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Molecular markers residing close to the *Rhg4* locus conferring resistance to soybean cyst nematode race 3 on linkage group A of soybean

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Abstract The restriction fragment length polymorphism (RFLP) clone pBLT65 is a 450-nt soybean cDNA encoding a portion of the bifunctional enzyme aspartokinase-homoserine dehydrogenase (AK-HSDH). pBLT65 maps within 3.5 cM of the *i* locus, conferring a pigmented seed coat, on linkage group A; hence, it is closely linked to the Rhg_4 locus conferring resistance to race 3 of the soybean cyst nematode. From this useful RFLP we developed a PCR reaction yielding polymorphic bands for use in marker-assisted breeding programs to select progeny containing the Rhg_4 allele. The polymorphic bands were sequenced to determine the cause of the polymorphisms. Using primers 548 and 563, PCR amplification of DNA from the soybean cultivar Peking (Rhg_4) yielded three DNA fragments, 1a (1160 bp), 1b (1146 bp) and 3 (996 bp). Amplification of DNA from the cultivar Kent (rhg_4) yielded DNA fragments 2 (1020 bp), 3 (996 bp) and 4 (960 bp). Fragments 1a, 1b, 2 and 4 were also polymorphic between the soybean lines PI 290136 and BARC-2(Rj_4). A segregating population of 80 F_2 and F_3 plants derived from the cross PI 290136 × BARC-2 (Rj_4) was used to confirm the map position of the PCR polymorphisms near the *i* locus, and hence the Rhg_4 locus on linkage group A. The nucleotide sequences of fragments 1b, 3 and 4 were determined. Large and small deletions in the intronic region were responsible for the size differences of the different fragments, whereas the exon was well conserved.

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Introduction

The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is a devastating pest of soybean and is responsible for significant losses in yield in the USA (Mulrooney 1988; Niblack et al. 1992). SCN causes root necrosis, reduced nodulation and decreased shoot vigor. Multiple races of SCN exist (Riggs and Schmitt 1988) and several genes involved in resistance to specific races have been identified (Caldwell et al. 1960; Matson and Williams 1965; Leudders 1987). These include rhg_1, rhg_2, rhg_3 and Rhg_4. In a cross between PI 437.654 and 'BSR 101' the Rhg_4 locus on linkage group A and two additional QTLs were identified; one was mapped to linkage group G and the other to linkage group M. Significant interaction between Rhg_4 and the QTL on G was noted as these provided complete resistance to race 3 of SCN (Webb et al. 1995). Neither locus alone conferred resistance to SCN race 3 much greater than that of the susceptible parent 'BSR 101'. Some of the genes conferring SCN resistance used in many cultivars today are derived from the cultivar Peking (Anand 1986). Attempts have been made to identify molecular markers closely linked to genes conferring SCN resistance (Weisemann et al. 1992; Concibido et al. 1994; Webb et al. 1995).

The Rhg_4 locus is located on linkage group A (Keim et al. 1990; Webb et al. 1995) and is very tightly linked with the *i* locus controlling black seed-coat pigmentation (Matson and Williams 1965). The Rhg_4 locus is 0.35 recombination units from the *i* locus. pBLT65 has been mapped to a position near the *i* locus and is a useful RFLP molecular marker that can be used to track the inheritance of the Rhg_4 locus in marker-assisted breeding schemes (Weisemann et al. 1992; Skorupska et al. 1994; Webb et al. 1995; Heer et al.

1998). It resides within 3.5 cM of the *i* locus and the Rhg_4 locus (Weisemann et al.1992; Webb et al. 1995). Seed-coat color in soybean is controlled by the *i* locus and in that region a family of genes encoding chalcone synthase is found (Todd and Vodkin 1998). Interestingly, deletions in this region increase levels of chalcone synthase mRNA and directly relate to seed-coat color. Chalcone synthase is a key enzyme involved in plant responses to pathogen attack and is important in phytoalexin and flavonoid synthesis (Welle et al. 1991).

pBLT65 has been used by several investigators to monitor the inheritance of the Rhg_4 locus (Webb et al. 1995; Heer et al. 1998). As an RFLP marker pBLT65 requires labor-intensive techniques, including Southern blots and probe hybridization. This paper describes a PCR method for identifying polymorphic bands with homology to the genomic sequence encoding the bifunctional enzyme, aspartokinase-homoserine dehydrogenase (AK-HSDH), indirectly derived from pBLT65. We confirmed that these PCR-amplified fragments also map close to the *i* locus, and hence to the Rhg_4 locus. Therefore this PCR assay should be useful in many marker-assisted breeding schemes to distinguish between progeny with a high likelihood of possessing the Rhg_4 or rhg_4 alleles.

Materials and methods

The soybean cross PI 290136 (Noir) × BARC-2 (Rj_4) and F_2 and F_3 seeds were produced at Beltsville Md. PI 290136 has a black pigmented seed-coat (*i–i*) and is from the USDA germplasm collection. BARC-2 (*Rj4*) carries *I–I* which inhibits seed-coat color expression and has a tan seed-coat (Devine and O'Niell 1986).

Segregation of the *i* locus was determined by examining seed from ten F_3 progeny each of the 131 plants from the F_2 generation, while segregation of the PCR products was examined using DNA from 80 plants from the F_2 generation. Both parents are susceptible to SCN race 3 and are rhg_4.

Soybean (*Glycine max* L. Merr.) cultivars PI 290136 (Noir), BARC2 (*Rj4*), Bedford, Century, Custer, Essex, Dyer, Forrest, Hartwig, Kent, Kingwa, Lee, NC55, Peking, Pickett, PI 437654, PI 88788, PI 90763, PI 588-1608 and PI 589-2122 were grown in the greenhouse. Leaves were harvested and genomic DNA extracted as described previously using the CTAB (hexadecyltrimethylammonium bromide) method (Weisemann et al. 1992).

PCR reactions were performed using the method of Williams et al. (1990) modified as described previously (Lin et al. 1996). Reactions of 25 µl contained 30–50 ng of genomic DNA,15 mM Tris HCl, pH 8.3, 6 mM KCl, 2.75 mM $MgCl_2$, 100 µM dNTPs, 0.8 µM of primer 548 and primer 563, and 1.8 units of *Taq* polymerase (Life Technologies, Rockville, MD., USA). Reactions were heated for 2 min and amplified for 35 cycles at 94°C for 1 min, 57°C for 30 s and 72°C for 1 min. Amplified DNAs were separated on a 2% agarose gel containing TBE and stained with ethidium bromide. DNA primers were synthesized on an Applied Biosystems DNA synthesizer. The 5′ to 3′ DNA sequence for primer 548 was GCA GAT ATC AAC AGT TGG GAC; primer 563 was GGA ATG GAC AGC TCG TAA AGC C; primer 803 was TGG AAT GAC TGC AAC CTG AGA G. Amplified fragments were excised from the agarose gel, eluted, and cloned into pT7 Blue (R) T vector (Novagen, Madison, Wis.).

DNA sequencing in both directions was performed on double-stranded plasmid using the dye terminator method with an automated ABI DNA fluorescence sequencer (Perkin Elmer Corp., Foster City, Calif.). The DNA sequences of the amplified fragments were analyzed using the University of Wisconsin GCG sequence analysis package running on a VAX 8250 system (Genetics Computer Group).

The data were tested for single-factor segregation and linkage using chi-square. Recombination frequency was estimated using the method of maximum likelihood (Mather 1951; Allard 1956). The bisection method was used to solve the maximum-likelihood equations (Yakowitz and Szidarovszky 1990).

Results

The RFLP probe pBLT65 is a cDNA clone encoding 450 nt of an isoform of the bifunctional enzyme AK-HSDH. DNA sequences for a cDNA encoding one isoform and a genomic clone encoding another isoform were known (Gebhardt and Matthews, unpublished). The location of primers 548 and 563 and the region amplified on the genomic DNA encoding AK-HSDH of soybean is depicted in Fig. 1. One primer hybridizes to a portion of intron 9 of the gene encoding AK-HSDH, while the other primer hybridizes to a portion of exon 11. The amplified fragments range from approximately 960 to 1150 nt.

PCR-amplification of genomic DNA of PI 290136 using primers 548 and 563 produced three fragments, 1a, 1b and 3, (M. Wt. 1160 bp, 1146 bp, and 996 bp, respectively), while amplification of genomic DNA of the parental line BARC-2 *(Rj4)* produced fragments 2, 3, and 4 (M. Wt. 1020 bp, 996 bp, and 960 bp, respectively), which could be separated by agarose-gel electrophoresis (Fig. 2). Fragments 1a, 1b, 2 and 4 were polymorphic between PI 290136 and BARC-2 (*Rj4*) and, therefore, could be used in mapping by monitoring differences in the amplified DNA from segregating F_2 and F_3 plants from a cross of PI 290136 and BARC-2(Rj_4). At times, PCR products produced using DNA from F_2 plants displayed a third high-molecular-weight band on agarose gels (band 1a′, Fig. 2) that was not always present, depending upon experimental conditions.

The segregation pattern for each band individually fits a 3 : 1 ratio (analyses not shown). Bands 1a and 1b

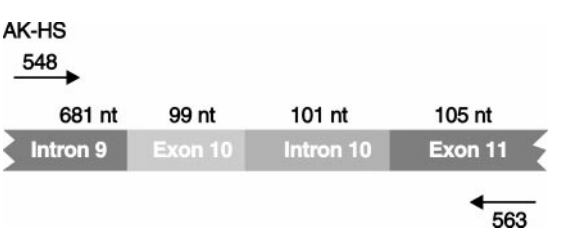

Fig. 1 Location and direction of primers 548 and 563 on intron 9 and exon 11 of the genomic sequence encoding the soybean bifunctional enzyme AK-HSDH

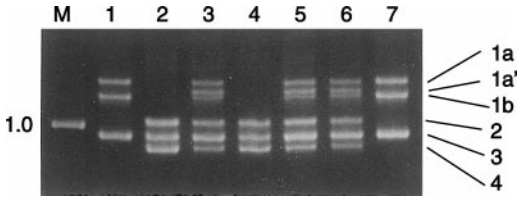

Fig. 2 PCR amplification products of DNA from PI 290136 (Noir), BARC-2(*Rj*$_4$) (*lanes 1 and 2*) and F_2/F_3 plant numbers 6, 10, 31, 81 and 120 (*lanes 3–7*). Products were separated on a 2% agarose gel in TBE. *Lane M* contains a molecular-weight DNA marker denoted in kb

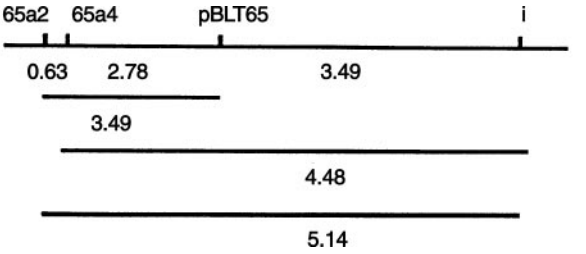

Fig. 3 The relative positions of loci defined by PCR products using primers derived from pBLT65, the RFLP marker pBLT65, and the *i* locus are depicted with relative map distances

always co-segregated, thus providing no evidence of recombination between them. Bands 2 and 4 co-segregated except for one F_2 plant of the 80 tested. This exception could be interpreted as a result of recombination or of a rare mutation that prevented primer extension in the PCR reaction. When each individual band was tested for linkage with the segregation data for the RFLP pBLT65, bands 2 and 4 mapped very near the *l* locus (less than 5% recombination), while bands 1a and 1b mapped to a position ten recombination units distant from pBLT65 (data not shown). Because bands 1a and 1b were derived from one parent and bands 2 and band 4 were derived from the other parent, it was postulated that band 1a could be allelic to either band 2 or band 4. When band 1a was considered as an alternative allelic form of band 2, with the presence of both bands 1a and 2 interpreted as the heterozygote, the monogenic ratio fit a 1:2:1 ratio. The locus thus defined was designated as pBLT65a2 and mapped more closely to the pBLT65 locus (less than 4% recombination, Table 1), designated 65a2 in Fig. 3. Alternatively, when band 1a was considered allelic with band 4, the observed monogenic ratio also fits a 1:2:1 ratio and the locus was designated as 65a4 (Fig. 3). This locus also mapped closely to the pBLT65 locus (less than 3% recombination). A reasonable interpretation of the recombination data permits the construction of the map shown in Fig. 3; thus, in either hypothetical case, the 65a2 or 65a4 locus maps close to the *l* locus and, consequently, the *Rhg*$_4$ locus.

Broad application of this PCR assay to the marker-assisted breeding of soybean requires that the assay identifies polymorphisms in numerous soybean cultivars. Genomic DNA was extracted from 26 different soybean cultivars and breeding lines and was PCR-amplified using primers 548 and 563 (Fig. 4). A banding pattern reflecting the genotype of each soybean cultivar was obtained, similar to the banding patterns of PI 290136 or BARC2 (*Rj*$_4$), indicating that the PCR assay can be reproduced with many cultivars (Table 2).

Table 1 Results of genetic linkage tests and linkage analysis for the *I* locus with molecular markers

Genes	Genotypic classes[a]										χ^2L[b]	$P(\chi^2$L)[c]	R[d]	SE[e]	Ratio
	e	f	g	h + i	j	k	l	m	n	Sum					
I, pBLT65	38	3	3	57	0	0	1	1	28	131	235.23	<0.001	3.49	1.16	121242121
I, pBLT65a2	0	0	2	38	19	2	16	2	1	80	115.8	<0.001	5.14	1.79	121242121
I, pBLT65a4	0	0	2	38	19	2	17	1	1	80	122.52	<0.001	4.48	1.67	121242121
I, pBLT65, pBLT65a2	0	0	2	36	18	2	14	1	0	73	110.84	<0.001	3.49	1.55	121242121
pBLT65, pBLT65a4	0	0	2	36	18	2	15	0	0	73	117.60	<0.001	2.78	1.38	121242121
pBLT65a2, pBLT65a4	16	1	0	41	0	0	0	0	22	80	150.38	<0.001	0.63	0.63	121242121
	a	b	c	d											
pBLT65a, pBLT65b	58	0	0	22						80	91.02	<0.001	0.00	3.9E-5	9331

[a] Class designations per Allard (1956)
[b] Linkage chi-square
[c] Chi-square probability
[d] Percent recombination
[e] Standard error for recombination estimate

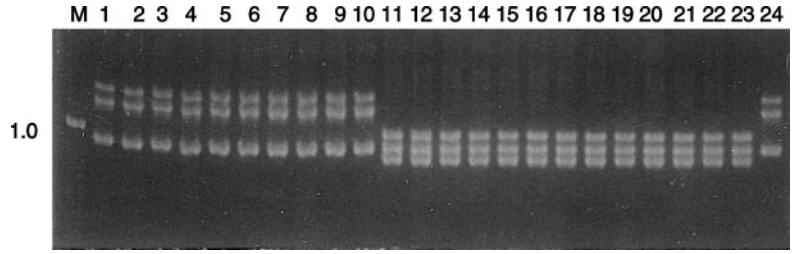

Fig. 4 The fragments amplified by primers 548 and 563 are also amplified using DNA from other soybean cultivars, indicating that these primers will be of general use for marker-assisted selection in breeding programs. Genomic DNA from 20 cultivars was subjected to PCR amplification with primers 548 and 563 and separated on a 2% agarose gel. *Lane M* denotes a 1-kb DNA marker. *Lane numbers and cultivars* are listed in Table 2

Amplified DNA fragments 1b, 3, and 4 were cloned into the pT7 Blue (R) T vector to identify the differences between the fragments and to provide the DNA sequences so that other investigators can design primers specific to their research interests (Fig. 5). The sequenced regions span from intron 9 through exon 11 of the genomic sequence of AK-HSDH. Therefore, exon 10 and exon 11 are part of the open reading frame encoding a portion of AK-HSDH. Several deletions and differences in DNA sequence are present among the sequences. The deletions are present in the introns. Most notable is a 185-nt sequence present in fragment 1b beginning at nt 352 that is deleted in fragments 3 and 4. Fragment 1b is closely related to fragment 4 and has only a 4-nt difference other than the 185-nt deletion. Fragment 1b is more distantly related to fragment 3 and has 42-nt differences in addition to the 185-nt section. Repeated attempts to clone and sequence fragments 1a and 2 failed.

To demonstrate that primers could be synthesized specifically for the PCR amplification of single bands in this area, producing a less complex banding pattern upon gel electrophoresis, primer 803 was synthesized and used (Fig. 6). This would allow an investigator to develop a PCR procedure with a less complex banding profile or to track a particular AK-HSDH allele.

Table 2 Soybean cultivar response to SCN race 3, seed-coat pigmentation and PCR banding pattern using primers 548 and 563

Lane	Variety	SCN response[a]	Seed-coat pigmentation[b]	Banding pattern				
1	Peking	R	S	1a	1b		3	
2	NC55	R	S	1a	1b		3	
3	PI88788	R	S	1a	1b		3	
4	PI89772	R	S	1a	1b		3	
5	PI90763	R	S	1a	1b		3	
6	PI437654	R	S	1a	1b		3	
7	Custer	R	N	1a	1b		3	
8	Pickett	R	N	1a	1b		3	
9	Bedford	R	N	1a	1b		3	
10	Hartwig	R	N	1a	1b		3	
11	S88-1608	R	N	1a	1b		3	
12	Essex	S	N			2	3	4
13	Lee	S	N			2	3	4
14	Hill	S	N			2	3	4
15	Kent	S	N			2	3	4
16	Scott	S	N			2	3	4
17	Bass	S	N			2	3	4
18	Williams82	S	N			2	3	4
19	Century	S	N			2	3	4
20	BARC2 (*Rj4*)	S	N			2	3	4
21	D62-7818	S	N			2	3	4
22	D49-2491	S	N			2	3	4
23	Blackhawk	S	N			2	3	4
24	PI290136 (Noir)	S	S	1a	1b		3	

[a] SCN response denotes the resistance response of the soybean cultivar to SCN race 3. (R) is resistant. (S) is susceptible

[b] The pigmentation is designated as (S) self-colored and (N) nonself-colored

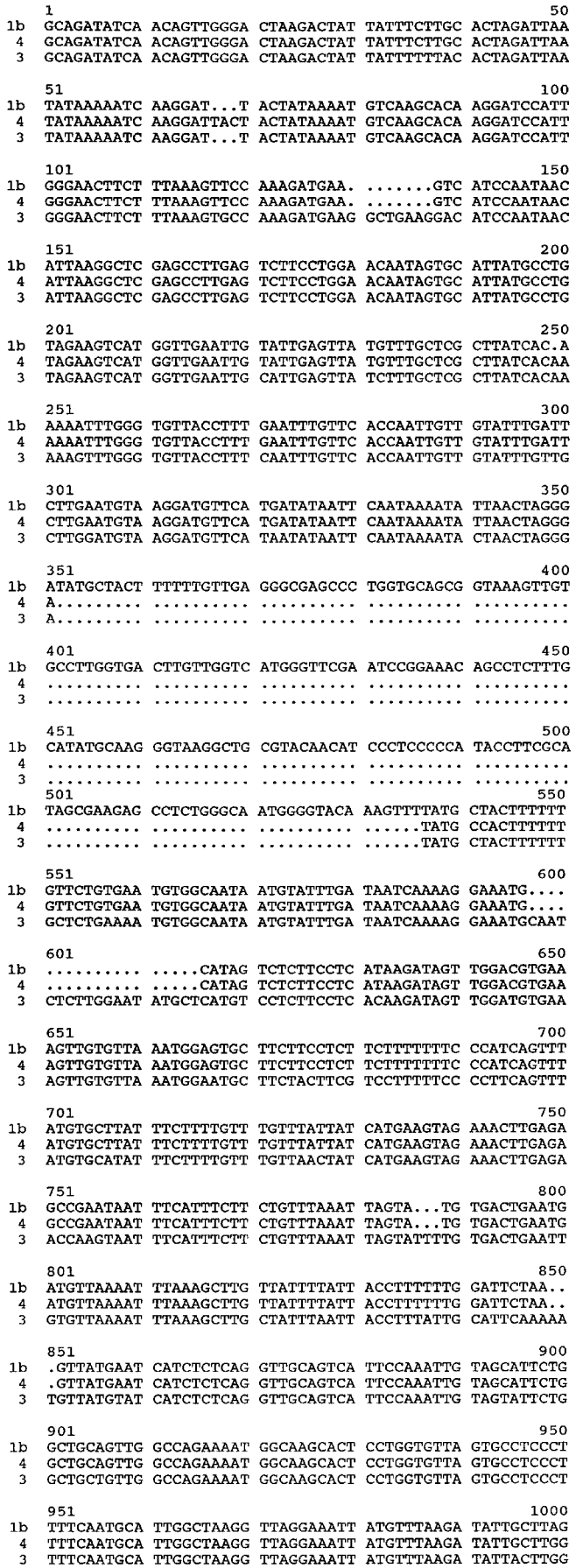

Fig. 5 DNA sequence alignments of amplified fragments 1b, 3, and 4. Absent nucleotides are depicted by (.)

Discussion

The PCR reaction of genomic soybean DNA containing primers 548 and 563 produces several DNA fragments. The polymorphic fragments map to the same region of linkage group A and map very close to the *i*, and hence the *Rhg*$_4$. locus. The banding patterns are readily distinguished in all cultivars examined; therefore they should be useful for molecular marker-assisted breeding with many different parental lines.

The PCR banding pattern and mapping data indicate that there are at least two related AK-HSDH genes located close to each other on linkage group A near the *i* locus. Molecular and biochemical evidence indicate that three or more isoforms of AK-HSDH are present in soybean (Matthews and Widholm 1979 a,b; Gebhardt and Matthews, unpublished), while electrophoretic gels of soybean extracts stained for HSDH activity indicate that there are four different isoforms of HSDH activity (Matthews and Widholm 1979 a,b). Three of these isoforms are closely related. Molecular evidence also supports this conclusion, because two independent, but highly homologous, genes encoding soybean AK-HSDH have been cloned and sequenced (Gebhardt, Weisemann and Matthews, unpublished). A third, highly homologous, gene was indicated by restriction enzyme analysis. The present paper provides additional evidence for the existence of at least one more AK-HSDH gene in soybean as indicated by fragment 3, which is presently unmapped.

Molecular markers based on the PCR are more rapidly assayed than RFLP-based markers (Lin et al. 1996). pBLT65 has been used by several investigators and appears to be polymorphic in a number of soybean cultivars. The PCR-based assay of this locus is also polymorphic among several soybean cultivars. By defining the DNA sequence of the polymorphism and its proximal regions, opportunities are available to design other valuable primers that capitalize on this polymorphism. Identifying recombinant plants for these PCR

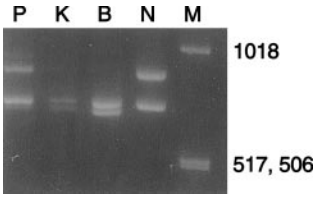

Fig. 6 Fragments amplified by primers 548 and 803 using DNA from (*P*) Peking, (*K*) Kent, (*B*) BARC2 (*Rj4*) and (*N*) PI 290136 (Noir). *Lane M* contains molecular-weight markers denoted in bp

fragments in mapping populations will establish the gene order and provide an orientation for the BAC and YAC clones in the region. This will aid in map-based cloning of the Rhg_4 locus and other important genes in the region.

Numerous attempts to clone and sequence bands 1a and 2 failed. Clones were obtained from excised agarose electrophoretic gels but, upon sequencing, clones putatively containing 1a had an identical sequence to 1b, while those putatively containing band 2 had a DNA sequence identical to band 3. At the present time we have no explanation for this.

The polymorphic fragments generated from primers 548/563 flank a polymorphism located in intron 9. A major deletion, as well as several small deletions and nucleotide changes, occur in this region. PCRs using templates from the 26 cultivars yielded reaction products suggesting that this assay can be used with DNA from many different soybean cultivars. By choosing one parental line with one marker pattern and another line with a different pattern, this PCR assay should be a useful molecular marker for this region.

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