a newly identified male-sterile, female-sterile mutant gene $St_?$ (from entry ASR-7-206). Distances are represented in centiMorgans (cM)

are associated with k_2, and all 25 are associated with *Mdh1-n*. Only one k_2 *Mdh1-n* *y20* mutant line (T253) has been reported (Chen and Palmer 1998b). Many of these mutants have arisen among progeny of germinal revertants of w_4-*m* (Palmer et al. 1989) or from studies designed to test the instability of the k_2 *Mdh1-n* *y20* chromosomal region (Chen et al. 1999; Xu and Palmer 2005a). Several were found as 'spontaneous mutations' (Chen and Palmer 1996), or after X-irradiation (Bernard et al. 1991). However, all k_2 *Mdh1-n* *y20* mutants are allelic. Similarly, all 24 necrotic root mutants derived from gene tagging studies, plus 6 additional necrotic root mutants are allelic (Palmer et al. 2008).

Mapping of the male-sterile, female-sterile mutants

Our strategy for this study was to molecularly map all 36 male-sterile, female-sterile mutants with SSR markers. Molecular mapping of the male-sterile, female-sterile mutant genes suggested that 35 of 36 genes are located in the *St8* region. Comparison of Fig. 1b with the consensus map for MLG J generated by Song et al. (2004) (Fig. 1a) and the map for the *St8* region generated by Kato and Palmer (2003) (Fig. 1c) confirms locations of the male-sterile, female-sterile mutants (Supplemental Table 1). Differences in genetic distances between markers may be a result of the use of smaller mapping populations. Complementation tests will be required to determine if any of the 35 mutant genes mapped to *St8*. Of the 36 mutant genes, only one mapped to a different linkage group. Male-sterile, female-sterile mutant from entry ASR-7-206 mapped to MLG G (Fig. 3).

Our alternative strategy was to use conventional cross-pollinations to do allelism tests. Molecular mapping was deemed more appropriate, that is, more efficient than to make traditional cross-pollinations. For the genetic allelism tests, the desired F_1 plants would need to have the recessive *st* allele from each parent. Because the homozygous recessive *st st* plants are male-sterile and female-sterile, fertile heterozygous *St st* plants must be used from both parents for the cross-pollinations. Heterozygous plants cannot be identified before flowering, so fertile plants are randomly selected, in segregating families (1 *St St*:2 *St st*:1 *st st*), from both parents. Thus the probability of selecting a heterozygous plant, from among the fertile plant phenotypes, is two-thirds from each parent. If one assumes equal gamete transmission of *St* and *st* alleles, then the probability of the recessive gamete being transmitted from each parent is $2/3 \times 1/2 = 1/3$. Thus, in the cross-pollinations for the allelism test, the probability of a *st* gamete from both parents uniting in fertilization is $1/3 \times 1/3$ or $1/9$. For every nine cross-pollinations, only 1 pollination on average, would be the desired F_1 plant genotype St_8 st_8 $St_?$ $st_?$ (non-allelic; fertile plant), or st_8 st_8 (allelic; sterile plant). And to be at the

99% probability level to determine allelism/non-allelism, 39 F_1 seed from each of the 36 parental combinations (unknown mutants) would need to be obtained (Mather 1951). Thus, a total of almost 1,000 cross-pollinations would need to be made. This is possible to do in one summer, but it would be a major effort. Now molecular markers of the *St8* region can be used to identify F_1 hybrids for complementation analyses.

Frequency and timing of reversion

The w_4-*m* allele can revert at different times during flower ontogeny (Groose et al. 1988). The size of the revertant sectors on mutable plants is dependent on the developmental timing of reversion of w_4-*m*. Purple stripes that extend the length of the hypocotyl are the result of reversion of w_4-*m* early in the development of mutable seedlings. Small purple flecks on mutable hypocotyls represent reversion of w_4-*m* late in development. Variability in developmental timing of reversion also was observed in flowers of mutable plants. Reversion very early in development resulted in large revertant sectors such as entire branches that produced only purple flowers (Groose et al. 1988). Reversion later in development produced somewhat smaller revertant sectors such as single racemes of purple flowers or individual purple flowers borne on otherwise mutable racemes. Reversions very late in the development of mutable flowers resulted in purple sectors on otherwise nonrevertant (w_4, near white) petals.

The w_4-*m* allele yields germinal revertants at a rate that varies from 5 to 10% per generation (Groose et al. 1988). Approximately 10% of the progenies derived from germinal revertant plants contained phenotypically observable mutations at other loci (Palmer et al. 1989). Reciprocal crosses of w_4-*m* with cultivar Harosoy-w_4 isoline produced mutable and wild-type F_1 plants. The wild-type F_1 plants were attributed to germinal reversion in the w_4-*m* w_4-*m* parent. Wild-type segregants occurred among F_2 progeny of mutable F_1 plants. These wild-type segregants were attributed to reversion in the germline of w_4-*m* w_4-*m* F_1 plants (Groose et al. 1990). The occurrence of somatic flower mutations, with the presence of new mutations among the progeny of germinal revertants, with the concommitant reversion of w_4-*m*, strongly suggests the activity of a functional transposon.

Preferential insertion/target specificity

The data on male-sterile, female-sterile phenotypes, as presented by mutable category, show that ASR-3 and ASR-10 have the largest number and the highest percentage of mutant phenotypes. Both ASR-3 and ASR-10 were in the late excision category, but ASR-3 was characterized by low

frequency and ASR-10 by high frequency of excision (Table 1). ASR-7, ASR-8, and ASR-9 were in the low frequency and early excision category and one, zero, or two male-sterile, female-sterile phenotypes were found. No male-sterile, female-sterile phenotype was found (ASR-6) in the high frequency, early excision category. Thirty-three of 36 mutant phenotypes were found in the late excision category.

For comparison, data are presented for the number of necrotic root phenotypes for the same ASR families (Table 1). The similarities are that no, or few sterility or necrotic root phenotypes were evident in the low frequency early excision mutable category, which was 1,277 entries (families) of about 50 plants per family. Interestingly, 35 of 36 male-sterile, female-sterile mutants reported in this study are most likely allelic as well as all 30 necrotic root mutants reported in soybean from spontaneous mutations and chemical mutagen (EMS) treatment (Palmer et al. 2008). Of the 37 male-sterile, female-sterile mutants from the two gene tagging studies (1 + 36), 36 are probably allelic at the St_8 locus, or are tightly linked to genes, based upon close linkage to the common SSR markers Satt132, Sct_065, and Satt414 on MLG J (Fig. 1).

Of the 37 male-sterile, female-sterile mutants from the two gene tagging studies, only one (from entry ASR-7-206), did not show linkage with SSR markers on MLG J. The ASR-7 mutable category family had 236 entries, but only one sterility mutant for a frequency of 0.42%. The mutant gene in entry ASR-7-206 was mapped to MLG G. These data strongly indicate that the putative transposon of the w_4-*m* allele showed target site specificity and most likely the element was transposed into the same gene required for male and female fertility.

Many studies have shown preferential insertion/target site specificity in maize. The timing of Mutator (*Mu*) activity was tested by Robertson (1980, 1981, 1985). The conclusion was that *Mu* excision did not occur throughout plant ontogeny, but seemed to be restricted to a time shortly before and/or during meiosis. Robertson et al. (1988) used three maize Mutator-induced a_1 mutant alleles to test for a relationship between somatic and germinal transposition activity. Somatic activity, as measured at the a_1 locus, was not a reliable predictor of germinal activity. *Mu* elements can insert into essentially any gene, but mutations at some loci do occur up to ten times more frequently than at others (Robertson 1985). Hardeman and Chandler (1993) have shown that different maize genes were preferentially targeted by different classes of *Mu* elements. They suggested that the differences in targeting were due to the differences in internal sequences among the various classes of *Mu* elements. Creese et al. (1995) have shown that *Mu-1*-like transposable elements insert primarily into chromosomal regions of low copy number DNA. Dietrich et al. (2002)

used the gl_8 locus and reported that the involvement of nucleotide sequences flanking the target site were important in *Mu* insertion-site selection.

However, for the *Ac* and *En/Spm* maize elements, specificity for insertion has been into unmethylated target sequences (Chen et al. 1987; Cone et al. 1988). *Ac* elements also preferentially insert at sites linked to the initial donor element site (Dooner and Belachew 1989). Kunze and Weil (2002) reported that the maize transposons *Ac/Ds, En/Spm,* and *Mu* preferentially insert into relatively hypomethylated, low-copy-number DNA.

For the generation of sterility mutants, late excision events seem to be a prerequisite, irrespective of low or high frequency of excision. The chronology of flower development in soybean has been summarized by Carlson and Lersten (2004). Soybean has a typical papilionaceous flower. About 25 days before flowering, the initiation of floral primordia begins. Next is sepal differentiation, followed by petal, stamen, and carpel initiation. About 14–10 days before flowering, ovule initiation, maturation of megasporocytes, meiosis, and then four megaspores are present. About 10–7 days before flowering, anther initiation begins, male archesporial cells differentiate, then meiosis and microsporogenesis (Johns and Palmer 1982).

Our observations/classifications were based upon mutable flowers, that is, somatic tissues. The germline, male and female organs are within the flowers. In soybean, self-pollinated progeny of foliage chimeric plants manifests a direct relationship between sector phenotype (somatic tissue) and nuclear or cytoplasmic genotypes (germline) (Cianzio and Palmer 1992; Palmer et al. 2000). With the w_4-*m* flowers, we believe that both the early and late transpositions have a direct relationship with both the occurrence and frequency of new mutants. There is preferential insertion/target specificity.

A total of 31 chlorophyll-deficient, tan-saddle seedcoat, malate dehydrogenase 1 mutations have been reported for their chromosome region on MLG H (Xu and Palmer 2005b). Several have been studied in detail and all show that the *Mdh1-n y20* mutants were missing a 5.5 kb *Eco*R1-band which corresponded to the soybean *Mdh1* gene (Imsande et al. 2001). This has been reported in *Mu* induced mutations in maize where the phenotypes of the "same" mutations can be quite different, either from gene to gene or between alleles of the same gene (Bennetzen et al. 1993).

In summary, 37 male-sterile, female-sterile mutations were independently generated in two germinal revertant experiments. One mutant, st_8 (T352) was non-allelic to all genetically characterized soybean synaptic mutants (Palmer and Horner 2000), and was mapped to MLG J (Kato and Palmer 2003). The second germinal revertant study produced 36 male-sterile, female-sterile mutants, of which 35

mapped to the *st8* region on MLG J and are assumed to be alleles at the *st8* locus, or at other loci adjacent to the *st8* locus. Complementation analyses should be able to determine which of these alternatives is correct. One male-sterile, female-sterile mutant, from entry ASR-7-206, mapped to MLG G. These 36 sterility mutants, 24 necrotic root mutants, and many of the 31 chlorophyll-deficient, tan-saddle seed coat, malate dehydrogenase 1 mutants, would be ideal for the molecular characterizations of target site-specific insertion of a putative transposon in soybean.

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