Identifying and Analyzing the Diversity of Resistance Gene Analogs in Colombian *Rubus* Genotypes

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Abstract

Afanador-Kafuri, L., Álvarez, E., Mejía, J. F., and González, A. 2015. Identifying and analyzing the diversity of resistance gene analogs in Colombian *Rubus* genotypes. *Plant Dis.* 99:994-1001.

Five Andean blackberry *Rubus* genotypes, three resistant and two susceptible to anthracnose, were used to identify regions in the *Rubus* genome with homology to disease-resistance genes found in other plant species. Polymerase chain reaction amplification with 12 pairs of primers and fragment cloning yielded 520 clones, of which 151 showed inserts between 500 and 700 bp long. When sequenced, 47 clones showed homology with two types of resistance genes, non-Toll/interleukin-1 receptor (TIR) nucleotide binding site (NBS) leucine-rich repeat (LRR) and TIR-NBS-LRR, thereby confirming their designation as resistance gene analogs (RGAs). The number of RGAs detected per *Rubus* genotype

ranged from 7 to 11, with the highest in a wild resistant and a cultivated susceptible genotype. *Rubus* RGAs were also homologous with several non-TIR- and TIR-type RGAs found in other members of the Rosaceae family (*Rosa* hybrid cultivar, *Rosa roxburghii*, *Malus × domestica*, *M. prunifolia*, *M. baccata*, *M. floribunda*, *Pyrus communis*, *Prunus persica*, *P. kansuensis*, *P. avium*, and *Fragaria vesca*). Three RGAs shared identity with two Rosaceae RGAs associated with the *CRPM1* locus for powdery mildew resistance in *R. roxburghii* and the *Rosa* hybrid cultivar. This is the first report on RGAs present in the Andean blackberry in Colombia.

The Andean blackberry (*Rubus glaucus* Benth.) belongs to the rose family (Rosaceae Juss.). It is a perennial shrub, with semierect or creeping stems that form clumps. Stems are of variable length and branched. They may or may not have thorns and constantly form buds at the base of the plant. The crop species, also known in Spanish as 'mora de Castilla', is widely grown in Colombia for domestic consumption and export, and is planted at altitudes between 1,800 and 3,200 m above sea level.

Despite its economic value, several constraints have prevented this species from realizing its full potential: widespread incidence of disease, commercial dependence on a limited number of varieties, and low genetic quality of planting materials. Although the Andean blackberry is widely adapted to growing regions of Colombia, it is highly susceptible to anthracnose (caused by *Colletotrichum* sp.), fruit rot or gray mold (caused by *Botrytis cinerea*), and downy mildew (caused by *Peronospora* sp.) (4,14–16)—all diseases that are also present in other South American countries, including Venezuela and Ecuador.

Since 1992, anthracnose has caused severe losses in blackberry crops in Colombia, where it has been reported as attacking fruits (5%) or stems and branches (50%) and generating losses of up to 40% (31,40,44,45). In the country's coffee-growing region, incidence of this disease ranges from about 25 to 73% (4,15,16,31,40,43–45). The disease is prevalent in areas with temperatures between 13 and 22°C and relative humidity of 92% or more (43–45).

Because of the costs and hazardous nature of agrochemical control of the disease, pesticide use on this fruit crop is considered socioeconomically and environmentally inappropriate. Integrated

strategies are needed to prevent the pathogen from developing fungicide resistance because anthracnose is highly variable and subject to developing new strains.

Breeding Andean blackberry for disease resistance is perhaps the best strategy for control of anthracnose in Colombia. However, due to a lack of research on the crop, no major genes for resistance have been identified by traditional genetic testing. Therefore, modern methods are important in the search for disease resistance, and one method is based on the use of molecular markers associated with resistance (*R*) genes such as resistance gene analog (RGA) markers.

Degenerate RGA targeting primers are playing a significant role in the search for *R* genes to several pathogens, including *Colletotrichum lindemuthianum* and *Uromyces appendiculatus* in bean (30,38); *C. acutatum* in strawberry (34); *C. truncatum* in lentil; *Hemileia vastatrix* in coffee (34); *Phytophthora infestans* in potato (20); and *Uncinula necator*, *Plasmopara viticola*, and *B. cinerea* in grape (19).

About 70 different *R* genes showing resistance to a highly diverse spectrum of major plant pathogens, including viruses, fungi, bacteria, and nematodes, have been isolated, cloned, and characterized in different plant species in the last 15 years (29). The genes can be classified according to their predicted protein structure (2,3,24,32). The largest class, comprising about 75% of *R* genes, encode cytoplasmic receptor-like proteins with the N-terminal nucleotide binding site (NBS) and the C-terminus leucine-rich repeat (LRR) domains (termed NBS-LRR-type genes).

The NBS-LRR type of genes can be further subdivided according to whether the genes contain structures such as leucine zippers, Toll/interleukin-1 receptors (TIRs), or coiled-coil (CC) sequences (34,36,47). The LRR region recognizes pathogens; the TIR and CC regions play roles in signal transduction in other cell processes, whereas the NBS is usually involved with programmed cell death (48).

Sequence analyses indicate that 150 to 175 NBS-LRR genes are found in the fully sequenced *Arabidopsis* genome (36), while about 600 of these genes are found in the rice genome (37). Examples of sequence-based markers for disease resistance include markers targeting the conserved sequences of NBS-LRR genes. These sequences are obtained from plant DNA using polymerase chain reaction (PCR) strategies and degenerate primers (49). Motifs in the NBS domains

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Accession numbers: GenBank JN671966 and JN990328 to JN990373.

*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary table is published online.

Accepted for publication 26 January 2015.

have been used to isolate RGAs from various families of plants, including legumes such as soybean (52) and common bean (38), as well as from maize (8) and other cereals, including rice, wheat, and barley (7,10). Other fruit and vegetable crops with characterized RGAs include potato, lettuce, apple (24–26,36), raspberry (41,51), strawberry (34), and melon (5). More specifically, in the Rosaceae family, RGA motifs have been used for phylogenetic and evolutionary analyses (51), marking resistance regions, and identifying parental materials in germplasm collections (3,6,13,33).

As far as we know, no studies have been conducted to identify sources of resistance to anthracnose in *Rubus* germplasm, or to clone RGAs from Andean blackberry genotypes. Detection of genes resistant to *Colletotrichum* spp. in this economically important crop is important and would be complemented by finding the RGA orthologs associated with anthracnose resistance. To meet these needs, our study aimed to identify RGAs in five Colombian *Rubus* germplasms varying for anthracnose resistance from wild and cultivated genotypes. We discuss our finding in terms of the utility of RGAs as molecular markers to understand the *R* genes that function against the disease and as an important step toward developing strategies to incorporate anthracnose resistance in susceptible clones of Andean blackberry.

Materials and Methods

Plant materials and pathogen inoculation. To identify RGAs in *Rubus* germplasm, DNA was first obtained from three highly resistant genotypes (ILS 2363, ILS 2372, and ILS 2377) and two susceptible ones (ILS 2270 and ILS 2371). The genotypes were selected for their resistance and susceptibility after inoculation of 58 *Rubus* spp. genotypes with 16 isolates belonging to *C. gloeosporioides* sensu lato (AN34a, AN40, C030, S035-1, VC130, H013, Q001-3, R002b, CD028), *C. acutatum* sensu lato (C026, S030-2, VC098, H064, Q044) and *C. boninense* sensu lato (R001, CD015-1). These isolates were selected based on their specific level of pathogenicity, with half (eight) being of low pathogenicity and the other half (another eight) being selected for high pathogenicity. The inoculation was followed by the method used by Stewart et al. (42) but modified to cover full plantlet infection (1). A complete block design was used with three replicates, with each experimental unit consisting of an individual plantlet. The plantlets were grown under greenhouse conditions for 4 months after transplanting from stem segments of the mother plants. Generally, Andean blackberry only develops one plantlet per segment, even if various nodes are included when the stem segment is planted horizontally in wet soil. Each plantlet was wounded at midplant height and an agar disk of fungal mycelia (5 mm) was applied at this wound site. For controls, plants were wounded and treated with an agar disk without mycelia. The fungal inoculum for each *Colletotrichum* spp. isolate was grown for 12 days on potato dextrose agar before the agar disks were removed for inoculation of the wound. Once a wound was inoculated, it was covered with Parafilm that covered the agar disk, fungal inoculum, and wound. The inoculated plants were incubated in humidity chambers for 5 days at an average temperature of 24°C, with 98% relative humidity. They were then taken to a greenhouse with the same average temperature and a relative humidity of 78% for 20 days to develop symptoms. The disease reaction was evaluated 10 days after inoculation and every 5 days thereafter.

Disease scoring. Resistance or susceptibility was scored on a per-plantlet basis using a 1-to-6 scale, where a score of 1 = 0% of the area infected with development of a callus around the wound and inoculation site; 2 = 3% area infected with development of a necrotic area around the inoculation site, 15% area infected with necrotic area and wilting of leaves; 3 = 25% area infected, yellowing and death of leaves of the inoculated stem; 5 = 45% area infected, death of leaves and plant apex; and 6 = 75% or more of the plant was infected and there was death of either a stem or the full plantlet. Any plants with scores of 1 or 2 were considered resistant whereas any with scores of 3 or 4 were intermediate and any with scores of 5 or 6 were susceptible. The area under the disease progress curve (AUDPC) was calculated from a combination of the scores and the measurements were made at 5-day intervals for severity of the disease. Statistical analyses

were carried out with the software package SAS (version 8.2; SAS Institute Inc., Cary, NC). Statistically significant differences between treatments and genotypes were determined using an analysis of variance (ANOVA) and means were compared using the Ryan-Einot-Gabriel-Welsch multiple range test ($\alpha \leq 0.05$).

DNA extraction and RGA amplification. DNA was extracted from newly expanded leaves of plants of the five selected blackberry genotypes, following the DNA extraction protocol for Diversity Arrays Technology analysis (11), with modifications by Mejía et al. (35). This DNA was used as template to test 12 pairs of primers previously reported to amplify RGA sequences from members of the Rosaceae (41) as well as other plant families (22,26,28,52; Table 1). All primers were synthesized by Integrated DNA Technologies (Corvallis OR) without high-performance liquid chromatography purification. PCR amplification was conducted in a 25- μ l reaction volume with 2.5 mM MgCl₂, 1 $\times$ *Taq* polymerase buffer, 0.2 mM dNTPs, 2.5 mM each primer, bovine serum albumin at 1 mg/ml, and 1.0 U of *Taq* polymerase (Bioline). In all, 10 ng of blackberry DNA was used per reaction.

Two amplification profiles were carried out using a PTC-100 thermal cycler (MJ Research Inc.). The first profile, for primers derived from the Rosaceae family, had initial denaturation at 94°C for 5 min; 45 cycles with denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min; with a final extension at 72°C for 7 min. The second profile, for primers not derived from Rosaceae plants, had initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 1 min, and extension at 72°C for 1 min; with a final extension at 72°C for 7 min. The amplified fragments were then separated in a 2% agar gel in 1 $\times$ Tris-acetate-EDTA buffer and visualized using ethidium bromide staining.

Cloning and sequencing of RGA PCR products. The amplification products were cloned using the pGEM-T Easy Vector System (Promega Corporation). From each ligation event, 10 colonies of recombinant bacteria were isolated. Insertion in the clones obtained was verified through PCR amplification by resuspending each recombinant colony in 7 μ l of ultrapure water in a PCR tube, then adding 18 μ l of a mixture that contained 2.5 μ l of 10 $\times$ *Taq* buffer, 0.75 μ l of 50 mM MgCl₂, 1 μ l of 2.5 mM dNTPs, 0.25 μ l of each primer (10 μ M each), 0.2 μ l of *Taq* polymerase at 5 U/ μ l, and 13.05 μ l of ultrapure water. Primers T7 and SP6 were selected for this mixture because they amplify near the insert site. The amplification program used had initial denaturation at 94°C for 3 min; 29 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min; with a final extension at 72°C for 5 min. The amplification products were separated in a 1.5% agar gel and visualized using ethidium bromide staining and UV light. Recombinant plasmids were selected based on having approximately 500 to 900 nucleotides of insert DNA and their DNA extracted, using the QIAprep Kit (Qiagen Inc.) following the manufacturer's instructions. The DNA was then sent for sequencing to the Iowa State University Biotechnology facility (Ames). Sequencing was done by automated di-deoxy sequencing (Applied Biosystems 3730xl DNA Analyzer), using the primers SP6 and T7 and a DNA-sequencing kit from Applied Biosystems.

Sequence analysis. The sequences were cleaned and assembled using the ChromasPro program (v.1.5; Technelysium Pty. Ltd.). The pGEM-T Easy Vector was then identified and removed using the VecScreen tool from the National Center for Biotechnology Information (NCBI). Similarity searches were then performed with the Blast2GO program (BioBam Bioinformatics S.L.) (9) and with the blastx and blast programs at the NCBI's Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov) to annotate genes and identify their best homologies with genes reported for disease resistance in other crops. The best BLAST hit and conserved domains were used as criteria to determine if a given *Rubus* amplicon sequence was an RGA gene and the type of RGA it could be. Homologous sequences were recorded and used in Gene Ontology searcher (<http://www.geneontology.org>) and conserved domains were sought using the InterProScan function (EMBL-EBI). For each sequence, the presence of an open reading frame (ORF) was also sought, using

the Geneious Pro 5.1 program (Biomatters Ltd.) to confirm its status as a potentially full-length gene rather than a truncated pseudogene which is common among RGA sequences (12).

The sequences that were highly homologous with NBS-LRR-RGA-type *R* genes reported in the NCBI database were then aligned among themselves and with sequences of *R* genes known for Rosaceae species (41). The deduced amino acid sequences of RGAs of these fragments were aligned using a constraint-based alignment tool for multiple protein sequences (39) and adjusted using the ClustalW program (v. 2.0; UCD Conway Institute of Biomolecular and Biomedical Research; 23). The amino acid sequences were also used to construct a phylogenetic tree by means of the neighbor-joining (NJ) method. Amino acid p-distances were also performed using the MEGA sequence alignment tool, version 5.1 (46). Bootstrap analysis was then carried out and replicated 1,000 times for estimation of stability and support for the identified clades and most significant nodes.

Results

Genotype resistance to *Colletotrichum* spp. AUDPC values showed significant differences for the 450 treatments resulting from 58 genotype by 7 isolate combinations tested during different periods of inoculation in the ANOVA (Supplementary Table S1). Three groups of *Rubus* spp. genotypes were identified according to their reactions to anthracnose. The first group had high susceptibility, the second was moderately susceptible, and the third group had resistant genotypes. The resistant genotypes included ILS1817, ILS2363, ILS2366, ILS2372, and ILS2377, which were resistant to all of the isolates evaluated, except in the case of ILS2372, which was intermediate after inoculation with isolate R002b. The remaining 53 genotypes were all highly or moderately susceptible against all the isolates of the *C. gloeosporioides* sensu lato used in the study but resistant to the isolates of *C. acutatum* sensu lato used. When the treatment effect was evaluated individually, significant differences were observed between genotypes when they were infected with individual isolates of *C. boninense* sensu lato and *C. gloeosporioides* sensu lato. A higher infectivity was observed with isolate Q001 (3) and a lower one with isolate R001. Meanwhile, genotypes did not show differences in response to all eight isolates of *C. acutatum* sensu lato.

Isolating RGAs using PCR. RGA amplification products were obtained, ranging in size from 100 to 1,200 bp, for 11 of the 12 primer combinations evaluated. Amplification products were not obtained with primer combination DegRosf/DegRosr. Strong bands were observed at 500 to 700 bp, especially in amplifications with the primers derived from the Rosaceae RGA sequences. For the non-Rosaceae group of primers, strong bands were also observed in this range and at 200 to 400 bp. In all, 10 clones were isolated per primer combination, resulting in a total of 520 amplicons for evaluation. Each colony was prescreened with a repeat of the PCR to confirm the presence of the expected insert, which resulted in 445 insert-positive clones (85%). The majority of these clones had been amplified with the primer combinations OLE 1121/OLE 1122, Pto1/Pto2, and WipKf/WipKr. In total, 151 of the insert-positive clones were selected for Sanger sequencing based on the primers used to amplify the fragment, the genotype template on which the amplicon was based, and the size of the cloned band, which was preferably large enough (500 to 700 bp long) for sequence ID. For some genotypes, smaller inserts representing RGA amplicons of 136 to 500 bp in length were selected when it was not possible to obtain the larger products normally considered within the range of NBS-LRR regions (7,26,28,52).

The identity searches at NCBI's GenBank database showed that only 47 of the 151 cloned sequences showed homology with the typical, conserved, internal motifs for *R* genes of the NBS-LRR type, in addition to the two motifs flanked by the corresponding primers. These clones were obtained with 6 of the 12 primer combinations evaluated (i.e., OLE1121/OLE1122, BP2f/BP2r, OLE1121/DegRos-r, OLE1121/BP2r, P-loop/OLE1122, and LM638/LM637). The fragments cloned from the other six primer combinations shared identity with sequences that were not of the NBS-LRR type and, therefore, were excluded from analysis.

The number of RGAs detected per genotype ranged from 7 to 11, being highest in genotypes ILS 2270 (susceptible) and ILS 2363 (wild resistant) (Table 2). For each of the 47 clones that presented the internal motif for NBS-LRR genes, an ORF was sought in their deduced amino acid sequence. Being full-length genes, these RGAs can be considered as likely to be true *R* genes. Consequently, these sequences may be considered as RGAs, which was in agreement with their identity matches shared with different *R* genes from other species.

Table 1. Primer pairs used to detect resistance gene analogs (RGAs) in Colombian *Rubus* germplasm

Plant, primer pair ^a	Sequences (5'–3')	Related <i>R</i> gene region ^b	References
Non-Rosaceae			
XLRRf/XLRRr	CCGTTGGACAGGAAGGAG/ CCCATAGACCGGACTGTT	LRR of the rice genes <i>Rps2</i> and <i>Xa21</i>	8
WipKf/WipKr	GGTCGTGGTGCTTATGGAAT/ CCATGAAGATGCAACCGAC	<i>MAK</i> kinase of parsley, tobacco, <i>Arabidopsis</i> , and <i>Medicago sativa</i>	26
NBS-F1/NBS-R1	GGAATGGGNGGNGTNGGNAARAC/ YCTAGTTGTRAYDATDAYYYTRC	NBS in tobacco <i>N</i> and <i>Arabidopsis RPS2</i>	52
PtoFen-S/PtoFen-AS	ATGGGAAGCAAGTATTTCAAGGC/ TTGGCACAAAATTCTCATCAAGC	<i>Pto</i> gene family conferring sensitivity to fenthion	25
LM638/LM637	GGIGGIGTIGGIAAIAIACIAC/ ARIGCTARIGGIARICC	NBS, P-loop of the genes <i>L6</i> , <i>N</i> , and <i>Rps2</i>	21
OLE1121/OLE1122	GGWATGGGWWGWRTHGGWAARACHAC/ ARNWYYTTVARDGCVARWGGVARWCC	NBS-LRR, P-loop, and hydrophobic domain from <i>Rps2</i> , <i>Rps5</i> , <i>L6</i> , and <i>Xa1</i>	24
Rosaceae			
BP2f/BP2r	GGNGDGTDDGSAARAC/ GCTAGTGGCAMNCCWCC	NBS-LRR of <i>Malus × domestica</i> Borkh.	3
DegRos-f/DegRos-r	MDTKSBDRRSSBDTTTWHRRMM/ RKDYWYDHMWHRDWKBWBMWK	NBS-LRR of <i>Rubus idaeus</i>	41
OLE1121/DegRos-r	GGWATGGGWWGWRTHGGWAARACHAC/ RKDYWYDHMWHRDWKBWBMWK	NBS-LRR of <i>R. idaeus</i> , P-loop, and hydrophobic domain from <i>Rps2</i> , <i>Rps5</i> , <i>L6</i> , and <i>Xa1</i>	24,41
BP2f/DegRos-r	GGNGDGTDDGSAARAC/ RKDYWYDHMWHRDWKBWBMWK	NBS-LRR of <i>Malus × domestica</i> Borkh. and <i>R. idaeus</i>	3,41
OLE1121/BP2r	GGWATGGGWWGWRTHGGWAARACHAC/ GCTAGTGGCAMNCCWCC	NBS-LRR of <i>Malus × domestica</i> Borkh., P-loop, and hydrophobic domain from <i>Rps2</i> , <i>Rps5</i> , <i>L6</i> , and <i>Xa1</i>	3,24
P-loop/OLE1122	GAATTCGGNGTNGGNAAGACAAC/ ARNWYYTTVARDGCVARWGGVARWCC	NBS-LRR, P-loop, and hydrophobic domain from <i>Rps2</i> , <i>Rps5</i> , <i>L6</i> , and <i>Xa1</i>	3,24

^a Primer pairs (forward/reverse) derived from non-Rosaceae and Rosaceae plants.

^b *R* gene = resistance gene, LRR = leucine-rich repeat, and NBS = nucleotide-binding site.

Analyzing diversity in *Rubus* RGA sequences. The diversity of *Rubus* RGAs was compared with (i) RGAs of other species available in the GenBank and (ii) RGAs of other Rosaceae species (41). The identity of amino acids shared among *Rubus* sequences ranged from 12 to 100%. An NJ tree was obtained with 1,000 bootstrapping replications. The tree grouped the *Rubus* RGAs into 14 clusters (A to N; Table 2), 6 of which were each formed by only 1 RGA (clusters B, C, D, G, K, and L). The identified clusters were supported by bootstrap probability values of 51 to 95% (Fig. 1).

Four clusters (E, G, H, and K) corresponded to RGAs of the TIR-NBS-LRR type, while the remaining clusters corresponded to RGAs of the NBS-LRR type. Clusters M and N appeared for all the genotypes evaluated, except genotype ILS 2377, in which RGAs from only the N cluster were detected (Table 2). One aspect that stood out was the presence in the resistant wild genotype (ILS 2363) of a high number of RGAs, some of which belonged to the two largest clusters (M and N). Only cluster A was always associated with resistance among the genotypes tested. Cluster I, K, and L were present only once in different resistance genotypes.

***Rubus* versus Rosaceae RGAs.** Analysis of the 47 *Rubus* sequences was carried out with 159 published NBS-LRR sequences from other Rosaceae (41).

The multiple alignments of amino acid sequences showed high similarity in the motifs P-loop, kinase-2, RNBS-A-TIR, RNBS-B, GPLAL, and RNBS-C. The distance index similarity between amino acids of *Rubus* RGAs and those of other Rosaceae RGAs ranged from 0.031 to 0.10, which was close similarity. Overall, the distance between amino acid sequences was 0.11 to 0.01.

The tree resulting from the comparative analysis among sequences (using the NJ method) showed the *Rubus* RGAs clustering with certain Rosaceae RGAs, leading to the identification of nine clusters (I to IX; Fig. 2) with 52 to 100% level of support based on bootstrapping. The *Rubus* RGAs separated into main types of genes: the TIR-NBS-LRR and the non-TIR (CC)-NBS-LRR genes, respectively.

These two major clades were evident in the structure of the entire tree, including Rosaceae and *Rubus* RGAs. We observed that clusters I to VII were formed by TIR-type *Rubus* RGAs with a clustering probability of 70 to 100%. Clusters VIII and IX were formed by *Rubus* RGAs of non-TIR-types, clustering at probabilities of 58 and 99%, respectively. TIR-type RGAs of several Rosaceae species clustered together and these were derived from *Rosa* hybrid cultivar, *Malus* × *domestica*, *Fragaria vesca* subsp. *vesca*, *Prunus kansuensis*, *Rosa roxburghii*, *P. avium* and *Malus floribunda*. Meanwhile, non-TIR-type RGAs derived from *M. baccata*, *M. prunifolia*, *F. vesca* subsp. *vesca*, *P. persica*, and *Pyrus communis* clustered together.

Discussion

The present article describes the results of analyzing 47 RGA fragments in Andean blackberry derived from 6 of the 12 primer pairs used. However, many more RGAs may exist in the species and could be analyzed through the development of additional primer pairs. *R* genes of the NBS class have been demonstrated to be abundant in plants. For example, *Arabidopsis thaliana* (genome size 150 Mb) is estimated to contain about 150 genes that code for NBS motifs (36). In other studies of less genome-reduced species such as common bean (600 Mb), around 400 RGAs have been identified by Garzón et al. (17). A similar situation probably exists for Andean

blackberry (41). Future studies need to clarify this issue, as well as the origin and evolution of *R* genes in the Andean blackberry in Colombia and their likely distribution in other *Rubus* spp. Results thus far indicate that, in the Andean blackberry genome as in the common bean genome, sequences of non-TIR types may predominate over TIR types.

This is the first report of RGAs for Andean blackberry in Colombia and it contributes significantly to the total of RGAs cloned and identified for different plant species, including several within the Rosaceae family (2,3,13,25,33,41,49–51). Most of the RGAs detected in Andean blackberry are related to known sequences of *R* genes and RGAs in species such as *Vitis vinifera* ($n = 2$), *Malus* × *domestica* ($n = 11$), *M. prunifolia* ($n = 3$), *M. floribunda* ($n = 2$), *Rosa* hybrid cultivar ($n = 5$), *R. roxburghii* ($n = 3$), *Populus trichocarpa* ($n = 4$), *P. tomentosa* × *P. bolleana*, *P. tomentosa* var. *truncata* ($n = 1$), *Prunus avium* ($n = 1$), and *Musa* group AA ($n = 1$). Of the 47 RGAs identified, 10 (i.e., 21%) were derived from genotype ILS 2363, which is resistant to anthracnose. The same 10 RGAs share similarity with 26% of RGAs found in *V. vinifera*. The uniqueness of ILS 2363 suggested that, as a wild genotype, it possessed a high proportion of the RGAs identified. Notably, the RGAs from the wild ILS 2363 genotype had a greater degree of diversity because both TIR and non-TIR types were found, with this last having three different classes (NBS-LRR, CC-NBS-LRR, and LRR).

Comparing RGA diversity of the four cultivated genotypes of Andean blackberry with that of the wild genotype showed only subtle differences. Most of the RGA classes identified were distributed equally between all genotypes evaluated, except for the TIR types, which were present only in the wild and two susceptible genotypes. These results agree with those reported by Lee et al. (25). By comparing apple RGAs, they found that no differences appeared for RGA diversity between species and that the RGAs were consistently distributed among wild and cultivated species. The authors also suggested that the low differentiation among RGAs may be caused by development of quantitative type tolerance to pathogen infection in apple species. Such mechanisms could remove any association between specific traits for disease resistance and species adaptability so that no differentiation would appear among their RGAs. If plants develop generalized pathogen tolerance, they reduce selection by isolate-specific resistance and alter the effect that pathogens have on RGA evolution. The role played by pathogen selection in plant evolution depends on the magnitude of genetic variation for traits of resistance and their correlation with the host–microbe interaction (29).

Comparative analysis between *Rubus* RGAs and Rosaceae *R* genes indicated that three Andean blackberry RGAs shared an identity of more than 50% with two Rosaceae RGAs that were closely associated with locus *CRPM1*, which contributes to resistance to powdery mildew in *R. roxburghii* and *Rosa* hybrid cultivar (50). Two *Rubus* RGAs shared an identity of more than 50% with another two members of the Rosaceae family that were closely associated with locus N-like for resistance to *Tobacco mosaic virus* (TMV) in *F. vesca* (wild strawberry). The similarity of an RGA for powdery mildew in *Rosa* spp. with one in Andean blackberry may correspond to a syntenic relationship of this NBS-LRR type gene in the species of *Rubus* and *Rosa* and possibly *Malus* and *Prunus*. The relationship between the *Rubus* and *Rosa* genes may also be a coincidence with the fact that most of the RGAs isolated from Andean blackberry were

Table 2. Clones obtained with primer pairs used to amplify resistance gene analog (RGA) sequences in *Rubus* germplasm

Andean blackberry genotype	Type	Anthracnose reaction	RGAs isolated (n)	RGA families represented ($n = 14$)	Primer pair ^a
M9 (ILS 2270)	Cultivated	Susceptible	11	C, E, H, F, J, M, N	Pp4, Pp7, and Pp8
M29 (ILS 2363)	Wild	Resistant	11	A, K, E, F, M, N	Pp1, Pp2, Pp4, and Pp6
M35 (ILS 2371)	Cultivated	Susceptible	10	B, D, G, H, M, N	Pp4, Pp7, and Pp8
M36 (ILS 2372)	Cultivated	Resistant	9	A, I, M, N	Pp1, Pp4, and Pp6
M39 (ILS 2377)	Cultivated	Resistant	7	A, J, L, N	Pp1, Pp2, Pp4, and Pp6

^a Pp1 = code number for primer pair OLE1121/OLE1122, Pp2 = BP2f/BP2r, Pp4 = OLE1121/DegRos-r, Pp6 = OLE1121/BP2r, Pp7 = P-loop/OLE1122, and Pp8 = LM638/LM637.

of the non-TIR type just as was found in *Rosa* spp. by Xu et al. (50). According to these studies, the two plant genera depend more on *R* genes of the non-TIR-type NBS-LRR class for defense against phytopathogens, which were probably amplified in the course of these species' evolution as opposed to other Rosaceae plants.

In crops such as Andean blackberry belonging to the Rosaceae family, improved resistance to disease is a major objective, especially for fruit-bearing trees and shrubs for which time and population size become principal problems. The existence of a syntenic relationship in the NBS regions that include certain *R* genes—as apparently

Fig. 1. Phylogenetic tree constructed with the neighbor-joining method and based on the amino acid sequence of the Toll/interleukin-1 receptor (TIR), coiled-coil (CC), nucleotide-binding site (NBS), leucine-rich repeat (LRR) domain, and potential disease-resistant proteins (PDRP) found in *Rubus* spp. and *Rubus glaucus*. It was constructed for 47 resistance gene analog (RGA) nucleotide sequences isolated by degenerate primers from five different Andean blackberry genotypes. GenBank accession numbers of nucleotides, amino acid sequences (in brackets), and the genotype to each RGA reported in this study are indicated. Numbers on the branches are bootstrap values obtained for 1,000 replicates. Only values of 50% or more are shown.

Fig. 2. Phylogenetic tree made by the neighbor-joining method and derived from distance analysis, showing the clustering of amino acid sequences of the Toll/interleukin-1 receptor (TIR), coiled-coil (CC), nucleotide-binding site (NBS), leucine-rich repeat (LRR) domain, and potential disease-resistant protein (PDRP) for resistance gene analogs (RGAs) identified through comparative analysis of *Rubus* spp., *Rubus glaucus*, and other Rosaceae plants (in bold). Names assigned to each RGA reported in this study and their GenBank accession number for nucleotides and amino acid sequences (in brackets) are indicated. The reliability of the tree topology was subjected to a bootstrap test; the numbers on the branches indicate the percentage of 1,000 bootstrap replicates that support the node. Only values of 50% or more are reported.

occurred with resistance to powdery mildew and TMV—may be useful in improving Rosaceae species for which classical genetic testing has not been conducted. Information that is available for some species may be cross referenced with information for others, thus providing useful genetic markers for molecular breeding of disease resistance. Using molecular markers, a preselection of resistant plants could be done in early years in the seedling state, long before expensive field evaluation was necessary, which would save time and space (51). In strawberry, Folta and Davis (13) used markers derived from RGAs to extend the use of genomic tools for the rapid appraisal of the structural and functional bases of traits of agronomic interest such as disease resistance, maturation, and production of volatile substances. These tools made possible the implementation of a marker-assisted selection program for the genus *Fragaria* (including the cultivated strawberry), the first of its kind in the Rosaceae family. Genomic tools such as the one developed in strawberry for Folta and Davis (13) could be developed in blackberry by using markers derived from RGAs of the A, I, K, and L families. These RGAs are unique among the 47 RGA fragments detected in blackberry because they are present only in the resistant genotypes ILS2363, ILS 2371, and ILS 2377. Family A RGAs were present in all three resistant genotypes. This makes them the most useful candidates for the future development of molecular markers in order to find markers close to genomic regions that contain genes resistant to anthracnose.

Plant disease resistance is the best control method from an economic and environmental point of view. Blackberry genotypes with resistance to anthracnose and other diseases are not available at the present time in Colombia, except for the genotypes identified in the present study. However, the only three resistance genotypes identified are not commercially available. Having a collection of RGAs identified in blackberry such as those in the A, I, K, and L families constitutes a useful tool for the characterization of genomic regions associated with anthracnose *R* genes and other disease *R* genes present in blackberry.

To classify the RGA fragments identified in the present study, we utilized sequences of Rosaceae that are publicly available (44). The strategy to follow could be the design of specific primers to the RGA sequences obtained from blackberry in order to develop PCR-based markers for genetic mapping in genus *Rubus*. The primers will enable discovery of sequence-characterized amplified regions (SCARs) or cleaved amplified polymorphic sequence type markers. These markers will allow the identification of polymorphic blackberry parentals, which differ in the anthracnose response based on the annealing site of the primer to the DNA of the genotypes tested. The mapping of the RGA-derived markers could be done on *Rubus* genotypes, particularly wild blackberry; then, these genotypes eventually could be part of a blackberry breeding program for resistance to disease, including anthracnose.

Linkage genetic maps for 84 Rosaceae species, including apple, strawberry, and raspberry (22), could be mapped to the genomic region of interest in which there are genes for resistance to anthracnose. These gene regions may be present in species such as apple and strawberry, where this disease has already been reported and on which *R* genes were previously identified (18,27). Lerceteau-Köhler et al. (28) used bulk segregant analysis to identify amplified fragment length polymorphism loci associated with resistance to *C. acutatum*, the causative agent of strawberry anthracnose, anthracnose fruit rot, or black spot. These loci were translated into SCAR markers that segregated faithfully with resistance to *C. acutatum* in 81.4% of accessions tested (27).

In conclusion, this study isolated, identified, and analyzed the diversity of RGAs in *Rubus* (Andean blackberry) germplasm in Colombia. To determine the benefits and applicability of RGAs detected in *Rubus* spp., further research is needed to develop specific primers derived from the RGAs identified. RGA types A, I, K, and L would then be the first molecular markers associated with resistance, identifying resistant and susceptible individuals in populations segregating for disease resistance. In this way, the germplasm screened here and the RGAs as molecular markers would contribute to the development of varieties resistant to anthracnose and other important diseases of Andean blackberry in Colombia.

Acknowledgments

This work was financed by the International Center for Tropical Agriculture (CIAT) and the Regional Fund for Agricultural Technology (FONTAGRO), ID 16 (2007 to 2010). We thank CORPOICA–Regional “La Selva” for providing the *Rubus* germplasm used in this research, A. Rodríguez (Vegetable Biotechnology, CIB, Colombia) for his assistance in analyzing the data, and M. W. Blair (Tennessee State University) for his editing help.

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