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Identification of a RAPD marker linked to the *Co-6* anthracnose resistant gene in common bean cultivar AB 136

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Abstract

The pathogenic variability of the fungus *Colletotrichum lindemuthianum* represents an obstacle for the creation of resistant common bean (*Phaseolus vulgaris* L.) varieties. Gene pyramiding is an alternative strategy for the development of varieties with durable resistance. RAPD markers have been proposed as a means to facilitate pyramiding of resistance genes without the need for multiple inoculations of the pathogens. The main aims of this work were to define the inheritance pattern of resistance present in common bean cultivar AB 136 in segregating populations derived from crosses with cultivar Rudá (susceptible to most *C. lindemuthianum* races) and to identify RAPD markers linked to anthracnose resistance. The two progenitors, populations F_1 and F_2, $F_{2:3}$ families and backcross-derived plants were inoculated with race 89 of *C. lindemuthianum* under environmentally controlled greenhouse conditions. The results indicate that a single dominant gene, *Co-6*, controls common bean resistance to this race, giving a segregation ratio between resistant and susceptible plants of 3:1 in the F_2, 1:0 in the backcrosses to AB 136 and 1:1 in the backcross to Rudá. The segregation ratio of $F_{2:3}$ families derived from F_2 resistant plants was 1:2 (homozygous to heterozygous resistant). Molecular marker analyses in the F_2 population identified a DNA band of approximately 940 base pairs ($OPAZ20_{940}$), linked in coupling phase at 7.1 cM of the *Co-6* gene. This marker is being used in our backcross breeding program to develop Rudá-derived common bean cultivars resistant to anthracnose and adapted to central Brazil.

INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is the main source of vegetable protein in most Latin American and African countries. The Brazilian population is the world's largest consumer, with an annual average intake of 22-23 kg/person (Ventura and Costa, 1992).

Fungal diseases drastically affect common bean yield, and anthracnose, caused by the fungus *Colletotrichum lindemuthianum* (Sacc. & Magn.) Scrib., is among the main diseases of the common bean in Brazil and in other bean-growing regions of the world (Pastor-Corrales, 1985). This disease may cause yield losses of up to 100% when infected seeds are sown in areas with high relative humidity and mild temperatures (Peloso, 1992).

Although breeding the common bean for resistance to anthracnose by traditional methods has created several resistant cultivars (Singh *et al.*, 1992; Vieira, 1983), new varieties have to be continually developed due to the high pathogenic variability of the fungus (Rava *et al.*, 1994). Gene pyramiding is an alternative strategy for the development of varieties with durable resistance, but traditional breeding procedures are inefficient for such purpose due to the difficulties brought about by the need for multiple inoculations (Michelmore, 1995). Pyramiding of resistance genes assisted by molecular markers has been proposed as an alternative solution for this type of problem (Haley *et al.*, 1993, 1994; Johnson and Gepts, 1994; Johnson *et al.*, 1995; Young and Kelly, 1996a).

A number of common bean lines have been evaluated for resistance to many pathotypes of anthracnose at the Centro Internacional de Agricultura Tropical (CIAT, Cali, Colombia), and among them, cultivar AB 136, selected as one of the 12 international differential cultivars for anthracnose, is one of the most resistant (Pastor-Corrales, 1992). Cultivar AB 136 showed resistance to 25 pathotypes of *C. lindemuthianum* collected in several common bean-growing regions in Brazil (Rava *et al.*, 1994).

Random amplified polymorphic DNA (RAPD) markers (Williams *et al.*, 1990) have been successfully used in common bean breeding programs to tag resistance genes for several important diseases (Haley *et al.*, 1993, 1994; Johnson and Gepts, 1994; Johnson *et al.*, 1995; Young and Kelly, 1996a; Alzate-Marin *et al.*, 1999a). This technique is being used in our common bean breeding program to facilitate the development of isolines containing disease resistance genes of interest.

The main aim of this work was to define the inheritance pattern of resistance present in cultivar AB 136 in segregating populations derived from crosses with cultivar Rudá

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(susceptible to most *C. lindemuthianum* races) and to identify RAPD markers linked to the resistance gene (*Co-6*) present in this cultivar.

MATERIAL AND METHODS

Source of *C. lindemuthianum* isolates and culture conditions

Race 89 of *C. lindemuthianum* was collected in Minas Gerais State (Brazil) and corresponds to race alpha-Brazil (group alpha), part of a group of 25 races collected in different regions of Brazil and identified by Rava *et al.* (1994). The original inoculum was kindly provided by Dr. Carlos A. Rava and Dr. Aloisio Sartorato (CNPAF/EMBRAPA, Goiânia, GO, Brazil). To increase the amount of spores the isolates were cultivated for approximately 10 days in sterile medium containing common bean green pods. To confirm the identity of the isolates, they were inoculated on the 12 bean anthracnose differentials according to the method of Pastor-Corrales (1992).

Genetic material

Seeds from differential cultivar AB 136 and "carioca type" cultivar Rudá were provided by CNPAF/EMBRAPA (Goiânia, GO, Brazil) (Table I). Cultivar AB 136 was used as male progenitor and crossed in a greenhouse with cultivar Rudá. The populations were maintained in the greenhouse.

Genetic analyses and evaluation of disease symptoms

Spores of *C. lindemuthianum* race 89 were inoculated onto plants derived from the cross Rudá x AB 136. The following number of seeds were sowed in the greenhouse in a completely randomized design: 30 seeds from each progenitor, 30 F_1 seeds, 256 F_2 seeds and 60 seeds from each backcross (BC_r and BC_s). Fourteen days after sowing the first expanded trifoliate leaf from each of the 466 plants was inoculated on the lower and upper leaf surfaces with spore suspensions (1.2×10^6 spores/ml), applied with a horse-hair paint brush. The plants were then incubated for seven days in a mist chamber, which was maintained at 20-22ºC and 100% relative humidity, after which the plants were evaluated using a 1- to 9-symptom scale (Rava *et al.*, 1993). Resistant (R) phenotype was assigned to plants with no or limited symptoms (grades 1 to 3), whereas plants graded 4 or greater were considered to be susceptible (S). Before inoculation, primary leaves from all plants were collected and kept at -80ºC for DNA extraction. The phenotypic class frequencies obtained were tested for goodness-of-fit to theorical ratios with chi-square tests.

One hundred and eighty-eight $F_{2:3}$ families derived from F_2 resistant plants of cross Rudá x AB 136, consisting of 12 plants each, were evaluated for resistance/susceptibility to *C. lindemuthianum* race 89. Inoculation conditions and symptom evaluation were performed as before except that in this case spores were sprayed onto the plants with the aid of a De Vilbiss apparatus.

DNA bulks

DNA from homozygous F_2 resistant individuals was used to make two contrasting bulks (resistant/susceptible) according to Michelmore *et al.* (1991). Two hundred and ten primers (Operon Technologies, Alameda, CA, USA) were used to test the bulks.

DNA extraction and amplification

DNA extraction was according to Doyle and Doyle (1990). Amplification reactions were performed in a thermocycler model 9600 (Perkin-Elmer, Norwalk, CT, USA). Each reaction (25 µl) contained: 25 ng DNA, 0.1 mM of each dNTP, 2.0 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.4 µM of one primer decamer (Operon Technologies, Alameda, CA, USA) and one unit of Taq DNA polymerase.

Each amplification cycle consisted of one denaturation step at 94ºC for 15 s, one annealing step at 35ºC for 30 s and one extension step at 72ºC for 1 min. After 40 cycles an extra extension step was performed for 7 min at 72ºC. Amplification products were analyzed on 1.2% agarose gels immersed in TBE buffer (90 mM Tris-borate, 1 mM EDTA, pH 8.0) containing 10 mg/ml ethidium bromide. DNA bands were visualized under UV light and pho-

Table I - Phenotypic characteristics of common bean progenitors Rudá and AB 136.

Progenitor	Growth habit	Seed size	Seed color	Race	Reaction to anthracnose
Rudá	Indeterminate prostrate (III)	Small	*carioca*	Meso-american	Susceptible to race 89
AB 136	Indeterminate (IV)	Small	Red	Meso-american	Resistant to all races identified in Brazil

tographed with the aid of an Eagle Eye II photosystem (Stratagene, La Jolla, CA, USA).

Linkage analyses

Chi-square analysis was used to test the phenotypic segregation of the F_1, F_2, $F_{2:3}$, BC_s and BC_r populations. To determine the genetic distance between the RAPD marker and the resistance gene, 239 F_2 plants were used. The genetic distance between the marker and the resistance gene was determined with the aid of MAP-MAKER III (Lander *et al.*, 1987) using a LOD score minimum of 3.0.

RESULTS AND DISCUSSION

Segregation analyses

Results of inoculations confirmed that cultivar AB 136 is resistant and that cultivar Rudá is susceptible to *C. lindemuthianum* race 89 (Table II) under greenhouse conditions. Rava *et al.* (1994) demonstrated that cultivar AB 136 is resistant to 25 *C. lindemuthianum* races detected in Brazil.

The segregation ratio (resistant:susceptible) of 3:1 in the F_2 and the good fit to the expected (homozygote:heterozygote) ratio in the $F_{2:3}$ families derived from F_2 plants (Table II) indicate that resistance of AB 136 to race 89 is controlled by a single dominant gene. This gene was identified in cultivar Catrachita, a breeding line derived from AB 136 and named *Co-6* (Young and Kelly, 1996b). Similar results were reported in previous work using the same resistance source (Vidigal, 1994; Alzate-Marin *et al.*, 1999a).

Identification of RAPD markers

Co-6 has proven to be effective against a large number of Latin American isolates of *C. lindemuthianum* and it is an important resistance source used by breeding programs in Central America (Pastor-Corrales, 1985, 1992; Young and Kelly, 1996b; Rava *et al.*, 1994). Young and Kelly (1997) identified two RAPD markers linked to, and flanking, the *Co-6* locus in cultivar Catrachita: marker $OPAH1_{780}$ is linked in coupling-phase at 12.3 cM of the gene and marker $OPAK20_{890}$ is linked in repulsion phase at 7.1 cM of the gene. The band produced by $OPAH1_{780}$ is polymorphic between Rudá and AB 136, but in co-segregation analyses in the F_2 population it mapped at 23.7 cM of *Co-6* (Table III, Figure 1).

We previously identified another marker ($OPZ04_{560}$) tightly linked in coupling to *Co-6* (Alzate-Marin *et al.*, 1999a). Using this marker it was possible to confirm that a single gene or a complex locus of tightly linked resistance genes present in cultivar AB 136 confers resistance to *C. lindemuthianum* pathotypes 73, 81, 89, and 64. The $OPZ04_{560}$ marker could be used in a marker-assisted breeding program to pyramid different resistance genes using cultivar AB 136 as a source for anthracnose resistance. In the case of the cross between cultivars Rudá and AB 136, however, $OPZ04_{560}$ was not polymorphic between the progenitors. For this reason it cannot be used in our breeding program aimed at the development of Rudá-derived bean varieties resistant to anthracnose.

One RAPD marker ($OPAZ20_{940}$) was identified in the F_2 plants inoculated with race 89. This marker, a 940-bp DNA band, is linked in coupling phase to the resistance gene

Table II - Segregation for resistance to race 89 of *Colletotrichum lindemuthianum* in the cross Rudá x AB 136.

Pedigree	Generation	No. of Plants		Expected ratio	χ^2	P
		Resistant	Susceptible			
Rudá	P_1	0	29	-	-	-
AB 136	P_2	30	0	-	-	-
Rudá x AB 136	F_1	29	0	-	-	-
Rudá x AB 136	F_2	206	50	3:1	3.79	0.10-0.05
F_1 x Rudá	BCs	17	43	1:1	10.42	0.01-0.00
F_1 x AB 136	BCr	60	0	1:0	0.00	1.00
Rudá x AB 136	$F_{2:3}$	59RR:129Rr	-	1:2	0.08	0.80-0.70

Table III - Linkage analysis between molecular markers and resistance genes in crosses involving cultivar AB 136.

	Locus tested	Expected ratio	Observed ratio	χ^2	P	cM[a]
Rudá x AB 136	*Co-6*[b]/$OPAZ20_{940}$	9:3:3:1	176:13:2:48	149.57	0.00	7.1
Rudá x AB 136	*Co-6*/$OPAH1_{780}$	9:3:3:1	155:34:8:42	84.77	0.00	23.7

[a]Distance in centimorgans in relation to *Co-6*. [b]Resistance gene present in cultivar AB 136.

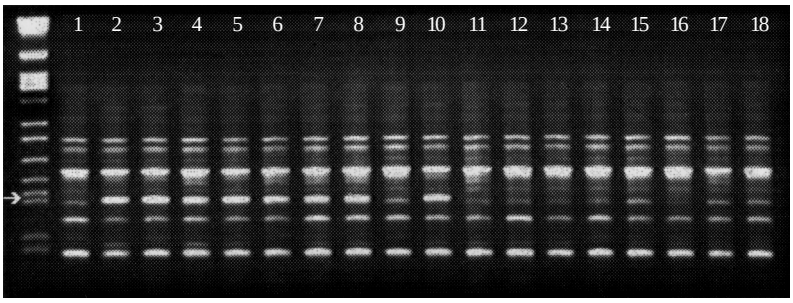

Figure 1 - Electrophoretic analysis of amplification products obtained with primer OPAH1. The first lane corresponds to lambda DNA cut with *Eco*RI, *Bam*HI and *Hin*dIII (size markers). Lanes 1 to 18 are as follows: 1, Rudá; 2, AB 136; 3-10, F_2 plants resistant to race 89; 11-18, F_2 plants susceptible to race 89. The arrow indicates a DNA band of 780 bp linked in coupling phase to the resistance gene.

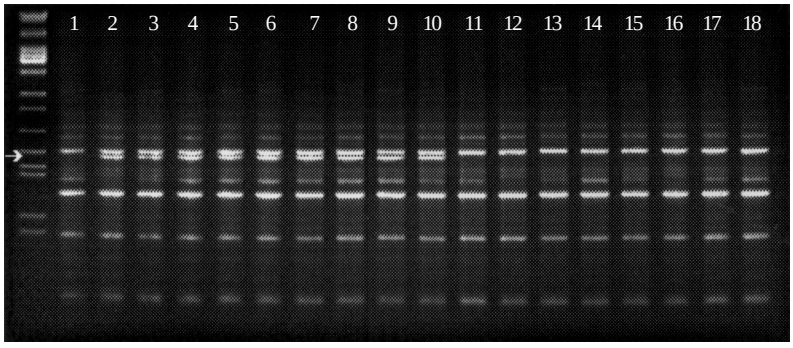

Figure 2 - Electrophoretic analysis of amplification products obtained with primer OPAZ20. The first lane coresponds to lambda DNA cut with *Eco*RI, *Bam*HI and *Hin*dIII (size markers). Lanes 1 to 18 are as follows: 1, Rudá; 2, AB 136; 3-10, F_2 plants resistant to race 89; 11-18, F_2 plants susceptible to race 89. The arrow indicates a DNA band of 940 bp linked in coupling phase to the resistance gene.

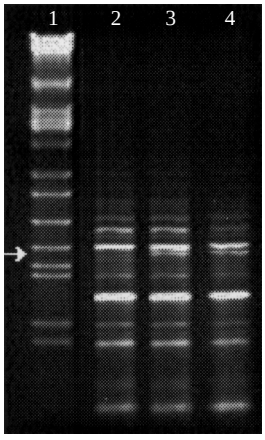

Figure 3 - Electrophoretic analysis of amplification products obtained with primer OPAZ20. Lanes are as follows: 1, lambda DNA cut with *Eco*RI, *Bam*HI and *Hin*dIII (size markers); 2, Rudá; 3, AB 136, and 4, Catrachita. The arrow indicates a DNA band of 940 bp linked in coupling phase to the resistance gene (*Co-6*).

(*Co-6*) (Figure 2). Co-segregation analyses in the F_2 population revealed that marker $OPAZ20_{940}$ was located 7.1 cM from the resistance gene (Table III). The presence of this marker in cultivar Catrachita (Figure 3) strongly supports the idea that the gene studied in this work is the same (*Co-6*) as that reported by Young and Kelly (1996b).

To determine if the other 11 differential cultivars carry the molecular marker OPAZ20 linked to the *Co-6* gene in cultivar AB 136, the DNA of these cultivars was extracted and amplified with the appropriate primers. Only differential cultivar AB 136 possessed the band $OPAZ20_{940}$. The allelism studies conducted so far do not indicate the presence of alternative alleles for *Co-6* in other differential cultivars for anthracnose (Young and Kelly, 1996b).

Marker $OPAZ20_{940}$ is being used to facilitate the development of Rudá-derived isolines containing the gene *Co-6* (Alzate-Marin *et al.*, 1999b), as well as to pyramid different resistance genes in common bean varieties as part of our breeding program toward the creation of commercial cultivars with long-lasting resistance to anthracnose.

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RESUMO

A antracnose do feijoeiro, causada por *Colletotrichum lindemuthianum*, é uma doença que ocasiona severas perdas, estando amplamente distribuída no Brasil. Trabalhos anteriores têm demonstrado que o cultivar AB 136, possuidor do gene *Co-6*, é uma importante fonte de resistência à antracnose no Brasil, uma vez que todos os patótipos identificados no país até o momento são incompatíveis com esse cultivar. O objetivo deste trabalho foi identificar um marcador RAPD ligado ao gene de resistência *Co-6* presente no cultivar AB 136. Para este fim, foi utilizada uma população do cruzamento entre o progenitor Rudá, suscetível à raça 89 de *C. lindemuthianum*, e o cultivar AB 136. Nas populações F_2 e F_3 deste cruzamento observou-se uma segregação de 3 plantas resistentes para 1 suscetível, e 1 planta homozigota para 2 heterozigotas, respectivamente, sugerindo que a resistência é determinada pela ação de um único gene. Na população F_2, foi identificado um marcador, de aproximadamente 940 pb (OPAZ20), ligado em fase de acoplamento a 7,1 cM do gene *Co-6*. Este marcador está sendo utilizado em nosso programa de melhoramento para desenvolver cultivares de feijoeiro derivados de Rudá resistentes à antracnose e adaptados ao Brasil Central.

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