Isolation and Characterization of Nucleotide-Binding Site Resistance Gene Homologues in Common Bean (*Phaseolus vulgaris*)

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ABSTRACT

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Common bean production is constrained by many fungal, viral, and bacterial pathogens. Thus, the identification of resistance (*R*) genes is an important focal point of common bean research. The main goal of our study was to identify resistance gene homologues (RGH) in the crop, using degenerate primers designed from conserved sequences in the nucleotide-binding site (NBS) domains of *R*-genes from the model legume *Medicago truncatula*. Total DNA of the Andean common bean genotype G19833 was used for amplification of over 500 primer combinations.

Sequencing of amplicons showed that 403 cloned fragments had uninterrupted open reading frames and were considered representative of functional RGH genes. The sequences were grouped at two levels of nucleotide identity (90 and 80%) and representative sequences of each group were used for phylogenetic analyses. The RGH sequence diversity of common bean was divided into TIR and non-TIR families, each with different clusters. The TIR sequences grouped into 14 clades while non-TIR sequences grouped into seven clades. Pairwise comparisons showed purifying selection, although some sequences may have been the result of diversifying selection. Knowledge about RGH genes in common bean can allow the design of molecular markers for pyramiding of resistance genes against various pathogens.

Common bean (*Phaseolus vulgaris* L.) is considered one of the most important sources of food protein especially in developing countries where per capita consumption is higher than 50 kg in some regions (8). The crop is cultivated in tropical, subtropical, and temperate regions in more than 100 countries around the world, with production of over 23 million tons of dry grain. Various diseases, such as angular leaf spot (caused by *Phaeoisariopsis griseola* (Sacc.), anthracnose (caused by *Colletotrichum lindemuthianum*) (Sacc. & Magnus) Lams.-Scrib.), rust (caused by *Uromyces appendiculatus*) (Pers.: Pers.)), and Ascochyta blight (caused by *Phoma exigua*) constitute fungal threats to common bean production (61). In addition, common bean is affected by halo blight (*Pseudomonas syringae* pv. *phaseolicola*), bacterial blight (*Xanthomonas axonopodis* pv. *phaseoli*), and a large number of viral pathogens.

In order to reduce the impact of these diseases, different biological and chemical management strategies have been developed. However, genetic resistance is considered the most useful and advisable in both economic and environmental terms since new resistant varieties are easily adopted by farmers and do not require extraneous inputs (43). The common bean genome is one of the smallest among the legumes (588 to 637 Mbp/haploid) and has more than 60 phenotypically described resistance genes (5,38). However, these genes have only been investigated in detail in just

a few cases, notably for the *I* gene virus resistance gene (63) and for *Co-2* anthracnose resistance gene (21).

Disease resistance against pathogens in plants includes several steps. A first line of defense is dependent upon the perception of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors. This first event has been denominated PAMP-triggered immunity. Next, defense responses are indirect and direct consequences of interaction between plant resistance proteins encoded by resistance (*R*) genes and pathogen effectors encoded by avirulence (*Avr*) genes. If this interaction occurs, the event is denominated “effector triggered immunity” (12). The relationship of *R* genes and *Avr* genes is commonly referred to as the “gene-for-gene” model of interaction (18).

To date, over 70 *R*-genes have been cloned from various plant species (35). Comparative analysis of these *R*-genes have revealed certain conserved amino acid domains that include nucleotide-binding sites (NBS), leucine-rich repeats (LRR), transmembrane domains, and serine/threonine protein kinases arranged as separate genes or parts of individual genes in different combinations of protein domains (12). In sequenced genomes such as *Arabidopsis thaliana* it is estimated that *R*-genes are abundant and may represent more than 1% of the total number of genes (41).

More specifically, the NBS-LRR family of *R*-genes can be grouped into TIR (*toll* and *interleukin-1 receptor*) and non-TIR subfamilies, referring to a TIR domain at the N-terminus of the protein that the gene encodes (40). Most of the proteins encoded by non-TIR type *R*-genes have a coiled coil (CC) motif also known as a leucine zipper in the N-terminus region of these proteins (50). The classification of a gene into TIR or non-TIR types can be predicted based on motifs and specific amino acid residues in the proteins (40). Thus, an aspartic acid (D) at the end of Kin-2 and the motifs (LQKKLLSKLL) and RNBS-D (FLHIACFF) are typical of the TIR subfamily. Meanwhile, non-

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TIR proteins have a tryptophan (W) at the end of the Kin-2 motif and the motifs RNBS-A (FDLxAWCCVSQxP) and RNBS-D (CFLYCELPED) inside the NBS domain (40,51,65).

Cloning of *R*-genes has been predominantly through a candidate gene approach either aided by genetic maps or by degenerate primers (1). In the latter case, highly conserved sequences in the P-loop and GLPL motifs of the NBS domain have been used to design degenerate primers which can be used to amplify TIR and non-TIR type *R*-genes in a variety of species. The sequences amplified by these primers are defined as putative resistance genes analogs (RGA) (31), resistance genes candidate (69), or resistance gene homologues (RGH) (9) are all terminologies that refer to similar amplified sequences from *R*-genes; however, we prefer to use the last of these names as it indicates that an ancestral sequence gave rise to orthologous sequences in derived species.

Taking into account that resistance genes have a conserved function even in very different species and remarkable similarity in the NBS domain, a phylogenetic approach to analysis of RGH genes is justified. However, because of their highly duplicated nature and existence as complex and clustered gene families, *R*-genes phylogeny must carefully distinguish orthologous and paralogous sequences. Phylogenetic analyses of this kind of sequences from different plant species strongly suggest that these genes/sequences existed in plant genomes prior to divergence of related species (42).

Many RGAs have been isolated in legume species including common bean, alfalfa (*Medicago sativa*), cowpea (*Vigna unguiculata*), chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), pea (*Pisum sativum*), peanut (*Arachis hypogaea*), and soybean (*Glycine max*) (6,9,11,28). In the model legume *Medicago truncatula*, between 400 to 500 NBS-LRR resistance genes have been identified (2) and RGH sequences from this species can be useful for the development of degenerate primers for RGH isolation in other legumes (B. Rosen and D. R. Cook, *unpublished data*).

In common bean, polymerase chain reaction (PCR)-based *R*-gene analog polymorphism (RGAP) markers were developed from the conserved kinase-1a (GVGKTT) and hydrophobic domains (GLPLAL) of known NBS-LRR type *R*-genes and from expressed sequence tags (EST) databases (46). In the latter study, 32 RGAP markers were mapped in the BAT 93/Jalo EEP558 mapping population, some of which were in the vicinity of known *R*-genes or quantitative trait loci (QTLs) that provided resistance to different diseases such as rust, common bacterial blight, anthracnose, bacterial brown spot, web blight, white mold, angular leaf spot, halo bacterial blight, and Fusarium wilt (43).

Other research by López et al. (37) isolated and evaluated common bean RGA amplicons derived from NBS and TIR domains. In that study, eight classes of RGAs were mapped in genomic regions containing QTL associated with resistance to anthracnose, angular leaf spot, and *Bean golden yellow mosaic virus* (BGYMV).

In a more recent study, the gene expression of some RGA genes in common bean was studied with a transcriptomic approach (16). That research used a data mining approach with 176,011 sequences from next generation or EST sequencing and a database of 50 *R*-genes. A total of 365 unique sequences were identified and named PvRGLs (resistance gene loci of *Phaseolus vulgaris*). The expression of 24 selected PvRGLs was confirmed through the reverse transcription-PCR method. The large number of

colocalization observed between RGAs and QTLs linked to resistance in other species (39) suggests that the further cloning and mapping of RGA sequences will be useful in common bean.

The objective of this study was to identify NBS-LRR diversity in common bean using primers derived from *Medicago* RGH sequences, which were amplified in the Andean genotype G19833 that has been used for a shotgun-sequencing and genome annotation project. Work in common bean is desirable to make comparative studies with other species from the genus *Phaseolus* or from the legume family (24). A considerable number of degenerate primers (over 500) were evaluated for this study, making it the most comprehensive study to date in putative sequences that encode for TIR and non-TIR proteins in common bean. A phylogenetic analysis and characterization of these sequences is carried out and discussed.

MATERIALS AND METHODS

Plant materials and DNA extraction. This study was carried out with the genotype G19833, which is a Peruvian landrace from the Andean gene pool with large yellow with red mottled seed and resistance to various fungal pathogens (37), that has been chosen for whole genome sequencing and annotation for the common bean genome. G19833 is a parent of a central mapping population that has been used to place simple sequence repeat- and single nucleotide polymorphism-based markers across the bean genome (7,19,20). In addition to having ESTs from cDNA libraries (55), a G19833 BAC library has been made (Blair and Muñoz, *unpublished data*) which was useful for physical map construction (60) and for BAC end sequencing in the whole-genome sequencing project. Genomic DNA from young leaves of this genotype was extracted using the cetyl-trimethyl-ammonium bromide method as described by Dellaporta et al. (13) and used by Vallejos et al. (64) for common bean DNA purification.

RGH sequences amplification. Amplification of candidate RGAs was carried out using degenerate primers designed at the University of California–Davis, based on conserved sequences in the P-loop and GLPL motifs of the NBS domain of RGH sequences based on confirmed and well-characterized *R*-genes from *M. truncatula* (S. Steiner, *personal communication*).

A total of 49 degenerate primers were used: 24 designed from conserved TIR type sequences (8 forward and 16 reverse) and 25 primers derived from conserved non-TIR type sequences (9 forward and 16 reverse). In total, 40.8% of the primers were degenerate by a factor of 192-fold, while only 22.4% were 96-fold degenerate. The remaining 36.8% were 72-, 128-, 256-, 288-, and 512-fold degenerate (Supplementary Table 1). All 544 possible primer combinations between the forward and reverse primers were used for PCR (Table 1). Each PCR was carried out in a final volume of 20 µl using an ExTaq enzyme kit for amplification (Takara Bio Inc., Japan).

PCR mix contained 40 ng of genomic template DNA, 0.15 U of ExTaq polymerase (5 U/µl), 1.25× of 10× ExTaq buffer (20 mM Mg2+ plus), 0.2 mM of ExTaq dNTPs, and 0.10 µM of each primer combination. Amplification was carried out in a Tetrad 2 DNA Engine Thermocycler (MJ Research, Bio-Rad Laboratories), programmed for an initial cycle of 95°C for 3 min and 50°C for 3 min, 72°C for 3 min, followed by 29 cycles of 94°C for 1 min,

TABLE 1. Primer combinations used to amplify bean resistance gene homologous genes

Type of primer	Primer direction	Primer series	Total number of primers	Primer combinations	Total number of primer combinations
TIR	Reverse	TIR-R	16	TIR-F + TIR-R	128
	Forward	TIR-F	8	TIR-F + non-TIR-R	128
non-TIR	Reverse	non-TIR-R	16	non-TIR-F + TIR-R	144
	Forward	non-TIR-F	9	non-TIR-F + non-TIR-R	144
Total primer evaluated					544

50°C for 1 min, 72°C for 1 min, and a final extension step of 72°C for 5 min. Amplicons were separated on 1.0% agarose gels run in Tris-borate-EDTA buffer and visualized by ethidium bromide staining.

Cloning and sequencing of RGH amplicons. PCR products between 400 and 700 bp were selected for cloning which was carried out with the TOPO TA Cloning Kit (Invitrogen). Ligation reactions were carried out by mixing 2 µl of PCR product, 0.5 µl of the pCR 4-TOPO cloning vector, and 0.5 µl of a salt solution. These reagents were incubated for 30 min at room temperature. TOPO10 competent cells of *Escherichia coli* (Invitrogen) were transformed using 2 µl of the ligation product and 37.5 µl of CBB80 medium. After 30 min on ice, cell transformation was carried out by heat shock at 42°C for 30 s and subsequently chilled on ice. The transformation mix was added to 250 µl of SOC medium and transferred to a shaker incubator for 1 h at 225 revolutions per minute. Finally, 250 µl of new SOC medium was added and the transformed cells were plated onto 200 µl of Luria broth agar containing 50 mg of kanamycin per liter. The plates were then incubated at 37°C for 24 h before colony picking was carried out with a Q-Bot (Genetix System, Inc.) robot.

A total of 192 colonies were picked for each primer pair. These were maintained in 384-well plates in a freezing-medium containing kanamycin (50 µg/ml) incubated at 37°C for 24 h and then frozen at -80°C. Plasmid DNA from 12 clones per primer pair were extracted using a Wizard SV-96 DNA Purification System DNA and sent for sequencing. The obtained sequences revealed the primer combinations with one or more positive clones for RGH fragments and these were chosen for deeper sequencing of 24 additional clones. All DNA sequencing was performed at the College of Agricultural & Environmental Sciences Genomics Facility (University California-Davis), on an automated ABI 3730 XL capillary sequencer (Applied Biosystems Inc., Foster City, CA). The M13 forward primer was used in all unidirectional sequencing reactions. Sequences were trimmed with Seq Trim so that only insert sequences were analyzed.

Phylogenetic analysis. Hidden-Markov model analyses were conducted with BioJava to identify vector contamination versus RGH sequences. Trimmed sequences were compared with a database of *M. truncatula* RGH and *R*-genes sequences from the National Center of Genomics Resources (NCGR) in order to identify *R*-gene homologues and to classify them into TIR or non-TIR subsets. The sequence analysis was performed using the Blastx algorithm and a cut off threshold of e^{-10} . Finally, the correct reading frame of the sequence and any stop codons indicating an incomplete open reading frame (ORF) were determined and then the gene sequence was trimmed to a full set of codons.

R-gene sequences were then aligned with MAFFT software with default options (<http://mafft.cbrc.jp/alignment/software>) (Supplementary Figures 1 and 2). Subsequent visual adjustment and editing was carried out in Jalview (<http://www.jalview.org>). The Phylip/Phylip4 sequence formats were obtained using Readseq (<http://www.ebi.ac.uk/cgi-bin/readseq.cgi>). Phylogenetic analyses were conducted with the maximum likelihood (ML) method specifically using the RAXML (randomized accelerated maximum likelihood) protocol. Computations were performed on the computer cluster of the Cyber-Infrastructure for Phylogenetic Research (CIPRES) project. The parameters were maintained at default settings with analyses conducted at nucleotide and amino acid levels. The program Fig Tree was used to import and draw trees from Newick format files (<http://tree.bio.ed.ac.uk/software/figtree>).

Phylogenetic analyses were carried out in two phases: first, considering only RGH sequences for common bean obtained in this work; and second, considering RGH sequences previously reported for common bean or other legume and plant species. In order to include all of the RGH sequences published for *Phaseolus vulgaris*, an exhaustive search was conducted through the GenBank

database of the National Center for Biotechnology Information (NCBI, Bethesda, MD). The terms used were as follows: NBS-LRR disease resistance-like protein, TIR-like putative resistance protein, putative resistance protein (RGA) gene, NBS-LRR like putative resistance protein, resistance-like protein, NBS-LRR type putative disease resistance protein, resistance protein analog, and disease resistance protein-like gene.

Cloned *R*-genes from other legumes corresponding to NBS-LRR type RGA sequences reported by Yaish et al. (66) in lentil and in *M. truncatula* and Palomino et al. (48) in faba bean (*Vicia faba*) and chickpea (*Cicer arietinum*) were also included. Sequences related to fully characterized resistance proteins from nonlegume plants species were included in a further analysis. These involved the cloned genes *RPS4* (AJ243468), *RPP1* (AF098962), *RPM1* (X87851), *RPP13* (AF209730), and *RPP8* (AF089711) from *Arabidopsis thaliana*; *L6* (LUU27081) from flax (*Linum utilissimum* L.); *N* (NGU15605) from tobacco (*Nicotiana tabacum* L.); *I2* (AF004878) from tomato (*Solanum lycopersicon* L.); *Xa1* (AB002266) from rice (*Oryza sativa* L.); and *Rp1* (AF107293) from maize (*Zea mays* L.).

The RPS2 sequence (EF560565.1) from *A. thaliana* (ecotype Ws-2) was used to root the phylogenetic tree made with common bean RGH sequences obtained in this work, while a mammalian sequence, APAF-1, was chosen to root the global phylogenetic tree.

Pairwise sequence alignments from representative sequences within the same family were made in order to evaluate the synonymous (Ks) and nonsynonymous (Ka) amino acid substitutions and to calculate the Ks/Ka value ω using the software program Codeml that is part of the PAML software package (<http://abacus.gene.ucl.ac.uk/software/paml.html>). In order to confirm the ω values, the same ratio was calculated using another software package, DNAsp (<http://www.ub.edu/dnasp/>) (33). Additionally, the MEME (Multiple EM for Motif Elicitation, <http://meme.sdsc.edu/meme/intro.html>) from Bailey and Gribskov (4) was used to discover conserved NBS motifs in amino acid sequences from TIR and non-TIR families. Motif probabilities were based on residues appearing at each possible position and were displayed in a graphic manner as “sequence LOGOS.”

RESULTS

Amplification of common bean RGH sequences. A total of 544 primer combinations based on 49 degenerate RGH oligonucleotide sequences were used to amplify common bean RGH sequences for this study. The amplifications showed band sizes between 50 and 3,000 bp in length. However, since the genomic region between the P-loop and GPLP of NBS-LRR type *R*-genes in *M. truncatula* is between 400 and 700 bp in length, we only cloned this approximate size for sequencing of RGH fragments. In order to ensure the identification of the highest numbers of putative RGHs, a two-step strategy was used. In the first step we sequenced 12 colonies from each primer combination. In the second step after this initial sequencing, the primer combinations that amplified one or more confirmed RGH sequences were chosen for deeper sequencing of an additional 24 colonies. Overall in the initial screening, 228 primer combinations (42%) produced amplicons with RGH sequences based on sequencing of a total of 2,736 clones (12 clones per primer combination). BlastX of the trimmed sequences against the NCGR database of *M. truncatula* *R*-genes was used to identify resistance gene homologues and to classify them into TIR and non-TIR families. This analysis allowed us to identify 155 sequences with full conserved motifs that encoded NBS type proteins from *R*-genes. This was equivalent to 5.7% of the total sequenced fragments. The positive sequences were obtained with 73 different primer combinations, corresponding to 13.4% of the 544 primer combinations evaluated.

The negative clones had mostly partial motifs, non-RGH sequences or had sequences with homology to transposons and retrotransposons. In deeper sequencing of the positive primer combinations, a total of 4,486 clones were sequenced of which 1,317 failed to sequence well and the majority of the remaining clones were eliminated because they showed homology with transposons and retrotransposons in blastn searches. The analysis of the remaining sequences showed 403 clones (9.0%) with homology to NBS-LRR domains of known *R*-genes or RGH sequences cloned from *M. truncatula*. The corresponding 403 NBS-containing amino acid sequences were screened for motifs characteristic of RGH proteins and the sequences were then classified as TIR or non-TIR type putative *R*-genes. Nearly half (47.9%) of the NBS sequences were of the TIR class corresponding to 193 sequences, with slightly more (52.1%) sequences being of the non-TIR class, corresponding to 210 sequences (Table 2).

ORF prediction was used to determine the correct codons of each putative *R*-gene and possible stop codons. A total of 158 TIR and 153 non-TIR sequences showed uninterrupted ORFs and were considered true RGH genes. The remaining sequences were not included in the phylogenetic analysis because they contained early stop codons and/or frame-shift mutations and were considered to be pseudogenes or PCR errors. The average size of the trimmed and selected RGH sequences was 462 nt (with a range between 100 and 517 nt). The shortest sequences from 100 to 370 nt were eliminated and as a result the remaining sequences varied from 406 to 517 nt. Redundant sequences were also eliminated using a nucleotide identity threshold value of 99%, where 56 TIR type sequences and 57 non-TIR type sequences were discarded.

Success rate of various primer combinations. A comparison of the sequence results and the primer combination used showed that the class of RGH genes identified did not exhibit an association with the primer combinations used. For example, primer combinations TIR+TIR, which are primers designed only from TIR sequences, amplified TIR type RGH but also non-TIR type RGH sequences. Likewise, non-TIR and non-TIR primer combinations detected both TIR type and non-TIR type RGH genes. The number of RGH sequences identified with the 73 primer combinations ranged from 1 to 15, with an average of 5 amplicons per primer combination (Table 3).

Most of the RGH sequences identified were amplified by 34 non-TIR+non-TIR primer pairs (182 sequences, corresponding to 46%), followed by 24 TIR+non-TIR primer pairs (141 sequences, corresponding to 32%). The other combination classes of TIR+TIR and non-TIR+TIR produced almost the same amount of RGH

sequences (12 and 9.5%, respectively). Some primer combination amplified both TIR and non-TIR sequences together. However, the non-TIR+TIR combination did not show this pattern. It was notable that TIR+TIR combination produced a mix of TIR and non-TIR sequences. In summary, the percentages of RGH sequences generated by each primer combination were variable, although the number of primers and their combinations were all very similar (16 reverse primers for TIR and non-TIR, 8 forward primers for TIR, and 9 forward primers for non-TIR). Finally, the primer combinations produced variable numbers of appropriately sized fragments ranging from 39 to 69 for each.

Phylogeny of newly discovered common bean RGH sequences. Phylogenetic analyses were performed using the common bean RGH sequences, considering only the common segments from the NBS regions found after trimming the primer sequences (P-loop and GLPL). Multiple alignments allowed us to group those sequences sharing 90% nucleotide identity. We selected this identity level because of our goal of identifying the maximum number of paralogous genes but we also followed the recommendations of Quirin et al. (53) to set gene family identity for RGH genes at 80% nucleotide identity. The number of sequences per group ranged from 1 to 21 for TIR sequences and from 1 to 25 for non-TIR sequences at 90% identity with higher limits at the 80% level. For each group, one sequence with the highest similarity values with the other sequences was chosen as a representative sequence to use in the phylogenetic analysis. After consolidating the data set to only the representative RGH sequences at the 90% identity level, 53 sequences were included in the phylogenetic tree (Fig. 1), 27 of which were TIR type and 26 of which were non-TIR type. These sequences were uploaded to GenBank with the accession numbers JQ313582 to JQ313634. At the 80% level, 17 TIR and 12 non-TIR clades or gene families were identified by phylogenetic analysis (Supplementary Figure 3). Because the phylogenetic trees obtained with nucleotide sequences and with deduced amino acid sequences had similar topologies, only the amino acid results are shown. Protein analysis was chosen because codon degeneracy can produce the same amino acid from different codons. Therefore, the pairwise comparison at the protein level is more reliable and the homology between amino acid sequences was generally higher than that of nucleotide sequences.

In the phylogenetical analysis at 90% identity, three sequences (JQ313603, JQ313604, and JQ313605) were eliminated because of their distance from the main branches of the tree. After this, three main clusters were evident for each family of RGH sequences (TIR and non-TIR). These clusters grouped a total of 14 clades of TIR sequences defined as clade 1 to clade 14 and 7

TABLE 2. Resistance gene homologues (RGH) and unique sequences found in common bean with RGH primer amplification along with the amount of genes and pseudogenes distinguished among unique sequences

<i>R</i> -gene class	ORF containing	non-ORF	Total sequences	Unique sequences		
				ORF containing	non-ORF	Total
TIR	158	35	193	105	33	138
non-TIR	153	57	210	116	48	164
Total	311	92	403	221	81	302

TABLE 3. Number of resistance gene homologous (RGH) sequences amplified by different types of primer combinations

Type of primer combination (forward + reverse)	Number of primer combinations evaluated	Number of primer combinations that amplified 400- to 700-bp fragments	Number of primer combinations that amplified RGH sequences					Final number of RGH	%
			non-TIR	TIR and non-TIR	TIR	Total	(%)		
TIR + TIR	128	51	0	4	5	9	12.0	44	11
TIR + non-TIR	128	69	6	4	14	24	32.0	141	35
non-TIR + TIR	144	39	2	0	5	7	9.5	36	9
non-TIR + non-TIR	144	69	6	4	24	34	46.0	182	45
	544	228	14	12	48	74		403	

clades of non-TIR sequences defined as clade 15 to clade 21. Ten of these clades, 7 for TIR genes and 3 for non-TIR genes, had only a single representative sequence each, showing that they were unique clades and that a diversity of RGH sequences exists in common bean. The phylogenetic tree showed TIR and non-TIR subfamilies as two major branches with high bootstrap values of 94 and 86%, respectively. The NBS domain of RPS2 from *A. thaliana* was a useful outgroup for anchoring the common bean

TIR and non-TIR groups. It was noted that *Phaseolus vulgaris* sequences were more similar amongst each other than with the RPS2 gene sequence.

Apart from the phylogenetic analysis, differentiation between TIR and non-TIR sequences was also evident in the Kin-2 and Kin-3 conserved motifs that are part of the NBS domain. The deduced amino acid sequence differences between 105 TIR-NBS and 116 non-TIR-NBS proteins are shown in Table 2. All non-

Fig. 1. Phylogenetic tree of *Phaseolus vulgaris* resistance gene homologue (RGH) sequences clustered at 90% identity and constructed with RAxML and using RPS2 from *Arabidopsis thaliana* to root the tree.

TIR amino acid sequences had a characteristic tryptophan residue (W); while TIR homologous sequences were more variable with aspartic acid (D), asparagine (N), serine (S), proline (P), or threonine (T) residues at the final amino acid position of the Kin2 motif. Relative entropy of these motifs is shown in the logos found in Figure 2. Apart from the Kinase motifs, the deduced TIR-NBS proteins exhibited a highly conservative sequence of SLEL[FL]SWHAF at the C-terminus (amino acids 159 to 168). Meanwhile, most of the non-TIR-NBS sequences exhibited the consensus sequence WVC[VI]SD[DH]F at the N-terminus (amino acids 32 to 39). Presence of these sequences is characteristic of NBS-LRR disease resistance proteins. The highest-scoring hits using the motifs present in our sequences were with RGAs from *Phaseolus vulgaris* and *Helianthus annuus* (data not shown).

Phylogenetic analysis with other legume RGH and non-legume R-genes. A total of 67 common bean sequences with similarity to NBS-LRR genes were found in GenBank and were included in a second phylogenetic analysis. These were classified into 27 Andean sequences from the genotypes G19833, JaloEEP558, and Sprite; and 22 Mesoamerican sequences from the genotypes BAT93, DOR364, and Negro Jamapa. A further nine sequences were found for Olathe and Sierra, also Mesoamerican beans but of race Durango. Seven sequences reported as RGA markers from a Mesoamerican genotype (27) were also included and two more common bean sequences (AF098970.1 and AF098971.1) without genotype information were incorporated. A complete description of all of these sequences is found in Table 4.

Sequences from the Andean genotype JaloEEP558 (EU856787.1, EU856786.1, EU856785.1, EU856784.1, EU856783.1, and EU856782.1) and from the Mesoamerican genotype BAT93 (EU856792.1, EU856791.1, EU856790.1, EU856789.1, and

EU856788.1) were not included in this analysis because they were cataloged as resistance-like pseudogenes.

Therefore, a total of 49 sequences obtained from GenBank were considered common bean RGH genes comparable to the new sequences we had obtained. For species comparisons, 123 RGA sequences from other legumes such as chickpea (25 sequences), faba bean (44 sequences), lentil (23 sequences), and *M. truncatula* (31 sequences) were also included along with resistance proteins from *Arabidopsis* (*RPS4*, *RPPI*, *RPM1*, *RPPI3*, *RPP8*), corn (*RPI*), flax (*L6*), rice (*Xa-1*), tobacco (*N*), and tomato (*I2*).

The sequences described above were grouped into two new phylogenetic trees for TIR and non-TIR clades (Figs. 3 and 4), considering only the NBS region without the P-loop and GLPL regions and using the nucleotide identity threshold of 90%. Based on this criterion only 12 of the 67 previously reported RGAs from common bean were included: EU856770.1, EU856776.1, AF072168.1, AF143553.1, AF363798.1, AF363804.1, AF478172.1, AF478173.1, AF478174.1, AF478175.1, AF478176.1, and AY237123.1. Similarly, only 43 of the legume sequences were used: 6 from chickpea, 5 from faba bean, 12 from lentil, and 20 from *M. truncatula* (Supplementary Table 2). Therefore, the new phylogenetic trees were constructed with 113 sequences in total including the 50 RGH sequences obtained in this work, 10 resistance genes from other species and APAF1 as an outgroup sequence to root the tree.

Nucleotide sequences obtained from GenBank and RGH sequences isolated in this work had an average length of 480 bp encoding 155 amino acids. Although the sequence AF478171 was over 500 bp it showed less than 90% nucleotide identity to other sequences and was eliminated from the phylogenetic analysis.

Fig. 2. Amino acid sequence logos. Relative entropy of conserved positions for Kin-2 and Kin-3 motifs across 105 TIR and 116 non-TIR nucleotide-binding sites (NBS) leucine-rich repeat sequences is shown. Numbers below sequence logo indicate residue positions.

The RGHS obtained from common bean formed defined clades and these RGHS were not clustered with the NBS domain of other plant species such as rice, flax, corn, tobacco, and tomato. Common bean sequences were clustered with sequences from the *Phaseolus vulgaris* sequences collected in genebank regardless of

the genotype. Other legume RGH sequences, were included along with common bean RGH sequences in some clades, most being from *M. truncatula*. Lentil, chickpea, and faba bean RGHS were largely separate except in a few cases where they were clustered with common bean RGH sequences.

TABLE 4. Description of resistance genes from *Phaseolus vulgaris* included in the phylogenetic analysis, with GenBank nucleotide and their protein identities (ID)

Genotype	ID nucleotide	ID protein	Gene pool	Description	Reference
G19833	AF084026.1	AAC33296.1	A	NBS type putative resistance protein gene	(37)
G19833	AF478170.1	AAO33123.1	A	NBS-like putative resistance protein (RGA2) gene	(37)
G19833	AF478171.1	AAO33124.1	A	NBS-like putative resistance protein (RGA3) gene	(37)
G19833	AF478173.1	AAO33126.1	A	NBS-like putative resistance protein (RGA5) gene	(37)
G19833	AF478175.1	AAO33128.1	A	NBS-like putative resistance protein (RGA7) gene	(37)
G19833	AF478176.1	AAO33129.1	A	NBS-like putative resistance protein (RGA8) gene	(37)
JaloEEP558	AF072168.1	AAC26763.1	A	Clone PRLJ1 putative resistance protein (RGA) gene	(23)
JaloEEP558	AF143551.1	AAF81615.1	A	Clone PRLJ4 resistance protein analog gene	na
JaloEEP558	AF143556.1	AAF81617.1	A	Clone PRLJ2 resistance protein analog gene	na
JaloEEP558	AF143557.1	AAF81618.1	A	Clone PRLJ3 resistance protein analog gene	na
JaloEEP558	AF306501.1	AAK61317.1	A	NBS-LRR resistance-like protein J71 (J71) mRNA	(16)
JaloEEP558	AF306502.1	AAK61318.1	A	NBS-LRR resistance-like protein J78 (J78) mRNA	(16)
JaloEEP558	AF306505.1	AAK61321.1	A	NBS-LRR resistance-like protein J71 (J71) gene	(16)
JaloEEP558	AF306506.1	AAK61322.1	A	NBS-LRR resistance-like protein J78 (J78) gene	(16)
JaloEEP558	AY237123.1	AAO62728.1	A	NBS-LRR resistance-like protein (JA1tr) gene isoforms	(17)
JaloEEP558	EU856778.1	ACJ22819.1	A	NBS-LRR type putative disease resistance protein CNL-J1 gene	(22)
JaloEEP558	EU856779.1	ACJ22820.1	A	NBS-LRR type putative disease resistance protein CNL-J3 gene	(22)
JaloEEP558	EU856780.1	ACJ22821.1	A	NBS-LRR type putative disease resistance protein CNL-J9 gene	(22)
JaloEEP558	EU856781.1	ACJ22822.1	A	NBS-LRR type putative disease resistance protein CNL-J5 gene	(22)
Sprite	DQ002468.1	ABA00702.1	A	Disease resistance protein gene	(63)
Sprite	DQ002469.1	NA	A	Disease resistance protein-like gene	(63)
Sprite	DQ002470.1	NA	A	Disease resistance protein-like gene	(63)
Sprite	DQ002471.1	NA	A	Disease resistance protein-like gene	(63)
Sprite	DQ002472.1	NA	A	Disease resistance protein-like gene	(63)
Sprite	DQ002473.1	NA	A	Disease resistance protein-like gene	(63)
Sprite	DQ002474.1	NA	A	Disease resistance protein-like gene	(63)
Sprite	DQ002475.1	NA	A	Disease resistance protein-like gene	(63)
BAT93	AF072167.1	AAC26762.1	MA	PRLB1 putative resistance protein (RGA) gene	(23)
BAT93	AF306499.1	AAK61315.1	MA	NBS-LRR resistance-like protein B8 (B8) mRNA	(16)
BAT93	AF306500.1	AAK61316.1	MA	NBS-LRR resistance-like protein B11 (B11) mRNA	(16)
BAT93	AF306503.1	AAK61319.1	MA	NBS-LRR resistance-like protein B8 (B8) gene	(16)
BAT93	AF306504.1	AAK61320.1	MA	NBS-LRR resistance-like protein B11 (B11) gene	(16)
BAT93	EU856769.1	ACJ22810.1	MA	NBS-LRR type putative disease resistance protein CNL-B7 gene	(22)
BAT93	EU856770.1	ACJ22811.1	MA	NBS-LRR type putative disease resistance protein CNL-B8 gene	(22)
BAT93	EU856771.1	ACJ22812.1	MA	NBS-LRR type putative disease resistance protein CNL-B11 gene	(22)
BAT93	EU856772.1	ACJ22813.1	MA	NBS-LRR type putative disease resistance protein CNL-B16 gene	(22)
BAT93	EU856773.1	ACJ22814.1	MA	NBS-LRR type putative disease resistance protein CNL-B17 gene	(22)
BAT93	EU856774.1	ACJ22815.1	MA	NBS-LRR type putative disease resistance protein CNL-B18 gene	(22)
BAT93	EU856775.1	ACJ22816.1	MA	NBS-LRR type putative disease resistance protein CNL-B19 gene	(22)
BAT93	EU856776.1	ACJ22817.1	MA	NBS-LRR type putative disease resistance protein CNL-B22 gene	(22)
BAT93	EU856777.1	ACJ22818.1	MA	NBS-LRR type putative disease resistance protein CNL-B23 gene	(22)
BAT93	EU856768.1	ACJ22809.1	MA	NBS-LRR type putative disease resistance protein CNL-B3 gene	(22)
BAT93	AF143553.1	AAF81616.1	MA	Clone PRLB5 resistance protein analog gene	na
BAT93	AF143555.1	NA	MA	Clone PRLB2 resistance protein analog-like gene	na
BAT93	AF143554.1	NA	MA	Resistance protein analog-like gene	na
BAT93	AF143552.1	NA	MA	Clone PRLB4 resistance protein analog-like gene	na
DOR364	AF478174.1	AAO33127.1	MA	NBS-like putative resistance protein (RGA6) gene	(37)
DOR364	AF478172.1	AAO33125.1	MA	NBS-like putative resistance protein (RGA4) gene	(37)
Jamapa	DQ002476.1	NA	MA	Disease resistance protein-like gene	(63)
Olathe	AF363801.1	AAK50046.1	NA	Disease resistance protein OB3 (RGA) gene	(57)
Olathe	AF363802.1	AAK50047.1	NA	Disease resistance protein OB5 (RGA) gene	(57)
Olathe	AF363803.1	AAK50048.1	NA	Disease resistance protein OB8 (RGA) gene	(57)
Olathe	AF363804.1	AAK50049.1	NA	Disease resistance protein OB9 (RGA) gene	(57)
Sierra	AF363796.1	AAK50041.1	NA	Disease resistance protein SB1 (RGA) gene	(57)
Sierra	AF363797.1	AAK50042.1	NA	Disease resistance protein SB3 (RGA) gene	(57)
Sierra	AF363798.1	AAK50043.1	NA	Disease resistance protein SB4 (RGA) gene	(57)
Sierra	AF363799.1	AAK50044.1	NA	Disease resistance protein SB5 (RGA) gene	(57)
Sierra	AF363800.1	AAK50045.1	NA	Disease resistance protein SB8 (RGA) gene	(57)
NA	AF098970.1	AAD13036.1	NA	NBS-LRR-like protein cD7 (CO-2) mRNA	(11)
NA	AF098971.1	AAD13037.1	NA	NBS-LRR-like protein cD8 (CO-2) mRNA	(11)
NA	HM126538.1	NA	NA	RGA marker RNF330 genomic sequence	(27)
NA	HM126539.1	NA	NA	RGA marker RNF356 genomic sequence	(27)
NA	HM126540.1	NA	NA	RGA marker RNF475 genomic sequence	(27)
NA	HM126541.1	NA	NA	RGA marker RRF380 genomic sequence	(27)
NA	HM126542.1	NA	NA	RGA marker RRF410 genomic sequence	(27)
NA	HM126543.1	NA	NA	RGA marker RKF310 genomic sequence	(27)
NA	HM126544.1	NA	NA	RGA marker RKR240 genomic sequence	(27)

DISCUSSION

A significant accomplishment of the present study was to obtain a large number of RGH sequences and to evaluate their similarity and relationships with each other or with other *R*-gene sequences from common bean. A total of 311 sequences were classified as successful RGH gene fragments based on having an uninterrupted ORF with high identity to *R*-genes from *M. truncatula* and containing conserved motifs such as Kin-2 or Kin-3 typical of NBS-LRR type genes. Other studies have had success at isolating bean RGH sequences with degenerate primers (37); however, in our study we used many primer pairs and size selection to target NBS-containing genes and isolate a fuller range of RGH genes.

In this study, a total of 73 primer combinations were efficient for amplifying RGH sequences from common bean. Of these, 13

amplified exclusively TIR sequences, 13 only non-TIR sequences and the remaining 48 primer combinations amplified TIR and non-TIR sequences. Therefore, cross-reaction of TIR and non-TIR primers producing both TIR and non-TIR genes was observed. This means that the family or the type of RGH sequence detected was largely independent of the primer combination used. Furthermore, the amplification of TIR or non-TIR sequences appear to be more related to the plant species genome than to primer specificity, and many primer combinations can amplify TIR sequences in one species and non-TIR sequences in a different species.

These results are not surprising, because both TIR and non-TIR genes contain conserved sequences at the P-loop and GLPL motifs. For example, one set of primers from 68 known to amplify TIR type sequences in this species only identified non-TIR

Fig. 3. Phylogenetic relationships between the newly obtained common bean non-TIR resistance gene homologue sequences and previous resistance genes downloaded from GenBank for common bean and other legume or nonlegume plant species. The GenBank name ID is preceded by two letters indicating the species: Ca: *Cicer arietinus*, Mt: *Medicago truncatula*, and Pv: *Phaseolus vulgaris*, and Vf: *Vicia faba*. Different colors in the online version represent RGH from different species with species abbreviation given at the beginning of the gene family name.

Fig. 4. Phylogenetic relationships between the newly obtained common bean resistance gene homologue (RGH) sequences and previous TIR RGH sequences downloaded from GenBank for common bean and other legume or nonlegume plant species. The GenBank name ID is preceded by two letters indicating the species: Ca: *Cicer arietinus*, Lc: *Lens culinaris*, Mt: *Medicago truncatula*, Pv: *Phaseolus vulgaris*, and Vf: *Vicia faba*. Different colors in the online version represent RGH from different species with species abbreviation given at the beginning of the gene family name.

sequences when used heterologously in *Brassica oleracea* (28). Studies in *Arachis* and soybean (*Glycine max* L.) have found that primers derived from P-loop and GLPL motifs appear to preferentially amplify TIR type genes rather than non-TIR type genes (6,53,68). Similarly, an association between the primer combination and the type of amplified sequence has been suggested in lentil (66). Meanwhile for a monocotyledonous plant, *Zingiber*, primers that previously amplified TIR and non-TIR sequences only obtained non-TIR NBS-LRR sequences (52).

Primer design is considered another factor which determines the success in amplification of the target products especially in the case of variable RGH sequence. The primer length, the nucleotide composition, and the position and number of degeneracy nucleotide within each primer as well as the prevalence of putative targets in the analyzed genomes analyzed are factors that determined the amount of RGH amplification (3,44). In this study, an important experimental strategy for increasing the number of common bean RGH sequences was the low level of degeneracy in most of the primer combinations, as was recommended by previous results (62).

The NBS-LRR class of resistance genes has been shown to be a very large and variable gene family among plant species. For example in *A. thaliana* (145 Mbp/1C [megabase pairs per genome]), approximately 200 genes encoding NBS related motifs are estimated to occur in the genome (40). Other plant species with more complex or larger genomes correspondingly can have more NBS-LRR genes. For example, Samuelian et al. (58), predicted 386 NBS encoding genes in their study of red raspberry (*Rubus* spp., 280 Mbp/1C); however, they only identified 75 genomic fragments with intermediate similarity to actual NBS-LRR genes. Successful RGH cloning is a question of targeting several hundred genes at once.

Based on calculations with *Arabidopsis*, common bean with a genome size of 450 to 650 Mbp/1C should have up to 850 NBS encoding genes. However, in this study we only obtained 403 RGH sequences and if we include 67 other independent previously cloned NBS-LRR sequences the number of 470 RGH would still be below that expected amount. However, *Arabidopsis*, due to recent duplication events in the genome, may have an excess of RGH sequences. A recent study of bioinformatically predicted RGH genes in common bean (36) found a total of 365 RGL genes possibly confirming that this legume species has fewer *R*-genes than *Arabidopsis*. The simple diploid nature of common bean relative to polyploidization events in *Arabidopsis* may explain this difference.

Some examples that show the difference among the number of RGHs in different fully sequenced plant genomes are as follows: *Populus trichocarpa* (550 Mb) with 400 RGH genes, *M. truncatula* (500 Mb) with 400, and *Oryza sativa* (430 Mb) with 600 (29, 41,45). These results could indicate that the number of RGHs is not proportional with the genome size. In some species the RGH number can be either lower or higher than expected. As a result, an in-depth study will need to be conducted with the whole genome sequence of G19833 to determine if our cloning efforts obtained almost the complete set of RGH genes found in common bean.

Apart from the number of RGH genes found in a given species, the ratio of the TIR and non-TIR type sequences has been variable among different plants. In *Arabidopsis* the ratio has been 2:1 (41), in peanut it was 3:1 (54), and in hop (*Humulus lupulus* L.) this ratio was 1:2 (30). The difference is especially notable when comparing monocotyledonous versus dicotyledonous plant genomes, because in dicot species TIR sequences are common but in monocots they are almost completely missing (44,50). This has been observed in a wild relative of potato, *Solanum bulbocastanum*, where TIR sequences were preferentially amplified (53). In general, non-TIR sequences are more common and heterogeneous than TIR sequences (44); however, our phylogenetic analysis for

common bean revealed an almost equal number of clades in the TIR and non-TIR subfamilies of common bean RGH genes at the 90% identity levels and 25% more TIR clades at the 80% identity level. This may be a pattern found in other legumes while in nonlegumes such as pear (*Pyrus* spp.), 93 TIR resistance gene families and only 5 non-TIR types were identified (69). When using 80% identity as a threshold to identify RGH families in *Solanaceae* species, Quirin et al. (53) found about 30% more non-TIR than TIR gene families. These results suggest that the proportion of TIR gene families is species- and perhaps genus-specific as a consequence of evolutionary processes such as gene-for-gene interaction with avirulence factors from specific plant pathogens.

For common bean, we found a large number of clades corresponding to different RGH families in the phylogenetic tree, which reflects a high polymorphism of these genes in this species. Point mutations and insertion or deletion mutations within the conserved motifs could be the cause of such sequence differentiation and creation of clades of homologous sequences. This diversity could be the adaptive response to the many different pathogens attacking common bean.

While repeated sequences were not taken into account for the phylogenetic analysis we performed, given that they can create confusion in the construction of gene trees and increase the time of analysis (26), similar sequences near 90% homology were included as individual RGH genes in part of our analysis. Sequences sharing more than 99% nucleotide identity were not included in the phylogenetic analysis but could be interesting for further study as members of the same families or paralogous genes that have new specificities in resistance (14). The most closely related sequences could be the result of recent duplication events from a common ancestral gene (59).

Grouping of common bean sequences according to their gene pool origins was not evident. However, the fact that some RGHs from the same gene pool grouped together could show that these sequences share conserved motifs inside the NBS domain or have other distinguishable characteristics, specifically in the sequences between the conserved motifs. These specificities could be the result of the differentiation in the evolution of RGH genes in each gene pool. Further sequencing of RGH genes from different bean genotypes might allow us to make more definitive conclusions on Andean versus Mesoamerican resistance genes. The TIR gene family of common bean seems to be extensive as the disease resistance genes RPS4, RPP1, L6, and N were distributed in different clades of the TIR subfamily. The other genes from *Arabidopsis*, tomato, rice, and corn belonging to non-TIR clades supported the division and classification of common bean RGH genes as non-TIR sequences.

Our results showed that not all common bean RGHs contained a complete ORF. Out of 403 sequences, one fourth exhibited stop codons or frame shift mutations, hence these sequences could be pseudogenes or partial sequences. Pseudogene identification in RGH studies has been important for various plant species. For example in the genus *Musa*, 25% of RGH sequences were pseudogenes (3). Dicotyledonous genomes contain a variable number of pseudogenes, ranging from 10% in tomato, *Solanum lycopersicum* L. (49), to 50% in *Zingiber* spp. (47) and *Pinus monticola* (34). Pseudogenes could be the result of high rates of recombination, point mutations and indels in RGH sequences as well as rearrangements by transposon that are often observed in *R*-gene families. All of this could be due to the rapid evolution of *R*-genes based on their tandem arrangement in certain parts of the genome (15,32,67). Pseudogenes might drive further evolution since they can be sources of sequences for recombination that allow gene conversion with functional *R*-gene paralogs (42). The variability found within the Kinase-2 motif of the NBS containing genes might also generate functional differences in the proteins these encode. While most TIR type NBS proteins have an aspartic acid (D) at the end of the Kin-2 domain and most non-TIR type NBS

proteins have a tryptophan residue (W) (40,49), we found additional variability for this amino acid position. These amino acid changes could determine the specificity of effector and NBS-LRR protein interactions as was found for chickpea and faba bean RGH sequences studied by Palomino et al. (48). Variability at the Kin-3 motif could also have an important role in changing *R*-gene function (49) especially compared with the putative anthracnose resistance genes identified for both gene pools of common bean by Ferrier-Cana et al. (16).

The ratio between synonymous and nonsynonymous mutations ($\omega = K_a/K_s$, sometimes termed dN/dS) is a good indicator of the type of selection acting on a specific coding region (63). If the ratio is higher than one, the region is under positive selection, but if the ratio is lower than one, then stabilizing selection is acting on the evolution of the sequence. If the ratio is near one, then neutral selection or a combination between positive and stabilizing selection is involved. Given this, the general values obtained for the common bean TIR and non-TIR gene comparisons ($\omega > 1$) reflected purifying selection. This type of selection is not surprising because the NBS domain is an important signaling component in plant defense activation and is highly conserved across different species and genus (56). However, pairwise comparisons inside the clades 11 and 13 showed values higher than 1 ($\omega < 1$) indicating positive selection. Sequences undergoing positive selection deserve a more detailed analysis because they may encode functional changes that drive evolution of a specific taxonomic lineage. Whole genome sequences for legumes could identify specific regions of the genome that have been strongly affected by selection using a sliding-window calculation of K_a/K_s ratio (10).

For previous studies in common bean RGH sequences, diversifying selection was found at the NBS domain of the candidate for I gene BCMV (*Bean common mosaic virus*) resistance, but not in the TIR domain of the same gene (62). Meanwhile, Ferrier-Cana et al. (16) found diversifying selection in the LRR domains of four linked NBS-LRR genes from common bean having to do with anthracnose resistance. In our study, the number of sequences analyzed was larger, producing more evidence that NBS domains are mainly subject to purifying selection. LRR domains meanwhile tend to be subject to diversifying selection as they directly interact with AVR factors that are constantly changing with pathogen evolution. In *Phaseolus vulgaris*, a coevolution process has been observed in the interaction with different pathogens such as *Colletotrichum lindemuthianum* (23), *Phaeoisariopsis griseola* (25), and *Uromyces appendiculatus* (59). Therefore, it is important to undertake more detailed studies of NBS genes in common bean, including genotypes from the Andean and Mesoamerican gene pool, in order to establish the role of pathogen pressure on the evolution of NBS disease-resistance genes in this species.

One further challenge will be to identify the role of each RGH gene and clade in specific disease resistances. Common bean is notoriously affected by multiple diseases such as angular leaf spot, anthracnose, Ascochyta blight, BCMV, BGYMV among many others for a total of at least 86 different diseases reported in *Phaseolus vulgaris*, as a result of infections with a high variety of pathogen species including bacteria, fungi, nematodes, phytoplasmas, and viruses.

The crop is one of the most ancient of the American cultigens and has been continuously exposed to pressure by different microorganism with the need to respond to new pathogen specificities. Plant defense strategies including effector triggered immunity along with several other types of resistance related proteins are important to understand for controlling disease in the crop. The RGH sequences obtained in the present work are a valuable source of molecular markers, which could be used to tag resistance genes of interest and then transfer them between genotypes by marker assisted selection. This would facilitate the generation

of new breeding varieties and contribute to common bean improvement.

The relatively small size of the common bean genome and its low physical to genetic map distance makes this crop species a good candidate for the isolation of *R*-genes via candidate gene techniques. The further exploration of RGH genes in common beans could be used to identify resistance genes in noncommercial genotypes, which may be introgressed into modern varieties through marker assisted selection. This is important in cases such as breeding for anthracnose resistance, where sources of resistance are known but some genes are uncharacterized or for Ascochyta blight resistance, where there are a limited number of sources of resistance in the primary gene pool and only secondary gene pool species are candidates for breeding to improve resistance. The spectrum of resistance offered by RGH sequences is wide across many species of plants and includes resistance to biotrophic or hemibiotrophic pathogens. Thus, there are many opportunities to use the strategy of RGH selection and association with *R*-genes in common bean.

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