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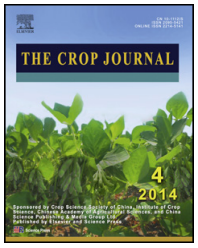

Development and mapping of SSR markers linked to resistance-gene homologue clusters in common bean

Luz Nayibe Garzon[a,b], *Matthew Wohlgemuth Blair*[b,c,d,*]

[a]*Universidad Nacional de Colombia, Dept. de Agronomia, Av. El Dorado, Bogotá, and Dept. de Ciencias Agricolas, Km. 12, Via Chapinero, Palmira, Colombia*

[b]*Universidad de Cundinamarca, Facultad de Ciencias agropecuarias, Programa Ingeniería Agronómica, Facatativá-Cundinamarca*

[c]*Universidad Nacional de Colombia, Dept. de Ciencias Agricolas, Km. 12, Chapinero, Palmira, Colombia*

[d]*Tennessee State University, Department of Agriculture and Natural Science, Lawson Hall, 3500 John A. Merritt Blvd., Nashville, TN 37219, USA*

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ABSTRACT

Common bean is an important but often a disease-susceptible legume crop of temperate, subtropical and tropical regions worldwide. The crop is affected by bacterial, fungal and viral pathogens. The strategy of resistance-gene homologue (RGH) cloning has proven to be an efficient tool for identifying markers and R (resistance) genes associated with resistances to diseases. Microsatellite or SSR markers can be identified by physical association with RGH clones on large-insert DNA clones such as bacterial artificial chromosomes (BACs). Our objectives in this work were to identify RGH-SSR in a BAC library from the Andean genotype G19833 and to test and map any polymorphic markers to identify associations with known positions of disease resistance genes. We developed a set of specific probes designed for clades of common bean RGH genes and then identified positive BAC clones and developed microsatellites from BACs having SSR loci in their end sequences. A total of 629 new RGH-SSRs were identified and named BMr (bean microsatellite RGH-associated markers). A subset of these markers was screened for detecting polymorphism in the genetic mapping population DOR364 × G19833. A genetic map was constructed with a total of 264 markers, among which were 80 RGH loci anchored to single-copy RFLP and SSR markers. Clusters of RGH-SSRs were observed on most of the linkage groups of common bean and in positions associated with *R*-genes and QTL. The use of these new markers to select for disease resistance is discussed.

Abbreviations: BAC, bacterial artificial chromosome; BES, BAC end sequences; RGH, resistance-gene homologue; SSR, simple sequence repeats; TIR, Toll–interleukin receptor.

* *Corresponding author at:* Tennessee State University, Department of Agriculture and Natural Science, Lawson Hall, 3500 John A. Merritt Blvd., Nashville, TN 37219, USA.
E-mail address: mwbeans@gmail.com (M.W. Blair).

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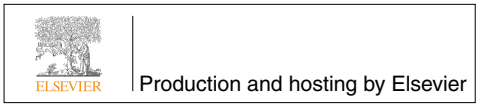

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1. Introduction

Common bean (*Phaseolus vulgaris*) is one of the most important legumes worldwide, with more than 20 million tons produced yearly in many countries, of which more than half is harvested in Brazil, Mexico, India, China, and the United States of America [1]. Two major genepools have been established, namely the Andean and Mesoamerican genepools [2]. This differentiation, as well as finer distinctions within each genepool, has been confirmed by morphological, agronomical, and biochemical traits including phaseolin protein patterns and isozymes [3], and molecular markers, especially microsatellite or simple sequence repeat (SSR) markers [4–6].

Production of common beans is constrained by pathogens that include bacteria, fungi, phytoplasms, and viruses. Anthracnose (*Colletotrichum lindemuthianum*), rust (*Uromyces appendiculatus*) and ascochyta (*Phoma exigua*) are considered the most important fungal diseases of this crop worldwide, with an angular leaf spot (*Phaeoisariopsis griseola*) important in tropical countries [7]. Genetic resistance is the most widely used management strategy for these pathogens [8]. Many major resistance (*R*) genes have been evaluated by linkage analysis, and many of these genes have been molecularly tagged in common bean, but mostly with older types of markers such as sequence characterized amplified region (SCAR) markers [9,10] rather than a newer type marker such as with SSR or single nucleotide polymorphism (SNP) markers, which are more reliable and polymorphic owing to their codominant and multi or bi-allelic nature, respectively [4].

Currently, there is wide interest in the use of resistance-gene homologues (RGHs) for identification of *R*-genes. This strategy is based on the design of degenerate primers from highly conserved sequence motifs characteristic of the nucleotide binding site (NBS) domain and has been applied in many crops [10–12]. The principle of RGH cloning is simple: if there is a PCR amplicon from RGH related degenerate primers with the desired size, it could be part of a resistance gene. RGH genes are also known as resistance-gene analogs (RGAs) [12], and sometimes as resistance-gene candidates (RGCs) [13–15]. Compared to the other domains common to *R*-genes, such as LRR repeats or Toll–interleukin receptor (TIR) domains, the NBS domain is associated almost exclusively with disease resistance [15].

After RGHs are identified, a subsequent step consists of their genetic mapping. This operation is difficult because of the high similarity among certain parts of RGH sequences. For this reason, finding specific markers near the RGH genes can be a better approach to genetic mapping of these genes. A commonly used approach is to develop RGH-SSR based on SSR markers that are physically associated with RGH genes on bacterial artificial chromosome (BAC) clones. RGH-SSR genes are often found in BAC sequencing projects but can also be found in the BAC end sequences (BES) of clones containing RGH genes.

In this study, we identified individual BAC clones with single or multiple RGH genes by a hybridization-based approach and found SSRs in the BES sequences of these or adjacent BAC clones. The RGH-SSRs thus identified were then located on a genetic map of common bean. To date, a high number of mapping populations have been developed [16], by means of which many *R*-genes or loci that respond and provide resistance to diseases or biotic stresses have been identified [9]. The combination of RGH-SSR markers with SSR-based maps allows the placement of disease resistance loci relative to microsatellite loci that have already been mapped [16–19]. In addition, RGH-SSR can be used for selection of marker and disease-resistance trait combinations [8,9]. The RGH-SSR is most likely to be polymorphic in populations from inter-genepool crosses such as DOR364 × G19833 which has a high level of polymorphism for most SSR markers [16–20].

The specific objectives of the present study were 1) to evaluate probes designed from RGH genes and pseudogenes of common bean found by hybridization to a BAC library for G19833 (a standard accession for full genome sequencing); 2) to identify positive BAC clones from the library, and 3) to determine whether SSR markers were localized in the BES sequences of positive or adjacent BAC clones. Once RGH-SSRs were identified, they were named as bean microsatellite RGA-associated (BMr) markers and their polymorphism was evaluated in the DOR364 × G19833 mapping population. The polymorphic markers were integrated into a microsatellite and RFLP based map as a tool for further identification of regions containing potential *R*-genes. In addition, the locations of the RGH-SSRs were compared to the known locations of *R*-genes for specific diseases in common bean. This study continues that of Garzón et al. [26] in which families of RGH sequences were identified in common bean by phylogenetic analysis.

2. Materials and methods

2.1. Probe design

Specific RGH sequences from common bean were identified based on 544 degenerate primers from *Medicago truncatula* *R*-genes followed by phylogenetic analysis [26]. Multiple alignment of the RGH bean nucleotide sequences was performed using MAFFT software (FFT-NS-i, slow iterative refinement method) [27]. TIR and non-TIR sequences were aligned independently in order to identify closely related sequences and to select a subset of unique sequences for designing hybridization probes. Clustering into clades of highly similar sequences (>90% nucleotide identity) was performed with the program JALVIEW [28]. One representative sequence of each clade was selected using CLUSTAL W [29]. These conserved sequences were used for probe design. Each probe was designed using Primer3 software [25], excluding the first and last 30 base pairs (bp) of each sequence. The probes were amplified using G19833 DNA as a template. The PCR products were sequenced with an ABI 3730 XL capillary sequencer, to validate the presence of respective TIR or non-TIR sequences.

2.2. BAC library filter evaluation

An aliquot of 60 ng of the purified PCR product was labeled with radioactive ^{32}P using the Ready-to-Go labeling protocol (Amersham, Biosciences Corp.). Pre-hybridization was

performed for 12 h at 65 °C in a solution containing 0.25 mol L^{-1} sodium phosphate buffer (pH 7.2); 7% SDS, and 1 mmol L^{-1} NaEDTA in a hybridization oven at rotation speed of 4 min^{-1}. After the filters were rinsed thoroughly with prehybridization buffer, 20–30 mL of fresh preheated hybridization buffer was added into the bottles followed by the denatured probes under denaturing conditions. Eight probes were hybridized together per bottle to reduce the number of hybridizations. In the first four assays only TIR probes were hybridized, and in the last six assays non-TIR probes were hybridized. The hybridization process was performed at 60 °C overnight at 3–4 min^{-1} rotation speed. Following the hybridization, the filters were rinsed with 40–50 mL of a solution containing 2 × SSC–0.1% SDS previously preheated to 60 °C. Two washes were performed for 30 min at 65 °C with rotation in large containers having 1 L each of 1 × SSC–0.1% SDS and 0.5 × SSC–0.1% SDS, respectively. After washing, the filters were covered with plastic wrap, transferred to phosphor image plates (FUJIFILM Company) for overnight exposure, and scanned with a Storm 820 detector (Molecular Dynamics). The positive clones were scored with the program ComboScreen [30] and ID number found at the common bean FPC website (http://Phaseolus.genomics.purdue.edu/WebAGCoL/Phaseolus/WebFPC), in order to determine whether the clone was part of a contig or was classified as a singleton.

2.3. Identification and evaluation of SSR markers

Three strategies were used to identify SSR markers. First, positive BAC clones were extracted from the G19833 BES database and clones associated with a RGH were evaluated for the presence of SSR loci [31]. The BES-SSR markers were cross-compared to RGH-positive BAC clones and these microsatellites were called primary hits. If the positive BAC clone did not contain a SSR marker within its BES, it was necessary to evaluate the presence of an SSR in other positions of the contig. If the result was positive, this SSR was called a secondary BES hit. The new SSR markers were named BMr markers and were evaluated for polymorphisms with the parents of the population DOR364 × G19833 [16]. Amplification reactions for SSR contained 25 ng of total DNA template, 1× buffer (500 mmol L^{-1} KCl, 10 mmol L^{-1} Tris–HCl, pH 8.8, 1% Tritron X-100, and 1 mg mL^{-1} bovine serum albumin), 0.10 μmol L^{-1} of each primer (Invitrogen Corp., Carlsbad, CA), 0.20 mmol L^{-1} of each dNTP, 2.5 mmol L^{-1} $MgCl_2$, 1 unit of *Taq* DNA polymerase, and HPLC grade H_2O. Each reaction was performed in a final volume of 15 μL. Amplification was performed on a PTC-200 thermocycler (MJ Research Inc., Watertown, MA), programmed for an initial denaturation at 94 °C for 3 min, followed by a touchdown program (55–45 °C) of 10 cycles at 94 °C for 30 s, 55 °C (with −1 °C decrease per cycle) for 30 s, 72 °C for 45 s, and then 25 cycles at 94 °C for 30 s, 45 °C for 30 s, and 72 °C for 45 s. The reaction was terminated after a final extension at 72 °C for 5 min. After SSR amplification, 5 μL of formamide containing 0.4% w/v bromophenol blue and 0.25% w/v xylene cyanol were added to each PCR sample. This mixture was denatured at 96 °C for 5 min and then loaded onto 4% polyacrylamide gels (29:1 acrylamide:bisacrylamide) on Owl Sequencing Chambers (Thermo Fisher Scientific Inc, Waltham MA), run at 100 W for 1 h in 0.5 × TBE buffer (pH 8.0), and silver stained to visualize the PCR products.

2.4. Genetic mapping

The polymorphic BMr markers were evaluated in the DOR364 × G19833 population of $F_{9:11}$ recombinant inbred lines (RIL) as described in Blair et al. [16]. DOR364 is a small red-seeded variety of the Mesoamerican genepool, grown in several countries of Central America. G19833 is a landrace originally collected in Peru of the Andean genepool with large, yellow and red-mottled seed and has been selected for genomic sequencing. An anchor genetic map for this population was built with single-copy RFLP and SSR markers (of the series BM, BMa and BMd) using the software Mapmaker 3.0 for Windows [31]. Genotyping results of the present work were recorded in a Microsoft Excel worksheet with female alleles as "A" and male alleles as "B". Heterozygotes or missing data was not considered. The BMr markers were added to the genetic map using the software program MapDisto v. 1.7 (http://mapdisto.free.fr/) with "find groups" at a minimum of LOD > 3.0. The "order sequence" and "compare all orders" commands were then used to identify the best marker order for each linkage group. The location of anchor markers was cross-checked with the map of Blair et al. [16]. Linkage groups were drawn according to the cytogenetic orientation of their corresponding chromosomes based on Fonsêca et al. [32]. R-genes or QTL were added to the map based on the estimated positions in Miklas et al. [9]. Genetic distances between markers in centiMorgans (cM) were obtained using the Kosambi function, which assumes crossover interference.

3. Results

3.1. RGH sequence selection and probe design

The first step in the detection of RGH-SSRs in common bean was probe design, which was based on singleton and assembled RGH gene and pseudogene sequences (Table 1). A total of 86 probes were amplified for screening of the G19833 BAC library. Based on the phylogenetic analysis of Garzón et al. [26], 38 of these represented TIR clades and 48 non-TIR clades. Some sequences with premature stop codon or with no evident open reading frame (ORF) were considered pseudogenes (22 out of 86) but were also used for probe design. If a probe was designed from two or more sequences, it was classified as from assembled sequences. Singleton probes were those designed from only one sequence sharing no more than 90% identity with any other sequence according to Garzón et al. [26]. Almost all the TIR probes were designed from assembled RGH gene sequences. Most of the non-TIR probes were designed from single RGH. Pseudogenes, even those with a low identity value with other sequences, were used for probe design because we were interested in identifying the maximum number of putative common bean RGHs.

3.2. Detection of RGH probe signals in the G19833 BAC clones

The next step was confirming probe amplification and deciding which probes to hybridize to the G19833 BAC library.

This was done by sequencing the PCR products of the probe amplification described above. After sequencing to confirm similarity and sequence identity to RGH genes, a total of 37 TIR and 43 non-TIR probes were validated for filter hybridization against the G19833 BAC library filters, which were in sets of three membranes per library and covering 12× genome equivalents. The original source of the BAC library was the Clemson University Genome Center, where 55.296 clones with an average insert size of 145 kb were distributed in 144 plates (384 wells). Preparation of the filters was done at the Purdue Genomic Center where a 10× genome equivalent number of clones was blotted onto the three nylon membranes. A total of ten hybridization assays were required for the 80 PCR-based probes to be evaluated, as eight probes were evaluated simultaneously. Hybridization of the G19833 BAC library with the 80 RGH probes identified 3202 positive BAC clones (Table 2). Variable numbers of positive BAC clones were observed for each hybridization assay. After redundant BAC clones were eliminated by ID number, the number of unique positive clones still varied. Differences were also observed depending on whether the probes analyzed were designed from TIR sequences (first four assays) or non-TIR sequences (last six assays) and the type of probe class used in the assay, namely if belonging to an assembled group of RGH sequences or a singleton RGH sequence. Some BAC clones hybridized with more than one probe, so that the positive clones were represented as from 1 to 5-fold, as shown in Table 3. We considered this classification useful, given that RGH loci occur as clusters of related genes.

Table 2 – Positive bacterial artificial chromosome (BAC) clones and unique positive clones identified by 80 RGH probes in the G19833 library.

Assay number	Positives BAC clone	Unique positive clone	% of unique positive BAC clone	Probe type
1	192	192	100.0	TIR
2	168	30	17.9	TIR
3	164	123	75.0	TIR
4	65	65	100.0	TIR
5	601	599	99.7	non TIR
6	573	195	34.0	non TIR
7	362	17	4.7	non TIR
8	406	99	24.4	non TIR
9	423	45	10.6	non TIR
10	248	86	34.7	non TIR
Total	3202	1451		

3.3. Discovery and selection of RGH-SSR markers

Of the 3202 positive clones, a total of 1451 were unique, nonredundant BAC clones. These positive hits from the hybridization process represented a total of 2902 BAC-ends on their 5′ and 3′ ends, although previous BAC-end sequencing was limited to the number of BAC clones representing only a 10× genome equivalent [33]. For this reason there was no actual BES sequence information for some positive hits. Analysis of the BAC-end sequence database for common bean allowed us to identify 2319 GenBank entries associated with the RGH-positive BAC clones. Of these, 1766 BES sequences were distributed in 164 BAC contigs and 553 were from singletons or non-overlapping BAC clones. We distinguished two types of positive BAC clones: primary hit BAC clones (the actual BAC with an RGH hybridizing to it) or secondary hits (an adjacent BAC from a contig containing the RGH-positive BAC). Following the procedures of Córdoba et al. [18,19], more than 600 BES-SSRs were identified in 2319 BAC-end sequences from the 3202 positive BAC clones (primary hits) or adjacent contigged BACs (secondary hits). This identification involved evaluations performed by three SSR discovery software pipelines: Batchprimer3 [22], SSRLocator [23], and AMMD [24] with TROLL [25], which found a total of 629 BES-SSR markers. These were named BMr markers (Table S1) to distinguish them from other series of markers that our laboratory has produced (BM, BMa, BMb, BMc, BMd, and BMe marker types). It is noteworthy that all the BMr markers can be considered also to belong to the BMb series, the series originally developed as BES-SSR markers [18,19]. However, given the importance of their association with RGH sequences, we decided to highlight them as being related to resistance genes and accordingly named them BMr markers. In a comparison of the different software engines, the program AMMD detected more total BES-SSR loci (319) than Batchprimer3 (257), while SSRLocator identified the fewest BES-SSR loci (53). Batchprimer3 identified 55 BES-SSR from the BAC-ends of primary BAC clones, distributed among 19 BAC contigs and 15 BAC singletons. Analysis of the secondary hits or adjacent BAC clones from

Table 1 – Number of probes designed for TIR and non-TIR sequences.

Probe class	TIR		Non-TIR		Total
	No.	%	No.	%	
Assembled RGH sequences	11	29	8	17	19
Singleton RGH sequence	7	18	16	33	23
Assembled RGH sequences, pseudogenes	11	29	11	23	22
Assembled pseudogenes[a]	5	13	5	10	10
Singleton pseudogenes[a]	4	11	8	17	12
Total general	38		48		86
Total validated probes	37		43		80

[a] Probes designed exclusively for pseudogene sequences.

Table 3 – Number of bacterial artificial chromosome (BAC) clones from the G19833 library hybridizing with different numbers of probes.

Number of probes	Number of positive BAC clones	%
1	657	21
2	548	17
3	732	23
4	460	14
5	805	25
Total	3202	100

RGH-containing BAC clones identified 202 SSRs distributed in 101 contigs. SSRLocator identified 20 primary hits, of which almost half were in BAC singletons, and 33 BES-SSRs from secondary hits distributed over 24 contigs. AMMD identified the most primary hits, with 103 SSR distributed in 46 BAC contigs and 35 BAC singletons, and 181 secondary hits distributed in 70 BAC contigs. In total, 629 BMr loci were found associated with RGH-containing BACs.

3.4. *Repeat types identified in RGH associated BAC-end sequences*

The breakdown of SSR motifs and their detection by various software programs for the 629 BMr loci are summarized in Tables 4 and 5. A total of 277 loci (44.0% of the total) were based on dinucleotide-based SSRs, 199 (31.6%) on trinucleotide, and 139 (22.1%) on tetra-, penta-, and hexanucleotide repeats. Based on previous evaluations [18,19], it was decided to target 476 mostly dinucleotide or trinucleotide repeat BES-SSR loci for testing. Primary hits identified with AMMD had a greater number of hexanucleotide or compound repeats than SSRLocator. However, AMMD did find dinucleotide (32%) and trinucleotide (21%) repeats in the primary BAC clones that were useful for marker development. The majority of secondary hits with SSR loci were of trinucleotide (54%) followed by dinucleotide (44%) repeat types. The use of three software programs to identify SSR loci was useful, given that each program complemented the other programs by detecting new loci. Compound repeats were infrequent in all evaluations, especially that of Batchprimer3, which did not find this repeat type. In other examples, Batchprimer3 detected no hexanucleotides in primary hits and SSRLocator detected no pentanucleotide repeats at all.

3.5. *Polymorphism and genetic mapping of BMr markers*

The full set of 629 BMr marker primer pairs (Table S1) was ordered, but only 200 were tested for polymorphism. In total, 63 BMr markers were observed to be mappable in the mapping population (Fig. 1). These were placed on the genetic map relative to 184 anchor markers (BM microsatellites and BNg or D single-copy RFLPs) from Blair et al. [16,17], as well as 14 RGH-RFLPs from López et al. [34] for a total of 264 loci and a genetic map of 1747.4 cM in length (Table 6). The average distance between markers was 6.6 cM and ranged from 5.4 cM on linkage group B02 to 9.8 cM on linkage group B05. The longest linkage groups were B06 (212 cM) and B09 (204.6 cM), while the shortest were B08 (104 cM) and B03 (109.5 cM). Chi-squared tests for an even distribution of marker types across all linkage groups were also used to show that BMr ($P \leq 0.0001$) and RFLP-RGH ($P \leq 0.0000$) markers were especially unevenly distributed. The largest numbers of BMr markers were concentrated on linkage groups B01 and B06 (>10 each) and also on B04 (8 markers) and B11 (7 markers). The linkage groups containing RGH-RFLPs were B10 (6 markers), B08 (4 markers), and B04 and B11 (1 marker each). The total number of markers varied from 15 (for B08) to 34 (for B02) with large numbers of markers also on B01 and B06 owing to the mapping of new BMr markers. Interestingly, although 18 loci were mapped as RGH-RFLPs [34], some of these were dominant bands and did not map in this study owing to low LOD scores; in particular, RGH4A, RGH4C, RGH5a, and RGH5b on linkage groups B01, B02, and B03 could not be confirmed. The other 14 RGH-RFLP did map to the correct locations and were closely linked to other BMr markers, including RGH4B, which mapped to the predicted position on linkage group B07.

4. Discussion

There were several major achievements of this study. First, we developed a reduced probe set for screening the G19833 common bean BAC library for RGH-like sequences. Of the 403 different RGH sequences identified by Garzón et al. [26], a total of 86 were developed as probes (38 TIR and 48 non-TIR). Most of these probes were NBS domains that were uninterrupted; however, pseudogenes were included in our probes, since they can result from rapid evolution and recombination in *R*-gene clusters [35], creating many adjacent paralogous sequences [36] that are reservoirs of variation [37]. Indeed, proper probe design was found to be an important factor for

Table 4 – Comparison of primary and secondary hits identified by Batchprimer3, SSRLocator, and AMMD programs.

BMr origin	Primary hits				Secondary hits		Total SSR
	Contig		Single BACs	Sub-total	Contig		
	Contig number	SSR number			Contig number	SSR number	
Batchprimer3	19	40	15	55	101	202	257
SSRLocator	9	11	9	20	24	33	53
AMMD	46	103	35	138	70	181	319
Total SSR	74	154	59	213	195	416	629

Table 5 – Motif types found at BMr loci identified as primary and secondary hits, using Batchprimer3, SSRL, and AMMD search engines.

Repeat type	Batchprimer3		SSRL		AMMD		Total
	Primary hit	Secondary hit	Primary hit	Secondary hit	Primary hit	Secondary hit	
Dinucleotide	24	89	7	33	44	80	277
Trinucleotide	13	57	3		29	97	199
Tetranucleotide	9	24	2		40	2	77
Pentanucleotide	9	32			9	2	52
Hexanucleotide			3		7		10
Compound			5		9		14
Total	55	202	20	33	138	181	629

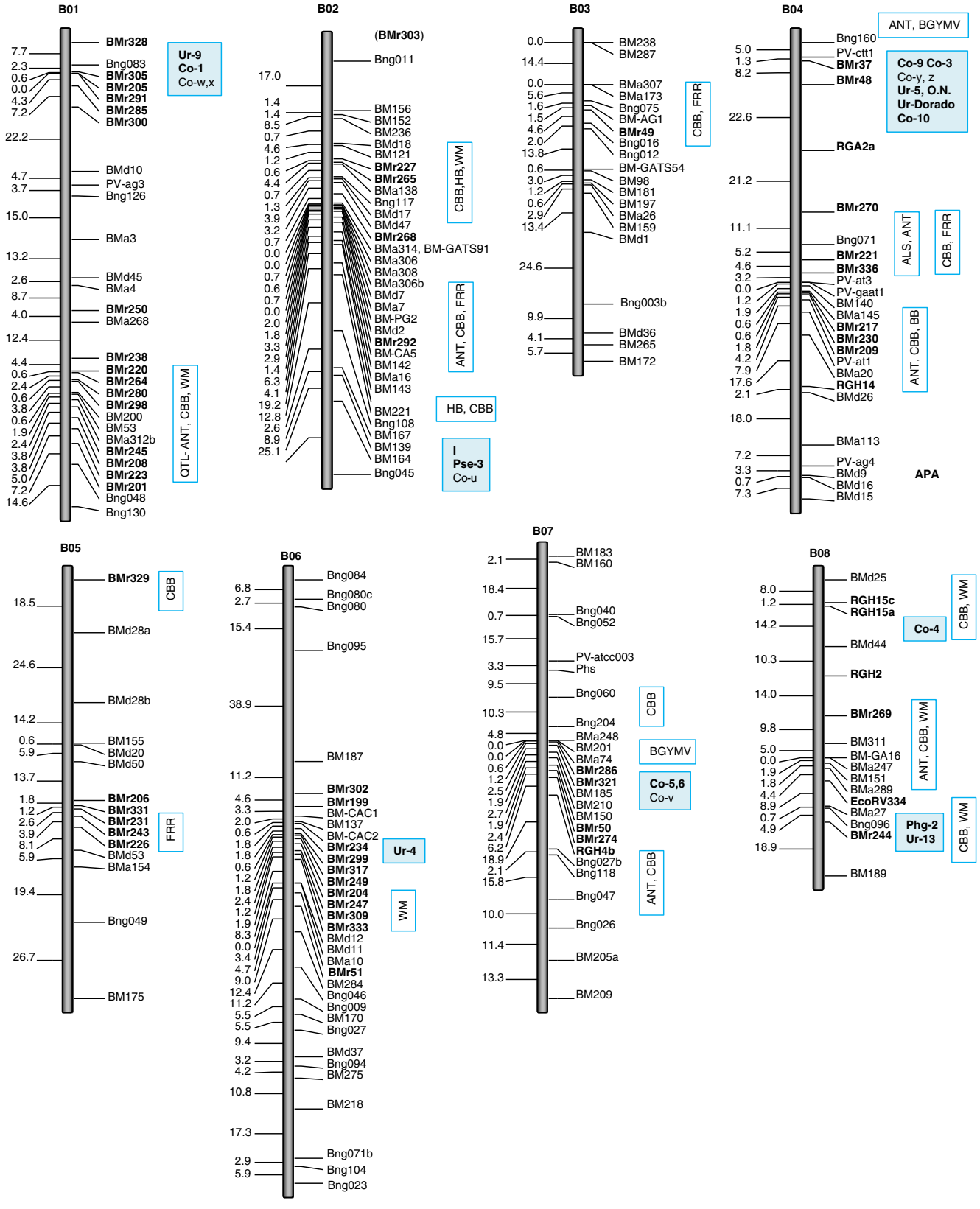
successful hybridization. In this study the primer pairs, designed for probe hybridization with the bean BAC library, had GC content of around 43% and average length of 22 bp, properties that were important for amplification of true *R*-gene homologues. Melting temperatures of forward and reverse primers were close to 60 °C. Expected product sizes, according to the positions of reverse and forward primers in the sequences, ranged from 240 to 666 bp with an average of 408 bp. Most probes contained the NBS domains with DNA sequences for Kin-2, Kin-3, P-loop, and GLPL protein polypeptide sequences characteristic of RGH genes [10–12], as confirmed by resequencing.

The second achievement of this work was the identification of BAC clones that contained RGH genes or pseudogenes using BAC filter hybridizations made efficient by pooling probes. Some redundancy of positive hits occurred between assays owing to RGH clustering [15]. This result also confirmed that TIR and non-TIR type *R*-genes could occur on the same BAC. However, specific clusters could be composed of large numbers of NBS genes of one type. David et al. [38] identified 26 non-TIR genes arranged in four clusters within a region of 650 kb at the end of chromosome B04, predicting that these had arisen from ectopic recombination between genomic regions. The high numbers of duplications in non-TIR genes may be explained by our results. In the four hybridization assays where only TIR probes were evaluated, mostly unique positive clones were identified. For example, for filters 1 and 3 all of the positive clones were unique sequences. However, in assays 5 to 10, performed with non-TIR probes, only 22% of the positive clones were unique sequences. The frequent hybridization of non-TIR probes to the BAC clones of the G19833 library suggests that the RGH sequences arose before the divergence between monocotyledonous and dicotyledonous plants and have an older evolutionary history [35,39]. In contrast, TIR domain sequences have hardly been identified in monocots but have evolved substantially in dicotyledonous plants [40].

Some probes hybridized with more than one BAC clone in the G19833 common bean genomic library. This result was expected, because this BAC library had a genome coverage of 12× haploid genome equivalents. In addition, duplicated genes or closely related paralogous sequences could account for the redundancy in hybridization. Also, it must be remembered that the probes were designed from sequences related to RGH genes, which represent a large and diverse gene family with many copies distributed throughout the genome [41]. If a higher number of gene duplication events have occurred in non-TIR sequences, then this could be the reason for finding more redundant sequences of this type in common bean.

The third major objective and achievement of this work was to develop and genetically map RGH-SSR sequences. This was achieved by identifying RGH-positive BAC clones or adjacent contigged BACs that were associated with SSRs in their BAC ends. The major point of this exercise was the physical linkage of the BES-SSR to the RGH sequence either as a primary hit in very close proximity within the length of a given BAC, or as a secondary hit within the length of a contig of BACs. The proportion of SSR in BES in regions near RGH genes (35.6%) appears to be higher than in previous estimates using the overall collection by Córdoba et al. [18,19]. The high frequency of SSRs in regions with RGH sequences may be a characteristic of genomic regions with RGH clusters. David et al. [38] observed that RGH clusters were interspersed with non-RGH genes, so that these EST providing regions may also be rich in SSRs [20,21]. It was also interesting that the proportion of hybridizing BACs falling in singleton BACs rather than contigs showing the difficulty of assembling regions with RGH sequences, owing to their characteristic presence in tandem repeats and their similar sequence domains [42,43]. The assembly of *R*-gene sequences and the search for RGH-SSR markers is made easier by long BAC clones, given that *R*-gene clusters tend to be large [44]. This trend is borne out in a study of the peanut (*Arachis hypogaea* L.) genome, where BES-SSRs linked to RGH sequences were helpful in genetic mapping [45]. On another point, the types of repeat motifs found near RGH genes appeared to be similar to those found in non-coding regions of the genome [46]. In both cases dinucleotide repeats were more common than trinucleotide repeats. One might assume, therefore, that the majority of RGH-SSRs reside around *R*-genes rather than inside them. In common bean, gene-coding regions are known to have a higher abundance of trinucleotide repeats versus other types of repeats [47,48].

The fourth achievement of the present study was the successful genetic mapping of a subset of BMr markers into a genetic map containing previously mapped anchor markers. Notably, all of the RFLP-RGH markers were mapped to the same locations as predicted in López et al. [34]. Similarly, the RFLP (BNg) and SSR markers were in the same approximate locations as in previous reports for the same population [17,49]. The phaseolin locus was mapped with a high LOD score to linkage group B07 in the expected



location on the short arm. Finally, the length of the genetic map, approximately 1750 cM in total, is similar to previous estimates for the D × G and many other RIL populations of common bean [16,17]. Dominant AFLP and RAPD markers were removed from the genetic map because they caused inflation [17].

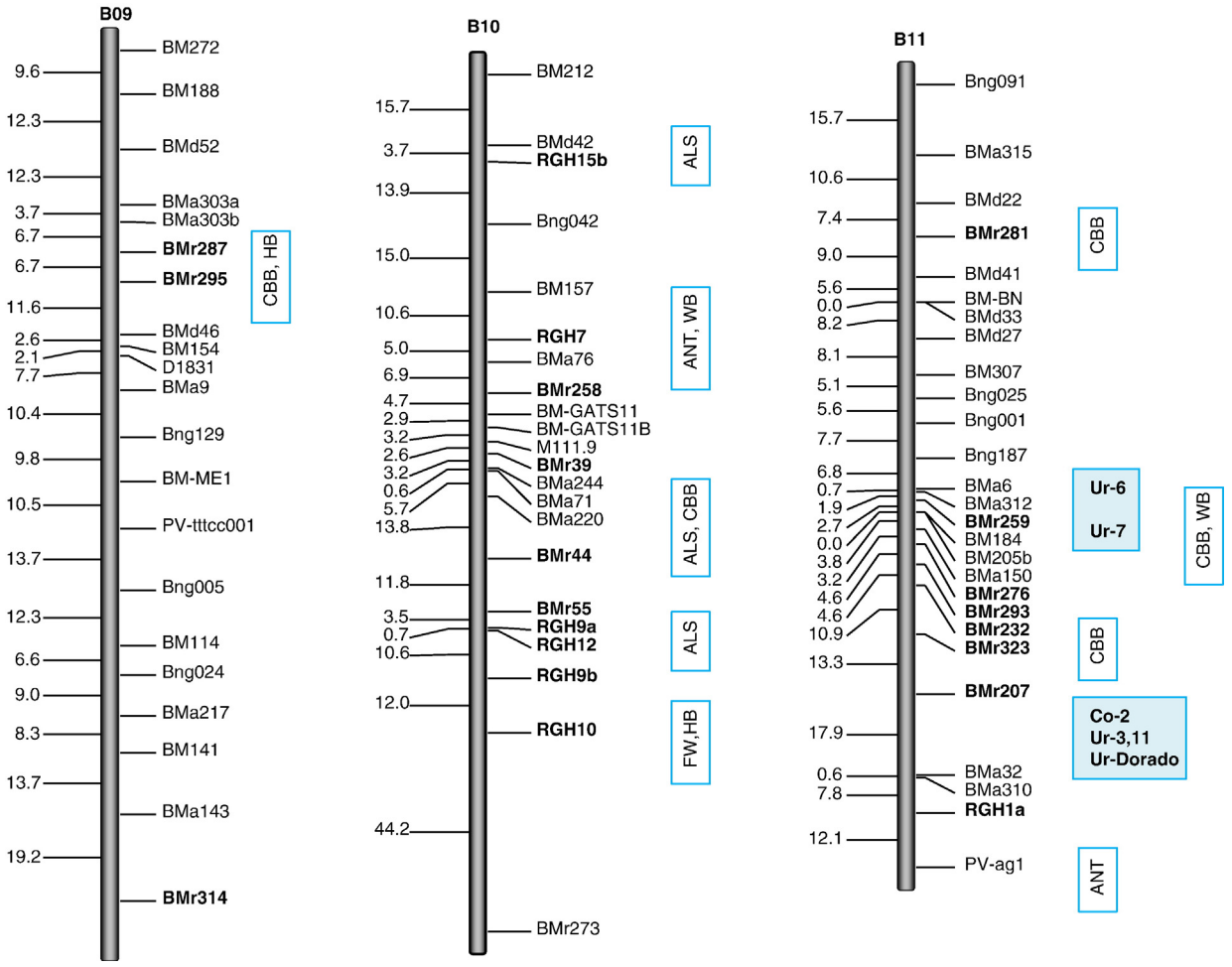


Fig. 1 – Genetic linkage map for the DOR364 × G19833 mapping population with anchor simple sequence repeat (SSR) and restriction fragment length polymorphism (RFLP) markers from Blair et al. [5,16,17] along with novel resistance-gene homologue (RGH) markers represented by RGH-SSR markers (BMr) and RGH-RFLPs from López et al. [34] highlighted in bold to the left of each linkage group. Map distances between markers are given in centiMorgans. Major resistance genes, known *R*-genes and quantitative trait loci (QTL) are in shaded and unshaded boxes, respectively, which are positioned based on Miklas et al. [9] using the same coding for diseases as in that publication: ALS, angular leaf spot, ANT, anthracnose; BB, bacterial brown spot; BGYMV, bean golden yellow mosaic virus; CBB, common bacterial blight; FRR, *Fusarium* root rot; FW, *Fusarium* wilt; HB, halo blight; WB, web blight; and WM, white mold.

Interestingly, the positions of the BMr microsatellites were mostly in clusters in few specific locations of the genome. The number of BMr markers was variable between linkage groups, just as numbers of *R*-genes and QTL for disease resistance have varied between linkage groups in a compiled map of results from studies in common bean [9]. There was an association of the positions of genes and QTL for disease resistance with the RGH-SSR clusters uncovered in this study with BMr markers. Apart from the previously observed associations between resistance to angular leaf spot and anthracnose with RGH-RFLP probes reported by López et al. [34], there were many further associations with the QTL shown in the map of Miklas et al. [9].

One of our goals in this study was to produce a genetic marker resource that would be useful for genetic mapping and characterizing the *R*-gene clusters in common bean. In this respect, the present RGH-SSR marker map is better for marker-assisted selection of *R*-genes than those based on RFLP markers, [34] dominant NBS-profiling markers [48], or RAPD type TRAP markers [50]. This superiority is due to the easy reproducibility and detection of codominant microsatellite markers in the BMr series compared to other technologies. Among the major genes mapped to locations near BMr markers are many of the most important genes useful in common bean breeding for disease resistance. Fig. 1 shows the alignment of bacterial, fungal and viral disease resistance genes with BMr markers and RGH-RFLP (RGH1 to 15). These associations are described below in order of chromosome, with the numbering of Fonsêca et al. [32].

On linkage group B01, the alignment of the anthracnose resistance genes *Co*-1, *Co*-w, and *Co*-x and the rust *R*-gene *Ur*-9 is evident at the top of the short arm of the equivalent chromosome near the RGH-SSR markers BMr205, BMr285, BMr291, BMr300, BMr305, and BMr328. A large number of QTL for

Table 6 – Genetic mapping and map distance in centiMorgans for each linkage group made up of BMr (RGH-SSR) markers associated with resistance genes, anchor markers, and RGA-RFLPs from previous studies [16,17].

Linkage group	BMr marker	Anchor marker	RGH-RFLP[b]	Total marker	Total distance	Average distance[c]
B01	16	13	0	29	159.4	5.5
B02	5	30	0	35	182.2	5.4
B03	1	18	0	19	109.5	5.8
B04	8	15	2	25	156.8	6.3
B05	7	8	0	15	147.1	9.8
B06	10	23	0	33	212.0	6.4
B07	4	19	1	24	155.7	6.5
B08	1	10	4	15	104.0	6.9
B09	3	19	0	22	204.6	9.3
B10	5	10	6	21	142.3	6.8
B11	7	19	1	27	173.8	6.4
Total	66	184	14	264	1747.4	6.62
Chi-square[a]	33.02	5.33	58.59	1.91	125 × 104	
Prob–Chi-Sq	0.0001	0.0056	0	0.0417	0	

[a] Chi-squared test for equal distribution.
[b] RGA-RFLP markers from López et al. [34].
[c] Total linkage group distance and average distance between markers on that linkage group in centiMorgans (cM).

resistance to anthracnose, common bacterial blight, and white mold are known to map to the long arm of this chromosome [9] and probably are associated with the ten RGH-SSR markers located in the interval between BMr201 and BMr250. All of these markers provide tools for marker-assisted selection for this linkage group and would assist in the dissection of the cluster of Andean anthracnose *R*-genes or alleles of *Co*-1 at the top of the short arm of this chromosome [51].

On linkage group B02, alignment of four BMr markers (BMr227, BMr265, BMr268, and BMr292) can be postulated with QTL for anthracnose, common bacterial blight, *Fusarium* root rot, halo blight, and white mold. However, it appears that no RGH-SSR was found for genes *I*, *Pse*-3, and *Co*-u [51]. The dominant *I* gene against bean common mosaic virus has been shown to lie within a cluster of NBS-LRR genes [52], but perhaps its sequence was not picked up by our library screening. Linkage group B03 had only one RGH-SSR in the region of QTL for common bacterial blight and *Fusarium* root rot resistance. Generally, this chromosome seems not to contain many RGH genes, although recessive virus *R*-genes such as *bc*-1^2, *bgm*-1 and perhaps *bc*-u have been mapped subtelomerically to the chromosome.

The map of linkage group B04 was among the most interesting, as this chromosome has been well characterized for many major *R*-genes and RGH sequences [53,54]. These include the anthracnose resistance genes *Co*-3, *Co*-9, *Co*-10, *Co*-x, and *Co*-y and rust resistance genes *Ur*-5, *Ur*-Ouro negro, *Ur*-Dorado, as well as many QTL against angular leaf spot, anthracnose, common bacterial blight, *Fusarium* root rot, and bean golden yellow mosaic virus [9]. This region has eight RGH-SSR and two RGH-RFLP (2a and 14) on the full chromosome, except at the end of the long arm, which contains the APA locus [55]. This is an example of a linkage group with well-characterized disease resistance factors coincident with panoply of potential *R*-gene markers. Fine mapping of *R*-genes, QTL and new markers are needed to determine the utility of the new RGH-SSR for marker assisted selection.

Linkage group B05 is an example of a chromosome that has been under-studied for resistance factors and yet had six RGH-SSR markers. So far, only QTL have been described for B05 with possible association between BMr329 a common bacterial blight QTL near the end of the short arm, as well as a cluster of five BMr markers in the middle of the linkage group associated with a QTL for *Fusarium* root rot resistance [9]. The promise of markers for root rot diseases is high compared to that for foliar diseases, which can be rapidly selected for phenotypically in the greenhouse. Linkage group B06 also has few major *R*-genes [9], with the notable exception of *Ur*-4, despite its apparent abundance of RGH sequences. The position of *bc*-3 was not considered, as this is a recessive *R*-gene that has been suggested to be related to a family of elongation initiation factors [56]. However, the *Ur*-4 gene, as well as a QTL, for white mold [9] was observed to lie in the same region as BMr51 to BMr302.

Linkage group B07 contained *Phs*, a phaseolin-encoding locus associated with a common bacterial blight QTL, as well as 4 RGH-SSR plus RGH4b, in the region suspected to contain the *R*-genes (*Co*-5, *Co*-6) and further QTL for anthracnose and common bacterial blight resistance. The three *R*-genes and multiple QTL on linkage group B08 aligned well with RGH genes. *Co*-4, although suspected to be a protein kinase gene, was near the loci RGH15a and RGH15c along with QTL for common bacterial blight and white mold resistance. QTL for resistance to the same diseases plus a QTL for anthracnose resistance were near RGH2, BMr244, and BMr269 and a previously unmapped RGH-RFLP named EcoRV334, which was in the region containing the *Phg*-2 (angular leaf spot) and *Ur*-13 (rust) resistance genes [9].

The next two linkage groups were contrasting, in that B09 had few RGH-SSR (3) and few QTL for resistance, while B10 had a large number of RGH genes (10) and many QTL for various diseases. Linkage group B10 has emerged as being very important for angular leaf spot resistance. One report cites anthracnose resistance in the middle of B10 although this is unconfirmed in other studies [34]. Major *R*-genes for angular leaf spot on B10 could be analyzed in relation to *Phg*-1, a new Andean *R*-gene on B01 [57]. The final chromosome-linkage group B11, especially the end of the

long arm, has been long known to be a hotspot for *R*-genes [9]. From the bottom of B11, there was alignment of BMr207 and RGH1a with *Co*-2, *Ur*-3, *Ur*-11, and *Ur*-Dorado [9]. Two other major *R*-genes for rust, *Ur*-6 and *Ur*-7, along with common bacterial blight and web blight QTL, are likely to be tagged by 5 RGH-SSR markers in a more proximal location on the chromosome B11 and in the upper part of the linkage group B11 another QTL for common bacterial blight may align with marker BMr281.

In summary, this work established the position of new RGH-SSR markers relative to known *R*-genes. A large number of RGH-related markers have been developed, including 32 from the BAT93 × Jalo EEP558 population [48], 21 from the Dorado × XAN 176 population [50], and 14 from the Calima × Jamapa population [58]. Mutlu et al. [59] coincidentally mapped 32 RGAP bands in the first of these populations and also detailed alignment with QTL and *R*-genes.

Finally, the 629 RGH-SSR markers created in this study are very important for dissecting not only RGH clusters, which tend to have high rates of recombination, but complex evolutionary dynamics [15]. The clustering of RGH-SSR markers near other *R*-gene and QTL clusters that was observed on almost every linkage group can be used to analyze well known clusters of *R*-genes. The large number of RGH-SSR markers will allow fine mapping of *R*-genes or QTL and perhaps their cloning via positional or association mapping approaches.

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Supplementary material

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