Co-segregation analysis and mapping of the anthracnose *Co-10* and angular leaf spot *Phg-ON* disease-resistance genes in the common bean cultivar Ouro Negro

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Abstract Anthracnose (ANT) and angular leaf spot (ALS) are devastating diseases of common bean (*Phaseolus vulgaris* L.). Ouro Negro is a highly productive common bean cultivar, which contains the *Co-10* and *Phg-ON* genes for resistance to ANT and ALS, respectively. In this study, we performed a genetic co-segregation analysis of resistance to ANT and ALS using an F_2 population from the Rudá × Ouro Negro cross and the $F_{2:3}$ families from the AND 277 × Ouro Negro cross. Ouro Negro is resistant to races 7 and 73 of the ANT and race 63-39 of the ALS pathogens. Conversely, cultivars AND 277 and Rudá are susceptible to races 7 and 73 of ANT, respectively. Both cultivars are susceptible to race 63-39 of ALS. Co-segregation analysis revealed that *Co-10* and *Phg-ON* were inherited together, conferring resistance to races 7 and 73 of ANT and race 63-39 of ALS. The *Co-10* and *Phg-ON* genes co-segregated and were tightly linked at a distance of 0.0 cM on chromosome Pv04. The molecular marker g2303 was linked to *Co-10* and *Phg-ON* at a distance of 0.0 cM. Because of their physical linkage in a *cis* configuration, the *Co-10* and *Phg-ON* resistance alleles are inherited together and can be monitored with great efficiency using g2303. The close linkage between the *Co-10* and *Phg-ON* genes and prior evidence are consistent with the existence of a resistance gene cluster at one end of chromosome Pv04, which also contains the *Co-3* locus and ANT resistance quantitative trait loci. These results will be very useful for breeding programs aimed at developing bean cultivars with ANT and ALS resistance using marker-assisted selection.

Keywords: Co-segregation analysis, linkage mapping, common bean, anthracnose, angular leaf spot

Introduction

The common bean (*Phaseolus vulgaris* L.) is a very important human food, particularly in many countries of the Americas and eastern and southern Africa. In these countries, the common bean is recognized as an especially important source of protein, complex carbohydrates, fiber, and minerals in the diet of millions of individuals (Broughton et al. 2003; Gepts et al. 2008). Moreover, the common bean is the most widely cultivated species of the genus *Phaseolus* and accounts for approximately 95% of the world's *Phaseolus* bean production. However, this crop is affected by several biotic, edaphic, and climatic factors that decrease its yields (Schwartz and Pastor-Corrales 1989). Among the biotic factors, diseases can cause severe yield losses and reduce the quality of dry and snap beans worldwide (Singh and Schwartz 2010). Anthracnose (ANT), caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib., and angular leaf spot (ALS), caused by *Pseudocercospora griseola* (Sacc.) Crous and Braun (Crous et al. 2006), are among the most widespread diseases of the common bean in the tropics. Both ANT and ALS are widespread in Brazil and eastern and southern Africa, causing crop losses of up to 100% due to ANT and up to 70% due to ALS (Correa-Victoria et al. 1989; Pastor-Corrales and Tu 1989; Singh and Schwartz 2010; Mahuku et al. 2009, 2011; Oblessuc et al. 2012). The pathogens that cause ANT and ALS are characterized by extensive diversity in their virulence; each pathogen has many virulent strains, which are known as races.

Host resistance is the most cost-effective strategy for controlling ANT and ALS in the common bean. Disease-resistant common bean cultivars are easily adopted by farmers and do not generate environmental risks. Resistance to ANT and ALS is conditioned primarily by single dominant genes. Fourteen genes for resistance to ANT with the *Co* designation have been identified (Kelly and Vallejo 2004; Gonçalves-Vidigal et al. 2008, 2009, 2011, 2012a). Similarly, seven independent dominant genes identified by the *Phg* symbol have been reported to confer resistance to *P. griseola* (Caixeta et al. 2003, 2005; Gonçalves-Vidigal et al. 2011; Mahuku et al. 2009, 2011). However, the names of many of these genes have not been submitted to the Bean Improvement Cooperative Genetics Committee (http://bic.css.msu.edu/Genetics.cfm). Ouro Negro is a black-seeded Mesoamerican cultivar derived from the CIAT accession G 3680, also known as Honduras 35. This cultivar, which was introduced in Brazil in 1991, is highly productive and exhibits desirable agronomic and cooking characteristics

(Alzate-Marin et al. 2003; Souza et al. 2011). In Brazil, Ouro Negro is very resistant to specific races of ANT, ALS, and rust and exhibits excellent morpho-agronomic traits. Thus, this cultivar has been recommended by the National Agricultural Research System (SNPA) for cultivation in several Brazilian states (Alzate-Marin et al. 2004).

Previous studies have identified the disease-resistance genes *Co-10* (ANT resistance), *Phg-ON* (ALS resistance), and *Ur-14* (rust resistance) in Ouro Negro. These three genes are considered very important for common bean breeding programs in Brazil (Alzate-Marin et al. 2003; Souza et al. 2011). Ouro Negro confers resistance to *C. lindemuthianum* races 7, 9, 23, 55, 64, 67, 73, 79, 81, 83, 87, 89, 95, 102, 117, 119, 343, 453, 1033, 1545, and 1600 and to *P. griseola* races 31-31, 31-55, 63-39, 63-47, 31-23, 63-55, and 63-63 (Faleiro et al. 2001; Alzate-Marin et al. 2003; Gonçalves-Vidigal and Kelly 2006; Gonçalves-Vidigal et al. 2009; Ragagnin et al. 2009). Thus, Ouro Negro is an important source of disease resistance in the bean breeding projects of Brazil and has been used to develop ANT-resistant dry bean cultivars (Alzate-Marin et al. 2003; Souza et al. 2011).

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 cowpea (*Vigna unguiculata* L. Walp). These authors reported the map locations of major resistance genes to bean rust, ANT, common bacterial blight, and white mold in gene clusters on chromosomes Pv01, Pv04, Pv07, and Pv11 in the common bean. Clusters of *Co*-ANT and *Ur*-rust resistance genes have been reported on Pv01, Pv04, and Pv011 (Geffroy et al. 1999; Miklas et al. 2002; Kelly and Vallejo 2004; Miklas et al. 2006; Geffroy et al. 2009). Among these, the Pv04 cluster, in which the *Co-10* (ANT) resistance gene resides, contains a large number of genes that confer resistance to various other common bean pathogens. The ANT resistance genes included in the Pv04 cluster are *Co-y*, *Co-z*, and *Co-15* of Andean origin and *Co-R*$_{VI}$, *Co-3* (previously also *Co-9*), and *Co-10* of Mesoamerican origin (Adam-Blondon et al. 1994; Geffroy et al. 1999, 2008; Rodríguez-Suárez et al. 2007; David et al. 2008; Gonçalves et al. 2010; Campa et al. 2011). Recent evidence has confirmed that *Co-15* is also located on Pv04 (Sousa 2012). Moreover, López et al. (2003) identified clusters of resistance genes on Pv04 that are effective against several strains of *C. lindemuthianum*, *P. griseola*, and the bean golden yellow mosaic virus.

Similar gene clusters have been described in other species. The Pv04 cluster containing the *Co-10* and *Phg-ON* genes has a homologous cluster in the soybean genome (Ashfield et al. 2012). Moreover, McClean et al. (2010) demonstrated that the Pv04 region was bounded by molecular markers g1375 and g2685, and the interval from 14-77 cM was homologous to regions on soybean chromosomes Gm13 and Gm19. Other examples of disease-resistance clusters in soybean include those identified by Meksem et al. (1999), Kang et al. (2012), and McHale et al. (2012). The presence of these clusters is widespread among higher plants, as they are also observed in members of the grass family, such as sorghum (Martin et al. 2011).

Previous studies on Ouro Negro have revealed that the *Co-10* ANT-resistance gene and *Ur-14* rust-resistance gene are positioned 12.3 cM apart on Pv04 (Corrêa et al. 2000); however, the relationship between the *Co-10* and *Phg-ON* ALS-resistance genes in Ouro Negro has not been established. To date, there has been only one report of a linkage between the *Co* and *Phg* genes. Gonçalves-Vidigal et al. (2011) described the co-segregation of the ANT *Co-1*^{4} and ALS *Phg-1* disease-resistant genes in the common bean cultivar AND 277, in which the genes were located in a cluster of resistance genes on Pv01.

The objectives of the present study were a) to investigate the linkage between the *Co-10* (ANT) and *Phg-ON* (ALS) disease-resistance genes and the existence of a possible gene cluster involving these genes, b) to verify the linkage between the newly identified molecular marker g2303 and the *Co-10* and *Phg-ON* genes, and c) to examine the value of the g2303 marker in comparison to the previously identified SF10 molecular marker (Corrêa et al. 2000) for the selection of common bean plants containing the *Co-10* and *Phg-ON* genes.

Materials and Methods

Genetic crosses and co-segregation analysis

To conduct the co-segregation and linkage studies, two different crosses were performed with the Ouro Negro cultivar, which contains the *Co-10* and *Phg-ON* disease-resistance genes that confer resistance to races 7 and 73 of *C. lindemuthianum* and race 63-39 of *P. griseola*. In the first cross, Ouro Negro was crossed with the common bean cultivar Rudá (susceptible to races 73 of *C. lindemuthianum* and 63-39 of *P. griseola*). In the second cross, Ouro Negro was crossed with the common bean cultivar AND 277 (susceptible to races 7 of *C. lindemuthianum* and 63-39 of *P. griseola*). Both AND 277 and Rudá were used as the female parents. The F_1 seeds were sown in polyethylene vases (48 × 30 × 11 cm) containing a mixture of previously fertilized and sterilized substrate. The plant vases were kept in a greenhouse until the F_2 seeds were produced. A total of 112 F_2 seeds derived from the Rudá × Ouro Negro cross were sown in plastic trays (50 × 30 × 9 cm) containing peat-based substrate. The seedlings were maintained in a greenhouse until the first trifoliolate leaves (stage V3; Gepts 1987) were fully expanded. At that time, the plants were inoculated with race 73 of *C. lindemuthianum* and race 63-39 of *P. griseola*.

A set of 63 F_2 seeds from the AND 277 × Ouro Negro cross was multiplied in pots to obtain the F_3 generation plants. The $F_{2:3}$ families, obtained by selfing individual F_2 plants, were used to characterize the corresponding F_2 plants for resistance to race 7 of *C. lindemuthianum* and race 63-39 of *P. griseola*. The dominant violet flower trait, which was inherited from the resistant male Ouro Negro parent, was observed in the F_1 plants, confirming that they were hybrids. The resistance genotype of each F_2 plant was inferred from the phenotypes of the corresponding $F_{2:3}$ families.

Inocula of Colletotrichum lindemuthianum *and* Pseudocercospora griseola

Races 7 and 73 of *C. lindemuthianum* were obtained from the mycology collection of NUPAGRI (Nucleo de Pesquisa Aplicada a Agricultura, Universidade Estadual de Maringá, State of Parana, Brazil), and *P. griseola* race 63-39 was kindly provided by Dr. M. Goreti de Almeida Oliveira of the "Instituto de Biotecnologia Aplicada à Agropecuária" (Bioagro), Universidade Federal de Viçosa, State of Minas Gerais,

Brazil. The classification as races 7, 73, and 63-39 was confirmed by inoculating two separate sets of 12 common bean differential cultivars used to characterize the virulence spectra of the ANT and ALS pathogens (Pastor-Corrales 1991). The initial inocula of races 7 and 73 of *C. lindemuthianum* and race 63-39 of *P. griseola* were obtained from monosporic cultures. The subsequent inocula of the ANT races were produced on young green common bean pod medium incubated at 22°C for 14 days. The inoculum of race 63-39 of ALS was first multiplied in Petri dishes containing 1-2 mL of a solution of 800 mL of sterilized water, 200 mL of commercial tomato sauce, 15 g of agar, 4.5 g of calcium carbonate ($CaCO_3$), and 10 μg mL^{-1} of streptomycin (Sanglard et al. 2009). Subsequent inocula of race 63-39 were produced in Petri dishes containing the tomato medium and maintained in a BOD incubator at 24°C for 15 days. Spore suspensions were adjusted to 1.2×10^6 and 1.2×10^4 conidia mL^{-1} for *C. lindemuthianum* and *P. griseola,* respectively.

Inoculation and evaluation of the Ouro Negro × Rudá F_2 population

The parental, F_1 generation, and 112 F_2 plants of the Rudá × Ouro Negro cross were simultaneously inoculated with race 73 of *C. lindemuthianum* and race 63-39 of *P. griseola* according to Gonçalves-Vidigal et al. (2001). After the expansion of the first trifoliolate leaf, the right leaflet was inoculated with *C. lindemuthianum* and the left leaflet with *P. griseola*. Each pathogen was inoculated separately using small brushes (Tigre® model 266, number 14). After inoculation, the plants were placed in a mist chamber for 48 h at a temperature of 20 ± 2°C with light controlled at 12 h of daylight and 12 h of darkness (light intensity of 300 μmoles m^{-2} s^{-1} at a height of 1 m) and a relative humidity of > 95%. After the incubation period, the inoculated plants were transferred to open-air benches at a temperature of 22°C with artificial light (12 h of daylight at 25°C), where they remained for 7 days before visual symptom assessment. The visual assessments of ANT and ALS symptoms were performed using the severity scales proposed by Pastor-Corrales et al. (1995) and Inglis et al. (1988), respectively, with scores of 1-9. Plants with disease reaction scores between 1 and 3 were considered resistant, whereas plants with scores from 4 to 9 were considered susceptible.

Evaluation of the population from the AND 277 × Ouro Negro cross

For the AND 277 × Ouro Negro cross, 12 plants from each of the 63 $F_{2:3}$ families derived from the F_2 population were also inoculated with races 7 of *C. lindemuthianum* and 63-39 of *P. griseola*. The seedlings were grown under natural light in greenhouses supplemented with 400 W high-pressure sodium lamps providing a total light intensity of 115 μmoles m^{-2} s^{-1} for 7 to 10 days until they reached the first trifoliate leaf stage. Twenty parental plants (Ouro Negro and AND 277), 20 F_1 plants, and 12 plants from each of the 63 $F_{2:3}$ families were separately inoculated with races 7 of *C. lindemuthianum* and 63-39 of *P. griseola*. A spore suspension containing 2.0×10^6 spores mL^{-1} of race 7 of *C. lindemuthianum* was inoculated using a DeVilbiss number 15 atomizer powered by an electric air compressor (Schulz, SA, Joinville, Santa Catarina, Brazil). A similar procedure was employed for the inoculation with race 63-39 of *P. griseola*. The procedures used after the bean plant inoculation with the ANT and ALS pathogens and for symptom evaluation were identical to those used for the Rudá × Ouro Negro population.

DNA extraction and bulked segregant analysis

The DNA extraction process was performed according to Afanador et al. (1993) with some modifications; the DNA was extracted from the central leaflet from the first trifoliolate leaf directly with 400 mL of CTAB extraction buffer. A total of 23 molecular markers, all mapping to chromosome Pv04 (PhaseolusGenes database: http://phaseolusgenes.bioinformatics.ucdavis.edu), were chosen for testing. The markers included 10 Sequence Tagged Sites (STS) (McConnell et al. 2010), eight microsatellites (Blair et al. 2003; Gaitán-Solís et al. 2002), and five SCARs (Corrêa et al. 2000; Corrêa et al. 2001; Queiroz et al. 2004). For each F_2 plant from the Rudá × Ouro Negro cross inoculated simultaneously with races 73 of *C. lindemuthianum* and 63-39 of *P. griseola*, the total genomic DNA was isolated from the newly emerged first trifoliate leaflet. The same procedure was used to isolate DNA from the F_2 plants from the AND 277 × Ouro Negro cross. The resistant or susceptible phenotype of these F_2 plants was inferred from the phenotypes of the 63 $F_{2:3}$ families that were separately inoculated with races 7 of *C. lindemuthianum* and 63-39 of *P. griseola*. Two contrasting DNA bulks were constructed by pooling equal volumes of fluorometrically standardized DNA from five F_2 plants that were homozygous RR for the resistant genotype and rr for the susceptible

genotype (Michelmore et al. 1991). The resistant and susceptible DNA pools were used for bulked-segregant analysis (Michelmore et al. 1991) for the identification of markers potentially linked to the *Co-10* ANT and *Phg-ON* ALS-resistance genes. All markers were tested on the parental plants and the resistant and susceptible bulks.

Of the tested STS and microsatellite markers, only the STS g2303 was polymorphic, with contrasting amplification patterns in the parental materials and the resistant and susceptible bulks and individuals from the bulks. The g2303 molecular marker was retained for subsequent studies. Among the tested SCARs, only SF10 was polymorphic; thus, SF10 was chosen for genotyping the F_2 population derived from the Rudá × Ouro Negro cross. In addition, the SF10 marker is linked to the *Ur-14* rust and *Co-10* ANT-resistance genes of Ouro Negro, and this marker has been mapped to Pv04 (Corrêa et al. 2000).

All amplification reactions were performed with a thermal cycler (MJ Research Inc., Waltham, MA). The polymerase chain reaction (PCR) program for g2303 consisted of 3 min at 95°C and 35 cycles of 30 s at 92°C, 1 min at 50°C, 60 s at 72°C, followed by a 5 min extension at 72°C and 4 min at 4°C. PCRs were performed in 25 µL total reaction volumes containing 40 ng total DNA; 0.2 mM each dNTP; standard *Taq* buffer containing 1.5 mM $MgCl_2$ and 0.2 µM forward primer and reverse primer; and 1 unit of *Taq* DNA polymerase. Following the addition of 2 µL loading buffer (30% glycerol and 0.25% bromophenol blue), the PCR products for g2303 were analyzed on 6% polyacrylamide gels stained with SYBR Safe (0.02%). The PCR for SF10 consisted of 3 min at 94°C, 35 cycles of 15 s at 94°C, 1 min at 65°C, and 90 s at 72°C, followed by a 7 min extension at 72°C and 4 min at 4°C. The PCR products from SF10 were visualized on agarose gels. The DNA bands were visualized under ultraviolet light, and digital images were recorded with an L-PIX Image EX model (Loccus Biotecnologia - Loccus do Brasil, Cotia, SP, Brazil).

Molecular mapping

The molecular markers g2303 and SF10 were both analyzed in the BAT93 × Jalo EEP558 (BJ: 71 lines; Freyre et al. 1998) recombinant inbred lines and in the F_2 populations from the AND 277 × Ouro Negro and Rudá × Ouro Negro crosses. The primer sequences for a segregation the g2303 marker were as follows: 'GGGGCGGAATCAGGTTCACCA' (forward) and

'GGTTTAGGACAACTAATGAGAGTGATGTACCGTGT' (reverse) (McConnell et al. 2010), as described in the PhaseolusGenes database (http://phaseolusgenes.bioinformatics.ucdavis.edu/markers/?ALL=g2303&format.html). The primer sequences for SF10 were 'GGAAGCTTGGTGAGCAAGGA' (forward) and 'GGAAGCTTGGCTATGATGGT' (reverse), according to the BIC database (http://www.css.msu.edu/bic/PDF/SCAR_Markers_2010.pdf).

Statistical analyses

Segregation analyses of the disease reactions of 112 F_2 plants from the Rudá × Ouro Negro cross were performed using the χ^2 test according to a Mendelian segregation hypothesis of 3 R (resistant) to 1 rr (susceptible). Additionally, this test was performed with the data from the 63 $F_{2:3}$ families from the AND 277 × Ouro Negro cross according to a segregation hypothesis of 1:2:1 (RR:Rr:rr). A goodness-of-fit test for a 1:1 segregation ratio was performed for the segregation of the g2303 and SF10 markers in the BJ population. Linkage analyses were performed using the MAPMAKER/EXP 3.0 (Lincoln and Lander 1993) computer software to estimate the genetic distances between the g2303 and SF10 markers and the *Co-10* and *Phg-ON* genes in the $F_{2:3}$ families derived from the AND 277 × Ouro Negro cross and F_2 population from the Rudá × Ouro Negro cross, respectively. A minimum likelihood of an odds ratio score of ≥ 3.0 and a maximum distance of 30 cM were used to test the linkages between these markers, as described by Freyre et al. (1998). The cluster containing the *Co-10*/*Phg-ON* and g2303 and SF10 markers was localized on Pv04 according to the standardized common bean linkage map nomenclature (Pedrosa-Harand et al. 2008). The map was drawn using MapChart software (Voorrips 2002).

Results

Interaction between the two pathogens used for bean plant co-inoculation

To detect a possible interaction between the two pathogens used in this study, we inoculated Ouro Negro (resistant to races 73 and 63-39) and Rudá (susceptible to races 73 and 63-39) singly with race 73 or race 63-39 of the ANT and ALS pathogens, respectively. We also inoculated these cultivars simultaneously with the same two races; the right leaflet was inoculated with *C. lindemuthianum*, and the left leaflet was inoculated with *P. griseola*. In both inoculation schemes, Ouro Negro was resistant and Rudá was susceptible to races 73 and 63-39. These results confirm previous observations by Gonçalves-Vidigal et al. (2012b). These authors inoculated singly the Ouro Negro (resistant to race 73 and susceptible to 63-23), Mexico 54 and Cornell 49-242 (susceptible to race 73 and resistant to race 63-23), and Rudá (susceptible to both races) cultivars with race 73 or 63-23 of the ANT and ALS pathogens. Simultaneous inoculation was also performed using the same cultivars with the 73 and 63-23 races. In both inoculation systems, Ouro Negro was resistant to race 73 and susceptible to 63-23; Mexico 54 and Cornell 49-242 were susceptible to race 73 and resistant to race 63-23, and Rudá was susceptible to both races. The results indicated that there was no interaction between these pathogens when inoculated simultaneously on the same bean plants.

Genetic resistance and co-segregation of the Co-10 and Phg-ON genes

A total of 112 F_2 plants derived from the Rudá × Ouro Negro cross and 20 plants each from the parental and F_1 generation were inoculated simultaneously with race 73 of *C. lindemuthianum* and race 63-39 of *P. griseola*. Nine days after the inoculation of the plants with both pathogens, a segregation was observed in the F_2 population, with 84 plants resistant and 28 susceptible to *C. lindemuthianum* (P = 1.0) and 83 plants resistant and 29 susceptible (P = 0.83) to *P. griseola* (Table 1). Except for one plant, the 112 F_2 plants from the Rudá × Ouro Negro cross exhibited identical resistant and susceptible phenotypes; that is, all but one of the 112 F_2 plants that were resistant to the ANT pathogen were also resistant to the ALS pathogen. Similarly, plants that were susceptible to the ANT pathogen were also susceptible to the ALS pathogen.

Insert Table 1

The segregation observed in the 63 $F_{2:3}$ families derived from the AND 277 × Ouro Negro cross inoculated separately with races 7 of *C. lindemuthianum* and 63-39 of *P. griseola* exhibited a similar co-segregation of resistance/susceptibility to both pathogens (Table 2). All plants that were resistant to ANT were also resistant to the ALS pathogen, and the plants that were susceptible to ANT were also susceptible to ALS. Thus, no recombinants were observed in these $F_{2:3}$ families, suggesting that the *Co-10* and *Phg-ON* genes are very tightly linked. The 63 $F_{2:3}$ families segregated into classes as follows: 14RR:34Rr:15rr ($P = 0.81$). These results revealed a co-segregation between the *Co-10* and *Phg-ON* genes that fit a 1R:2Rr:1S ratio for a single dominant gene.

As noted earlier, except for one plant, the 112 F_2 plants from the Rudá × Ouro Negro cross also exhibited identical resistant or susceptible phenotypes. Thus, the segregation results from both the Rudá × Ouro Negro cross and from the AND 277 × Ouro Negro cross suggest that the *Co-10* and *Phg-ON* genes are very tightly linked. These segregation patterns fit the model of a 3R:1S ratio of monogenic, dominant resistance. Therefore, it was concluded that the resistance to races 7 and 73 of *C. lindemuthianum* and 63-39 of *P. griseola* in the Mesoamerican common bean cultivar Ouro Negro is conferred by the tightly linked *Co-10* and *Phg-ON* genes. Other researchers have previously conducted separate ANT and ALS inheritance studies and observed that Ouro Negro exhibited dominant and monogenic inheritance of resistance to each of the pathogens causing these diseases (Corrêa et al. 2001; Alzate-Marin et al. 2003).

Insert Table 2

Marker analysis and mapping of the Co-10 and Phg-ON genes

In order to identify a molecular marker linked to the *Co-10* and *Phg-ON* resistance genes and determine the chromosomal location of these genes, 23 molecular markers previously mapped on Pv04 were evaluated using bulked segregant analysis (Michelmore et al. 1991). Of the 23 tested molecular markers, only g2303 and SF10 exhibited polymorphisms characterized by contrasting amplification patterns in the parental materials and the resistant versus susceptible bulks or individuals and were

chosen for further studies. The SF10 marker amplified a band of 1072 bp in the genomic region of interest in the resistant Ouro Negro parental plants and the resistant bulk. The g2303 marker amplified a 350 bp fragment in the resistant parental Ouro Negro plants and in all resistant F_2 plants from the Rudá × Ouro Negro and AND 277 × Ouro Negro crosses. The presence of the 350 bp amplicon of g2303 in Ouro Negro (*Co-10/Phg-ON*), Mexico 222 (*Co-3*), and BAT93 (*Co-3*[3]) is presented in Figure 1; conversely, this marker was absent in the Corinthiano (*Co-15*) and Rudá cultivars.

As illustrated in Table 3, the co-segregation of resistance versus susceptibility to ALS and ANT and the SF10 marker in the F_2 population from the Rudá × Ouro Negro cross revealed a segregation pattern of 92(+):20(-), indicating that SF10 is linked to the *Co-10* and *Phg-ON* loci at a distance of 7.8 cM.

Insert Table 3 and Figure 1

The results of the co-segregation analyses of the $F_{2:3}$ families from the AND 277 × Ouro Negro cross inoculated separately with ANT and ALS and the molecular analysis using the g2303 marker are depicted in Table 4. The genetic linkage analysis resulted in a segregation of 48(+):15(-), indicating a good fit to the expected ratio of 3R:1S (P = 0.83). All 48 resistant plants possessed the g2303 molecular marker, and the marker was absent in the 15 susceptible plants. The lack of recombinants among the *Co-10* and *Phg-ON* genes and the g2303 marker indicated that g2303 is tightly linked to these genes and can be used to map the *Co-10* and *Phg-ON* resistance genes of Ouro Negro.

Figure 2 presents the distances and locations of the *Co-10* (ANT resistance) and *Phg-ON* (ALS resistance) genes and the g2303 and SF10 markers on Pv04. The linkage analysis revealed that the g2303 marker was linked to the ANT and ALS genes at a distance of 0.0 cM on Pv04 and that the SF10 marker was linked at a distance of 7.8 cM.

Insert Table 4 and Figure 2

Marker g2303, which was previously mapped to chromosome Pv04 (McConnell et al. 2010), was tested in the BAT93/Jalo EEP558 (BJ) RI population, resulting in a segregation of 36(+):37(-) (χ^2 = 0.013; P = 0.90), for a good fit to a 1R:1S ratio. In addition, the SF10 marker, positioned on chromosome Pv04 (Corrêa et al. 2000), was

383 also assessed in the same mapping population, yielding a ratio of 38(+):35 (-) (χ^2 =
384 0.12; P = 0.72) and tight linkage with g2303 (Figure 2).
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Discussion

In this study, to better understand the existing association between the *Co-10* and *Phg-ON* genes and the trait of simultaneous resistance to two major common bean pathogens, *C. lindemuthianum* and *P. griseola*, we performed co-segregation and linkage analyses of the F_2 population from the Rudá × Ouro Negro cross and the $F_{2:3}$ families from the AND 277 × Ouro Negro cross.

The co-segregation between the *Co-10* and *Phg-ON* resistance genes for the ANT and ALS pathogens, respectively, was detected when the F_2 population from the Rudá (S) × Ouro Negro (R) cross was simultaneously inoculated with both pathogens. We observed only one recombinant in this F_2 population, suggesting a tight linkage (0.9 cM) between the *Co-10* ANT and *Phg-ON* ALS-resistance genes in Ouro Negro. These results also indicated that the resistance of Ouro Negro to race 73 of *C. lindemuthianum* and race 63-39 of *P. griseola* was linked to the g2303 and SF10 molecular markers. As expected, the genotyping of the SF10 marker in this F_2 population revealed that it is linked to the *Co-10* and *Phg-ON* loci at a distance of 7.8 cM. A previous study reported that the SF10 marker was linked to the *Ur-14* rust and *Co-10* ANT-resistance genes on chromosome Pv04 at a distance of 6.0 ± 1.3 cM (Corrêa et al. 2000).

Additionally, to obtain more precise phenotypic results, we evaluated 63 $F_{2:3}$ families from the AND 277 (S) × Ouro Negro (R) cross inoculated separately with race 7 of *C. lindemuthianum* and race 63-39 of *P. griseola*. Each of the 63 $F_{2:3}$ families exhibited identical responses to both pathogens. The data for the segregation of these $F_{2:3}$ families revealed a tight linkage between the *Co-10* and *Phg-ON* genes. The segregation obtained for both pathogens fit the expected ratio of 1RR:2Rr:1rr. No recombinants were observed during the evaluation of the $F_{2:3}$ families, revealing a tight linkage of the two genes.

The molecular analysis of F_2 population corresponding to the above mentioned $F_{2:3}$ families revealed close linkage between the resistance genes and g2303 maker (0.0 cM). To date, SF10 is the only mapped marker linked to Ouro Negro resistance genes (Corrêa et al. 2000). However, we identified g2303 as a molecular marker that is much more tightly linked to the *Co-10* ANT and *Phg-ON* ALS disease-resistance genes.

A previous study involving ANT and ALS established that the ANT *Co-1*^{4} and ALS *Phg-1* resistance genes, present in the AND 277 cultivar, co-segregated to form a cluster of resistance genes on Pv01 (Gonçalves-Vidigal et al. 2011). In the course of the present

study, we identified a tight linkage between the resistance genes *Co-10* and *Phg-ON*. These genes are also closely linked to a third gene, the *Ur-14* rust-resistance gene (Corrêa et al. 2000), suggesting the existence of a resistance gene cluster on chromosome Pv04. Prior studies have suggested that the majority of R (resistance) genes reside in clusters, and the frequency of recombination between clustered genes can vary strikingly, even within a single cluster (McDowell and Simon 2006). David et al. (2008) confirmed that the B4 (Pv04) R gene cluster is very large, spanning several megabases. Moreover, Oblessuc et al. (2012) reported the existence of different ANT-resistance genes clustered in the bean genome. In addition, David et al. (2008) observed that this Pv04 cluster contained genes conferring resistance not only to *C. lindemuthianum* but also to other pathogens, such as *Uromyces appendiculatus* (rust) and *Pseudomonas syringae* pv. *phaseolicola* (halo blight).

Geffroy et al. (1999, 2000) and Méndez-Vigo et al. (2005) were among the first to describe the *Co-3* gene cluster, which confers specific resistance to different races of *C. lindemuthianum* in the Mesoamerican cultivars Mexico 222 and BAT93. According to Campa et al. (2011), the gene present in the Andean cultivar Michigan Dark Red Kidney confers resistance to race 1545 of *C. lindemuthianum* and is located within the *Co-3* cluster on Pv04. The *Co-10/Phg-ON* resistance cluster present in Ouro Negro may correspond to the ANT-resistance cluster *Co-3*, as both gene clusters are very closely linked to the molecular marker g2303 on Pv04. However, previously conducted tests in an F_2 population from the Ouro Negro (*Co-10*) × Mexico 222 (*Co-3*) cross, inoculated with race 23 of *C. lindemunthianum*, revealed a lack of allelism (Alzate-Marin et al. 2003). In addition, the resistance spectrum of *Co-10* to 21 races of *C. lindemuthianum* shows only 28.5% similarity with the resistance of *Co-3*.

To verify the independence of the newly identified gene cluster *Co-10/Phg-ON*, electrophoretic analysis of the amplification products was conducted to test the association of the g2303 marker with the *Co-10/Phg-ON* and *Co-39* gene clusters and the *Co-15* resistance gene. The g2303 marker was assessed in the following cultivars: Corinthiano (*Co-15*), Ouro Negro (*Co-10/Phg-ON*), BAT93 (*Co-3*[3]), Mexico 222 (*Co-3*), and Rudá. These results revealed an association between g2303 and the *Co-10*/*Phg-ON* and *Co-3* gene clusters but not with the *Co-15* gene. The *Co-15* gene, present in Corinthiano, is linked to $g2685^{150}$, a marker that also maps on Pv04 (Sousa, 2012). A previous study conducted by McConnell et al. (2010) determined that the two markers $g2303^{350}$ and $g2685^{150}$ are unlinked (58 cM) on chromosome Pv04. The physical

position in bp on chromosome 04 also confirms a loose association between the two markers as g2303 maps at position 3,356,300 bp and g2685 at position 9,078,200 bp (out of a total chromosome length of 45,960,019 bp; PhaseolusGenes). Similarly, allelism tests conducted by Gonçalves et al. (2010) confirmed that *Co-15* is distinct from the *Co-3*, *Co-3*^{3}, and *Co-10* resistance genes. These data suggest the presence of a large gene cluster containing the resistance genes *Co-3*, *Co-9* (now re-labeled *Co-3*^{3}), *Co-10*, and *Phg-ON*,. An independent locus, *Co-15*, is placed on a distinct chromosome region of Pv04.

The main objective of this study was to verify the presence of a resistance gene cluster in Ouro Negro that not only confers resistance to ANT but also to ALS, regardless of how it should be properly named. In addition, a much more effective molecular marker, g2303, was found linked to the above mentioned gene clusters (*Co-3/ Co-10/Phg-ON*) and it should be recommended to breeding programs. Whether *Co-10* should be considered an additional allele of the *Co-3* locus (*Co-3*^{4}?) and *Phg-ON* should be re-labeled as *Phg-3*, should be decided by the Genetics Committee of the Bean Improvement Cooperative.

The results of this study will be very useful for breeding programs wishing to develop bean cultivars with combined ANT and ALS resistance using marker-assisted selection. The g2303 marker, in particular, will reduce the time and cost of pyramiding the *Co-10* and *Phg-ON* genes into commercial common bean cultivars.

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1

Table 1 Segregation for resistance to races 73 of *Colletotrichum lindemuthianum* and 63-39 of *Pseudocercospora griseola* in common bean F_2 plants from the Rudá × Ouro Negro cross

Parental cross	Generation	Observed Ratio (R:S) [a]	Expected Ratio (3R:1S)	χ^2	*P* value (1 df)
Race 73 of *Colletotrichum lindemuthianum*					
Ouro Negro	RP[b]	20:0			
Rudá	SP[c]	0:20			
Rudá × Ouro Negro	F_1	20:0			
Rudá × Ouro Negro	F_2	84:28	84:28	0.00	1.0
Race 63-39 of *Pseudocercospora griseola*					
Ouro Negro	RP[b]	20:0			
Rudá	SP[c]	0:20			
Rudá × Ouro Negro	F_1	20:0			
Rudá × Ouro Negro	F_2	83:29	84:28	0.04	0.83

[a]R = Resistant; S = Susceptible; RP[b] = Resistant Parent; SP[c] = Susceptible Parent.

2

3

1

Table 2 Segregation for resistance to races 7 of *Colletotrichum lindemuthianum* and 63-39 of *Pseudocercospora griseola* in 63 $F_{2:3}$ plants from the AND 277 × Ouro Negro cross

Parental cross	Generation	Observed Ratio (RR:RS:SS) [a]	Expected Ratio (1RR:2RS:1SS)	χ^2	*P* value (2 df)
Race 7 of *Colletotrichum lindemuthianum* – the ANT pathogen of common bean					
Ouro Negro	RP[b]	20:0:0			
AND 277	SP	0:0:20			
AND 277 × Ouro Negro	F_1	20:0			
AND 277 × Ouro Negro	$F_{2:3}$	14:34:15	15.75:31.5:15.75	0.43	0.81
Race 63-39 of *Pseudocercospora griseola* – the ALS pathogen of common bean					
Ouro Negro	RP	20:0:0			
AND 277	SP	0:0:20			
AND 277 × Ouro Negro	F_1	20:0			
AND 277 × Ouro Negro	$F_{2:3}$	14:34:15	15.75: 31.5: 15.75	0.43	0.81

[a] $F_{2:3}$ families were classified as having all individuals resistant (RR), individuals resistant and susceptible (RS), and all individuals susceptible (SS);
[b] RP = Resistant parent; SP = Susceptible parent.

2

Table 3 Reaction of F_2 plants from the Rudá × Ouro Negro common bean cultivar cross inoculated with races 73 of *Colletotrichum lindemuthianum* and 63-39 of *Pseudocercospora griseola* and the presence [f] (+) or absence (-) of the SF10 molecular marker

	Observed numbers of plants resistant to races 73 and 63-39[a]							Linkage distance (cM)[e]
Generation	$R^{ANT\ b}$ R^{ALSc}	$R^{ANT\ b}$ S^{ALSc}	$S^{ANT\ b}$ R^{ALSc}	$S^{ANT\ b}$ S^{ALSc}	Expected ratio 3:0:0:1[d]	χ^2	*P* value (3 df)	*Co-10/Phg-ON*
F_2	83	1	0	28	84:0: 0:28	0.023	0.92	0.9
SF10	92 (+)	0	0	20 (-)	84:0:0:28	3.047	0.09	7.8

[a] R = Resistant plants; S = Susceptible plants;

[b] ANT = Anthracnose;

[c] ALS = Angular leaf spot;

[d] Two dominant genes in absolute linkage;

[e] Linkage distance between the *Co-10/Phg-ON* genes indicates one recombinant was observed for the reaction to both pathogens;

[f] Marker present (+); absent (-).

Table 4 Reaction of $F_{2:3}$ plants from the AND 277 × Ouro Negro cross of common bean cultivars inoculated with races 7 of *Colletotrichum lindemuthianum* and 63-39 of *Pseudocercospora griseola* and the presence (+) or absence (-) [d] of the g2303 molecular marker

	Observed numbers of plants							Linkage distance (cM)[c]
	Observed ratio to race 7		Observed ratio to race 63-39				*P* value	
Generation	1RR:RS:SS[a]		RR:RS:SS[a]		Expected ratio[b]	χ^2	(3 df)	0.0
$F_{2:3}$	14:34:15		14:34:15		15.75:31.5: 5.75	0.43	0.81	
(+) Presence and (-) absence of marker in F_2 plants	48 (+)	0	0	15 (-)	47.25: 0: 0:15.75	0.05	0.83	0.0

[a] R = Resistant plants; S = Susceptible plants;

[b] Two dominant genes in linkage;

[c] Linkage distance between the g2303 marker and the *Co-10/Phg-ON* genes indicates no recombinants were observed.

[d] Marker present (+); absent (-).

Figure legends

Fig. 1 Electrophoretic analysis of the amplification products from the g2303 marker. Lanes: L, 100 bp ladder; 1, Corinthiano; 2, Ouro Negro; 3, BAT93; 4, Mexico 222; 5, Rudá. The arrow indicates the DNA band of 350 bp linked to the resistance cluster *Co-10/Phg-ON*

Fig. 2 Genetic distances and locations of the *Co-10* gene for resistance to common bean ANT, the *Phg-ON* gene for resistance to ALS, and the molecular markers g2303 and SF10 on linkage group Pv04 of *Phaseolus vulgaris* L. using the populations from the AND 277 × Ouro Negro and Rudá × Ouro Negro crosses. The map was drawn with MapChart (Voorrips 2002)

Figure 1

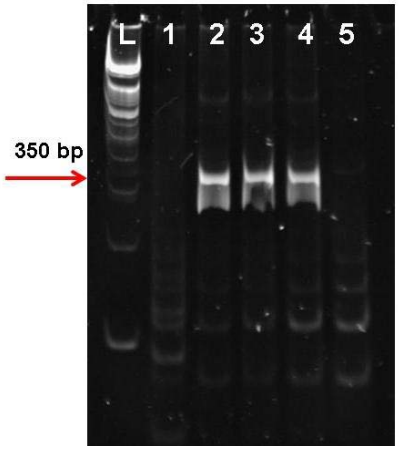
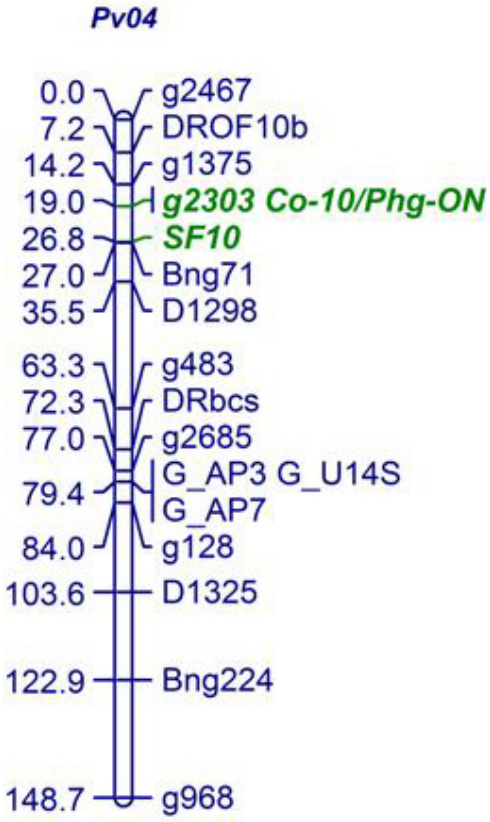


Figure 2


Pv04
0.0
7.2
14.2
19.0
26.8
27.0
35.5
63.3
72.3
77.0
79.4
84.0
103.6
122.9
148.7
g2467
DROF10b
g1375
g2303 Co-10/Phg-ON
SF10
Bng71
D1298
g483
DRbcs
g2685
G_AP3 G_U14S
G_AP7
g128
D1325
Bng224
g968