Polymerase chain reaction detect polymorphisms and trait association in soybean

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Abstract

Five sets of synthetic oligonucleotide (20-to 24-mers containing no intenal repeats) primers of known gene sequences [yellow lupin nodule specific (hydroxyl) proline-rich protein, pearl millet alcohol dehydrogenase, *Pisum sativum* heat shock proteins, *Drosophila* homeobox, and tRNA] were used to differentiate 73 soybean accessions, including 56 *Glycine max* (L.) Merr. and 17 *G. soja* Zucc. & Sieb. The amplified genetic markers revealed polymorphic bands for most genotypes studied. The χ^2-analyses of the results showed that several fragments produced with these gene primers were associated non-randomly with resistance to *Phytophothora*, maturity, seed size, flower colour, seed coat colour, seed hilum colour, growth type, and leaf shape. These markers appear to be valuable for differentiation of *G. max* and *G. soja* species and genotypes within these species.

Additional key words: Glycine max, Glycine soja, homeobox.

Introduction

Soybean breeders can accelerate progress in selecting desired plants if they are able to identify superior alleles and thus superior genotypes by selecting for the alleles themselves rather than their expression. Unfortunately, the majority of agronomic characteristics are controlled by genes which have no known products, and therefore, these genes are currently detected through linkage to a specific chromosome segment. Techniques such as restriction fragment length polymorphisms (RFLPs) and DNA fingerprinting with polymerase chain reaction (PCR) have provided powerful tools for mapping any segment of the genome in any desired species;

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moreover, linkage map construction is a straight-forward process and more rapid than developing linkage maps through breeding and subsequent segregation of traits.

Direct analysis of polymorphisms in soybean genomic DNA can provide information on genetic diversity and gene linkage. Until a few years ago, the most commonly used DNA markers in soybean were RFLPs. For example, soybean ribosomal gene variation (Doyle 1988, Doyle and Beachy 1985), soybean quantitative traits and hard seededness were analyzed using RFLPs (Keim *et al.* 1990a.b). Restriction fragment and restriction site mapping analyses require restriction endonuclease digestion and hybridization with specific cloned probes to compare homologous DNA fragments within or between species, and thus can be time-consuming, laborious, expansive, and potentially dangerous due to the use of radioactive isotopes and their highly regulated disposal.

Simple procedure for the detection of DNA polymorphisms based on the PCR, which are not dependent on hybridization analysis with isotopically labeled probes, have been described for various plant, animal, and bacterial species (Caetano-Anolles *et al.* 1991, Welsh and McClelland 1991, Williams *et al.* 1990). Detection of polymorphisms by using random amplified polymorphism DNA (RAPD), (Williams *et al.* 1990), arbitrarily primed polymerase chain reaction (AP-PCR) (Welsh and McClelland 1990, Welsh *et al.* 1991), or DNA amplification fingerprinting (DAF) (Caetano-Anolles *et al.* 1991) is faster and less laborious than using RFLPs, and the polymorphisms are useful as genetic markers.

Polymorphisms generated by RAPD and AP-PCR have been applied to the identification of molecular markers for the nematode resistance gene *Mi* in tomato (Klein-Lankhorst *et al.* 1991, Martin *et al.* 1991), for the identification of inbred parents in maize (Welsh *et al.* 1991), and location of 5S-rRNA gene clusters on specific chromosomes in hexaploid wheat (Cox *et al.* 1992). Weining and Langridge (1991) synthesized specific primers from an α-amylase gene designed to target the α-amylase gene family and observed polymorphisms between wheat and barley genomes using PCR. Welsh and McClelland (1991) used PCR with consensuus tRNA gene primers to identify species and genera since most products in the fingerprinting are conserved between closely related species.

In this study, we utilized six sets of synthetic primers (20 to 24 bp) based on sequences of the known genes-yellow lupin nodule specific (hydroxyl) proline-rich protein, pearl millet alcohol dehydrogenase (*Adh*), *Pisum sativum* heat shock protein (*Hsp*), *Drosophila* homeobox (*Dfd*), consensus tRNA, and soybean glycinin (A_2B_1a) - to characterize genomic DNA of diverse soybean genotypes and to identify molecular markers associated with agronomic traits.

Materials and methods

Soybean accessions: Seventy-three soybean accessions including 56 *Glycine max* (L.) Merr and 17 *G. soja* Zucc. & Sieb species were selected for this study. These genetic materials were obtained from various sources as follows: Nine *G. max* commercial grain-type cultivars were obtained from the soybean breeding program, Department

of Plant and Soil Science, Alabama A & M University, Normal, Alabama. Twenty-nine *G. max* cultivars and 17 wild *G. soja* genotypes were obtained from the USDA Soybean Germplasm Collection, Urbana, Illinois. Nineteen vegetable-type lines of *G. max* obtained from Harbin, Nanjing, and Taiwan were available from our breeding program.

Characteristics of these accessions are: Soybean flower colour (FC) (purple and white), growth type (GT), and leaf shape (LS) (broad and narrow); they were based on observations of 120 to 150 plants grown in a field at the Winfred Thomas Agricultural Research Station, Alabama A & M University. One-hundred seed mass (SS) and seed coat (SCC) and hilum colours (SHC) (yellow, black, buff, green, and gray) were recorded after harvest. Three classes of 100-seed mass were established: small ≤ 10.9 g, medium 11.0 to 17.9 g, and large ≥ 18.0 g. The classification for maturity group (MG) and response to Race 1 of *Phytopthora megasperma* f. sp. *glycinea* (Phyt.) (resistant, medium resistant, sensitive) were obtained from Athan (1987) and Keeling (1982), respectively.

DNA extraction: Total cellular DNA of soybean was isolated from fresh young leaves of one plant from each accession as described by Saghai-Maroof *et al.* (1984). The plants were grown in individual pots in the greenhouse for to 4 weeks and fully expanded leaves were harvested for DNA extraction.

Synthesis of oligonucleotide primers of known genes: Specific oligonucleotide gene primers were synthesized by standard phosphoramidate chemistry on a *Pharmacia LKB Gene Assembler Plus* DNA synthesizer. The specific primers were all oligodeoxynucleotide 20- to 24-mers containing no internal repeats. The protecting groups of the synthesized primers were removed by placing them in 5 % ammonium hydroxide at room temperature for 24 h and then desalted with *Pharmacia NAP* columns containing *Sephadex G-25*.

These primers were designed according to published cDNA seqences. They were *1*) the nodule-specific (hydroxyl) proline-rich protein LENOD2 gene of yellow lupin (Szczygolowski and Legocki 1990), *2*) the alcohol dehydrogenase gene (Adh1) of pearl millet (Ha *et al.* 1990), *3*) the heat shock protein (Hsp) gene of *Pisum sativum* (Lauzon *et al.* 1990), *4*) the homeobox sequence from the *Drosophila* deformed gene (Dfd) consisting of 24-mers encoding for amino acids 1 to 8 and 54 to 61, respectively (Regulski *et al.* 1985), *5*) the consensus tRNA gene (Welsh and McClelland 1991), and *6*) the soybean glycinin A_2B_1a gene (Kitamura *et al.* 1990). Primers synthesized from a white clover *Adh* gene (Ellison *et al.* 1990), *Neurospora* clock gene (McClung *et al.* 1989), *Pisum sativum* phytochrome gene (Sato 1990), and wheat alpha/beta gliadin cDNA (Garcia-Maroto *et al.* 1990) were also examined, however, they failed to detect any polymorphism in soybean, therefore are not discussed further. Similarly, fragments amplified by the glycinin A_2B_1a gene were mostly monomorphic and also not useful.

The root nodule protein gene was selected because root nodules are highly organized structures formed on leguminous plants as a result of interaction with *Rhizobium*. The formation of root nodules involves differential expression of nodule-specific plant genes. Proline-rich protein genes represent a group of genes the

expression of which is induced at the early stages of root nodule development (Fuller *et al.* 1980. Alcohol dehydrogenase (ADH) is a cytocylic enzyme that breaks down alcohol. In most plants species, including maize and pearl millet, two genes (*Adh-1* and *Adh-2*) encode ADH. Each gene has two different allelic forms (F and C) in cultivated species. Heat shock proteins (hsp) are induced during high temperature stress. The *Hsp* gene belongs to a multiple low-molecular-mass gene family, and its function is to provide thermotolerance. It has been reported that high temperature induces the synthesis of a novel set of *Hsp* genes in soybean (Key *et al.* 1981).

Using *Drosophila* homeobox sequences as probes, previously unknown homeotic genes have been isolated from other higher organisms, including vertebrates, indicating that homeobox sequences have been highly conserved during evolution. The gene *Dfd* used in this study is part of the antennapedia complex and is homologous to the mouse gene *hox2.6* and human gene *Hox4.2*. An examination of these genes revealed that nine amino acids in the homeodomain are invariant in 16 *Drosophila* domains and 22 other domains from sea urchins to human (Regulski *et al.* 1985). As homeobox sequences influence development in both animals and plants, the question of whether soybean exhibits diversity for DNA sequences when primed by a homeobox motif was pursued in this study.

tRNA genes have multiple copies throughout the genome in most species. They are also found in mitochondria and chloroplasts of higher plants. tRNA genes seem to change relatively slowly (Welsh and McClelland 1991) during evolution. Due to their conservative nature and abundance, they are good candidates for germplasm evaluation, gene mapping, and phylogenetic analyses. The glycinin A_2B_1a sequence was obtained from the soybean cultivar Bonminori (Kitamura *et al.* 1990), of which 5' flanking region reaches to the 3'-end of another glycinin subunit gene A_1aB_1b which is located 28806 bp upstream of the cap site of this gene. The transcribed regions are undefined. The repeat (-1920 to -1969) is composed of 25 repeats of 'AT' pair.

PCR amplification conditions: PCR amplifications were performed in reaction volumes of 0.1 cm^3 in a 0.5 cm^3 microcentrifuge tube (*Perkin-Elmer Cetus*) containing the following: sterile distilled water, 1 × *Taq* DNA polymerase buffer (*Promega*), 200 μM of dNTP (*Promega*), 0.2 μM of each primer, 1.25 mM of $MgCl_2$ (*Promega*), 70 ng of total genomic DNA of soybean, and 2.5 units of Taq DNA polymerase (*Promega*). Amplification was carried out of 1 min at 94 °C for DNA denaturation, 1 min at 50 °C for primer annealing, and 2 min at 72 °C for primer extension. At the end of the cycle program, fragments were extended for 10 min at 72 °C. Amplified PCR products were resolved by electrophoresis on 1.5 % agarose gels followed by staining with ethidium bromide.

Data collection and statistical analysis: The DNA patterns generated from each set of primers were evaluated with the assistance of a computer-integrated laser densitometer (*Ultrascan XL*; *LKB products AB*, Bromma, Sweden) by using *Gelscan XL*, version 2.0 software (*Pharmacia LKB Biotechnology*, Uppsala, Sweden). Each reproducible band was examined for size in base pairs and recorded as '+' or '-' for the presence and absence of a fragment, respectively. The contingency χ^2-test was

used to test for randomness of association of DNA fragments with soybean plant characteristic.

Results

Of the six primer sets used in this study, five were polymorphic in the population of *Glycine* accessions studied (Table 1).

Yellow lupin nodule-specific (hydroxyl) proline-rich protein gene primer: The primer synthesized from the nodule-specific (hydroxyl) proline-rich protein gene of yellow lupin revealed 11 polymorphic bands in soybean ranging in size from 330 to 1750 bp. The number of fragments amplified in each accession ranged from 1 (IAC-100 and Blue Side) to 10 (PI91160). Most of the accessions tested by the nodule-specific gene primer set contained fragments of 400, 500, 650, 800 and 1300 bp (Fig. 1). The frequencies of individal fragments (number of accessions with a particular fragment/total number of accessions) present in individual accessions are shown in. Fragments 400, 500, 650, 800 and 1300 bp were the most common (0.76 to 0.98), while fragments 330, 1000 and 1750 bp were the least common 0.02 to 0.16).

Table 1. Synthetic gene primers.

Gene	Primer sequence
Yellow lupin nodule-specific (hydroxyl) prolin-rich protein	5' TCTCCGCCTGTGCACCCCCA-3'
Pearl millet *Adh1*	5'-GTGATCAAGTGCAAAGCCGCC-3'
	3'-GATGCAGCGGATGCCCTCCCC-5'
Pisum sativum Hsp	5'-CGTGCGACAAACACAAAATCATCC-3'
	3'-GTAAATGTAACATTTTCTTTTCTT-5'
Drosophila homeobox	5'-CCAAAACGCCAACGCACCGCCTAC-3'
	3'-CTTGTTGTCCTTCTTCCACTTCAT-5'
Consensus tRNA	5'-AGTCCGGTGCTCTAACCAACTGA-3'

Some fragments generated by the nodule-specific gene primer were highly specific to the commercial grain-type cultivars and vegetable-type *G. max*. For example, a 900-bp fragment was amplified in 38 of the *G. max* lines and vegetable-types and *G. soja*, while none of the commercial cultivars contained the 900-bp fragment. Two fragments of 1100 and 1750 bp were amplified in some of the germplasm lines but were not amplified in any of the commercial, grain-type cultivars, vegetable-type or *G. soja* accessions. We also found that identical DNA patterns were produced in some soybean accessions. For example, all commercial cultivars produced six identical fragments (400, 500, 650, 800, 1300, and 1500 bp), and 8 of the 19 vegetable-type lines produced seven identical fragments (400, 500, 650, 800, 900,

1300, and 1500 bp). Three of *G. soja* accessions produced seven identical fragments, which were the same as those produced by eight vegetable lines.

Pearl millet *Adh* gene primer set: The primers synthesized from the pearl millet alcohol dehydrogenase (*Adh1*) gene revealed 11 polymorphic bands in soybean ranging in size from 150 to 1150 bp. The number of bands of each accession ranged from three (SNB 128 and Heilong 33) to 10 (*Leflore*, Davis, Tracy-M, Centennial, Bedford, Forrest, Heihe 3, and N1589). The fragments 150, 270, 400, 500, and 800 bp were the most frequent (0.67 to 0.970 and fragments 330, 600, 700, and 1150 bp were the least frequent (0.35 to 0.43).

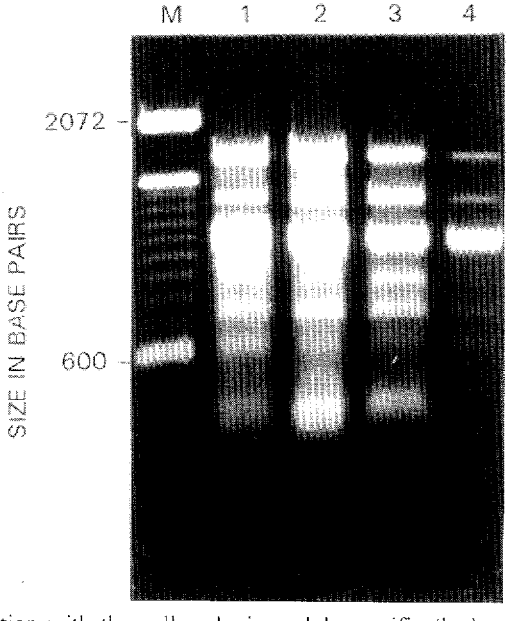

Fig. 1. DNA amplification with the yellow lupin nodule-specific (hydroxyl) proline-rich protein gene primer. M: 100 bp DNA ladder. Lanes 1 to 4: PI 91160, PI 172901, and PI 381674 (accessions 24, 27, 31, and 32, respectively).

More fragments were generated in the commercial cultivars (8 to 10) than in wild soybeans (4 to 6) and vegetable lines (3 to 10). Among the amplified fragments, some were uniquue to *G. max* and *G. soja*, as well as to commercial and vegetable soybeans. For example, the 800-bp fragment was amplified in *G. max* but was not amplified in *G. soja*; the 200 bp fragment was amplified in all commercial cultivars but only one vegetable-type, N7788. Furthermore, DNA amplification with *Adh* gene primers showed that most accessions (56 %) of the commercial cultivars had identical DNA patterns, while the rest of commercial cultivars had one or two fragment differences in comparison with the vegetable and wild soybeans.

***Pisum sativum Hsp* protein gene primer set:** The primer synthesized from Hsp gene of *Pisum sativum* revealed eight polymorhic bands ranging in size from 240 to 1250 bp.

Table 2. PCR fragments significantly associated with agronomic and morphologic traits based on contengency χ^2 tests. * - $P \leq 0.05$, ** - $0.01 \leq P \leq 0.05$.

Gene primer	Trait	Fragment size	χ^2	df	No. accessions
Nodule specific protein	Flower colour	500	5.50*	1	73
	Maturity group	900	24.60**	7	49
	Seed coat colour	900	13.90*	4	73
Alcohol dehydrogenase	Seed hilum colour	150	11.10*	4	73
	Seed hilum colour	200	11.20**	4	73
	Seed hilum colour	270	14.00**	4	73
	Seed coat colour	200	9.50**	4	73
	Seed coat colour	600	15.10**	4	73
	Seed coat colour	800	16.55**	4	73
	Seed coat colour	950	9.92*	4	73
	Seed size	600	11.74**	2	73
	Seed size	700	17.78*	2	73
	Seed size	800	17.14**	2	73
	Seed size	950	7.35*	2	73
	Maturity group	700	18.31*	7	73
	Maturity group	800	26.02**	7	49
	Maturity group	950	17.18*	7	49
	Maturity group	1150	27.55**	7	49
	Growth type	800	11.07**	1	55
	Growth type	950	5.48*	1	55
	Growth type	1150	5.48*	1	55
	Lef shpe	800	4.31*	1	55
	Species	600	3.96*	1	73
	Species	700	5.16*	1	73
	Species	800	12.82**	1	73
	Species	950	5.38*	1	73
Heat shock protein	Seed coat colour	240	12.28*	4	73
	Seed coat colour	280	12.34*	4	73
	Seed coat colour	800	15.06*	4	73
	Seed coat colour	870	11.13*	4	73
	Seed hilum colour	240	15.83**	4	73
	Seed hilum colour	280	12.34*	4	73
	Seed hilum colour	470	11.63*	4	73
	Growth type	800	4.79*	1	55
	Phytophthora	450	8.49*	2	23
Homeobox	Maturity group	900	23.05**	7	49
	Seed size	100	6.16*	2	73
	Seed size	900	6.66*	2	73
	Seed coat colour	900	11.68*	4	73
	Growth type	100	5.20*	1	55
	Leaf shape	100	5.09*	1	55
tRNA	Seed size	820	9.57**	2	73
	Seed size	905	6.61*	2	73
	Seed size	1600	8.59*	2	73
	Seed coat colour	820	12.05*	4	73
	Seed coat colour	1600	10.65*	4	73
	Seed coat colour	2350	15.24**	4	73
	Growth type	820	5.71*	1	55

The number of bands in each accession ranged from 1 to 9. Tracy-M, Bay, SNB128, SNT 180, and SNIH 620904 produced nine fragments while TN 4-86 produced only one fragment. The smallest fragment, 125 bp, was monomorphic in all accessions while the largest fragment, 1250 bp was amplified only in six samples.

Most of the accessions (73 to 100 %) produced six common fragments (125, 240, 280, 360, 470 and 800 bp). Among the 64 accessions, 13 produced 8 identical fragments (125, 240, 280, 360, 470, 620, 800 and 870 bp). In addition, an 870 bp fragment was amplified in most commercial cultivars and germplasm lines. Fragments 125, 240, 280, 360, and 800 bp were the most frequent (0.78 to 1.00), while fragments 870 and 1250 bp were the least frequent (0.1 to 0.45).

***Drosophila* homeobox gene (*Dfd*) primer set:** The primers synthesized from the homeobox gene of *Drosophila* revealed 11 polymorphic bands ranging in size from 100 to 1500 bp.

Three to eight fragments were amplified in each accession. For example, commercial cultivar TN 4-86 and vegetable line G 9053 produced eight fragments, while germplasm SNB 112 and SNB 128, and vegetable line Heilong 33 produced three fragments. Most of the soybean accessions had 1 to 2 fragments in addition to for common fragments (200, 280, 330, and 590 bp).

The polymorphisms generated by homeobox gene primers showed that some fragments can be used to identify different groups of soybean. For example, the 900 bp fragment was amplified in eight of nine commercial cultivars, but it was not amplified in germplasm cultivars with the exception of PI 547834 and HARD 5272. All of the nine commercial cultivars had six fragments in common (100, 200, 280, 330, 590, and 900 bp), except Leflore which lacked the 900 bp fragment. Among the commercial cultivars, five produced the same six identical fragments as well as a 400 bp fragment.

tRNA gene primer: The tRNA gene primer revealed 17 polymorphic bands ranging in size from 250 to 2720 bp. Three to 12 fragments were amplified in each accession. Vegetable lines G 9053, G 84136 - P 418, Blue Side, and N 7788 produced 12 fragments, while vegetable lines AGS 129 and AGS 290 produced only three fragments. DNA patterns varied in the rest of the soybean accessions, which had common fragments (250, 450, 630, and 905 bp). Fragments 250 and 630 bp were the most frequent (0.95 and 0.87, respectively), while fragments 500, 750, 1500, 2100, and 2350 bp were the least frequent (0.03 - 0.13).

Relationship between amplified fragments and plant characteristics: The results demonstrated that some genes produced more polymorphisms than others and that some polymorphisms could be closely associated with a particular group or genotype, whereas others were unique to a given accession or genotype.

The frequency distribution for each amplified fragment was determined in association with each group of individual characteristics, *e.g.*, flower colour, maturity group, seed size, growth type, and leaf shape.

Some fragments generated by the primer set of five known genes were significantly associated with the following soybean characteristics (Table 2): *1*) resistance to *Phytophthora megasperma* with the 450-bp fragment generated by

the heat shock protein gene primer set; *2*) maturity with six fragments: a 900-bp fragment generated by the nodule-specific gene primers, as well as four fragments (700, 800, 950, and 1150 bp) generated by the *Adh* gene primers, and one fragment (900 bp) generated by the homeobox gene primers; *3*) seed size with the 600, 700, 800, and 900-bp fragments generated by the *Adh* gene primers, and a 1600-bp fragment generated by the tRNA gene primer; *4*) flower colour with the 500-bp fragment generated by the nodule-specific gene primer set; 5) seed coat colour also with 13 fragments including a 900-bp fragment generated by the nodle-specific gene primer, four fragments (200, 600, 800, and 950 bp) generated by the *Adh* gene primers, four fragments (240, 280, 8800, and 870 bp) generated by the *Hsp* gene primers, one fragment (900 bp) generated by the homeobox gene primers, and three fragments (820, 1600, and 2350 bp) generated by the tRNA gene primer; *6*) seed hilum colour with three fragments (150, 200, and 270 bp) generated by the *Adh* gene primers, and three fragments (240, 280, and 470 bp) generated by the *Hsp* gene primer set; *7*) growth type with three fragments (800, 950, and 1150 bp) generated by the *Adh* gene primers, one 800-bp fragment generated by the *Hsp* gene primers, one 100-bp fragment generated by the homeobox gene primers; *8*) leaf shape with the 100 bp fragment generated by the homeobox gene primers; and *9*) species with four fragments 9600, 700, 800, and 950) generated by the *Adh* gene primers.

Some amplified fragments (Table 2) were simultaneously associated with several characteristics in soybean. For example, the 800-bp fragment generated by the *Adh* gene primers was associated not only with maturity but also with seed size, seed coat colour, growth type, leaf shapx and species. Since fragments generated by the *Adh* gene primers were significantly associated with seven of the characteristics studied, this gene may be useful for detecting genetic linkage with the associated traits.

Discussion

Primers synthesized from known gene sequences from *Drosophila*, *Pisum sativum*, yellow lupin, and pearl millet have the ability to detect soybean genomic DNA variation, suggesting that homologous sequences can be identified in soybean under the conditions of amplification employed in this study. The results also showed that some of these homologous sequences may be highly conserved as measured by the low degree of polymorphism generated in these soybean accessions. For example, the soybean glycinin gene primer set was largely monomorphic for the most part, reinforcing the conclusion that this gene may be highly conserved.

The nodule-specific (hydroxyl) proline-rich protein, *Adh*, *Hsp* and homeobox genes are reported to be conserved in other species. In our study, a relatively large number of similar fragments were generated by these four gene primers, but only a few polymorphic bands were generated among soybean accessions studied.

Since segregating populations were not used in this study, the associations observed here may not have been due to genetic linkage. Instead, these associations may infer genetic associations due to interactions between alleles at different loci. This kind of linkage disequilibrium can be maintained by inbreeding which restricts

recombination. These kinds of associations may represent character combinations desired in breeding but they may be dispersed by segregation when hybrid populations are made. The specific gene primer assay method used here has proven its applicability for the identification of individual accessions and they are highly effective and convenient when examining a large number of germplasm accessions in germplasm evaluation or in searches for redudancies in genetic resource collections. We believe that these genes and the soybean polymorphisms revealed can have application in plant breeding to enhance breeders ability to use molecular markers as selection criteria in crop improvement.

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