Improved Selection with Newly Identified RAPD Markers Linked to Resistance Gene to Four Pathotypes of *Colletotrichum lindemuthianum* in Common Bean

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ABSTRACT

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Three F₂ populations derived from crosses between the resistant cultivar AB 136 and the susceptible cultivar Michelite (MiA), and one F₂ population derived from a cross between AB 136 and Mexico 222 (MeA), were used to identify markers linked to anthracnose resistance genes present in cultivar AB 136. Primer OPZ04 produced a DNA band (OPZ04₅₆₀) linked in coupling phase to the resistance gene for pathotype 89 (8.5 ± 0.025 cM) in one population derived from the cross MiA. In the

same population, primer OPZ09 produced one band (OPZ09₉₅₀) linked in repulsion phase (20.4 ± 0.014 cM) to the same resistance gene. The simultaneous use of markers in coupling and in repulsion phases allowed the identification of the three genotypic classes. In the other two populations from cross MiA, OPZ04₅₆₀ was linked in coupling phase to resistance genes for pathotypes 73 (2.9 ± 0.012 cM) and 81 (2.8 ± 0.017 cM). In population MeA, OPZ04₅₆₀ was linked in coupling phase (7.5 ± 0.033 cM) to resistance to pathotype 64. These data suggest that a single gene or complex locus of linked resistance genes present in cultivar AB 136 confers resistance to all four pathotypes of *C. lindemuthianum*.

Additional keywords: MAS, *Phaseolus vulgaris*.

Anthracnose, caused by the fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) 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 (12). This disease may cause yield losses of up to 100% when infected seeds are sown in areas with high relative humidity and mild temperatures (14).

Although common bean breeding for resistance to anthracnose by traditional methods has created several resistant cultivars (19,21), new cultivars have to be developed continuously due to the high degree of pathogenic variability of the fungus (17). Cultivar AB 136, one of the 12 differentials for anthracnose in common beans, has been shown to be resistant to all 25 races of *C. lindemuthianum* identified in Brazil. Vidigal (20) suggested that a single gene confers anthracnose resistance in AB 136 to pathotypes delta and capa. Populations derived from crosses with Catrachita, a resistant breeding line derived from AB 136, produced similar results when inoculated with pathotypes 23, 64, and 73 (24). Recently, 3:1 ratios were obtained in F₂ populations from the crosses MeA and MiA when inoculated with pathotypes 64 and 89 (1). These results suggest that a single gene, designated *Co-6*, governs the anthracnose resistance of AB 136. However, not all pathotypes of *C. lindemuthianum* have been tested, and recent findings suggest that more than one gene may be involved in the expression of resistance, depending on the parent crossed with AB 136 (1). For this reason, it would be interesting to challenge populations de-

rived from crosses involving AB 136 with different anthracnose pathotypes and to determine the mode of inheritance of resistance to these pathotypes.

Nelson (11) recommended the use of gene pyramiding as strategy for developing stable resistance and for preventing the outbreak of new pathotypes of a pathogen. However, traditional breeding procedures are inefficient for pyramiding resistance genes due to the need for multiple inoculations (9). Pyramiding resistance genes using molecular markers should permit a more efficient selection of disease-resistant beans (3–6,23,25). Random amplified polymorphic DNA (RAPD) markers (22) could facilitate the pyramiding of resistance genes into commercial cultivars of common bean. Markers that are close to the locus containing the resistance allele are more useful. Marker-assisted selection (22) has been successfully used in common bean breeding to screen bean lines for resistance to several important diseases (3–7,23,25). Unfortunately, RAPD markers are inherited in a dominant fashion, preventing the identification of heterozygous individuals (22). However, this limitation can be bypassed by using an RAPD marker linked to a resistance allele in coupling phase in association with another marker linked in repulsion phase to the same allele (3,5,25).

The identification of a specific molecular marker linked to a resistance gene for different pathotypes of a pathogen, in populations which have one resistant parent in common, may be useful as evidence for allelism. For instance, it was possible to use molecular markers to confirm that the single dominant resistance gene in Catrachita (*Co-6*) is the same resistance gene present in AB 136 (24). It was also possible to confirm that gene *Mexique 3* (*Co-5*) from cultivar TU is one of the three resistance genes proposed for G 2333, one of the anthracnose differential cultivars (26).

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The main goal of this work was to identify RAPD markers linked to the gene(s) present in cultivar AB 136 that confers resistance to *C. lindemuthianum* pathotypes 64 and 89. Another objective was to study the possible linkage between these RAPD markers and resistance to pathotypes 73 and 81. This information will facilitate pyramiding of resistance genes from cultivar AB 136 into cultivars susceptible to the four *C. lindemuthianum* pathotypes.

MATERIALS AND METHODS

Source of *C. lindemuthianum* isolates and cultural procedure. Characteristics of the four *C. lindemuthianum* pathotypes used in this work are listed in Table 1. They are part of a group of 25 pathotypes collected in different regions of Brazil and identified by Rava et al. (17). The original inoculum was kindly provided by Carlos A. Rava and Aloisio Sartorato (EMBRAPA Arroz e Feijão, Goiânia, GO, Brazil). Inoculum of the isolates was increased by cultivating the fungus for approximately 10 days in sterile medium containing young green pods of common bean (15). The identity of the isolates was confirmed by inoculation onto the 12 bean anthracnose differentials cultivars (13).

Common bean genetic materials, crosses, and seed production. Seed from differential cultivars Michelite, Mexico 222, and AB 136 were provided by EMBRAPA (Goiânia, GO, Brazil). Characteristics of these cultivars are summarized in Table 2. Cultivar AB 136 was used as male parent in crosses with Michelite and Mexico 222. All populations were grown in the greenhouse.

Genetic analyses and evaluation of disease symptoms. Three F₂ populations derived from the cross Michelite × AB 136 (MiA) consisted of 81, 84, and 171 plants; and one F₂ population from the cross Mexico 222 × AB 136 (MeA) consisted of 190 plants. The plants from each of the three populations from cross MiA were inoculated separately with *C. lindemuthianum* pathotypes 73, 81, and 89, respectively. The population MeA was inoculated with pathotype 64. Spores (1.2 × 10⁶ conidia per ml) were applied with a paint brush to one primary leaf on 14-day-old plants. The plants were incubated in a mist chamber (20 to 22°C, 100% relative humidity) for 7 days, and disease symptoms were scored visually using a 1 to 9 scale (16) 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 mid- 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 stems and petioles; and 9 = severely diseased or dead plants.

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, leaves from all plants were collected and frozen at -80°C for DNA extraction.

DNA extraction and amplification. DNA extraction was conducted according to Doyle and Doyle (2). Amplification reactions were performed in a thermocycler (Model 9600, Perkin-Elmer, Norwalk, CT). Each reaction (25 µl) contained: 25 ng of DNA, 0.1 mM 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, Alameda, CA), and one unit of *Taq* DNA polymerase (22).

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 TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0) containing 10 mg of ethidium bromide per ml (22). DNA bands were visualized under UV light and photographed with the Eagle Eye II photosystem (Stratagene, La Jolla, CA).

Identification of RAPD markers. The Bulk Segregant Analysis technique (10) was used to identify RAPD markers linked to a resistance gene(s) for pathotype 89 in population MiA. Homozygosity of F₂ resistant plants, to be used in the bulks, was determined by inoculating 27 F_{2:3} families (20 plants each). Inoculation conditions and symptom evaluation were performed as mentioned before; however, in this case, spores were sprayed onto plants with a De Vilbiss no. 15 apparatus. Based on these results, two contrasting bulks were prepared, each containing DNA from 10 resistant or susceptible F₂ individuals.

Preliminary amplification of DNA samples from each of the three progenitors with 980 different primers identified 374 that could reveal polymorphisms (data not shown). The MiA F₂ populations and the MeA population were inoculated with pathotypes 73, 81, and 64, in order to verify if the RAPD marker linked to resistance to pathotype 89 was also linked to resistance to these three pathotypes.

Linkage analyses. Chi-square analyses were used to test the phenotypic segregation of the F₂ populations and to determine possible linkages between the RAPD marker and the resistance gene(s). For this purpose, DNA samples of 81, 84, and 117 F₂ plants from cross MiA, and of 79 plants from cross MeA, were amplified with primers revealing specific RAPD markers. Genetic distances between the markers and the resistance gene(s) were determined with the aid of the program MAP-MAKER III (8), using a LOD score minimum of 3.0.

Selection efficiency analyses. The efficiency of using RAPD markers for the identification of resistant/susceptible plants was determined by analyzing phenotypic data obtained for the 117 F₂ plants from cross MiA inoculated with pathotype 89. The 27 F_{2:3} families derived from F₂ resistant plants from this cross were used

TABLE 1. *Colletotrichum lindemuthianum* pathotype identification and origins^a

Pathotype (binary nomenclature)	Group/pathotype (classic nomenclature)	Origin (Brazilian state)
64	Mexican I/Mexican I	Espirito Santo
73	Alpha/Alpha Brazil	Espirito Santo
81	Alpha/Eta	Pernambuco
89	Alpha/Alpha Brazil	Minas Gerais

^a Rava et al. (17).

TABLE 2. Phenotypic characteristics of the parental cultivars Michelite, Mexico 222, and AB 136

Progenitor	Growth habit	Seed size ^a (100 seed weight in g)	Seed color	Gene pool	Reaction to anthracnose
Michelite	Indeterminate weak-stemmed (III)	Small (19.65)	White	Middle-American	Susceptible to pathotypes 73, 81, and 89
México 222	Determinate (I)	Medium (34.66)	White	Durango	Susceptible to pathotype 64
AB 136	Indeterminate (IV)	Medium (27.23)	Red	Middle-American	Resistant to pathotypes 64, 73, 81, and 89

^a Singh et al. (18).

to test the selection efficiency of the simultaneous use of markers as a “codominant” marker.

RESULTS

Segregation analyses and identification of RAPD markers linked to resistance to *C. lindemuthianum* in cultivar AB 136. Segregation analyses in the F₂ populations derived from MiA crosses revealed a 3:1 ratio suggesting the presence of a single dominant resistance gene for *C. lindemuthianum* pathotype 89 (Table 3) in these crosses. A total of 374 primers that revealed polymorphic bands between the parental cultivars were used to test the two contrasting DNA bulks. Two RAPD markers were identified in these bulks. One marker was linked in coupling phase to the resistance gene, a 560-bp fragment designated OPZ04₅₆₀ amplified with primer OPZ04 (AGGCTGTGCT); the other was linked in repulsion phase, a 950-bp fragment designated OPZ09₉₅₀ amplified with primer OPZ09 (CACCCCAGTC) (Figs. 1 and 2). Cosegregation analyses revealed that marker OPZ04₅₆₀ was located at 8.5 ± 0.025 cM from the resistance gene in population MiA inoculated with *C. lindemuthianum* pathotype 89. Marker OPZ09₉₅₀ was located at 20.4 ± 0.014 cM from the resistance gene in population MiA inoculated with pathotype 89 (Table 3).

Cosegregation analysis among the RAPD markers and the resistance gene is described in Table 3. Chi-square analyses strongly suggest that both RAPD markers are linked to the resistance gene present in cultivar AB 136 (Table 3).

Linkage between OPZ04₅₆₀ marker and the resistance gene for *C. lindemuthianum* pathotypes 64, 73, and 81. In the F₂ populations that were inoculated with *C. lindemuthianum* pathotypes 64, 73, and 81, the phenotypic ratio between resistant and susceptible plants was 3:1, suggesting that resistance to these pathotypes is governed by a single gene (Table 3). Marker OPZ04₅₆₀ was also tested in these populations inoculated with pathotypes 64, 73, and 81. In the F₂ population from the cross MeA inoculated with pathotype 64, primer OPZ04 also mapped a 560-bp band in coupling phase at 7.5 ± 0.033 cM from the resistance gene. In the F₂ population inoculated with pathotype 73, marker OPZ04₅₆₀ mapped in coupling phase at 2.9 ± 0.012 cM from the resistance gene. In the population inoculated with pathotype 81, marker OPZ04₅₆₀ mapped in coupling phase at 2.8 ± 0.017 cM from the resistance gene (Table 3).

DISCUSSION

Among the 12 differential cultivars for anthracnose (13), cultivar AB 136 has resistance to all *C. lindemuthianum* pathotypes

identified in Brazil (17). A single dominant gene named *Co-6* was identified in cultivar Catrachita, a breeding line derived from AB 136 (24). The genetic resistance conferred by *Co-6* has proven to be effective against a large number of Latin American isolates of *C. lindemuthianum* and is an important resistance source used by breeding programs in Central America (1,12,13,24,25).

Phenotypic analysis of F₂ populations derived from crosses between AB 136 and cultivars susceptible to *C. lindemuthianum* pathotypes 73, 81, and 89 (Michelite) and 64 (Mexico 222) demonstrates that a single dominant gene (*Co-6*) confers resistance to these pathotypes. Our data support the findings of Young and Kelly (24) and elucidate the inheritance mode of AB 136 resistance to two additional pathotypes of *C. lindemuthianum* (81 and 89).

Due to the wide use of AB 136 as a source for resistance to anthracnose in Brazil (17) and other parts of the world (12), the identification of markers tightly linked to *C. lindemuthianum* re-

Fig. 1. Electrophoretic analysis of amplification products obtained with primer OPZ04₅₆₀. Lanes are as follows: 1, lambda DNA cut with *Eco*RI, *Bam*HI, and *Hind*III (size markers); 2, Michelite; 3, AB 136; 4 to 10, F₂ plants resistant to pathotype 89; 11 to 17, F₂ plants susceptible to pathotype 89. The arrow indicates a DNA band linked in coupling phase to the resistance gene.

Fig. 2. Electrophoretic analysis of amplification products obtained with primer OPZ09₉₅₀. Lanes are as follows: 1, lambda DNA cut with *Eco*RI, *Bam*HI, and *Hind*III (size markers); 2, Michelite; 3, AB 136; 4 to 10, F₂ plants resistant to pathotype 89; 11 to 17, F₂ plants susceptible to pathotype 89. The arrow indicates a DNA band linked in repulsion phase to the resistance gene.

TABLE 3. Inheritance study and linkage analysis between molecular markers and resistance genes in crosses involving cultivar AB 136

Population	Pathotype	Locus tested (<i>Co-6</i>)	Expected ratio (F ₂)	Observed ratio	χ ²	P	cM ^a (±SE)
Mexico222 × AB136	64	<i>Co-6</i> ^b	3:1 ^c	145:45	0.175	0.70	—
Michelite × AB136	73	<i>Co-6</i>	3:1	63:18	0.27	0.70	—
Michelite × AB136	81	<i>Co-6</i>	3:1	67:17	0.90	0.50	—
Michelite × AB136	89	<i>Co-6</i>	3:1	129:42	0.031	0.90	—
Mexico222 × AB136	64	<i>Co-6</i> /OPZ04	9:3:3:1 ^d	50:3:4:22	12.82	0.00 ^e	7.5 ± 0.033
Michelite × AB136	73	<i>Co-6</i> /OPZ04	9:3:3:1	63: 0:1:17	63.23	0.00	2.9 ± 0.012
Michelite × AB136	81	<i>Co-6</i> /OPZ04	9:3:3:1	65:2:0:17	60.70	0.00	2.8 ± 0.017
Michelite × AB136	89	<i>Co-6</i> /OPZ04/OPZ09 ^f	27:9:9:9:3:3:3:1 ^g	62:19:4:0:2:3:27:0	30.36	0.00	8.5 ± 0.025 20.4 ± 0.014

^a Distance in centimorgans in relation to *Co-6* (resistance gene).
^b Resistance gene present in AB 136 cultivar.
^c Ratio expected for a single gene governing resistance.
^d Ratio expected if *Co-6* and two markers (OPZ04₅₆₀ and OPZ09₉₅₀) are independently inherited (27R++:9R+--:9R+-:3R---:3r++:3r+-:1r--). The symbols ++ represent bands present and linked to markers OPZ04₅₆₀ and OPZ09₉₅₀, respectively. The symbols --- represent bands absent for the same markers, respectively.
^e The markers and gene analyzed (*Co-6*) are not segregating independently.
^f OPZ04₅₆₀/OPZ09₉₅₀ are molecular markers linked in coupling and repulsion phases, respectively, to the resistance gene *Co-6*.
^g Ratio expected if *Co-6* and two markers (OPZ04₅₆₀ and OPZ09₉₅₀) are independently inherited (27R++:9R+--:9R+-:3R---:3r++:3r+-:1r--). The symbols ++ represent bands present and linked to markers OPZ04₅₆₀ and OPZ09₉₅₀, respectively. The symbols --- represent bands absent for the same markers, respectively.

sistance gene *Co-6* is an important contribution for breeding programs pyramiding resistance genes to anthracnose in common beans. Two RAPD markers were reported to be linked to and flanking the *Co-6* locus: OAH1₇₈₀ linked in coupling phase at 12.3 ± 1.9 cM and OAK20₈₉₀ linked in repulsion phase at 7.3 ± 1.5 cM (25). We have been able to identify two other RAPD markers, OPZ04₅₆₀ and OPZ09₉₅₀, linked to *Co-6* in distinct populations challenged by pathotypes different from those tested by Young and Kelly (25). In populations inoculated with pathotypes 73 and 81, marker OPZ04₅₆₀ was shown to be around 2.8 cM from the resistance gene (Table 3). This marker is more useful in breeding programs, because it is closer to *Co-6* than the marker previously identified by Young and Kelly (25). Besides, it is important to consider that one marker detected in one population involving one specific resistant parent is not necessarily polymorphic in other populations, even if the same resistant parent is used. In our case, for instance, primer OAH1₇₈₀ identified by Young and Kelly (25) did not express polymorphisms in crosses between cultivar AB 136 and susceptible cultivars Michelite and Mexico 222. OPZ04₅₆₀ was present in Catrachita (data not shown), which suggests that the marker could be used in crosses with this cultivar.

Using OPZ04₅₆₀, we showed 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, and 89 in population MiA and 64 in population MeA. This confirms previous results for other populations and pathotypes (24).

This information can be used in breeding programs using marker assisted selection (MAS) to pyramid anthracnose resistance genes in common bean varieties. The availability of a molecular marker tightly linked to *Co-6* can save a considerable amount of time and avoid laborious inoculation procedures when screening lines for resistance.

In the MiA F₂ population inoculated with *C. lindemuthianum* pathotype 89, it was possible to identify markers OPZ04₅₆₀ and OPZ09₉₅₀ linked to *Co-6* in coupling and repulsion phases, respectively. Selec-

tion efficiency based on markers OPZ04₅₆₀/OPZ09₉₅₀ as a “codominant” marker to identify resistant plants within the MiA F₂ population was 95%. In the case of selection against marker OPZ09₉₅₀ for resistant plants, the efficiency dropped to 22%. Despite this low efficiency, it is highly probable that the individuals that do not harbor this marker are homozygous and resistant, and individuals with this marker are heterozygous and resistant. Selection efficiency for susceptible plants using these two markers as a “codominant” marker was 84% (Table 4).

Analyses of F_{2:3} populations showed that selection efficiencies for resistance for the *Co-6Co-6* genotype were 100, 73, and 73% using, respectively, the marker linked in coupling, the marker linked in repulsion, and the two types of markers simultaneously (Table 5). Although selection efficiencies based on the marker linked in repulsion only and on the pair linked in coupling and repulsion were the same, use of the RAPD markers as a codominant marker allows the precise identification of the plant genotypes (Figs. 1 and 2) and the discarding of double recombinants (25).

Selection of plants resistant to common bean mosaic virus (3), rust (5), and anthracnose (25) based on the simultaneous use of markers linked in coupling and repulsion phases to the resistance gene has been shown to be efficient. Selection efficiency of *Co-6Co-6* resistant plants was reported to be 95% with the simultaneous use of markers OAH1₇₈₀ and OAK20₈₉₀ linked in coupling and repulsion phases to *Co-6*, respectively (25). This value dropped to 33 and 92% when only the marker linked in coupling phase or the marker in repulsion phase was used, respectively (25).

The selection efficiency of *Co-6Co-6* resistant plants in this study using marker OPZ04₅₆₀ was higher than that obtained with marker OAH1₇₈₀ (25), possibly because marker OPZ04₅₆₀ is more tightly linked to *Co-6*. Selection efficiency of *Co-6Co-6* resistant plants using OPZ04₅₆₀/OPZ09₉₅₀ (this study) and OAH1₇₈₀/OAK20₈₉₀ (25) markers showed the importance of the simultaneous use of two markers for selection and identification of genotypes in segregating populations. The differences in the selection efficiencies between

TABLE 4. Selection efficiency for resistance to pathotype 89 in 117 F₂ individuals from cross Michelite × AB 136, based on molecular markers

Phenotype	Observed frequency ^a	Selection OPZ04 ₍₅₆₀₎ (coupling)	Selection OPZ09 ₍₉₅₀₎ (repulsion)	Selection OPZ04/OPZ09	Selection efficiency OPZ04/OPZ09 (%) ^b
Resistant plants	R ₋ :85.0	R ₋ :81.0 (+) ^c	RR:19.0 (–) ^d Rr:66.0 (+)	RR:19.0 (+/–) Rr:62.0 (+/+)	R ₋ (95)
Recombinants		[R ₋ : 4.0 (–)] ^e		[R ₋ : 4.0 (–/+)] ^e	
Susceptible plants	rr:32.0	rr:27.0 (–)	r ₋ :29.0 (+)	rr:27.0 (–/+)	Rr (84)
Recombinants		[rr: 5.0 (+)] ^e	[rr: 3.0 (–)] ^f	[rr: 2.0 (+/+)] ^e [rr: 3.0 (+/–)] ^f	

^a Observed frequency 3:1 (R₋, rr). RR corresponds to *Co6 Co6*, R₋: *Co6*_, Rr: *Co6 co6*, and rr: *co6 co6* individuals.

^b Selection efficiency using two markers in the nonrecombinant individuals. The selection efficiency for RR or Rr individuals is only possible using information of F_{2:3} families.

^c Band present.

^d Band absent.

^e Resistant (– or –/+) and susceptible (+ or +/+) individuals are recombinant using coupling and coupling/repulsion markers, respectively.

^f Susceptible individuals (– and +/–) are recombinant and double recombinant, respectively.

TABLE 5. Selection efficiency^a for resistance to pathotype 89 in 540 plants of 27 F_{2:3} families from cross Michelite × AB 136 based on molecular markers

Phenotype	Observed F _{2:3} ^c	Selection OPZ04 ₍₅₆₀₎ (coupling)	Selection OPZ09 ₍₉₅₀₎ (repulsion)	Selection OPZ04/OPZ09	Selection efficiency ^b (%)		
					OPZ04	OPZ09	OPZ04/OPZ09
Resistant plants	RR: 11 Rr: 16	R ₋ :27	RR:8 (–) ^d Rr:15 (+) ^e	RR:8 (+/–) Rr:15 (+/+)	R ₋ :100	RR:73 Rr:94	RR:73 Rr:94
Recombinants			[Rr:1 (–) RR:3 (+)] ^f	[Rr:1 (+/–) RR:3 (+/+)] ^g			

^a Selection efficiency in F₂ generation.

^b Observed ratio in resistant families F_{2:3}. RR correspond to *Co6 Co6*, R₋: *Co6*_, and Rr: *Co6 co6* individuals.

^c Using two markers in the nonrecombinant individuals.

^d Band absent.

^e Band present.

^f Resistant RR (+) or Rr (–) individuals are recombinant, respectively.

^g Resistant Rr (+/–) or RR (+/+) individuals are recombinant, respectively.

the two studies are probably due to the different distances between locus *Co-6* and the markers identified.

Identification of one marker in repulsion phase to the resistance gene depends on the correct phenotypic evaluation of the F_2 plants from the resistant bulk according to the segregation of each $F_{2,3}$ family. The presence of many heterozygous individuals in the resistant bulk may interfere with the proper identification of this type of marker (Fig. 2, lanes 5 and 8). This problem can be avoided using advanced recombinant inbred lines (RILs) if they are available.

Molecular markers not only have proved to be an important tool for the identification of resistant individuals but also help to confirm the number of genes that confer resistance to any pathotypes of *C. lindemuthianum*. This type of information is essential to pyramid resistance genes for the most important pathotypes of *C. lindemuthianum*.

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