Molecular markers linked to the resistance to race 3 of the soybean cyst nematode *Heterodera glycines*

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ABSTRACT

The objective of this study was to identify molecular markers associated with the resistance to race 3 of the SCN. Two microsatellites (Satt187 and Satt309) and three RAPD markers (OPAG-05$_{946}$, OPF-04$_{1038}$, and OPAQ-01$_{1987}$) were found which explained 31.3%, 28.9%, 13.8%, 11.4% and 9.9%, respectively, of the phenotypic resistance variation to race 3 of the SCN. However, by multiple regression analysis, with the elimination of markers which least contributed to an explanation of the resistance, the most significant combination occurred with the inclusion of the markers Satt187 and Satt309, which together explained 75.2% of the resistance. These markers were mapped in two distinct regions. One located in the linkage group G with the markers OPAG-05$_{846}$, OPF-04$_{1038}$, OPAQ-01$_{1987}$ and Satt309 at an interval of 34.7 cM, and another located in the group A$_2$ with the marker Satt187. Inheritance studies have shown those two dominant genes control resistance to SCN, in the population analyzed.

KEY WORDS: RAPD, microsatellite, bulked segregant analysis, soybean, breeding.

INTRODUCTION

The soybean cyst nematode (SCN), [*Heterodera glycines* (Ichinohe)] has soybean *Glycine max* (L) Merril as its main host. This nematode was reported for the first time in Japan in 1915, causing a disease denominated soybean yellow dwarf, due to the symptoms presented by infested plants (Riggs, 1977). In Brazil, it was found in 1992 in soil samples from the States of Mato Grosso, Minas Gerais, and Mato Grosso do Sul collected after the 1991/92 growing season (Mendes and Dickson, 1993). Since its identification, this disease has spread very rapidly and nowadays it is established in several soybean production regions of the country causing severe losses (EMBRAPA, 1998). The SCN is very difficult to eradicate since it possesses resistance structures, and the cysts remain viable in the soil for long periods of time. However, it can be controlled by phytosanitation means (EMBRAPA, 1998) and crop rotation between resistant and susceptible soybean cultivars and non-host cultivated crop species (Noel and Edwards, 1996). In Brazil, although selection pressure due to resistant cultivars has not yet occurred, races 1,2,3,4,5,6,9,10 and 14 and the races 4+ and 14+, which are able to break the resistance of the

cultivar Hartwing, until now considered resistant to all races, have been identified (EMBRAPA, 1999).

The resistance to SCN is a complex and oligogenic characteristic. Besides the cultivar Peking, genes for resistance were found in several genotypes as PI90763, PI438489, PI88788, PI404166 and PI404198 (Rao-Arelli and Anand, 1988). Although sources of resistance to several races of SCN exist, the selection of resistant plants is very time and labor intensive. However, with the improvement of techniques in molecular biology it is possible to directly analyze the DNA of the organisms to obtain markers that facilitate the selection of resistant plants.

Inheritance studies initiated in the 1960s have shown that the resistance to SCN in the cultivar Peking is conditioned by three recessive genes *rhg1*, *rhg2* and *rhg3* (Caldwell et al., 1960) and one dominant gene *Rhg4* (Matson and Williams, 1965). Weisemann et al. (1992) identified two loci near the gene *Rgh4*. These loci are located respectively at 4.4 and 4.0 cM of the locus I, which is responsible for the hilum color of the seed. According to Rao-Arelli and Anand (1988) the resistance to race 3 of the SCN is controlled by one dominant and one recessive gene. Concibido et al. (1996) suggested that the majority of the loci for resistance to SCN are located in the

linkage group G. Mahalingan and Skorupka (1995) identified three RAPD markers linked to the resistance to race 3 of the SCN, located in the linkage groups A and F and two markers of RFLP, located in the linkage groups A and C. Webb et al. (1995) localized two QTLs related to the resistance to race 3 of the SCN, identified by RFLP markers, located in the linkage groups G and M. Mudge et al. (1997) found two markers of microsatellites located in the linkage group G, linked to alleles of resistance to the SCN in the genotypes PI209332, PI88788, PI90763, PI437654, and Peking.

The objective of this study was to identify molecular markers associated with the resistance to race 3 of the SCN.

MATERIAL AND METHODS

Experiments with SCN

Sixty soybean lines $F_{4:5}$, obtained from the crossing Centennial x {[BR16 x (BR37-555 x OCEPAR 9)] x Sharkey}, from the Soybean Breeding Program of Embrapa Soybean were used in this study.

The population of *Heterodera glycines*, used in this research, originated from a soybean field in Irai de Minas municipality, State of Minas Gerais, Southeast Brazil, reared under *Heterodera glycines* race 3.

A completely randomized experimental design with seven replications was used. Seeds of each line and the differential host race cultivars germinated in the field. Seven seedlings of each genotype were transferred to clay pots, containing a sand soil previously treated with methyl bromide and kept in a greenhouse under controlled conditions of temperature (25º C). Seven days after transplanting, each seedling was inoculated with 4.000 eggs of *Heterodera glycines*, pipeting the suspension in a soil hole 2 cm from the seedling stem.

The test of race using differential cultivars was conducted to verify the occurrence of race changes in the nematode population used as inoculum.

Thirty days after inoculation, each plant was collected and the root systems were carefully separated from the aerial parts, accommodated in plastic bags and stored in a refrigerator at 4º C. The females extracted from the roots by a flow of pressurized water were then counted under stereomicroscope.

The classification of lines as resistant or susceptible was performed considering the index of parasitism (I) according to the methodology of Golden et al. (1970). This index was obtained as follows:

$$I = \frac{\text{Mean number of females in the lines}}{\text{Mean number of the females in the Lee cultivar}} \times 100$$

The lines presenting I ≥ 10% in relation to Lee cultivar, considered as a susceptible standard, were classified as susceptible. The lines that showed I < 10%, was classified as resistant.

DNA analysis

The DNA was extracted from the leaves according to Keim et al. (1988). The extraction buffer was composed of 50 mM Tris-HCl (pH 8.0), 50 mM of EDTA (pH 8.0), 07M of NaCl, CTAB 1%, PVP 1%, and b-mercaptoethanol 1%.

The soybean population was analyzed by RAPD and microsatellite markers technique using the strategy of Bulk Segregant Analysis (BSA) (Michelmore et al., 1991). Two resistant and two susceptible bulks were formed, each one formed by equivalent mixture of DNA of four plants highly resistant or highly susceptible, respectively. These plants were selected in the population after the classification made by artificial inoculation of the SCN. For the resistant bulks, two plants with a low index of parasitism were used, while for the susceptible bulks, plants with high index of parasitism were used.

In the reactions of amplification a total of 1000 decamer primers were tested (Operon Technologies) by the RAPD technique and seven microsatellite primers, selected from regions of the soybean genome previously mapped for genes of resistance to SCN. The primers of the microsatellites Satt187, Satt315, Satt589, Satt038, Satt309, Satt001, and Satt082 were chosen based on the soybean genetic map of the USDA/Iowa State University (Cregan et al., 1999). The primers, which originated polymorphism between resistant and susceptible bulks were tested in the 60 lines.

Conditions of amplification and electrophoresis

The reactions of amplification of RAPD were performed in a total volume of 25 ml, containing 10 mM Tris-HCl (pH8.3), 50 mM KCl, 2 mM $MgCl_2$, 100 mM of each one of the deoxyribonucleotides (dATP, dTTP, dGTP, dCTP), 0.4 mM of primer, one unit of the enzyme Taq-polymerase (Gibco BRL, Life Technologies) and 25 ng of DNA. The amplifications occurred in a thermal cycler programmed for 45 cycles of 15 seconds at 94ºC, 30 seconds at 35ºC and 1 min. at 72ºC, followed by a final

step of 7 min. at 72ºC. The microsatellite reactions were performed in a total volume of 20 ml, containing 12.5 mM Tris-HCl (pH 8.3), 62.5 mM KCl, 2.5 mM MgCl$_2$, 0.125 mM of each one of the deoxyribonucleotides, 0.7 mM of primer, one unit of Taq-polymerase (Gibco BRL, Life technologies) and 30 ng of DNA. The amplifications were performed in a thermal cycler programmed for an initial step of 7 min. at 72ºC, followed by 30 cycles of 1 min. at 94ºC, 1 min. at 50ºC, 2 min. at 72ºC and a final step of 7 min. at 72ºC. The RAPD and microsatellite Satt187 amplified fragments were separated by electrophoresis in agar gel 1.3% and 3%, respectively, containing ethidium bromide and buffer TBE 1x. The Satt309 microsatellite amplified fragments were separated by eletroforese in polyacrylamide gel 9.0% containing buffer TAE 1x end ethidium bromide. After running the electrophoresis, the plates were photographed under ultraviolet light, with a Polaroid camera and film 667 type.

Data analysis

Different hypotheses for the inheritance of resistance to race 3 of the SCN were tested applying the χ^2 test on the number of observed and expected lines classified by artificial inoculation of SCN, within the resistant and susceptible classes.

The linkage of the molecular markers obtained with the characteristic resistance to race 3 of SCN was checked by multiple regression analysis, eliminating the markers with less explicability based on the level of significance of each marker, using the SAS program (1985).

The identified markers were mapped using the MAPAMAKER/EXP program (version 3.0 b). For mapping purposes, the population of 60 lines F$_{4:5}$ were considered as recombinant inbred line (RIL).

The efficiency of selection was determined for each marker considering its classification based on the markers and the resistant phenotypic reaction of the lines determined by artificial inoculation of SCN.

RESULTS AND DISCUSSION

Artificial inoculation of SCN, conducted in greenhouse, presented good results in the multiplication of the SCN. The mean number of females obtained on the cultivar Lee, the susceptibility standard, was 259.7, while in all the differentials it was zero. According to Riggs and Schmitt (1988) methodology, theses results showed that there was

no race shift and that the population of SCN was homogeneous for the race 3. The average obtained on "Lee" made possible a safe classification of the lines within the resistant and susceptible classes.

The RAPD and microsatellite techniques combined with the BSA strategy were efficient, since they are practical techniques that allowed the analysis of a large number of samples in a short period of time.

Inheritance of resistance of SCN

The classification of the lines into resistant and susceptible, using the Index of parasitism limit of 10% of the obtained on "Lee" was adequate to separate these phenotypic classes, since it was a point of inflection of the distribution frequency curve.

The hypothesis of one dominant gene or one recessive gene alone determining the resistance to race 3 of the SCN was rejected (P < 0.001), but on the other hand, the hypothesis with two genes was accepted (Table 1).

The most probable hypothesis is that the characteristic is controlled by two dominant genes (P = 0.9956). However the hypothesis of one dominant gene and one recessive gene together was also accepted (P = 0.2044), agreeing with the results obtained by Rao-Arelli and Anand (1988). These authors identified one dominant gene and one recessive gene controlling the resistance to race 3 of SCN in a genotype originated from a crossing between the cultivar Peking and the line PI88788. The control of the resistance to SCN in the cultivar Peking by three recessive genes, was reported by Caldwell et al. (1960), while Matson and Williams (1965) identified only one dominant gene. Mauro et al. (1999) identified one dominant gene and two recessive genes together controlling the resistance to race 3 in one line derived from the crossing of the cultivar FT-Cristalina (susceptible) and the line BR-904722, with resistance to race 3 acquired from the cultivar Peking.

Although not yet reported in the literature, a model of inheritance with two dominant genes for the resistance to race 3 of SCN, with genotypes originated from the cultivar Peking, was the hypothesis that presented the highest probability of explaining the data showed in this manuscript.

Molecular markers for resistance to race 3 of the SCN

The population studied was also evaluated by molecular markers. The search for molecular markers

linked to genes for resistance to race 3 of the SCN was performed by the Bulk Segregant Analysis strategy (Michelmore et al., 1991). From the 1000 RAPD and seven microsatellites primers tested, three RAPD and two microsatellite markers were polymorphic among the bulks (Figure 1) and presented correlation with the parasitism index evaluated in the lines. RAPD markers OPAG-05$_{946}$, OPF-04$_{1038,}$ and OPAQ-01$_{1987}$ and microsatellite markers Satt309 and Satt187, which explain, 13.8%, 11.4%, 9.9%, 31.3% and 28.9%, respectively, of the phenotypic variation of the resistance to race 3 of the SCN were identified. When these markers were combined, using the multiple regression procedure with the stepwise method of elimination, the most significant combination occurred between the markers Satt309 and Satt187, which explained 75% of the phenotypic variation of the resistance (Table 2).

These results show that an additive effect occurred when these markers were combined and agree with the soybean microsatellite map developed by the USDA/Iowa State University (Cregan et al., 1999), where these markers were mapped in distinct regions of the soybean genome, located respectively in the linkage groups G an A$_2$.

In mapping the markers identified, using the MapMaker/QTL, two genomic regions were identified. One with the three RAPD markers and the microsatellite Satt309 were mapped within an interval of 34.7 cM. The QTL was located between the OPAG-01 end Satt309 markers (Figure 2). The Satt309 marker is located in the linkage group G of the soybean genetic molecular map (Cregan et al., 1999). Several other authors have also already reported the existence of genes for resistance in this linkage group. The other

genomic region, not linked to the previous region, is marked by the marker Satt187, located in the linkage group A$_2$ (Cregan et al., 1999). In same linkage group, Matthews et al. (1998) identified one RFLP marker, the pBLT65, mapped at 3.5 cM of the locus *i* which is linked to the gene *Rhg4*. Weisemann et al. (1992) also identified RFLP markers linked to the gene *Rhg4* and Mahalingan and Skorupska (1995) using RFLP and RAPD markers, identified a QTL for the
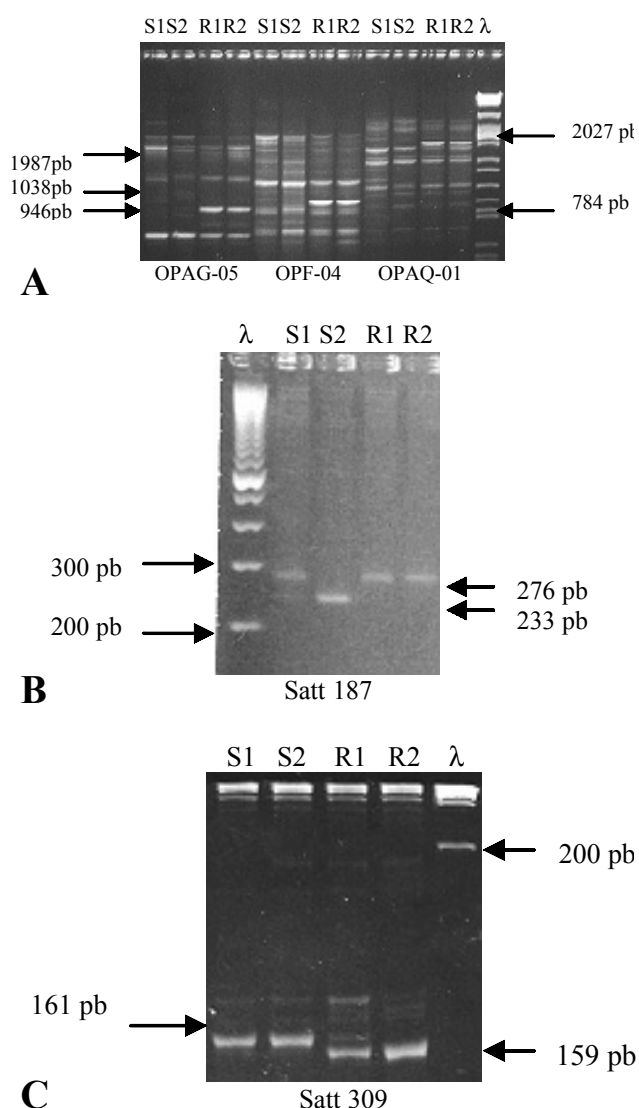
A   OPAG-05   OPF-04   OPAQ-01

B   Satt 187

C   Satt 309

Figure 1. Band pattern produced by amplification reactions of RAPD and microsatellite. (A) agar gel 1.3% showing bands of 946 pb, 1038 pb, and 1987 pb, amplified by the primers OPAG-05, OPF-04 and OPAQ-01, respectively, in the resistant bulks and absent in the susceptible ones. (B) agar gel 3.0% showing bands of 276 pb and 233 pb amplified by the primer Satt187, respectively in the resistant and susceptible bulks. (C) polyacrylamide gel 9.0% showing bands of 159 pb and 161 pb amplified by the primer Satt309, respectively in the resistant and susceptible bulks.

Table 1. χ^2 test for the hypothesis of two genes determining resistance to race 3 of the Soybean Cyst Nematode in 60 soybean lines F$_{4:5}$.

Two dominant genes		
Lines	Observed frequency	Expected frequency
Resistant [1]	19	18.98
Susceptible	41	41.02
		$\chi^2 = 0.00003$ (P=0.995)
One dominant end one recessive gene		
Lines	Observed frequency	Expected frequency
Resistant	19	14.76
Susceptible	41	45.24
		$\chi^2 = 1.61$ (P=0.204)

[1] Classification by artificial inoculation of the SCN: resistant PI < 10% and susceptible PI > 10%.

Table 2. Phenotypic proportion of the resistance to the soybean cyst nematode explained by the markers identified based on the regression analysis among the markers, combination of markers in relation to index of parasitism.

Marker	Explained variation (%)	F	Pr > F
Satt309	31.3	12.99	0.0001
Satt187	28.9	11.72	0.0001
OPAG-05	13.88	9.35	0.0034
OPF-04	11.46	7.51	0.0082
OPAQ-01	9.91	6.38	0.0143
Satt187 + Satt309	75.2	19.40	0.0001

resistance to race 3 of the SCN.

Regression analysis data (Table 2) show that the marker Satt309, located in the linkage group G, was the one which best explained the characteristic resistance to race 3 of the SCN and is in accordance with Concibido et al. (1996), who concluded that the majority of the genes for resistance to the SCN are located in the linkage group G.

Results obtained in this work confirm those described in the literature for the microsatellite markers Satt187 and Satt309, mapped in regions of the soybean genome which contains genes for resistance to the SCN and show that RAPD markers identified, mapped near the marker Satt309, are also located in genomic region near to the genes for resistance to race 3 of the SCN.

Figure 2. Mapping the molecular markers identified, using the MapMaker/QTL. On the right side are molecular markers identified and on the left the distances, in cM. The asterisk indicates the probable location of the QTL.

Application in plant breeding

The lines classified as resistant with the markers OPAG-05$_{946}$, OPF-04$_{1038}$, OPAQ-01$_{1987}$, Satt187, and Satt309, showed a reduction of 44.7% (Figure 3A), 41.7% (Figure 3B), 39.9% (Figure 3C), 58.9% (Figure 3D) and 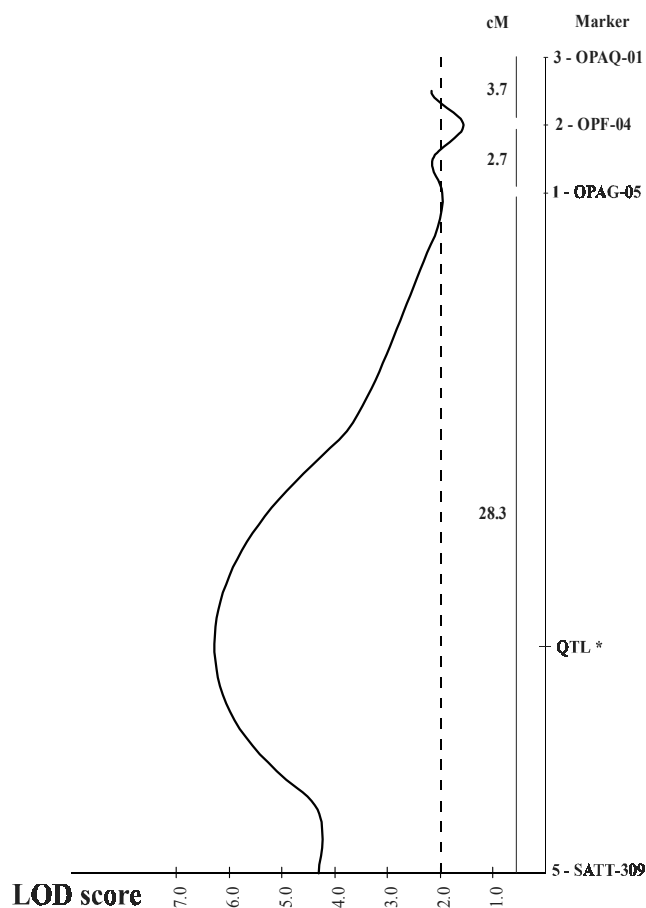62.8% (Figure 3E), respectively, in the mean number of females. However, the combinations including the markers Satt187 and Satt309 and Satt187, Sartt309, OPAG-05$_{946}$, OPF-04$_{1038}$ andOPAQ-01$_{1987}$, showed a reduction of 98.7% (Figure 3F) and 99.8% (Figure 3G), respectively, in the mean number of females.
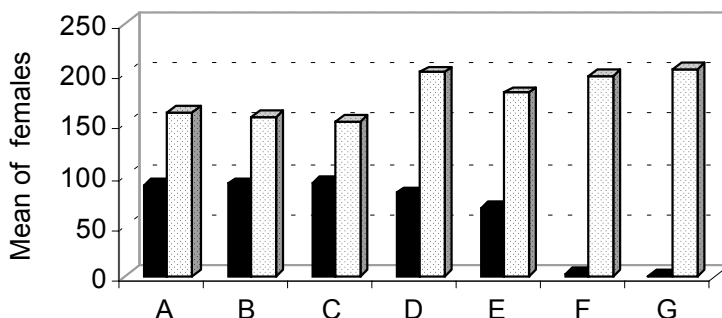

Figure 3. Effect of the markers in the mean number of soybean cyst nematode females extracted from soybean roots. (A, B and C) OPAG-05$_{946}$, OPF-04$_{1038}$, and OPAQ-01$_{1987}$ markers, respectively. (D and E) Satt309 and Satt187, respectively. (F) combination between the markers Satt187 and Satt309. (G) combination of all markers. Black bars represent the presence of the allele which reduces the character mean of females.

Table 3. Efficiency of the selection using the markers for to select resistant plants to Soybean Cyst Nematode.

Molecular markers	Selected plants			Efficiency (%)*
	Resistant	Suceptible	Total	
Satt187 and OPAG-05	13	1	14	92.8
Satt187 and OPAQ-01	11	1	12	91.6
Satt187 and Satt309	16	0	16	100

* Efficiency (%) = Number of plants resistant x 100/Number of plants selected.

The efficiency of the selection obtained with the combination, including the markers Satt187 and Satt309, was 100% once all the lines selected with this combination were resistant. Other combinations including the markers Satt187 and OPAG-05$_{946}$; and Satt187 and OPAQ-01$_{1987}$ also presented high selection efficiency with 92.8% and 91.6%, respectively (Table 3).

The low mean number of females presented by the lines classified with the markers Satt187 and Satt309 previously mapped (Cregan et al., 1999) confirm that these mark regions containing genes for resistance to race 3 of the SCN and can be used together with the RAPD markers identified in this research, in breeding programs for selection of resistance plants, from crossings involving sources of resistance acquired from the cultivar Peking and may contribute to obtaining resistant cultivars.

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RESUMO

Marcadores moleculares ligados à resistência a raça 3 do nematóide de cisto da soja *Heterodera glycines*

O objetivo desse trabalho foi identificar marcadores moleculares associados à resistência ao Nematóide de Cisto da Soja (NCS), *Heterodera glycines*, raça 3. Foram identificados dois marcadores de microssatélite (Satt187 e Satt309) e três de RAPD (OPAG-05$_{946}$, OPF-04$_{1038}$ e OPAQ-01$_{1987}$) que explicaram 31,3%, 28,9%, 13,8%, 11,4% e 9,9%, respectivamente, da variação fenotípica da resistência à raça 3 do NCS. Entretanto, por análise de regressão múltipla com eliminação de marcadores que menos contribuíram para a explicação da resistência, a combinação mais significativa ocorreu com a inclusão dos marcadores Satt187 e Satt309 que explicaram 75,2% da resistência. Esses marcadores foram mapeados em duas regiões distintas. Uma, localizada no grupo de ligação G com os marcadores OPAG-05$_{946}$, OPF-04$_{1038}$, OPAQ-01$_{1987}$ e Satt309 num intervalo de 34,7 cM e outra, localizada no grupo A_2 com o marcador Satt187. Estudos de herança mostram que a característica resistência à raça 3 do NCS, na população analisada, é controlada por dois genes dominantes.

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