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Inheritance of two independent isozyme variants in soybean plants derived from tissue culture

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Summary. Soybean [*Glycine max* (L.) Merr.] plants were regenerated via somatic embryogenesis from nine soybean cultivars. Our objective was to identify and characterize genetically novel mutations that would further our understanding of the soybean genome. Variant isozyme patterns were observed in two independent tissue culture-derived lines. Genetic analyses were conducted on these two isozyme variants, and they were heritable. No variant isozyme patterns were evident in control (parental) soybean lines. In the cultivar BSR 101, a mutation of *Aco2-b* (aconitase) to a null allele was detected. The *Aco2-bn* mutant, Genetic Type T318, had not been previously observed in soybean. In the Chinese cultivar Jilin 3 (PI 427.099), a chlorophyll-deficient plant was identified that also lacked two mitochondrial malate-dehydrogenase (*Mdh* null) isozyme bands. These two mutant phenotypes, chlorophyll-deficient and *Mdh* null, were found to cosegregate. The Jilin 3 mutant, *Mdh1-n* (Ames 1) *y20* (Ames 1) Genetic Type T317, was allelic to three chlorophyll-deficient, *Mdh1* null mutants [*Mdh1-n* (Ames 2) *y20* (Ames 2) (T323), *Mdh1-n* (Ames 3) *y20* (Ames 3) (T324), and *Mdh1-n* (Ames 4) *y20* (Ames 4) (T325)] previously identified from a transposon-containing soybean population, and to a chlorophyll-deficient, *Mdh1* null mutant [*Mdh1-n* (Urbana) *y20* (Urbana) *k2*, Genetic Type T253] which occurred spontaneously in soybean. The recovery of two isozyme variants from progeny of 185 soybean plants regenerated from somatic embryogenesis indicates the feasibility of selection for molecular variants.

Key words: Somaclonal variation – Tissue culture – *Glycine max* (L.) Merr. – Isozymes

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Introduction

Tissue culture-induced genetic variation has been observed in numerous plant species (Larkin and Scowcroft 1981; de Klerk 1990). Culture-induced mutant traits that exhibit stable genetic variation from the parental source (referred to as somaclonal variation) have been observed in maize (*Zea mays* L.) (McCoy and Phillips 1982; Lee and Phillips 1988), geranium (*Pelargonium* spp. L.) (Skirvin and Janick 1976), alfalfa (*Medicago sativa* L.) (Reisch and Bingham 1981), tomato (*Lycopersicon esculentum* L. Mill.) (Evans and Sharp 1983), potato (*Solanum tuberosum* L.) (Landsmann and Urig 1985), wheat (*Triticum aestivum* L. em Thell.) (Larkin et al. 1984; Brettell et al. 1986b), as well as soybean (*Glycine max* L. Merr.) (Barwale and Widholm 1987; Graybosch et al. 1987; Freytag et al. 1989; Amberger et al. 1992).

Mechanisms involved in generating genetic variability among regenerated plants remain uncertain. Many reports of genetic variation among tissue culture-regenerated plants suggest some type of DNA modification such as methylation changes (Brettell and Dennis 1991; Peschke et al. 1991). Evans and Sharp (1983) and Lee and Phillips (1988) demonstrated that genetic variation in tissue culture-derived plants can result from changes in chromosome number and/or chromosome rearrangement. Landsmann and Urig (1985) provided evidence that changes in the copy number of repeated sequences in potato generate stable mutants deficient in 25*s*-rRNA genes. Brettell et al. (1986a) conducted molecular analyses of an electrophoretic mobility variant for an alcohol dehydrogenase (*Adh1*) isozyme in maize. They found that this variant was generated by a single-base change in the *Adh1* gene sequence, resulting in substitution of one amino acid for another. This implies that some tissue culture-induced DNA alterations may not be readily dis-

tinguishable by screening for changes in gross plant morphology. Therefore, analyses of regenerated plants for detection of somaclonal variants should include specific molecular markers as well as gross morphological characteristics.

Genomic stress, which might result from tissue culture, has been suggested to activate or release latent transposable elements (Burr and Burr 1981). Peschke et al. (1987) reported that ten regenerated plants from two independent maize embryo cell lines contained an active *Ac* transposable element, whereas no active *Ac* elements were detected in original explant sources. The presence of an *Ac*-homologous DNA fragment that cosegregated with *Ac* genetic activity was suggested as evidence for the molecular basis of a transposable element activated by tissue culture (Peschke et al. 1991). The transposable element *Suppressor-mutator* (*Spm*) was also activated and detected in regenerated maize plants (Peschke and Phillips 1991). James and Stadler (1989) found that maize tissue culture lines containing an active Mutator system generated novel restriction fragments of "unmodified" *Mu* elements. No evidence was found for activation of inactive maize *Mu* elements as a result of a tissue-culture cycle (Planckaert and Walbot 1989).

We previously reported morphological variants in soybean regenerated via somatic embryogenesis (Amberger et al. 1992). We also identified two new isozyme markers in R2 generation plants. Our current objective was to genetically characterize these two independently derived isozyme variants.

Materials and methods

Tissue culture

Nine soybean cultivars [BSR 101, Clark 63, Harosoy 63, Hodgson 78, Maple Arrow, Medium Green, Jilin 3 (PI 427.099), Preston, and Williams 82] were used as explant sources of immature cotyledons for direct induction of 475 R0 plants by somatic embryogenesis. All R0 plants were evaluated phenotypically, self-pollinated, and single-plant threshed (Amberger et al. 1992).

Electrophoresis

Fifty seed from each of the parental cultivars and three R2 seed from a representative sample of 500 individual R1 plants representing 185 R0 plants were germinated in a lighted growth chamber at 28 °C (Table 1). A sample for electrophoretic analysis was taken from the cotyledons of 4 day-old seedlings by using a 100 µl micropipette. Samples were stored at −70 °C until assayed. Starch-gel electrophoresis techniques described by Cardy and Beversdorf (1984a, b) were used to evaluate isozyme patterns of the nine parental cultivars and to determine if the tissue culture-derived progeny expressed variant patterns. Nine isoenzymes, encoded by more than 20 loci, were assayed (Table 2).

Starch gels were prepared by using starch concentrations of 11.78% with "B" or "D" gel and electrode buffer systems (Cardy and Beversdorf 1984a). Gels were run at a constant power of 9.5 W for 5.5 h in a 4 °C chromatography cooling chamber. The "B" gel system was used to assay for endopeptidase (ENP, EC 3.4.99), phosphoglucoisomerase (PGI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 2.7.5.1), and malate dehydrogenase (MDH, EC 1.1.1.37). The "D" gel system was used to assay for diaphorase (DIA, EC 1.6.4.3), isocitrate dehydrogenase (IDH, EC 1.1.1.42), acid phosphatase (AP, EC 3.1.3.2), malic enzyme (ME, EC 1.1.1.38), and aconitase (AC0, EC 4.2.1.3). Stain recipes were modified from Cardy and Beversdorf (1984a) to stain two gel slices with one staining solution. This was accomplished by doubling the amount of reagent in a single, one-slice, volume of appropriate staining buffer solution. The gel slices were incubated at 38 °C for 30–60 min. Then, each gel was screened to determine if there were any deviations in the isozyme patterns of the R2 generation tissue culture-derived plants from the control or parental genotypes.

Table 1. Number of individual R0 and R1 plants, representing each soybean [*Glycine max* (L.) Merr.] cultivar, used for isozyme analysis of R2 seed

Cultivar	No. R0 plants	No. R1 plants	No. R2 seed
BSR 101	17	81	243
Clark 63	30	78	234
Harosoy 63	7	24	72
Hodgson 78	13	66	198
Jilin 3	31	83	249
Maple Arrow	38	44	132
Medium Green	22	49	147
Preston	15	27	81
Williams 82	12	48	144
Total	185	500	1,500

Table 2. Number of alleles of isozyme loci in soybean observed in the somaclonal variation study

Isozyme locus	No. alleles	Inheritance
Ap	2	Codominant
Aco1	1	Codominant
Aco2	2	Codominant
Aco3	1	Codominant
Aco4	2	Codominant
Aco5	1	Codominant
Dia1	2	Codominant
Dia2	2	Codominant
Dia3	2	Dominant/recessive
Enp	2	Codominant
Idh1	2	Codominant
Idh2	2	Codominant
MDH[a]	Unknown	–
ME[a]	Unknown	Codominant
Pgm1	2	Codominant
Pgm2	2	Codominant
Pgm3	2	Codominant
Pgi1	2+[b]	Codominant
Pgi2	2+[b]	Codominant
Pgi3	2+[b]	Codominant

[a] Inheritance has not been determined
[b] Two or more alleles

Genetic evaluation

After isozyme analysis, seedlings that expressed isozyme patterns that differed from the parental cultivar were saved and transplanted into pots containing a standard greenhouse soil mix. These seedlings were maintained in the USDA-ARS greenhouse where they were allowed to self-pollinate.

Three variant banding patterns were detected on zymograms. Two heritable isozyme variants, aconitase (*Aco2*) and malate dehydrogenase (*Mdh1*), were observed. Each of these mutants has been assigned Genetic Type Collection numbers and gene symbols. The aconitase mutant has been assigned Genetic Type Collection Number T318 and a gene symbol of *Aco2-bn* since it lacks an isozyme encoded by the *Aco2-b* allele (Table 3). The malate-dehydrogenase mutant has been assigned Genetic Type Collection Number T317 and a gene symbol of *Mdh1-n* (Ames 1) *y20* (Ames 1) because the plants are both malate-dehydrogenase null and chlorophyll-deficient (Table 3). The third isozyme variant, a diaphorase null in cultivar Jilin 3 progeny, was not heritable (Amberger et al. 1992). Additional soybean lines were used for allelism and inheritance studies with these isozyme mutants and these lines are listed in Table 3.

The *Aco2-bn* variant, T318, was found among regenerated plants of cultivar BSR 101. The variant plant was self-pollinated and backcrossed to BSR 101. The F_1, F_2 and F_3 seed were analyzed electrophoretically. The F_3 progeny from all F_2 plants were analyzed to determine the genotype (homozygous or heterozygous) of dominant F_2 plants and to confirm stable inheritance of the null genotype from recessive F_2 plants. Five F_3 seed from each F_2 plant were analyzed to achieve a minimum 95% probability of identification of segregating families.

To allow for better interpretation of the aconitase isozyme patterns, T318 was crossed to PI 437.477B. The F_1 and F_2 seed were analyzed for aconitase isozyme patterns.

The chlorophyll-deficient and malate-dehydrogenase (*Mdh1*) variant from Jilin 3, T317, was crossed reciprocally with Minsoy to determine the mode of inheritance of the chlorophyll mutation (cytoplasmic or nuclear), the inheritance patterns of the malate-dehydrogenase variant, and to test for linkage with four marker loci. Minsoy has purple flowers (W_1, W_1), non-fluorescent roots under UV light (fr_1, fr_1), a sharp pubescence tip (*Pb*, *Pb*), and tawny pubescence (*T*, *T*) at maturity. Jilin 3 has white flowers (w_1, w_1), fluorescent roots (Fr_1, Fr_1), a blunt pubescence tip (*pb*, *pb*), and gray pubescence (*t*, *t*). This combination of characteristics enabled detection of possible linkages between the chlorophyll-deficient phenotype, the malate-dehydrogenase null phenotype, and the four marker loci. This cross was also used to confirm inheritance of both mutant traits, chlorophyll-deficiency and malate-dehydrogenase null.

The F_1 plants were grown in the greenhouse during Spring 1989 and F_2 plants were field grown in 1989 at Ames, Iowa. At the time of evaluation of the F_1 plants and the F_2 progeny, we had not determined that the chlorophyll-deficient Jilin 3 mutant was missing two malate-dehydrogenase bands. Thus, F_3 seed from individual F_2 plants, from the cross Minsoy × T317, were analyzed electrophoretically. The seedlings were transplanted to pots in the greenhouse, and the plants were classified for plant color. These F_2 plant-progeny rows gave us data for determining the inheritance of plant color and our first data for determining the mode of inheritance of malate dehydrogenase.

Plants from T317 were crossed to two unrelated lines, cultivar Harosoy and Genetic Type T219. F_1 and F_2 seed were evaluated for malate dehydrogenase, and the seedlings scored for plant color.

Previous studies had shown that three chlorophyll-deficient mutants (T323, T324, and T325), from an active transposable element-containing line, and the chlorophyll-deficient mutant, T253, of spontaneous origin, were missing the same two malate-dehydrogenase bands as T317 (Table 3). The T323, T324, T325, and T253 mutants were allelic, based on F_1, F_2, and F_3 data (Hedges 1989; Palmer et al. 1989).

Reciprocal crosses were made between T317 and the T323, T324, T325 and T253 mutants. F_1 and F_2 data were obtained for plant color and malate-dehydrogenase patterns.

Results

Aconitase variant in BSR 101

An isozyme variant was found in tissue culture-regenerated plants from the cultivar BSR 101. This variant was characterized by a missing isozyme band corresponding to the *Aco2-b* allele (*Aco2-bn*) (Fig. 1). Both the parental

Table 3. Description of soybean lines used for allelism and inheritance studies

Genetic type	Strain no.	Gene symbol	Phenotype	Reference
T219	A691-1	*Y11*	Homozygous dominant has green leaves, heterozygote has greenish yellow leaves, and homozygous recessive is a lethal yellow	Weber and Weiss 1959
T253	L67-4415A	*Mdh1-n* (Urbana) *y20* (Urbana) *k2*	Malate-dehydrogenase null, yellowish green leaves (weak plant), tan saddle on seed coat	Palmer 1984
T317	LA45-1-5-1	*Mdh1-n* (Ames 1) *y20* (Ames 1)	Malate-dehydrogenase null, yellowish green leaves (reduced vigor)	Amberger et al. 1992
T318	LA2-3	*Aco2-bn*	Aconitase 2-b null	Amberger et al. 1992
T323	CD-1	*Mdh1-n* (Ames 2) *y20* (Ames 2)	Malate-dehydrogenase null, yellowish green leaves (reduced vigor)	Hedges 1989
T324	CD-2	*Mdh1-n* (Ames 3) *y20* (Ames 3)	Malate-dehydrogenase null, yellowish green leaves (reduced vigor)	Hedges 1989
T325	CD-3	*Mdh1-n* (Ames 4) *y20* (Ames 4)	Malate-dehydrogenase null, yellowish green leaves	Hedges 1989

BSR 101 and the variant have an isozyme pattern containing a faint band that migrates between the *Aco2-b* and *Aco3-a* isozymes. This faint band seems to be associated with the *Aco3* isozyme because it is present in both normal and mutant lines. PI 437.477B contains both *Aco2-a* and *Aco3-a* isozymes and shows an identical faint band in correspondence with the migration of *Aco3-a* (see Fig. 1).

F_2 segregation data from BSR 101 × T318 indicate that the *Aco2-b* null allele behaves like a recessive allele in combination with *Aco2-b* (Table 4). By using the F_3 data, individual F_2 plant genotypes were identified as 19 homozygous dominant (*Aco2-b*, *Aco2-b*), 66 heterozygous (*Aco2-b*, *Aco2-bn*), and 23 homozygous null (*Aco2-bn*, *Aco2-bn*) and fit a 1:2:1 segregation ratio (Table 4). Data from F_2 heterozygous plants fit a 3:1 segregation ratio (Table 4) and confirmed single-gene inheritance of *Aco2-bn*. Additional F_2 seed analyzed from T318 × PI 437.477B fit a 3:1 segregation ratio [χ^2 (3:1) = 0.06, $P > 0.80$, 1 df] and also confirmed single-gene inheritance of *Aco2-bn*.

Table 4. F_2 segregation data (BSR 101 *Aco2-b* × T318) and F_3 progeny for aconitase variants in BSR 101

Genotype		No. F_2 plants	χ^2 (1:2:1)		P
F_2 progeny:	Dominant (*Aco2-b Aco2-b*)	19			
	Heterozygous (*Aco2-b Aco2-bn*)	66			
	Null (*Aco2-bn Aco2-bn*)	23			
	Total	108	5.63		>0.05
F_3 progeny (from heterozygous F_2 plants):	F_3 progeny	Dominant	Null	χ^2 (3:1)	P
	F_2 Family 1	202	55	1.78	>0.10
	F_2 Family 2	226	93	2.94	>0.05
	F_2 Family 3	270	97	0.40	>0.50
	Total	698	245	0.48	>0.25

χ^2 value of 5.99 is significant at the 0.05 probability level, 2 df and a χ^2 value of 3.84 is significant at the 0.05 probability level, 1 df

Chlorophyll-deficient, malate-dehydrogenase variant in Jilin 3

An isozyme variant was observed in the Jilin 3 tissue culture-derived plants. This variant, T317, was initially characterized by a chlorophyll deficiency and was later analyzed for isozyme patterns. This chlorophyll-deficient variant was also lacking a nuclear-encoded mitochondrial malate-dehydrogenase isozyme (Fig. 2).

Results from T317 and Minsoy crosses indicated that the non-lethal chlorophyll-deficient phenotype was caused by a dominant-to-recessive nuclear gene mutation. The F_2 progeny segregation ratio was 252 green:70 non-lethal chlorophyll-deficient plants [χ^2 (3:1) = 1.84, $P > 0.10$, 1 df]. The chi-square tests of F_2 data indicate that this mutation is not linked to any of the four marker loci tested (Table 5).

Seventeen F_2 plants were individually threshed, and F_3 seed and plants were scored for malate dehydrogenase and plant color, respectively. The F_2 green plants that gave rise to all green F_3 seedlings had wild-type isozyme patterns for malate-dehydrogenase bands. The F_2 chlorophyll-deficient plants had a true-breeding phenotype and were missing two malate-dehydrogenase bands on the zymograms. Eight green F_2 plants segregated 107 green:28 chlorophyll-deficient F_3 plants [χ^2 (3:1) = 1.31, $P > 0.20$, 1 df], and all chlorophyll-deficient plants were malate-dehydrogenase nulls. There was cosegregation of the malate-dehydrogenase null phenotype with the plant color phenotype.

The F_2 segregation from the cross Harosoy × T317 was 88 green plants:38 chlorophyll-deficient plants [χ^2 (3:1) = 1.79, $P > 0.10$, 1 df]. The F_2 segregation from the cross T219 × T317 was 102 green plants:44 chlorophyll-deficient plants [χ^2 (3:1) = 2.06, $P > 0.10$, 1 df]. All green F_2 plants from both crosses were malate-dehydro-

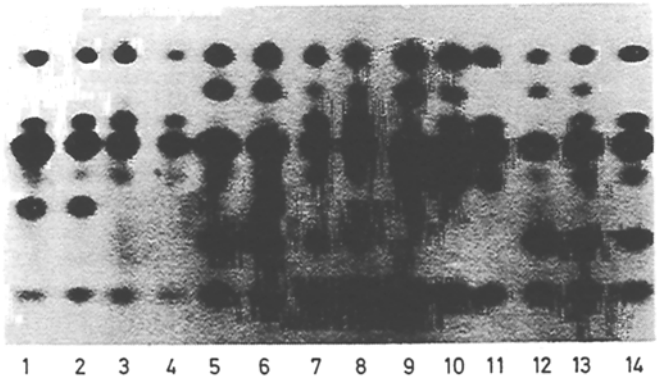

Fig. 1. A zymogram of aconitase isozyme patterns of BSR 101 parental (*lanes 1–2*), T318 *Aco2-bn* (*lanes 3–4*), PI 437.477B (*lanes 5–6*), F_1 progeny of PI 437.477B × T318 (*lanes 7–8*) and segregating F_2 progeny from PI 437.477B × T318 (*lanes 9–14*)

Table 5. F_2 linkage tests from reciprocal crosses of T317 × Minsoy

Marker genes	Observed phenotypes				No.	χ^2 value (9:3:3:1)	*P*
	A[a]-Y[b]-	A-yy[c]	aa[d]Y-	aayy			
Fr_1 Root fluorescence	199	47	53	23	322	5.87	>0.10
Pb Pubescence tip	151	50	67	11	278	6.83	>0.05
W_1 Flower color	178	48	70	18	314	4.16	>0.10
T Pubescence color	190	48	56	19	313	3.32	>0.25

χ^2 value of 7.81 is significant at the 0.05 probability level with 3 df
[a] Dominant for marker gene
[b] Dominant for plant color (green)
[c] Recessive for plant color (chlorophyll deficient; yellow-green)
[d] Recessive for marker gene

Table 6. Segregation data from allelism tests of F_1 and F_2 generations from reciprocal crosses of T317 × three independently derived chlorophyll-deficient, malate-dehydrogenase nulls (T323, T324, T325) from a transposable element containing population, and × T253, a chlorophyll-deficient malate-dehydrogenase null mutant isolated in the cultivar Harosoy

Cross	F_1 plants			F_2 plants		
	No.	Plant color[a]	MDH[b]	No.	Plant color[a]	MDH[b]
T323 × T317	3	CD	Null	101	CD	Null
T317 × T323	3	CD	Null	89	CD	Null
T324 × T317	3	CD	Null	117	CD	Null
T317 × T324	3	CD	Null	99	CD	Null
T325 × T317	5	CD	Null	201	CD	Null
T317 × T325	3	CD	Null	109	CD	Null
T253 × T317	4	CD	Null	186	CD	Null
T317 × T253	4	CD	Null	177	CD	Null

[a] CD, chlorophyll-deficient plants
[b] Null, malate dehydrogenase-null phenotype

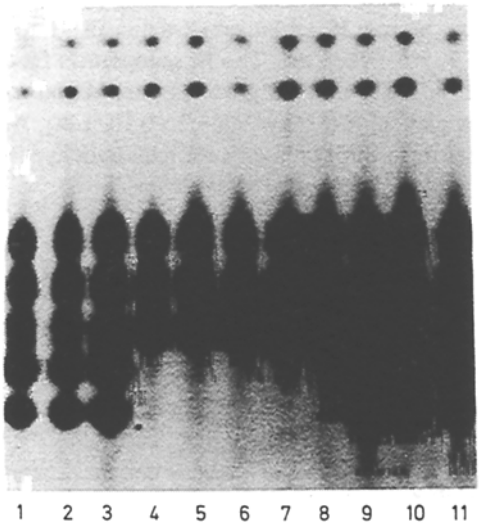

Fig. 2. A zymogram of malate-dehydrogenase isozyme patterns of Jilin 3 (PI 427.099) parental (*lanes 1–3, 9–11*) and T317 malate-dehydrogenase null (*lanes 4–8*)

genase wild-type, and all chlorophyll-deficient F_2 plants were malate-dehydrogenase nulls. There were no exceptions.

Allelism tests were conducted between T317 and four independently derived single-gene recessive, chlorophyll-deficient, malate-dehydrogenase null mutants. The F_1 and F_2 progeny from these reciprocal crosses indicated allelism in all cross combinations for plant color and for malate dehydrogenase (Table 6). There was consensus correspondence of the chlorophyll-deficient plant phenotype with the malate-dehydrogenase null phenotype.

Discussion

The frequency of soybean somaclonal isozyme mutants in this study was two out of 185 R0 plants. Brettell et al. (1986a) isolated one stable mutant of *Adh1* from 645 primary-regenerant maize plants tested. Dennis et al. (1987) identified one stable mutant of *Adh1* from 1,382 cultured maize embryos. There have been few reports where regenerated plants have been screened for isozyme or protein variants. Only nine isozyme systems in soybean were screened for somaclonal variants. The high frequency of variants observed in the present study is surprising because soybean is considered a diploidized polyploid (Hymowitz and Singh 1987).

Aconitase variant in BSR 101

The *Aco2-bn* isozyme variant, T318, had not been reported previously in soybean. Our genetic tests indicate that this variant is inherited as a single-gene recessive. Future analyses of T318 will, therefore, be directed towards molecular screening techniques that detect changes in base pair composition and deletions or alterations of the DNA sequence encoding the *Aco2-bn* variant allele.

Chlorophyll-deficient, malate-dehydrogenase variant in Jilin 3

The second variant, chlorophyll-deficient Jilin 3, T317, was initially recognized by its yellow-green plant color

but after isozyme analysis was found to be missing two nuclear-encoded mitochondrial malate-dehydrogenase bands. Our inheritance and linkage studies indicated that the chlorophyll-deficient phenotype was inherited as a single-gene recessive and was not linked to root fluorescence (Fr_1 of linkage group 12), to pubescence tip (*Pb* of linkage group 14), to flower color (W_1 of linkage group 8), or to pubescence color (*T* of linkage group 1).

In allelism tests of T317 with T323, T324, T325, and T253 (known nuclear chlorophyll-deficient, malate-dehydrogenase null mutants), all F_1 and F_2 plants were chlorophyll-deficient and malate-dehydrogenase null. Hedges (1989) determined that each of the four chlorophyll-deficient mutants (T323, T324, T325, and T253) lacked two of three NAD^+-dependent mitochondrial malate-dehydrogenase enzymes, whereas green sibling plants had all three enzymes. Since malate dehydrogenase is a dimeric enzyme in most plant systems, the silencing of one locus is expected to result in the loss of two isozymes, a homodimer, and an interlocus heterodimer.

The parental line of the T323, T324, and T325 mutants is *w4-m*, a line homozygous for mutable alleles at the *w4* locus. The mutable phenotype, a high rate of change from a mutable allele to a functional wild-type allele or pale allele, possible changes of state, and new mutations at other loci, all suggest that an active transposable element exists in the *w4-m* soybean line (Groose et al. 1988, 1990; Palmer et al. 1989).

The fourth soybean chlorophyll-deficient malate-dehydrogenase null mutant was Genetic Type T253. The chlorophyll-deficient phenotype is either tightly linked to or else has a pleiotropic effect with tan saddle seed coat. Palmer (1984) evaluated about 25,000 F_2 and F_3 plants, but the chlorophyll-deficient phenotype could not be separated genetically from the tan saddle phenotype. He suggested that the mutant phenotypes were the result of a small chromosomal deletion caused by a transposable element.

Evidence from two maize transposable-element systems, *Ac*/*Ds* and *Spm*, supports the hypothesis that genomic stress, such as tissue culture, activates or releases latent transposable genetic-element systems (Peschke et al. 1987, 1991). These workers identified two independent maize embryo cell lines that contained an active *Ac* transposable element. Due to the presence of *Ac* from the tissue-culture cell-line parents, the maize embryo cell line × tester genetic stocks produced variegated kernels. *Ac* activity was not found in uncultured control kernels for any of the cell lines; thereby, providing evidence for tissue culture-induced activation of latent transposable elements. The presence and cosegregation of *Ac* activity with an *Ac*-homologous *Sst*I restriction fragment and *Ac*-homologous *Bgl*II restriction fragment provides documentation at the molecular level for transposable-element activation by tissue culture. In their 1991 study, *Spm*-homologous sequences were detected in each of the inbreds used to initiate tissue cultures. *Spm* activity was observed in two R1 progeny of a single regenerated plant. The authors presumed that one of the *Spm*-homologous sequences became activated during tissue culture, giving rise to *Spm* activity in the R1 progeny.

James and Stadler (1989) initiated cell culture lines from F_1 progeny of two maize crosses with one parent containing an active *Mu*. Transposition events were indicated by the observation of novel *Mu* homologous restriction fragments in three of eight culture sublines. Uniformity of DNA hybridization profiles from control sublines indicated no discernible genetic alterations. Planckaert and Walbot (1989) did not observe any activation of latent *Mu* elements, but occasionally, the unmethylated *Mu* sequences in callus from active lines became methylated.

Chromosome breakage in tissue-culture cell lines frequently occurs in or near chromosome regions known to be highly heterochromatic (McCoy et al. 1982; Johnson et al. 1987). Peschke et al. (1987) suggested that, in maize, the late-replicating chromosome regions, which are highly heterochromatic, may break more frequently than euchromatin in cell-culture lines. This chromosome breakage could lead to transposable-element activation.

Soybean has $2n=2x=40$ chromosomes. Several chromosomes (numbers 4, 6, 7, and 18) have more than 50% of their length as heterochromatic regions (Singh and Hymowitz 1988). Perhaps chromosome breaks have resulted in activation of a transposable element that has excised, transposed, and inserted elsewhere in the genome to generate new mutants. Alternatively, chromosome breaks, caused by genomic stress during tissue culture, may have resulted in deletion of part of the chromosome. The chlorophyll-deficient malate-dehydrogenase nulls may be a direct manifestation of these deletions.

The chlorophyll-deficient phenotype and the malate-dehydrogenase null phenotype observed in the soybean mutants could result from a deletion of heterochromatic regions of the chromosome. The T323, T324, and T325 mutants, which occurred in the T322 (*w4-m*) genetic background, can be distinguished phenotypically, based upon different degrees of chlorophyll deficiency; yet, they are allelic. The independent origin of these mutants and their different phenotypes suggest that differenes among them exist at the molecular level. The occurrence of three alleles at the same locus implies non-random mutation, which may reflect some transposition specificity such as the recognition of specific target-site sequences by a transposable element.

Genetic type, T253, was found in the cultivar Harosoy, and T317 was isolated from the Chinese cultivar Jilin 3 (PI 427.099). It is possible that these three different genetic lines (T322, Harosoy, and Jilin 3), share a com-

mon parent, which could be the source of the same, or a related, transposable element family.

We have presented genetic analyses of two independent tissue culture-derived mutants in soybean. The T318 mutant, *Aco2-bn*, is a null allele at the *Aco2* locus. Molecular analysis of T318 is necessary to determine the mechanism responsible for the null phenotype. The T317 mutant, *Mdh1-n* (Ames 1) *y20* (Ames 1), is both chlorophyll-deficient and malate-dehydrogenase null. Tissue culture could have induced genomic stress, thus resulting in chromosome breakage. This breakage may have caused chromosome segment losses, activated transposable elements, or induced other mechanisms that produce somaclonal variation. The five independently derived chlorophyll-deficient, malate-dehydrogenase nulls in soybean (T253, T317, T323, T324, and T325) indicate that this chromosome region is quite vulnerable to events that generate variants.

Our results demonstrate that somatic embryogenesis in soybean generates isozyme variants which are heritable. Of the two heritable variants observed, one variant, *Aco2-bn*, has not been identified previously in soybean. The other variant, *Mdh1-n* (Ames 1), *y20* (Ames 1), was allelic to a known spontaneous mutant and to three mutants from a transposon-containing soybean population. Molecular analyses of these allelic mutants may provide insight into both transposable-element and tissue culture-induced variation in soybean.

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