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ORIGINAL PAPER

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Characterization and mapping of NBS-LRR resistance gene analogs in apricot (*Prunus armeniaca* L.)

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Abstract Genomic DNA sequences sharing homology with the NBS-LRR (nucleotide binding site-leucine-rich repeat) resistance genes were isolated and cloned from apricot (*Prunus armeniaca* L.) using a PCR approach with degenerate primers designed from conserved regions of the NBS domain. Restriction digestion and sequence analyses of the amplified fragments led to the identification of 43 unique amino acid sequences grouped into six families of resistance gene analogs (RGAs). All of the RGAs identified belong to the Toll-Interleukin receptor (TIR) group of the plant disease resistance genes (R-genes). RGA-specific primers based on non-conserved regions of the NBS domain were developed from the consensus sequences of each RGA family. These primers were used to develop amplified fragment length polymorphism (AFLP)-RGA markers by means of an AFLP-modified procedure where one standard primer is substituted by an RGA-specific primer. Using this method, 27 polymorphic markers, six of which shared homology with the TIR class of the NBS-LRR R-genes, were obtained from 17 different primer combinations. Of these 27 markers, 16 mapped in an apricot genetic map previously constructed from the self-pollination of the cultivar Lito. The development of AFLP-RGA markers may prove to be useful for marker-assisted selection and map-based cloning of R-genes in apricot.

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Introduction

Genes conferring resistance (R-genes) to a wide range of diseases caused by viruses, bacteria, fungi and nematodes have been cloned and studied from mono- and dicotyledonous plants (Hammond-Kosack and Jones 1997; Hulbert et al. 2001). These genes fall into five main classes (Baker et al. 1997), the most frequent of which is the class comprising R-genes containing the nucleotide binding site-leucine-rich repeat (NBS-LRR) domain structure (Bent et al. 1994; Whitham et al. 1994). The other four classes consist of: (1) genes with extracytoplasmic LRR domains and a C-terminal membrane anchor (Dixon et al. 1996); (2) R-genes encoding a serine/threonine protein kinase with no LRR domains (Martin et al. 1993) (3) genes having both the extracellular LRR domain and the protein kinase (Song et al. 1995); (4) the *HM1* gene encoding a toxin reductase (Johal and Briggs 1992).

The NBS domain is present in numerous ATP-binding and GTP-binding proteins, such as ATP synthetase or elongation factors (GTPase) (Saraste et al. 1990), suggesting that R-genes need ATP or GTP to function (Traut 1994). Within the major conserved motifs identified in the NBS region, there is a kinase motif, called the phosphate-binding loop (P-loop), with the consensus sequence GxGxxGR(T/S), a hydrophobic domain (sequence GLPLxL) and two additional kinase domains, kinase 2 and kinase 3a. The kinase 2 domain coordinates the metal ion binding required for phospho-transfer reactions, and the kinase 3a domain contains an arginine that in other proteins interacts with the purine base of ATP (Traut 1994). With respect to its potential function, the NBS domain seems to be involved in signaling events to induce defense responses through the activation of kinases or G proteins (Hammond-Kosack and Jones 1997).

The LRR domain consists of a repeated sequence motif (24 amino acids on average) with leucine and,

occasionally, asparagine and proline residues at conserved positions. This domain has been proposed to participate in protein-protein interactions (Kobe and Deisenhofer 1994), and it is an obvious candidate for the pathogen-specific recognition function (Takken and Joosten 2000).

Phylogenetic analyses have divided the NBS-LRR R-gene family into two subfamilies based on a group of motifs located within the N-terminal domain (Meyers et al. 1999; Pan et al. 2000). In the R-genes belonging to the first subfamily, this N-terminal region shows homology to the *Drosophila* Toll and mammalian Interleukin-1 receptor (TIR) proteins (Meyers et al. 1999; Young 2000), whereas in the second group, designated as the non-TIR subfamily, a coiled-coil motif is generally found instead of the corresponding TIR domain (Pan et al. 2000).

Sequences including the NBS-LRR domains have been called resistance gene analogs (RGAs) (Kanazin et al. 1996) or resistance gene homologs (RGHs) (Leister et al. 1998). Using the conserved motifs located in the NBS domain, degenerate primers have been designed to amplify RGAs in a broad range of plant species, such as soybean (Kanazin et al. 1996), potato (Leister et al. 1996), citrus (Deng et al. 2000), almond (Bliss et al. 2002), grapevine (Di Gaspero and Cipriani 2002) and apple (Baldi et al. 2004). Genetic analysis has shown that RGAs are widely distributed in the genome, often organized in clusters and sometimes strongly linked to known resistance loci (Kanazin et al. 1996; Meyers et al. 1999). Therefore, the development and mapping of RGA markers is an effective strategy by which to identify genomic regions linked to known R-genes (Aarts et al. 1998; Quint et al. 2002).

In this paper, we report the cloning and characterization of apricot RGA sequences using degenerate primers designed from the P-loop and the hydrophobic motifs of the NBS domain. The RGA families found allowed us to design specific primers in order to develop amplified fragment length polymorphism (AFLP)-RGA markers based on the techniques of Hayes and Saghai Maroof (2000) and Egea-Gilabert et al. (2003). These markers were mapped in an F_2 population linkage map segregating for plum pox virus (PPV) resistance (Vilanova et al. 2003). PPV is an important limiting factor of crop production in apricot and other *Prunus* species, and most breeding programs currently in progress are focused on the PPV resistance. However, marker-assisted selection (MAS) is not yet possible, since this trait has only been tentatively mapped to date (Hurtado et al. 2002; Salava et al. 2002; Vilanova et al. 2003). AFLP-RGA markers may be a very useful tool to identify genomic regions containing PPV resistance genes or genes conferring resistance to other important apricot diseases.

Materials and methods

Plant material and DNA isolation

Two apricot (*Prunus armeniaca* L.) cultivars, Goldrich and Lito, kept at the germplasm collection of the Instituto Valenciano de Investigaciones Agrarias (IVIA) (Valencia, Spain), were used in this study. An apricot intraspecific F_2 population ($n = 76$) generated by self-pollination of the cultivar Lito and designated as Lito × Lito (L×L) family, also maintained at the IVIA, was used to map the AFLP-RGA polymorphic markers.

DNA was extracted from 50 mg of young leaves following the CTAB (hexadecyltrimethylammonium bromide) method of Doyle and Doyle (1987). DNA quantification was performed by comparison with λDNA (Promega, Madison, Wis.).

PCR amplification of RGA sequences

Two combinations of degenerate primers were used to amplify RGA sequences by PCR from cvs. Goldrich and Lito. Combination I used the s1 (5′-GGTGGGGTTGGGAAGACAACG-3′) (Leister et al. 1996) and LM637 (5′-ARIGCTARIGGIARICC-3′) primers (Kanazin et al. 1996), and combination II, LM637 and the forward primer (5′-GGAATGGGKGGAGTYGGYAARAC-3′) developed by Gentzbittel et al. (1998). The forward primer of Gentzbittel et al. (1998) and s1 were developed from the P-loop [GxGxxGR(T/S)] motif of the NBS domain, and LM637 was developed from the hydrophobic (GLPLxL) motif of the NBS domain.

PCRs were performed in a total volume of 25 µl containing 20 m *M* Tris-HCl, pH 8.4, 50 m *M* KCl, 1.5 m *M* $MgCl_2$, 0.1 m *M* of each dNTP, 1 μM of each primer, 50 ng DNA and 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, Calif.) using a GeneAmp PCR System 9700 thermal cycler (Perkin-Elmer Foster City, Calif.). The following cycling conditions were used: for combination I, there was a denaturation step at 94°C for 2 min, followed by ten cycles of 94°C for 20 s, 50°C for 45 s and 72°C for 1 min, and 20 cycles of 94°C for 20 s, 45°C for 45 s and 72°C for 1 min, with a final elongation step at 72°C for 8 min; for combination II, there was a denaturation step at 94°C for 2 min, followed by 30 cycles at 94°C for 20 s, 45°C for 45 s and 72°C for 1 min, and a final elongation step at 72°C for 8 min. PCR products were separated by electrophoresis on a 0.8% (w/v) agarose (Pronadisa, Madrid, Spain) using 1× TBE (89 m *M* Tris-HCl, 89 m *M* boric acid and 2 m *M* EDTA pH 8.0) buffer, stained with ethidium bromide (0.8 µg/ml), and visualized under UV light. The molecular sizes of the amplified fragments were estimated by comparison with a 100-bp ladder (Invitrogen).

RGA cloning and restriction analysis

Fragments with the expected size were gel-purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, Calif.) and cloned into the pGEM-T easy plasmid vector (Promega) following the manufacturer's instructions. Colony-PCR amplifications followed the protocol described by Di Gaspero and Cipriani (2002).

DNA from 200 clones (50 per cultivar and primer combination) was digested with five restriction enzymes: *Hae*III, *Hin*fI, *Cfo*I, *Sau*3AI (Roche, Basel, Switzerland) and *Rsa*I (Promega). The restriction reactions were performed overnight at 37°C in a total volume of 25 µl, with each aliquot containing 20 µl of the PCR reactions, 2.5 µl of 10× restriction buffer and 1 U of enzyme. Fragments were separated by electrophoresis on a 3% (w/v) MS-8 agarose (Pronadisa) with 1× TBE buffer, stained and visualized as described above.

A similarity matrix was obtained from the restriction pattern with the Nei's genetic distance (Nei 1972) followed by the unweighted pair group method with arithmetic mean (UPGMA) grouping approach (Sneath and Sokal 1973) using the NTSYS software (Rohlf 1993).

RGA sequence analysis

Two to four clones from each defined group were sequenced at the Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC, Valencia, Spain) using an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, Calif.) and the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems).

Nucleotide sequences were translated, and a multiple alignment of amino acid sequences was carried out with CLUSTALX software (Thompson et al. 1997). A dissimilarity matrix and a phylogenetic tree were obtained from this alignment following the neighbor-joining method (Saitou and Nei 1987) with the Poisson correction distance (Nei and Kumar 2002) and the bootstrap analysis with 500 replications (Felsenstein 1985) using the MEGA software (Kumar et al. 2001). A consensus sequence was obtained from each of the defined groups using BIOEDIT software (Hall 1999). Comparisons of the apricot consensus sequences with GeneBank accessions were performed using BLASTX (Altschul et al. 1990). These consensus sequences were also aligned along with the NBS domain of R-genes previously reported in other species (*RPS2*, *RPP5*, *RPM1*, *RPS5* and *RPP8* from *Arabidopsis*; *N* from tobacco; *L6* from flax), and a dissimilarity matrix and a phylogenetic tree were subsequently constructed as described above.

AFLP-RGA procedure

Two specific apricot RGA primers were designed from each consensus sequence using PRIMER3 software (Rozen and Skaletsky 2000). The forward primer amplifies downstream towards the C-terminal region and the reverse primer upstream to the N-terminal region (Table 3).

DNA digestion and adaptor ligation were carried out following the standard AFLP protocol (Vos et al. 1995) according to the manufacturer's recommendations (Invitrogen). This template DNA was then pre-amplified using primers homologous to the *Eco*RI or *Mse*I adaptor with a selective nucleotide (*Eco*RI + A, *Mse*I + C) and an apricot-specific RGA primer (Table 3). Pre-amplification was performed in a total volume of 40 µl containing 2 µl of the template DNA, 50 ng of each primer, 0.2 m *M* of each dNTP, 20 m *M* Tris-HCl, pH 8.4, 50 m *M* KCl, 1.5 m *M* $MgCl_2$ and 1 U of *Taq* DNA polymerase (Invitrogen). PCRs were carried out in a GeneAmp PCR System 9700 thermal cycler (Perkin-Elmer) using the following cycling conditions: a denaturation step at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, and a final elongation step at 72°C for 5 min. Selective amplifications were performed from 2 µl of the pre-amplified DNA with the same RGA primers and with the *Eco*RI or *Mse*I primers with two and three selective nucleotides, respectively (*Eco*RI + AA, *Mse*I + CAT and *Mse*I + CTG) using the touchdown PCR protocol described by Vos et al. (1995). The PCR products were dried, and 3 µl of denaturing loading buffer (95% formamide, 0.01 *M* NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue) was added to each one. The samples were then denatured by incubation at 95°C for 3 min, placed directly on ice and run on a 6% denaturing polyacrylamide gel that was stained with silver nitrate (Sambrook et al. 1989). Molecular sizes of the amplified fragments were estimated using a 10-bp ladder (Invitrogen).

AFLP-RGA sequencing and mapping

The AFLP-RGA polymorphic bands were excised from the acrylamide gel and re-amplified in a total volume of 100 µl containing 20 m *M* Tris-HCl, pH 8.4, 50 m *M* KCl, 1.5 m *M* $MgCl_2$, 0.2 m *M* of each dNTP, 50 ng of each primer and 1 U of *Taq* DNA polymerase (Invitrogen). The PCR was performed in a GeneAmp PCR System 9700 Thermal Cycler (Perkin-Elmer) with the following cycling conditions: a denaturation step at 94°C for 2 min, followed by ten cycles of 94°C for 10 s, 60°C for 10 s and 72°C for 10 s, and 20 cycles of 94°C for 10 s, 55°C for 10 s and 72°C for 10 s, with a final elongation step at 72°C for 7 min. PCR products were separated by electrophoresis in 2% (w/v) MS-8 agarose (Pronadisa, Madrid, Spain) using 1× TBE buffer, stained with ethidium bromide (0.8 µg/ml) and visualized under UV light. The fragments were gel-purified using a QIAquick Gel Extraction Kit (Qiagen) and cloned into pGEM-T easy plasmid vector (Promega) following the manufacturer's instructions. Plasmid DNA was isolated with the Rapid Plasmid Miniprep System (Marligen, Ijamsville, Md.), and fragments were then sequenced as previously described.

Polymorphic AFLP-RGAs were mapped in the F_2 L×L segregating population (Vilanova et al. 2003). The linkage analysis was carried out using JOINMAP 3.0 software (Van Ooijen and Voorrips 2001), setting F_2-type data, with a LOD value threshold of 3.0 and the Kosambi mapping function (Kosambi 1944).

Results

Identification of apricot NBS-LRR RGAs

Two combinations of degenerate primers previously designed from the P-loop and the hydrophobic motifs of the NBS conserved domain of soybean (Kanazin et al. 1996), potato (Leister et al. 1996) and sunflower (Gentzbittel et al. 1998) amplified a major band of approximately 500 bp in the apricot cultivars Lito and Goldrich (data not shown). This was the expected size based on the sequences of the *N*, *L6* and *RPS2* genes (Mindrinos et al. 1994; Whitham et al. 1994; Lawrence et al. 1995). These bands were cloned, and the restriction analysis of the 200 clones obtained (50 clones per cultivar and primer combination) showed different restriction patterns, indicating that they were composed of heterogeneous fragments. Thus, a single endonuclease such as *Hin*fI discriminated up to 33 classes of inserts from the cultivar Goldrich when the combination II was used. Nonetheless, much lower levels of polymorphism were observed using *Cfo*I or *Rsa*I that only identified three to five classes of fragments with the combination I in both cultivars (Table 1). Based on these restriction patterns, most clones were classified by the UPGMA method (Sneath and Sokal 1973) into several main groups. With the primer combination I, six and eight different main groups were defined in Goldrich and Lito, respectively. Similarity within these groups was higher than 95% and between them lower than 90%. Using the primer combination II, seven and six groups were obtained for Goldrich and Lito, respectively (Table 1). In this case, similarity within each group was higher than 70% and between groups lower than 70%.

RGA sequence analysis and classification

Two to four clones from each of the 27 groups identified (a total of 86 clones) were sequenced, obtaining 71 unique DNA sequences. These sequences were compared with the GenBank database using BLASTX (Altschul et al. 1990), and all of them shared homology with putative R-genes (data not shown). When several sequences were identical at the amino acid level, only one was considered for the phylogenetic analysis, and those containing stop codons (7) were excluded. The alignment of the remaining 43 sequences using CLUSTALX (Thompson et al. 1997) produced a dissimilarity matrix and a phylogenetic tree based on the neighbor-joining method (Saitou and Nei 1987). This analysis grouped the 43 sequences into six families (designated as RGA1–RGA6) using a 70% identity threshold value (Fig. 1). The number of sequences in each RGA family ranged from 1 (RGA4) to 17 (RGA1), and the amino acid

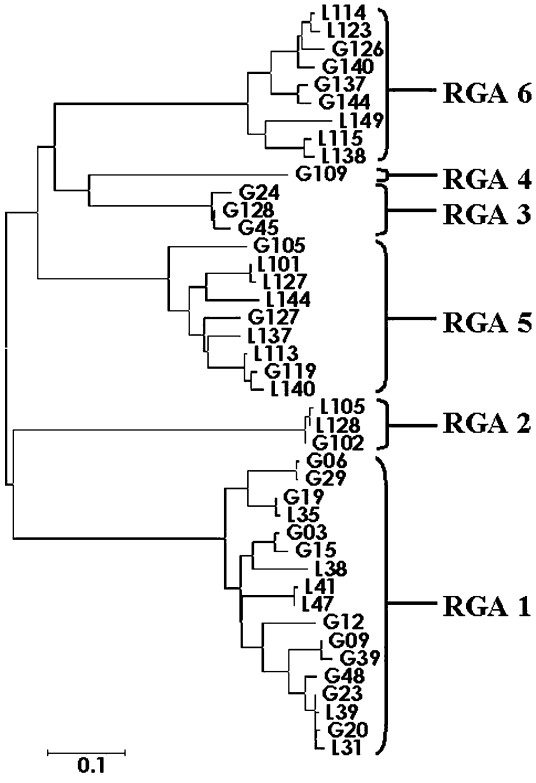

Fig. 1 Phylogenetic tree, based on the neighbor-joining method (Saitou and Nei 1987), of the deduced amino-acid sequences of 43 apricot RGA clones. Six RGA families (*RGA1–RGA6*) were obtained using a 70% identity threshold value. The SCALE BAR displays a distance corresponding to 0.1 amino acid substitutions per site

Table 1 Number[a] of different restriction patterns found in cvs. Goldrich and Lito following digestion of the 200 clones obtained with five restriction enzymes

Combination	Goldrich					Lito					Groups	
	*Hae*III	*Hin*fI	*Sau*3AI	*Rsa*I	*Cfo*I	*Hae*III	*Hin*fI	*Sau*3A	*Rsa*I	*Cfo*I	Goldrich	Lito
I	7	11	10	4	5	8	7	7	3	5	6	8
II	7	33	15	13	6	5	30	12	11	3	7	6

[a]The number of main groups, based on the restriction analysis in both cultivars, was determined by the UPGMA method (Sneath and Sokal 1973)

Table 2 Results of similarity searches between apricot RGA sequences and GenBank accessions using *blastx*

RGA family	Highest similarity with GenBank accessions	Percentage identity[a]	E^{b}
RGA1	Putative disease RGA NBS-LRR *Malus* × *domestica*	47	$2e^{-38}$
	Putative disease RGA NBS-LRR *Malus prunifolia*	49	$5e^{-37}$
RGA2	RCa4. *Manihot esculenta*	59	$2e^{-49}$
	Putative disease RGA NBS-LRR *Malus* × *domestica*	54	$4e^{-45}$
RGA3	Unknown *Cicer arietinum*	57	$2e^{-49}$
	MRGH63. *Cucumis melo*	55	$1e^{-48}$
RGA4	Resistance-gene protein *Vigna unguiculata*	54	$8e^{-45}$
	Resistance-like protein KNBS2 *Glycine max*	55	$2e^{-44}$
RGA5	MRGH63 *Cucumis melo*	44	$9e^{-33}$
	Putative resistance gene homologue. *Cucumis melo*	44	$7e^{-32}$
RGA6	RCa2. *Manihot esculenta*	55	$6e^{-46}$
	Putative disease RGA NBS-LRR *Malus prunifolia*	55	$1e^{-43}$

[a] Percentage of amino acid identity
[b] The expected (*E*) value refers to the number of matches expected by chance alone. The lower *E*-value, the more significant the match is

identities for members of a given family ranged between 80% and 99%.

A consensus sequence was developed from each RGA family and compared with GenBank accessions using BLASTX (Altschul et al. 1990). The amino acid identities found ranged from 44% between RGA5 family and RGHs of *Cucumis melo* (muskmelon) to 59% between RGA2 family and a putative resistance gene candidate of *Manihot esculenta* (cassava) (Table 2).

The consensus sequences of the six apricot RGA families were aligned by *clustalx* (Thompson et al. 1997) together with the NBS domain sequence of known resistance genes: *N* from tobacco (Whitham et al. 1994), *L6* from flax (Lawrence et al. 1995), *RPS2* (Mindrinos et al. 1994), *RPM1* (Grant et al. 1995), *RPP5* (Parker et al. 1997), *RPP8* (McDowell et al.1998) and *RPS5* (Warren et al. 1998) from *Arabidopsis* (Fig. 2). L6, N and RPP5 sequences belong to the TIR type of the NBS-LRR R-genes, and RPS2, RPM1, RPS5 and RPP8 to the non-TIR type. The six apricot consensus sequences showed basically the NBS domain-specific motifs and residues of the TIR group reported by Pan et al. (2000). For example, a phenylalanine (F) is located 15 residues downstream of the P-loop motif, and the tryptophan residue (W) characteristic of non-TIR class found at the final position of the kinase-2 motif (Meyers et al. 1999) was not present in any of the 43 apricot RGA sequences analyzed (Fig. 2).

A dissimilarity matrix and a phylogenetic tree (Fig. 3) grouping apricot RGA sequences and known resistance genes were constructed from this alignment. The highest similarity was detected between RGA3 and RGA4 families and the lowest between RGA2 and RGA5 families with similarity percentages of 71.7% and 49.6%, respectively. The *N* gene from tobacco also showed a significant degree of similarity (54.3%) with apricot RGA3 and RGA4. In the phylogenetic tree of Fig. 3, two main clusters can be observed. The first cluster grouped all the apricot RGA classes along with those genes (*L6*, *N* and *RPP5*) belonging to the TIR subfamily (Parker et al. 1997). The second cluster grouped the non-TIR-class genes from *Arabidopsis* (*RPS2*, *RPS5*, *RPM1* and *RPP8*). Clustering of TIR-

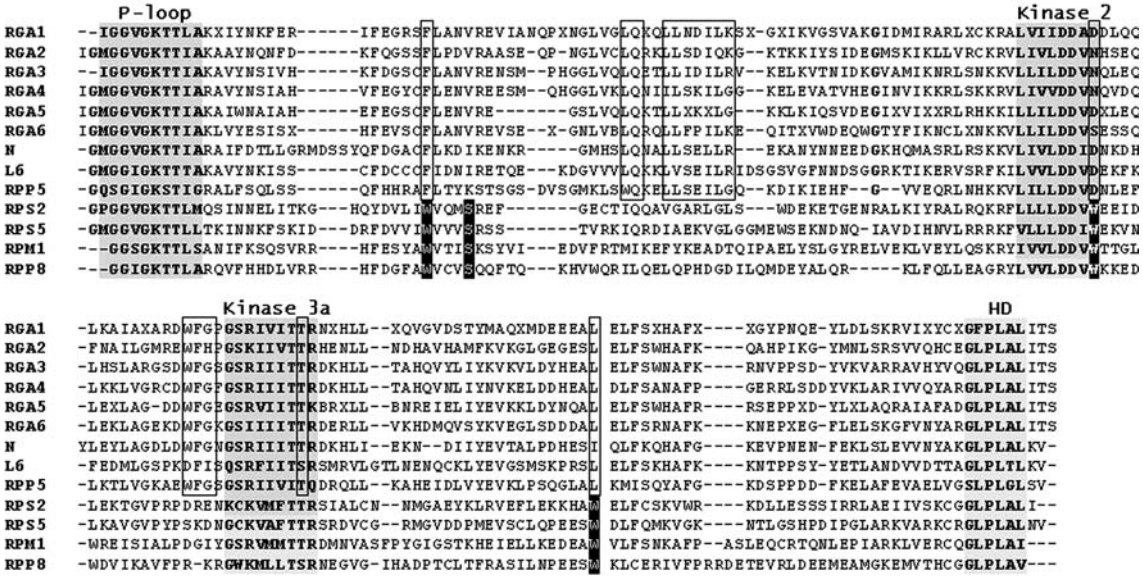

Fig. 2 Alignment of the translated apricot RGA consensus sequences, along with the NBS domain of known resistance genes from other species, using CLUSTALX (Thompson et al. 1997). EMBL/DDJB/GenBank Nucleotide Sequence Databases, accession numbers: *N* (U15605) from tobacco, *L6* (U27081) from flax, *RPS2* (U14158), *RPP5* (U97106), *RPM1* (X87851), *RPS5* (AF074916), *RPP8* (AF089710) from *Arabidopsis*. Conserved motifs of the NBS domain are *shaded* (Meyers et al. 1999), TIR subfamily-specific residues are *boxed*, non-TIR residues are *black boxed* (Pan et al. 2000). *HD* hydrophobic motif

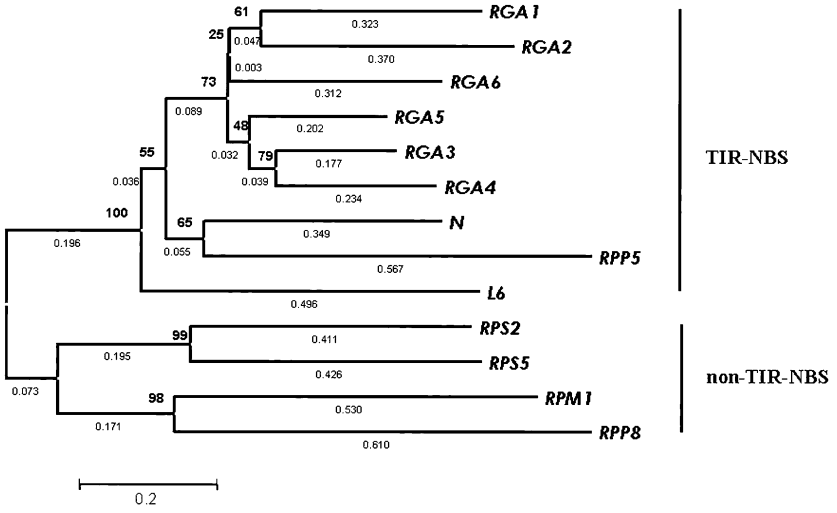

Fig. 3 Phylogenetic tree based on the alignment of the consensus amino acid sequences of each apricot RGA family and the NBS domain of seven known resistance genes using the neighbor-joining method (Saitou and Nei 1987). Branch lengths are indicated *under lines*, and bootstrap values based on 500 replications are *in bold*. The *scale bar* in the *bottom left corner* displays a distance corresponding to 0.2 amino acid substitutions per site

NBS and non-TIR-NBS clades was strongly supported on the basis of the bootstrap analysis, but apricot RGA tree nodes were only moderately supported (Fig. 3).

Development and mapping of AFLP-RGAs

To develop AFLP-RGA markers, we designed two specific apricot RGA primers from the NBS divergent region of each RGA family consensus sequence. These primers allowed us to amplify downstream the C-terminal region and upstream the N-terminal region of the NBS domain (Table 3).

The AFLP selective amplifications were performed combining the apricot-specific RGA primers and three AFLP primers (*Eco*RI + AA, *Mse*I + CAT and *Mse*I + CTG) selected on the basis of the high number of polymorphisms obtained with them in the apricot L×L population (Vilanova et al. 2003). Of the 26 primer combinations used, 17 resulted in 27 AFLP-RGA polymorphic markers, an average of 4.5 markers per RGA family (ranging from three in the RGA1 to seven in the RGA6 family) (Table 4). Figure 4 shows the *aflp-rga 6-03* marker segregating in the L×L population.

These markers were sequenced and shown to contain both the AFLP and the specific RGA primers. Therefore, unspecific AFLP-AFLP bands were not detected. BLASTX analyses (Altschul et al. 1990) showed that six markers (*aflp-rga 1-01*, *aflp-rga 4-03, aflp-rga 5-01, aflp-rga 6-02, aflp-rga 6-03* and *aflp-rga 6*-04) shared homology with the TIR class of the NBS-LRR R-genes (Table 4). Another one (*aflp-rga 2-04*) showed significant homology to a glucosyltransferase expressed during fire blight infection in *Malus*×domestica and therefore was putatively involved in the pathogen response mechanism. Six were homologous to other proteins, including ATP-binding proteins, with an unknown function or not specifically related to disease resistance. Four additional markers were similar to probable retroelement pol polyproteins. Finally, no significant similarity to any databank accession was found for ten of the AFLP-RGA markers obtained (Table 4).

Of the 27 AFLP-RGAs obtained, 16 were located in the F_2 L×L map (Fig. 5) developed by Vilanova et al. (2003). These markers mapped in five linkage groups,

Table 3 Sequences of family-specific RGA primers used to develop AFLP-RGA markers

Name	Sequence
RGA1-R	5′-CAATTACTTCCCTCACGTTTG-3′
RGA1-F	5′-CAAGTTGGAGTGGATAGCAC-3′
RGA2-R	5′-GAAGCTGCTCTAACATCTGG-3′
RGA2-F	5′-CAAGCGCATCCTATCAAAGG-3′
RGA3-R	5′-GCGACTCCTTTATCAATATTAG-3′
RGA3-F	5′-CGTGCAGTACACTATGTTC-3′
RGA4-R	5′-CAGACCTCCATGTTGCATTG-3′
RGA4-F	5′-GCTTGCTAGAATTGTAGTGCAG-3′
RGA5-R	5′-CAAACTCATGGGCAATTGC-3′
RGA5-F	5′-CACAACGTGCAATAGCTTTTG-3′
RGA6-R	5′-GAAATGCTCTCATAATCTAGC-3′
RGA6-F	5′-GTTAAGCATGATATGCAGGTATC-3′

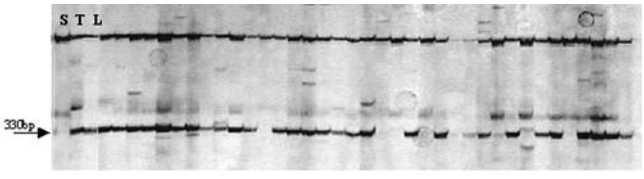

Fig. 4 Band pattern of the *aflp-rga 6-03* amplified using the primer combination RGA6-R and *Mse*I-CAT. From *left to right S* SEO, *T* Thyrintos, *L* Lito and individuals 1 to 37 of the L×L progeny. The *arrow* indicates the size of the *aflp-rga 6-03* marker (330 bp)

Table 4 Polymorphic bands identified using the AFLP-RGA procedure, indicating the primer pair combination used, the size of the marker, the linkage group where they were mapped and the closest homolog found in the GenBank accessions using BLASTX

Name	Primer pair	Size (bp)	Linkage group	Closest homolog	BLASTX *E*-value	*blastx* identity (%)
aflp-rga 1-01	RGA1-R + *Mse*I-CAT	506	1	Putative TIR-NBS type R protein 4 (*M. baccata*)	$5e^{-06}$	45
aflp-rga 1-02	RGA1-F + *Eco*RI-AA	469	6	SEC14 cytosol. factor fam. prot (*A. thaliana*)	$5e^{-11}$	43
aflp-rga 1-03	RGA1-F + *Mse*I-CTG	129	NM[b]	NS[c]	–	–
aflp-rga 2-01	RGA2-R + *Eco*RI-AA	138	3	USP family (*O. sativa*)	2.8	56
aflp-rga 2-02	RGA2-R + *Mse*I-CAT	106	NM	NS	–	–
aflp-rga 2-03	RGA2-F + *Eco*RI-AA	273	6	Putatove retroelement pol polyprot. (*A. thaliana*)	$6e^{-22}$	58
aflp-rga 2-04	RGA2-F + *Eco*RI-AA	202	4	Glucosyltransferase (*M. domestica*)	$4e^{-16}$	70
aflp-rga 2-05[a]	RGA2-F + *Eco*RI-AA	165	3	Putative retroelement pol polyprotein (*A. thaliana*)	$2e^{-09}$	62
aflp-rga 2-06[a]	RGA2-F + *Eco*RI-AA	129	1	Putative retroelement pol polyprotein (*A.thaliana*)	0.44	43
aflp-rga 3-01	RGA3-R + *Mse*I-CAT	227	NM	Hyp. prot. MMP0913 (*M. maripaludis*)	6.4	34
aflp-rga 3-02	RGA3-R + *Mse*I-CAT	186	NM	NS	–	–
aflp-rga 3-03	RGA3-F + *Eco*RI-AA	228	NM	ATP-binding protein ycf2 (*A. thal iana*)	$2e^{-33}$	90
aflp-rga 3-04	RGA3-F + *Eco*RI-AA	164	1	NS	–	–
aflp-rga 4-01	RGA4-R + *Mse*I-CAT	204	8	NS	–	–
aflp-rga 4-02	RGA4-F + *Eco*RI-AA	191	NM	ATP-binding HA1F transport. (*C. elegans*)	8.4	40
aflp-rga 4-03	RGA4-F + *Mse*I-CAT	317	8	Putative RGA protein (*L. culinaris*)	$5e^{-14}$	38
aflp-rga 5-01	RGA5-R + *Eco*RI-AA	138	4	TIR-NBS-LRR type R protein 7 (*M. baccata*)	$9e^{-07}$	55
aflp-rga 5-02	RGA5-R + *Mse*I-CAT	162	8	NS	–	–
aflp-rga 5-03	RGA5-R + *Mse*I-CAT	223	6	Dihydroflavonol 4 reductase (*C. sinensis*)	$2e^{-06}$	62
aflp-rga 5-04	RGA5-F + *Eco*RI-AA	124	4	NS	–	–
aflp-rga 6-01	RGA6-R + *Eco*RI-AA	209	NM	Putative polyprotein (*O. sativa*)	$3e^{-14}$	57
aflp-rga 6-02	RGA6-R + *Eco*RI-AA	167	NM	Putative RGA protein (*L. culinaris*)	$5e^{-05}$	76
aflp-rga 6-03[a]	RGA6-R + *Mse*I-CAT	274	8	Putative RGA protein (*L. culinaris*)	$3e^{-05}$	80
aflp-rga 6-04	RGA6-F + *Eco*RI-AA	190	NM	Putative LRR protein kinase (*A. thaliana*)	0.005	43
aflp-rga 6-05	RGA6-F + *Eco*RI-AA	174	NM	NS	–	–
aflp-rga 6-06	RGA6-F + *Eco*RI-AA	94	NM	NS	–	–
aflp-rga 6-07	RGA6-F + *Eco*RI-AA	94	NM	NS	–	–

[a] Sequences containing stop codons
[b] NM, Not mapped
[c] NS, Not significant similarity found

ranging from four AFLP-RGAs in groups 1 and 8, to 2 in group 3. Although most of the sequences seem to be randomly distributed in the apricot genome, linkage groups 6 and 8 showed the presence of putative clusters grouping AFLP-RGA markers in a relatively short map distance (three markers in 10 cM and four in 9 cM, respectively).

Discussion

RGAs in apricot

PCR approaches with degenerate primers designed from conserved motifs of the NBS domain of the R-genes have enabled the amplification of analog sequences from several plant species (Leister et al. 1996; Deng et al. 2000; Di Gaspero and Cipriani 2002; Baldi et al. 2004). In this study, we have successfully identified RGAs in apricot using degenerate primers from soybean (Kanazin et al. 1996), potato (Leister et al. 1996) and sunflower (Gentzbittel et al. 1998). The discrete band of 500 bp amplified with these primers showed heterogeneous fragments by restriction analysis, suggesting the existence of different apricot candidate resistance genes. Similar results have been observed previously in many other species (Kanazin et al. 1996; Leister et al. 1996; Mago et al. 1999). The cluster analysis based on the restriction patterns grouped clones in terms of their similarity. This al-

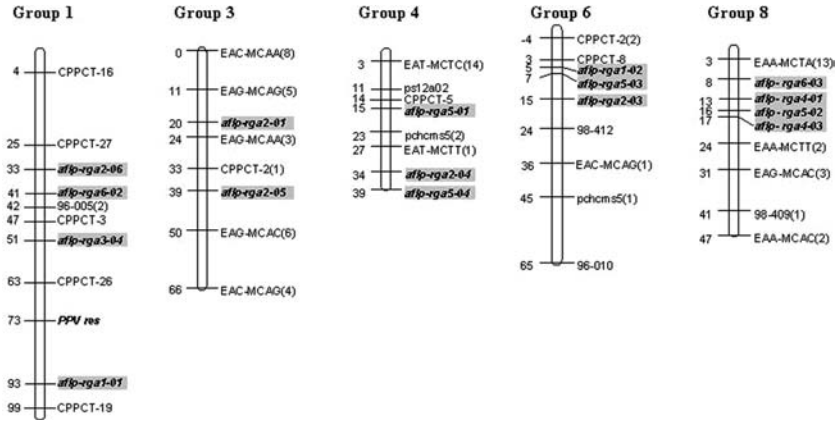

Fig. 5 Distribution of AFLP-RGA markers in the apricot F_2 L×L linkage map. Only SSRs and a few AFLP markers are shown (for more details see Vilanova et al. 2003). AFLP-RGAs are *shaded*

lowed the selection of representative clones from each defined group for sequencing, thereby avoiding the possible bias due to random sampling.

Phylogenetic analysis of the 43 unique amino acid sequences identified revealed the presence of six apricot RGA families with a variable number of members using a 70% identity threshold value. The number of RGA families found in apricot (after analyzing 200 clones and 86 sequences) was similar to the number of RGA families observed in other plant species. Thus, Kanazin et al. (1996) found nine families in soybean (analyzing 450 clones and one to five sequences from each class), Cordero and Skinner (2002) found six in alfalfa (analyzing the sequences of 94 clones) and Di Gaspero and Cipriani (2002) could differentiate three families in grape (analyzing 71 clones and 29 sequences).

The alignment and the phylogenetic tree of the apricot RGA consensus amino acid sequences, along with the NBS domain of known R-genes, indicated that the six apricot RGA families defined belong to the TIR subfamily.

The reported ratio of non-TIR:TIR-type NBS sequences in the complete *Arabidopsis* genome is 1:2 (Initiative 2000). However, this ratio can vary among different species. Thus, Kanazin et al. (1996) in soybean, Tian et al. (2004) in sugar beet and Martínez Zamora et al. (2004) in strawberry identified mainly TIR-subfamily sequences, but Donald et al. (2002) using in grapevine a similar PCR strategy with degenerate primers found a high proportion of non-TIR:TIR sequences (6:1). Although the PCR degenerate primers used in this study were designed from the conserved motifs of the NBS domain across the non-TIR:TIR subfamilies, slight differences in sequence might explain why non-TIR type sequences were not amplified. In fact, Baldi et al. (2004) found mainly non-TIR-class RGHs in apple when the LDD-AS primer located at the end of the kinase2 motif was used. However, with primers designed from the P-loop and from the hydrophobic motif the result was the opposite and most RGHs obtained were the TIR-type. In conclusion, the absence of apricot non-TIR class RGAs may reflect an unequal distribution of TIR/non-TIR sequences also found in other species or may be due to a bias in the PCR strategy used.

Apricot AFLP-RGA mapping

RGAs have been usually converted into RFLP markers to map them (Leister et al. 1996; Seah et al. 1998; Mago et al. 1999) and more recently into cleaved amplified polymorphic sequences (CAPS) (Deng et al. 2000; Quint et al. 2002) and single strand conformation polymorphisms (SSCPs) (Kuhn et al. 2003; Baldi et al. 2004). Alternately, Hayes and Saghai Maroof (2000) in soybean and Egea-Gilabert et al. (2003) in pepper used modified AFLPs, based on degenerate primers designed from the NBS domain, to detect polymorphisms linked to resistance to soybean mosaic virus (SMV) and to *Phytophtora capsici*, respectively.

In this work, we developed AFLP-RGA markers according to the methodology of Hayes and Saghai Maroof (2000) and Egea-Gilabert et al. (2003), with the exception of the use of specific apricot RGA family primers instead of the NBS degenerate primers. These markers were aimed at studying the distribution of the distinct RGA TIR families throughout the apricot genome. The results obtained showed that markers developed from the same family (i.e. *aflp-rga 4-01* and *4-03*) may be found tightly linked or lying on different chromosomes (i.e. *aflp-rga 6-02* and *6-03*). In addition, markers originating from different families can be located in the same region (i.e. *aflp-rga 6-03* and *5-02*). These results are consistent with those obtained by Baldi et al (2004) in apple where mapping and phylogenetic distance do not seem to be correlated. However, in order to confirm this it would be necessary to analyze a larger number of markers.

Six AFLP-RGA markers were homologous to the NBS-LRR TIR type R-genes and another one to a glucosyltransferase putatively involved in the pathogen response mechanism. Interestingly, four markers were similar to putative retroelement pol polyproteins, which seem to co-localize locally with disease resistance genes in *Arabidopsis* and poplar (Lescot et al.

2004). Therefore, all of these markers could indicate the presence of genomic regions containing candidate resistance genes.

RGAs are widely distributed in plant genomes and often organized in clusters (Kanazin et al. 1996; Meyers et al. 1999; Young 2000). In this study, 16 AFLP-RGAs were mapped in a previously developed apricot genetic map (Vilanova et al. 2003) covering five out of the eight linkage groups. Two putative clusters have been detected in linkage groups 6 and 8, although only the group 8 contains NBS-LRR homologous sequences (*aflp-rga 4-03* and *6-03*). The rest of the markers grouped in clusters did not show homology to R-genes. However, we can not discard the hypothesis that these sequences were related to pseudogenes present during the generation process of novel resistance specificities (Quint et al. 2003). In fact, it has been proposed that a significant proportion of NBS-encoding genes may be pseudogenes (Meyers et al. 1999; Dilbirligi et al. 2004).

In *Prunus*, Bliss et al. (2002) mapped seven RGAs in an almond × peach population. Interestingly, these authors found a putative RGA cluster with three markers in the linkage group 8 and mapped three additional RGAs in the group 1. These results are similar to those obtained in this work, as four AFLP-RGAs clustered together in the group 8 and four more are located in group 1. Furthermore, AFLP-RGA markers homologous to NBS-LRR R-genes (*aflp-rga 1-01* and *6-02*) have been found in the apricot G1 linkage group near a region where a powdery mildew resistance QTL was mapped in *P. davidiana* (Foulongne et al. 2003), and the plum pox virus resistance trait was tentatively located in a *P. armeniaca* map (Vilanova et al. 2003) (Fig. 5). Nevertheless, although some AFLP-RGA markers seem to be present near resistance regions in *Prunus*, further studies are necessary to confirm these associations.

These findings suggest that the development and mapping of AFLP-RGAs in *Prunus* may contribute to the identification of genomic regions containing putative resistance genes and facilitate the long process of map-based cloning.

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