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Genetic mapping of the resistance allele *Co-5²* to *Colletotrichum lindemuthianum* in the common bean MSU 7-1 line

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Abstract

Anthracnose is a devastating fungal disease of common bean (*Phaseolus vulgaris* L.), the causal agent of which is *Colletotrichum lindemuthianum*. MSU 7-1 is a common bean breeding line that contains the *Co-5²* and *Co-7* anthracnose resistance genes. In this study, phenotypic analyses were conducted in an F_2 population derived from the Mexico 222 (S) × MSU 7-1 (R) cross that was inoculated with race 64 of *C. lindemuthianum*. Segregation analysis revealed a 3R:1S ratio, indicating that only *Co-5²* confers resistance to race 64 once the *Co-7* gene has been defeated by this race. The molecular marker $g1233_{3250}$, in linkage group Pv07, was linked in coupling phase to *Co-5²* at a distance of 1.2 cM. The presence or absence of this marker was also determined in the G 2333, TU, H1 and PI 207262 cultivars. It was found that the $g1233_{3250}$ molecular marker was present in G 2333 and absent in the other cultivars. Because the allele and marker are physically linked in a *cis* configuration, the *Co-5²* resistance allele present in MSU 7-1 and G 2333 cultivars can be monitored with great efficiency using $g1233_{3250}$. These results will be very useful for breeding programs aimed at developing anthracnose-resistant bean cultivars via marker-assisted selection.

Keywords: Anthracnose, genetic resistance, linkage mapping, molecular markers, *Phaseolus vulgaris* L.

Introduction

The common bean (*Phaseolus vulgaris* L.) is the most widely grown grain legume used for direct human consumption and is highly favored in many parts of Africa, Latin America and southern Europe (Broughton et al., 2003). Anthracnose, which is caused by the fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara, is one of the most hazardous diseases of common bean. This fungal disease can cause severe yield losses, and its development depends mostly on the susceptibility of the particular cultivar involved and environmental conditions favorable to fungal growth and spread (Pastor-Corrales, 1992; Mendéz-Vigo et al., 2005; Genchev et al., 2010). Host resistance is the most cost-effective strategy for controlling anthracnose in the common bean. Disease-resistant common bean cultivars are easily adopted by farmers and do not pose environmental risks (Gonçalves-Vidigal et al., 2013).

Currently, the 21 anthracnose resistance loci are identified using the *Co* symbol (Kelly and Vallejo, 2004), and there are four allelic series that confer resistance to *C. lindemuthianum.* Some of these alleles have been mapped in the common bean genome and are currently widely used in common bean breeding programs (Kelly and Vallejo, 2004; Gonçalves-Vidigal et al., 2012). On the common bean map, most of these genes are allocated to 11 linkage groups, referred to as Pv groups.

According to Balardin et al. (1997), the *Co-5* gene has a wide resistance spectrum and confers resistance to 31 races of *C. lindemuthianum*. This resistance allele was first reported in the differential cultivar TU by Fouilloux (1976) and was designated *Mexique 3*. In 1998, Young and collaborators detected the *Co-5* gene in the differential cultivar G 2333, and Vallejo and Kelly (2009) subsequently confirmed the presence of this gene in MSU 7-1. Later studies conducted by these authors concluded that the *Co-5* allele present in G 2333 is actually a different allele, which was designated as *Co-5²*.

The *Co-7* resistance allele was first described in the differential cultivar G 2333 (Young et al., 1998) and was the third independent anthracnose resistance gene to be identified in this cultivar. The *Co-7* gene was distinguished from other anthracnose resistance genes through analyses carried out by Young et al. (1998), in which several tests involving marker-assisted selection and inoculation with different races of *C. lindemuthianum* were applied. In these tests, individuals were screened using race 521 (Hd 16.1 from Honduras) to identify resistant lines that likely possessed the *Co-7* gene, as race 521 defeats the *Co-5* gene, while the *Co-7* gene confers resistance to this race. Pereira and Santos (2004) have also reported the presence of *Co-7* in line H1, which is derived from a CI 140 (susceptible) × [ESAL 696 (*Co-5*) × G 2333 (*Co-4², Co-5, Co-7*)] cross and is susceptible to races 2047 and 73 but resistant to race 1545, thus confirming the presence of *Co-7*.

Line MSU 7-1 was derived from a Black Magic × SEL 111 cross, and it has been shown to exhibit a wide spectrum of resistance to *C. lindemuthianum* races 7, 9, 23, 55, 64, 65, 73, 89, 448 and 453 (Gonçalves-Vidigal et al., 2008, 2009;

Vallejo and Kelly, 2009). It is known that MSU 7-1 possesses a resistance gene at the *Co-5* locus. However, it is not clear whether the *Co-5* allele present in MSU 7-1 corresponds to the resistance gene found in TU. Therefore, the objectives of this work were to utilize molecular marker and segregation analyses in order to verify whether the allele found in MSU 7-1 is *Co-5* or *Co-5*^{2} and to identify an efficient molecular marker associated with the resistance allele present in MSU 7-1.

Results and Discussion

Genetic resistance

Phenotypic evaluation of the 90 F_2 individuals from the Mexico 222 × MSU 7-1 cross inoculated with *C. lindemuthianum* race 64 revealed a segregation pattern of 69 resistant plants and 21 susceptible plants (χ^2 = 0.13; p = 0.71), thus fitting a 3R:1S ratio. This result indicates the presence of a single dominant allele, *Co-5*^{2}, in MSU 7-1 that confers resistance to race 64 once the *Co-7* locus has been overcome by this race. The $F_{2:3}$ families derived from the Mexico 222 × MSU 7-1 cross were also evaluated for resistance to *C. lindemuthianum* race 64. The segregation pattern observed in this case fit a 1RR:2Rr:1rr ratio. This result is in agreement with the hypothesis that a single dominant allele confers resistance to race 64.

Previous studies have revealed that the *Co-5* gene is present in the TU cultivar (Fouilloux, 1976). In 1998, Young and collaborators also identified a *Co-5* gene in the differential cultivar G 2333, and Vallejo and Kelly (2009) subsequently confirmed the presence of this gene in MSU 7-1. Later studies performed by these authors revealed that the *Co-5* allele present in G 2333 is actually a different allele, which was then designated *Co-5*^{2}. Therefore, the data obtained in the present work confirm that the monogenic segregation of resistance to *C. lindemuthianum* race 64 is conferred by the *Co-5*^{2} allele in MSU 7-1 (Vallejo and Kelly, 2009). A previous study involving the differential cultivars TU, G 2333 and SEL 1360 revealed the presence of the *Co-5* allele in these cultivars (Young and Kelly, 1996). In the course of the present study, we identified the same *Co-5*^{2} allele in MSU 7-1 and G 2333.

Linkage analysis

As illustrated in Fig 1, molecular analyses showed that the g1233 marker amplified a 3,250-bp fragment that was present in the resistant parent and in all five resistant plants from the F_2 population. The *Co-5*^{2} resistance allele and the g1233 marker co-segregated in the F_2 population from Mexico 222 × MSU 7-1 at a ratio of 68 (+):22 (-), indicating a good fit to the expected 3:1 ratio (χ^2 = 0.0148; p = 0.90). The g1233 marker was linked in coupling phase to *Co-5*^{2} at a distance of 1.2 cM within linkage group Pv07 on the common bean consensus map.

The g1233 marker was also tested in the cultivars TU (*Co-5*), G 2333 (*Co-4*^{2}, *Co-5*^{2}, *Co-7*), H1 (*Co-7*) and PI 207262 (*Co-4*^{3} and *Co-3*/*Co-9*). The obtained results revealed the presence of the 3,250 bp g1233 amplicon in G 2333. However, this marker was absent in the Mexico 222, TU, H1 and PI 207262 lines. These data suggest that the anthracnose resistance exhibited by MSU 7-1 is dominant, monogenic and conferred by the resistance allele *Co-5*^{2} (Fig 2.).

The BAT 93/Jalo EEP 558 (BJ) recombinant inbred line (RIL) population was assayed with the g1233 marker and displayed a segregation ratio of 39 (+):35 (-) (χ^2= 0.22; p = 0.64), corresponding to a 1:1 ratio. This result confirmed the linkage of this marker with the *Co-5*^{2} resistance allele in this mapping population. Therefore, according to the literature discussed above and earlier studies, a map was constructed using MapChart software (Voorrips 2002) and the common bean consensus map, employing the appropriate nomenclature. The obtained results allowed the *Co-5*^{2} resistance allele to be positioned within Pv07 (Fig 3.).

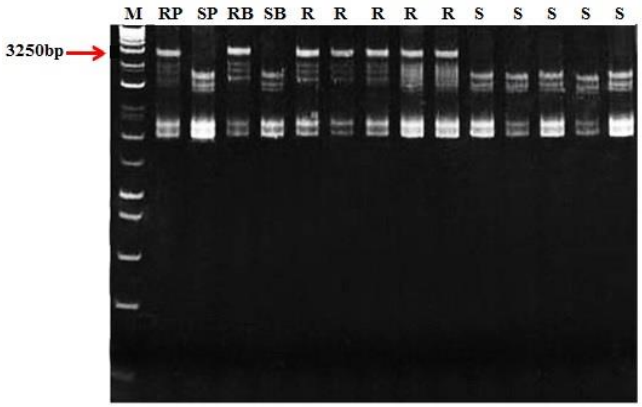

Fig 1. Electrophoretic analysis of the amplification products obtained with the g1233 marker. Lanes: M, 1-kb ladder; RP, MSU 7-1; SP, Mexico 222; RB, resistance bulk; SB, susceptible bulk; R, individuals resistant to *C. lindemuthianum*; S, individuals susceptible to *C. lindemuthianum*. The arrow indicates the 3250-bp amplification product linked to the *Co-5*^{2} resistance allele.

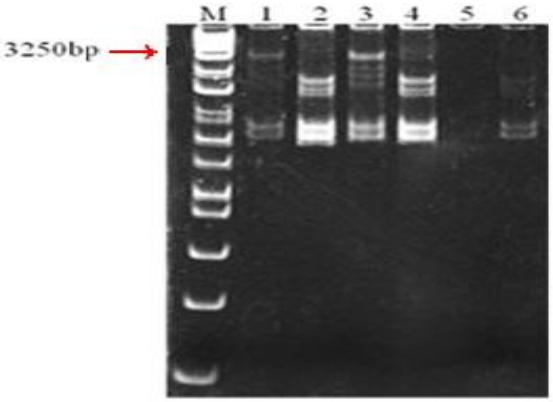

Fig 2. Electrophoretic analysis of the amplification products obtained with the g1233 marker. Lanes: M, 1-kb ladder; 1, MSU 7-1; 2, Mexico 222; 3, G 2333; 4, TU; 5, H1; 6, PI 207262. The arrow indicates the 3250-bp amplification product linked to the *Co-5*^{2} resistance allele.

The results of molecular analysis revealed that the g1233 marker was tightly linked to the *Co-5*^{2} allele (1.2 cM), thus confirming the presence of this allele in MSU 7-1 and demonstrating that it is the same *Co-5*^{2} allele identified in G 2333, as reported by Vallejo and Kelly (2009).

By observing the relationship between the pathogen and host, it was possible to infer that the resistance conferred by the *Co-5* locus would extend to races that carry the same number of virulence alleles, which display a genotypic constitution value of 2^9 = 512 (Robinson, 1987). Additionally, Sousa et al. (2009) conducted studies on F_2 populations from a G 2333 × MSU 7-1 cross that were inoculated with *C. lindemuthianum* race 7 to determine the number of genes segregating with anthracnose resistance. The obtained results suggested allelism between MSU 7-1

and G 2333 (*Co-5*^{2}) because these lines carry resistance alleles at the same locus.

Similar results were obtained when the F_3 families from a H1 (*Co-7*) × Mexico 222 (*Co-3*) cross were inoculated with races 8 and 65 of *C. lindemuthianum* (Lima et al., 2008). The results revealed that families possessing the *Co-7* gene were susceptible and were overcome by races with virulence allele 64 (2^6), which is similar to the spectrum of resistance observed in Mexico 222. The same authors found that the resistance conferred by the *Co-7* gene was not overcome by virulence allele 1 or 8; however, the resistance allele was defeated by race 64. Furthermore, allelism studies conducted by Gonçalves-Vidigal et al. (2008) on the MSU 7-1 and Jalo Vermelho cultivars and by Sousa et al. (2009) on the MSU 7-1 and Mexico 222 cultivars showed that a single gene confers resistance to *C. lindemuthianum* races 64 and 65 in MSU 7-1.

An examination of the inheritance of resistance and allelism tests conducted in MSU 7-1 by Sousa et al. (2009) demonstrated that MSU 7-1 contains two alleles that confer resistance to race 7 of *C. lindemuthianum*. If MSU 7-1 does in fact possess two alleles that confer resistance to anthracnose, both races 64 and 65 of *C. lindemuthianum* would be expected to overcome one of these resistance alleles, most likely *Co-7*. Through allelism tests, Sousa et al. observed that the alleles present in MSU 7-1 are independent of *Co-1*^{5}, *Co-2*, *Co-6*, *Co-10*, *Co-11*, *Co-12* and *Co-13*. The lack of segregation observed in the F_2 populations derived from the crosses between MSU 7-1 and the cultivars Mexico 222, PI 207262, G 2333 or H1 suggested that the *Co-7* allele found in MSU 7-1 is allelic to *Co-3* (Therefore, the *Co-7* in MSU 7-1 should be renamed as *Co-3*^{5}). Previous results have demonstrated that the resistance conferred by the *Co-3*^{5} gene in MSU 7-1 can be overcome by races 64, 65 and 73 of *C. lindemuthianum* (Alzate-Marin et al., 2000; Gonçalves-Vidigal et al., 2008; Sousa et al., 2009; Vallejo and Kelly, 2009).

Mahuku and Riascos (2004) observed that the *Co-5* allele present in the differential cultivar TU confers resistance to races 3481 and 3545 of *C. lindemuthianum*. However, the same tests conducted with the differential cultivar G 2333 revealed that this cultivar is susceptible to these races, indicating that the gene found in G 2333 is different from the one present in TU (*Co-5*). Vallejo and Kelly (2009) suggested that the *Co-5* allele found in SEL 1360 and G 2333 is distinct from that found in TU, as TU is resistant to race 3481, whereas SEL 1360 is susceptible.

The *Co-5* gene has been used by breeders to develop gene pyramids combining two *Phaseolus vulgaris* gene pools: Mesoamerican and Andean (Young and Kelly, 1997). The use of pyramided genes conferring resistance against the races that are predominant in a region is a breeding strategy that is utilized to improve the durability of major resistance genes that combat pathogens with high genetic variability (Miklas et al., 2006).

The common bean (2n = 2x = 22) consensus map is organized into 11 chromosomes (Nodari et al., 1993; Freyre et al., 1998; Pedrosa Harand et al., 2008). Gepts (1999) and Kelly et al. (2003) reviewed the development of integrated consensus maps of the 11 chromosomes in both the common bean and the cowpea (*Vigna unguiculata* L. Walp). These authors reported the map locations of major resistance genes against bean rust, ANT, common bacterial blight and white mold in gene clusters on chromosomes Pv01, Pv04, Pv07 and Pv11 in common bean.

The Pv07 linkage group includes the locus involved in the genetic control of phaseolin as well as two lectin genes that are not linked to phaseolin (Campa et al., 2011), loci coding

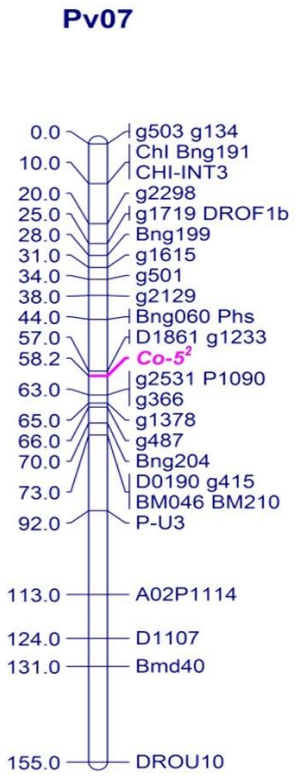

Fig 3. Genetic distance and location of the *Co-5*^{2} allele, which confers resistance to common bean anthracnose, and the g1233 molecular marker within linkage group Pv07 of *Phaseolus vulgaris* L. using the population obtained from the Mexico 222 × MSU 7-1 cross. The map was constructed with MapChart (Voorrips 2002).

for seed proteins, seed coat color (*Asp* and *P* genes) (Pérez-Vega et al., 2010, 2012) and various genes conferring resistance to different pathogens, including *C. lindemuthianum*, *Bct* (Beet curly top virus), *Agm-* (*Apion godmani*-bean weevil) and *Ur-12* (*Uromyces appendiculatus*) (Garza et al., 1996; Young and Kelly, 1996; Miklas et al., 2000, 2002; Kelly et al., 2003; Blair et al., 2006; Geffroy et al., 2009, Pérez-Vega et al., 2010, 2012). Additionally, Pv07 contains a cluster of genes conferring specific resistance to races 3, 6, 7, 31, 38, 39, 102 and 449 of *C. lindemuthianum* (Campa et al., 2009).

Young and Kelly (1997), Castanheira et al. (1999) and Vallejo and Kelly (2001) identified three molecular makers linked to the *Co-5* locus in coupling phase. Due to the difficulty in amplifying the RAPD marker $OAB3_{450}$ in the BJ RIL mapping population, a SCAR marker ($SAB3_{400}$) was developed by Vallejo and Kelly (2001). Additionally, the $SAB3_{400}$ marker is linked to the *Co-5* locus at a distance of 12.98 cM, and Campa et al. (2005) localized the *Co-5* locus to Pv07.

The *Co-5* resistance locus may correspond to the anthracnose resistance cluster located within Pv07. The Mesoamerican cultivars TU, SEL 1360, G2333 and AB136 were found to carry genes associated with resistance to different races of *C. lindemuthianum* that map near the center of Pv07. Within the Pv07 group, there is an anthracnose resistance cluster that confers specific resistance to different races of *C. lindemuthianum* in the Mesoamerican genotypes TU, SEL1360, G2333 and AB136 (Campa et al., 2007, 2009).

Another locus situated within Pv07 is *Co-6*, which was identified in the differential cultivar AB 136 and was described by Schwartz et al. (1982). The molecular marker $OPZ04_{560}$ was mapped in Pv07, which was later converted to SCAR $Z04_{567}$ linked in repulsion phase with *Co-6* gene (Freyre et al., 1998; Alzate-Marin et al., 1999a, b; Mendéz-Vigo et al., 2002; Kelly et al., 2003; Queiroz et al., 2004).

Campa et al. (2007) identified a resistance gene specific to race 81 of *C. lindemuthianum* in $F_{2:3}$ families derived from a Michelite × AB 136 cross. However, these authors also found a second dominant gene or cluster in the AB 136 cultivar that was independent of the *Co-6* locus, conferred resistance to races 357 and 453 of *C. lindemuthianum* and was located within Pv07 in the common bean consensus map. The independent segregation of the cluster and the resistance marker present in TU, $OAK20_{890}$ (distance of 7.3 cM), confirmed that the *Co-5* anthracnose resistance locus is independent of *Co-6* (Young and Kelly 1996; Campa et al., 2009).

Materials and Methods

Plant materials

This work was conducted in a greenhouse and at the Laboratório de Biotecnologia do Núcleo de Pesquisa Aplicada á Agricultura (Nupagri) of the Universidade Estadual de Maringá, Paraná, Brazil. A seed sample of line MSU 7-1 was kindly provided by Dr. James D. Kelly (Department of Plant, Soil and Microbial Sciences, Michigan State University). The seeds of the Mexico 222 cultivar used in this study were provided by the Germplasm Bank of Nupagri.

Parental and F_1 seeds derived from each cross were planted in pots with soil that was previously sterilized and fertilized. The pots were kept in the greenhouse until pod maturation, and F_2 seeds were harvested. The F_2 seeds were sown in trays containing soil. Plants were maintained in the greenhouse until the first trifoliate leaves were fully developed.

Genetic crosses and segregation analysis

The genetic and molecular analyses were carried out in the F_2 population and in $F_{2:3}$ families derived from a cross between cultivars Mexico 222 (susceptible to race 64) and MSU 7-1 (resistant to race 64). Mexico 222 was used as the female parent, and MSU 7-1 served as the male parent. The dominant violet flower trait, which was inherited from the resistant male parent (MSU 7-1), was observed in the F_1 plants, thus establishing that they were hybrids. The F_1 seeds were sown in polyethylene vases (48 × 30 × 11 cm) containing a previously fertilized and sterilized substrate mixture. The plants were maintained in a greenhouse until F_2 seeds were produced. For inoculation, a total of 90 F_2 seeds derived from this cross and 15 seeds from each of the parents were sown in plastic trays (50 × 30 × 9 cm) containing a peat-based substrate. The seedlings were maintained in a greenhouse until the first trifoliate leaves were fully expanded at stage V3 (Gepts, 1987). The first trifoliate leaf of each parent and of each F_2 plant was then individually collected and stored in a freezer at -21ºC for subsequent DNA extraction.

Inheritance test

The parental plants and 90 F_2 individuals were inoculated with *C. lindemuthianum* race 64. Among the 90 inoculated F_2 individuals, a set of 69 plants were found to be resistant and were grown in a greenhouse. The $F_{2:3}$ plants were obtained by selfing each of the remaining F_2 individuals. Resistance of the F_2 plants to *C. lindemuthianum* race 64 was inferred from the phenotypes of the corresponding $F_{2:3}$ families

Inoculation

The identity of race 64 was confirmed through an inoculation test performed using 12 seedlings from each of 12 differential cultivars according to the methodology proposed by Pastor-Corrales (1991). Inocula were prepared following the method described by Cárdenas et al. (1964), which involves multiplying the spore pathotype of *C. lindemuthianum* in tubes containing sterile young green common bean pod medium. The tubes were kept in a BOD incubator at 22°C for 14 days. The *C. lindemuthianum* spore suspension was adjusted to 1.2×10^6 conidia mL^{-1}.

The *C. lindemuthianum* inoculum was prepared with distilled and sterile water to obtain an adjusted suspension of up to 1.2×10^6 spores mL^{-1}. This suspension was spread onto the seedlings using a DeVilbiss atomizer (n° 15) powered by an electric compressor. The inoculated plants were placed in a mist chamber for 72 h and maintained at 20± 2°C with >95% relative humidity under a 12-h photoperiod (light intensity of 300 μmol/m^{-2} per s at 1 m height). Ten days after inoculation, visual evaluations were performed using a scale from 1 to 9; a score of 1-3 indicates resistant plants, and a score of 4-9 indicates susceptible plants (Pastor-Corrales et al., 1995).

Molecular marker analyses

The fully expanded trifoliate leaf tissue samples collected from the parents and 90 F_2 individuals derived from the Mexico 222 × MSU 7-1 cross were stored in a -20°C freezer until DNA extraction. Genomic DNA was extracted from the F_2 population and the parents using the methodology proposed by Afanador et al. (1993) with the following modification: The DNA was extracted using 400 μL of hexadecyl trimethyl ammonium bromide (CTAB) extraction buffer. Bulked segregant analysis was performed to identify DNA markers linked to the *Co-5* allele in the F_2 population derived from the Mexico 222 × MSU 7-1 cross. Equal amounts of DNA from five homozygous resistant (RR) F_2 plants were combined to form the R DNA bulk, while DNA from five susceptible F_2 plants were combined to form the S DNA bulk (Michelmore et al., 1991). Characterization of the R and S plants was based on the $F_{2:3}$ phenotypic data.

Amplifications were performed in a thermal cycler (model TC-412), and the polymerase chain reaction (PCR) program used for all the STS molecular markers consisted of 3 min at 95°C followed by 35 cycles of 30 s at 92°C, 1 min at 50°C and 1 min at 72°C, with a final 5 min extension at 72°C and a subsequent decrease to 4°C for 4 min. PCR was performed in a total reaction volume of 21 μL containing 40 ng of DNA, 0.2 mM each dNTP, a standard *Taq* buffer containing 1.5 mM $MgCl_2$, 0.2 μM forward and reverse primers and 1 unit of *Taq* DNA polymerase. The PCR products were analyzed on 6% polyacrylamide gels stained with SYBR® Safe (0.02%). DNA bands were visualized under ultraviolet light, and digital images were recorded with an L-PIX Image EX model (Loccus Biotecnologia-Locus do Brazil, Cotia, SP, Brazil).

Molecular mapping

Among the screened markers (g1175, g1233, g1378, g2416, g2459, g2531 and SAB3) located within the Pv07 linkage

group in the common bean consensus map (McConnell et al., 2010), only the g1233 STS marker was polymorphic in the parental plants as well as in resistant bulks derived from five F_2 homozygous resistant plants and susceptible bulks derived from five F_2 susceptible plants. The g1233 molecular marker was analyzed in the BAT93/Jalo EEP558 RILs (BJ: 71 lines; Freyre et al., 1998) and in the F_2 population from the Mexico 222 × MSU 7-1 cross. The primer sequences used to determine the segregation pattern of the g1233 marker were as follows: TGAAGGTGGATGTACAGGAAGACA (forward) and TACCTTCATTGGCTTGGTCAGCTA (reverse) (McConnell et al., 2010). These sequences are also provided in the PhaseolusGenes database (http://phaseolusgenes.bioinformatics.ucdavis.edu/markers/766).

Statistical analyses

Segregation analyses of resistance phenotypes were performed using χ^2 tests according to a Mendelian segregation hypothesis of 3 R (resistant) to 1 rr (susceptible). A goodness-of-fit test for a 1:1 segregation ratio was conducted to analyze the segregation pattern of the g1233 marker in the BJ population. Linkage analyses were carried out using Mapmaker/Exp 3.0 software (Lincoln and Lander, 1993) to estimate genetic distances between the g1233 marker and the *Co-5* allele in the F_2 population derived from the Mexico 222 × MSU 7-1 cross.

A minimum likelihood of odds (LOD) score of ≥ 3.0 and a maximum distance of 30 cM were used to test the linkages between these markers (Freyre et a., 1998). The cluster containing *Co-5* and g1233 was localized to Pv07 according to standardized common bean linkage map nomenclature (Pedrosa-Harand et al., 2008). The distance between the loci under investigation was determined by converting the recombination rate to cM using the Kosambi mapping distance (Kosambi, 1944). Additionally, a map was generated using MapChart software (Voorrips, 2002).

Conclusion

In conclusion, the results obtained in this work and in previous studies suggest that the resistance allele present in MSU 7-1 is homologous to the *Co-5*^{2} allele found in G 2333. The linkage between the $g1233_{3250}$ marker and the *Co-5*^{2} allele identified in MSU 7-1 will be of great importance for marker-assisted introgression of this gene into commercial cultivars and elite cultivars, which could expand the resistance spectrum of common bean cultivars. Thus, the $g1233_{3250}$ marker may become an efficient tool for use in bean breeding programs.

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