Identification of Random Amplified Polymorphic DNA Markers Linked to the *Co-4* Resistance Gene to *Colletotrichum lindemuthianum* in Common Bean

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ABSTRACT

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New cultivars of the common bean (*Phaseolus vulgaris*) with durable resistance to anthracnose can be developed by pyramiding major resistance genes using marker-assisted selection. To this end, it is necessary to identify sources of resistance and molecular markers tightly linked to the resistance genes. The objectives of this work were to study the inheritance of resistance to anthracnose in the cultivar TO (carrying the *Co-4* gene), to identify random amplified polymorphic DNA (RAPD) markers linked to *Co-4*, and to introgress this gene in the cultivar Rudá. Popu-

lations F₁, F₂, F_{2,3}, BC₁S, and BC₁R from the cross Rudá × TO were inoculated with race 65 of *Colletotrichum lindemuthianum*, causal agent of bean anthracnose. The phenotypic ratios (resistant/susceptible) were 3:1 in the F₂ population, 1:1 in the BC₁S, and 1:0 in the BC₁R, confirming that resistance to anthracnose in the cultivar TO was monogenic and dominant. Six RAPD markers linked to the *Co-4* gene were identified, four in the coupling phase: OPY20_{830C} (0.0 centimorgan [cM]), OPC08_{900C} (9.7 cM), OPI16_{850C} (14.3 cM), and OPJ01_{1,380C} (18.1 cM); and two in the repulsion phase: OPB03_{1,800T} (3.7 cM) and OPA18_{830T} (17.4 cM). OPY20_{830C} and OPB03_{1,800T}, used in association as a codominant pair, allowed the identification of the three genotypic classes with a high degree of confidence. Marker OPY20_{830C}, which is tightly linked to *Co-4*, is being used to assist in breeding for resistance to anthracnose.

Anthracnose, caused by the fungus *Colletotrichum lindemuthianum* (Sacc. & Magn.) Lams.-Scrib., is among the most important diseases of the common bean (*Phaseolus vulgaris* L.) in Brazil and in other growing regions of the world (17). This disease can cause yield losses of up to 100% when infected seed is sown in areas with high relative humidity and mild temperatures (21).

Although breeding for resistance to anthracnose has created several resistant common bean cultivars (27,28), new cultivars have to be developed continuously due to the high degree of pathogenic variability of the fungus (24). Cultivar TO, one of 12 differentials used in the identification of races of the anthracnose fungus in common beans (18), has been shown to be resistant to 22 of 25 *C. lindemuthianum* pathotypes identified in Brazil (24). The differential cv. TO, which originated from the cross between susceptible cv. Tenderette and the Mexican line Acapulco, carries a dominant resistance gene, originally designated *Mexique II* and renamed *Co-4* (5), that is different and independent of other anthracnose resistance genes *Co-1*, *Co-2*, *Co-3*, *Co-5*, and *Co-6* (7,34). The resistance conferred by *Co-4* has proven to be effective against a large number of Latin American isolates of *C. lindemuthianum* and is an important resistance source used by several breeding programs (1,7, 17–20,32,33).

Nelson (16) recommended the use of gene pyramiding as a strategy for developing stable resistance and for preventing the out-

break of new pathotypes of a pathogen. However, traditional breeding procedures are inefficient for pyramiding resistance genes due to the need for multiple inoculations (14). Pyramiding resistance genes using molecular markers should allow a more efficient selection of resistant plants in segregating populations. Random amplified polymorphic DNA (RAPD) markers (30) linked to anthracnose resistance genes are currently being used by several bean-breeding groups (2,8–11,31–33) to aid the development of disease-resistant cultivars. RAPD markers can facilitate the pyramiding of resistance genes into commercial common bean cultivars, especially if they are tightly linked to the locus containing the resistance allele. Unfortunately, RAPD markers are inherited in a dominant fashion, preventing the identification of heterozygous individuals. However, this limitation can be bypassed by using a RAPD marker linked to a resistance allele in the coupling phase in association with another marker linked in the repulsion phase to the same allele (2,8,10,33).

The main goal of this work was to identify RAPD markers linked to gene *Co-4* present in cv. TO using a segregating population derived from the cross Rudá × TO. Rudá is a commercial cultivar with a carioca seed highly acceptable to consumers in Brazil, but susceptible to most *C. lindemuthianum* pathotypes. The information obtained will be used by our breeding program to pyramid several disease-resistance genes into cv. Rudá.

MATERIALS AND METHODS

Source of *C. lindemuthianum* isolates and cultural procedures. Pathotype 65 of *C. lindemuthianum* used in this study was

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collected in the state of Minas Gerais, Brazil. It is part of a group of 25 pathotypes collected in different regions of Brazil and identified by Rava et al. (24). The original inoculum was provided by C. A. Rava and A. Sartorato (Rice and Bean Research Center—EMBRAPA, Goiânia, GO, Brazil). Inoculum was increased by cultivating the fungus for approximately 10 days in sterile medium containing young green pods of common bean (22). The identity of the pathotype was confirmed by inoculation onto the 12 bean anthracnose differential cultivars (18).

Parents, crosses, and seed production. Seed from cvs. Rudá and TO was provided by EMBRAPA (Goiânia, GO, Brazil) and CIAT (Tropical Agriculture International Center, Cali, Colombia), respectively. TO is a Mesoamerican cultivar, determinate, with medium dark beige seeds. Rudá is also a Mesoamerican cultivar, indeterminate, with small carioca-type seeds (26). All crosses were performed in the greenhouse. 'TO' was used as the male parent in all crosses. Backcrosses were made to both 'TO' (BCr) and the susceptible parent (BCs). F₁ plants were selfed to generate F₂ seed. Individual F₂ plants were harvested to generate F_{2:3} families.

Genetic analyses and evaluation of disease symptoms. The following number of seeds was sown in the greenhouse in a completely randomized design: 27 seeds from each parent, 16 F₁ seeds, 171 F₂ seeds, 32 seeds from BCr, and 46 seeds from BCs. Fourteen days after sowing, the first expanded trifoliate leaf from each of the 405 plants were inoculated with spores of *C. lindemuthianum* pathotype 65. Spores (1.2×10^6 conidia per ml) were applied with

a paint brush to one of the primary leaves of 14-day-old plants. The plants were incubated and maintained in a mist chamber (20 to 22°C, 100% relative humidity) for 7 days and then the disease symptoms were scored visually using a scale of 1 to 9 (23), in which 1 = plants with no visible symptoms; 2 = up to 1% of small lesions in midveins on the lower leaf surface; 3 = up to 3% of small lesions in midveins on the lower leaf surface; 4 = up to 1% of lesions present in the midvein on the lower and upper leaf surfaces and occasionally in secondary leaf veins; 5 = up to 3% of small lesions scattered in midveins and secondary veins on both leaf surfaces; 6 = more than 3% of small lesions as described in grade 5 on both leaf surfaces and in the stems and petioles; 7 = many large lesions scattered on both leaf surfaces and many lesions in the stems and petioles; 8 = many large coalesced lesions accompanied by tissue breakdown and chlorotic or abscised leaflets, reduced plant growth, and many lesions in the stems and petioles; and 9 = severely diseased or dead plants.

The resistant (R) phenotype was assigned to plants with no or limited symptoms (grades 1 to 3), whereas plants graded 4 or greater were considered susceptible (S). Before inoculation, the healthy primary leaf from each plant was collected and frozen at -80°C for DNA extraction.

DNA extraction and amplification. DNA extraction was conducted according to Doyle and Doyle (6). Amplification reactions were performed in a thermocycler (model 9600; Perkin-Elmer Corp., Norwalk, CT). Each reaction (25 µl) contained 25 ng of DNA; 0.1 mM of each dNTP; 2.0 mM MgCl₂; 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.4 µM of one primer decamer (Operon Technologies, Inc., Alameda, CA); and one unit of *Taq* DNA polymerase (30).

Each amplification cycle consisted of one denaturation step at 94°C for 15 s, one annealing step at 35°C for 30 s, and one extension step at 72°C for 1 min. After 40 cycles, an extra extension step was performed for 7 min at 72°C. Amplification products were analyzed on 1.2% agarose gels immersed on Tris-borate-EDTA buffer (TBE; 90 mM Tris-borate and 2 mM EDTA, pH 8.0) containing ethidium bromide at 10 mg/ml (30). DNA bands were visualized under UV light and photographed with the Eagle Eye II photosystem (Stratagene Inc., La Jolla, CA).

Identification of RAPD markers. The Bulk Segregant Analysis technique (15) was used to identify RAPD markers linked to the resistance gene or genes for pathotype 65. Two contrasting bulks were prepared, each containing DNA from eight homozygous resistant or susceptible F₂ individuals. The homozygosity of F₂ resistant plants used in the bulks was determined by inoculating all F_{2:3} families (11 to 22 plants each). Inoculation conditions and symptom evaluation were performed as mentioned before; however, in this case, spores were sprayed onto plants with a DeVilbiss No. 15 apparatus (DeVilbiss Co., Somerset, PA). For DNA amplification, 567 different primers were used (Operon Technologies, Inc.).

Linkage analyses. Chi-square analyses were used to test the phenotypic segregation of the populations originating from the

TABLE 1. Segregation for resistance to race 65 of *Colletotrichum lindemuthianum* in the cross Rudá × TO

Pedigree	Generation	No. of plants		Expected ratio	χ^2	P
		Resistant	Susceptible			
Rudá	P ₁	0	27	...	...	...
TO	P ₂	27	0	...	...	...
Rudá × TO	F ₁	16	0	...	...	...
Rudá × TO	F ₂	125	46	3:1	0.3295	56.60
F ₁ × Rudá	BCs	23	23	1:1	0.0870	76.80
F ₁ × TO	BCr	32	0	1:0	0.0000	100.00
Rudá × TO ^a	F _{2:3}	43 RR:82 Rr	46 rr	1:2:1	0.3905	82.30

^a RR corresponds to genotype *Co4 Co4*, Rr to *Co4 co4*, and rr to *co4 co4*.

Fig. 1. Relative positions of random amplified polymorphic DNA markers linked to gene *Co-4* identified in a segregating population derived from the cross Rudá × TO.

TABLE 2. Segregation analyses of random amplified polymorphic DNA OPY20_{830C}, OPC08_{900C}, OPJ01_{1380C}, OPI16_{850C}, OPB03_{1800T}, and OPA18_{830T} markers and the *Co-4* gene in the F₂ and F_{2:3} populations in the cross Rudá × TO

Locus tested	Generation	Observed ratio	Expected ratio	χ^2	P	cM ^a
<i>Co-4</i>	F ₂	125:46	3:1	0.3295	56.60	...
<i>Co-4</i>	F _{2:3}	43:82:46	1:2:1	0.3905	82.30	...
OPY20 _{830C} ^b	F ₂	125:46	3:1	0.3295	56.60	0.0 ± 1.1
OPC08 _{900C}	F ₂	123:48	3:1	0.8596	35.40	9.7 ± 2.1
OPI16 _{850C}	F ₂	120:51	3:1	2.1228	14.51	14.3 ± 2.5
OPJ01 _{1380C}	F ₂	123:48	3:1	0.8596	35.40	18.1 ± 2.8
OPB03 _{1800T} ^c	F ₂	126:45	3:1	0.1579	69.10	3.7 ± 1.5
OPA18 _{830T}	F ₂	120:51	3:1	2.1228	14.51	17.4 ± 2.7

^a Distance in centimorgans in relation to *Co-4* (resistance gene).

^b The 830-bp molecular marker linked in the coupling phase (*cis*) to the *Co-4* gene.

^c The 1,800-bp molecular marker linked in the repulsion phase (*trans*) to the *Co-4* gene.

TABLE 3. Amplification of DNA from differential cultivars for common bean anthracnose with random amplified polymorphic DNA primers flanking markers linked to the resistance gene *Co-4*

Molecular marker	Differential cultivars ^a											
	1	2	3	4	5	6	7	8	9	10	11	12
OPY20 _{830C}	–	–	–	–	–	–	–	+	+	–	–	+
OPC08 _{900C}	+	–	+	+	+	–	+	+	+	–	+	–
OPJ01 _{1,380C}	–	–	–	–	–	–	+	+	+	–	+	+
OPB03 _{1,800T}	–	–	–	–	–	–	–	–	–	–	–	–
OPI16 _{850C}	+	–	–	–	–	–	+	+	+	+	–	–
OPA18 _{830T}	–	–	–	–	–	–	–	–	–	–	–	–

^a 1 = ‘Michelite’, 2 = ‘MDKR’, 3 = ‘Perry Marrow’, 4 = ‘Cornell 49-242’, 5 = ‘Widusa’, 6 = ‘Kaboon’, 7 = ‘México 222’, 8 = ‘PI 207262’, 9 = ‘TO’, 10 = ‘TU’, 11 = ‘AB136’, and 12 = ‘G 2333’; – = band absent and + = band present.

TABLE 4. Selection efficiency for resistance to *Colletotrichum lindemuthianum* pathotype 65 in 171 F₂ individuals from the cross Rudá × TO based on molecular markers

Phenotype	Genotype ^a	Selection ^b			Selection efficiency (%)		
		OPY20 _{830C} ^c	OPB03 _{1,800T} ^d	OPY20/OPB03	OPY20	OPB03	OPY20/OPB03 ^e
Resistant plants	RR:43 Rr:82	RR:43 (+) Rr:82 (+)	RR:43(–) Rr:80 (+) Rr:2 (–) ^f	RR:43 (+/–) Rr:80 (+/+) Rr:2 (+/–) ^f	R _– :100	RR:95	RR:100 Rr:98
Recombinant plants							
Susceptible plants	rr:46	rr:46 (–)	rr:46 (+)	rr:46 (–/+)	rr:100		rr:100

^a Observed ratios in F₂ plants based on corresponding F_{2,3} families. RR corresponds to *Co4 Co4*, Rr to *Co4 co4*, and rr to *co4 co4* individuals.

^b + = band present, – = band absent, and +/- = marker OPY20_{830C} present and marker OPB03_{1,800T} absent.

^c Coupling.

^d Repulsion.

^e Selection efficiency with combined use of markers in the coupling and repulsion phases to the *Co-4* gene.

^f Resistant individuals Rr (–) or Rr (+/–) are recombinants.

cross Rudá × TO and to determine possible linkages between the RAPD marker and the resistance gene. Genetic distances between the markers and the resistance gene were determined with the aid of the program MAP-MAKER III (12), using a log of the likelihood ratio (LOD) score minimum of 3.0.

Selection efficiency analyses. The efficiency of RAPD markers to identify resistant or susceptible plants and to determine the genotypic constitution of the resistance locus was determined in the F₂ plants based on phenotypic data derived from the respective F_{2,3} families.

RESULTS AND DISCUSSION

Cv. TO resistance to *C. lindemuthianum* race 65. Resistance of cv. TO to race 65 appears to be controlled by a single dominant gene. Chi-square values revealed that a good fit was obtained for the segregation ratio of 3:1 in the F₂, 1:2:1 in the F_{2,3}, 1:1 when the F₁ was backcrossed to ‘Rudá’, and 1:0 when the F₁ was backcrossed to ‘TO’ (Table 1). These results support previous work (4,7, 13,20,28,34).

Identification of RAPD markers linked to *Co-4*. The following RAPD markers were linked in the coupling phase to the *Co-4* gene in the F₂ population: OPY20_{830C} (0.0 ± 1.1 centimorgans [cM]), OPC08_{900C} (9.7 ± 2.1 cM), OPI16_{850C} (14.3 ± 2.5 cM), and OPJ01_{1,380C} (18.1 ± 2.8 cM). Two RAPD markers linked in the repulsion phase to the same gene were also identified: OPB03_{1,800T} (3.7 ± 1.5 cM) and OPA18_{830T} (17.4 ± 2.7 cM). The positions of these markers in relation to gene *Co-4* is shown in Figure 1. The segregation analyses for each marker is shown in Table 2. This is the first report of molecular markers tightly linked to the *Co-4* gene present in cv. TO.

DNA was extracted and amplified with the appropriate primers (Table 3) to determine if the other 11 differential cultivars carry the molecular markers linked to the *Co-4* gene in cv. TO. Differential cvs. PI 207262 and G 2333 possess the band OPY20_{830C}, which is tightly linked to *Co-4*. The presence of this marker in ‘G 2333’ reinforces the finding that this cultivar carries an allele of the *Co-4* gene that was designated *Co-4*² (34). ‘PI 207262’ affords resistance levels greater than ‘TO’ (24); therefore, two hypotheses can be raised: ‘PI 207262’ carries more than one anthracnose resis-

tance gene or it carries a different allele at the *Co-4* locus (34). Published data show that ‘PI 207262’ carries duplicated dominant genes for resistance to *C. lindemuthianum* race delta and that only one gene confers resistance to race kappa (29). On the other hand, if ‘PI 207262’ carries an allele at the *Co-4* locus, this allele would be different from *Co-4*². This is suggested by the different resistance indexes (RI) between Selection 1308 (RI = 97), derived from ‘G 2333’, and ‘PI 207262’ (RI = 79) (3). Marker OPC08_{900C} (9.7 ± 2.1 cM) was present in most differential cultivars, both Andean and Mesoamerican, suggesting that these cultivars might carry different alleles from gene *Co-4*. Although this marker is relatively distant from the *Co-4* locus, it has been recently detected in the bean line P45, a Brazilian line derived from cv. TO (25).

Selection efficiency. The simultaneous use of RAPD markers in the coupling and repulsion phases to the same gene allows the identification of the three genotypic classes (2,33). Markers OPY20_{830C} and OPB03_{1,800T}, linked to the *Co-4* gene in the coupling and repulsion phases, respectively, were used for indirect selection of resistant plants as individual markers and as a codominant pair.

The phenotypic and genotypic data of 171 F₂ plants derived from the cross Rudá × TO were obtained by direct analyses of the F₂ plants and F_{2,3}-derived families and compared with those obtained with the two markers (Table 4). The selection efficiency for resistant and susceptible plants was 100% with marker OPY20_{830C}. This is certainly due to its close linkage to locus *Co-4*. RAPD marker OPY20_{830C} would be very effective identifying phenotypes of resistant and susceptible plants. This marker would be most useful for screening advanced generation (F₆ or later) lines that are nearly homozygous. The phenotypic efficiency selection of resistant homozygous plants with marker OPB03_{1,800T} (identified by the absence of a band) was 95%. However, OPB03_{1,800T} cannot distinguish between heterozygous resistant and susceptible plants. This marker would be most useful to breeders making selections in early generation populations in which one would expect a high frequency of heterozygous plants. The combined use of markers OPY20_{830C} and OPB03_{1,800T} as a codominant pair allowed the identification of homozygous and heterozygous resistant plants with selection efficiencies of 100% and 98%, respectively (Table 4). Our results validate the use of RAPD markers linked in the repulsion and cou-

Fig. 2. Electrophoretic analysis of amplification products obtained with primers **A**, OPY20 and **B**, OPB03. Lane 1, lambda DNA digested with *Eco*RI, *Bam*HI, and *Hind*III (size markers); lane 2, 'Rudá'; lane 3, 'TO'; lanes 11, 16, 18, 20, 21, and 24, homozygous dominant F_2 plants resistant to *Colletotrichum lindemuthianum* pathotype 65 (R); lanes 4–6, 8, 10, 12–15, 17, 19, 22, 23, 25, and 26, heterozygous F_2 plants resistant to pathotype 65 (H); and lanes 7 and 9, homozygous recessive F_2 plants susceptible to pathotype 65 (S). The arrows indicate markers OPY20_{830C} and OPB03_{1,800T} linked to *Co-4* in the **A**, coupling and **B**, repulsion phase, respectively.

pling phases to the gene of interest as a codominant pair to predict specific genotypes in segregating populations (2,8,9,33). DNA amplification patterns of 26 F_2 individuals analyzed simultaneously with markers OPY20_{830C} and OPB03_{1,800T} are shown in Figure 2.

Due to the wide use of the *Co-4* gene as a source for resistance to anthracnose in Brazil (24) and in other parts of the world (17), the identification of markers tightly linked to this major gene is an important contribution for breeding programs interesting in pyramiding resistance genes to anthracnose in common beans.

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