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Two simple sequence repeat markers to select for soybean cyst nematode resistance coditioned by the *rhg1* locus

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Abstract The soybean cyst nematode (SCN) (*Heterodera glycines* Inchinoe) is the most economically significant soybean pest. The principal strategy to reduce or eliminate damage from this pest is the use of resistant cultivars. Identifying resistant segregants in a breeding program is a difficult and expensive process which is complicated by the oligogenic nature of the resistance and genetic variability in the pathogen. Fortunately, resistance at one SCN-resistance locus, *rhg1*, is generally accepted as a necessity for the development of resistant genotypes using any source of resistance and when challenged by any SCN race. Thus, the development of SCN resistant cultivars would be expedited if an effective and rapid system were available to identify breeding lines carrying a resistance allele at the *rhg1* locus. In this study we report two simple sequence repeat (SSR) or microsatellite loci that cosegregate and map 0.4 cM from *rhg1*. Allelic variation at the first of these loci, BARC-Satt309, distinguished most, if not all, SCN-susceptible genotypes from those carrying resistance at *rhg1* derived from the important SCN-resistance sources 'Peking', PI 437654, and PI 90763. BARC-Satt309 was also effective in distinguishing SCN resistance sources PI 88788 and PI 209332 from many, but not all, susceptible genotypes. BARC-Satt309 cannot be used in marker-assisted selection in populations developed from typical southern US cultivars crossed with the important resistance sources PI 88788 or PI 209332 because these genotypes all carry the identical allele at the BARC-Satt309 locus. A second SSR locus, BARC-Sat_168, was developed from a bacterial artificial chromosome (BAC) clone that was identified using the primers to BARC-Satt309. BARC-Sat_168 distinguished PI 88788 and PI 209332 from southern US cultivars such as 'Lee', 'Bragg' and 'Essex'. Both BARC-Satt309 and BARC-Sat_168 were used to assay lines from SCN-susceptible×SCN-resistant crosses and proved to be highly effective in identifying lines carrying *rhg1* resistance from those carrying the allele for SCN susceptibility at the *rhg1* locus.

Key words Simple sequence repeats · Microsatellites · Soybean cyst nematode · Genetic mapping · Marker-assisted selection

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Introduction

The soybean cyst nematode (*Heterodera glycines* Inchinoe; SCN) is among the most economically destructive soybean [*Glycine max* (L.) Merr.] pests. The use of resistant cultivars is very effective in reducing crop loses to SCN and, thus, a number of sources of genetic resistance have been identified and used in the development of resistant cultivars over the past 40 years. These include germplasm lines 'Peking', PI 88788, PI 437654, PI 90763, and PI 209322. Breeding for resistance has been complicated by the genetic heterogeneity of SCN populations (Niblack 1992) and the oligogenic nature of resistance (Caviness 1992). Fortunately, the allele for partial resistance at the *rhg1* resistance locus has been demonstrated to control more than 50% of the variation for resistance (Concibido et al. 1996, 1997) and appears to effectively control a number of SCN races (Concibido et al. 1997; Webb et al. 1995).

Molecular marker-assisted selection provides an efficient method to identify genotypes that carry alleles at genetic loci of interest without the need to assess phenotype. Quantitative trait locus (QTL) analyses have detected restriction fragment length polymorphism (RFLP)

loci linked to the *rhg1* resistance locus and have placed the locus on molecular linkage group G (Concibido et al. 1996, 1997; Webb et al. 1995). To expedite the process of marker-assisted selection, simple sequence repeat (SSR) or microsatellite DNA markers have a number of advantages over RFLP markers. SSR markers are based upon the polymerase chain reaction (PCR) making analysis relatively rapid. In addition, while RFLP loci in soybean are generally dimorphic, SSR loci with as many as 26 alleles have been reported (Rongwen et al. 1995), thus greatly increasing the likelihood that a locus will be informative in any given population.

Mudge et al. (1997) recently reported two SSR loci (BARC-Satt038 and BARC-Satt130) that flank the *rhg1* locus. These were fortuitously identified as part of an effort to develop and map approximately 600 soybean SSR loci (Cregan et al. 1999b). Five different alleles were observed at both of these loci among a group of SCN susceptible and resistant genotypes. The SCN resistance sources 'Peking', PI 88788, PI 437654, PI 90763, and PI 209322 could each be distinguished from some or all susceptible cultivars at both SSR loci. However, neither locus could distinguish all SCN-susceptible from all resistant genotypes. In addition, neither of these loci was in very close proximity to *rhg1*. BARC-Satt038 was estimated to be 3 cM distal (telomeric) to *rhg1*, while BARC-Satt130 was 20 cM proximal. Concurrent selection of SSR alleles associated with resistance would permit selection of the genomic segment carrying the *rhg1* resistance allele with a high level of probability, but it would also necessitate a significant amount of linkage drag. The use of either locus alone would select at least some susceptible genotypes (false positives) as a result of recombination between the SSR locus and *rhg1*. One possible solution to reduce the rate of false positives would be the identification of one or more SSR loci in very close proximity to *rhg1*. The use of only one locus in marker-assisted selection would also reduce the level of linkage drag. In the present study we report two SSR loci in very close proximity to *rhg1*. Using one or the other of these loci, we were able to distinguish each of the five commonly used SCN resistance sources from all of the SCN-susceptible genotypes that we examined.

Materials and methods

Plant Materials

One of the SSR loci, BARC-Satt309 (subsequently referred to as Satt309) was mapped in two populations. The first population consisted of 98 $F_{4:5}$ lines from a cross of 'Evans'×PI 209332 previously described by Concibido et al. (1996) and characterized for SCN resistance. The second consisted of 110 $F_{5:6}$ lines from 'Evans'×'Peking' which were derivatives of the $F_{2:3}$ lines described by Concibido et al. (1997). In addition, Satt309 was examined in two different sets of genotypes whose SCN phenotype was previously determined. One set of genotypes was $F_{6:7}$ lines derived from 'Evans'×'Peking' and the other was $F_{4:5}$ lines from 'Parker'×A92–526007. The latter set was segregating for SCN resistance derived from 'Peking'. The second SSR locus, BARC-Sat_168 (subsequently referred to as Sat_168), was mapped in a subset of 17 lines derived from the 'Evans'×PI 209332 population that were recombinant near *rhg1* on soybean molecular linkage group G.

The alleles present at the Satt309 SSR locus were determined in the SCN-susceptible and resistant soybean genotypes listed in Table 1. A subset of these genotypes was assayed for allelic varia-

Table 1 The allele present at the Satt309 simple sequence repeat locus in soybean cyst nematode (SCN)-resistant and SCN-susceptible genotypes

Allele 1 (125 bp)	Allele 2 (131 bp)	Allele 3 (134 bp)	Allele 4 (149 bp)
SCN resistant	SCN susceptible	SCN resistant	SCN susceptible
PI 88788	Mandarin	Peking	AK (Harrow)
PI 209332	Fiskeby V	PI 90763	Amsoy
Bedford	Harosoy	PI 437654	Clark
Delsoy 4210	Jackson	Cordell	Evans
Delsoy 4710	Minsoy	Custer	Illini
Fayette	Mukden	Doles	Lincoln
Faribault	Richland	Dyer	Williams
Jack	Roanoke	Forrest	
Jeff	S-100	Hartwig	
LN89–5699	Tokyo	Hartwig 2000	
Linford		J87–233	
Nathan		M70–187	
Pyramid		Manokin	
S89–2122		Nile	
		Pickett	
		Pickett71	
SCN Susceptible			
Archer			
CNS			
Bragg			
Dunfield			
Essex			
Hill			
Lee			
Noir 1			
Ogden			

Table 2 Forward and reverse primer sequences of Satt309 and Sat_168, two SSR loci located 0.4 cM proximal to the *rhg1* soybean cyst nematode resistance locus on soybean linkage group G

Locus	Primer	Primer sequence (3' to 5')
Satt309	Forward	GCGCCTTCAAATTGGCGTCTT
	Reverse	GCGCCTTAAATAAAACCCGAAACT
Sat_168	Forward	TGTGGATAAAAGAGCATTCAAAATG
	Reverse	GCGATCCTTGTTTATCTCAAAAAAGTGT

tion at the Sat_168 locus. Seeds of the cultivars 'Delsoy 4210', 'Delsoy 4710', 'S89–2122', and 'Hartwig 2000' were obtained courtesy of Dr. Sam Anand, University of Missouri, Portageville. Seeds of M70–187 were supplied by Dr. James Orf, Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul. The remaining soybean cultivars and Plant Introductions (PI) were obtained from Dr. Randall Nelson, (USDA-ARS, University of Illinois, Urbana).

SCN Phenotype

Greenhouse assays were conducted as described by Concibido et al. (1994). Twelve F4:6 plants or F5:7 plants were tested for each of the 'Evans'×PI 209332 or 'Evans'×'Peking' lines, respectively. If the average number of cysts recovered from the roots of the plants in a line was less than 30% of the susceptible check, 'Evans', lines were classified as resistant. Otherwise, they were classified as susceptible.

DNA extraction

Genomic DNA was extracted from the lines of the mapping populations by the method of Dellaporta et al. (1983) from bulked leaf tissue of a row of plants for each line. To assay the alleles present at the two SSR loci in the various SCN-susceptible and -resistant genotypes, we extracted DNA from bulked leaf tissue of 25–35 plants of each genotype as described by Keim et al. (1988).

SSR marker development

The two SSR loci described here were developed by slightly different procedures. Satt309 was developed as described by Cregan et al. (1994). Briefly, this procedure involved the creation of a plasmid library of 500- to 700-bp fragments of 'Williams' soybean genomic DNA, screening of the library for clones containing trinucleotide (ATT/TAA) SSRs, sequencing of the SSR-containing clones, and selection of PCR primers to the regions flanking the SSR. The primers were then tested in PCR amplifications of genomic DNA of a diverse set of soybean cultivars to identify primer sets that amplified a single product that varied in size among the soybean cultivars. In contrast, Sat_168 was developed using an approach to specifically target the *rhg1* region of soybean linkage group G. This approach is described in detail by Cregan et al. (1999a). Briefly, the procedure involved the identification of a Bacterial Artificial Chromosome (BAC) clone(s) from the *rhg1* genomic region followed by random subcloning of the BAC to create a plasmid library that was screened for the presence of ATT/TTA and AT/TA SSR-containing clones. Positive clones were sequenced, and PCR primers to SSR flanking regions were selected and tested as described above. In the development of Sat_168, the primers to the Satt309 locus were used to identify a BAC clone from the UMN BAC library that consists of approximately 30,000 clones with an average insert size of 120 kbp (three genome equivalents) generated from DNA of soybean cv 'Faribault' (Danesh et al. 1998). The Satt309 primers were used in PCR amplifications of a series of BAC pools as suggested by Green and Olson (1990) and identified BAC clone UMN-K4. PCR amplification of BACs was carried out as described by Mudge et al. (1997) except that 15 pg of BAC template was used, reactions were scaled to 15 ul and run in the absence of radioactivity, and the products electrophoresed on 3.5% Metaphor agarose (FMC BioProducts, Rockland ME). The forward and reverse primers to Satt309 and Sat_168 are listed in Table 2.

SSR mapping and allele sizing

The PCR amplification of SSR alleles followed the two alternative methods described by Mudge et al. (1997). In the case of the mapping of the two loci, both forward and reverse primers were end-labeled with γ-[^{33}P]. In the characterization of alleles at the Satt309 and Sat_168 loci in the various SCN-susceptible and -resistant genotypes and in a subset of SCN-resistant and -susceptible lines from 'Evans'×'Peking', SSR alleles were [^{32}P]-labeled by the incorporation of 0.1 µml of 3,000 Ci/mmole α-[^{32}P]-dATP in the reaction mix as described by Mudge et al. (1997). Radiolabeled products were separated on a vertical polyacrylamide gel [6% polyacrylamide, 5.6 *M* urea, 30% formamide, and 1×TBE (0.09 *M* TRIS-borate, 0.002 *M* EDTA, pH 8.3]. Further characterization of the Satt309 locus was conducted on the set of recombinant inbreds from the cross of 'Parker'×A92–526007. In this instance, reactions were run in the absence of radiolabeled nucleotides, the reaction volumes were scaled to 21 µl, DNA was in disk rather than liquid form (Lange et al. 1998), and products were analyzed by 3.5% Metaphor agarose electrophoresis and visualized by staining with ethidium bromide.

Statistical analysis

Molecular genotype and SCN reaction classification data from the lines of the 'Evans'×PI 209332 and 'Evans'×'Peking' populations were analyzed using JOINMAP (Stam 1993) to estimate the position of Satt309 in relation to *rhg1*.

Results and discussion

Satt309 was previously mapped in three populations as part of an effort to develop and map approximately 600 soybean SSR loci (Cregan et al. 1999a). In two of the three populations, Satt309 mapped 1.9 cM proximal to Satt038 on molecular linkage group G, and in the third it was estimated to be 3.5 cM proximal to Satt038. Mudge et al. (1997) has previously estimated the location of *rhg1* as 3 cM proximal to Satt038. Together these data suggested the likelihood that Satt309 was very close to *rhg1*. The close proximity was confirmed by mapping in the 'Evans'×'Peking' and 'Evans'×PI 209332 populations and the subsequent JOINMAP analysis that estimated Satt309 to be 0.4 cM distal to the SCN resistance locus. In the subset of the 'Evans'×PI 209332 population selected for crossovers near *rhg1*, Sat_168 cosegregated with Satt309, thereby confirming its proximity to *rhg1* as would be anticipated with a marker developed from a BAC clone that contained the Satt309 locus. Both the Satt309 and Sat_168 primer sets amplified a product from BAC K4 that was the same size as that amplified from genomic DNA of cv 'Faribault'.

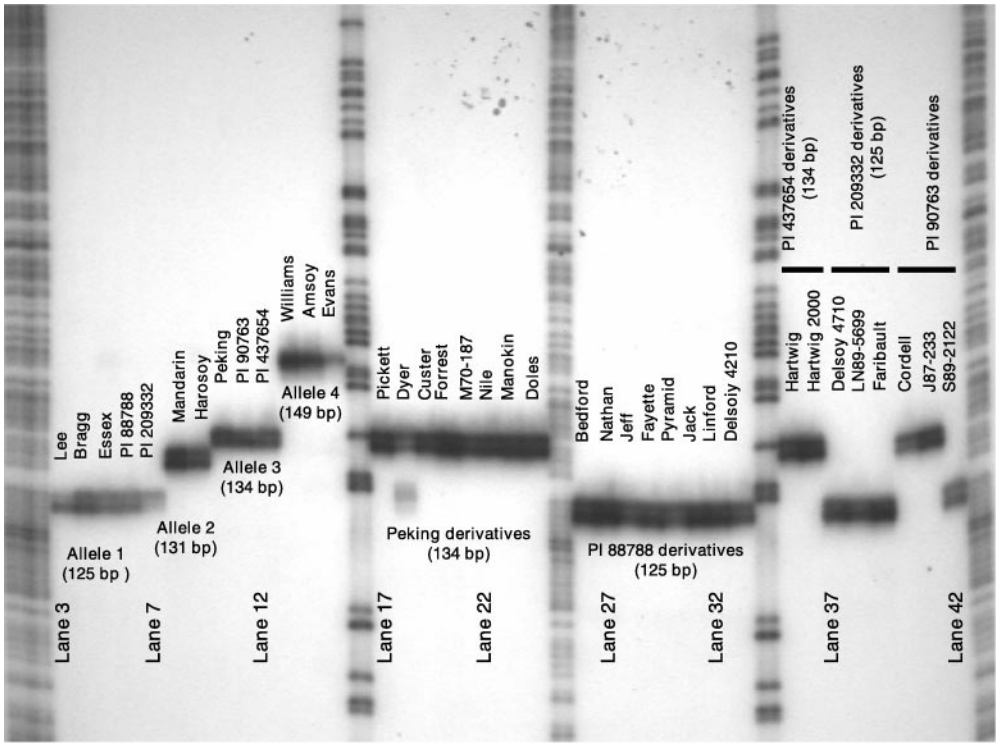

Fig. 1 Four alleles at the Satt309 simple sequence repeat locus in soybean cyst nematode (SCN)-susceptible and -resistant genotypes including derivatives of the major sources of SCN resistance 'Peking', PI 88788, PI 437654, PI 209332, and PI 90763. *Lanes 1, 2, 16, 25, 34,* and *43* contain DNA size standards derived from sequencing reactions of M13 single stranded DNA. *Lanes 1, 2, 25,* and *43* are the combined reactions using ddTTP, ddGTP, and, ddCTP terminators and *lanes 16* and *34* contain the sequencing reaction with the ddATP terminator

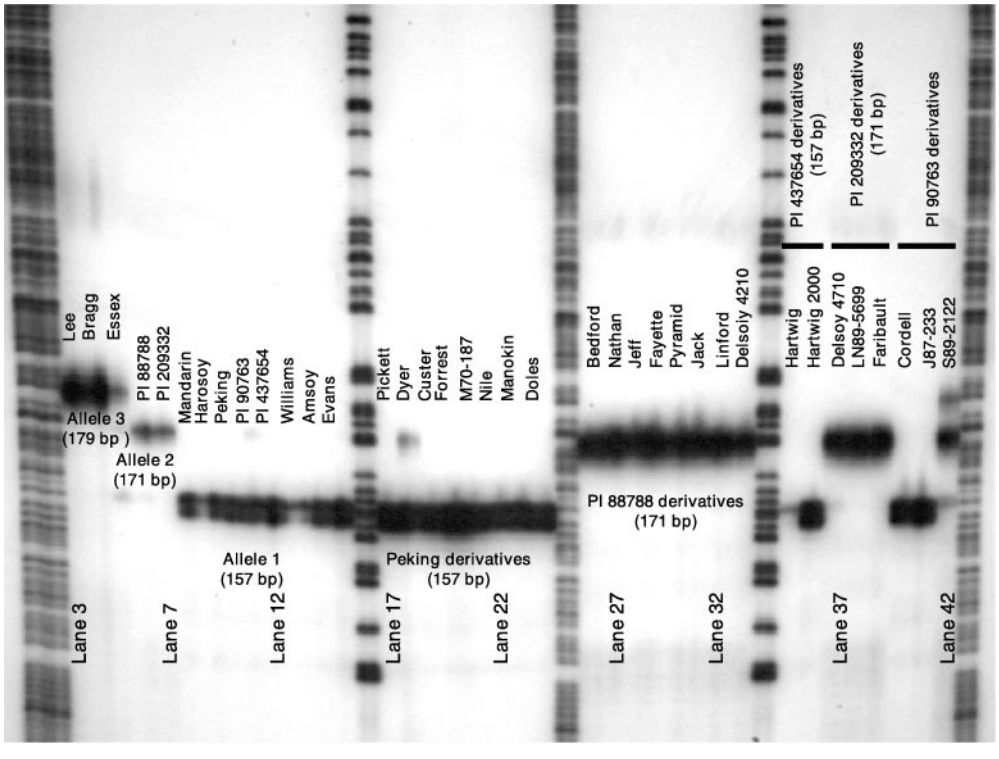

Fig. 2 Three alleles at the Sat_168 simple sequence repeat locus in soybean cyst nematode (SCN)-susceptible and -resistant genotypes including derivatives of the major sources of SCN resistance 'Peking', PI 88788, PI 437654, PI 209332, and PI 90763. *Lanes 1, 2, 16, 25, 34,* and *43* contain DNA size standards derived from sequencing reactions of M13 single-stranded DNA. *Lanes 1, 2, 25,* and *43* are the combined reactions using ddTTP, ddGTP, and ddCTP terminators, and *lanes 16* and *34* contain the sequencing reaction with the ddATP terminator

Allelic variation at the Satt309 SSR locus

Analysis of SCN-susceptible and -resistant soybean genotypes revealed four SSR alleles at the Satt309 locus (Fig. 1). Allele 1, with an estimated length of 125 bp, was present in SCN-susceptible cultivars typical of those grown in the southern US, such as 'Lee', 'Bragg', and 'Essex', as well as earlier maturing genotypes such as 'Archer', 'Dunfield' and 'Noir 1' (Table 1). Allele 1 was also present in the SCN resistance sources PI 88788 and PI 209332 and all SCN-resistant cultivars whose resistance was derived from these plant introductions. Alleles

2 and 4 were present in a genetically diverse group of SCN-susceptible cultivars. Allele 3 was unique to the three resistance sources ‘Peking’, PI 90763, and PI 437654 and genotypes that were developed using these resistance sources. Of the 25 SCN-resistant cultivars or experimental lines examined, there was only one instance in which a resistant cultivar did not carry the allele at the Satt309 locus that was present in the SCN-resistance source from which it was developed. The exception was S89–2122, which is derived from ‘Essex’×PI 90763 (Anand et al. 1994). Either S89–2122 does not carry the *rhg1* resistance allele derived from PI 90763 or there has been a recombination between Satt309 and *rhg1*. Because of the importance of *rhg1* in conditioning resistance in lines derived from PI 90763 (Concibido et al. 1997) recombination would seem the more likely alternative.

Allelic variation at the Sat_168 SSR locus

Allelic variation at the Sat_168 locus was assayed in the genotypes included in Fig. 2 as well as in a diverse group of SCN-susceptible cultivars that included ‘Archer’, ‘Clark’, ‘Fiskeby V’, ‘Jackson’, Noir 1, ‘Minsoy’, and ‘Tokyo’. Allele 1 was present in the SCN-susceptible indeterminate cultivars typical of those grown in the northern US. Allele 1 was also present in the three resistance sources ‘Peking’, PI 90763, and PI 437654 and all the resistant cultivars developed using these sources, with the exception of S89–2122. Allele 2 was only present in PI 88788 and PI 209332 and SCN-resistant cultivars developed using these sources of resistance, while Allele 3 was only present in ‘Lee’, ‘Bragg’, and ‘Essex’, cultivars typical of those grown in the southern US. As noted above, S89–2122 was exceptional in not carrying the allele from PI 90763, its source of SCN resistance. Figure 2 indicates that S89–2122 carries Allele 2, which is not present in either parent. In addition, two fainter bands, one which was the same size as Allele 1 and the other identical to Allele 3, can be noted in Fig. 2. This may be due a mixed seed lot. A second seed sample of S89–2122 is being obtained for further analysis of this genotype. Aside from this anomaly, the significance of the allelic variation at the Sat_168 locus is the ability to distinguish typical southern US cultivars such as ‘Lee’, ‘Bragg’, and ‘Essex’ that are SCN-susceptible from the important SCN resistance sources PI 88788 and PI 209332. Allelic variation at the Satt309 locus could not distinguish these two sets of genotypes.

Marker-assisted selection using Satt309

A selected group of SCN-resistant and -susceptible lines from ‘Evans’×‘Peking’ were assayed at the Satt309 locus using [^{32}P]-labeled PCR products separated on denaturing polyacrylamide gel (Fig. 3). ‘Evans’ carries Allele 4 (149 bp), while ‘Peking’ carries Allele 3 (134 bp). Each of the lines that was classified as susceptible (S) in the greenhouse SCN screening is homozygous for Allele 4, while the resistant (R) lines all carry Allele 3. The one exception was line 62 which appears to carry Allele 1 and was subsequently found to be the result of a seed mixture.

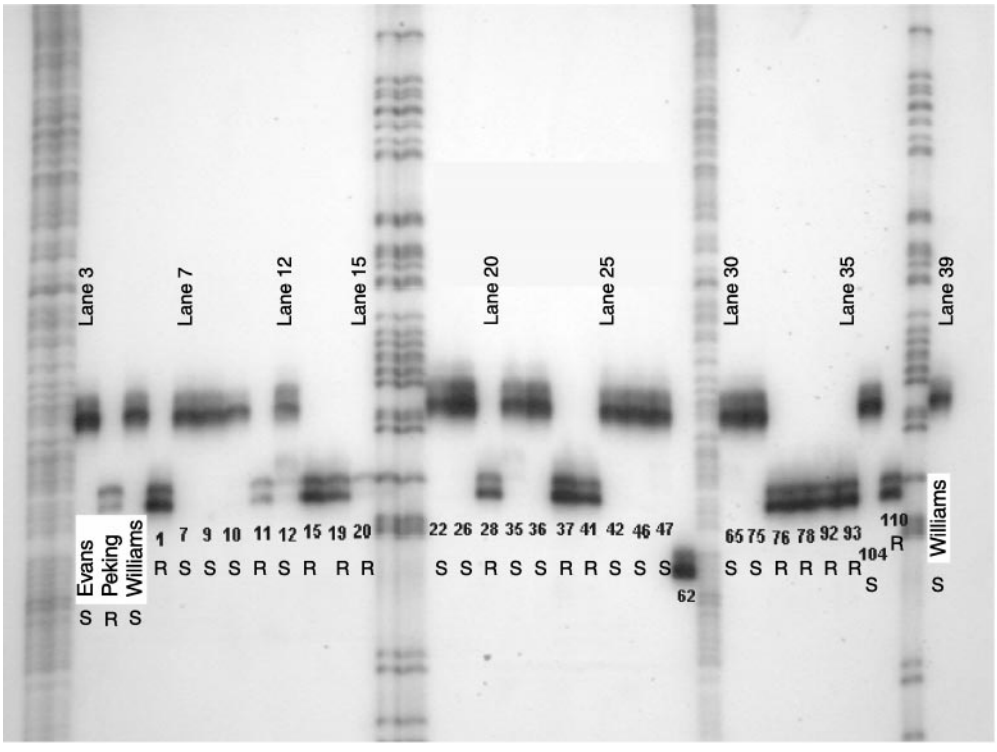

Fig. 3 Alleles present at the Satt309 locus in selected $F_{6:7}$ lines with defined soybean cyst nematode (SCN) resistance phenotype (*S*, susceptible, *R*, resistant) from ‘Evans’×‘Peking’. *Lane 3* contains the SCN-susceptible parent ‘Evans’, *lane 4* the SCN-resistant parent ‘Peking’, and *lanes 5* and *39* the cultivar ‘Williams’. *Lanes 1, 2, 16, 17, 29,* and *38* contain DNA size standards derived from sequencing reactions of M13 single-stranded DNA. *Lanes 1, 2,* and *29* are the combined reactions using ddTTP, ddGTP, and ddCTP terminators, and lanes *16, 17,* and *38* contain the sequencing reaction with the ddATP terminator

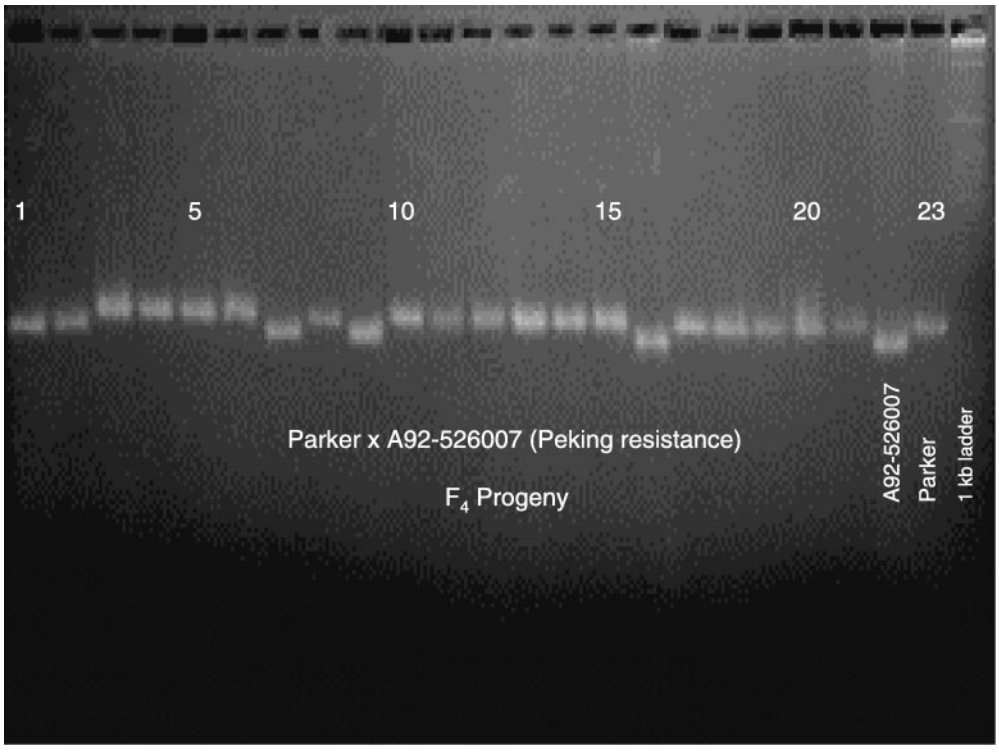

Fig. 4 Amplification products from the Satt309 locus in selected $F_{4:5}$ lines with defined soybean cyst nematode (SCN) resistance phenotype (*lanes 1, 2, 7, 9,* and *16* from SCN-resistant lines and *lanes 3–6, 8, 10–15,* and *17–21* from susceptible lines) from 'Parker'×A92–526007. *Lane 22* is from A92–526007 which carries SCN resistance from 'Peking', and *lane 23* from the SCN-susceptible parent 'Parker'. PCR products were separated on a 3.5% Metaphor agarose gel followed by staining with ethidium bromide

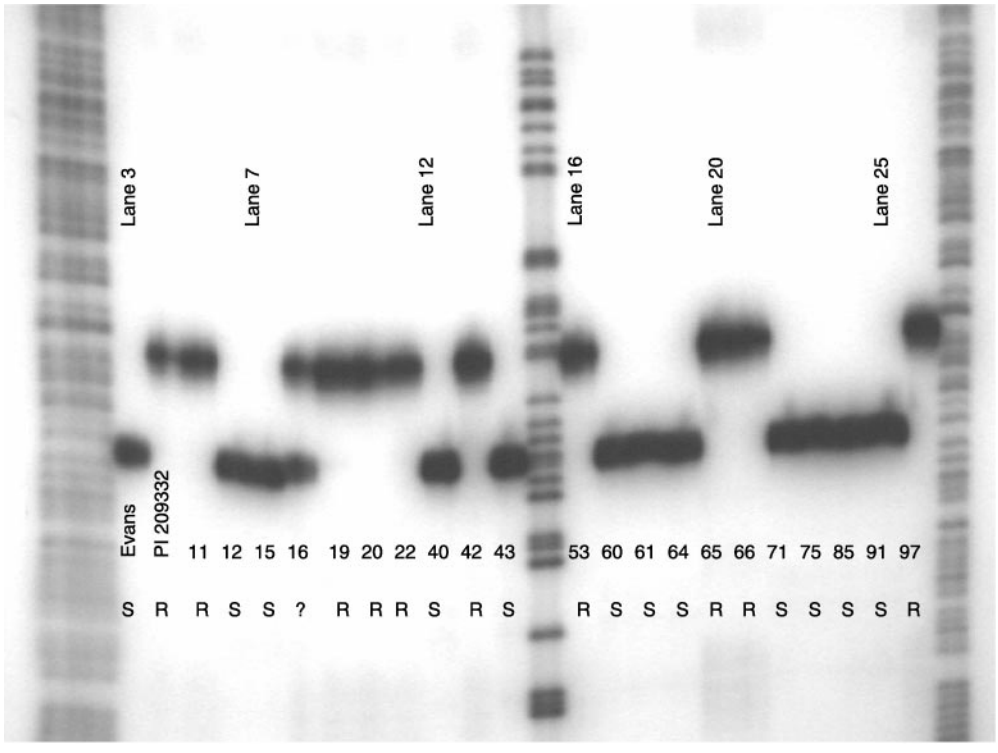

Fig. 5 Alleles present at the Sat_168 locus in selected $F_{4:6}$ lines with defined soybean cyst nematode (SCN) resistance phenotype from 'Evans'×PI 209332. *Lane 3* contains the SCN-susceptible parent 'Evans', *lane 4* the SCN-resistant parent PI 209332. *Lanes 1, 2, 15,* and *27* contain DNA size standards derived from sequencing reactions of M13 single-stranded DNA. *Lanes 1, 2,* and *27* are the combined reactions using ddTTP, ddGTP, and ddCTP terminators, and *lane 15* contains the sequencing reaction with the ddATP terminator

A technically less demanding assay system to discern SSR allelic variation is demonstrated in Fig. 4 in which variation at the Satt309 locus was used to distinguish SCN-resistant from -susceptible $F_{4:5}$ progeny in a cross of 'Parker'×A92–526007. The larger PCR product on 3.5% Metaphor agarose is the Satt309 allele that is generally associated with susceptibility and the smaller fragment with SCN resistance. In the case of the lines assayed in Fig. 4, only one of the five genotypes with the smaller Satt309 allele was found to be SCN-resistant. None of the genotypes with the larger fragment was resistant. Thus, allelic differences at the Satt309 locus do not allow com-

plete discrimination between resistant and susceptible progeny. Selection using Satt309 is particularly useful for discarding susceptible lines and greatly reducing the number of lines that must be further tested in the greenhouse for resistance. This result is anticipated based upon previous reports indicating that SCN resistance is under multigenic control and that resistance at *rhg1* is only one of the genes required to condition resistance to SCN (Concibido et al. 1994, 1997; Webb et al. 1995).

Both the polyacrylamide and the agarose assay systems demonstrate the effectiveness of Satt309 to function in marker-assisted selection of genotypes that possess 'Peking' resistance at the *rhg1* locus. Based upon our examination of SCN-susceptible and -resistant genotypes (Table 1), marker-assisted selection for resistance at *rhg1* using Satt309 should be effective to select for SCN resistance at *rhg1* derived from 'Peking', PI 90763, and PI 437645 in crosses with most, if not all, SCN-susceptible soybeans.

Marker assisted selection using Sat_168

A selected subset $F_{4:6}$ lines from the 'Evans'×PI 209332 mapping population, whose SCN phenotype was previously determined, were characterized at the Sat_168 locus (Fig. 5). The SCN susceptible lines carried Allele 1 (157 bp), while those classified as resistant carried the 171-bp Allele 2. Line 16, classified as resistant, is heterozygous at Sat_168. However, an earlier DNA extraction and genoytpe analysis indicated that Line 16 was homozygous at Sat_168 and other flanking loci, suggesting that the discrecepancy in genotype may be the result of a seed or DNA mixture. The 14-bp difference between Allele 1 and Allele 2 at the Sat_168 locus could be readily distinguished to identify genotypes that possess the PI 209332 resistance allele at the *rhg1* locus. Furthermore, Fig. 2 indicates that Alleles 2 and 3, that differ by 8 bp in length, can be readily distinguished. This assay permits marker assisted selection for *rhg1* in populations derived from PI 88788 and PI 209332 when crossed with typical southern US cultivars derived from genotypes such as 'Lee', 'Bragg' and 'Essex'.

Selection of genotypes carrying resistance from any source of SCN resistance at the *rhg1* locus can be accomplished with a high degree of success using marker-assisted selection with Satt309 and/or Sat_168. In addition to a high level of genetic resistance to SCN, a second important research objective is the development of SCN-resistant cultivars with maximal productivity under SCN-free conditions. One obvious approach to attaining this goal is the reduction, to as great an extent as possible, of linkage drag. Mudge et al. (1996) suggested the presence of a "yield depression gene" in the same region as *rhg1*. If we assume that yield depression is the result of linkage drag and not a pleiotrophic effect of *rhg1*, it should be possible to select genotypes in which the yield depression gene has been eliminated. The presence of SSR loci Satt309 and Satt038, 0.4 and 3.0 cM distal of rhg1 and Sat_163, Sat_141, and Satt610, approximately 1.0, 1.5, and 2.5 cM proximal of *rhg1*, respectively (Cregan et al. 1999a) should allow the selection of genotypes in which linkage drag is quite limited. Ultimately, we anticipate developing a similar saturation of SSR loci in most regions in which SCN QTL are present, thereby allowing the selection of the resistance QTL and the concomitant reduction in the level of linkage drag. The result will be the selection of genotypes with high levels of SCN-resistance and minimal levels of linkage drag. Such genotypes should perform as well as the best SCN-susceptible genotypes under SCN-free conditions and show no yield reduction when grown in SCN-infested fields.

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