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High-Throughput Genotyping for a Polymorphism Linked to Soybean Cyst Nematode Resistance Gene *Rhg4* by Using TaqMan™ Probes

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Abstract

An individual soybean breeder can generate over one hundred thousand new genotypes each year. The efficiency of selection in these populations could be improved if these genotypes were effectively screened with one DNA marker that identified an important gene, and if laboratory throughput was high and costs were low. Our aim was to develop a rapid genotyping procedure for resistance to the soybean cyst nematode. A high-throughput genotyping method was developed with fluorogenic probes to distinguish between two insertion polymorphisms in alleles of an AFLP marker that is located about 50 kbp from the *Rhg4* gene candidate. The assay uses the 50 exonuclease activity of *Taq* polymerase in conjunction with fluorogenic probes for each allele. The method can be used for scoring the polymorphism in a recombinant inbred line population and for screening parent lines in a breeding program. The TaqMan™ method of determining genotype was accurate in 90% of scores in the RIL population compared to 95% accuracy with electrophoresis. Among 94 cultivars that are parents in our breeding program allele 2 that is derived from the sources of resistance to SCN was common in resistant cultivars (30 of 56) but rare in

susceptible cultivars (3 of 38). Therefore, this method can be applied to automated large-scale genotyping for soybean breeding programs.

Keywords: TaqMan™, genetic mapping, marker-assisted breeding, SCN, *Rhg4*

Abbreviations: E × F – Essex × Forrest, FAM – 6-carboxyfluorescein, FI – female index, QTL – quantitative trait loci, SCN – soybean cyst nematode, TAMRA – 6-carboxy-N,N,N′5N′-tetrachlorofluorescein, TET – 6-carboxy- 4,7, 2′,7′-tetrachlorofluorescenin, PE – Perkin-Elmer

Introduction

Soybean cyst nematode (SCN, *Heterodera glycines* L.) causes annual crop losses in soybean (*Glycine max* (L.) Merr.) estimated at 4% of the total yield worldwide, representing about $800 million in losses to US production (Wrather et al. 1995, 1996). Yield loss to SCN is limited by an iterative cycle of development of high yielding cultivars incorporating genetic resistance to SCN (Brown 1987; Bernard et al. 1988). Several sources of resistance to SCN, including 'Peking' and 'PI437654' (Myers and Anand 1991; Rao-Arrelli and Anand 1992), have been used to develop cultivars. SCN resistance is simply inherited, but field resistance is oligogenic due to the existence of variation among SCN populations that are described as 'races' (Riggs and Schmidt 1988).

One gene, *rhg1*, provides the major portion of resistance to SCN race 3 across many genotypes derived from Peking (Mahlingam and Skorupska 1995; Chang et al. 1997; Mathews et al. 1998; Meksem et al. 2001b), PI437654 (Webb et al. 1995; Prabhu et al. 1999), 'PI88788' (Concibido et al. 1997; Bell-Johnson et al. 1998; Cregan et al. 1999a, b, c), 'PI209332' (Concibido et al. 1996), or 'PI90763' (Concibido et al 1997). A second gene for SCN resistance, *Rhg4*, provides an equal portion of resistance to SCN race 3 across genotypes derived from Peking (Mahalingam and Skorupska 1995; Chang et al. 1997; Mathews et al. 1998; Meksem et al. 2001b) and PI437654 (Webb et al. 1995; Prabhu et al. 1999) but not PI88788, PI209332, or PI90763 (Concibido et al. 1996, 1997). Cytological studies suggest PI437654 and Peking derived resistances share mechanisms (pronounced necrosis and cell wall appositions) not seen in PI88788 in response to race 3 (Mahalingam and Skorupska 1996). These differences in mechanism may derive from distinct alleles at *Rhg4*, *rhg1* and/or other defense-associated loci.

The SCN race 3 resistance genes *rhg1* and *Rhg4* have been surrounded with DNA markers (Brown, 1987; Weiseman et al. 1992; Mahlingam and Skorupska 1995; Webb et al. 1995; Concibido et al. 1996, 1997; Cregan et al. 1999a, b, c; Meksem et al. 1999, 2001b) that allow selection of alleles of the two genes in new combinations in many breeding programs. The markers described herein, A2D8, is derived from an AFLP band that maps within the interval that appears to contain *Rhg4* in RIL populations (Meksem et al. 2001b). The marker is also found within BACs that encompass the genetic region and both flanking markers (Zobrist et al. 2000) (fig. 1). Markers physically linked to the genes for resistance to SCN allow the direct selection of recombination events close to the genes which may be useful for the reduction of linkage drag on yield (Mudge et al. 1996; Njiti et al. 1997) and the selection of clustered resistance genes (Meksem et al. 1999). While selection with physically

linked markers will be inferior to selection with markers derived directly from the *Rhg4* gene sequences, those sequence derived gene markers are not unequivocally established (Meksem et al. 2001b). SCN resistance alleles can be efficiently selected with linked molecular markers (Bell-Johnson et al. 1998; Cregan et al. 1999a; Prabhu et al. 1999) at lower cost than by phenotype determination in the greenhouse (Rao-Arrelli et al. 1992; Prabhu et al. 1999). However, the cost of marker selection and limited throughput capability still limit the degree to which breeders can afford to use the techniques.

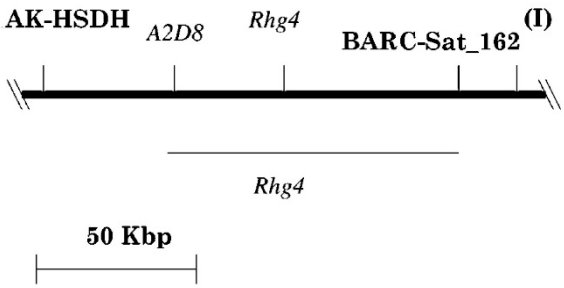

Figure 1. A genetic and physical map showing the location of an *Rhg4* candidate relative to DNA markers. The location of the aspartokinase serine dehydrogenase (AK-HSDH) and the A2D8 marker are indicated as determined by restriction mapping of BAC DNA (not shown). The A2D8 sequences for Essex and Forrest alleles are deposited in GenBank (AF286701 and AF286700). The *I* locus (I) position was estimated by relation to BARC-SAT_162 (Cregan et al. 1999c). Genetic mapping shows *Rhg4* and A2D8 are both within the interval shown by the horizontal line and within a large insert clone, 100B10, that contains a 140 kb insert (Zobrist et al. 2000).

Each year individual breeders can generate over 100 000 new genotypes and select about 14 000 at the first visual selection step of recurrent selection. Selection methods based on PCR are being used to process this high, seasonal throughput (Prabhu et al. 1999). Gel electrophoresis, which is necessary to score many markers, consumes about half the cost of the selection method (Gu et al. 1995; Prabhu et al. 1999). The TaqMan™ allelic discrimination assay, PCR-OLA, molecular beacons, padlock probes and well fluorescence have been developed to determine genotypes without gel electrophoresis (see Landegren et al. 1998). The TaqMan™ allelic discrimination assay is based on the 5′ nuclease activity of *Taq* polymerase and detection of a fluorescent reporter during or after PCR reactions (Livak et al. 1995a, b). Each TaqMan™ probe consists of a 25–35 base oligonucleotide complementary to one of two alleles with a 3′ quencher dye attached (6-carboxy-N,N,N′5N′-tetrachlorofluorescein, TAMRA). The oligomer complimentary to allele 1 is linked covalently to a 5′ reporter dye (6-carboxy-4,7,2′,7′-tetrachlorofluorescenin, TET) while allele 2 is linked to a dye that fluoresces at a distinct wavelength (6-carboxyfluorescein; FAM). PCR directed by flanking oligomers of 18–20 bases causes degradation during the extension phase of the oligomer that hybridizes most efficiently to the polymorphic site(s) in the sample. TaqMan™ is useful for high-throughput genotyping of important polymorphisms, in man (Shi

et al. 1999), soybean (this work) and for the detection of microbes (Shin et al. 1999). It reduced the exposure of personnel to ethidium bromide and other hazards. The TaqMan™ programs eliminate human scoring error and data entry errors as results are returned in spreadsheet format (Livak et al. 1995b). Adaptations can make the assay chemistry suitable for multiplexing (Nararabadi et al. 1999) and miniaturization (Kalinina et al. 1997) to reduce cost and increase throughput.

In the present study we report a PCR marker for an insertion polymorphism close to *Rhg4* and the TaqMan™ probe we derived. Using this locus we are able to detect, in a high throughput format, Peking and PI437654 (SCN resistance) alleles among SCN-resistant alleles and distinguish these resistance alleles from the majority of SCN susceptibility alleles in susceptible soybean cultivars.

Materials and methods

Plant material

The mapping population consisted of 100 $F_{5:13}$ recombinant inbred lines (RIL 1-100) derived from a cross between 'Forrest' (Hartwig and Epps 1973) and 'Essex' (Smith and Camper 1973). These soybean cultivars contrast for disease resistances (Hartwig and Epps 1973; Smith and Camper 1973; Cang et al. 1997), response to water deficit (Cho et al. 1999), yield potential (Njiti et al. 1997), and phytoestrogen content (Njiti et al. 1999). The derived recombinant inbred line (RIL) population was advanced to the $F_{5:13}$ generation from never less than 300 plants per RIL per generation. The 78 lines shown for gel electrophoresis (RIL1–RIL78) and 86 lines used for TaqMan™ assays (RIL1–RIL86) were overlapping subsets from the 100 RILs represented in the population. The numbers of RILs shown were selected to allow for appropriate controls while fitting a high-throughput slab gel system (84 wells) or assay plate (96 wells). Forrest is resistant to the soybean cyst nematode (SCN) race 3 and Essex is susceptible to all races of SCN (Hnetkovsky et al. 1996; Chang et al. 1997; Meksem et al. 1999). SCN resistance in Forrest derives from Peking (Hartwig and Epps 1973) and results exclusively from alleles of *rhg1* and *Rhg4* (Chang et al. 1997; Meksem et al. 2001b).

Oligonucleotides

PCR primers and TaqMan™ probes were designed with the primer express program (Perkin-Elmer/Applied Biosystems, Foster City, CA) and were custom-synthesized by Perkin-Elmer (PE). Two TaqMan™ probes were designed to encompass the A2D8 (fig. 1) insertion polymorphisms (underlined). That A2D8 SCAR was derived from the codominant AFLP bands ECCG-MAAC417 (Essex, allele 1, GenBank AF286701) and ECCG-MAAC409 (Forrest, allele 2, AF286700) that contain a homologue (P = 2e-05) of one component (*Tic22*; AAC64606.1) of the protein import apparatus of the chloroplast inner envelope membrane (Meksem et al. 2000b). Allele 1: 5′-TET-TTG**CAGATA TTTTAGTTGATT**GGCC-TAMRA. Allele 2: 5′-6FAM-AGTTGATTG**GCTCAAACCA**TGGCC-TAM RA. Reverse primer: 5′-d-TTGCGTGTGATCGGTA TTAC-3′. Forward primer: 5′-d-TACCTGAGTTCTCT CAAGTC-3′

DNA isolation

Soybean genomic DNA was extracted from a pooled sample of leaves from 5 plants per genotype and purified using the Qiagen Plant Easy DNA Extraction Kit (Qiagen, Hilden, Germany).

SCN female index (FI) determination

The number of white female cysts was compared on each genotype to the number of white female cysts on a susceptible control to determine the female index (FI) for each population (Meksem et al. 1999). The FI was performed on seedlings at the University of Missouri–Colombia by inoculating the genotypes with 2000 ± 25 eggs from a homogenous isolate of *H. glycines*. The population was 'race 3' as determined by the FI on Peking (1.2%), 'Pickett' (1.8%), PI88788 (3.0%), PI90763 (1.2%) and PI437654 (1.2%). All experiments used five single-plant replications per line. The mean number of white female cysts on each genotype and the susceptible control were determined and FI was calculated as the ratio of the mean number of cysts on each genotype to the mean number of cysts on the susceptible check.

TaqMan™ genotyping assay

TaqMan™ reactions were performed essentially as the PE TaqMan™ PCR Reagent Kit protocol describes except the PCR reaction was performed in 384 well plates to reduce assay volume and cost. Briefly, each reaction contained 10 ng of the extracted DNA, 0.025 units/μl of AmpliTaq Gold (PE), 400 nM of the forward and reverse primers (Research Genetics, Huntsville, AL), 50 nM of FAM fluorescent probe, and 150 nM of TET fluorescent probe (PE) in 1 × universal master mix (PE). The above ratio of primers and probes was optimized using a series of primer/probe combinations to reach a maximal signal and the balance of the two probes by reading in an ABI 7200 sequence detector. The TaqMan™ universal PCR master mix is a premix of all the components, except primer and probes, necessary to perform a 5′ nuclease assay. The final optimized conditions represented a two-step PCR protocol, with two holds followed by cycling, on a 384-well thermal cycler (GeneAmp PCR System 9700, PE). The two holds were 50°C for 2 min and 95°C for 10 min. The 35 cycles were at 95°C for 15 s, 60°C for 1 min. After amplification the plates were cooled to room temperature and samples were transferred from a 384-well plate to a 96-well MicroAmp™ optical tray and fluorescence was detected on an ABI Prism™ 7200 Sequence Detector (PE).

The results were analyzed by allelic discrimination of the sequence detection software (PE). Two grouping methods were used to attempt to accurately separate heterogeneous lines from homogeneous lines at each allele. In grouping method 1 (TaqMan™ 1) a stringent cut-off for FAM (>7) was used for allele 1 compared to heterogeneous scores. This served to reduce the number called as potentially heterogeneous to about the percentage expected from the breeding method used for RIL development (6%). Fluorophore ratios were as follows; no amplification (FAM and TET both less than 6 units); allele 1 homozygous (FAM less than 7, TET greater than 7); allele 2 homozygous (FAM greater than 10, TET less than 5); and heterogeneous for allele 1 and allele 2 (FAM greater than 7, TET 5–8). For TaqMan™ selection grouping method 2 ratios were; no amplification (FAM and TET

both less than 6 units); allele 1 homozygous (FAM less than 5, TET greater than 7); allele 2 homozygous (FAM greater than 10, TET less than 5); and heterogeneous for allele 1 and allele 2 (FAM greater than 5, TET 5–9). The FAM and TET signals were stable in the dark for 2 days after PCR.

Gel electrophoresis markers

PCR reactions were performed with DNA from the recombinant inbred lines. The 114 and 120 bp PCR products were generated using the forward and reverse primers (above). The final optimized conditions were 94°C for 10 min, then 35 cycles of 94°C for 25 s, 56°C for 30 s and 72°C for 60 s. After the PCR reactions were completed, the plates were cooled to room temperature and the PCR products separated by electrophoresis on a 4% w/v agarose gel.

Results

Direct screening of A2D8 alleles with TaqMan™ probes

Genomic DNA samples were analyzed according to the TaqMan™ PCR protocol (fig. 2) (Livak et al. 1995a, b). Using the raw fluorescence signals of the reporter dyes FAM and TET from the 'dye component' field of the sequence detection software, two grouping methods were performed. Each method detected four distinct populations (fig. 2). The four populations could be assigned according to the FAM:TET ratio based on where the heterogeneous class cut-off was placed.

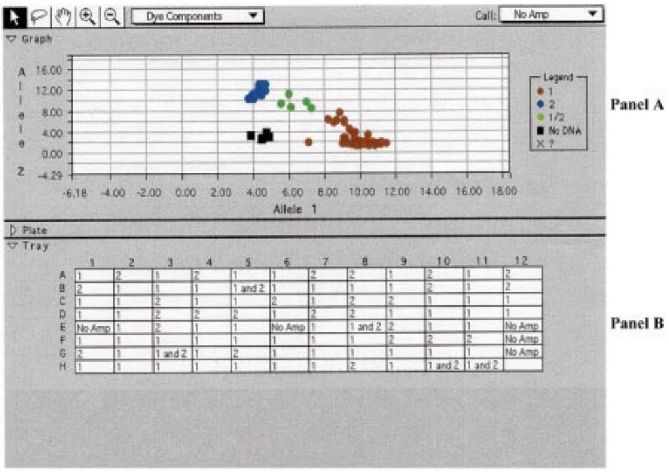

Figure 2. Detection of the A2D8 marker polymorphism by TaqMan™ Allelic discrimination of soybean genotypes with manual selection of genotypes. A total of 86 individuals from an F5 derived population of recombinant inbred lines from the cross of Essex × Forrest that segregate for resistance to SCN are shown. Panel A. The fluorescent signals viewed under the 'dye component' field of the sequence detection software and the A2D8 genotypes were manually selected based on the ratio of FAM and TET signals. Allele 1 homozygous, Forrest type; FAM<< TET. Allele 2 homozygous, Essex type; TET<< FAM.

Alleles 1 and 2 heterogeneous, Essex and Forrest type; TET less than 2-fold greater or lesser than FAM. Two selections were used; in the first (TaqMan™ assay1) the group of genotypes FAM 6–8 and TET 8–9 were considered susceptible, while in the second (TaqMan™ assay 2) they were considered heterogeneous. Panel B. The Excel spreadsheet that contains scores (allele designations) for the samples as they were arranged in the 96-well plate. There was no DNA in wells E12, F12, and G12 (negative controls). There was Essex DNA in wells A1, C12, and D12. There was Forrest DNA in wells B2, A12, and B12. The RIL DNA was in well A3 to H11 in order by row from RIL1-RIL86 except samples E1 (RIL3) and E6 (RIL 43) that did not amplify. The RILs resistant to SCN had an index of parasitism FI <10% of the susceptible check resistant lines.

For the TaqMan™ selection two grouping methods were arbitrarily selected to attempt to accurately separate heterogeneous lines from homogeneous lines at each allele. For grouping method 1 (TaqMan™ 1) a stringent cut-off was used to reduce the number called as potentially heterogeneous. Fluorophore ratios were as follows: no amplification (FAM and TET both less than 6 units); allele 1 homozygous (FAM<7, TET >7); allele 2 homozygous (FAM >10, TET <5); and heterogeneous for allele 1 and allele 2 (FAM >7, TET 5–8). For TaqMan™ selection grouping method 2 (TaqMan™ 2), a lower stringency cut-off value was used to increase the number classified as potentially heterogeneous. Ratios were: no amplification (FAM and TET both less than 6 units); allele 1 homozygous (FAM <5, TET >7); allele 2 homozygous (FAM>10, TET <5); and heterogeneous for allele 1 and allele 2 (FAM >5, TET 5–9).

Based on the FI of the ExF RIL population, the 86 selected individuals were classified into 3 classes: 15 resistant, 60 susceptible and 11 segregating lines. TaqMan™ analysis of 86 individuals from the RILs by method 1 (high stringency) shows a strong agreement between allele 1 and susceptibility to SCN (56 from the 60 susceptible lines were allele 1 type). However, there was lesser agreement between allele 2 and resistance to SCN (only 15 lines from the 23 lines showing the presence of allele 2 were resistant by phenotype) due to the segregation of rhg1, the second gene necessary for resistance to SCN in Forrest (Meksem et al. 2001b). Of the 11 lines known to be heterogeneous for the resistance to SCN phenotype, five should segregate at *Rhg4*. TaqMan™ method 1 identified one among the five classified as heterogenous (the 5 include 4 misclassified lines, see below). TaqMan™ method 2 identified all five among the 11 classified as heterogenous, but the 11 include 6 misclassified lines.

Genotyping by PCR and gel electrophoresis

To validate the specificity of TaqMan™ genotyping samples of each of the RILs classified by the TaqMan™ method (fig. 2) were rescored by PCR and gel electrophoresis (fig. 3). The classifications produced by the two methods agreed with TaqMan™ assay 1 most closely but with eight exceptions. The mis-scores were as follows (annotated as RIL number; FI phenotype; allele with TaqMan™ grouping method 2; allele with TaqMan™ grouping method 1; allele by gel marker score): 4;S;H;H;S, 21;R;H;H;R, 32;R;H;H;R, 44;S;S;S;H, 51;S;S;S;H, 59;R;H;H;R, 63;S;S;S;R, and 78;R;H;H;R.

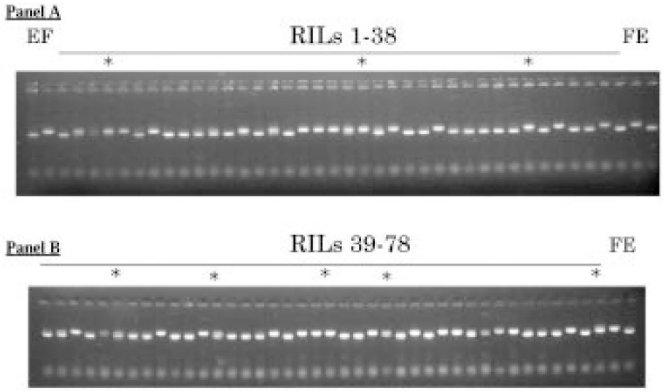

Figure 3. Detection of the A2D8 marker polymorphism by PCR amplification and gel electrophoresis of soybean genotypes. Seventy-eight individuals from an F5 derived population of recombinant inbred lines from the cross of Essex × Forrest that segregate for resistance to SCN are shown. Panel A. Lane 1, 42 Essex; Lanes 2 and 41 Forrest; Lanes 3–40, RILS 1–38. Panel B. Lane 42 Essex; Lane 41 Forrest; Lanes 1–40 RILS 39–78. Asterisks indicate disagreements with the TaqMan™ assay 1.

The majority of disagreements resulted from resistant lines that were scored as heterogeneous by TaqMan™ but not gel electrophoresis or phenotype (4 of 8) and phenotypically susceptible lines that were scored incorrectly by gel electrophoresis (3 of 8). One genotype (RIL84) was mis-scored relative to phenotype (84SRRR) by all the allele genotyping methods and may represent a recombination event between A2D8 and *Rhg4*.

Allele distribution in soybean germplasm

Genotypes at A2D8 were determined from the genomic DNA of 94 cultivars that represented the parents of populations in the SIUC soybean breeding program from 1997 to 1999 (table 1). There were 38 cultivars susceptible to SCN and 56 cultivars resistant to SCN race 3. Allele 2 (R) was found in 32 of 94 cultivars tested. There were very few susceptible genotypes with allele 2 (3 of 32) and the majority of genotypes with allele 2 (29 of 32) were resistant to SCN. In contrast, allele 1 (S) was found in 62 cultivars but frequently in both resistant cultivars (27 of 56) and susceptible cultivars (35 of 38).

Table 1. Cultivars, plant introductions (PI), breeding lines and germplasm releases that were parents in the SCN molecular breeding program at SIUC from 1997 to 1999 separated by their soybean cyst nematode resistance phenotype and allele at A2D8.

	Resistant	Susceptible
Allele 2	Forrest, Hartwig, Fayette, Pharaoh, Picket, Accomac, Bedford, Delsoy4710, Peking, PI88788, PI209332, PI90763, PI437654, LS92-1088, LS92-4173, LS94-3207, LS95-0259, LS95-0709, LS95-1454, LS96-1631, LS90-1920, LS94-3545, S92-1679, S92-2711A, S94-2086, LN94-10527, A5560, K1390, K1425	MD93-5298, Pace, Holladay
Allele 1	Manokin, Mustang, Dwight, Pana, Ina, PI 398680, IA2036, IA3005, LS92-3660, LS93-0292, LS93-0375, LS94-2435, LS96-0735, LS96-3813, LS96-5009, LN92-10725, GX93-1573, SS94-7546, SS94-4337, S95-1908, A4138, A95-483010, M92-1645, M92-1708, M90-184111, K1423, K1424	Essex, Bragg, Dunfield, Hill, CNS, Lee, Noir1, Ogden, Calhoun, Chesapeake, Choska, Stressland, Macon, Misuzudaiza, Nakasennari, PI 520733, PI567445B, PI567583C, PI567650B, PI 567374, PI 567650B, IA3010, IA1006, TN96-58, N96-180, LN93-11632, LN93-11945, LN95-5417, A94-674017, A94-774021, A96-494018, C1963, HC93-2690, HS93-4118, K1410

Discussion

Direct screening of A2D8 alleles with TaqMan™ probes

The divergent 10 bp and 18 bp insertions in the alleles of the AFLP band represented by A2D8 provide a suitable substrate for many assays of polymorphism. Here we report the development of a robust, high-throughput genotyping method based on the TaqMan™ assay. Despite reducing reaction volumes to 15 μl instead of the recommended 50 μl, the raw fluorescence signals of the reporter dyes FAM and TET from the 'dye component' field of the sequence detection software allowed four distinct populations to be detected (fig. 2). The method may be further reduced in volume to reduce costs (Kalinina et al. 1997). The method has been shown to be effective with impure DNA preparations extracted by high throughput methods (Gu et al. 1995; Bell-Johnson et al. 1998; Prabhu et al. 1999). Therefore, the method is suitable for marker assisted breeding.

The genoytpe and phenotype were in close agreement among the 86 genomic DNA samples analyzed with the TaqMan™ PCR protocol. The lesser agreement between allele 2 and resistance to SCN (15 of 23) was shown to be due to the segregation of *rhg1*, by scoring of the BARC-Satt 309 marker (Meksem et al. 1999). The bias toward a higher frequency of allele 1 is caused by sampling error (Chang et al. 1997). The accuracy of genotyping was high by the TaqMan™ assay and was better than one pass gel electrophoresis (Prabhu et al. 1999). Even compared to a highly optimized gel electrophoresis assay reported here the

assays were not significantly different in accuracy for detecting the genotypes within the F5 derived RILs in a single-pass assay. Exactly 78 of the 86 tested with both TaqMan™ and gel electrophoresis agreed. There were 5 errors with TaqMan™ (94% accurate) and 3 errors with gel electrophoresis (96% accurate) judged by replicated genotyping (not shown) and the phenotype. Low frequencies of error are important to the accurate selection of resistance (Cregan et al. 1999a; Prabhu et al. 1999) and in the generation of accurate genetic maps (Cregan et al. 1999b).

The ability to distinguish heterozygotes and their derived heterogeneous lines is important to early generation selection (before the F5) in soybean breeding programs when within population variability is high (Brown 1987; Bernard et al. 1988). The lower-stringency assay 2 was most effective for identifying most of the heterogeneous lines in this population. However, the cutoff values of FAM and TET for the efficient identification of heterogeneous lines (or heterozygous F2 lines) is likely to vary across assays and should be set arbitrarily according to expectations of the number of lines that are expected to contain both alleles. The assay has subsequently proven robust for analyzing 2 000 lines derived from specific cultivar crosses over 3 days. A single researcher can process 768 sample per day (8 × 96 samples) since the reading time of the machine is 15 min for one 96 well plate and the thermal cycler stage takes 2 h.

Allele distribution in soybean germplasm

Table 1 shows that with genomic DNA from 94 cultivars the standard TaqMan™ allelic discrimination assays and PCR assays (not shown) provided allele scores that were in good agreement with the cultivar phenotypes (Bernard et al. 1988; Concibido et al. 1997). The prevalence of allele 1 was in good agreement with allele frequencies for markers that are closely linked to *Rhg4* (Mahalingan and Skorupska 1995; Mathews et al. 1998; Cregan et al. 1999c). Those resistant cultivars sharing allele 1 with the susceptible lines may not require the presence of *Rhg4* for resistance to SCN or have derived their resistance to SCN at the *Rhg4* locus from alleles derived from cultivars other than Forrest. In addition, some soybean breeders may have been effective in separating even the most closely linked marker from resistance genes using phenotypic selection. However, this is probably infrequent since selection to generate the resistance allele 2 in susceptible cultivars has not occurred frequently. Only three cultivars with allele 2 were susceptible.

Automated, high-throughput, rapid genotyping of DNA polymorphisms is highly desirable for selection among the millions of genotypes generated annually by plant breeding programs (Prabhu et al. 1999). Currently, selection of resistance to SCN is conducted in the greenhouse or field by counting cyst number on soybean roots (rao-Arrelli and Anand 1988; Chang et al. 1997). This approach is time-consuming and labor-intensive and requires either significant greenhouse space or large field plots and a stable source of pathogen populations. Marker assisted selection for resistance to SCN has been possible for several years (Webb et al. 1995). PCR followed by gel electrophoresis is the most common method for genotyping polymorphism in soybean (Bell-Johnson et al. 1998; Mathews et al. 1998; Cregan et al. 1999a; Prabhu et al. 1999). While this approach requires no greenhouse space it is still time-consuming and labor-intensive and requires a small laboratory. The procedures involved in gel electrophoresis are moderately hazardous and difficult to automate.

Gel electrophoresis requires highly skilled staff to spend half their time involved in gel electrophoresis and data entry. In contrast TaqMan™ allelic discrimination allows automated high sample throughput with fewer chemical hazards. The method simplifies the assay by eliminating the need for gel electrophoresis, visual assessment of bands and manual data input and therefore allows accurate detection of polymorphism (Shi et al. 1999). The method can be carried out in a closed tube to reduce the risk of contamination (Lirak et al. 1995b). Fluorescence signals can be detected within minutes of reactions being performed and reaction sizes can be reduced to 15 μl without compromising the signal ratios.

The cost of TaqMan™ compared to a gel assay are almost the same, about $1.5 per data point. Reduction in the costs of primer labeling will reduce the assay cost for genotyping to half of a similarly multiplexed gel-based marker assay. The A2D8 marker is currently being adapted to allow miniaturization of the assay to nanolitre scale (Kalinina et al. 1997; Landegren et al. 1998) and modifications for multiplexing (Nasarabadi et al. 1999) to facilitate ultra-high-throughput, low-cost assays.

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