Genetics and Molecular Biology, 25, 3, 317-321 (2002)

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Research Article

RAPD and SCAR markers linked to resistance to frogeye leaf spot in soybean

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Abstract

The soybean (*Glycine max* (L.) Merrill) frogeye leaf spot is caused by the fungus *Cercospora sojina* Hara and is a widespread disease in Brazil and other countries, causing severe losses in grain yield and also affecting seed quality. The availability of DNA markers linked to genes for resistance to this disease would accelerate breeding programs, particularly when other traits are also being evaluated. Bulked segregant analysis was applied to 3 F_2 populations derived from crosses between the resistant cultivars Parana, Cristalina and Uberaba, and the susceptible cultivar Bossier. In the cross 'Parana' x 'Bossier', 2 RAPD markers were identified, $CSOPA1_{800C}$ and $CSOPA2_{1,250C}$, located at 4.4 ± 1.8 centiMorgans (cM) and 3.4 ± 1.7 cM respectively from the resistance locus. DNA fragments of similar molecular weight were observed in the population derived from the cross 'Cristalina' x 'Bossier' at 2.3 ± 1.2 and 4.7 ± 1.5 cM from the resistance locus, respectively. In the offspring of the cross 'Uberaba' x 'Bossier', a DNA fragment corresponding to marker $CSOPA1_{800C}$ was detected at 5.6 ± 2.1 cM from the resistance locus. Although marker $CSOPA2_{1,250C}$ was not observed in this population, an additional marker was detected ($CSOUB1_{1,100C}$) at 6.7 ± 2.2 cM from the resistance locus. The 1,250 bp fragment of $CSOPA2_{1,250C}$ was cloned and converted into a SCAR marker, which amplified a single fragment whose size corresponded to the cloned segment of the crosses involving cultivars Cristalina and Parana. Markers $CSOPA1_{800C}$, $CSOPA2_{1,250C}$ and $CSOUB1_{1,100C}$ were mapped to soybean linkage group J with the aid of known SSRs linked to the Rcs3 locus, indicating that the RAPD and SCAR markers identified in our research also tag this resistance gene.

Key words: molecular markers, RAPD, SCAR, soybean, *Cercospora sojina*, frogeye leaf spot.

Received: July 19, 2000; accepted: June 6, 2002.

Introduction

Frogeye leaf spot, caused by the fungus *Cercospora sojina* Hara, is a worldwide important soybean disease, causing both yield losses and seed deterioration. The use of resistant cultivars is the most efficient and cost-effective means of controlling this disease, but the existence of several *C. sojina* races (Yorinori, 1989b) demands the permanent search for new sources of resistance and their incorporation into resistance breeding programs. Another factor to be considered is that the evaluation of soybean crops with regard to this disease is a time-consuming process, which requires expertise for the precise distinction between susceptible and resistant plants.

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These problems can be better managed in breeding programs by identifying and using DNA markers linked to resistance genes. DNA markers are abundant and essentially independent from environmental conditions (Keim *et al.*, 1989), and several research groups have been using this tool in breeding programs (Shoemaker *et al.*, 1992; Young and Kelly, 1996; Young *et al.*, 1998). In this paper, we report on the identification of DNA markers linked to soybean loci responsible for resistance to *C. sojina* in soybean cultivars Cristalina, Parana and Uberaba, and also on the confirmation of the resistance locus identity.

Material and Methods

Genetic material and crosses

Seeds of the soybean cultivars Bossier, Cristalina, Paraná and Uberaba were provided by the Soybean

Breeding Laboratory (SBL) of the Department of Plant Sciences of the Federal University of Viçosa, Minas Gerais, Brazil. The Bossier cultivar is susceptible to the race 4 *C. sojina* fungus, while 'Cristalina', 'Parana' and 'Uberaba' are resistant to it. A number of allelism tests involving these resistant cultivars and/or their resistant progenitors indicate that they harbor the same resistance locus, Rcs3, which is also present in cultivar Davis (Arias *et al.*, 1996).

Origin and cultivation of *C. sojina* isolate

The monosporic isolate (provided by the SBL) used in all inoculations was collected in the Alto Paranaíba region of the Brazilian State of Minas Gerais, and identified as race 4 (Machado *et al.*, 1997), its isolation and cultivation being carried out as described by Veiga (1973) and Cordeiro (1986).

Evaluation of symptoms

The crosses performed were 'Cristalina' x 'Bossier' (CB), 'Parana' x 'Bossier' (PB), and 'Uberaba' x 'Bossier' (UB), with 'Bossier' as the male parent in all crosses. F_1 plants were selfed, and the F_2 seeds were planted in a greenhouse. In addition to the parents, 219 CB, 126 PB, and 124 UB F_2 plants were tested for resistance/susceptibility to *C. sojina*. The primary leaf from each plant was collected and kept at -80 °C. When the first trifoliolate leaf was fully expanded, the plants were inoculated with the pathogen by spraying approximately 4 x 10^5 conidia on each plant, on the abaxial and adaxial sides of the leaves (Casela *et al.,* 1979). The plants were then transferred and kept for 3 days in a mist chamber maintained at 20 to 22 °C and 95% relative humidity.

Symptoms were assessed 20 days after inoculation, based on the scale proposed by Yorinori (1989a). To suit our purpose better, the scale was modified as follows: grade 1 = leaf without lesions; 2 = 1 to 10% of leaf area infected (LAI); 3 = 11 to 25% LAI; 4 = 26 to 50% LAI; and 5 = more than 50% LAI. Plants with scores from 1 to 3 were considered resistant, and those which scored 4 and 5 were considered susceptible.

DNA bulks and amplification

In order to identify homozygous F_2 plants for the construction of DNA bulks, 20 F_3 seeds were taken from each of 30 F_2 resistant plants (with score 1) and planted in a greenhouse; the resulting F_3 plants were inoculated with the pathogen and evaluated for symptoms, as described above.

For each cross, two DNA bulks (Michelmore *et al.*, 1991) were constructed, one containing DNA from 6 homozygous susceptible F_2 plants, and the other containing DNA from 6 resistant plants. The DNA was extracted by the method described by Doyle and Doyle (1990), and RAPD DNA amplification was carried out by the method of Williams *et al.* (1990), using primers from Operon Technologies (Alameda, CA, USA) and a model 9600 thermocycler (Perkin-Elmer, Norwalk, CT, USA). 1,200 primers were tested for DNA amplification. Amplification conditions were as follows: 40 cycles, each consisting of a 15 s denaturation step at 94 °C, a 30 s annealing step at 35 °C, and a 1 min extension step at 72 °C. After the 40^{th} cycle, a final extension step of 7 min at 72 °C was performed. The amplification products were separated on 1.2% agarose gel containing 0.2 µg/mL ethidium bromide, immersed in pH 8.0 TBE (90 mM Tris-borate buffer, 1 mM EDTA), and the DNA bands were observed under UV light and photographed; only the most intense and reproducible bands were used for analysis. Primers generating DNA bands which were polymorphic between the bulks were individually tested against the components of each bulk and then tested against the F_2 population, to determine the genetic distances between the markers and the resistance loci.

Development of SCAR markers

One of the RAPD bands linked to the resistance locus identified in population CB was excised from the gel, purified with the aid of the Glass Max^{tm} DNA Isolation Matrix System (BRL) and cloned in the vector pGEM-T Easy (Promega). White colonies were grown in 2 mL LB medium containing 100 µg/mL ampicillin, and the plasmid was purified with the QIA Prep Spin Miniprep kit (Qiagen). The clone was partially sequenced by automated sequencing using M13 universal primers. The sequence information was used to design two primers, each one containing 18 nucleotides including the sequence of the original RAPD primer.

The PCR reaction to amplify the SCAR marker consisted of 35 cycles, each one consisting of a 30 s step at 94 °C, a 1 min step at 62 °C, and a 1 min 30 s step at 72 °C. The amplified bands were analyzed as described for the RAPD amplification products.

Linkage analysis

Student's chi-square (χ^2) test was used to analyze the phenotypic segregation of the three populations and to determine possible linkages between the RAPD markers and the resistance loci.

For linkage analysis, 219 CB, 126 PB, and 124 UB F_2 plants were used, and the progenitors of these crosses were also tested with primer-pairs for SSR Satt-547 and Satt-431, which were shown to be linked to the Rcs3 locus in soybean linkage group J (Mian *et al.*, 1999). Where polymorphic bands were detected, the contrasting bulks and the F_2 plants were also analyzed. The distances between the molecular marker loci and the resistance locus were estimated using version 3.0 of the MAPMAKER/EXP program (Lander *et al.*, 1987; Lincoln *et al.*, 1992), with a minimum *lod score* of 3.0 and a recombination setting of 50%.

Results and Discussion

Identification of RAPD markers

Figure 1a and 1b show the two polymorphic DNA bands which distinguished the contrasting bulks from the PB cross. These bands were present in all resistant individuals of the bulks and absent in all susceptible individuals. These markers were designated CSOPA1$_{800C}$ and CSOPA2$_{1,250C}$, because they are linked to a locus which controls resistance to *C. sojina* (CSO) originally detected in cultivar Parana (PA), contain approximately 800 and 1,250 bp, respectively, and are in the coupling-phase (C) with regard to the resistance gene. These two markers also co-segregated with resistance in the CB cross. Marker CSOPA1$_{800C}$ also co-segregated with resistance in the UB cross, but locus CSOPA2$_{1,250C}$ was monomorphic in that cross. A third marker, CSOUB1$_{1,100C}$, was detected only in the UB cross (Figure 1c).

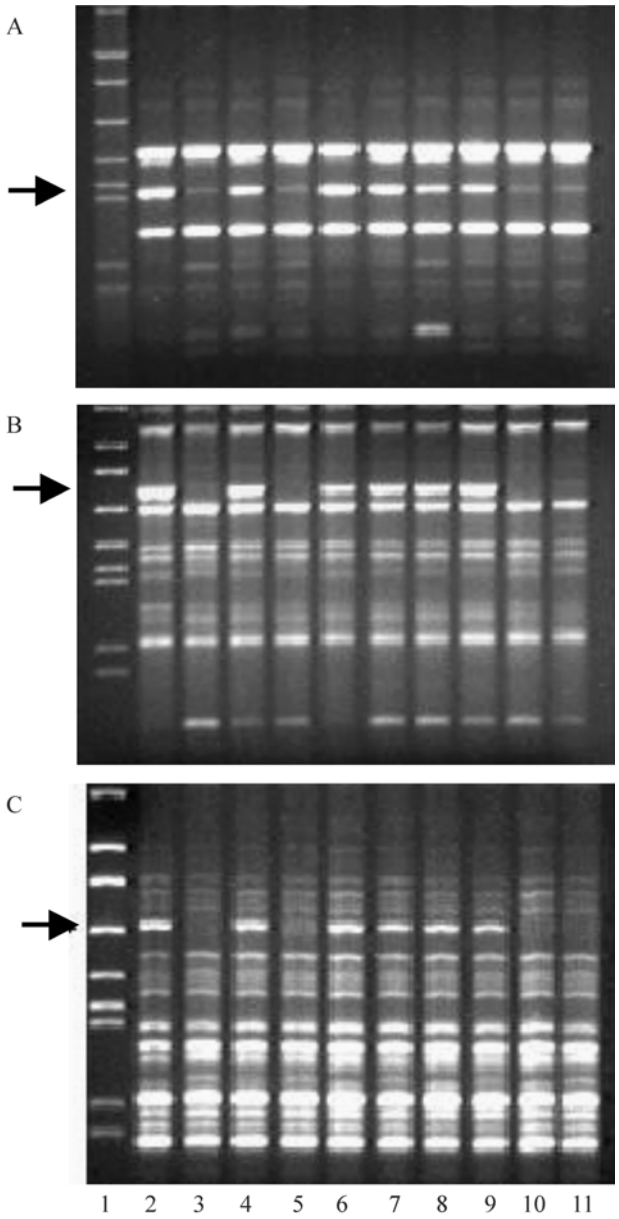

Figure 1 - Gels of DNA amplification products from soybean plants with different susceptibility to frogeye leaf spot: a) marker CSOPA1$_{800C}$ in the PB cross, b) marker CSOPA2$_{1,250C}$ in the PB cross, c) marker CSOUB1$_{1,100C}$ in the UB cross. Lanes are as follows: 1, lambda phage DNA digested with *Eco*RI, *Bam*HI and *Hin*dIII (size markers); 2, resistant cultivar; 3, susceptible cultivar; 4, resistant *bulk*; 5, susceptible *bulk*; 6-9, resistant F_2 plants; and 10-11, susceptible plants. Arrows indicate the polymorphic DNA band.

The segregation ratio 3:1 (resistant**:**susceptible or band presence**:**absence) in the F_2 populations was consistent with the single-factor dominant inheritance of the resistance locus and of the RAPD markers in the CB and PB crosses (Table I). In the UB cross, resistance to *C. sojina* segregated at a 13:3 ratio (Table I), indicating that at least two independent loci control resistance in the Uberaba cultivar, one being dominant and the other recessive. Cordeiro (1986) also detected two resistance loci to *C. sojina* race 4 in a cross between 'Santa Rosa' and 'Bossier', 'Santa Rosa' being the progenitor of 'Uberaba'.

In view of the high χ^2 values, the hypothesis of independent linkage (9:3:3:1) could be discarded, indicating that the RAPD markers were linked to the resistance locus (Table II). The estimates of the genetic distance between the RAPD locus CSOPA1$_{800C}$ and the resistance locus varied between 2.3 cM for the CB cross and 5.6 cM for the UB cross. The estimated genetic distances between RAPD locus CSOPA2$_{1,250C}$ and the resistance locus varied between 3.4 cM for the PB cross and 4.7 cM for the CB cross. CSOUB1$_{1,100C}$ was 6.7 cM from the resistance locus.

It is conceivable that the locus tagged by CSOPA1$_{800C}$ in all three crosses corresponds to locus Rcs3, a resistance locus previously identified in the cultivars Parana, Cristalina, Santa Rosa and Davis (Arias *et al.*, 1996). To confirm this hypothesis, two SSR markers, Satt 431 and Satt 547, previously shown to be linked to locus Rcs3 (Mian *et al.*, 1999), were tested in the three populations used in this study (Table I). The results show that, in the CB population, Satt 431 and Satt 547 co-segregated with resistance and with the CSOPA1$_{800C}$ and CSOPA2$_{1,250C}$ markers. However, in the PB population, both SSR markers were monomorphic, Satt 547 alone being polymorphic in the UB population and co-segregating with resistance and with the CSOPA1$_{800C}$ and CSOUB1$_{1,100C}$ markers. These SSR and RAPD marker data strongly suggest that the resistance locus we mapped in the three populations corresponds to the resistance locus Rcs3.

It is noteworthy that this conclusion could only be reached by the combined analysis of the two types of markers. In addition, our results confirm the usefulness of the integrated SSR map for soybean (Cregan *et al.*, 1999) as a main guide for breeders and emphasize that the optimal use of molecular markers in plant breeding will depend on a specific analysis of each individual cross.

Table I - Segregation analysis of molecular markers and the *C. sojina* resistance locus in F_2 populations derived from the crosses 'Cristalina' x 'Bossier' (CB), 'Parana' x 'Bossier' (PB) and 'Uberaba' x 'Bossier' (UB)[1/].

Population	Locus tested	Observed frequency	Expected frequency	χ^2	p
CB	Rcs3[2/]	162:57	164.25:54.75	0.0745	0.79
CB	$CSOPA1_{800C}$	159:60	164.25:54.75	0.5495	0.46
CB	$CSOPA2_{1,250C}$	155:64	164.25:54.75	1.8645	0.17
CB	Satt-547	155:64	164.25:54.75	1.8645	0.17
CB	Satt-431	159:60	164.25:54.75	0.5495	0.46
PB	Rcs3	98:28	94.50:31.50	0.3809	0.54
PB	$CSOPA1_{800C}$	95:31	94.50:31.50	0.0000	0.99
PB	$CSOPA2_{1,250C}$	94:32	94.50:31.50	0.0000	0.99
PB	Satt-547	-[3/]	-	-	-
PB	Satt-431	-	-	-	-
UB	Rcs3	103:21	100.75:23.25	0.1621	0.69
UB	$CSOPA1_{800C}$	97:27	93.00:31.00	0.5269	0.47
UB	$CSOUB1_{1,100C}$	98:26	93.00:31.00	0.8709	0.35
UB	Satt-547	99:25	93.00:31.00	1.5484	0.21
UB	Satt-431	-	-	-	-

[1/]Expected proportion for acceptance of the independence hypothesis was 3:1. resistance or presence of DNA band**:**susceptibility or absence of band. In the case of population UB locus Rcs3, the expected proportion for independence was 13:3.
[2/]Resistance to *C. sojina* locus.
[3/]Monomorphic for this cross.

Conversion of RAPD marker into a SCAR

The DNA fragment corresponding to RAPD marker $CSOPA2_{1,250C}$ was cloned and partially sequenced. Two 18-nucleotide-long primers were synthesized based on the sequencing data: SCARBG5F (5' GCC GTG AGA AAG GCG AAG 3') and SCARBG5R (5' AGC CGT GAA TTA TCC GAT 3'). These primers were tested in the CB and PB F_2 populations, and Figure 2 shows that the polymorphism of the amplifications is identical to the one revealed by the RAPD marker. This SCAR marker can be used for marker-assisted selection in programs aiming at the development of cultivars which are resistant to frogeye leaf spot, particularly when other traits are also being evaluated.

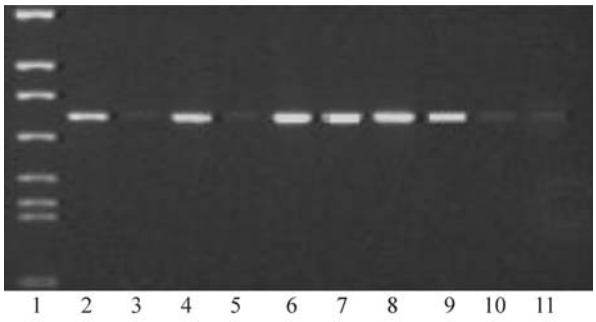

Figure 2 - Transformation of RAPD marker $CSOPA2_{1,250C}$ into SCAR marker. Lanes are as follows: 1, lambda phage DNA digested with *Eco*RI, *Bam*HI and *Hin*dIII (size markers); 2, resistant Parana cultivar; 3, susceptible Bossier cultivar; 4, resistant *bulk*; 5, susceptible *bulk*; 6-9, resistant F_2 plants; and 10-11, susceptible plants.

Table II - Analyses of linkage between RAPD markers and resistance to *Cercospora sojina* Hara locus in F_2 populations derived from the crosses 'Cristalina' x 'Bossier' (CB), 'Parana' x 'Bossier' (PB) and 'Uberaba' x 'Bossier' (UB)[1/].

Population	Locus tested	Observed frequency	χ^2	p	CM ± SD[2/]
CB	Rcs3 / $CSOPA1_{800C}$	158:4:1:56	214.63		2.3 ± 1.2
CB	Rcs3 / $CSOPA2_{1,250C}$	154:8:1:56	211.49		4.7 ± 1.5
PB	Rcs3 / $CSOPA1_{800C}$	94:4:1:27	92.98		4.4 ± 1.8
PB	Rcs3 / $CSOPA2_{1,250C}$	94:4:0:28	99.99		3.4 ± 1.7
UB	Rcs3 / $CSOPA1_{800C}$	97:6:0:21	69.81		5.6 ± 2.1
UB	Rcs3 / $CSOUB1_{1,100C}$	97:6:1:20	64.53		6.7 ± 2.2

[1/]Expected proportion for acceptance of the independence hypothesis was 9:3:3:1 presence of resistance locus and marker: presence of resistance locus and absence of marker: absence of resistance locus and presence of marker**:** absence of resistance locus and marker.
[2/]Genetic distance in centiMorgans ± standard deviation.

Acknowledgments

The authors thank BID/FINEP and FAPEMIG for financial support. SMF was supported by a fellowship from CNPq.

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