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Development of SCAR marker linked to stem canker resistance gene in soybean

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ABSTRACT - *Stem canker caused by the fungus* Diaporthe phaseolorum *f. sp.* meridionalis *is a disease that limits soybean cultivation. Phenotypic evaluations aiming at disease resistance require labor-intensive processes, as for instance handling and transport of phytopathogens. The use of DNA markers in the selective procedures eases certain phases, besides being practical, safe and reliable. A RAPD fragment of 588pb was identified among bulks of resistant and susceptible plants in the cross BR92-15454 (R) x IAC-11 (S). Through co-segregation, the distance between the resistance locus and the fragment was estimated at 7.4 ± 2.1 cM, with a* $Lod_{max.}$ *of 23.072 (first year) and at 6.0 ± 3.4 cM with a* $Lod_{max.}$ *of 7.806 (second year). The fragment was converted into a SCAR marker and digested with enzyme Hinc II, which made the classification in homozygous resistant, heterozygous resistant and susceptible plants possible. This SCAR marker is suitable for use in the improvement program conducted in Jaboticabal.*

Key words: RAPD, *Glycine max* , *Diaporthe phaseolorum* f. sp. *meridionalis*, assisted selection.

INTRODUCTION

One of the most important soybean diseases is stem canker with a highly destructive potential, as the first occurrences in the State of Paraná demonstrated. The nationwide losses in the period from 1989 to 1995 were estimated at over US$ 350 million (Yorinori 1996). Nowadays the disease caused by the fungus *Diaphorte phaseolorum* f. sp. *meridionalis* (Dpm) is disseminated across all producing regions in Brazil.

First studies aiming at the determination of resistance inheritance were performed with the fungus *Diaphorte phaseolorum* var. *caulivora* (Dpc). Kilen et al. (1985) inoculated F_2 plants (Tracy-M x J77-339) and identified two dominant genes of disease resistance in the cultivar Tracy-M. Wendel and Allen (1986) identified one dominant and another partially dominant gene in Tracy-M, *Dpc1* and *Dpc2*, respectively. Kilen and Hartwig (1987) verified that the lines X404 and X412 presented the genes *Rdc1* and *Rdc2*, respectively, identified earlier in cultivar Tracy-M by Kilen et al. (1985). Bowers Jr et al. (1993) confirmed that inheritance in Tracy-M is controlled by two resistance genes.

Later the fungus *Diaporthe phaseolorum* f. sp. *meridionalis* (Dpm), causal agent of the disease in the

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south of the United States as well as in Brazil, was used in research. Kilen and Hartwig (1995) investigated the Dpm fungus and observed two stem canker resistant lines (D85-10404 and D85-10412), carriers of the genes *Rdc1* and *Rdc2*, respectively. Bárbaro et al. (2005) worked with F_2 and F_3 populations of crosses involving cultivar Tracy-M. The authors concluded that the resistance is controlled by one gene with two alleles and dominant action.

Molecular markers can be used to characterize a genotype based on tissue samples. Williams et al. (1990) proposed the RAPD (*Random Amplified Polymorphic DNA*) method for the identification of molecular markers. To associate a phenotypic trait to molecular markers Michelmore et al. (1991) developed the BSA (*Bulked Segregant Analysis*) methodology.

Carvalho et al. (2002) identified two molecular RAPD markers, linked to the gene of resistance to soybean stem canker, amplified by a same primer: $OPAB19_{1320}$ present in all plants classified as homozygous resistant (coupling phase) and $OPAB19_{1150}$ present in the susceptible plants (repulsion phase), while the plants classified as heterozygous resistant presented both fragments. The distance between the gene and the markers was estimated at 4.7 cM.

RAPD markers have been cloned, sequenced and converted into SCAR markers (*Sequence Characterized Amplified Region*) as proposed by Paran and Michelmore (1993). The loss of polymorphism is common in this process due to the amplification of two alleles of a same locus, which inhibits plant differentiation. The authors reported that the polymorphism observed in the RAPD reaction may be caused by differences in the nucleotide sequence of the annealing site or by a rearrangement in the internal sequence of the amplified fragment. The base-pairing error, mainly at the 3'-end of a primer, could inhibit the fragment amplification in one of the genotypes. The SCAR primers, synthesized at the 3' end of the primers used in the RAPD reactions, have a larger number of nucleotides. This end is in the central region of the SCAR primer and cannot influence the fragment amplification.

The loss of polymorphism can be solved by restriction enzymes that can promote the digestion enzyme in only one of the alleles of a particular locus. The technique has been used successfully in different species (Lahogue et al. 1998, Weng et al. 1998, Dax et al. 1998 and Zhang and Stommel 2001).

The objective of this study was to develop a SCAR marker linked to a gene that controls plant resistance to soybean stem canker.

MATERIAL AND METHODS

Resistance was phenotypically evaluated in F_2 populations of the crosses: BR92-15454 (R) x IAC-11 (S) and Tracy – M (R) x IAC-8 (S) as well as in backcross populations. The parentals classified as resistant (R) and susceptible (S) presented the following genealogies: Tracy-M - selection in Tracy (D61-618 x D60-9647); BR92-15454 - [F81-2129 x (Kirby x Tracy-M)]; IAC-8 - [Bragg x (Hill x PI 240664)]; IAC-11 - {Paraná x [Davis x (Hill x PI 240664)]}.

The seeds were placed individually to germinate in five liter plastic pots with substrate. The seedlings were inoculated in the V_1 stage (Fehr and Caviness 1977), according to the methodology described by Yorinori (1996). After inoculation, each pot was covered with a transparent plastic bag and sealed, forming a separate moist chamber, for 72 hours. In the end of this period, the plants were uncovered and sprayed in an alternate system for seven days, and thereafter irrigated directly in the pots. The pathogen inoculum, isolated from infested plants in the municipality of Dourados, Mato Grosso do Sul, was provided by the Embrapa Centro Nacional de Pesquisa de Soja (Londrina, Paraná). The resistance was phenotypically evaluated 30 days after inoculation and scored on a scale proposed by Ito et al. (1997), according to the appearance of the lesions: 1- healthy plants; 2-plants with lesions around the inoculated internode only; 3-plants with lesions covering more than one internode and 4-dead plants. The inoculated populations were monitored daily and the plant death date recorded (score 4).

The greenhouse experiment was conducted in two separate years: in the first year plants of the F_2 generation of the two crosses were inoculated and evaluated. After evaluation, the seeds of the F_2 plants, classified with score one, were individually harvested. These seeds generated F_3 plants, which were inoculated and evaluated according to the methodologies described above. The results in the F_3 families were used to separate the F_2 plants in segregating homozygous and segregating heterozygous. In the second year the F_2 population of the cross BR 92-15454 x IAC-11 and the backcross populations F_1 (BR 92-15454

x IAC-11) x IAC-11 and F_1 (Tracy - M x IAC-8) x IAC-8 were evaluated.

In the phenotypic evaluations, the F_1 plants and respective parentals were inoculated in the same conditions as the study populations. In the first year 15 plants of each parental and 10 F_1 plants were evaluated and in the second year only 15 plants of each parental.

The results of the score scale in the F_2 and backcross populations were analyzed statistically, to confirm the hypotheses of segregation, using the software GQMOL (Cruz and Schuster 2001). The plants were separated in two groups: live plants - LP (scores 1, 2 and 3) and dead plants - DP (score 4).

Prior to inoculation one unifoliolate leaf was taken from each plant for DNA extraction according to the protocol described by Ferreira and Grattapaglia (1995). The samples were quantified in a biophotometer. Two bulks were composed - one with equimolar DNA quantities of seven homozygous resistant F_2 plants (score 1) and the other with the DNA of seven susceptible F_2 plants (score 4).

For the RAPD reactions, a PTC-100 thermocycler was used considering a final volume of 25 mL in each reaction. The protocol of Williams et al. (1990) was applied with some modifications: 2.5 mL buffer (10X), 2 mM $MgCl_2$, 100 mM of each dNTPs, 10 pmoles of the primer, 100 ng DNA and 1.0 U Taq DNA polymerase, in the following amplification conditions: 94 °C for 2 minutes, followed by 40 cycles of 94 °C for 1 minute, 35 °C for 30 seconds, 72 °C for 1.5 minute, plus a final extension step at 72 °C for 10 minutes. For each one of the bulks within the two crosses 400 decamer primers (Operon Technologies) (A-R, AA and AB) were used.

In the end of the amplification cycles 20 µL of each reaction were applied on agarose gel (1.5%), ethidium bromide-stained (0.15 µg mL^{-1}) and subjected to 100 V for 2 hours. TBE 1X buffer (89 mM Tris base, 89 mM boric acid and 1mM EDTA pH 8.0) was used for the migrations on horizontal electrophoresis cubes. Each gel was photographed under UV light and the images recorded by the Kodak system.

With the underlying data of the phenotypic evaluation and the polymorphic marker, the hypothesis of independent segregation and of recombination frequency was tested by the co-segregation analysis using GQMOL (Cruz and Schuster 2001). Primer OP-AB04 was evaluated with the DNA of stem canker-resistant and susceptible cultivars.

The polymorphic RAPD fragment was isolated from the gel, purified (SNAP Kit, Invitrogen), ligated to the plasmid pCR® 2.1-TOPO (Invitrogen) and used in the transformation of *Escherichia coli* (DH10β) bacteria through electroporation (Sambrook et al. 1989). The fragment was sequenced according to the methodology of Sanger et al. (1977). With the program Gene Runner (available at http://www.generunner.com), two primers (forward and reverse) were designed, containing the additional RAPD primer, at the 3' end of the 5 adjacent internal bases.

The annealing temperature of the new SCAR primers (JAB-01 and JAB-02) was determined in the thermocycler (Master cycler gradient), using DNA of the parentals BR92-15454 and IAC-11 The primers were evaluated (jointly) following the original RAPD protocol. The amplified monomorphic fragment was digested (at 37 ºC for 3 hours) with the restriction enzyme *Hinc II*.

The amplified sequence with the SCAR primers was aligned with identified sequences and included in the databank National Center for Biotechnology Information (NCBI) (www.ncbi.com), by the program BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1997).

RESULTS AND DISCUSSION

In the first phase of the research, related to the phenotypic evaluation of the F_2 plants of the crosses BR92-15454 x IAC-11 and Tracy - M x IAC-8, it was possible to visualize symptoms in some plants soon after removing the plastic bags over the pots. This had been expected, since in some studies the evaluation was performed between 15 and 20 days after inoculation (Siviero and Menten 1995, Siviero et al. 1997, Carvalho et al. 2002).

The period of 30 days after inoculation was considered as a limit for the final evaluation of the plants, in agreement with Kilen et al. (1985), Kilen and Hartwig (1987), Weaver et al. (1988), Bowers Jr. et al. (1993). In the χ^2 test the results indicated the presence of one gene with dominant action in the parentals Tracy-M and BR92-15454, governing plant resistance to stem canker (Table 1). Bárbaro et al. (2005) concluded that the resistance to stem canker in Tracy-M is controlled by one gene with two alleles and dominant action. The

Table 1. Values calculated by the χ^2 test for four populations, considering the hypothesis of segregation 3:1 for F_2 and 1:1 for backcross

Population	χ^2 test for the evaluated populations							
	First year				Second year			
	LP	DP	χ^2	P (%)	LP	DP	χ^2	P (%)
F_2 - Tracy - M x IAC-8	78	22	0.4800	48.8422	-	-	-	-
F_2 - BR92-15454 x IAC-11	123	45	0.2857	59.2980	41	12	0.1572	69.1717
F_1(Tracy - M x IAC-8) x IAC-8	-	-	-	-	29	35	0.5625	45.3254
F_1(BR92-15454 x IAC-11) x IAC-11	-	-	-	-	12	10	0.1818	66.9815

LP = live plants, DP = dead plants

grouping of the plants in two classes (resistant and susceptible) only is apparently sufficient for research that focuses on the determination of the resistance inheritance type to the causal fungus of soybean stem canker. It is however known that there are intermediary reactions which are in some cases difficult to understand. Kilen and Hartwig (1987), Bowers Jr. et al. (1993), Tyler (1996) and Carvalho et al. (2002) grouped the plants in two classes.

The RAPD reactions obtained a marker of 588 pb, amplified by primer OP-AB04, only present in the bulk of resistant F_2 plants of the cross between the parentals BR92-15454 x IAC-11. The individual evaluation of the plant components of the bulks confirmed the amplification of the previously identified fragment. The same result was obtained in the reactions when using DNA of the resistant parental BR92-15454 and F_1 plants (Figure 1).

Owing to the genealogy of parental BR92-15454, the marker had been expected to be present in Tracy-M as well. Since the marker is normally not the gene, but rather a region near it, amplification occurred in the parental BR92-15454 that had not been observed in cultivar Tracy-M, which can be explained by mutations or rearrangements between the two sites, or right in the hybridization site of the primer (Paran and Michelmore 1993). Differences in only one base pair can be enough to inhibit amplification, mainly in the 3' position (Williams et al.1990).

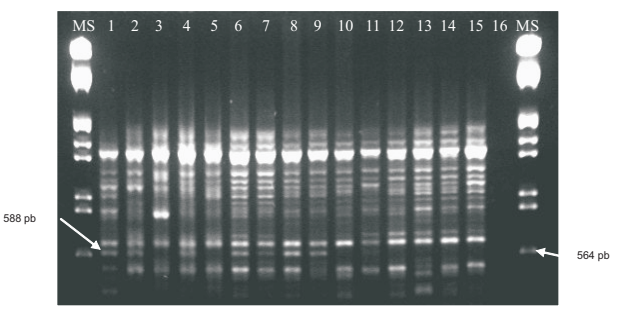

Figure 1. Polymorphic RAPD fragment of 588 pb observed in: 1. Resistant parental BR 92-15454, 2. F_1 Plant, 4. Resistant bulk and 6-9. Resistant F_2 plants, and absent in: 3. Susceptible parental IAC-11, 5. Susceptible bulk and 10-15. Susceptible F_2 plants; 16. Negative control; MS - Molecular standard (phage λ digested with the enzymes *Eco* RI and *Hind* III)

The F_2 plants of the complete population of the cross between BR92-15454 and IAC-11 (first year) were evaluated with the polymorphic RAPD primer for the confirmation of the presence of the fragment identified in the bulks. The plants scored 1, 2 and 3 presented the polymorphic fragment demonstrating that the RAPD marker can identify plants with some resistance degree. Independently of the disease severity, it was possible to separate live from dead plants. The RAPD marker could therefore be used to reduce the number of plants evaluated in a population (Poulsen et al. 1995).

The observed as well as the expected values of the phenotypic and molecular evaluations are presented in Table 2. The results of the χ^2 test for the co-segregation analysis were significant for both years of evaluation, which refutes the hypothesis of independent segregation 9:3:3:1, that is, the marker and the resistance gene are located in the same chromosome. The values for the partitioning of the degrees of freedom are also shown (Table 2). Based on the data of recombinant plants (resistant without band and susceptible with band), the distance between the marker and the gene was estimated: 7.4±2.1 cM, with $Lod_{max.}$ of 23.072 (first year) and 6.0±3.4 cM with $Lod_{max.}$ 7.806 (second year).

The evaluation of stem canker-resistant cultivars with primer OP-AB04 confirmed the amplification pattern in BRSMG Renascença - [F81-2129 x (Kirby x Tracy-

Table 2. Observed and expected values (independent segregation 9:3:3:1), between the 588pb RAPD marker and the locus that controls stem canker resistance in soybean for two F_2 populations of the cross BR92-15454 x IAC-11 and partitioning of the degrees of freedom of the χ^2 test (chi-square)

Plant Classification	First year		Second year	
	Observed Value	Expected Value	Observed Value	Expected Value
Resistant with band (R+)	120	94.5000	39	29.8125
Resistant without band (R-)	3	31.5000	2	9.9375
Susceptible with band (S+)	9	31.5000	1	9.9375
Susceptible with band (S-)	36	10.5000	11	3.3125
Total	168	168	53	53

Segregation	df	First year		Second year	
		χ^2	P (%)	χ^2	P (%)
Total	3	110.6666	-	35.0503	-
*Segregation R/S	1	0.2857	59.298	0.1572	69.1717
**Segregation +/-	1	0.2857	59.298	0.0062	93.6789
Joint Segregation	1	110.0952	-	34.8867	-

* (R/S) – phenotypic classification for resistant or susceptible plants
**(+/-) – molecular classification for presence or absence of the 588 pb RAPD fragment

M)] x Forrest and MG/BR-46 Conquista – (Lo 76-4484 x Numbaíra), giving rise to a fragment of equal molecular weight to the amplified in parental BR92-15454. The susceptible cultivars Paraná [Hill x (Roanoke x Ogden)], BR-16 (D69-B10-M58 x Davis) and Ocepar-4 (Iguaçu) (R70-733 x Davis) did not amplify the fragment, similarly to parental IAC-11. These results suggested that the primer used to amplify the polymorphic RAPD fragment in the population of F_2 plants of the cross BR92-15454 X IAC-11 could be used with other cultivars.

The SCAR reactions with the primers JAB-01 and JAB-02, at annealing temperatures between 53 ºC and 59 ºC, evidenced the loss of the initial polymorphism. Other temperatures were evaluated as well as the time of pairing, but the 588 pb fragment appeared to be a single band, present in the parentals BR92-15454 and IAC-11. Likewise, the loss of polymorphism was evidenced in studies of Paran and Michelmore (1993), Lahogue et al. (1998), Weng et al. (1998), among others.

After cloning and sequencing of the monomorphic fragments generated by the SCAR reaction, a restriction site was identified in position 531 for enzyme *Hinc* II, present only in the sequence amplified with the resistant parental DNA. SCAR reactions were carried out with the DNA of the resistant and susceptible parentals, as well as with the DNA of one F_1 plant. An aliquot of each one of the reactions was used for enzyme digestion.

The technique was effective for plant separation, since the alleles of the resistant parental were digested by the enzyme *Hinc* II, producing two fragments (531 pb and 57 pb), while the susceptible parental maintained the 588 pb fragment. The F_1 plants, heterozygous for the locus as well as the F_2 plants classified as resistant, although in heterozygosis, presented a pattern of three bands (588, 531 and 57 pb) (Figure 2). Due to the low molecular weight, the 57 pb band is very clearly represented on the gel. The same was observed for the 588 pb band of the heterozygous F_1 and F_2 plants, probably due to little amplification of the susceptibility allele in the presence of the resistance allele.

After the SCAR reaction and enzyme digestion, the F_2 plants (second year) of the cross BR92-15454 x IAC-11 were separated in homozygous resistant, heterozygous resistant and stem canker-susceptible

plants. The statistical analysis in the χ^2 test presented results that confirmed the hypothesis of segregation 1:2:1 ($\chi^2 = 0.6225$ with probability of 73.2478%).

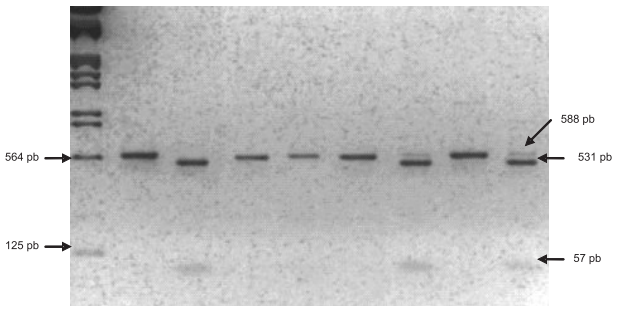

Figure 2. Monomorphic SCAR fragment of 588 pb before and after enzyme digestion (*Hinc* II): 1. Resistant parental BR92-15454; 2. Resistant parental BR92-15454 (digested); 3. Susceptible parental IAC-11; 4. Susceptible parental IAC-11 (digested); 5. F_1 plant; 6. F_1 plant (digested); 7. heterozygous resistant F_2 plant; 8. heterozygous resistant F_2 plant (digested); MS - Molecular standard (phage ë digested with the enzymes *Eco* RI and *Hind* III)

The results obtained by aligning presented significant values (e-value = $3e^{-29}$) in relation to gene accB-2 (Reverdatto et al. 1999) in the region located between the bases 265-357. The region of the polymorphic fragment between the bases 266-357 presented similarities (e-value = $2e^{-18}$) to cDNA expressed in plants attacked by fungus *Fusarium solani* f. sp. *glycines* (Vodkin et al. 2002), supposedly cDNA sequences related to the plant defense mechanisms.

The region between the bases 442-586 (e-value = $3e^{-15}$) and 404-559 (e-value = $8e^{-22}$) were similar to the clones of libraries of large genome inserts (Shultz et al. 2003). Similarities were also observed to cDNA expressed in salicylic acid-treated plants (Tian et al. 2004) (bases 300-350, e-value = $2e^{-11}$; bases 265-310, e-value = $2e^{-08}$; bases 265-325, e-value = $9e^{-08}$). This acid is an activator of the phenylpropanoid pathway, with glyceollin as one of the end products, a compound related to the plant defense mechanism against pathogens. The restriction site for the enzyme *Hinc* II is located in a region of the polymorphic fragment with similarities with clones of cDNA libraries of plants attacked by the fungus *Fusarium solani* f. sp. *glycines* (Shoemaker et al. 2002) were observed.

The SCAR marker developed here could be useful as a tool for assisted selection in the soybean improvement program conducted in Jaboticabal.

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Desenvolvimento de marcador SCAR ligado ao gene de resistência ao cancro da haste em soja

RESUMO - *O cancro da haste causado pelo fungo* Diaporthe phaseolorum *f. sp.* meridionalis *constitui-se em doença limitante para o cultivo da soja. Avaliações fenotípicas visando resistência a doenças envolve processos trabalhosos, como manipulações e transporte de fitopatógenos. A utilização de marcadores de DNA nos processos seletivos facilita certas etapas, além de ser prática, segura e confiável. Identificou-se um fragmento RAPD de 588 pb entre bulks de plantas resistentes e suscetíveis no cruzamento BR92-15454 (R) x IAC-11 (S). Através da co-segregação estimou-se a distância entre o locus da resistência e o fragmento em 7,4±2,1 cM, com* $Lod_{máx.}$ *de 23,072 (primeiro ano) e 6,0±3,4 cM com* $Lod_{máx.}$ *7,806 (segundo ano). O fragmento foi convertido em marcador SCAR, que digerido com a enzima Hinc II, possibilitou a classificação das plantas em resistentes homozigotas, resistentes heterozigotas e suscetíveis. O marcador SCAR desenvolvido poderá ser utilizado dentro do programa de melhoramento desenvolvido em Jaboticabal.*

Palavras-chaves: RAPD, *Glycine max* , *Diaporthe phaseolorum* f. sp. *meridionalis*, seleção assistida.

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