Fine-mapping of angular leaf spot resistance gene \textit{Phg-2} in common bean and development of molecular breeding tools

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Abstract

**Key message** The Common Bean Angular Leaf Spot Resistance Gene \textit{Phg-2} was fine-mapped to a 409-Kbp region, and molecular markers for breeders were developed and validated in field experiments.

**Abstract** Common bean (\textit{Phaseolus vulgaris} L.) is an important food legume in Latin America, Asia and Africa. It is an important source of protein, carbohydrates and micro-minerals, particularly for smallholder farmers. Common bean productivity is affected by angular leaf spot (ALS) disease caused by the pathogenic fungus \textit{Pseudocercospora griseola}, resulting in significant yield losses, particularly in low-input smallholder farming systems in the tropics. The ALS resistance gene \textit{Phg-2}, which was found in several highly resistant common bean genotypes, was investigated in crosses between Mesoamerican pre-breeding lines and elite Andean breeding lines. Next-generation sequencing (NGS) data sets were used to design new SNP-based molecular markers. The \textit{Phg-2} locus was confirmed to be the major locus providing ALS resistance in these crosses. The locus was fine-mapped to a 409-Kbp region on chromosome 8. Two clusters of highly related LRR genes were identified in this region, which are the best candidate genes for \textit{Phg-2}. Molecular markers were identified that are closely linked to the \textit{Phg-2} resistance gene and also highly specific to the donor germplasm. Marker-assisted selection (MAS) was used to introgress the \textit{Phg-2} resistance locus into Andean breeding germplasm using MAB lines. The usefulness of molecular markers in MAS was confirmed in several field evaluations in complex breeding crosses, under inoculation with different ALS pathotypes. This project demonstrates that NGS data are a powerful tool for the characterization of genetic loci and can be applied in the development of breeding tools.

**Introduction**

Common bean (\textit{Phaseolus vulgaris} L.) is the most important food legume for direct human consumption around the world (Broughton et al. 2003). Common bean is grown in the tropics in Eastern/Southern Africa as well as Latin America and...
Asia. In Africa and the Americas alone, production exceeds 13.5 million t/year (FAO 2016). Beans are of particular importance to smallholder farmers that depend on the staple for its calories, protein and nutritional value.

Bean production is affected by many constraints including pests and diseases as well as abiotic stresses such as drought or low soil fertility. Smallholder farming systems with low inputs of agrochemicals are particularly vulnerable to these stresses. Angular leaf spot (ALS), caused by Pseudocercospora griseola (Sacc.) Crous and Braun [previously known as Phaeoisariopsis griseola (Sacc.) Ferrari (Crous et al. 2006)], is a major production constraint of common bean in the tropics (Wortmann et al. 1998). Being a widespread and devastating disease, ALS can cause yield losses up to 80% (Schwartz et al. 1981; Correa-Victoria et al. 1989).

Common bean germplasm is divided into two gene pools that were domesticated individually, the mainly large-seeded Andean gene pool, and the small-to-medium-seeded Mesoamerican gene pool (Gepts and Bliss 1985; Bitocchi et al. 2012). Accordingly, P. griseola pathotypes can also be classified into two groups, namely Andean and Mesoamerican, which co-evolved with the gene pools of its host (Guzmán et al. 1995). The Andean class of isolates are mainly pathogenic on Andean beans, while the Mesoamerican isolates are more pathogenic on Mesoamerican beans but also affect Andean beans, showing a greater diversity of virulence (Pastor-Corrales et al. 1998).

Many efforts have increased the knowledge about pathotypes, have characterized resistance genes and have generated molecular markers for marker-assisted selection (MAS) (recently reviewed by Nay et al. 2019). Five major resistance genes have been confirmed, named Phg-1 to 5, identified in different resistance sources from both gene pools of common bean. Phg-2 at the end of chromosome 8 was reported in several highly resistant genotypes and probably represents the strongest ALS resistance locus characterized so far providing resistance to many isolates. It was originally discovered in the Mesoamerican cultivar Mexico 54 as a single-dominant gene and named Phg-2 by Sartorato et al. (1999) studying a cross between Mexico 54 × Rudá. Genetic mapping revealed that the RAPD markers OPN02, OPAC14, OPAC15, and OPE04 were linked to Phg-2, at 5.9, 6.6 and 11.8 cM, respectively (Sartorato et al. 2000). A resistance locus at the end of chromosome 8 was also found in the Mesoamerican lines Cornell 49-242, MAR 2, G10474, BAT 332 and G10909 (Ferreira et al. 2000; Nietsche et al. 2000; Caixeta et al. 2003; Mahuku et al. 2004, 2011), which may represent allelic variations of Phg-2 to Mexico 54. Further SCAR markers PF5, PF9, and PF13 were developed during these efforts, which are also linked to the Phg-2 gene.

Breeding for disease resistance is considered the most sustainable method to reduce production losses due to diseases. Breeding for ALS resistance has mainly relied on phenotypic selection (Arantes et al. 2010), but occasionally MAS has been employed (Namayanja et al. 2006). A variety of molecular marker systems has been used to study disease resistance in common bean. Recently, SNP markers are becoming the dominant marker type as they are the most abundant and amendable to high-throughput genotyping platforms. SNP-based markers tagging ALS resistance have been converted from previous marker systems. Markers that are tightly linked to the resistance gene and highly specific for the resistance source are available for Phg-4 (Keller et al. 2015), and whole-genome sequencing data were used to develop germplasm-specific markers for five ALS resistance loci (Lobaton et al. 2018).

This study aimed to characterize and fine-map the Phg-2 gene and to develop molecular breeding tools to utilize this ALS resistance gene in breeding. Mesoamerican pre-breeding lines carrying Phg-2 from the resistance source G10474 were available to introgress the resistance locus into Andean elite germplasm. Genetic fine-mapping was used to delimit the Phg-2 locus. Breeder-ready markers were developed and validated in complex genetic backgrounds evaluated for disease resistance in several field trials.

Materials and methods

Plant material

Several Mesoamerican pre-breeding lines coded MAB (after “mancha angular” the Spanish word for ALS) with the pedigree (MD 23-24 × (G4691 × G10474)) × (G4090 × 9824-56-2) were developed at the International Center for Tropical Agriculture (CIAT, Colombia). The elite Mesoamerican breeding line MD 23-24 was crossed with ALS resistance sources G10474 and G4691 and sources for Bean Common Mosaic Virus. Different resistance sources were used in the crosses, following a strategy to pyramid several resistance genes in the same germplasm. The MAB lines selected for this study were: MAB 348, MAB 349, MAB 351, MAB 352, MAB 353, MAB 354, and MAB 484, due to their observed resistance to the disease in the field (CIAT location Santander de Quilichao, Colombia). Only the line MAB 484 has a different pedigree ((MAB 163 × SER 31) × (SEN 22 × SER 7)) compared to the other MAB lines described above.

To further introgress ALS resistance into beans with Andean seed type, MAB lines were crossed to CAL 96 and other elite Andean breeding germplasm. The populations used for fine-mapping in this project are crosses between the seven MAB lines and CAL 96 (pedigree: MAB × CAL 96). CAL 96 is a commercial variety from the Andean gene pool valued for its high-quality grain and its wide adaptation in East and Southern Africa, which is susceptible to ALS. As
generations were advanced, plants that were heterozygous for associated markers were identified to create residual heterozygous lines (RHL), as a tool for fine-mapping.

**ALS phenotyping**

Phenotypic evaluations under greenhouse conditions were carried out at CIAT headquarters in Palmira, Colombia (latitude 3° 29′ N; longitude 76° 21′ W; 965 masl, average temperature 23 °C).

Four different isolates of the pathogen *P. griseola* were tested to evaluate the response of the samples to the infection. Colombian isolate Pg331-1 from Santander de Quilichao belonging to race 63–63 was selected for the screening of the population because the disease response showed clear differentiation between resistant and susceptible plants. For field inoculations, isolates are used that have been collected at those same locations.

For phenotypic evaluations during the fine-mapping process, plants were grown in the greenhouse and inoculated with the isolate 17 days after sowing following CIAT’s standard evaluation procedures (Castellanos et al. 2011). Disease development was recorded 10, 12, 14 and 17 days after inoculation using a standard visual scale where scores 1–3 represent resistant, 4–6 intermediate and 7–9 susceptible plants (van Schoonhoven and Pastor-Corrales 1987). The area under disease progress curve (AUDPC) was calculated with the isolate 17 days after sowing following CIAT’s standard evaluation procedures (Castellanos et al. 2011). Every read was scanned for the first 6-mer of the adapter sequence, and reads were then trimmed to only retain the common bean sequence. Trimmed reads were aligned to the *Phaseolus vulgaris* v2.1 reference genome (https://phytozome.jgi.doe.gov/pz/portal.html, accessed 2017) (Schmutz et al. 2014), using bowtie2 with default parameters (Langmead and Salzberg 2013). Alignments were coordinate-sorted and indexed using SAM Tools (Li et al. 2009). Variant discovery was performed by running the NGSEP FindVariants module. The maximum base-quality score was set to 30, and the minimum quality for reporting a variant was set to 40; five bases at the 5′ end and five bases at the 3′ end of each read were ignored. Distinct to GWS, as required for GBS data, the detection of repetitive regions, copy number variants (CNVs) and other structural variants was turned off. Correspondingly, due to the nature of GBS experiments, the maximum number of reads allowed to start at the identical position which was raised to 100. The prior heterozygosity rate was set to 10^{-4}, and minor allele frequency was set to 0.05 as in other *Phaseolus* populations (Perea et al. 2016; Lobaton et al. 2018). In order to obtain the list of variant sites from GBS populations in combination with the WGS sequencing data, the variant (VCF) files developed for WGS and GBS data sets were combined using the MergeVariants module. Afterward, all samples were genotyped at the variant sites by running the FindVariants module again, keeping all parameters unchanged except for the minimum variant quality score, which we set to 40 to retain higher-quality genotype calls. Finally, the MergeVCF module was used to join all the VCFs into a single file gathering the genotyping information of the whole experimental population.

**Read alignment, variant detection, genotyping and merging of variants**

SNP-based molecular markers were used to fine-map the *Phg*-2 gene locus. Based on the physical position of SCAR marker PF5 (Mahuku et al. 2004), SNPs were initially selected covering a region of 1.74 Mbp, which represents ~5 cM up- and downstream of the reported position for PF5. Further markers were developed at different stages of the
fine-mapping process, based on improved information on the Phg-2 location.

Taking advantage of several sets of next-generation sequencing (NGS) data, SNPs were either selected from a panel of common bean genotypes sequenced by WGS (Lobaton et al. 2018), or from a panel of 600 breeding lines genotyped by GBS using the ApeKI enzyme (Elshire et al. 2011). The genotyped panel included three of the parental MAB lines MAB 348, MAB 349 and MAB 484, as well as G10474 and CAL 96.

For breeding marker development, SNPs were sought that were unique for the MAB lines and/or G10474 in a panel of Mesoamerican and Andean genotypes. To develop markers for the fine-mapping study, the extensive list of SNPs was utilized that differentiates Mesoamerican from Andean gene pools (Lobaton et al. 2018), or from a panel of 600 breeding lines genotyped by GBS using the ApeKI enzyme (Elshire et al. 2011). The genotyped panel included three of the parental MAB lines MAB 348, MAB 349 and MAB 484, as well as G10474 and CAL 96.

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A combined panel of 600 lines genotyped by WGS and GBS was utilized to select further candidate SNPs for breeding markers. MAB 348, MAB 349 and G10474 were defined as resistance source lines, and 22 lines that had previously been reported to show ALS resistance were set aside as extended positive controls (Online Resource 5). Andean resistance sources G5686 and AND 277 were ignored, because Andean germplasm may have other resistance genes and thereby confuse the association. The remaining lines were used as the susceptible set. Polymorphisms in the region between 61,879,947 and 62,139,256 bp on chromosome 8 were filtered, selecting SNPs (1) with homozygous genotype calls for the source lines, (2) the same genotype call for all source samples, (3) that were biallelic, and (4) with a source allele frequency in the rest of the panel below 10%.

### DNA extraction and genotyping

Total genomic DNA from all the plants sampled in the greenhouse was extracted either from young trifoliate leaves or from seed powder in 96-well plates using liquid nitrogen and an SDS/NaCl-buffer-based DNA extraction Mini-Prep protocol (modified from King et al. 2014). DNA quality and concentration were visually estimated by electrophoresis on 1% agarose gels. DNA was then diluted 1:10 with distilled water for PCR.

For the field experiments, DNA was extracted from young tissue in 96-well plates. One disk from young trifoliate leaves from each plant was obtained using a hollow punch and then placed into a well of the plate and stored at −80 °C. DNA was extracted with the alkaline protocol, 100 µl extraction buffer (50 mM NaOH, 1% Tween 20) was added to each well containing the frozen tissue and the plate was placed in a water bath with boiling water for 10 min. Then, 50 µl neutralization buffer (100 mM Tris HCl, 1.7 mM EDTA, pH 7.3) was added to each well and the plate mixed by vortexing at moderate speed. DNA was diluted 1:10 with distilled water for PCR.

### SCAR genotyping

The SCAR marker PF5 was amplified by PCR in 15 µl reaction volumes containing 5 µl of genomic DNA, 1X PCR Buffer [10 mM Tris–HCl pH 8.8, 50 mM KCl, 0.8% (v/v) Nonidet P40 (Fermentas)], 2.5 mM of MgCl2 (Fermentas), 0.4 mM of dNTPs mix (Promega), 0.2 µM of each primer (forward and reverse), and 0.15 µl of Taq polymerase (laboratory made). The PCR was performed on an Eppendorf Mastercycler under following conditions: a first denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s, and then a final extension step at 72 °C for 10 min before cooling down to 4 °C. PCR products were visualized on 1.5% agarose gels in 1X BS Buffer [10 mM Tris–HCl pH 7.3] was added to each well and the plate mixed by vortexing at moderate speed. DNA was diluted 1:10 with distilled water for PCR.

### SNP genotyping by melting temperature Tm-shift (Tms)

Primers were designed according to Wang et al. (2005) using the software Primer 3 (Untergasser et al. 2012). Amplification and melting point analysis for allele determination were performed on a fluorescence-detecting thermocycler (CFX384 Real-Time System, Bio-Rad) with EvaGreen fluorescent dye (Biotium, Fremont, CA, USA). The PCR volume was 15 µl containing 5 µl of genomic DNA, 1X PCR Buffer, 2 mM of MgCl2, 0.2 mM of dNTP mix, 0.2 µM of each primer (two allele-specific forward primers and the common reverse primer), 0.8X EvaGreen, and 0.1 µl of Taq polymerase (laboratory made) under the following thermal profile: a first denaturation step at 94 °C for 3 min, then 35 cycles of denaturation at 92 °C for 20 s, annealing for 20 s (temperature specific to each primer trio), extension at 72 °C for 20 s, and finally, 1 min at 95 °C. The melting curve step ramping from 70 to 95 °C and increments of 0.5 °C/20 s under fluorescence detection followed the amplification.

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SNP genotyping by high-resolution melting (HRM) analysis

HRM primers were designed flanking a SNP with an amplicon size not exceeding 100 bp. PCR amplification was carried out as described before for Tm-shift genotyping on the CFX384 Real-Time System with the difference that the amplification product was melted ramping from 65 to 95 °C and incrementing 0.2 °C/20 s. HRM analysis was performed using the software Bio-Rad Precision Melt Analysis 1.2 (Bio-Rad).

SNP genotyping using LGC KASP markers

KASP™ chemistry is based on using three primers, two allele-specific and a common reverse primer, similar to Tm-shift, whereas different tags are used to incorporate fluorescence-labeled oligos. KASP assays were purchased from the commercial provider LGC genomics (Hertfordshire, UK) (http://www.lgcgroup.com/products/kasp-genotyping-chemistry) and run on the CFX384 Real-Time System following the provider’s instructions for 5 µl reaction volumes.

Identification of candidate genes

Annotations of genes in the fine-mapped region on chromosome 8 were downloaded from the phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html, accessed 2017). Annotations were evaluated for their reported involvement in disease resistance, and such genes were identified as candidates.

Results

Phg-2 segregates as a single gene in crosses with MAB lines

The MAB lines are pre-breeding lines that were selected for bush growth habit, small red seed and good agronomic performance, combined with repeated phenotypic ALS resistance under field conditions. Most MAB lines were found to be resistant to several ALS strains (Table 1). To introgress ALS resistance into beans with Andean seed type, MAB lines were crossed to CAL 96 and other Andean elite lines. The inheritance of ALS resistance was characterized in these crosses evaluating seven MAB × CAL 96 cross populations in F3 generation (Table 2). A Chi-squared test was conducted to investigate whether the ratio of resistant and susceptible individuals deviates from the 5:3 ratio expected for a single-dominant resistance locus. In five populations, the observed ratio was not significantly different to the expected 5:3 ratio, in line with a single-dominant resistance gene. Two populations deviated significantly (p < 0.05) from the expected ratio, which may indicate involvement of other loci or insufficient sample size. In all cases, ALS resistance was transferred from the MAB sources to the new populations.

Co-segregation of ALS resistance with the Phg-2 locus was evaluated in these 179 F3 plants (50 F2-derived families). Crosses were evaluated phenotypically and genotypically with marker ALS_08_62193174 (Fig. 1). Disease scores largely fell into two distinct classes of resistant and susceptible lines suggesting single-gene resistance. The genotype of the Phg-2 marker clearly co-segregated with the phenotype, while the few non-concordant samples may be phenotypic escapes or due to recombinations. Data demonstrate that the Phg-2 gene confers ALS resistance in this population.

To investigate the source ALS resistance, the parental lines of the crosses were evaluated phenotypically and genotyped with the SSR marker PF5, previously published to be linked with Phg-2 (Mahuku et al. 2004), and 10 additional SNP-based markers in the vicinity (Table 1). CAL 96 is susceptible to all P. griseola strains; other lines show resistant disease scores (1–3) to some or all of the isolates of the pathogen. Based on the markers tested here, the Phg-2 locus in all MAB shows the same haplotype as G10474 and not that of the other resistance source G4691 employed in the crosses.

Phg-2 was fine-mapped to a 409-Kbp region on chromosome 8

To fine-map the Phg-2 region, we selected lines from the different MAB crosses that showed recombinations in this region for further analysis. In three of the seven MAB × CAL 96 crosses, useful recombination events were identified in the Phg-2 region evaluating F4 lines (CAL 96 × MAB 484, CAL 96 × MAB 348, and CAL 96 × MAB 349). In further fine-mapping steps, only the latter two crosses were used for the analyses as descendants showed a clear phenotypic segregation and strong phenotype-genotype association for the Phg-2 locus. Recombinants were identified in further generations, and if possible, several segregating descendants of each recombinant were phenotyped in the following generation, to determine whether markers on either side of the recombination were associated with the phenotype (i.e. on which side of the recombination event the resistance gene locates). Plants heterozygous at the Phg-2 locus and those carrying interesting recombinations were advanced and evaluated as follows (depicted in Online Resource 1): 703 F4 seeds from 46 F3 recombinant plants were genotyped with four SNP markers covering a region of 1.6 Mbp on chromosome 8, and from those, 72 seeds were selected to be sown and evaluated phenotypically and again genetically. Sixteen new recombination
### Table 1  Phenotypic ALS evaluations and genotyping data of MAB lines and other parental germplasm

| Line   | Pg3 | Pg331 | Pg335 | Phenotype | Phenotypic   | ALS_08_6156896 | ALS_08_61705350 | ALS_08_61730261 | ALS_08_61731208 | ALS_08_61825915 | ALS_08_61901622 | ALS_08_62047925 | ALS_08_62139256 | ALS_08_62170288 | ALS_08_62193174 |
|--------|-----|-------|-------|-----------|--------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| MAB 348 | 1.0 | 2.7   | 2.9   | 2.2       | R +          | C              | T              | A              | G              | A              | T              | C              | A              | G              | G              |
| MAB 349 | 1.1 | 2.1   | 3.7   | 2.2       | R +          | C              | T              | A              | G              | A              | T              | C              | A              | G              | G              |
| MA B 351 | 1.2 | 1.8   | 2.2   | 1.6       | R +          | C              | T              | A              | G              | A              | T              | C              | A              | G              | G              |
| MAB 352 | 1.2 | 1.1   | 2.3   | 2.9       | R +          | C              | T              | A              | G              | A              | T              | C              | A              | G              | G              |
| MAB 353 | 1.2 | 1.9   | 3.4   | 2.3       | R +          | C              | T              | A              | G              | A              | T              | C              | A              | G              | G              |
| MAB 354 | 1.0 | 2.4   | 2.7   | 3.4       | R +          | C              | T              | A              | G              | A              | T              | C              | A              | G              | G              |
| MAB 484 | 1.3 | 2.3   | 8.0   | 8.6       | Isolate      | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       |
| G10474 | 1.0 | 1.0   | 1.0   | 1.0       | R +          | C              | T              | A              | G              | G              | T              | C              | A              | G              | G              |
| G4691  | 1.2 | 5.2   | 7.3   | 2.7       | Isolate      | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       |
| MD 23-24 | 1.0 | 5.1   | 7.3   | 2.5       | Isolate      | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       |
| G4900  | 1.0 | 8.0   | 4.7   | 9.0       | Isolate      | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       | Isolate       |
| 9824-56-2 | –   | –     | –     | –         | No data      | T              | T              | G              | G              | G              | C              | G              | T              | T              | T              |
| CAL 96 | 6.2 | 6.7   | 8.5   | 8.4       | S –          | T              | C              | G              | C              | G              | T              | G              | T              | T              | T              |

*R* resistant, *S* susceptible, *NA* no amplification
†Mesoamerican isolates
*Andean isolate*
events were found and those lines were advanced to the F5 generation. Results confirmed that the Phg-2 locus is flanked by markers ALS_08_60980148 and ALS_08_62721133 (Fig. 2); hence, more markers were developed in this region. Further 63 plants were evaluated phenotypically and genotypically in F5 and 320 descendant plants in F6 generation.

The Phg-2 region was progressively delimited, and more SNP-based molecular markers were developed and genotyped during the process to further characterize recombinants, based on the positional information obtained in previous generations. Combining all data sets, the Phg-2 locus was delimited to a 408,995-bp region flanked by markers ALS_08_61730261 and ALS_08_62139256 (Fig. 2).

**Candidate genes for ALS resistance on chromosome 8**

The 409-Kbp Phg-2 region identified here encompasses 70 genes (Online Resource 3). Eighteen of those are annotated as members of gene families that have been reported to be involved in plant defense; 14 are annotated to harbor LRR domains. LRR genes have been reported to directly detect and bind to pathogen proteins activating a resistance response (DeYoung and Innes 2006). Other candidate genes are annotated to encode kinases, which play a role in many signaling networks, including pathogen defense (Romeis 2001). Hence, these genes represent the likeliest candidates encoding the Phg-2 gene (Table 3). The LRR genes at the start of the fine-mapped region, from Phvul.008G273600 to Phvul.008G274100, represent a cluster of closely related genes. A second group of closely related LRR genes is found between Phvul.008G276100 and Phvul.008G277352.

Analysis of WGS data comparing G10474 with a panel of genotypes (Lobaton et al. 2018) revealed a total of 36,960 polymorphisms in the 409-Kbp region composed of SNPs, INDELs and copy number variants (CNVs). One hundred and seventeen SNPs differentiate G10474 against > 90% of the other genotypes, 101 of these SNPs are genic, 20 represent missense mutations, of which 3 appear in the likely candidates (Online Resource 4). Missense SNPs in G10474 were found in Phvul.008G275000 (positions 61,853,763 and 61,855,007 bp) and in Phvul.008G275100 (61,859,887 bp). These represent the best available candidate causal variants for the observed phenotype. However, G10474 may have further copies of this gene family, which are not present in the reference G19833 and contribute to the resistance. With the available depths and type of sequencing data, these cannot be identified.

**Phg-2 markers for marker-assisted selection (MAS)**

Marker ALS_08_62193174 was used to tag the Phg-2 locus for the marker-assisted introgression from Mesoamerican MAB sources into Andean breeding lines. This marker does not specifically tag Phg-2 in the Mesoamerican gene pool, but it can be utilized to tag this locus in an Andean background. Ninety-seven F4 lines derived from simple crosses

### Table 2 Segregation ratios of ALS resistance in seven F3 populations of CAL 96 × MAB crosses

| Cross                | # Plants per population | Expected ratios resistant/susceptible (5:3) | Observed ratios resistant/susceptible | $\chi^2$ | p value |
|---------------------|-------------------------|---------------------------------------------|---------------------------------------|----------|---------|
| CAL 96 × MAB 348    | 38                      | 24:14                                       | 30:8                                  | 4.3860   | 0.036   |
| CAL 96 × MAB 349    | 35                      | 22:13                                       | 22:13                                 | 0.0019   | 0.965   |
| CAL 96 × MAB 354    | 15                      | 9:6                                         | 13:2                                  | 3.7378   | 0.053   |
| MAB 352 × CAL 96    | 27                      | 17:10                                       | 12:15                                 | 3.7556   | 0.053   |
| MAB 351 × CAL 96    | 19                      | 12:7                                        | 14:5                                  | 1.0140   | 0.314   |
| MAB 353 × CAL 96    | 11                      | 7:4                                         | 5:6                                   | 1.3636   | 0.243   |
| CAL 96 × MAB 484    | 34                      | 21:13                                       | 27:7                                  | 4.1490   | 0.042   |
| All crosses         | 179                     | 112:67                                      | 123:56                                | 2.9501   | 0.086   |

Plants were inoculated with ALS isolate Pg331-1. Single-dominant gene hypothesis was evaluated using the Chi-squared test ($\chi^2$).
of 9 resistance sources with 8 elite Andean genotypes (Fig. 3a) were evaluated for ALS response in a non-replicated field trial, after inoculation with a mix of either Mesoamerican or Andean ALS isolates (Fig. 3b). Lines that carried the resistance-associated allele of ALS_08_62139256 showed higher levels of resistance in both leaves and pods for both pathotype mixes. This demonstrates the utility of the MAB resistance sources and new SNP-based markers for MAS in breeding, as the association holds up in complex genetic backgrounds and provides resistance in field evaluations with complex pathotype compositions.

Marker–trait associations were also validated in field trials with early-generation breeding lines of the Mesoamerican gene pool. Lines derived from 3-, 4-, 5-, and 6-way crosses were genotyped in the final F1 generation with the marker ALS_08_62139256. At the Quilichao field station, 720-derived F1:2 families were evaluated for ALS (Fig. 3c). Practically, all families that were genotyped homozygous for the resistance allele showed high levels of field resistance (Fig. 3d). However, many lines that carried the susceptible allele for this marker also proved to be resistant, suggesting that other alleles of Phg-2 or other resistance genes also confer resistance here. Seventy-five lines from individual selections were re-evaluated in the following generation at Darien field station (Fig. 3e). Here, only lines homozygous for the resistance-associated marker allele were
resistant, indicating disease pressure from pathotypes different to the Quilichao experiment; in this case, only the Phg-2 allele tagged by the marker confers resistance. In summary, these results clearly confirm the marker–trait association in complex genetic backgrounds under field conditions and demonstrate its utility for MAS in breeding.

Sequencing data provide a rich polymorphism resource that can be mined for marker design. Available whole-genome re-sequencing (WGS) and genotyping-by-sequencing (GBS) data were analyzed to identify further polymorphisms for marker design with optimal positioning and differentiation between resistance sources and susceptible materials.

Polymorphisms in the region between 61,730,261 bp and 62,139,256 bp on chromosome 8 were investigated in 600 breeding lines, varieties and landraces, to identify new SNPs for markers, as described in M&M (Online Resource 5). Interestingly, sequencing data show that G10474 and MAB seem to have a different haplotype up to about 61,879,947 bp, as several markers are observed to be polymorphic upstream of that position. GBS data are not completely conclusive, but the available data suggest that Phg-2 is located in the remaining interval of 259 Kbp (61,879,947 to 62,139,256 bp).

An ideal SNP would have a high allele frequency in the group of positive controls while having a very low frequency of the resistance source allele in the remaining panel and a high number of SNP calls in the whole panel. Table 4 lists further 14 polymorphisms that differentiate resistance sources from the panel of breeding lines. These are promising to design further markers to tag the Phg-2 locus in breeding crosses, in case the known markers are not suitable for certain genotyping platforms or specific genetic backgrounds.

**Discussion**

**Phg-2 was fine-mapped to a 409-Kbp region containing LRR resistance gene clusters**

The Phg-2 locus is the most effective ALS resistance locus reported with best cross-pathotype stability. The resistance gene Phg-2 was first identified by Sartorato et al. (1999) in the resistance source Mexico 54 and subsequently discovered in several resistance sources, such as G10474 (Mahuku et al. 2004), MAR 2 (Ferreira et al. 2000), Cornell 49–242 (Nietsche et al. 2000), Ouro Negro (Queiroz et al. 2004) and G10909 (Mahuku et al. 2011). At this point, the authors assume that all of these represent the same resistance locus using the same gene name Phg-2. In case an entirely different gene confers resistance in Mexico...
54 compared to G10474, e.g. a different LRR gene copy, this may have to be corrected at a later stage.

Previous studies reported the location of Phg-2 at a distance of about 5 cM from marker PF5 (61,251,610 bp) (Mahuku et al. 2004), or at 3 cM distance of marker g796 (61,514,592 bp) (Miller et al. 2018). In this project, the Phg-2 locus was identified much more precisely to a region of 409 Kbp. WGS data on G10474 (Lobaton et al. 2018) and lower-quality GBS data on MAB lines suggest that Phg-2 is located in a 259-Kbp interval (61,879,947– 62,139,256 bp) where G10474 and the MAB lines share the same haplotype (Online Resource 5). However, GBS data should be interpreted with care and results confirmed by other genotyping methods.

Marker data show that the origin of the Phg-2 locus in all MAB lines is G10474. Resistance sources G10474 and G4691 that were used in the crosses may harbor additional resistance genes, but probably these were not selected for under the applied ALS screening conditions.

Fine-mapping identified a locus with clusters of LRR genes belonging to two closely related LRR gene families. Similarly, previous resistance gene mapping efforts resulted in regions with repetitive gene clusters (Vallejos et al. 2006; Keller et al. 2015). Out of the 70 genes in the candidate region, 15 LRR genes represent the likeliest candidate genes for Phg-2. WGS data were used to identify non-synonymous SNPs in candidate genes that are associated with resistance which are candidate causal variants for...
the resistance. However, a further characterization of these principal candidates is challenging, as G10474 may have other or different copies of these LRR family genes. Due to the short-read lengths of the available G10474 WGS data, it is not possible to assemble such a repetitive locus, which would be required to identify the actual Phg-2 gene. Long-read sequencing platforms, such as PacBio, could facilitate the assembly of such repetitive regions.

NGS data sets are invaluable tools supporting fine-mapping strategies

The fine-mapping strategy applied in this project used residual heterozygous lines (RHLs), identifying and evaluating recombinants in consecutive generations. RHLs have been successfully employed in genetic mapping in common bean and other crops (Haley et al. 1994). This RHL method has less phenotypic noise due to the more homogeneous genetic background and is more resource efficient requiring less phenotypic evaluations. Hence, it can be applied in fine-mapping projects with lower heritability phenotypes or resource-demanding phenotyping requirements. However, this method is also more time-consuming than one-step approaches for recombinant identification, which were reported, for example, for bean common mosaic virus resistance mapping in common bean (Vallejos et al. 2006) or in soybean (Wu et al. 2018).

The growing availability of large data sets of sequencing data has a profound effect on marker design. Large databases of millions of SNPs and INDELs (Lobaton et al. 2018) contain ample polymorphisms that can be used for marker design, to achieve high marker density for nearly any genomic region. Sequencing data allowed this project to proceed with higher marker densities, where comparable previous fine-mapping effort stalled due to lack of markers (Vallejos et al. 2006; Oblessuc et al. 2013). Hence, with the availability of such data resources the success of fine-mapping is largely delimited by the genetic resolution, i.e. the size of the population and thereby the number of recombination events the can be identified and evaluated.

Deploying Phg-2 in breeding

The MAB pre-breeding lines are presented here as good sources for ALS resistance in breeding. Resistance alleles were introgressed from lines with undesirable climbing growth habits and non-commercial grain types into Mesoamerican bush breeding germplasm.

Resistance has now been transferred into a first generation of large-seeded Andean bush beans, termed AAB lines (Andean ALS-resistant bush). These lines were generated by MAS using an early marker ALS_08_62193174. Resistance was evaluated in complex crosses under field conditions in leaves and also in pods where the disease causes most economic damage in snap bean production. Pod disease scores are often not measured as screening of leaves after a few weeks is faster and easier. AAB lines are also considered pre-breeding lines as they do not yet meet the

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**Table 4 Candidate polymorphisms for ALS marker development tagging the Phg-2 locus**

| Position (bp) | Ref | Alt | Positive controls | Extended positive controls | Remaining lines |
|---------------|-----|-----|-------------------|---------------------------|----------------|
|               |     |     | MAB 348, MAB 349, G10474 | Resistance allele frequency (%) | n | Resistance allele frequency (%) | n |
| 61,901,182    | G   | T   | T                 | 4.76                      | 21 | 0.00                        | 356 |
| 61,999,071    | G   | A   | A                 | 5.26                      | 19 | 7.64                        | 471 |
| 61,999,210    | A   | G   | G                 | 5.00                      | 20 | 7.08                        | 551 |
| 62,043,108    | A   | T   | T                 | 75.00                     | 16 | 1.65                        | 484 |
| 62,043,144    | G   | A   | A                 | 75.00                     | 16 | 1.83                        | 491 |
| 62,043,203    | A   | C   | C                 | 83.33                     | 18 | 7.17                        | 488 |
| 62,043,204    | GTT  | T   | T                 | 82.35                     | 17 | 7.55                        | 437 |
| 62,047,897    | C   | T   | T                 | 58.33                     | 12 | 2.78                        | 396 |
| 62,047,952    | G   | A   | A                 | 58.33                     | 12 | 2.98                        | 369 |
| 62,048,236    | G   | A   | A                 | 85.71                     | 14 | 6.45                        | 124 |
| 62,048,237    | T   | C   | C                 | 85.71                     | 14 | 3.25                        | 123 |
| 62,048,255    | G   | T   | T                 | 88.24                     | 17 | 4.84                        | 124 |
| 62,139,186    | A   | G   | G                 | 66.67                     | 18 | 1.46                        | 481 |
| 62,139,256    | G   | A   | A                 | 61.11                     | 18 | 3.30                        | 273 |

A panel of 600 lines with available GBS and WGS sequencing data was evaluated; results are filtered from a larger set shown in Online Resource 5

Ref reference allele, alt alternative allele, n indicates the number of lines in the respective group with genotype calls
Resistance genes explaining its observed broad resistance. Other ALS resistance genes are known, and also, G10474 may harbor further polymorphisms (reviewed by Mundt 2018). Other ALS resistance genes are reported to lentils, potatoes, and several cereals against different pathogens. However, single-gene resistance to ALS is generally not expected to provide durable resistance, as pathogen evolution is a continuous, rapid process and resistance genes into one variety, provide better long-term resistance. This is the most effective ALS resistance gene in common bean. The locus was fine-mapped to a 409-Kbp region on chromosome 8, where two clusters of highly related LRR genes represent the best candidate genes for Phg-2.

NGS data sets have allowed to identify SNP markers for mapping and breeding, which are well positioned as well as highly specific to the donor germplasm. Introgression of the resistance locus using MAB lines has been demonstrated; highly specific markers are available that will most likely work in other genetic backgrounds. These tools and germplasm will aid future ALS resistance breeding activities. The NGS resources described here will be widely used to improve breeding markers for other diseases and traits. This project is an example on using NGS data as a powerful tool for the characterization of genetic loci and for their application in the development of breeding tools.

**Conclusion**

Phg-2 is the most effective ALS resistance gene in common bean. The locus was fine-mapped to a 409-Kbp region on chromosome 8, where two clusters of highly related LRR genes represent the best candidate genes for Phg-2.

NGS data sets have allowed to identify SNP markers for mapping and breeding, which are well positioned as well as highly specific to the donor germplasm. Introgression of the resistance locus using MAB lines into Andean material has been demonstrated; highly specific markers are available that will most likely work in other genetic backgrounds. These tools and germplasm will aid future ALS resistance breeding activities. The NGS resources described here will be widely used to improve breeding markers for other diseases and traits. This project is an example on using NGS data as a powerful tool for the characterization of genetic loci and for their application in the development of breeding tools.

**Author contribution statement** JG is involved in fine-mapping and writing; DS performed field evaluations; JDL NGS analyzed the data; VM is involved in supervision of field evaluations and Diana’s thesis; SB performed field evaluations in Quilichao; CJ performed supervision pathology; SB contributed to the development of MAB lines; BR is involved in writing and supervision.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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**References**

Arantes LDO, Abreu ÂDFB, Ramalho MAP (2010) Eight cycles of recurrent selection for resistance to angular leaf spot in common bean. Crop Breed Appl Biotechnol 10:232–237
Bitocchi E, Nanni L, Bellucci E et al (2012) Mesoamerican origin of the common bean (Phaseolus vulgaris L.) is revealed by sequence data. Proc Natl Acad Sci 109:E788–E796. https://doi.org/10.1073/pnas.1108973109
Broughton WJ, Hernández G, Blair M et al (2003) Beans (Phaseolus spp.)—model food legumes. Plant Soil 252:55–128. https://doi.org/10.1023/A:1024146710611
Caixeta ET, Bórèm A, De Azevedo Fagundes S et al (2003) Inheritance of angular leaf spot resistance in common bean line BAT 332 and identification of RAPD markers linked to the resistance gene.
Euphytica 134:297–303. https://doi.org/10.1007/BF00000494.41083.1f

Castellanos G, Jara C, Mosquera G (2011) Manejo del hongo en el laboratorio. Phaeoisariopsis griseola. In: Guías Prácticas de Laboratorio para el Manejo de Patógenos del Frijol. Centro Internacional de Agricultura Tropical (CIAT), Cali-Palmira

Cichy K, Porch T, Beaver J et al (2015) A Phaseolus vulgaris diversity panel for Andean bean improvement. Crop Sci 55:2149–2160. https://doi.org/10.2135/cropsci2014.09.0653

Correa-Victoria FJ, Pastor-Corrales MA, Saettler AW (1989) Angular leaf spot. In: Bean production problems in the tropics. Centro Internacional de Agricultura Tropical, Cali, Colombia, p 75

Crous PW, Liebenberg MM, Braun U, Groenewald JZ (2006) Re-evaluating the taxonomic status of Phaeoisariopsis griseola, the causal agent of angular leaf spot of bean. Stud Mycol 55:163–173. https://doi.org/10.3114/sim.55.1.163

DeYoung BJ, Innes RW (2006) Plant NBS-LRR proteins in pathogen sensing and host defense. Nat Immunol 7:1243–1249. https://doi.org/10.1038/nimi1410

Duitama J, Quintero JC, Cruz DF et al (2014) An integrated framework for discovery and genotyping of genomic variants from high-throughput sequencing experiments. Nucleic Acids Res 42:e44. https://doi.org/10.1093/nar/gkt1381

Elshire RJ, Glaubitz JC, Sun Q et al (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS ONE 6:1–10. https://doi.org/10.1371/journal.pone.0019379

FAO (2016) FAOSTAT. http://www.fao.org/faostat/en/#data/QC. Accessed 24 Mar 2018

Ferreira CF, Borém A, Carvalho GA et al (2000) Inheritance of angular leaf spot resistance in common bean and identification of a RAPD marker linked to a resistance gene. Crop Sci 40:1130–1133. https://doi.org/10.2135/cropsci2000.40411130x

Gepts P, Bliss FA (1985) F1 hybrid weakness in the common bean. J Hered 76:447–450. https://doi.org/10.1093/journal.HERE.76.5.447

Guzmán P, Gilbertson RL, Nodari RO et al (1995) Characterization of variability in the fungus Phaeoisariopsis griseola suggests coevolution with the common bean (Phaseolus vulgaris). Phytopathology 85:600–607. https://doi.org/10.1094/Phyto-85.600

Haley SD, Afanador LK, Braun U, Groenewald JZ (2006) Increasing the density of markers around a major QTL controlling angular leaf spot resistance in common bean and genome-wide analysis of dual domestications. Theor Appl Genet 128:813–826. https://doi.org/10.1007/s00122-010-9461-x

Keller B, Manzanares C, Jara C et al (2015) Fine-mapping of a major QTL controlling angular leaf spot resistance in common bean accession G 10474 and identification of an AFLP marker linked to the resistance gene. Crop Sci 44:1817–1824. https://doi.org/10.2135/cropsci2004.1817

Maiden GM, Henriquez MA, Montoya C et al (2011) Inheritance and development of molecular markers linked to angular leaf spot resistance genes in the common bean accession G10909. Mol Breed 28:57–71. https://doi.org/10.1007/s11032-010-9461-x

McDonald BA, Linde C (2002) Pathogen population genetics, evolutionary potential, and durable resistance. Annu Rev Phytopathol 40:349–379. https://doi.org/10.1146/annurev.phyto.40.12050.1.101443

Miller T, Gepts P, Kimmo S et al (2018) Alternative markers linked to the Phg-2 angular leaf spot resistance locus in common bean using the Phaseolus genes marker database. African J Biotechnol 17:818–826. https://doi.org/10.5897/AJB2018.16493

Mundt CC (2018) Pyramiding for resistance durability: theory and practice. Phytopathology 108:792–802. https://doi.org/10.1094/PHTY-12-17-0426-RVW

Namanya A, Buruchara R, Mahuku G et al (2006) Inheritance of resistance to angular leaf spot in common bean and validation of the utility of resistance linked markers for marker assisted selection outside the mapping population. Euphytica 151:361–369. https://doi.org/10.1007/s10681-006-9158-8

Nay MM, Souza TLPO, Raatz B, Mukanuski CM, Gonçalves-Vidigal MC, Abreu AFB, Melo LC, Pastor-Corrales MA (2019) A review of angular leaf spot resistance in common bean. Crop Sci (accepted)

Nietsche S, Borém A, Carvalho GA et al (2000) RAPD and SCAR markers linked to a gene conferring resistance to angular leaf spot in common bean. J Phytopathol 148:117–121

Oblessuc PR, Cardoso Perseguiuni JMK, Baroni RM et al (2013) Increasing the density of markers around a major QTL controlling resistance to angular leaf spot in common bean. Theor Appl Genet. https://doi.org/10.1007/s00122-013-2146-1

Pastor-Corrales MA, Jara C, Singh SP (1998) Pathogenic variation in, sources of, and breeding for resistance to Phaeoisariopsis griseola causing angular leaf spot in common bean. Euphytica 103:161–171

Pereira C, De-La-Hoz J, Cruz D, et al (2016) Bioinformatic analysis of genotype by sequencing (GBS) data with NGSEP. In: Proceedings of ISBRA 11th international symposium on bioinformatics research and applications, vol 17. pp 539–551. https://doi.org/10.1186/s12864-016-2027-7

Queiroz YT, Sousa CS, Costa MR et al (2004) Development of SCAR markers linked to common bean angular leaf spot resistance genes. Annu Rep Bean Improv Coop 47:237–238

Romeis T (2001) Protein kinases in the plant defence response. Curr Opin Plant Biol 4:407–414. https://doi.org/10.1016/S1369-5266(00)00193-X

Sartorato A, Nietsche S, Barros EG, Moreira MA (1999) SCAR marker linked to angular leaf spot resistance gene in common bean. Annu Rep Bean Improv Coop 43:23–24

Sartorato A, Nietsche S, Barros E, et al (2000) Rapid and Scar Markers Linked To Resistance Gene To Angular Leaf Spot in Common Beans

Schmutz J, McClean PE, Mamidi S et al (2014) A reference genome for common bean and genome-wide analysis of dual domestications. Nat Genet 46:707–713. https://doi.org/10.1038/nge.3008

Schwartz HF, Correa V, Pineda D et al (1981) Dry bean yield losses caused by Ascochyta angular, and white leaf spots in Colombia. Plant Dis 65(6):494–496

Song Q, Jia G, Hyten DL et al (2015) SNP assay development for linkage map construction, anchoring whole genome sequence and other genetic and genomic applications in common bean. G3 G3(5):2285–2290. https://doi.org/10.1534/g3.115.020594

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Untergasser A, Cutcutache I, Koressaar T et al (2012) Primer3—new capabilities and interfaces. Nucleic Acids Res 40:e115. https://doi.org/10.1093/nat/gks596
Vallejos CE, Astúa-monge G, Jones V et al (2006) Genetic and molecular characterization of the I locus of Phaseolus vulgaris. Genetics 172:1229–1242. https://doi.org/10.1534/genetics.105.050815
van Schoonhoven A, Pastor-Corrales MA (1987) Standard system for the evaluation of bean germplasm. CIAT, Palmira
Wang J, Chuang K, Ahluwalia M et al (2005) High-throughput SNP genotyping by single-tube PCR with Tm-shift primers. Biotechniques 39:885–892. https://doi.org/10.2144/000112028
Wortmann CS, Kirkby RA, Eledu CA, Allen DJ (1998) Atlas of Common Bean (Phaseolus vulgaris L.) Production in Africa. Pan-Africa Bean Research Alliance Centro Internacional de Agricultura Tropical (CIAT)
Wu M, Wu W-P, Liu C-C et al (2018) A bean common mosaic virus (BCMV)-resistance gene is fine-mapped to the same region as Rsv1-h in the soybean cultivar Suweon 97. Theor Appl Genet 131:1851–1860. https://doi.org/10.1007/s00122-018-3117-3

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