Revista Ceres, 48(279):565-571,2001

RAPD MARKER FOR RESISTANCE TO CYST NEMATODE (RACE 3) IN SOYBEAN[1]

Antonio Orlando Di Mauro[2]
Roberto Carlos de Oliveira[3]
Sonia Marli Z. Di Mauro[2]

ABSTRACT

The soybean cyst nematode (*Heterodera glycines* Ichnohe, 1952) is considered to be the most important problem for soybean cultivation. The use of rotation between resistant cultivars, susceptible cultivars and non-host species is the most economic and reliable control method. To develop resistant cultivars, the breeding program has to evaluate soybean genotypes in infested fields or in artificially inoculated areas. To avoid transportation and possible contamination of SCN free areas, the identification of molecular markers linked to the genes conferring resistance to SCN is highly desirable. Thus, crosses between the resistant lines BR 90-4722, BR 92-15-440 and BR 90-4617 with the susceptible cultivar FT-Cristalina were made, and the BSA technique was used to identify RAPD markers linked to the genes conferring SCN (race 3) resistance. The marker OPR-15_{220pb} produced a band capable of distinguishing resistant and susceptible F_2 plants, however further studies are necessary to develop a SCAR marker that can be efficiently used in marker-assisted selection.

Key words: *Glycine max*, *Heterodera glycines*, RAPD marker.

---

[1] Accepted for publication on July 2, 2001.
[2] Departamento de Produção Vegetal. Faculdade de Ciências Agrárias e Veterinárias. 14884-900 Jaboticabal, SP.
[3] Aluno do curso de Pós-Graduação em Genética e Melhoramento de Plantas da Faculdade de Ciências Agrárias e Veterinárias. 14884-900 Jaboticabal, SP.

RESUMO

MARCADOR RAPD PARA RESISTÊNCIA AO NEMATÓIDE DO CISTO (RAÇA 3) EM SOJA

O nematóide do cisto da soja (*Heterodera glycines* Ichnohe, 1952) é considerado o mais importante problema para a cultura da soja. A utilização de cultivares resistentes em rotação com cultivares suscetíveis e espécies não-hospedeiras é o método mais eficaz para o controle do patógeno. Para o desenvolvimento de cultivares resistentes, o programa de melhoramento necessita avaliar os genótipos em áreas infestadas com o nematóide ou através de inoculações artificiais. Para evitar o transporte e a possível contaminação de áreas livres do fitopatógeno, a identificação de marcadores moleculares ligados aos genes que conferem resistência ao nematóide do cisto é altamente desejável. Assim, cruzamentos das linhagens resistentes BR-90-4722, BR 92-15440 e BR 90-4617 com o cultivar suscetível FT-Cristalina foram efetuados e a técnica BSA foi empregada para identificar marcadores RAPD ligados aos genes que conferem resistência ao fitopatógeno. O iniciador OPR-15_{220pb} produziu uma banda capaz de distinguir as plantas resistentes das suscetíveis, entretanto mais estudos são necessários para o desenvolvimento de marcadores SCAR, que poderão ser eficientemente utilizados em programas de seleção assistida por marcadores moleculares, objetivando o desenvolvimento de genótipos de soja resistentes ao nematóide do cisto, raça 3.

Palavras-chaves: *Glycine max*, *Heterodera glycines*, marcador RAPD.

INTRODUCTION

The soybean cyst nematode (*Heterodera glycines* Ichinohe, 1952) is considered to be the most important problem for soybeans, causing considerable economic damages (*10*). Since its occurrence was first reported in Brazil, there has been a unanimous concern by the main Brazilian Soybean Research Centers with this nematode. In areas of occurrence, losses can reach 100%, depending on the nematode populational density (*4*). The use of resistant cultivars in rotation with susceptible ones and non-host species is the most economical and reliable method for controlling soybean cyst nematode (SCN). Thus, genetic improvement assumes great importance as a technology capable of minimizing the damages caused by SCN.

To develop resistant cultivars, a breeding program must evaluate genotypes in an infested field or through artificial inoculation. To avoid transportation and possible contamination of SCN free areas, the identification of molecular markers linked to the gene conferring resistance to SCN is highly desirable. To be used in a breeding program, the molecular marker should be closely linked to the genes of interest and the technique must be efficient and with low cost per reaction, allowing the evaluation of a great number of genotypes as a routine. The technique also has to be reproducible and easy to use.

According to Williams et al. (*15*), RAPD reactions involve the amplification of small DNA fragments, using PCR with arbitrary oligonucleotide primers. Observed polymorphism can result from chromosomal changes in the amplified regions or base changes that alter primer binding. The procedure is rapid, easy to use, involves no radioactivity, and requires small amounts of DNA. The RAPD technique has been successfully used to map several characteristics in many crops and also in marker-assisted selection (*7*, *9*).

The BSA method was proposed as a rapid procedure for identifying markers linked to genes of interest. The method involves comparing two pooled DNA samples taken from segregating individuals (F_2), originated from a single cross between contrasting parentals. Polymorphic markers between the pools are supposed to be genetically linked to the loci or gene responsible for the trait of interest. Further analysis of DNA samples taken from F_2 individuals and/or cultivars previously screened for the studied trait will determine whether or not the selected marker can separate contrasting genotypes for the trait (*11*).

The objectives of this work were to identify RAPD markers linked to the gene that confers resistance to SCN, in a soybean population developed in Brazil.

MATERIAL AND METHODS

Plant material

To obtain the segregating populations, crosses between resistant lines BR 90-4722, BR 92-15440 and BR 90-4617 with the susceptible cultivar FT-Cristalina were made. These lines and the cultivar were studied previously by Arantes *et al.* (*2*), who classified them according to their reaction to SCN, race 3. Part of the F_1 seeds was used to obtain the segregating F_2 generation. Parents (resistant and susceptible) along with their F_2 generation were sowed in boxes containing sterilized sand as a substrate. After the complete expansion of cotyledonar leaves, seedlings were transferred to three-liter pots containing a sterile 2:1 soil:sand mixture.

Plant inoculation

The inoculum was obtained from infested fields in the county of Iraí de Minas. Thirty-five-day-old plants were collected from these fields and the females were extracted from the roots and macerated in a system of sieves. The race of the nematode was confirmed through tests in differential soybean cultivars performed according to suggestions of Arantes (*1*, *2*).

To evaluate the resistance of F_2 plants to SCN, race 3, two days after

transferring the seedlings to the pots, each pot was inoculated with a suspension containing approximately 11,000 eggs and juveniles. The inoculation was performed by applying the suspension near the stem of each seedling and plants were irrigated after inoculation to avoid the drying out of the nematodes.

Plants were carefully taken from the pots 34 days later, and the number of females and cysts in the roots of the plants was counted. Plants were classified as resistant or susceptible according to the rating system used by Hartwig (*5*), as follows: 0 - no cysts on roots; 1 - 1 to 5 cysts; 2 - 6 to 10 cysts; 3 - 11 to 20 cysts; 4 - more than 20 cysts. Plants scored 2 to 4 were considered susceptible.

DNA extraction and BSA analysis with RAPD

Leaf samples were collected from parents and F_2 plants prior to the inoculation with *Heterodera glycines*, race 3, and stored in a –80 °C freezer. DNA was extracted from all F_2 plants using the CTAB method (*12*) with modifications for soybean as outlined by Keim et al. (*8*). DNA concentration in the working solution was approximately 25 ng/µl, as confirmed by spectrophotometric analysis.

Based on the results from the inoculations, DNA samples were classified as resistant or susceptible to SCN, and two bulks were established. PCR reactions were carried out on bulks and F_2 individual DNA samples using 10 - bp oligonucleotide primers (100 ng) with arbitrary sequence (Operon Technologies), on a MJ thermal cycler, model PTC 100. The optimal mix for a 25 µl reaction was: 10X buffer - 2.5 µl [5.75 mL of glycerol, 2.00 mL EDTA 0.5 M, 0.5 mL de SDS (Sodium Dodecyl Sulfate) 20%, 1.75 mL of H_2O and bromophenol blue], $MgCl_2$ - 2.5 mM, Taq DNA polymerase - 1 unit, primers - 10 pmols and dNTPs - 2.5 mM. The template DNA was initially denatured at 96 ºC for 2 minutes and then subjected to 40 cycles at 94 ºC for 1 minute, 35 ºC for 1 minute and 72 ºC for 2 minutes.

Scoring and analyzing RAPD data

Following amplification, the samples were subjected to electrophoresis in 1.5% agarose gels, which were subsequently stained with ethidium bromide (10 mg/ml) and viewed under ultra-violet light. When a primer was found to be polymorphic between bulks, it was used to amplify DNA samples from F_2 individuals, previously classified as

resistant or susceptible, to verify if the PCR product was linked to the target gene.

RESULTS AND DISCUSSION

Total number of F_2 plants screened for resistance or susceptibility to SCN, race 3, and derived from all crosses was 105. Plants were considered resistant when scored 0 or 1 (*5*). From the cross between the parentals BR 90-4722 and FT-Cristalina, 5 resistant and 21 susceptible plants were initially taken for the reactions. Therefore, resistant and susceptible bulk DNA samples were constructed, respectively, from 5 and 21 plants.

A total of 360 primers were used for amplifications of resistant and susceptible DNA bulks. Polymorphism between bulks was observed for 56 of the tested primers. Table 1 contains all the primers that revealed polymorphisms with their respective code numbers and the kits from which they were taken.

TABLE 1 - Kits and codes found to be polymorphic between resistant and susceptible bulks.

Kit	Codes
OPA	2, 5, 15, 16, 18 and 19
OPB	2, 4, 5, 8, 13, 14, 16, 17 and 18
OPC	1, 7 and 8
OPD	7, 8 and 18
OPE	1, 2, 3, 6 and 10
OPG	4, 5, 8, 13, 14 and 16
OPI	1, 3, 4, 7, 9 and 11
OPJ	4, 9, 15, 16 and 20
OPM	3 and 13
OPN	1, 5 and 10
OPR	2, 5, 7, 8, 9, 11, 12, 13, 14 and 15

DNA taken from 74 susceptible and 29 resistant F_2 plants, previously screened for resistance or susceptibility to SCN in the greenhouse, were submitted to amplifications with the primer OPR-15, the one that showed polymorphism among F_2 plants. Figure 1 shows the results of PCR amplifications for 5 resistant and 21 susceptible F_2 plants, with the random primer OPR-15. It is possible to verify the presence of a polymorphic band of approximately 0.220 Kb which was designated $OPR\text{-}15_{220}$, according to the system used by Michelmore et al. (*11*). Further amplifications using the DNA from the remaining 53 susceptible and 21 resistant samples also showed that all resistant plants possessed the

polymorphic band, suggesting that the band was linked to genes conferring soybean resistance to SCN, race 3.

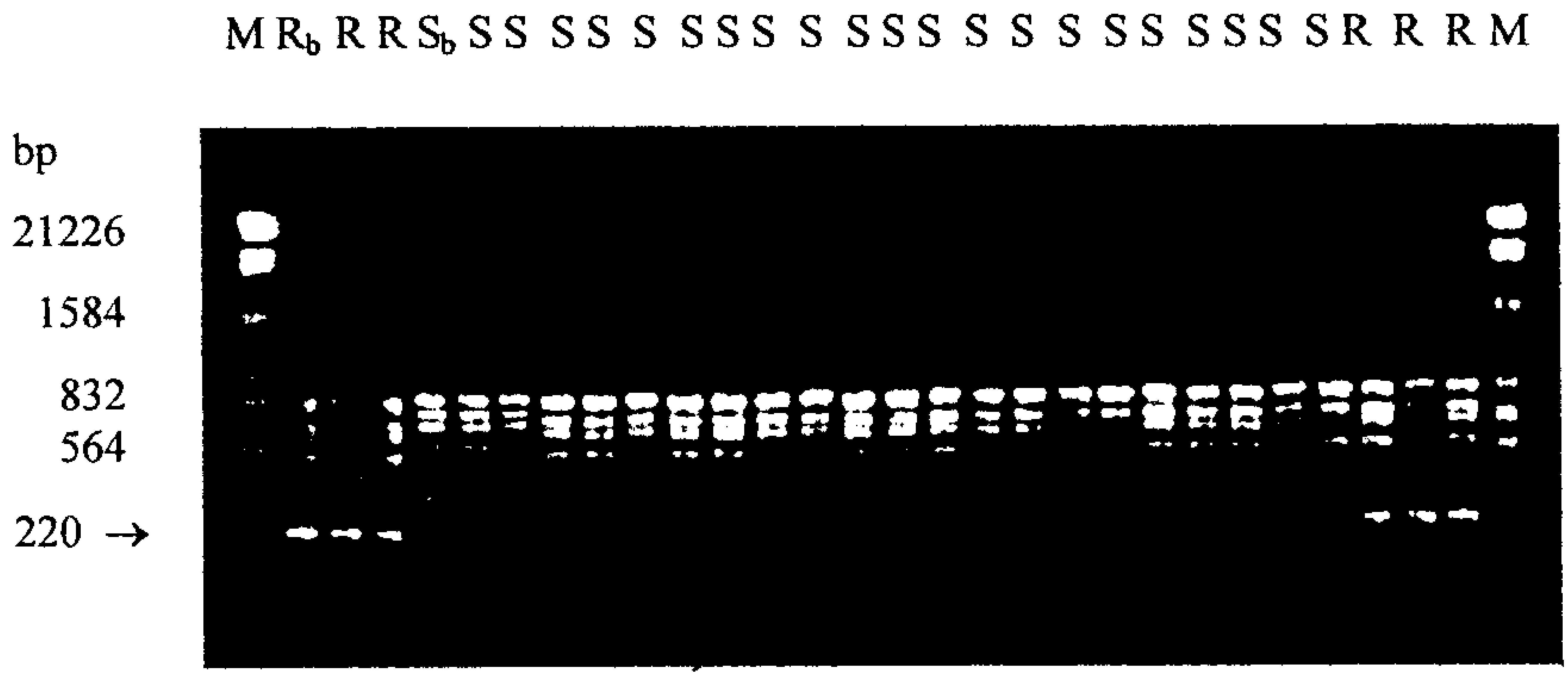

FIGURE 1 - Amplification of resistant (R) and susceptible (S) F_2 individual F_2 DNA samples, R_b-resistant bulk, S_b-susceptible bulk. M-marker (λ DNA *Eco* RI, *Hin*d III). Arrow indicates the marker OPR-15_{220}.

Further amplifications of resistant and susceptible DNA samples taken from resistant and susceptible soybean varieties showed the same pattern of bands, confirming that the primer is capable of distinguishing resistant and susceptible genotypes. Several authors (*3*, *13*, *14*) have also reported the identification of RFLP or RAPD molecular markers closely linked to the gene underlying resistance for soybean cyst nematode.

Heer et al. (*6*) compared 6 RFLPs, 2 RAPDs and 1 SCAR marker for resistance to SCN and found the SCAR548/$563_{1100/1025.975}$ marker to be associated with resistance to SCN races 1 and 3. According to Lawson et al. (*9*), the use of RAPDs and bulk segregant analysis for the development of trait-specific markers, such as SCARs, provides an efficient method of marker development. Thus, although primer OPR-15_{220} produced a polymorphic band capable of separating resistant and susceptible F_2 individuals, further studies are necessary in order to develop a SCAR marker, which is more reliable and functional for marker-assisted selection.

REFERENCES

1. ARANTES, N.E. Subsídio ao desenvolvimento de genótipos de soja resistente ao nematóide do cisto (*Heterodera glycines Ichinohe*). Jaboticabal, Fac. de Ci. Agrárias e Veterinárias, 65p. 1997. (Tese de Doutorado).
2. ARANTES, N.E.; MAURO, A.O. & TIHOHOOD, D. An alternative field method for screening soybean genotypes for resistance to *Heterodera glycines*. Journal of

Nematology, 30:542-6, 1998.
3. CONCIBIDO, V.C.; LANGE, D.A.; DENNY, R.L.; ORF, J.H. & YOUNG, N.D. Genome mapping of soybean cyst nematode resistance genes in "Peking", PI 90763 and PI 88878 using DNA markers. Crop. Sci., 37:258-64, 1997.
4. EMBRAPA. Centro Nacional de Pesquisa de Soja. Recomendações técnicas para a cultura da soja na Região Central do Brasil. 1996/97. Londrina, 1996. 119p.
5. HARTWIG, E.E. Breeding productive soybean with resistance to soybean cyst nematode. In: World Soybean Conference, 2°, Ames, 1984. Proceedings... Boulder, Westview Press, 1985. p. 394-9.
6. HEER, J.A.; KNAP, H.T.; MAHALINGAM, R.; SHIPE, E.R. & MATTHEWS, B.F. Molecular markers for resistance to *Heterodera glycines* in advanced soybean germplams. Molecular Breeding. 4:359-67, 1998.
7. JOHNSON, E.; MIKLAS, P.N.; STAVELY, K.J.; RAFALSKI, J.A. & TINGEY, S.V. DNA polymorphims amplified by arbritary primers are useful as genetic markers. Nucleic Acid Research., 18:6531-5, 1990.
8. KEIM, P.; SHOEMAKER, R.C. & PALMER, R.G. Restriction fragment length polymorphism diversity in soybean. Theor. Appl. Genet., 77:786-92, 1989.
9. LAWSON, W.R.; GOULTER, K.C.; HENRY, R.J.; KONG, G.A. & KOCHMAN, J.K. Marker-assisted selection for two rust resistance genes in sunflower. Molecular Breeding., 4:227-34, 1998.
10. NOEL, G.R. History, distribution and economics. In: Riggs, R.D. & Wrather, J.A. (eds.). Biology and management of the soybean cyst nematode. St. Paul, APS Press, 1992. p. 15-26.
11. MICHELMORE, R.W.; PARAN, I. & KESSELI, R.V. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci., 88:9828-32, 1991.
12. ROGERS, S.O. & BENDICH, A.G. Extraction of DNA from milligram amount of fresh, herbarium and mummified plant tissue. Plant Mol. Biol., 5:69-76, 1985.
13. WEBB, D.M.; BALTAZAR, B.M.; RAO-ARELLI, A.P.; SCHUPP, J.; CLAYTON, K.; KEIM, P. & BEAVIS, W.D. Genetic mapping of soybean cyst nematode race-3 resistance loci in the soybean PI 437654. Theor. Appl. Genet., 91:574-81, 1995.
14. WEISEMANN, J.M.; MATTHEWS, B.F. & DEVINE, T.E. Molecular markers located proximal to the soybean cyst nematode resistant gene, Rhg4. Theor. Appl. Genet., 85:136-8, 1992.
15. WILLIAMS, J.G.; KUBELIK, A.R.; LIVAK, K.J.; RAFALSKI, L.A. & TINGEY, S.V. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18:6531-5, 1990.