

Molecular Characterization of Parental Lines and Validation of Snp Markers for Disease Resistance in Common Bean

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Abstract

The implementation of molecular tools that help the early selection of genotypes carrying target alleles increases efficiency and reduces the time and costs of breeding programs. The present study aimed the molecular characterization and validation of SNPs targeting disease resistance alleles for assisted selection. A total of 376 common bean lines with contrasting responses for anthracnose and angular leaf spot resistance were used, as well as 149 F₂ plants from the cross between BRS Cometa x SEL 1308 (carrying the Anthracnose resistance gene *Co-4²*). Seven of the ten SNP markers evaluated showed potential for assisted breeding: snpPV0025 (*Phg-2*), snpPV0027 (*Phg-5*), snpPV0079 (*Phg-5*), snpPV0046 (*Co-u*), snpPV0068 (*Co-4²*), snpPV0070 (*Co-4²*) and snpP8282v3-817 (*Co-4²*). Markers snpPV0070 and snpP8282v3-817 showed high efficiency of selection (99.7 and 99.8%, respectively). These markers exhibit great potential to assist in the selection at different stages of the breeding program and may be readily incorporated into marker-assisted selection.

1. Introduction

Brazil is the world's largest producer and consumer of common beans (*Phaseolus vulgaris*), an important nutritional source and socioeconomically significant crop (Kotue et al. 2018; Embrapa Rice and Beans 2021). This legume is widely cultivated in the country, being produced in three growing seasons and grown by farmers with different technological profiles, at different scales and production systems (Brusamarello et al. 2017). In this scenario, numerous challenges limit production potential, one of the main causes being biotic stresses (Basavaraja et al. 2020).

The common bean is host to a wide range of diseases caused by different pathogens that affect, to a greater or lesser extent, all cultivars recommended by genetic improvement programs (Assefa et al. 2019). Numerous studies made it possible to deepen knowledge on the variability of pathogens (Padder et al. 2017; Nietsche et al. 2002), inheritance of disease resistance (Souza et al. 2016; Kelly and Vallejo 2004), inoculation methods (Bigirimana and Höfte 2008; Rezende et al. 2018), standardization of disease response rating scales (Pastor-Corrales et al. 1992; Van-Schoonhoven and Pastor-Corrales 1987), in addition to identifying numerous resistance loci to different pathogen lines throughout genomes (QTL and genes with greater effect) using molecular markers (Keller et al. 2015; Bassi et al. 2017).

In Brazil, at least 15 epidemiologically important diseases can potentially cause severe damage (Wendland et al. 2018). Among the diseases that affect common bean shoots, anthracnose (*Colletotrichum lindemuthianum*) and angular leaf spot (*Phaeoisariopsis griseola* Sacc.) stand out due to the significant production losses in major producing regions worldwide (Singh and Schwartz 2010). These diseases are caused by pathogens that exhibit pathotype diversity and a monogenic and/or quantitative inheritance pattern of resistance (Padder et al. 2017; Nay et al. 2019b), which hinder the development of genetically resistant cultivars. Depending on the level of susceptibility of the cultivar, these diseases can cause losses of 80-100% (Singh and Schwartz, 2010).

Genomic advances incorporated into breeding programs have enabled the identification of molecular tools for more effective marker-assisted selection (MAS). The strategy of identifying markers linked to disease resistance loci in the common bean has been widely used and once validated, many of the markers show significant MAS application potential (Burt et al. 2015; Perseguini et al. 2016; Zuiderveen et al. 2016; Wu et al. 2017; Lobaton et al. 2018; Gil et al. 2019; Nay et al. 2019a; Fritsche-Neto et al. 2019). In regards to the potential impacts, the implementation of molecular tools that aid the selection of resistant genotypes in breeding programs is a strong ally in the early selection of target alleles, increasing efficiency and reducing time and costs in the selection of superior genotypes for breeding programs (Alvares et al. 2019).

For anthracnose, currently, 12 genes are characterized and described in the BIC, of which four have allelic series: *Co-1* (*Co-1²*, *Co-1³*, *Co-1⁴* and *Co-1⁵*); *Co-2*, *Co-3* (*Co-3²*, *Co-3³*, *Co-3⁴*); *Co-4* (*Co-4²*, *Co-4³*); *Co-5* (*Co-5²*); *Co-6*, *Co-7*, *Co-8*, *Co-11*; *Co-12*, *Co-13* and *Co-14* (Souza et al. 2014). For angular stain, three resistance loci were mapped and named according to the BIC as *Phg-1* (AND 277), *Phg-2* (Mexico 54) and *Phg-3* (Ouro Negro). The *Phg-1* locus, of Andean origin, was mapped in the subtelomeric region of chromosome Pv-01 in the AND 277 cultivar (Meziadi et al. 2016; Gonçalves-Vidigal et al. 2011) and has been used by breeding programs in Brazil. More recently, two QTLs ALS 4.1 (G5686) and ALS 10.1 (G5686 and CAL143) associated with the *Phg-4* and *Phg-5* locus, respectively, are being proposed (Souza et al. 2016).

The main objective of this study was to evaluate several SNP markers previously associated with alleles linked to anthracnose (*Co-4²* and *Co-u*) and angular leaf spot resistance (*Phg-1*, *Phg-2* and *Phg-5*). These SNPs were evaluated in a diverse panel of common bean

genotypes, including important sources of resistance and parent plants used in Embrapa's (Brazilian Agricultural Research Corporation) bean breeding program, in order to characterize elite parent lines for the presence of disease resistance alleles, as well as validate or indicate SNP markers to be incorporated into MAS.

2. Material And Methods

2.1 Genetic material

We used 376 diverse common bean genotypes from several national and international breeding programs that show contrasting resistance responses to anthracnose and angular leaf spot, including cultivars, elite parent plants and sources of resistance (donor parent plants). Information on the institution of origin, gene *pool*, grain type and reaction to anthracnose and angular leaf spot are presented in Table S1. Phenotypic information regarding the reaction to the two diseases was obtained for 240 of the 376 lines in this study, of which 139 were phenotyped for anthracnose and 101 for angular leaf spot (Table S1).

Ten seeds from each line were deposited on sheets of gemitest paper, moistened with autoclaved distilled water and then placed in a germinator (Mangelsdorf) at a constant temperature of 25°C and 27% humidity for seven days. Two leaf discs obtained from the primary leaves with a puncher were individually collected and transferred to 96 sterile deep well plates. The plates were stored in a freezer at -80°C for 24 hours and then submitted to lyophilization for six hours using a freeze dryer (Liotop® model L101). Finally, the plates were sent to Intertek Agritech (Sweden) for genotyping. This genotyping service provider performs KASP marker analysis (Rasheed et al. 2016).

2.2 Molecular analysis with SNP markers

Nine SNPs associated with disease resistance in common bean were selected from a portfolio of molecular markers made available by the "High Through-Put Genotyping (HTPG)" project (Bohar et al. 2020) [<https://cegsb.icrisat.org/marker-panels-2/>].

Three of these are associated with anthracnose (two with the *Co-4²* allele and one with the *Co-u* genes) (Cieslak et al. 2015; Oblessuc et al. 2015; Burt et al. 2015; Zuiderveen et al. 2016) and six with angular spot (one with the *Phg-1* gene, three with *Phg-2* and two with *Phg-5*) (Gonçalves-Vidigal et al. 2011; Lobaton et al. 2018; Nay et al. 2019a, Gil et al. 2019), as described in Table 1. Genotyping was performed at the genotyping provider Intertek AgriTech, through an agreement with HTPG (Bohar et al. 2020).

Table 1

SNP markers associated with common bean disease resistance genes and their respective identification, position in the genome and indication of genotypes carrying the target alleles.

SNP	Allele	Chromosome	Pathogen	Pool	Resistant parent	Susceptible parent	Reference
snpPV0051	<i>Phg-1</i>	Pv01	MA	A	AND 277	-	GonçalvesVidigal et al. (2011)
snpPV0025	<i>Phg-2</i>	Pv08	MA	M	G10474 e México 54	Sprite e VAX1	Lobaton et al. (2018)
snpPV0033	<i>Phg-2</i>	Pv08	MA	M	G10474	Sprite e VAX1	Lobaton et al. (2018)
snpPV0071	<i>Phg-2</i>	Pv08	MA	M	G10474	-	Nay et al. (2019)a
snpPV0027	<i>Phg-5</i>	Pv10	MA	A	G5686	Sprite	Lobaton et al. (2018)
snpPV0079	<i>Phg-5</i>	Pv10	MA	A	G5686	-	Nay et al. (2019)a
snpPV0068	<i>Co-4²</i>	Pv08	AN	M	SEL 1308 e B09197	Black Magic e Nautica	Oblessuc et al. (2015); Burt et al. (2015)
snpPV0070	<i>Co-4²</i>	Pv08	AN	M	SEL 1308 e B09197	Black Magic e Nautica	Oblessuc et al. (2015); Burt et al. (2015)
P8282v3-817	<i>Co-4²</i>	Pv08	AN	M	SEL 1308	BRS Cometa	Cieslak et al. (2014)
snpPV0046	<i>Co-u</i>	Pv02	AN	M	Montcalm G	-	Zuiderveen et al. (2016)

R: Resistant; S: Susceptible; ALS: Angular leaf spot; AN: Anthracnose; M: Mesoamerican; A: Andean

Additionally, the snpP8282v3-817 (GRAF1) associated with the *Co-4* resistance locus (Cieslak et al. 2015) was evaluated through experiments with a TaqMan® hydrolysis probe (ThermoFisher) (Shen et al. 2009; Applied Biosystems 2021) conducted at the Biotechnology Laboratory at Embrapa Rice and Beans, as described in item 2.3.3.

A simple linear regression analysis was performed using R software, version 4.1.0 (R Development Core Team 2021), at significance level of $p < 0.05$, based on the genotypes of the SNP markers associated with phenotypic values.

2.3 Validation of snpPV0070, snpP8282v3-817 (*Co-4²*) and snpPV0025 (*Phg-2*) markers

From the nine SNP markers, genotyped by Intertek Agritech, four SNPs that showed an association between the marker and disease resistance were submitted to an additional validation step as described below. These markers were converted into TaqMan® (ThermoFisher) probes (Shen et al. 2009; Applied Biosystems 2021). In order to design the probes, the sequences containing target SNPs were aligned to the common bean reference genome (Schmutz et al. 2014) using the BLAST command, and a search for repetitive elements was conducted using RepeatMasker, with both analyses available on the Phytozome platform (Phytozome v12.1: Home (doe.gov)). Probes were designed using the online Custom TaqMan Assay Design Tool (Thermo Fisher) available at [<https://www.thermofisher.com/order/custom-genomic-products/tools/genotyping/>].

2.3.1 Segregating population and contrasting lines

The TaqMan® SNP markers associated with anthracnose (*Co-4²*) were validated using an F₂ population of 149 plants from the cross between BRS Cometa ♀ (female parent, susceptible to pathotype 73) and SEL 1308 (male parent, harboring the allele *Co-4²*, resistant to *C. lindemuthianum* pathotype 73) (Table S2), which was phenotyped for the reaction to *C. lindemuthianum* pathotype 73. The TaqMan® SNP marker associated with angular leaf spot (*Phg-2*) was validated in a set of 30 contrasting common bean genotypes for the angular leaf spot reaction (Table S3), evaluated under field conditions in different tests performed in the Embrapa Rice and Beans breeding program.

2.3.2 Obtaining *Colletotrichum lindemuthianum* inoculum

The *C. lindemuthianum* isolate CL1869 (pathotype 73) was used to inoculate 149 F₂ plants from the cross between BRS Cometa x SEL 1308. F₂ seeds, as well as ten seeds from each parent plant and the susceptible control (Rosinha G2) were sown in polystyrene seedling trays. Plants were inoculated seven days after sowing, at stage V2 (fully expanded primary leaves) (Pastor-Corrales et al. 1992). The spore solution (1.2×10^6 spores mL⁻¹) was applied to the abaxial and adaxial surfaces of the primary leaves with the aid of a hand sprayer (De Vilbiss, No. 15). After inoculation, the plants were incubated in a humidity chamber for 48 hours, at 20±2°C, with relative humidity of around 95%, controlled by a misting system, and a 12-hour light/dark photoperiod. Next, misting was interrupted and the inoculated plants were kept in a controlled environment under the same temperature and photoperiod described above, where they remained until the disease symptoms were evaluated.

Symptoms were assessed seven days after inoculation, based on a grading scale proposed by Pastor-Corrales and Tu (1989), in which grade 1 represents no symptoms and 9 dead plants due to fungal disease. Plants with grades between 1 and 3 were considered resistant and the others susceptible (Table S2).

2.3.3 DNA extraction and determination of target alleles

Genomic DNA from 149 F₂ plants (BRS Cometa x SEL 1308) (Table S2) and from the set of 30 contrasting genotypes related to angular leaf spot reaction (Table S3) was extracted using the CTAB method, according to the protocol proposed by Doyle and Doyle (1990), modified by Ferreira and Grattapaglia (1998). DNA concentration was estimated using a Qubit® (Thermo Scientific®, Waltham, USA) fluorometer, and integrity visualized by electrophoresis on 1% agarose gel stained with ethidium bromide.

TaqMan® SNP genotyping assays were amplified with the Taqman® GTXpress™ (Thermo Fisher Scientific, Waltham, MA, USA) reagent, according to the manufacturer's guidelines. Amplification was conducted using the QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems) under the following conditions: 60°C for 30 seconds, 95°C for 20 seconds, followed by 50 cycles of 95°C for 3 seconds and 60°C for 30 seconds, and a final extension of 60°C for 30 seconds. This was followed by allele analysis using the Genotyping Analysis Module, V.3.7.

2.3.4 Genetic-statistical analysis

The phenotypic data and the genotypes of the anthracnose-associated markers in the F₂ generation (BRS Cometa × SEL 1308) were submitted to the chi-square test (χ^2) to test the 3R:1rr and 1RR:2Rr:1rr segregation hypotheses (R: resistant; rr: susceptible), respectively, adopting a 5% significance level. Linkage analysis between the marker locus and the *Co-4²* allele was performed using the OneMap package (Margarido et al. 2007) and the estimated recombination frequency converted into genetic distance (cM). All analyses were conducted using R software, version 4.1.0 (R Development Core Team 2021).

The selection efficiency (SE) for codominant markers was estimated according to the methodology described by Liu (1998), using the following estimator:

$SE(\%) = (1 - 4rf^2) \times 100$, where rf is the recombination frequency.

3. Results And Discussion

3.1 Molecular analysis with SNP markers

Eight of the nine SNPs that were selected out of the HTPG project data and genotyped at Intertek (88.8%) were polymorphic and were considered suitable for genotyping analysis. The snpPV0071 marker, associated with the *Phg-2* locus (Nay et al. 2019a), was the only one that displayed a monomorphic profile for the susceptible associated allele (G:G) and was discarded from the analysis. This marker is specific to tag the resistance *Phg-2* allele at G10474 line, not genotyped in this study.

Table 2

Gene annotation performed on the Phytozome platform for the five SNPs associated with common bean disease resistance genes selected for analysis, validation and marker-assisted selection.

SNP	Allele	Primary transcript	Description
snpPV0025	<i>Phg-2</i>	Phvul.008G280700.2	PTHR11132:SF38 - GB
snpPV0046	<i>Co-u</i>	Phvul.002G328300.1	Mitogen-Activated Protein Kinase 16-Related
snpPV0068	<i>Co-4²</i>	Phvul.008G028000.1	MYB Transcription Factor
snpPV0070	<i>Co-4²</i>	Phvul.008G028400.1	Domínio Serine Threonine Kinase/homólogo ao gene <i>COK-4</i>
snpP8282v3-817	<i>Co-4²</i>	Phvul.008G028200	Domínio Serine Threonine Kinase

3.1.1 SNP markers associated with the *Phg loci*

The snpPV0051 (*Phg-1*) marker is located in a region that contains repeats in the genome (identified via RepeatMasker). Of the seven markers that exhibit a technically adequate genotyping profile, snpPV0033 did not amplify the target allele in the sources carrying the *Phg-2* allele.

The target allele was amplified by the snpPV0051 (A:G, where the resistance associated allele is the first in bold) marker in only 103 (27.4%) of the 376 lines evaluated. This SNP is located in a repetitive region of the common bean genome, identified using the RepeatMasker tool (Table 3), which may explain why the fragment was not amplified well. Of the 103 genotyped lines, the "A" allele associated with *Phg-1* was amplified in 11 lines (Table S4), including AND 277, the source of the *Phg-1* allele (Souza et al. 2016) and resistant to the 11 most important *P. griseola* pathotypes in Brazil, such as 63-23, 63-31, 63-47 and 63-63 (Damasceno-Silva et al. 2015; Balbi et al. 2009). Pathotype 63-63 is considered the most aggressive, causing susceptibility symptoms in all differential cultivars from the Andean and Mesoamerican gene pool (Nay et al. 2019b). The angular spot-resistant CAL 143 line was extracted from the cross between G12229 and AND 277 (Nay et al. 2019b) and contains the "A" allele associated with *Phg-1* resistance in its genome (Table S4). Furthermore, the *Phg-1* and *Co-1⁴* alleles were reported to be strongly linked, at a distance of 0.0 cM (Gonçalves-Vidigal et al. 2011), jointly conferring resistance to the *P. griseola* pathotype 63-23 and *C. lindemuthianum* pathotypes 65, 73 and 2047 (Gonçalves-Vidigal et al. 2011). Due to the inconsistent amplification of this marker in the evaluated lines, it was not validated in a reduced set of contrasting lines for resistance to angular leaf spot. However, the snpPV0051 marker has potential for use in the assisted selection of populations from crosses with known sources of the *Phg-1* allele.

Table 3
Genotyping summary of 10 SNP markers associated with common bean disease resistance genes.

SNP	Allele	Polymorphism	Allele 1	Allele 2	Amplified?	NA's	%NA	Monomorphic?	RepeatMasker?
snpPV0051	<i>Phg-1</i>	A:G	A:A (R)	G:G	Yes	273/376	72.60%		Yes
snpPV0025	<i>Phg-2</i>	G:T	G:G (R)	T:T (S)	Yes	0/376	0.00%		No
snpPV0033	<i>Phg-2</i>	T:C	T:T (R)	C:C (S)	Yes	0/376	0.00%		No
snpPV0071	<i>Phg-2</i>	T:G	T:T (R)	G:G (S)	Yes	0/376	0.00%	Yes	No
snpPV0027	<i>Phg-5</i>	T:C	T:T (R)	C:C (S)	Yes	1/376	0.26%		No
snpPV0079	<i>Phg-5</i>	A:G	A:A (R)	G:G (S)	Yes	2/376	0.53%		No
snpPV0068	<i>Co-4²</i>	G:C	G:G (R)	C:C (S)	Yes	0/376	0.00%		No
snpPV0070	<i>Co-4²</i>	G:T	G:G (R)	T:T (S)	Yes	25/376	6.60%		No
snpP8282v3-817	<i>Co-4²</i>	A:G	A:A (R)	G:G (S)	Yes	1/181	0.55%		No
snpPV0046	<i>Co-u</i>	A:G	A:A (R)	G:G (S)	Yes	2/376	0.53%		No

(R): Resistant; (S): Susceptible; NA: No Amplification; The alleles in bold are associated with resistance.

The snpPV0025 marker (G:T) associated with the *Phg-2* allele, located on the Pv-08 chromosome, exhibited the “G” allele in the Mexico 54 and Cornell 49-242 breeding lines (Table S5), which are known sources of *Phg-2* (Souza et al. 2016; Nay et al. 2019b). This *Phg-2* allele, which has a wide diversity of functional haplotypes for a resistance locus in common bean (Nay et al. 2019b), confers resistance to pathotypes 63-19 and 63-39, and was found to be the most relevant allele in Brazil (Bassi et al. 2017). It was also detected in the PT 65 line, which has shown a high level of resistance to angular leaf spot under controlled inoculation and in the field (Pereira et al. 2019; Pereira et al. 2016). The line MAIII 16.159 from the recurrent selection program for angular leaf spot resistance conducted at the Federal University of Lavras (Pereira et al. 2019) and 11 lines from Embrapa’s program also displayed the “G” allele of the snpPV0025 marker, suggesting the presence of the resistant *Phg-2* allele (Table S5). In addition, studies showed that *Phg-2* was responsible for the resistance found in breeding lines evaluated under field and greenhouse conditions, and effective against *P. griseola* isolates from Colombia, Uganda and Brazil (Sartorato et al. 2000; Nay et al. 2019b). *Phg-2* different resistance haplotypes, which confers a broad spectrum of resistance to different *P. griseola* pathotypes from the Andean and Mesoamerican gene pool were described by Nay et al. (2019a).

Due to its importance for breeding programs, the use of *Phg-2* allele sources has been frequently adopted in crossing blocks, in addition to studies that seek to develop markers strongly linked to *Phg-2* (Gil et al. 2019; Miller et al. 2018; Sartorato et al. 2000). For example, the improved MAB 348, MAB 349, MAB 351, MAB 352, MAB 353, MAB 354 and MAB 484 lines, with high angular leaf spot resistance, contain the G10474 line, source of *Phg-2*, as one of their parents (Gil et al. 2019). The results of this study indicate that the *Phg-2* allele is present in elite germplasms developed by Embrapa, the Federal University of Lavras, IAC, IAPAR, Agropecuária Terra Alta and CIAT, and have been detected in 72 lines/cultivars (Table S5). However, as pointed out by Gi et al. (2019), care should be taken when using the snpPV0025 (ALS_08_62193174) for MAS, since it has been used to tag and introgress the *Phg-2* locus from Mesoamerican MAB sources into Andean breeding lines, and do not tag specific meso alleles. Nay et al. (2019a) shed greater light on this by identifying pathotype-specific haplotypes at the *Phg-2* and offering new molecular markers to be tested and used in MAS.

The markers snpPV0027 (T:C) and snpPV0079 (A:G), both for the *Phg-5* gene, contained the "T" and "A" alleles, respectively, only in the G5686 line identified as a *Phg-5* source (Keller et al. 2015). The *Phg-5* allele, found in the line G5686, originates from the Andean gene pool and confers resistance to several *P. griseola* pathotypes from the Andean and Mesoamerican gene pools. Mahuku et al. (2009) studied the reaction of the G5686 line against 15 *P. griseola* pathotypes and reported resistance for 53 and 72% of the Andean and Mesoamerican isolates tested, respectively. However, for pathotypes of Mesoamerican origin, such as 63-23, 63-31, 63-47 and 63-63, which occur more frequently on Brazilian farms, the line G5686 was moderately susceptible or susceptible (Mahuku et al. 2009), justifying the limited use of *Phg-5* allele sources in crossing blocks of the main common bean breeding program in Brazil.

In the common bean - *P. griseola* pathosystem, combining the *Phg-1* (Andean) and *Phg-2* genes (Mesoamerican) would be important to produce plants resistant to pathotypes 63-19, 63-23 and 63-39 (Bassi et al. 2017). The molecular characterization of Embrapa's elite germplasm allowed the identification of elite lines containing pyramided alleles, such as CNFC 16636 (*Phg-1 + Phg-2*) (Table S4) and the differential varieties of anthracnose pathotypes G2333, G2858 and PI 207262 (*Co-u + Co-4²*) (Table S6). This demonstrates the need to obtain lines that simultaneously combine alleles that confer resistance to anthracnose and angular leaf spot, in addition to the other agronomic characteristics demanded by the market.

3.1.2 SNP markers associated with the *Co-4* and *Co-u* loci

The *Co-4* locus is located close to a telomeric region of the chromosome Pv-08, characterized by containing about 18 copies of the *COK-4* gene and described as being associated with anthracnose resistance in the common bean (Oblessuc et al. 2015). In the present study, four SNP markers associated with the anthracnose-resistant locus were analyzed. In case of the markers snpPV0068 (G:C) and snpPV0070 (G:T), the "G" alleles (of both SNPs) were identified in the G-2333, SEL 1308, PI 207262, K-10, K-13, and CNFC 5547 lines (Table S6), which are known sources of the resistant allele of *Co-4²* (Kelly and Vallejo 2004; Vieira et al. 2018). Another 22 black grain lines also displayed this same allele (Table S6). Some of these lines, already characterized in terms of resistance/susceptibility, are part of the differential varieties of anthracnose pathotypes or originated in the anthracnose resistance breeding program (Table S6). The presence of "G" alleles in the two SNPs also coincided with the polymorphisms identified in the snpP8282v3-817 marker (GRAF1, A:G) (Cieslak et al. 2015) in detecting the *Co-4²* allele of the *Co-4* locus in lines K-10, K-13 and CNFC 5547 (Table S6). However, there was no "A" allele amplification of the snpP8282v3-817 marker in the 22 black bean lines amplified by snpPV0070 (Table S6). This result indicates that snpPV0070 is not specific for the *Co-4²* allele, but capable of detecting resistance at the *Co-4* locus. An alternative to snpPV0070 are the markers snpPV0068 and snpP8282v3-817, both amplified in lines that are known to carry the *Co-4²* allele (Table S6). Portilla et al (2021) when evaluating the *Co-4* marker snpPV0069 (closely linked to the snpPV0068 and snpPV0070), developed from G2333 genotype (Lobaton et al. 2018) and available at HTPG portfolio, did not identify any significant effect of association, which was suggestive of a race specific resistance gene interaction. The *Co-4²* is a dominant allele of the *Co-4* locus and has been used by breeding programs worldwide (Kelly and Vallejo 2004; Vieira et al. 2018) due to its broad spectrum of resistance to several *C. lindemuthianum* pathotypes (Balardin and Kelly 1998; Silvério et al. 2002).

For snpPV0046 (A:G, *Co-u* locus), located in the exon of the Phvul.002G328300 gene, the "A" allele is associated with the *Co-u* resistant allele, which also confers resistance to anthracnose (Zuiderveen et al. 2016; Oblessuc et al. 2014; Geffroy et al. 2008). In this study, the "A" allele was identified in 51 of the 376 lines genetically characterized with snpPV0046, including the BAT 93 breeding line (Table S7), which contains the parental genotype PI 207262 (Geffroy et al. 2008) and is a source of the *Co-u* allele (Geffroy et al. 2008). In the present study, the "A" allele was amplified in PI 207262, suggesting that *Co-u* may come from this parent or from a mutation that occurred during the evolutionary process of BAT 93. Of the 51 lines that contain the "A" allele, 13 are from the black bean group, 4 from the Carioca group and the remainder belong to different commercial classes, such as brindle, jalo, white, calima (Table S7), which are detected in Andean and Mesoamerican gene pools. Of these, 12 are resistant cultivars that belong to differentiating varieties of the anthracnose pathotypes (Table S7). The *Co-u* allele is located in the Pv-02 linkage group, very close to *I* locus, conferring resistance to important common bean viruses, such as the bean common mosaic virus, potyviruses and comovirus (Meziadi et al. 2016; Geffroy et al. 2008). At the molecular level, these two loci may have a common origin, since most resistance genes in plants encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins, responsible for recognizing several pathogens (Geffroy et al. 2009). Recent information confirms this hypothesis, since the functional annotation of resistance genes has revealed common protein motifs, such as: Leucine Zippers-LZ, Leucine Rich Repeat-LRR and protein kinase domains (Protein Kinase - PK) (Fritsche-Neto et al. 2019; Nogueira et al. 2019; Queiroz et al. 2019; Nay et al. 2019a; Banoo et al. 2020; Gonçalves-Vidigal et al. 2020; Costa et al. 2021). Thus, validating the snpPV0046 marker for assisted selection is particularly important for programs aimed at incorporating multiple resistance into elite germplasms.

In addition, the introgression of resistance alleles from different gene pools (Andean and Mesoamerican) in the same line through assisted selection by molecular markers is an important strategy for developing bean cultivars with broad and long-lasting resistance (Miller et al. 2018; Vieira et al. 2018; Miklas et al. 2006; Kelly and Miklas 1998; Guzmán et al. 1995). In Brazil, an example of an important combination of anthracnose resistance alleles is that of the allele of Mesoamerican origin *Co-4²* ('SEL1308' and 'K13') and its Andean counterpart *Co-1* (*Co-1^A*) (Vieira et al. 2018; Souza et al. 2014).

3.1.3 Simple linear regression analysis of marker effects

Of the eight SNP markers analyzed, five (snpPV0046, snpPV0068, snpPV0070, snpP8282v3-817 and snpPV0025) showed potential for indirect selection of common bean genotypes containing the *Co-4²* and *Phg-2* alleles and were therefore submitted to simple linear regression analysis. Only for the marker snpPV0046 the regression model was significant (Table S8), but they do not explain much of the variability (low R-square of 3%). Although marker effects were not significant in the sample set of lines containing information regarding the reaction to anthracnose and angular leaf spot (snpPV0046 = 133; snpPV0068 = 134; snpPV0070 = 115; snpP8282v3-817 = 46 and snpPV0025 = 78), all lines carrying the target SNPs showed resistance, suggesting that these alleles can be monitored from these markers in the lines of interest. The absence of significance in the regression test is certainly due to the fact that many lines, even though resistant, did not exhibit the target alleles of this study, suggesting the presence of other anthracnose and angular leaf spot-resistant alleles. When the regression analysis was applied in a subset of genotypes contrasting for the resistance/susceptibility to the anthracnosis and ALS diseases (Table S9), the *Co-4²* and *Phg-2* marker effects were significant (Table 4) and the slope values were negative for all markers (Table 4), revealing an association between SNPs and resistance to anthracnose and ALS. The introgression of resistance alleles into elite bean germplasms assisted by molecular markers is a promising strategy in breeding programs, given that it reduces time and costs in the initial selection stages (Sakiyama et al. 2014). Fig. 1 illustrates the importance of resistance alleles *Co-1* ("A"), *Co-4²* ("G") and *Phg-2* ("G") in reducing mean phenotypic values in the lines that contain them.

Table 4

Summary of the regression analysis framework between SNP markers and resistance reaction to anthracnose or angular leaf spot of common bean genotypes.

snpPV46 (Co-u) - Anthracnose							
Source of variation	Df	SS	MS	F-value	p-value	R ²	Inclination ⁽²⁾
A vs G ⁽¹⁾	1	283.9	283.9	255.7	2.2E-16	0.86	-2.54
Residual	42	46.6	1.1				
snpPV0068 (Co-4²) - Anthracnose							
Source of variation	Df	SS	MS	F-value	p-value	R ²	Inclination
G vs C	1	158.5	158.5	175.7	2.97E-12	0.88	-2.95
Residual	23	20.7	0.9				
snpPV0070 (Co-4²) - Anthracnose							
Source of variation	Df	SS	MS	F-value	p-value	R ²	Inclination
G vs T	1	167.9	167.9	93.0	2.14E-10	0.77	-2.41
Residual	28	50.6	1.8				
snpP8282v3-817 (Co-4²) - Anthracnose							
Source of variation	Df	SS	MS	F-value	p-value	R ²	Inclination
A vs G	1	112.2	112.2	78.2	2.45E-07	0.83	-3.03
Residual	15	21.5	1.4				
snpPV0025 (Phg-2) – Angular Leaf Spot							
Source of variation	Df	SS	MS	F-value	p-value	R ²	Inclination
G vs T	1	66.2	66.2	72.2	4.52E-08	0.78	-1.76
Residual	20	18.3	0.9				
The alleles in bold are associated with resistance; ⁽¹⁾ Contrast considered in the regression analysis between marker locus alleles and the severity of anthracnose or angular leaf spot infection; ⁽²⁾ Angular coefficient of the linear regression equation. The negative sign on the slope indicates that the allele is associated with resistance.							

Additionally, gene annotation revealed that snpPV0046 (*Co-u*), snpPV0068 (*Co-4²*), snpPV0070 (*Co-4²*) and snpP8282v3-817 (*Co-4²*) are located in gene regions that encode defense proteins in plants (Table 2). The snpPV0046 marker is found in the gene that encodes the Mitogen-Activated Protein Kinase (MAPK) protein, which interacts with salicylic acid, a plant hormone known to play a role in plant-acquired resistance against pathogen infection (Jagodzik et al. 2018). The snpPV0068 marker is located in the MYB transcription factor (MYB Transcription Factor) coding region, playing an essential role in the control of cellular processes in response to biotic and abiotic stresses (Ambawat et al. 2013), such as resistance to *Pseudomonas syringae* pv. *tomato* (Pst DC3000) in *Arabidopsis* (Zhang et al. 2019). The snpPV0070 and snpP8282v3-817 markers are located in the Phvul.008G028400 and Phvul.008G028200 transcripts, respectively, homologous to the *COK-4* locus (STK domain) (Table 2), previously described as an anthracnose resistance source in common bean (Melotto and Kelly 2001).

3.2 Validation of the snpPV0070, snpP8282v3-817 and snpPV0025 markers

TaqMan® hydrolysis probes were developed from the snpPV0070 and snpP8282v3-817 markers, targeting the *Co-4²* allele. 149 F₂ plants from the cross between BRS Cometa and SEL1308 were genotyped with two probes and phenotyped for reaction to *C. lindemuthianum* pathotype 73 (Table S2). Of these, 110 were characterized as resistant and 39 as susceptible, in line with the expected ratio of 3R:1S ($\chi^2 = 0.11$; $p = 0.74$) (Table 5). Previous studies support the hypothesis that resistance to anthracnose contained in SEL 1308 is controlled by a single locus, with complete dominance (Young et al. 1998; Oblessuc et al. 2015). Markers snpPV0070 and

snpP8282v3-817 maintained 1RR:2Rr:1rr ratio (Table 5), segregating as expected for codominant markers. Linkage analysis revealed that the snpPV0070 and snpP8282v3-817 markers used in F₂ population genotyping (BRS Cometa x SEL 1308) are strongly linked to the *Co-4* locus, with a recombination frequency of 0.026 (2.6 cM) and 0.019 (1.9 cM), respectively (Table 5). Markers snpPV0070 and snpP8282v3-817 showed selection efficiency (SE) of 99.7% and 99.8% respectively, indicating the high potential value of molecular markers in strong linkage disequilibrium in MAS programs.

Table 5

Genotypic and phenotypic segregation of individuals from the F₂ population, originated from the crossing between BRS Cometa x SEL 1308, evaluated for reaction to *Coletotrichum lindemuthianum* pathotype 73, the causal agent of anthracnose in the common bean.

Genotype/Phenotype		Observed	Expected	Hypothesis	χ^2	P-value	rf	Distance ¹	SE
snpPV0070	T:T	40	37	1:2:1	0.72	0.70	0.026	2.6 cM	99.7%
	G:T	76	75						
	G:G	33	37						
P8282v3-817	G:G	39	37	1:2:1	0.65	0.72	0.019	1.9 cM	99.8%
	A:G	77	75						
	A:A	33	37						
Class	Resistant (1-3)	110	112	3:1	0.11	0.74	-	-	-
	Susceptible (4-9)	39	37						
Total		149	149	-	-	-	-	-	-

The alleles in bold are associated with resistance; ^{rf} Recombination fraction; ^{SE} Selection efficiency; ¹Distance in centiMorgans; P-value associated to the null hypothesis not rejected (1:2:1 for molecular markers data and 3:1 for phenotype data); snpPV0070: "G:G" dominant homozygous for the *Co-4*² allele, "G:T" heterozygous for the *Co-4*² allele and "T:T" recessive homozygous for the *Co-4*² allele; snpP8282v3-817: "A:A" dominant homozygous for the *Co-4*² allele, "A:G" heterozygous for the *Co-4*² allele and "G:G" recessive homozygous for the *Co-4*² allele.

The TaqMan® probe derived from snpPV0025 (G:T) associated with the *Phg-2* allele was evaluated in a set of contrasting elite lines for resistance to angular leaf spot (Table S3). This target SNP was able to detect the *Phg-2* locus, despite its amplification in varieties that are susceptible to angular leaf spot (Table S3). This is in accordance with previously reported that snpPV0025 would only effectively tag *Phg-2* in Andean backgrounds. Additional SNP markers have been identified as associates with *Phg-2* and should be tested (Gil et al. 2019; Nay et al. 2019a). On the other hand, the complementary analysis performed with RAPD_SE04 (Sartorato et al. 1999) and STS_g796 (Miller et al. 2018) markers resulted in specific markers for the *Phg-2* locus, amplifying in resistance sources Mexico 54 and MAR-2 (Table S3). These two markers have been frequently used in studies aimed at *Phg-2* allele introgression (Sanglard et al. 2016; Miller et al. 2018). In addition, SE04 also amplified in lines DM 108, CNFC 17142, CNFC 17395 and CNFC 18710; while g796 amplified in lines CNFC 15086 and CNFC 17153 (Table S3). Among the parental lines used by the breeding program in crossing blocks, DM108 stands out, originating from the cross between BRS Rudá and MAR-2 (Sanglard et al. 2016). The MAR-2 line contains the resistant *Phg-2* locus in its genome; however, the allelic variation of this locus in this variety is still not clear (Nay et al. 2019a). Thus, the presence of the *Phg-2* locus in the elite lines of Embrapa's common bean breeding program may be related to the previous use of the DM108 line as parental line in the crossing blocks. The *Phg-2* locus is particularly important in angular leaf spot resistance, since it confers resistance to the most prevalent pathotypes in Brazilian common bean crops (Bassi et al. 2017).

4. Conclusion

Seven of the nine SNP markers provided by the HTPG project showed potential for routine use in MAS at Embrapa Rice and Beans (snpPV0025 - *Phg-2*; snpPV0027 - *Phg-5*; snpPV0046 - *Co-u*; snpPV0068 - *Co-4*²; snpPV0070 - *Co-4*²; snpP8282v3-817 - *Co-4*²; snpPV0079 - *Phg-5*) and other programs interested in these resistance alleles.

The markers snpPV0025 and snpPV0070 associated with the *Phg-2* and *Co-4* loci, respectively, are indicated to monitor the presence of target alleles in crosses involving well-characterized parental lines, such as Mexico 54 (*Phg-2*) and SEL1308 (*Co-4*²).

The genotyping systems based on hydrolysis probes developed in this study (TaqMan® SNP) for the snpPV0070, snpP8282v3-817 (*Co-4*²) and snpPV0025 (*Phg-2*) markers enabled the specific amplification of target alleles and are therefore suitable for use in MAS activities for the common bean.

The snpPV0070 and snpP8282v3-817 markers showed high selection efficiency (99.7 and 99.8%, respectively) for the allele that confers anthracnose resistance located in the *Co-4* locus and may considerably improve efficiency in identifying superior genotypes in the common bean breeding program for resistance to anthracnose and angular leaf spot.

Declarations

Author contribution

Lucas Gomes-Messias, Rosana Vianello and Thiago Souza planned the study; Lucas Gomes-Messias, Luana Rodrigues, Joney Júnior were responsible for the laboratory analysis; Ana Paula Mota developed and phenotyped the F₂ population; Bodo Raatz provided the sequence and expertise of the SNP markers; Lucas Gomes-Messias was responsible for the data analysis; Lucas Gomes-Messias and Rosana Vianello drafted the manuscript; The final version of the manuscript was revised by all authors.

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Journal relevant subheadings: plant genetics; resistances; and tropical crops.

This paper is a cooperative contribution on validation of SNP markers linked to disease resistance genes for their effective use in the routine of Embrapa (Brazilian Agricultural Research Corporation) common bean breeding program. The main goal was to characterize and select elite parent lines according the presence of disease resistance alleles, as well as validate SNP markers for MAS of superior genotypes from populations derived from these parent lines. It reports an elegant example of cross validation and effective use of SNP markers for MAS in an applied breeding program of a very social important orphan crop (common bean or dry bean).

Data Availability

Relevant data are included in this paper and its associated Online Resources.

Ethics approval and consent to participate

Not applicable

Consent for publication

The authors give consent for the publication.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

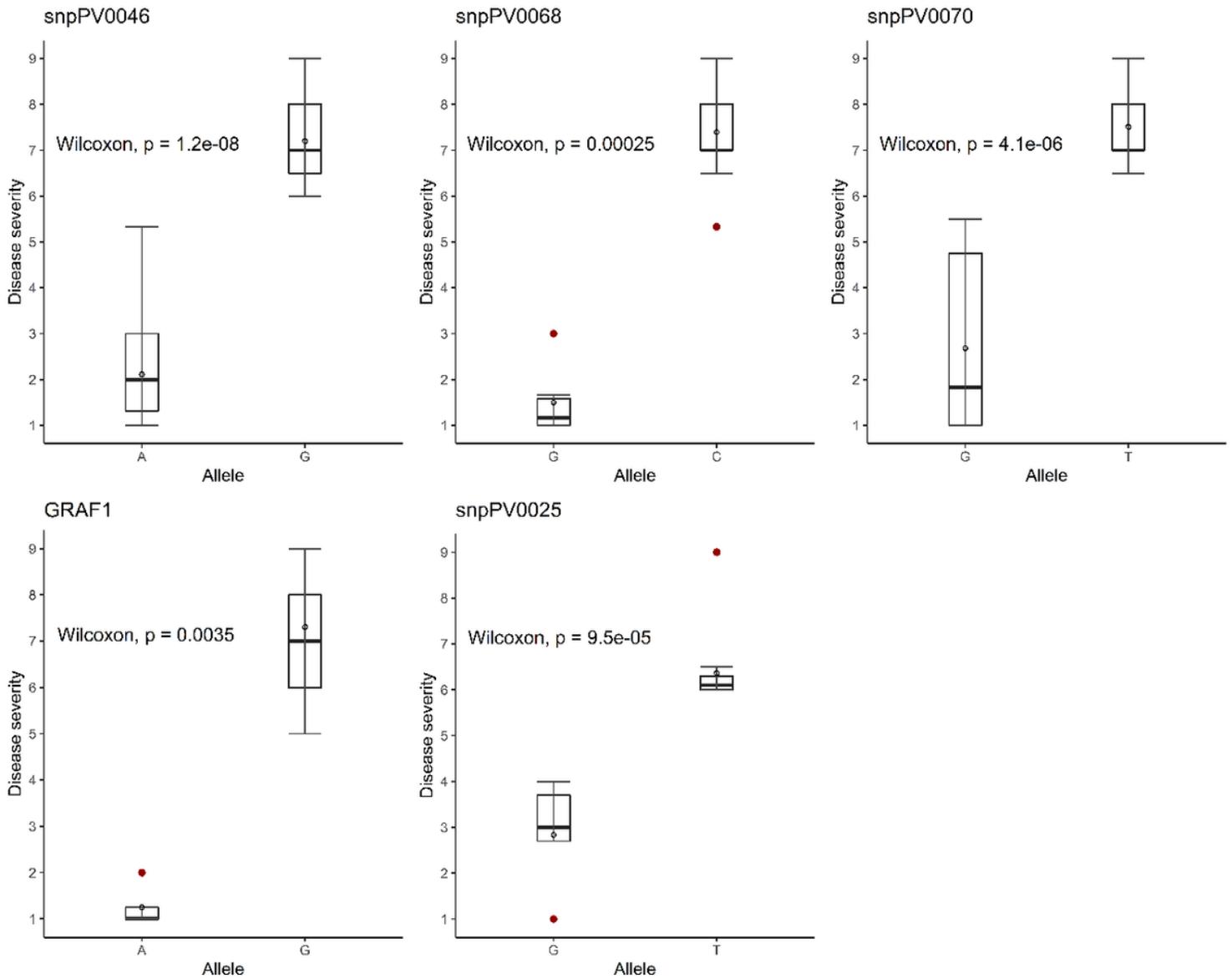


Figure 1

illustrates the importance of resistance alleles Co-u ("A"), Co-42 ("G") and Phg-2 ("G") in reducing mean phenotypic values in the lines that contain them.

Supplementary Files

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