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Esterase polymorphisms for analysis of genetic diversity and structure of soybean (*Glycine max*) cultivars

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ABSTRACT. We used native polyacrylamide gel electrophoresis to identify polymorphism levels in α- and β-esterase loci from leaf tissues of Brazilian soybean cultivars for the analysis of population genetic diversity and structure, and to investigate relationships between conventional and genetically modified cultivars. The cultivars included lines developed by a soybean-grower cooperative (CD), by EMBRAPA (BR), and "Roundup Ready" (RR) cultivars. Esterase isozymes recorded with α-naphthyl acetate and β-naphthyl acetate were produced from 14 loci. Two to three allelic variants were detected in leaves from 420 plants of 21 CD, BR, and RR cultivars at *Est-1*, *Est-2*, *Est-3*, *Est-5*, and *Est-14* loci. The estimated proportion of polymorphic loci in CD cultivars was 21.4%, and in BR and RR cultivars it was 28.6%. High and low H_O and H_E values were observed within CD and BR cultivars and a very high cultivar differentiation level was evident in the plants of the 21 CD, BR, and RR cultivars (F_{ST} = 0.3865). A low level of differentiation (F_{ST} = 0.0289) was detected between conventional and RR cultivars. Plants from cultivar BR37 had the highest level of genetic differentiation compared to the other cultivars. The genetic basis of BR cultivars (0.5538-0.9748) was found to be broader than the genetic

basis of CD cultivars (0.7058 for CD205 and CD209 and 0.9995 for CD205 and CD208). Higher genetic identity was detected between plants of CD and CDRR cultivars (I = 0.9816). Understanding the genetic structure of these populations can help provide specific culture strategies for each cultivar, depending on its level of heterozygosity.

Key words: Soybean; Esterase polymorphism; Genetic diversity

INTRODUCTION

Protein or enzyme electrophoresis is still the simplest tool to analyze a population's genetic structure in order to detect allele fixation and reproductively isolated and genetically divergent populations (Allendorf and Luikart, 2007). For example, esterase isozyme polymorphism (EC 3.1.1._) has been documented in plants since the 1960s (Schwartz, 1967); their specificity for different substrates allows simultaneous analysis of various loci in studies on genetic variations in plant populations. They are adequate co-dominant markers for the analysis of genetic structure in populations. Esterases are often found in multigene families (Oakeshott et al., 1993; Robin et al., 1996); 14 to 16 esterase isozymes have been detected by polyacrylamide gel electrophoresis (PAGE) in different plant species (Pereira et al., 2001; Carvalho et al., 2003; Orasmo et al., 2007; Frigo et al., 2009). A large number of esterase loci can be used simultaneously for the identification of genetic variation and for polymorphism analyses.

The PAGE system has been used for analysis of genetic diversity in peroba trees (*Aspidosperma polyneuron*; Carvalho et al., 2003), cassava (*Manihot esculenta*; Pereira et al., 2001), grapes (*Vitis vinifera*; Orasmo et al., 2007), and wild poinsettia (*Euphorbia heterophylla*; Frigo et al., 2009), but no such studies have been made of soybean (*Glycine max*) cultivars. Genetic diversity in soybean has been assessed using morphological traits (Perry et al., 1991), pedigrees (Zhou et al., 2000), geographic origins (Gorman, 1984), parentage coefficient (Miranda et al., 2007), isozymes (Yu and Kiang, 1993; Griffin and Palmer, 1995; Hirata et al., 1999), and DNA markers (Abdelnoor et al., 1995; Li and Nelson, 2001; Ude et al., 2003; Xu and Gai, 2003; Priolli et al., 2002, 2004; Bonato et al., 2006; Yamanaka et al., 2007; Fu et al., 2007; Hwang et al., 2008). An overview of genetic diversity in Chinese (Li et al., 2008; Wang et al., 2008), Japanese (Hwang et al., 2008), and Korean (Yoon et al., 2009) soybeans has been made using DNA markers.

Brazilian soybean cultivars have also been genetically characterized, mainly with parentage coefficient and DNA markers (Priolli et al., 2002, 2004; Bonato et al., 2006; Miranda et al., 2007). Brazilian agriculture uses a large number of improved cultivars adapted to the very diverse producing regions in Brazil. Brazil is the second largest soybean producer in the world, with a cultivated area of 24.7-25.0 million hectares, and a prevision of 72.2-73.3 million tons produced in the 2011/2012 season (http://www.conab.gov.br).

Development of new conventional and transgenic cultivars of soybean by breeding programs requires genetic markers for analysis of genetic polymorphism. Esterase polymorphism in the PAGE system has potential as a biochemical marker for the genetic characterization of soybean cultivars. The PAGE system may be used to estimate the propor-

tion of polymorphic loci, number of alleles, proportion of alleles per polymorphic locus, frequency of alleles at each esterase locus, effective number of alleles, mean observed and expected heterozygosity, and to determine genetic differentiation within and among different soybean cultivars. Similarity among cultivars can also be determined. We used native PAGE to identify polymorphism in α- and β-esterase loci in leaf tissues of different soybean cultivars for analysis of genetic diversity and structure of populations, and to examine relationships between conventional and genetically modified cultivars.

MATERIAL AND METHODS

Seeds of the soybean cultivars CD and BR were obtained from COODETEC (Cooperativa Central de Pesquisa Agrícola) and EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária), respectively. The seeds were superficially sterilized with 15% sodium hypochloride for 10 min, and after extensive washing (4-5 times) with sterile water under aseptic conditions the seeds were distributed for germination on germtest paper. Germination occurred in a growth chamber at 25 ± 2°C under 14.9 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light radiation, provided by cold-white fluorescent lights, on a 12-h light-dark cycle for 120 h. The plantlets obtained from germinated seeds were maintained at room temperature in a hydroponic system for 10 days, and leaves of each cultivar were used for the electrophoresis analysis.

The samples consisting of leaves collected from 20 plants of CD201, CD202, CD204, CD205, CD206, CD208, CD209, CD215, CD216, BR37, BR48, BRS133, BRS137, BRS184, BRS230, CD212RR, CD213RR, CD214RR, CD219RR, BR244RR, and AntaRR cultivars were evaluated by electrophoresis. Leaf pieces (50 mg) were separately homogenized with a glass rod in a microcentrifuge tube using 50 µL extraction solution prepared with 1.0 M phosphate buffer, pH 7.0 (890 µL), containing 5% PVP-40 (50 mg), 1.0 mM EDTA (10 µL), 0.5% β-mercaptoethanol (5 µL), and 10% glycerol solution (50 µL) and maintained in an ice bath. After homogenization, the samples were centrifuged at 25,000 rpm (48,200 *g*) for 30 min, at 4°C, in a Sorval 3K-30 centrifuge and the supernatant (45 µL) was used from each sample.

Polyacrylamide gels (12%) were prepared with 0.375 M Tris-HCl, pH 8.8, as buffer (Ceron et al., 1992), 6.2 mL acrylamide/bis-acrylamide solution (30 g acrylamide and 0.8 g bis-acrylamide dissolved in 100 mL twice-distillated water), 4.0 mL 1.5 M Tris-HCl, pH 8.0, 6.2 mL twice-distilled water, 320 µL 2% ammonium persulfate, and 16 µL TEMED was used to separate the gel. The stack gel was prepared with 3.0 mL acrylamide/bis-acrylamide (5 g acrylamide and 0.25 g bis-acrylamide dissolved in 50 mL twice-distilled water), 3.0 mL 0.24 M Tris-HCl, pH 6.8, 30 µL twice-distilled water, 250 µL ammonium persulfate (2%), and 3 µL TEMED. Electrophoresis was performed for 10 h, at 4°C, at a constant voltage of 200 V (Pereira et al., 2001). Running buffer was 0.125 M Tris/0.0959 M glycine, pH 8.3.

Esterases were identified by staining techniques described in Pereira et al. (2001). Gels were soaked for 30 min in 50 mL 0.1 M sodium phosphate, pH 6.2, at room temperature. Esterase activity was visualized by placing the gels for 1 h in a staining solution prepared with 50 mL sodium phosphate solution, 30 mg β-naphthyl acetate, 40 mg α-naphthyl acetate, 60 mg Fast Blue RR salt, and 5 mL N-propanol. For identify the α- and β-esterases, the gels were separately incubated with: i) 40 mg α-naphthyl acetate; ii) 40 mg β-naphthyl

acetate; iii) 40 mg α-naphthyl and 30 mg β-naphthyl acetate; iv) 30 mg α-naphthyl acetate and 40 mg β-naphthyl acetate. The esterases of the different bands have characteristic colors: α (black), β (red), and αβ (a mixture of black and red); these hydrolyze, respectively, α-naphthyl acetate, β-naphthyl acetate, and both α- and β-naphthyl acetate.

Polyacrylamide gels, dried as described by Ceron et al. (1992), were kept at room temperature for 1 h in a mixture of 7.5% acetic acid and 10% glycerol embedded in 5% gelatin. They were further placed between two sheets of wet cellophane paper stretched on an embroidering hoop and left to dry for 24-48 h.

Genetic variability in the CD, BR, and RR (Roundup Ready) cultivars was analyzed with the POPGENE 1.32 Computer Program (Yeh et al., 1999) for the analysis of allele frequencies, observed and expected mean heterozygosity (H_O and H_E) and mean number of alleles per locus (N_A), mean number of alleles per polymorphic locus ($N_{A/PL}$), percentage polymorphic loci (%P), χ^2 test for deviation from Hardy-Weinberg equilibrium, fixation index (F_{IS}), genetic similarity or distance coefficients, and Wright's (1965) *F*-statistic values (F_{IT} and F_{ST}) for the CD and BR cultivars. Genetic identity (Nei, 1978) and distances among cultivars were calculated by UPGMA grouping.

RESULTS

Native PAGE analysis for esterase isozymes in leaves of the soybean cultivar plants, showed α-preferential esterases (preferentially hydrolyzed α-naphthyl acetate in the presence of α- and β-naphthyl acetate) and α/β-esterases (equally hydrolyzed α- and β-naphthyl acetate) produced from 14 loci. The α- and α/β-esterases were numbered in sequence, starting from the anode, according to decreasing negative charge (Figure 1). The characteristic band colors for the esterase isozymes correspond to the hydrolysis of either α-naphthyl acetate (black) or both (a mixture of black and red). Esterases produced from *Est-1*, *Est-10*, *Est-11*, *Est-12*, and *Est-13* loci hydrolyzed preferentially α-naphthyl acetate (α-esterase; black bands), and esterases produced from *Est-2*, *Est-3*, *Est-4*, *Est-5*, *Est-6*, *Est-7*, *Est-8*, *Est-9*, and *Est-14* loci utilized the α- and β-naphthyl acetates (α/β-esterase) to the same extent (bands showing a mixture of black and red).

Two to three allelic variants were detected in leaves of the 21 CD, BR, and RR cultivars at *Est-1*, *Est-2*, *Est-3*, *Est-5*, and *Est-14* loci in 420 plants analyzed. Allele frequencies were analyzed for *Est-1*, *Est-2*, *Est-3*, *Est-5*, and *Est-14* loci and estimated proportion of polymorphic loci (%P) in CD cultivars is 21.4% and in BR and RR cultivars is 28.6%.

Table 1 shows the number of polymorphic loci (N_{PL}), %P, N_A, $N_{A/PL}$, effective number of alleles (N_E), H_O and H_E in each of the cultivars. The estimated values in BR cultivars were higher than those detected in CD and RR cultivars. Whereas in the BR37 cultivar the highest H_O and H_E values were 0.27 and 0.29, respectively, a lower value was recorded in BRS184 (H_O = 0.02) and BRS230 (H_O = 0.06). In the CD cultivars, the highest H_O value was 0.20 in the CD216 plants, and the lowest (0.00) was recorded in CD215 plants. In the RR cultivars the highest H_O and H_E values were 0.22 and 0.2705, respectively, in the CD212RR cultivar, and the lowest value was recorded in the CD214RR cultivar (H_O = 0.00 and H_E = 0.1330).

Departure from Hardy-Weinberg equilibrium was observed in 14 of the 45 tests done

on CD cultivars, in 12 of the 30 tests done in BR and RR cultivars (results not shown), resulting from a deficit in heterozygous plants. The F_{IS} value was negative only in both CD206 (F_{IS} = -0.1715) and BRS133 (F_{IS} = -0.2121) cultivars (Table 1), showing an excess of heterozygous plants. Since H_O was higher than H_E only in CD206 and BRS133 cultivars, excess homozygous plants (or deficit of heterozygous plants) occurred in the soybean cultivars.

A very high cultivar differentiation level (F_{ST} >0.25; Wright, 1978) was evident in the plants of the 21 CD, BR, and RR cultivars (F_{ST} = 0.3865; Table 2). Accordingly, estimates of gene flow calculated from F_{ST} were below the N_m = 1 level (Table 2). In contrast, a relatively low level of differentiation (F_{ST} <0.05) was detected between CD and BR cultivars (F_{ST} = 0.017) and between CD and CDRR cultivars (F_{ST} = 0.0261), which suggested a common genetic basis of founder soybean genotypes in the different breeding programs. The common genetic origin of soybean genotypes introduced in Brazil may be the reason for the low level of population differentiation in CD and BR plants. A low level of differentiation (F_{ST} = 0.0289) was also detected between conventional cultivars (9 CD and 6 BR) and RR cultivars (CD212RR, CD213RR, CD214RR, CD219RR, BRS244RR, and AntaRR). On the other hand, a high differentiation level was evident among the nine CD (F_{ST} = 0.3630) and among the six BR (F_{ST} = 0.4517) cultivars. These results are typical of the genetic structure of self-pollinated species, where most genetic variation is observed among populations.

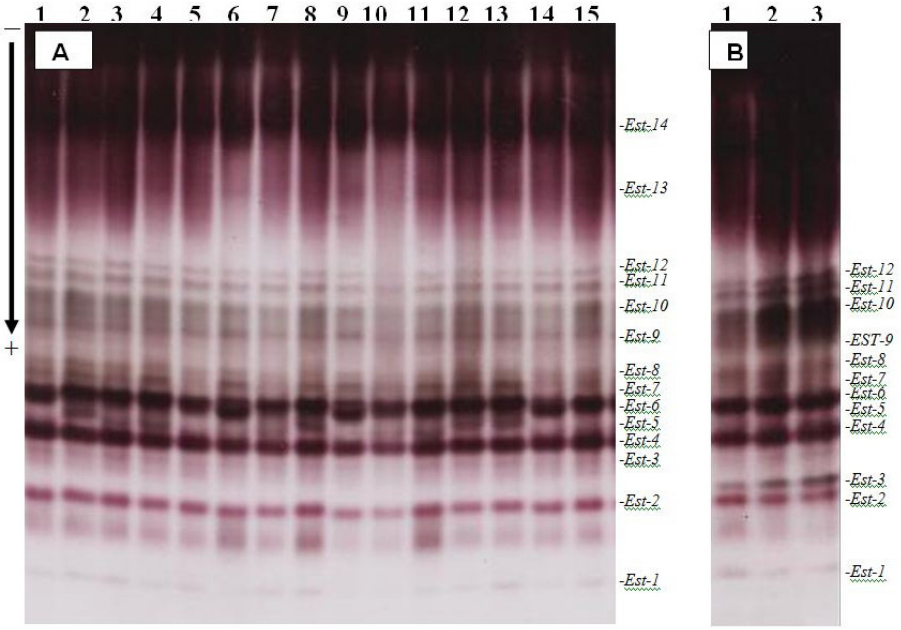

Figure 1. Isozymes α- and β-esterases of soybean leaves in a polyacrylamide gel electrophoresis system showing the alleles *Est-1*^{1} (*lanes 6-11*; gel A) and *Est-1*^{2} (*lanes 1-5* and *12-15*, gel A; *lanes 1-3*, gel B), *Est-2*^{1} (*lanes 6*, *9*, *10*, and *14*, gel A) and *Est-2*^{2} (*lanes 1-5*, *7*, *8*, *11-13*, and *15*, gel A; *lanes 1-3*, gel B), *Est-3*^{1} (*lanes 1-3*, gel B) and *Est-3*^{2} (*lanes 1-15*, gel A; *lane 3*, gel B), *Est-5*^{1} (*lanes 1*, *3-5*, *7*, *10*, *11*, *15*, gel A; *lanes 1-3*, gel B), *Est-5*^{2} (*lanes 2*, *8*, *12*, *13*, gel A) and *Est-5*^{3} (*lanes 1*, *6*, *9*, *14*), *Est-6*^{1} (*lanes 1*, *6*, *9*, *14*, gel A) and *Est-6*^{2} (*lanes 2-5*, *7*, *8*, *10-13*, *15*, gel A; *lanes 1-3*, gel B), and the genotype *Est-14*$^{1/2}$ (*lanes 1-15*, gel A) in the BR37 (gel A) and BRS230 (gel B) cultivars.

Table 1. Number of polymorphic loci (N_{PL}), proportion of polymorphic loci (%P), number of alleles (N_A), proportion of alleles per locus ($N_{A/PL}$), effective number of alleles (N_E), mean observed (H_O) and expected (H_E) heterozygosity, and F_{IS} values in plants of 21 soybean cultivars.

Cultivar	N_{PL}	%P	N_A	$N_{A/PL}$	N_E	H_O	H_E	F_{IS}
CD201	2	14.28	8	1.60	1.35	0.080	0.187	0.5238
CD202	2	14.286	8	1.60	1.56	0.020	0.227	0.8959
CD204	3	21.42	8	1.60	1.55	0.140	0.289	0.4871
CD205	2	14.28	7	1.40	1.26	0.080	0.150	0.3107
CD206	2	14.28	7	1.40	1.12	0.110	0.088	-0.1715
CD208	2	14.28	7	1.40	1.30	0.020	0.171	0.8666
CD209	3	21.42	8	1.60	1.29	0.130	0.1958	0.3084
CD215	2	14.28	7	1.40	1.21	0.000	0.1390	1.0000
CD216	2	14.28	7	1.40	1.32	0.200	0.1750	0.0000
BR37	4	28.57	9	1.80	1.52	0.270	0.2908	0.5858
BR48	4	28.57	9	1.80	1.26	0.150	0.1337	0.2798
BRS133	1	7.14	6	1.20	1.08	0.070	0.0578	-0.2121
BRS137	4	28.57	10	2.00	1.57	0.190	0.2808	0.3222
BRS184	3	21.42	9	1.80	1.36	0.020	0.1950	0.6491
BRS230	3	21.42	8	1.60	1.15	0.060	0.1210	0.6078
CD212RR	3	21.42	8	1.60	1.50	0.220	0.2705	0.2498
CD213RR	4	28.57	9	1.80	1.43	0.140	0.2645	0.3917
CD214RR	2	14.28	8	1.60	1.23	0.000	0.1330	1.000
CD219RR	2	14.28	8	1.60	1.38	0.000	0.1480	1.000
BR244RR	4	28.57	9	1.80	1.33	0.090	0.2187	0.3970
AntaRR	2	14.28	7	1.40	1.24	0.090	0.1448	0.2517
Total	5	35.71	12	2.40	1.68	0.0995	0.3061	0.3571

Table 2. Fixation coefficients *F* (F_{IS}, F_{IT}, F_{ST}; Wright, 1965) and gene flux value (N_m) in soybean cultivars.

Locus	F_{IS}	F_{IT}	F_{ST}	N_m
Est-1	0.0041	0.1704	0.1669	1.2473
Est-2	-0.1087	-0.0157	0.0839	2.7298
Est-3	0.9514	0.9709	0.4028	0.3707
Est-5	0.6726	0.8302	0.4814	0.2693
Est-14	-0.5665	-0.1781	0.2479	0.7585
Total	0.470	0.6749	0.3865	0.3968

Nei's identity (I) values varied between 0.5538 (plants of the BR37 and BRS133 cultivars) and 0.9995 (plants of CD205 and CD208 cultivars). The genetic basis of BR cultivars (0.5538-0.9748) was found to be broader than the genetic basis of CD cultivars (0.7058 for CD205 and CD209 and 0.9995 for CD205 and CD208). A higher genetic identity value was also detected between plants of CD and CDRR cultivars (I = 0.9816).

DISCUSSION

Our results confirm that PAGE can be a powerful procedure for analysis of α- and β-esterase isozymes from leaf tissues of soybean plants. Fourteen loci for isoesterases were simultaneously evident in the same electrophoresis, using only one enzymatic system. Isozyme studies in other soybean genotypes have revealed 13 and 15 loci from analysis of 8 and 9 enzymatic systems, respectively (Griffin and Palmer, 1995; Hirata et al., 1999). The analysis of various different enzymatic systems generally requires higher cost and time investments. PAGE system α- and β-esterase isozyme analysis can be used for studies to

detect genetic diversity in soybean cultivars at a relatively low cost. Esterase polymorphism in soybeans has only been reported for the *Est-1* locus (Abe and Ohara, 1992; Hirata et al., 1999). The *Est-1* locus was used as an additional marker to differentiate the soybean Japanese population from populations of other countries (Hirata et al., 1999).

The α- and β-esterase isozyme analysis in the PAGE system showed that the proportions of polymorphic loci (%P) in the CD, BR, and RR Brazilian cultivars (35.7%) is lower than that in the Japanese (69%), Korean (56%), and Chinese (75%) populations, but the number of alleles per locus (2.4) was similar to the values reported for the Japanese (2.31) and slightly higher than the values of the Korean (2.19) and Chinese (2.13) populations tested by Hirata et al. (1999), in an analysis of 16 loci (protein and isozyme loci). The value of H_E in CD, BR, and RR soybean cultivars (0.306) was also relatively higher than for the Japanese (0.248), Chinese (0.249), and Korean (0.209) populations. These Japanese, Korean, and Chinese populations, analyzed by Hirata et al. (1999) using isozyme loci, are principally accessions of soybean landraces and local varieties.

Soybean cultivars, including the Brazilian cultivars, have been characterized mainly by DNA markers (Abdelnoor et al., 1995; Priolli et al., 2002, 2004; Bonato et al., 2006; Yamanaka et al., 2007; Fu et al., 2007; Hwang et al., 2008). The microsatellite markers or SSR (simple sequence repeat) loci have been used as tool for cultivar identification, pedigree analysis and the evaluation of genetic distance among Brazilian cultivars (Priolli et al., 2002, 2004; Yamanaka et al., 2007). The number of alleles per locus and proportion of polymorphic loci have been reported from SSR loci analysis, but the observed and expected heterozygosity in SSR loci have not been reported for Brazilian cultivars.

The α- and β-esterase polymorphisms in the PAGE system in our study had high and low values for observed and expected proportions of heterozygous loci in the 21 different soybean cultivars, indicating that CD, RB, and RR cultivars are genetically structured. A larger number of heterozygous plants in CD202 (H_E = 0.227), CD204 (H_E = 0.289), CD216 (H_O = 0.20), BR37 (H_O = 0.27 and H_E = 0.29), BRS137 (H_E = 0.2808), CD212RR (H_O = 0.22 and H_E = 0.2705), and CD213RR (H_E = 0.2645) cultivars may be a result of different reactions to the environment, preventing uniform plant responses. High heterozygosity indicates that the plant population probably has substantial adaptive genetic variation to overcome environmental changes (Allendorf and Luikart, 2007). Alternatively, reduced heterozygosity reduces the fitness of individuals at loci in which the heterozygous individuals have a relative advantage over homozygous specimens.

Differences in allele frequencies and proportions of heterozygous loci in different populations demonstrated genetic divergence among the 21 cultivars (F_{ST} = 0.3865). According to Wright (1978), F_{ST} values between 0.15 and 0.25 indicate high interpopulational divergence levels, or high genetic differentiation level between populations. Limited spatial dispersal and populations frequently disturbed by human interference may cause the high level of differentiation in soybean cultivars. Self-pollination as a primary form of reproduction in soybeans may explain the high degree of genetic structure in the populations. Additionally, high selection pressure adopted in conventional cultivar management may cause the selection of particular genotypes and may create increased spatial heterogeneity. High differentiation levels was evident among the nine CD and six BR (F_{ST} = 0.4517) cultivars. Analysis of genetic diversity of 184 soybean cultivars from six different Brazilian breeding programs using 12 microsatellite loci also showed that most of the variance occurred among cultivars within

breeding programs, and only 5.3% of the whole variance was due to differences between breeding programs (Priolli et al., 2004). In our study, only 1.7% of the total variance was due to differences between the COODETEC (CD cultivars) and EMBRAPA (BR cultivars) breeding program cultivars.

The α- and β-esterase polymorphism in the soybean cultivars also showed low genetic differentiation between CD and CDRR cultivars (F_{ST} = 0.0261), indicating that gene insertion and *in vitro* tissue culture to obtain genetically modified plants have not produced highly differentiated plants. Plants regenerated from tissue culture are frequently genetically altered; somaclonal variation has been included to produce plants with new traits that are genetically stable and inherited in a Mendelian manner as either dominant or recessive traits (Larkin and Scowcroft, 1981). Tissue cultures prepared from roots of individual soybean plants developed RFLP allelic differences at various loci (Roth et al., 1989). However, new alleles for α- and β-esterase isozymes in RR cultivars were not detected, and other possible mutations induced by *in vitro* system culture were not sufficient to produce genetic differentiation among original-conventional (CD) and RR cultivars.

Allele frequency analysis in CD, BR, and RR cultivars showed that the *Est-1*^{1}, *Est-2*^{1}, and *Est-14*^{2} alleles were the most frequent alleles at *Est-1*, *Est-2*, and *Est-14* loci in all soybean cultivars. Although the physiological role and specific substrate for α- and β-esterase isozymes *in vivo* are unknown for soybean, it is possible that the selection processes used by breeding programs to develop important agronomic characteristics have also selected the most frequent alleles at *Est-1*, *Est-2*, and *Est-14* loci.

Esterase analysis in our study showed high and low genetic diversity within CD and RB cultivars and high genetic diversity among the 21 soybean cultivars. Understanding the genetic structure of these populations is important and may provide specific and differential culture strategies for each cultivar, depending on whether they have high or low heterozygosity. α- and β-esterase loci are functional-coding regions of the soybean genome and their analysis in the PAGE system is a conventional, relatively simple procedure that can be used to quickly determine the genetic structure of soybean cultivars.

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