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Alternative markers linked to the *Phg-2* angular leaf spot resistance locus in common bean using the *Phaseolus* genes marker database

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Common bean (*Phaseolus vulgaris*) is a rich source of protein, vitamins, and micronutrients and is an important crop for food security throughout Latin America, Asia, and Africa. Among tropical and subtropical regions of the world, where the majority of beans are grown, yield losses due to the pathogenic fungus *Pseudocercospora griseola*, causing angular leaf spot (ALS), can be as high as 80%. The strategic use of marker-assisted selection (MAS) to pyramid multiple resistance genes into a single genetic background with preferred morphological and cooking characteristics is being implemented by six research groups throughout East Africa that make up the African Bean Consortium (ABC). Identifying unique markers that are polymorphic among multiple parents is a major source of marker attrition. In this study, an illustration of how 22 DNA sequences physically linked to the *Phg-2* ALS resistance locus were identified using the PhaseolusGenes marker database and checked for amplification and polymorphism among 16 ABC breeding parents are given. Only three polymorphic markers could be identified following this procedure; one (g796), showed a polymorphism present only in the ALS resistance donor, Mexico 54. The PCR protocol developed to identify the g796 polymorphism was validated among five laboratories. Furthermore, co-segregation analysis of the marker and ALS resistance phenotype in a population of 100 F_2 individuals from the cross between French bean (that is, green bean) genotype Amy and ALS resistance donor Mexico 54 showed the marker is genetically linked (3 cM) to the *Phg-2* locus, in addition to being physically linked. This study suggests that in the near future, genetic resequencing data of diverse common bean accessions, compiled within an easily accessible database, will facilitate identification of markers for MAS, marker/trait association, and candidate gene identification.

Key words: Angular leaf spot resistance, *Phaseolus vulgaris,* marker-assisted selection.

INTRODUCTION

Marker-assisted selection (MAS) is a breeding approach, based on the existence of DNA markers in or closely linked to a gene coding for an important phenotypic trait. Instead of directly selecting for the phenotype, selection

takes place for the linked marker; in a later step, the presence of the phenotype is then verified. This procedure enables the selection process to proceed in the absence of conditions promoting the phenotype for example, low heritability or absence of the pathogen or pest; (Collard and Mackill, 2008) or when phenotypic screening is cumbersome for example, salt tolerance (Ashraf and Foolad, 2013).

While MAS does not replace phenotypic selection, it is an adjunct tool that had been used to facilitate and accelerate breeding, for example in common bean (*Phaseolus vulgaris*). In this crop, MAS has been used mainly to introduce and combine genes of resistance to various fungal, viral, and bacterial diseases (Kelly et al., 2003; Miklas et al., 2006; Duncan et al., 2012; Tryphone et al., 2013; Souza et al., 2014; Meziadi et al., 2016). These diseases are one of the major causes of yield reductions in the bean production regions throughout the world (Schwartz and Pastor-Corrales, 1989; Wortmann et al., 1998). MAS has been used recently in the development of improved common bean germplasm in the U.S. (Singh and Miklas, 2015), Canada (Durham et al., 2013), Brazil (Costa et al., 2010; Souza et al., 2014) and Tanzania (Tryphone et al., 2013).

Common bean is the economically most important domesticated species of the genus *Phaseolus* (Gepts et al., 2008). It originated from two geographically separate domestications, from an already diverged wild ancestor (Gepts et al., 1986). Numerous molecular investigations have confirmed the existence of two geographically diverged gene pools: Andean and Mesoamerican, most recently based on SSR data (Kwak and Gepts, 2009; Okii et al., 2014), SNP data (Cortés et al., 2011; Ariani et al., 2016), and DNA sequences (Schmutz et al., 2014; Vlasova et al., 2016; Rendón-Anaya et al., 2017). A consequence of this diversity organization is that genetic distances within each of the two gene pools are considerably smaller than between gene pools. Hence, it is more difficult to detect polymorphisms within the gene pools. Yet, the sharply increased availability of DNA sequences since the introduction of next-generation sequencing (NGS) and the existence of the PhaseolusGenes genetic marker database make the identification of new markers possible as is illustrated in this article.

The existence of two major geographic gene pools in the common bean host plant is mirrored in the genetic diversity and virulence pattern of some pathogens, including angular leaf spot (*Pseudocercospora griseola)* (Guzmán et al., 1995), anthracnose (*Colletotrichum lindemuthianum)* (Geffroy et al., 1999, 2000), and rust (*Uromyces appendiculatus*) (Araya et al., 2004). These pathogens also have Andean and Mesoamerican gene pools, each of which tends to be more virulent on the bean host from the same geographic area. Thus, breeders seeking to obtain resistance for the Andean gene pool tend to use this resistance in the Mesoamerican gene pool and vice-versa.

Several common bean accessions had been identified from Andean and Mesoamerican gene pools and dominant, monogenic, loci conferring qualitative ALS resistance to specific pathogen races have been mapped in the bean genome. The *Phg-1* locus, identified in AND277, is located on chromosome 1 at a distance of 1.3 cM from marker TGA1.1 (Queiroz et al., 2004; Gonçalves-Vidigal et al., 2011). Mesoamerican resistance locus, *Phg-2*, mapped qualitatively in Mexico 54 is located on chromosome 8 at a distance of 5.9 and 11.8 cM from markers SN02 and OPE04, respectively (Sartorato et al., 2000). The Mesoamerican ALS resistance locus, *Phg-3*, mapped in accession Ouro Negro, is linked to marker g2303 at a distance of 0 cM (Gonçalves-Vidigal et al., 2013). Two dominant, monogenic, resistance loci were also identified in G10909: $Phg_{G10909A}$ and $Phg_{G10909B}$ (Mahuku et al., 2011). These loci are inherited independently from all other known resistance genes, conferring resistance to ALS race 63-63, which overcomes all other known resistance sources. Quantitative trait locus (QTL) mapping had also been used to identify multiple resistance loci in Andean germplasm. Seven QTLs on five chromosomes were identified in a IAC-UNA × CAL 143 recombinant inbred population and named ALS2.1, ALS3.1, ALS4.1, ALS4.2, ALS5.1, ALS5.2 and ALS10.1 (Oblessuc et al., 2012, 2013, 2015). Additionally, three major R genes identified in Andean G5686 (Phg_{G5686A}, Phg_{G5686B} and Phg_{G5686C}) (Mahuku et al., 2009) were later treated as quantitative loci by Keller et al. (2015) and mapped to the same loci as ALS4.1, ALS4.2 and ALS9.1 (Oblessuc et al., 2012; Keller et al., 2015).

The transfer of resistance genes can be facilitated by MAS. Until recently, one of the constraints to marker-assisted selection was the availability of tightly linked DNA sequences from which markers could be developed. The identification of linked RAPD markers and their subsequent conversion into SCAR markers (Adam-Blondon et al., 1994; Melotto et al., 1996; Johnson et al., 1997), while effective in the long run, was slow and generally provided only dominant markers.

This situation has changed markedly with increasing availability of genome sequences, especially whole-genome sequences arranged in pseudomolecules for

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each chromosome and anchored on a dense genetic map (Morrell et al., 2012). In common bean, two whole genome sequences are available, corresponding to the Andean (accession G19833; Schmutz et al. 2014) and Mesoamerican (accession BAT93; Vlasova et al., 2016) gene pools. The Andean whole-genome sequence has been used as the current backbone for a "bean breeder' toolbox", that is, a marker database – Phaseolus Genes (http://phaseolusgenes.bioinformatics.ucdavis.edu), which combines information from both legacy molecular markers and more recent markers resulting from genomic analysis of the common bean genome. Of importance is the anchoring of markers in their respective chromosomal locations via a genome browser (http://abcgb.genomecenter.ucdavis.edu), thus allowing the identification of tightly linked markers in specific regions of the bean genome.

Angular leaf spot is a major driver of common bean yield reduction in Central and South America as well as East Africa, necessitating the need to develop markers linked to resistance genes, which can be used during breeding (Schwartz and Pastor-Corrales, 1989).

In this study, the main goal was to identify new marker alternatives to the SN02 marker tagging the *Phg-2* locus on chromosome Pv08, which confers resistance to Angular Leaf Spot (Nietsche et al., 2000; Sartorato et al., 2000). The SN02 marker is monomorphic in certain crosses, requiring the search for alternative markers as detailed subsequently. The first objective was, therefore, to conduct an *in silico* study in the PhaseolusGenes database to identify tightly linked potential markers to replace SN02. The second objective was to test these potential markers for their suitability across a representative sample of bean lines, including target varieties and disease resistance donors, both at UC Davis and in collaborating laboratories in East Africa. Thirdly, the study sought to demonstrate that the new marker identified was indeed linked to the disease resistance locus, *Phg-2*. In spite of the challenges, mainly the need to identify polymorphisms among evolutionarily close lines, the study is able to identify a new marker exhibiting a robust, easy to score polymorphism and tightly linked to the *Phg-2* resistance gene.

MATERIALS AND METHODS

Plant materials

The 16 parental materials analyzed in this study are part of the breeding projects of the African Bean Consortium (ABC). This consortium groups several breeding projects with a common goal, namely to develop a multiple-disease-resistant version of a local variety that is popular with farmers and consumers (target or preferred variety). Table 1 shows different ABC target varieties and sources of resistance. Seeds of the 16 parental materials used inhybridizations to develop multiple-disease-resistant improved target varieties were obtained from the respective breeding programs and were imported into the U.S. under USDA APHIS Small Seed Quantities permit no. P37-16-00357.

Marker segregation from *Phg-2* locus

After putative alternative markers were identified by PCR amplification of the 16 ABC breeding parents, a F_2 segregating population between a *P. griseola*-susceptible French bean Amy and the resistant parent Mexico 54 was used to confirm the linkage between the g796 marker and the *Phg-2* locus. Amy is a commercially preferred French bean (that is, green bean) genotype with white seeds and round, straight, smooth pods; it is susceptible to ALS. The Mexico 54 parent possesses known resistance to most *P. griseola* races in East Africa; however, it is unadapted to dual growing cycles in Kenya as it only gives flowers under short days. Mexico 54 has medium-sized, pink seeds and is a landrace from Southern Mexico, near the border with Guatemala (CIAT germplasm accession G2595, collected at 16.1167º N. Lat., -92.05º W. Long.). A total of 100 F_2 progeny were genotyped with the g796 STS marker and phenotyped with ALS race 63-39, which was identified by inoculating 12 ALS differential cultivars in triplicate (Pastor-Corrales et al., 1998). Race 63-39 was collected from diseased bean leaves at University of Embu, Kenya and inoculum was produced by isolating spores from single fungal synnemata, grown on V8 medium for 12 days (CIAT, 1987) before inoculating the Amy x Mexico 54 F_2 population. Inoculation of the F_2 population took place in a screenhouse at the University of Embu. Following inoculation, plants were placed underneath screenhouse benches, which were covered with vapor-resistant plastic to maintain relative humidity at 90 to 100% for three days. The temperature during infection and disease development was approximately 20 to 22°C. The phenotypic data for ALS infection with race 63-39 were taken as scores on a 1 to 9 CIAT scale, whereby plants with a disease score of 1 to 3 were considered resistant and those with scores of 4 to 9 were susceptible (CIAT, 1987).

Amy X Mexico 54 F_2 marker genotyping

Leaf tissue from fully expanded first trifoliate leaves were harvested from 100 F_2 plants and their parents, Mexico 54 and Amy, and used for DNA extraction. The harvested leaves were crushed to form a paste and placed on a Whatman FTA® plant card (Sigma Z719730). The cards were air dried at room temperature (20-25°C) for one and half hour then washed with FTA purification reagent as per the manufacturer's procedure. Ten 2 mm FTA® discs were obtained from each sample using a Harris® core punch and placed in 1.5 ml micro-centrifuge tubes. Five hundred microliters of FTA® purification reagent was added to the discs, and incubated at room temperature for five minutes with moderate mixing. The procedure was repeated twice. The discs were washed twice with 500 µl TE before drying for 1.5 h at room temperature. DNA obtained from the discs was adjusted to a concentration of 10 to 50 µg/ul prior to its usage for PCR. The g796 STS marker was amplified from DNA of the 100 F_2 lines and parents (Amy and Mexico 54) to test the linkage relationship between the marker and *Phg-2* resistance locus. PCR amplification of the g796 marker was done using puReTaq ready to go PCR beads®. The PCR reaction was prepared by adding the following to lyophilized PCR beads to make a final volume of 25 µl: 1 µl of each forward and reverse primers (g796 F – 5' GAGAAACTACGGGCTGTTTTACCC 3', g796 R2 – 5' AGTTAAGACCGTTCTGAAGCTTC 3'), 22 µl of sterile water and 1 µl of DNA at concentration of 10 to 50 µg/ul. Marker PCR amplification was performed in an Eppendorf Thermal Cycler® with initial DNA denaturation at 94°C for 3 min followed by 35 cycles of

Table 1. African bean consortium target varieties and sources of disease resistance included in this study.

Target parents to be improved			
Name	**Gene pool**	**Country**	**Attributes**
Kablanketi	Andean	Tanzania	Dry bean; most valuable market class in Tanzania (Mishili et al. 2011); climbing type grown often as bush bean; short cooking time; purple-speckled over cream colored seeds
NABE12 C	Andean	Uganda	Dry bean; climbing type for S.W. Uganda; sugar type (red streaks over cream colored seed
NABE13	Andean	Uganda	Dry bean; bush bean
NABE14	Andean	Uganda	Dry bean; bush bean
Red Wolayta	Mesoamerican	Ethiopia	Dry bean; bush bean
Ibaddo	Andean	Ethiopia	Dry bean; bush bean
Hawassa-Dume	Mesoamerican	Ethiopia	Dry bean; bush bean
Gasilida	Andean	Rwanda	Dry bean; climbing bean
RWV3006	Mesoamerican	Rwanda	Dry bean; climbing bean
Amy	Andean	Kenya	Green bean (that is, French bean) with superior pod quality traits; bush bean
Donor parents			
Name	**Gene pool**		**Disease resistance[1,2] (Genes; Source reference)**
Mexico 54	Mesoamerican		ALS (*Phg-2*; Sartorato et al., 2000)
G2333	Mesoamerican		ANT (*Co-4*^{2}, *Co-5*, *Co-7*; Young et al., 1998)
RWR719	Mesoamerican		Root Rot (*Pythium* spp.) (Nzungize et al., 2011)
VAX3 VAX4 VAX6	Mesoamerican, with introgression from *Phaseolus acutifolius*		CBB (SAP6, SU91; Singh and Muñoz, 1999; Singh et al., 2001; Singh and Miklas, 2015)
Mshindi	Andean		BCMV (*bc-1*^{2}; Nchimbi-Msolla et al., 2008)

1ANT, Anthracnose (C*olletotrichum lindemuthianum);* ALS, Angular leaf spot (P*seudocercospora griseola); B*CMV, Bean Common Mosaic Virus; CBB, Common bacterial blight (X*anthomonas axonopodis);* 2In parentheses, resistance genes targeted by marker-assisted selection.

melting at 94°C for 15 s, primer annealing at 52°C for 20 s, DNA polymerization at 68°C for 20 s, and a final extension at 68ºC for 5 min. After 35 cycles, the amplification products were separated by gel electrophoresis on a 2% (w/v) agarose (Thermo Scientific TopVision Agarose: R0491) gel, stained with ethidium bromide, and visualized under a standard UV transilluminator and photographed using a mounted digital camera. Following PCR, electrophoresis and visualization of marker amplification products, marker genotypes were scored for the parents and 100 Amy x Mexico 54 F_2 individuals, with PCR bands of size 209 base pairs corresponding to the allele from Amy (ALS susceptible parent) and those of size 233 base pairs corresponding to the marker allele from Mexico 54 (ALS resistant parent).

F_2 phenotyping with *P. griseola* race 63-39

Prior to evaluating the F_2 population for symptoms to ALS inoculation, the ALS isolate was identified as race 63-39 by inoculating spores of the fungus onto the ALS differential set of cultivars (Pastor Corrales et al., 1998). Seventeen days after planting, spores of race 63-39 at a concentration of 2×10^4 were sprayed onto the first fully expanded trifoliolate leaves of each of the F_2 and parental plants. Inoculated plants were kept under screenhouse benches covered in vapor resistant plastic to maintain 100% humidity and a range of 20 to 22°C for three days. Plants were then moved to the benchtop and subsequently evaluated for disease symptoms 18 days post inoculation. Phenotypic evaluation of inoculated plants and uninoculated controls were scored on a 1 to 9 scale (CIAT, 1987) with 1 to 3 considered a resistant reaction and 4 to 9 considered a susceptible reaction. A chi-squared analysis was used to test the independence between the marker and gene controlling the host reaction to race 63-39 with an expected segregation ratio of 3:6:3:1:2:1, consistent with independent assortment between a co-dominant marker locus and a dominant resistance gene.

RESULTS

Identification of candidate alternative markers around the SN02 marker

Markers used for MAS in a breeding program must be polymorphic among the parental genotypes and be

cheaply and reproducibly assayed. The SN02 marker previously used to map the *Phg-2* ALS resistance locus (Sartorato et al., 2000) is monomorphic among some of the 16 breeding parents used in the ABC, necessitating the identification of an alternative polymorphic marker. The PhaseolusGenes marker/QTL database is a compilation of all previously identified marker datasets described in *Phaseolus* species (http://phaseolusgenes.bioinformatics.ucdavis.edu/) and is a resource that the bean breeding community can use to predict the physical genetic locations of markers linked to trait-associated loci.

In a first step, the SN02 marker was searched for on the PhaseolusGenes homepage. The marker sequence provided by the database (obtained by sequencing the SN02 amplicon in the common bean breeding line BAT93; J. Kami and P. Gepts, unpublished results) was then aligned to the G19833 reference sequence (*Phaseolus vulgaris* V1.0, Schmutz et al., 2014) using the blast algorithm (Altschul et al., 1990). The physical location of SN02 in the common bean genome was viewed in the UCSC Genome Browser, as implemented in PhaseolusGenes. The UCSC Genome Browser displays all previously published marker datasets aligned to the reference genome, as well as newly developed markers, such as Indel markers (S. M. Moghaddam and P. McClean, pers. comm.) and SSR-motif-containing sequences. The SN02 marker is physically located on chromosome 08 of the G19833 V1.0 reference genome from position 58,535,516 to 58,536,215 bp.

Using PhaseolusGenes, twenty-two alternative SSR, STS, SCAR, Indel and SNP markers were located with an overall bracket of approximately 2.5 Mbp around the SN02 marker (Table 2). These potential alternative markers were chosen due to their close physical linkage to the SN02 SCAR marker, which was previously found to be linked to the *Phg-2* ALS resistance locus at a genetic distance of 5.9 cM in a biparental population derived from the cross between Rudá and Mexico 54 (Sartorato et al., 2000). The markers included seven STSs, six SSRs, five Indels, four SCARs and one SNP spaced at an average distance of ~107,000 base pairs (Figure 1; Mapchart, Voorrips, 2002)

Amplification and polymorphism of potential markers

The 22 markers and SN02 were first tested for PCR amplification and polymorphisms between the ALS resistant parent, Mexico 54, and the three other parents (NABE12C, RWR719, and G2333) utilized in the ABC Ugandan breeding program. NABE12C is a target variety from the Uganda project: It is a climbing variety with large 'sugar'-type seeds (elongated; cream background with red streaks). RWR719 and G2333 are donors of resistance for *Pythium* root rot and anthracnose, respectively. Subsequently, the 23 markers were also analyzed in all 16 lines included in this study (Table 1).

PCR amplification of the 22 alternative markers as well as the SN02 SCAR marker in the Ugandan breeding parents, NABE12C, RWR719, G2333 and Mexico 54 resulted in identification of 13 markers, robustly amplified in all four parents (Table 2). All PCRs were performed using the same cycling parameters which were: 1. 94°C for 3 min; 30 cycles of 2. 94°C for 15 s, 3.50°C for 30 s, 4.68°C for 30 s and 5.68°C for 7 min. Two Indels, NDSU_IND_8_58.8634 and NDSU_IND_8_58.2730, and 1 sequence-tagged site marker, g796, had visible molecular weight differences between Mexico 54 and the other parents. The g796 marker showed a 24-base-pair insertion only in Mexico 54, which was easily assayable by visualization on a gel. The g796 marker was tested in all 16 ABC breeding parents, confirming that Mexico 54 has an insertion in the marker, whereas the other 15 parents do not (Figure 2). The 24-base-pair insertion in the marker of Mexico 54 confers the addition of eight additional amino acids in the predicted protein. The marker (primers and protocol) had been distributed to projects in Uganda, Tanzania, Kenya, and Ethiopia, where the polymorphism has been reproducibly observed (data not shown).

Analysis of linkage relationship between the g796 Marker and the *Phg-2* resistance locus

A test for independent assortment between the g796 marker and the *Phg-2* ALS resistance locus was performed using an F_2 population consisting of 100 individuals from the cross Amy x Mexico 54. First, of the 100 F_2 individuals phenotyped with race 63-39, 73 had phenotypic scores of 1 to 3 (resistant) and 27 had scores of 4 to 9 (susceptible) (Table 3). The observed number of resistant and susceptible individuals in the F_2 was not significantly different than the 3:1 ratio predicted with a single dominant resistance gene conferring resistance ($\chi^2 = 0.213$, df = 1, p = 0.64). Second, the segregation for the g796 marker was tested for an expected 1:2:1 ratio corresponding to a co-dominant locus. No significant departure from the tested ratio was observed ($\chi^2=3.58$, df = 2, p = 0.17). A third chi-square test was then conducted to check for independent assortment between the co-dominant g796 marker and dominant ALS resistance gene using an expected ratio of 3:6:3:1:2:1 (Table 4). The Chi square test statistic was equal to 70.63 with $p < 0.00001$ (df = 5), indicating the marker and resistance gene are linked on chromosome 08 (Table 3). Three individuals display recombination between the marker and resistance gene, which corresponds to an approximate map distance of 3 cM between the marker

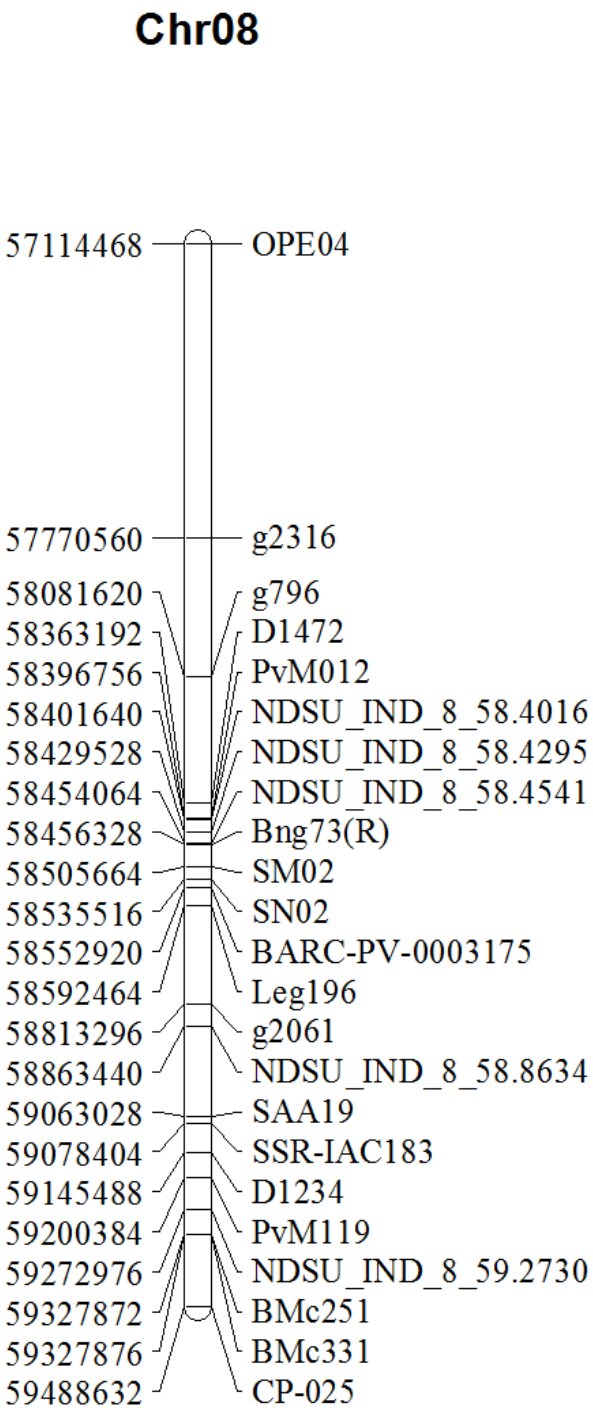

Figure 1. Partial map of chromosome Pv08 of common bean showing twenty-three markers used to screen the Ugandan breeding parents (NABE12C, Mexico 54, RWR719 and G2333) for an alternative to the SN02 marker. On the left of the chromosome are distances expressed in bp according to the G19833 reference map (Schmutz et al., 2014). To the right of the chromosome are the marker sequence names as shown in the PhaseolusGenes gBrowse portal. Markers span a 2.4 Mb region around SN02. More information is given in Table 2.

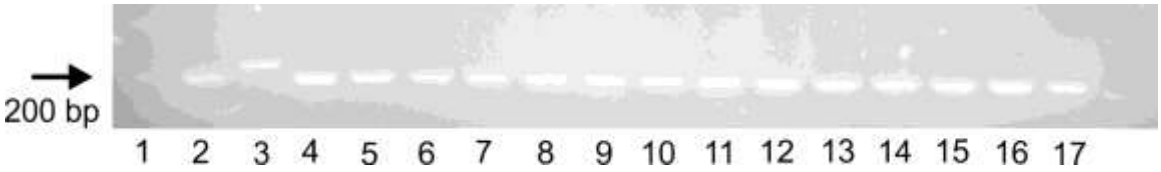

Figure 2. g796 PCR products for 16 ABC parents. Left to right: 1. 1 Kb DNA ladder, 2. NABE12C, 3. Mexico 54, 4. G2333, 5. NABE13, 6. NABE14, 7. Kablanketi, 8. Mshindi, 9. VAX3, 10. VAX4, 11. Ibaddo, 12. HawassaDume, 13. Redwolayta, 14. VAX6, 15. RWR719, 16. Gasilida, 17. TARS-VR-7s (Cultivar Amy is not included in the figure, but shows the same fragment size as the other parents, except Mexico 54).

and gene (3 recombinants / 100 F_2 individuals * 100 = 3 cM).

DISCUSSION

Although the legacy SN02 marker was polymorphic between parents used to identify the *Phg-2* resistance locus (Sartorato et al., 2000), the marker was shown to be monomorphic among the ABC breeding parents from Uganda (Namayanja et al., 2006), and later among all 16 parents, necessitating an alternative marker, which could be used for MAS. A crucial element in the identification of such marker is information on the genetic relationships between parents, which help determine the frequency of polymorphic markers that can be utilized for MAS. In common bean, the well-established divergence between the Andean and Mesoamerican gene pools predicates a preference for Andean x Mesoamerican crosses to maximize polymorphisms at the molecular level (Kwak and Gepts, 2009; Vlasova et al., 2016). However, most crosses in bean breeding often involve parents from the same gene pool or even closely related parents to increase progress from selection and avoid hybrid weakness (Beaver and Osorno, 2009), thereby limiting the potential for polymorphism.

In the ABC project, the multi-parent crosses in each project involve one target (preferred, recurrent) parent and two to three resistance donor parents. Most preferred varieties in each of the projects are of Andean origin, whereas most resistance donors are of Mesoamerican origin. While this situation would seemingly facilitate the identification of markers, polymorphisms should also be identified among the Mesoamerican parents in each project, so that the contribution of each Mesoamerican resistance donor can be monitored in the progenies. The need for polymorphism even within gene pools can be partially be satisfied by the availability of a large number of potential markers as included in the PhaseolusGenes database. It is only such a large number of markers that can withstand the strong attrition imposed by the needs for polymorphism and strong and reproducible amplification, as illustrated in this study. Of the 22 markers selected initially in a region of some ~2.4 Mbp, one - or at most three markers - were found to be suitable as an alternative marker for the SN02 marker because they provided a robust amplification and were polymorphic between the donor parent of the resistance and the three other parents (whether the target recipient or the two other resistant donors).

The PhaseolusGenes marker database has become an essential tool for cataloging bean marker diversity so that the breeding community can utilize marker information from multiple sources to facilitate MAS (Gonçalves-Vidigal et al., 2011, 2013; Reinprecht et al., 2013; Oblessuc et al., 2013, 2015; Aranda et al., 2014; Keller et al., 2015; Sousa et al., 2015; Coimbra-Gonçalves et al., 2016; Perseguini et al., 2016). Because genomic resequencing of breeding parents is today a more routine practice with the lowered cost of sequencing, the PhaseolusGenes database should evolve from a marker database into a sequence database, which includes not only the current markers but also the increasingly large number (> 2,000) of genotypes that have been sequenced, including the 16 parental genotypes of the ABC projects (T. Miller and P. Gepts, unpubl. data).

The development using NGS of markers that are specific to a breeding population has become common practice in crop breeding because the development of reference genome sequences facilitates efficient identification of a large number of physically mapped new markers, as well as different types of markers. *P. vulgaris* reference genomes, based on the Andean landrace G19833 (Chaucha Chuga, Peru), the Mesoamerican breeding line BAT93, and the Mesoamerican variety OAC Rex, introgressed with *P. acutifolius*, have been released recently (Schmutz et al., 2014; Vlasova et al. 2016; http://www.beangenomics.ca/). These resources offer a means (in conjunction with the PhaseolusGenes database and linkage mapping of phenotypic traits by genetic and QTL analyses) to develop new markers for use in MAS and map-based gene isolation. Short genomic sequences for each breeding parent can be mapped to the reference genome and new polymorphisms can be identified, such as SNP, INDELs, or SSR. Moreover, since different breeding programs

Table 2. Twenty-three markers (22 potential alternatives as well as SN02), linked to the *Phg-2* ALS resistance locus on chromosome 8 identified in the *Phaseolus* Genes database (database accessed December 16, 2016).

Marker	Type[a]	Start position (bp)[b]	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplified In	Polymorphic
OPE04	SCAR	57,114,468	TGTGACATGCCAACAACCAC	TGTGACATGCCAACAACC	NMRG	No
g2316	STS	57,770,560	TGAAGGCAATGTTCACAAATATGG	GAGAAACAAGAGTTGGCAACCAAG	NMRG	No
g796	STS	58,081,620	GAGAAACTACGGGCTGTTTTACCC	AGTTAAGACCGTTCTGAAGCTTC	NMRG	Yes
D1472	STS	58,363,192	AGCTTTGCCTCCTGTTGCTA	CACCAATTGATGTGTCGAAAA	NMRG	No
PvM012	SSR	58,396,756	AGCCATTCCCTAAGCCTGTT	CCTTGAACCAAAGGAAACCA	MR	No
NDSU_IND_8_58.4016	InDel	58,401,640	TTATTTGATGGGGGATCCAA	CATCTCTCCAGCATTGCACT	MR	No
NDSU_IND_8_58.4295	InDel	58,429,528	GGGTGTTTTGCAGGTCTT	TCCCCATGATTGTTACCC	NMRG	No
NDSU_IND_8_58.4541	InDel	58,454,064	TCAGGTTGGCAACGGATATT	GGTGCAGTGAATGTGGAAGA	NMRG	No
Bng73 (R)	STS	58,456,328	TTCCCAGTCACGACGTTG	GAATTCGAGCTCGGTACC	none	No
SM02	SCAR	58,505,664	TCTCTTTGCCATCGTTGTTG	AACGCCTCTAAACGGGAGAA	NRG	No
SN02	SCAR	58,535,516	TTTGGAAGTGTGAGCAGTGG	TAGAGGGGCTGAGGACTGAA	NMRG	No
BARC-PV-0003175	SNP	58,552,920	CCGTTGGCATTGGCAATCTGC	AATCTTGGGATGCCTGTCAC	NMRG	No
Leg196	SSR	58,592,464	GTTTTTGCTTGAGCCTCTGC	CTGGTGCGAAGACAATCTGA	NRG	No
g2061	STS	58,813,296	CAAAGCTCTCAATGATCACCATGT	TCAGAAGCAAATGCTTAGACTGTATCA	none	No
NDSU_IND_8_58.8634	InDel	58,863,440	AAATTCTACGGAAATTGAAAGTATAAA	TGCTAGTGAAAGCAAATGACACAGT	NMRG	Yes
SAA19	SCAR	59,063,028	GTCGGGCCAAACTAACAAAG	CAGGCTGGTGGACCTAAAAA	NMRG	No
SSR-IAC183	SSR	59,078,404	TACGCGTGGACTATCCCTCT	GTGACCATCATCTTCGAGCA	MR	No
D1234	STS	59,145,488	TGTTGCAGTGCTCTTCTGCT	TGCTAGTGTTTAAGTGTGTGCCTA	NMG	No
PvM119	SSR	59,200,384	GGGGAGGATAAACCAGAAGG	GTGTAATCCCCTCCAGCAAA	none	No
NDSU_IND_8_59.2730	InDel	59,272,976	CCAGAATGGGAAACAGGATG	AGAGTGAGTTTGGCAGCCAT	NMRG	Yes
BMc251	SSR	59,327,872	TTCAAGGAGGACGTTTGGTC	CATTAACCCCAGCTTTCTCC	none	No
BMc331	SSR	59,327,876	AGGAGGACGTTTGGTCCAG	CCCAGCCTTCTCCCAAAC	NMRG	No
CP-025	STS	59,488,632	GACTTTGGCCTCTCCGTTTTCTTC	ATCTATATGTCCCTTCAATACTGC	NMRG	No
Total genomic range (bp)		2,374,164				

[a] Indel, Insertion-deletion; SCAR: Sequence-characterized amplified region; SNP,Single-nucleotide polymorphism; STS, Sequence-tagged site; bThe starting map position of each marker on chromosome 08 is based on the G19833 Andean whole-genome reference sequence V1.0 (Schmutz et al., 2014) as implemented in the PhaseolusGenes database (phaseolusgenes.bioinformatics.ucdavis.edu; accessed 16 December 2016). Markers were screened for polymorphism between breeding parents NABE12C (N), Mexico 54 (M), RWR719 (R), and G2333 (G). Polymorphic sequences are denoted as those that showed a visible molecular weight difference between Mexico 54 (M) and the other three parents in the Ugandan breeding program (N, NABE12 C; R, RWR719; G, G2333). After confirming the g796 marker was polymorphic between the Ugandan breeding parents, the total 16 parents used in the ABC breeding programs were screened to confirm the polymorphism.

Table 3. F_2 of Amy x Mexico 54 phenotypic and genotypic counts.

Phenotype	n	*g796* genotype		
		$R^{Mex54}r^{Amy}$	$R^{Mex54}R^{Mex54}$	$r^{Amy}r^{Amy}$
Total	χ^2 = 3.58 and 2 d.f., ns	43	33	24
R	73	40	33	0
S	27	3	0	24
χ^2 = 0.21 and 1 d.f., n.s.				

Table 4. Test of independence between marker g796 (A, segregating in co-dominant fashion according to a 1:2:1 ratio) and resistance to *Pseudocercospora griseola* race 63-39 due to *Phg-2* (B, segregating in dominant fashion, 3:1 ratio) in the Amy x Mexico 54 F_2 population.

Class	Genotype	Observed	Expected	χ^2
3	$A^{Mex54}A^{Mex54}B^{Mex54}B^{Mex54}$ $A^{Mex54}A^{Mex54}B^{Mex54}b^{Amy}$	33	18.75	10.83
6	$A^{Mex54}a^{Amy}B^{Mex54}B^{Mex54}$ $A^{Mex54}a^{Amy}B^{Mex54}b^{Amy}$	40	37.50	0.17
3	$a^{Amy}a^{Amy}B^{Mex54}B^{Mex54}$ $a^{Amy}a^{Amy}B^{Mex54}b^{Amy}$	0	18.75	1.0
1	$A^{Mex54}A^{Mex54}b^{Amy}b^{Amy}$	0	6.25	1.0
2	$A^{Mex54}a^{Amy}b^{Amy}b^{Amy}$	3	12.50	7.22
1	$a^{Amy}a^{Amy}b^{Amy}b^{Amy}$	24	6.25	50.41
	Total	**100**	**100**	**70.63**

$P_{df=5}$<0.00001.

have different technologies available to them to detect markers, the development of gel-based, size-polymorphism markers, which are the type routinely used by the ABC breeding programs, can be achieved. Alternative polymorphic markers are useful not only for the selection of loci influencing a specific trait (foreground selection), but also for identifying the genetic composition of recombined individuals after selection (background selection).

Newly developed varieties result from recombination of the genomes of the selected breeding parents Using information about polymorphism present among the parents can allow breeders in the ABC programs to characterize the genetic makeup of the varieties they release into a seed system to estimate their impact. Besides the utility of using polymorphic markers to pyramid multiple genes into a single genetic background (foreground selection), background genomic selection in backcross breeding can be used to select genetic recombinants with the largest amount of their genetic makeup derived from the recurrent parent. Furthermore, improved cultivars with known introgression alleles can be genotyped to verify the identity of an improved cultivar based on its specific genetic signature, or for further improvement during breeding.

The procedure followed here demonstrates the steps to follow to identify new polymorphic markers useful for MAS and the utility of the PhaseolusGenes marker database in a breeding program that seeks to introgress several known resistance alleles into a single genetic background. Furthermore, the combined genomic and transcriptomic resources that are now available to the bean breeding community allow for the identification of genes functionally related to particular traits, such as resistance to ALS. By using the physical location of known markers within annotated genes in reference genome sequences, genes with different putative functions can be identified based on their homology to proteins with known function in other species, such as soybean or Arabidopsis. Polymorphisms identified in annotated genes can then be tested for their association to a particular trait of interest using a population segregating for the trait or with methods such as Virus Induced Gene Silencing to transcriptionally silence

the candidate gene. Overall, the bean community should seek to systematically compile and catalog the corresponding genetic and phenotypic diversity available in *Phaseolus* beans.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

ABC, African Bean Consortium; **ALS,** angular leaf spot; **BLAST,** basic local alignment search tool; **InDel,** insertion-deletion; **MAS,** marker-assisted selection; **PAMP,** pathogen associated molecular pattern; **PCR,** polymerase chain reaction; **PTI,** PAMP triggered immunity; **RAPD,** random amplification of polymorphic DNA; **SCAR,** sequence-characterized amplified fragment; **SNP,** single-nucleotide polymorphism; **SSR,** simple sequence repeat; **STS,** sequence-tagged site.

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