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ORIGINAL PAPER

Isolation and linkage mapping of NBS-LRR resistance gene analogs in red raspberry (*Rubus idaeus* L.) and classification among 270 Rosaceae NBS-LRR genes

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Abstract Plant *R* genes confer resistance to pathogens in a gene-for-gene mode. Seventy-five putative resistance gene analogs (RGAs) containing conserved domains were cloned from *Rubus idaeus* L. cv. 'Latham' using degenerate primers based on RGAs identified in Rosaceae species. The sequences were compared to 195 RGA sequences identified from five Rosaceae family genera. Multiple sequence alignments showed high similarity at multiple nucleotide-binding site (NBS) motifs with homology to Drosophila Toll and mammalian interleukin-1 receptor (TIR) and non-TIR RNBSA-A motifs. The TIR sequences clustered separately from the non-TIR sequences with a bootstrap value of 76%. There were 11 clusters each of TIR and non-TIR type sequences of multiple genera with bootstrap values of more than 50%, including nine with values of more than 75% and seven of more than 90%. Polymorphic sequence characterized amplified region and cleaved amplified polymorphic sequence markers were developed for nine *Rubus* RGA sequences with eight placed on a red raspberry genetic linkage map. Phylogenetic analysis indicated four of the mapped sequences share sequence similarity to groupTIR I, while three others were spread in non-TIR groups. Of the 75 *Rubus* RGA sequences analyzed, members were placed in five TIR groups and six non-TIR groups. These group classifications closely matched those in 12 of 13 studies from which these sequences were derived. The analysis of related DNA sequences within plant families elucidates the evolutionary relationship and process involved in pest resistance development in plants. This information will aid in the understanding of *R* genes and their proliferation within plant genomes.

Keywords Disease resistance · Resistance gene analog

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Introduction

Plant resistance to pathogens is often governed by a specific interaction between a pathogen *Avr* (avirulence) gene locus and an allele of the corresponding plant disease resistance (*R*) locus (Dangl and Jones 2001). This concept of matching an *R* gene in the host and an *Avr* gene in the pathogen is referred to as the gene-for-gene hypothesis (Flor 1971). A number of plant genes conferring resistance to various plant pests have been isolated and characterized from a wide range of divergent species (Bent 1996; Hammond-Kosack and Jones 1997). Their products share

striking structural similarities (Jones 1996), which led to the hypothesis that certain signaling events commonly occur during plant defense (Baker et al. 1997). Several *R* gene classes have been identified on the basis of specific conserved functional domains. The most common belong to the leucine-rich repeat (LRR) family encoding proteins that contain an LRR domain near the C terminus. The LRR domain is highly variable in length and is thought to be involved in the recognition of the invading pathogen and/or the biochemical signals it produces. In addition to the LRR domain, the majority of the plant disease *R* genes cloned to date (approximately 70%) also encode a putative tripartite nucleotide-binding site (NBS) near the N terminus. The NBS region is characterized by the presence of several highly conserved domains regardless of the diversity of pathogens against which they act. The P-loop (kinase-1a) and the kinase-2 domains are found in both adenosine-triphosphate- and guanosine-triphosphate-binding proteins (Saraste et al. 1990), while additional motifs found in the NBS region are the kinase-3a and the Gly-Leu-Pro-Leu (GLPL, also known as "hydrophobic domain"), a putative membrane spanning domain. NBS-LRR genes have also been shown to possess additional domains at their N terminus. A cytoplasmic signaling domain has been identified in several plant resistance genes that shares homology to the Drosophila Toll protein and the mammalian interleukin-1 receptor protein (TIF; Hammond-Kosack and Jones 1997). This motif has been given the acronym TIR and is speculated to be involved in cell signaling. Most of the non-TIR NBS-LRR *R* genes have been reported to contain a coiled-coil motif or a leucine zipper motif proposed to facilitate protein interactions (Pan et al. 2000).

Previous approaches for identifying candidate genes controlling resistance against different pathogens have used highly saturated genetic maps for map-based cloning. The markers used for such maps generated polymorphic data based on restriction sites (restriction fragment length polymorphism [RFLP] and amplified fragment length polymorphism [AFLP]), random sequences (random amplified polymorphic DNA [RAPD]), and repetitive elements (simple sequence repeats). Such markers may not represent the segregating gene, and the likelihood of identifying a marker linked to the target gene is a function of the distribution of the marker type and location of the gene in the genome.

The conserved backbone of both TIR and non-TIR NBS-LRR-class proteins has led to the development of polymerase chain reaction (PCR)-based strategies for isolating putative resistance gene analogs (RGAs). By the use of degenerate and/or specific primers targeted to the particular conserved amino acids in the NBS motifs at low annealing temperatures (35°C to 55°C), a remarkable number of RGA sequences have been identified from many plant species including soybean [*Glycine max* (L.) Merr.] (Kanazin et al. 1996; Yu et al. 1996), potato (*Solanum tuberosum* L.; Leister et al. 1996), barley (*Hordeum vulgare* L.) and rice (*Oryza sativa* L.; Leister et al. 1998), *Arabidopsis thaliana* (L.) Heynh. (Aarts et al. 1998), pea (*Pisum sativum* L.; Timmerman-Vaughan et al. 2000), grape (*Vitis vinifera* L.; Donald et al. 2002; Gaspero and Cipriani 2002), cotton (*Gossypium* spp.; Tan et al. 2003), tomato (*Lycopersicum esculentum* L.; Zhang et al. 2003), apple (*Malus* × *domestica* Borkh.; Baldi et al. 2004), and chestnut rose (*Rosa roxburghii* Tratt.; Xu et al. 2005). NBS sequences tend to be clustered in the genome, and isolated RGAs are frequently located at or near previously identified resistance loci or might even be parts of known *R* genes (Kanazin et al. 1996; Yu et al. 1996; Aarts et al. 1998; Collins et al. 1998; Donald et al. 2002). Thus, molecular markers generated through this approach are useful to saturate regions of the genome where clusters of resistance genes are located and facilitate their map-based cloning.

Phytophthora species cause billions of dollars in annual losses to agricultural and forest species in the USA and worldwide (Erwin and Ribeiro 1996). *Phytophthora* root rot (PRR) of red raspberry is an economically important disease in nearly all temperate regions. The causal agent *P. fragariae* var. *rubi* Wilcox and Duncan (referred earlier as *P. erythroseptica*, *P. erythroseptica* var. *erythroseptica*, *P. megasperma* "type 2", and *P. fragariae*; Wilcox et al. 1999) is subterranean, soil persistent, and polycyclic. It is capable of rapidly spreading within the plant to cause severe root and crown rot in the absence of suppressing measures. Host resistance is the most effective control practice from both the environmental and economic perspective. Cultivars with known resistance include 'Latham', 'Newburgh', 'Durham', and 'Chief', while moderate resistance has been observed in 'Taylor', 'Haida', 'Chilcotin', and others (Barritt et al. 1979). Susceptible cultivars include 'Titan', 'Canby', 'Willamette', and 'Skeena' (Barritt et al. 1979; Wilcox et al. 1999). Unfortunately, many resistant cultivars are not commercially accepted in contrast to many moderately resistant and susceptible cultivars because of fruit quality requirements.

Analysis of the distributional extremes and quantitative trait loci (QTL) mapping of a backcross population (B_1) [('Titan' × 'Latham') × 'Titan'] using RAPD, AFLP, CAPS, and SCAR markers revealed two major genomic regions associated with PRR resistance in red raspberry (Pattison et al. 2007). A collection of RGA sequences from red raspberry would be an effective tool for the characterization of these regions and other disease-related genes (Leister et al. 1996, 1998; Yu et al. 1996). This study reports the identification and characterization of 75 RGAs developed through the use of degenerate primers designed to bind the P-loop, kinase-2, and the GLPL elements of the NBS

region. Multiple and diverse RGAs are shown to exist in the red raspberry genome, and eight RGAs were mapped using a linkage map developed from the red raspberry cultivars 'Titan' and 'Latham'. To classify the *Rubus* gene fragments identified in this study, NBS-LRR sequences from Rosaceae that were publicly available were collected and analyzed. This allowed placement of the *Rubus* sequences in context with others identified within this plant family.

Materials and methods

Plant material, PCR amplification of RGA sequences

NBS-LRR sequences that had been previously identified in Rosaceae species and entered into GenBank were analyzed for the purpose of identifying suitable primers for the amplification of RGAs in red raspberry (Table 1). Seven degenerate primers from species in this family were utilized in this study (1) P-loop: 5′-GAATTCGGNGTNGGNAA GACAAC-3′ (forward; Shen et al. 1998); (2) BP2f: 5′-GGN GGDGTDGGSAARAC-3′ (forward; Baldi et al. 2004); (3) BP2r: 5′-GCTAGTGGCAMNCCWCC-3′ (reverse; Baldi et al. 2004); (4) OLE 1121: 5′-GGWATGGGWGGW RTHGGWAARACHAC-3′ (forward; Lee et al. 2003); (5) OLE 1122: 5′-ARNWYYTTVARDGCVARWGG-VARWCC-3′ (reverse; Lee et al. 2003); (6) DegRos_f: 5′-MDTKSBDR RRSSBDTTTWHRMM-3′ (forward); (7) DegRos_r: 5′- RKDYWYDHMWHRDWKBWBMWK- 3′ (reverse). Primers DegRos_f and DegRos_r were designed during the course of this study.

Genomic DNA from newly expanded leaves from the resistant cultivar 'Latham' was extracted for RGA amplification using the cetyl trimethylammonium bromide method as described by Pattison et al. (2007). PCRs were carried out in a total volume of 50 µl with a 100-ng template DNA and 0.4 µM of each primer in 49.3 mM Tris–HCl (pH=8.3), 2.5 mM $MgCl_2$, 1 mM tartrazine, 1.5% Ficoll, 125 µM deoxynucleotide triphosphates, and 0.5 U *Taq* polymerase. PCRs were performed in a MJ Research PTC-100 thermocycler (Watertown, MA, USA) including initial denaturation for 2 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C, and a final extension at 72°C for 5 min. Amplified DNA fragments were separated on a 2% TAE agarose gel and visualized by ethidium bromide staining.

Cloning and sequence analysis

DNA bands generated from the PCR reactions were excised from the gels and the DNA retrieved with a Sephaglas BandPrep Kit (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturer's instructions. The eluted fragments were cloned using the pGEM-T Vector System (Promega, Madison, WI, USA). Recombinant plasmids were extracted with the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany), and the DNA was sequenced at the Cornell Sequencing Biotechnology Resource Center (Cornell University, Ithaca, NY, USA).

DNA and amino acid sequences were analyzed with the Laser Gene software package (DNASTAR, Madison, WI, USA) and the GeneDoc software, version 2.5.000 (www.psc.edu/biomed/genedoc). DNA similarity (basic local alignment search tool [BLAST]) searches were performed against nucleotide and protein sequence databases at the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov; Rehm 2001). Nucleotide sequences were conceptually translated using sequence utilities at the Baylor College of Medicine Search Launcher (http://searchlauncher.bcm.tmc.edu; Smith et al. 1996). In nine cases, amino acid frames were interrupted by one or two mis- or non-sense mutations. Correction to the original frame was done based on related published NBS-LRR amino acid sequences for the following nine

Table 1 Clones generated with the degenerate primers used for amplification of RGA sequences in red raspberry and number of fragments revealing homology to publicly available NBS-LRR sequences

Primer comb.	Degenerate primer		Total number of clones	NBS-LRR homologous clones	Group name	Class	GenBank ID
	Forward	Reverse					
1	OLE1121	OLE1122	48	43	N4	TIR-NBS-LRR	BV681230-271
2	BP2f	BP2r	13	9	N6	non TIR-NBS-LRR	BV681272-280
3	DegRos_f	OLE1121	16	5	N9	non TIR-NBS-LRR	BV681281-285
4	OLE1121	DegRos_r	15	9	N14	non TIR-NBS-LRR	BV681287-296
5	BP2f	DegRos_r	16	1	N16	non TIR-NBS-LRR	BV681297
6	OLE1121	BP2r	13	5	N19	non TIR-NBS-LRR	BV681298-302
7	P-loop	OLE1122	12	3	N23	TIR-NBS-LRR	BV681303-305
Total			133	75			

sequences: 15_Ri_19-7, 20_Ri_4-1, 23_Ri_4-12, 34_Ri_4-23, 46_Ri_4-40, 49_Ri_4-53, 50_Ri_4-54, 65_Ri_6-31, and 72_Ri_9-11 (Supplemental Table S1).

All public NBS-LRR amino acid fragments from Rosaceae available at the time of analysis were downloaded from the NCBI GenBank (http://www.ncbi.nlm.nih.gov; Supplemental Table S1). These sequences were aligned together with the conceptually translated *Rubus* fragments generated in this study, using hidden Markov models with the Sequence Alignment and Modeling Software System (SAM-T2K; Karplus et al. 1998) and formatted for analysis with the Phylip phylogenetic inference package (Supplemental File "Samuelian et al Rosa NBS-LRR S2.phy"). Seqboot in the Phylip package (Felsenstein 2006) was used to generate 1,000 bootstraps of the dataset, and Protdist was used to construct 1,000 bootstrapping distance matrices using the Jones–Taylor–Thornton calculation, with one category of substitution rates. A neighbor-joining tree of the 1,000 bootstraps was constructed (jumbling the sequence input order twice) and a majority-rule consensus tree determined.

Marker development and RGAs mapping

Primers specific to the cloned raspberry RGA sequences were designed utilizing a manual analysis of the clone sequences to develop PCR-based markers for genetic mapping. Three criteria were followed for primer design: (1) 50–70% guanine–cytosine (GC) content, (2) predominant GC content at the 3′ end, and (3) a primer length of 20 to 24 bp to ensure similar melting temperatures (Table 2). The oligonucleotides were synthesized by MWG-Biotech (High Point, NC, USA). Each primer pair was tested with genomic DNA from 'Titan', 'Latham', and the F_1 parent from the previously mapped B_1 population (Pattison et al. 2007) to identify polymorphisms. Amplification was performed under the same conditions already described, only with an annealing temperature of 62 °C instead of 50 °C. Markers generated using this technique are referred to as sequence characterized amplified regions (SCARs; Paran and Michelmore 1993). When a polymorphism between the parents was not identified based on primer site annealing, a restriction digestion was performed using 20 µl of each PCR reaction to identify sequence differences resulting in restriction site polymorphisms. Polymorphisms identified through PCR amplification with specific primers followed by restriction digestion are referred as to cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel 1993). PCR products and their digestions were visualized on 3% MetaPhor agarose gels (Cambrex, Rockland, ME, USA) stained with ethidium bromide. The specific primers for the development of the SCARs and the specific primers together with the restriction enzymes used for the development of the CAPS markers are shown in Table 2.

Mapping of markers derived from RGAs was performed using 68 individuals from a B_1 population previously screened for PRR resistance (Pattison et al. 2007). Identified polymorphisms were mapped using JoinMap 3.0® (Van Oijen and Voorrips 2001) with linkage groups assigned at a minimum logarithm of the odds of 3.0.

Results

Cloning of RGAs and sequence analysis

All 12 possible primer combinations between the forward and reverse primers described were investigated. From each primer combination, approximately 15 clones were sequenced. Searches of the GenBank database using the BLASTN and the BLASTX algorithms showed that clones generated with seven of the primer combinations revealed homology to

Table 2 Specific primers based on cloned RGA sequences from 'Latham' red raspberry that generated polymorphic SCAR and CAPS markers in a ('Titan × 'Latham') × 'Titan' B_1 red raspberry population with the corresponding restriction enzyme for the CAPS markers

RGA clone	Primers		Enzyme revealing polymorphism	LG (Fig. 1)	Marker type
	Forward	Reverse			
55_Ri_4-6	AAAACTACCATCGCCACAGCTG	AGCACACTCTATGGCAAGACC		7	Dominant
23_Ri_4-12	AGAATCGACGGTACTCTGTCGA	TTCACAAAGAAGGGTGAGACAG	*Alu*I	2	Co-dominant
27_Ri_4-16	ACCTTGGTAAAGCAAGCGTACG	TTCCCCTGAAATGTCTTCCTGC	*Mse*I	2	Co-dominant
32_Ri_4-20	CTGCACGGCTTGAGAGATGATC	TTCGGGAACCAAGTACGTATGC		3	Co-dominant
42_Ri_4-34	AAGACTACCATCGCTAGAGCTG	AGTTGAACAGCTTCACCATCGC	*Eco*RI	6	Dominant
58_Ri_4-62	TTGGCAGTTTGAACACTGTTGC	GACACGCCTTGAGAGATGATTG	*Mse*I	4	Dominant
70_Ri_6-7	GATGAGGTGGTTATGGTACTCG	CCACACTTCTCAGCTACTTTGG	*Taq*I		Dominant
67_Ri_6-33	GATGTGATTATCCTACTAGATG	CATCTTTCGGCTACTTTGATTG	*Mse*I	1	Dominant
9_Ri_14-36	GACGGTAGCTTAACCATAGAGC	AAGACTACCCTTGCTAAGCTCG	*Taq*I	1	Dominant

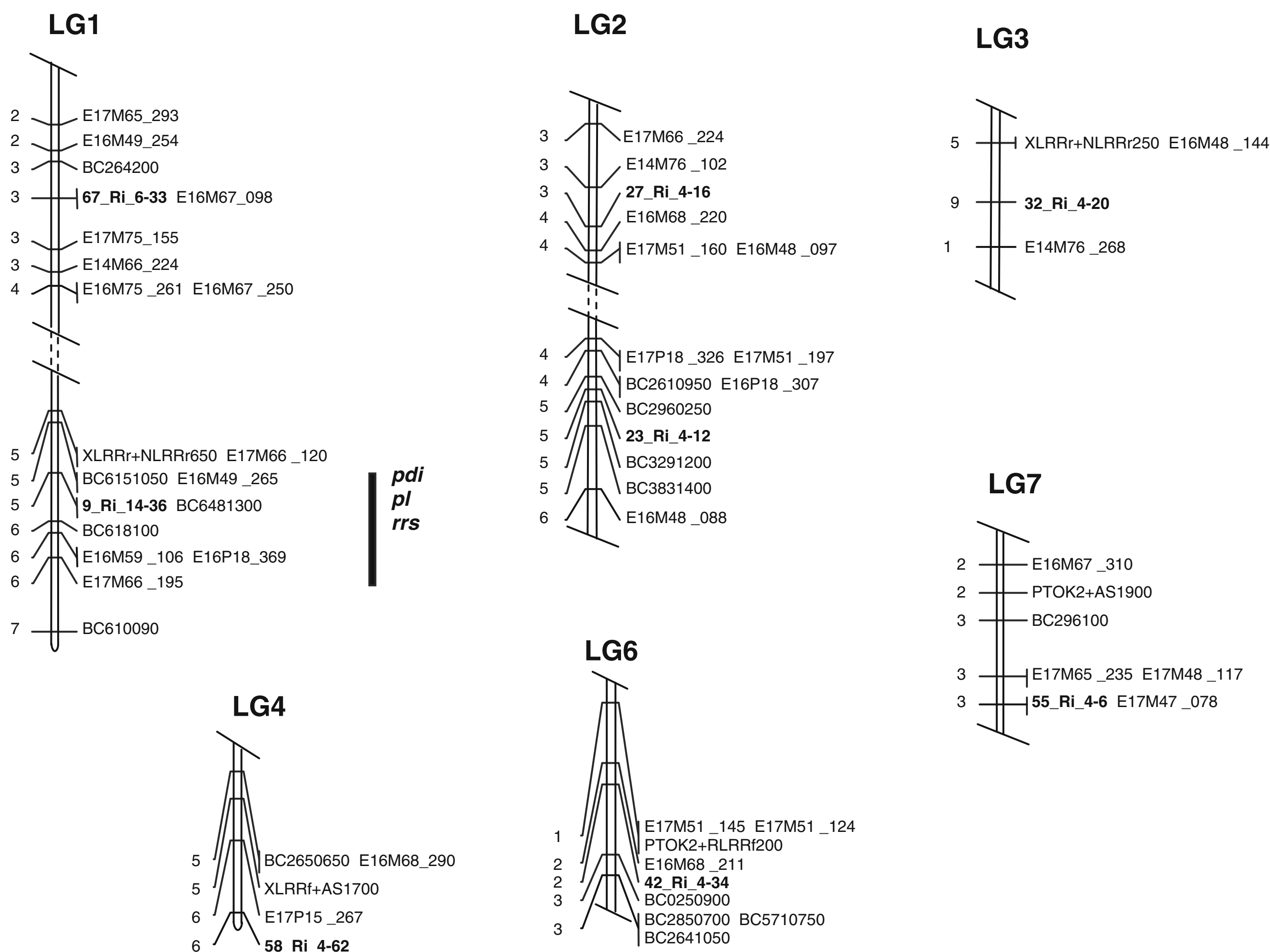

Fig. 1 Genetic linkage map showing the position of the RGA loci (*in bold letters*) on red raspberry linkage groups. Mapping was done on a ('Titan' × 'Latham') × 'Titan' B_1 population. Relative locations of putative QTLs for plant disease index (*pdi*), petiole lesion (*pl*), and root regeneration score (*rrs*) are shown as *vertical bars on the right of the linkage group* and were assigned by Pattison et al. (2007). The marker distances are indicated in centimolar calculated in Kosambi units (Lander et al. 1987)

NBS-LRR sequences (Table 1). Fragments cloned from the remaining five primer combinations did not reveal homology to NBS-LRR sequences and were excluded from further analysis. Approximately 93% of the clones generated with primer pair OLE1121/OLE1122 showed strong overlapping similarity to RGA sequences. Therefore, an additional set of 33 clones from that group was sequenced bringing the total number of clones analyzed for NBS-LRR homologies to 133 (Table 1). BLASTX searches of the SwissProt and GenBank NR databases revealed that 62 of them were highly homologous to house-keeping genes or did not show homology to any sequences in the database and were excluded from further analysis. The remaining 75 sequences with sizes between 197 and 702 bp were highly similar to RGA sequences cloned from other plant species using similar PCR-based approaches.

Marker development and linkage mapping

Specific primers were designed for each RGA sequence (Table 2) to identify polymorphic markers that could be placed on a red raspberry genetic linkage map (Pattison et al. 2007). The map was developed using a segregating B_1 population that was screened for resistance to *P. fragariae* var. *rubi* and was analyzed for QTL associated with resistance (Pattison et al. 2007). When genomic DNA of 'Titan', 'Latham', and F_1 parent of the mapping population were amplified, a dominant presence/absence polymorphism was revealed for two RGAs, 55_Ri_4-6 and 32_Ri_4-20 (Table 2). A single band of the expected size was observed for all other RGA fragments. To identify polymorphisms in the monomorphic products, a set of restriction enzymes (mainly four basepair cutters) was

utilized to detect restriction site polymorphisms. Polymorphisms were identified in seven of the RGAs, thus allowing the development of CAPS markers. These markers were screened in the mapping population for placement on the map. The RGA markers were present on six of the seven linkage groups in red raspberry with no clustering seen (Fig. 1). The RGA fragment 9_Ri_14-36 was mapped on LG1 within the first putative QTL for PRR resistance (Pattison et al. 2007; Fig. 1), and 67_Ri_6-33 was mapped elsewhere on the same linkage group. No NBS-LRR loci were placed on LG5 where the second major QTL for PRR was positioned. Two sequences, 23_Ri_4-12 and 27_Ri_4-16, were mapped on LG2, and the remaining four sequences 32_Ri_4-20, 58_Ri_4-62, 42_Ri_4-34, and 55_Ri_4-6 on linkage groups 3, 4, 6, and 7, respectively (Fig. 1). Marker 70_Ri_6-7 could not be placed on the genetic linkage map.

Classification of Rosaceae RGAs

Additional published NBS-LRR protein fragments from other Rosaceae were downloaded from GenBank, conceptually translated, and aligned with the 75 *Rubus* sequences obtained in this study. These include 16 *Fragaria* (Martinez Zamora et al. 2004), 76 *Malus* (Lee et al. 2003; Lee and Lee 2003, 2006; Baldi et al. 2004; Varshochi 2006), 8 *Prunus* (Lalli et al. 2005; Liang et al. 2005), 11 *Pyrus* (Afunian et al. 2006), and 78 *Rosa* (Hattendorf 2005; Hattendorf and Debener 2007; Xu et al. 2005). Six additional *Prunus* consensus NBS-LRR fragments representing sequences that have not been deposited in the public domain were added (Soriano et al. 2005; Supplemental Table S1). Sequences from some related studies for peach (Liang et al. 2005) and apple (Calenge et al. 2005) were not available.

Fig. 2 Alignment of conceptually translated *Rubus* TIR sequences. The RNBS-A-TIR motif is characteristic of plant TIR-NBS-LRR RGAs. Identical residues with 50% or greater frequency in a column have a *dark grey background*. Similar residues with 50% or greater frequency have a *light grey background*

```
                 P-loop                                          RNBS-A-TIR
             ----------------------                            ---------------
20_Ri_4-1    GMGGVGKTTLAKLVFERISHHHFEVSK-FLVNVREVSAKHG--TVVDLQKQLLSPILKEN
34_Ri_4-23   GMGGVGKTTLAKVVFERIS-HHFEVSK-FLVNVSEVSAKHG--TLVELQKQLLSPILKKN
17_Ri_23-1   EFG-VGKTTLARLIFERIS-HHFEVSN-FLLNVREVSAKHG--SLVDLQKQLLSPILKEN
57_Ri_4-61   GMGGIGKTTLAKVLFDGIS-HQFEFSS-FVSYVRN-NEEKS--GLVHLQETLISRILG-K
16_Ri_19-9   GMGGIGKTTVAKALYNKFCH-SFEASS-FLADVRETMQK-D--GKVSLQESLLSDISKTT
44_Ri_4-37   GMGGVGKTTIAKFVYNSNFQ-RFERCSSFLENIREVSEQSN--GLLKLQKQLLNDILTGR
60_Ri_4-8_   GMGGVGKTTIAKFVYNSNFQ-SFERYS-YLENIREVSEQPN--GLLRLQKQLLNDILTGS
21_Ri_4-10   GMGGIGKTTIAKFVYNSNYE-KFERCS-FLENIREVSEQAN--GLVQLQKQLLYDILNGK
19_Ri_23-9   -EFGVGKTTIAKVVYNSNFR-RFEASS-FLENIREISENPN--GLVQLQRQLLADILN-R
36_Ri_4-25   --GGIGKTTIAKVVYNSNFR-RFEASS-FLENVREISENPN--GLVQLQRQFISDILN-R
29_Ri_4-18   GMGGVGKTTIAKVVYNSNFR-RFEASS-FLENIREISENPN--GLVQLQRQLLADILN-R
61_Ri_4-9    GMGGVGKTTLAQCFVDKMAN-QYDATS-FLNNVREVSAERHGTGIVTLQEKLLSDAQMG-
18_Ri_23-6   EFG-VGKTTIARAVYGKIHQ-QFEHFC-FLDNVKEEFLTKH-----KVTEALLSKILKVN
45_Ri_4-4    GMGGIGKTTIARAVYGKIHQ-QFEHFC-FLDNVKEEFLTKN-----KVTEALLSKILKVN
55_Ri_4-6    GMGGVGKTTIATAVYNKIEG-QFDHCC-FLENIKDRFRATNG--DIHTLEELLSRMLKEE
39_Ri_4-29   -------TTIARAVYDQLVC-QFEHHC-FLENVKEGFKNNG---AIHMQEELLSRIFDKR
22_Ri_4-11   GMGGVGKTTIAKAVYDEIAY-QFDHCC-FLDNVKEGFTEKG---EAEMQEELLSRILNEK
56_Ri_4-60   GMGGIGKTTIAKAVYDEIAY-QFDHCC-FLDNVKEGFTEKG---EAEMQEELLSRILNEK
31_Ri_4-2    GMGGIGKTTIAKAVYDEIAC-EFDHCC-FLDDVKEGFTKKG---KTQIQEDLLSRILKEK
35_Ri_4-24   GMGGVGKTTIAKAVYDEIAC-QFDHYC-FLDDVKEGFTKKG---KAQILEDFLSRILKKK
43_Ri_4-36   GMGGVGKTTIAKAVFDEIAC-QFDHCC-FLENVKEGFT-KD---KAQIQEDLLSRILKDK
48_Ri_4-5    GMGGVGKTTIAKAVFDEIAC-QFDHCC-FLENVKEGFT-KD---KAQIQEDLLSRILKDK
54_Ri_4-59   GMGGVGKTTIAKAVFDEIAC-QFDHCC-FLENVKEGFT-KD---KAQIQENLLSRILKDK
41_Ri_4-33   GMGGVGKTTIAKAFYDEIAY-QFNHCC-FLDDVKEVFTKRD---KAQIQEDLISRILKEK
37_Ri_4-26   GMGGVGKTTIARAVYDEIAC-QFQHSC-FLDNVKEGFAKKG---EAQMKEELLSTILREK
38_Ri_4-28   GMGGVGKTTIARAVYEKLAC-QFEHYC-FLDNVKEGFTKKG---GIQMQEELLCRILMEK
42_Ri_4-34   GMGGVGKTTIARAVYEKLAC-QFEHYC-FLDNVKEGFTKKG---GIQMQEELLCRILMEK
47_Ri_4-41   GMGGVGKTTIARAVYEKIAC-QFEHCC-FLDNVKEGFTKKG---GIQMQEELLCRILMEK
23_Ri_4-12   GMGGIGKTTVARAVYDKIAC-QFEG---FLENVKEGFTKKG---ETEMRKELLSRILTVP
46_Ri_4-40   GMGGVGKTTVARAVYDKIAC-QFEYHC-FLENVKEGFTKKG---ETEMRKELLSRILTVQ
40_Ri_4-3    GMGGVGKTTIASVVYDEIAS-QFEYHC-FLENVKEGFTKKG---ETQMQKDLLSRILTEK
52_Ri_4-57   GMGGIGKTTIASAFYDKMAC-QFEYCC-FLENITGGFTKKG---ETQMRKELLSRIYSDK
32_Ri_4-20   GMGGIGKTTIATEVYDEIRS-QFSHCY-FLPNVKEGFGNQV-----RMQEQLLSGILKKE
49_Ri_4-53   GMGGIGKTTIAKAVYDKIAY-QFVHKC-FLGDVKEGFAKKG---EAQMRGEFLSEILKDK
50_Ri_4-54   GMGGVGKTTIARAVYGKIAY-QFEHKC-FLGDVKEGFAKKG---EAQMRGEFLSEILKDK
58_Ri_4-62   GMGGVGKTTIAKAVYDEIAW-QFEHCC-FLVNVKEAFANKR---EVQLQEELLSRMLKEK
28_Ri_4-17   GMGGVGKTTITRAVYDEIAC-QFEHVC-FLDHVDYHFVNKR---EVKLQEKLLSGLLKDK
33_Ri_4-22   GMGGAGKTTIARAVYDKWSR-KFEACC-FLENVRKRSS------IVQMQEELLFRILKEK
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Fig. 2 (continued)

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                                          Kinase-2              RNBS-B
                                         ----------          -------------
20_Ri_4-1   -IAQVWDEQEGTLFIRKCLFNKKVLLVVDNVDHYNQLE-ILVGNKSWFGKGSRVIITTRE
34_Ri_4-23  -IAQVWDEQEGTLFIRNCLFNKKVLLIVDNVDHDKQLE-ILVGDKSWFGEGSRVIITTRD
17_Ri_23-1  -IAQVWNEQEGTLFIKNFFFNKKALIVVDDVDHYNQLA-ILVGNRSWFGEGSRIIITTRD
57_Ri_4-61  -ETKICDIHEGATMIKRLLHHKKVLLILDDVNQWRQLE-YLAGKQDWFGFGSIIIITTRD
16_Ri_19-9  -RTKVGHVDRGINVIKNRLGCRRVFVVIDDVDQVEQLE-ALAIDRDSFGPGSRIPITTRD
44_Ri_4-37  -KVKIHNISEGIAKIEDAVSSRRVFLVLDDVDHVDQLA-ALLRMQNRFHPGSKIIITSSC
60_Ri_4-8_  -KVKIHSISEGIAKIENAVSSRRVFLVLDDVDHVDQLA-ALLRMQNRFYPGSKIIITSSC
21_Ri_4-10  -KVEIHSISEGIAKIEDVVSSKRVLLVLDDVDHVDQLD-ALLRIQDRLYPGSKIIITTSC
19_Ri_23-9  -KVRVHSVSQGTSKIKDVVSSKKVLLVLDDVIHKDQFD-AILEMKSGFRAGSKIIITTRD
36_Ri_4-25  -KVKVHSVSQGTSKIKDVISSKKVLLVLDDVIHKDQFD-AILEMKSGFRAGSKIIITTRD
29_Ri_4-18  -KVKVHSVSQGTSKIKDVVSSKKVLLVLDDVIHKDQFD-AILEMKSNFKAGSKIIITTRD
61_Ri_4-9   TGTKKLDVYKGMNEIKHRLSHKKVLIVIDDVDHIKQLE-ALVGSHEWFGRGSRIIITTRN
18_Ri_23-6  DRHI--LDGGLN-MMQ--------------------------------------------
45_Ri_4-4   DRHI--LDGGLN-MIRERLGKKKVLIVLDDVDNLDQIET---------------------
55_Ri_4-6   RRILGTLDKGLN-MIRKKLGKKKVVLVLDDVDNLDQIEALIGKKPS-FGGGSRIIITTRD
39_Ri_4-29  VCSLGTLSRGSK-IIMERLSKKKVLLVLDDVENFAQIEALLGKQYS-FGSGSRIVVTTRD
22_Ri_4-11  VPSTGSLNRGFN-MIMKRLGKKKVLLVLDDLDDIAQFEILLGDQPS-FGGGSRIILTTRD
56_Ri_4-60  VPSTGSLNRGFN-MIMKRLGKKKVLLVLDDLDDIAQFEILLGDQPS-FGGGSKIILTTRD
31_Ri_4-2   VPSTGILNRGSN-MIMERLGKKKVLLVLDDLDDIAQIETLLGEKPS-FGGGSRIILTTRY
35_Ri_4-24  VPSTGILNRGSN-MIMKSLGKKKVLLVLDDLDDIAQIETLLGEQHS-FGGGSRIILTTRY
43_Ri_4-36  VPSTGILSRGSI-MIMERLGKKKVLIVLDDLDNIAQIETLLGDPYS-FGGGSRIILTTRY
48_Ri_4-5   VPSTGILSRGSI-MIMERLGKKKVLIVLDDLDNIAQIETLLGDPYS-FGGGSRIILTTRY
54_Ri_4-59  VPSTGILSRGSI-MIMERLGKKKVLLVLDDLDNIAQIETLLGDPYS-FGGGSRIILTTRY
41_Ri_4-33  VPSTGILNRGFK-MIMERLGKKKVLLVLDDLDDIAQFETLLGEQPS-FGGGSRIILTTRY
37_Ri_4-26  VRST-RLKRGST-MIMERLGKKKVLLVLDDVDDISQIESLLGKQLA-FGGGSRIIITTRD
38_Ri_4-28  VPTVGTLNRGSN-MIMERLGKKKVLIVLDDVDDVAQIEFLLGKEHS-FGGGSRIILTTRD
42_Ri_4-34  VPTVGTLNRGSN-MIMERLGKKKVLIVLDDVDDVAQIEFLLGKEHS-FGGGSRIILTTRD
47_Ri_4-41  VPTVGTLNRGSN-MIMERLGKKKVLIVLDDVDDVAQIEFLLGKEHS-FGGGSRIILTTRD
23_Ri_4-12  SVYRDSMKEVIQGKKKVKLGTKKVLLVLDDVDDIAQIDALLGLL---------LIIATRD
46_Ri_4-40  SVYRDSMKEVIQGKKKVKLGTKKVLLVLDDVDDIAQIDALLGLLYS-FGGGSRLIITTRD
40_Ri_4-3   VHSVGTLNRGSN-MIMENLGKKKVLLVLDDVDDIAQIETLLGHQYS-FGGGSRIIVTTRD
52_Ri_4-57  VQNVGTLNRCSN-MIMENLGKKKVLLVLDNVDNIAQIETLLGQQYS-FGGGSRIIVTTRD
32_Ri_4-20  VR-IDTLNDGFK--IMKSLSEKKVLVVLDDVDNLDQIEALLGPEPS-FGGESRIIVTTRD
49_Ri_4-53  VQTVGILNEGSN-MTLERLGEKKVLVVLDDVESSAQIEALLGN-LDSFGVGSRIIITTRD
50_Ri_4-54  VQTVGILNEGSN-MIMERLGKKKVLVVLDDVETVAHIRDLLGN-LHSFGVGSRIIITTRN
58_Ri_4-62  VQGLGILSRGWN-MIMERLSKKKILLVLDDVDDVAQIETLLG--KHSFGGGSRIIITTRD
28_Ri_4-17  GK-IECLSRGRN-LIIQKLGKKKVLVVLDDVENPTQIENILGNNQDSFGVGSRIIITTRD
33_Ri_4-22  VQNLGTLSIDSV-LIKEMLSKKKIFVVLDDVDSLDQVEDLLGTRCS-FGNGSKVIITTRD

                                RNBS-C
                      ----------------------
20_Ri_4-1   ERLLIEHD---IERSF--KVDGLNDSTA-LELFSHNAFRKD--EPREDFSELSNCFVDYV
34_Ri_4-23  ERLLIKHD---IERSF--EVQGLNASTA-LELFSHNAFRKG--EPQEGFSKLSKHFVDYA
17_Ri_23-1  ERLLIEHD---IERSFTVELEGLNASTA-LELFCHNAFRKD--EPPEGFLELSKCFVDYA
57_Ri_4-61  EHLLVKRG---VTRRF--QVQGLHTDEA-LKLFCRKAFKKD--SPEQSYLVLSNRVVNYA
16_Ri_19-9  GHLLKQLG---VDAIYR--AREMNEEEA-LELFSLHAFKAC--CPNEGYLELTRSVVDCC
44_Ri_4-37  AGLLEA-HC-QFVKVHD--VRILDNCES-LALFSWHAFGQD--YPFQSYKDHSNRVVDHC
60_Ri_4-8_  AGLLEA-HC-QFVKVHE--VRILDPSES-LALFSWHAFGQE---PFQSYKDHSNRVVHHC
21_Ri_4-10  VGLFEA-HH-QFVEVHK--VETLSYDES-LALFSWHAFGQD--HPIHSYWDHSKRLIDHC
19_Ri_23-9  AGLLTA-LQ-VVDYVHT--VETLDIKES-LELFSMHAFGQV--HPIESYMEVSKKVMSHC
36_Ri_4-25  AGLLTA-LQ-VVDYVHT--VETLDVKES-LELFSMHAFGQV--HPIESYMEVSKKVMSHC
29_Ri_4-18  AGLLTA-LQ-VVDYVHM--VETLSDNES-LELFSRHAFRQV--HPIKGYTQLSQQVVSHC
61_Ri_4-9   EHLLTTHGVD---VIYE--AQKLRTDEA-LKLFSCKAFKNRNHDDKEKYMVLSDKFVKYT
18_Ri_23-6  ------------------------------------------------------------
```

Multiple alignments showed that the similarity was especially high at various NBS motifs: P-loop, kinase-2, RNBS-B, and GLPL. The TIR and non-TIR RNBS-A motifs are clearly visible (Figs. 2 and 3). The full alignment of all 270 amino acid sequences is available in format suitable for input to Phylip as supplemental data (Supplemental File "Samuelian et al Rosa NBS-LRR S2.phy"). Amino acid distances between the *Rubus* RGA fragments and those from other Rosaceae ranged from 0.1303 to 9.5287. Among the entire dataset, the range of amino acid distances was 0.0058 to 9.2587.

The resulting neighbor-joining tree displays well-supported classification of the Rosaceae NBS-LRR sequences both between TIR and non-TIR and within each category. The

Fig. 2 (continued)

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45_Ri_4-4  -----------------------------------------------------------------
55_Ri_4-6  KHLLA-GY-----VMYE--PKLFTDEKA-LELFRQYAFRTK--PPSGNYDGLLGLAIECA
39_Ri_4-29 IQSLSGVN-----ARYS--PMFLSDDEA-LELFMQYAFRTN--KPTREYDPLSRRAVEYA
22_Ri_4-11 KQSLSGVEY----QLYN--PKCLSYDKA-HELFMKYAFRTK--KPSGEYDHLSRRAIKYA
56_Ri_4-60 KQSLSGVEY----QLYN--PKCLSYDKA-HELFMKYAFRTK--KPSGEYDHLSRCAIKYA
31_Ri_4-2  IQSLSGVKY----RLYK--LKCLSYYKA-HELFMKYAFRTN--KPSGEYDHLSRRAIKYA
35_Ri_4-24 IQSLSGVEY----RLYM--PTCLSYDKA-HELFMKYAFRTN--KPSGEYVHLSRRAIEYA
43_Ri_4-36 IQSFSGVEY----RLYK--PKCLSYDKA-HKLFMKYAFRTN--KPSGEYDHLPRRAIEYA
48_Ri_4-5  IQSFSGVEY----RLYK--PKCLSYDKA-HKLFMKYAFRRN--KPSGEYNHLPRRAIEYA
54_Ri_4-59 IQSFSGVEY----RLYK--PKCLSYDKA-RKLFMKYAFRTN--KPSGEYNHLPRRAIEYA
41_Ri_4-33 IQSLSRVEY----RLYK--PKCLSYDKA-YELFIKYAFRTN--KPSGEYYHLSRCAIEYA
37_Ri_4-26 IQSLSGVEY----VMYK--PKCLRYSEA-YELFRQYAFRTN--EPSAEFDHLSRCAIEYA
38_Ri_4-28 TQLLRRVD-----QIYK--PNLLSDGEA-VQLFRQYA-----------------------
42_Ri_4-34 TQLLRRVD-----QIYK--PNLLSDGEA-VQLFRQY------------------------
47_Ri_4-41 TQSLRRVD-----QIYK--PNLLSDGEA-VQLFRQYAFRTN--KPSGQYDNLSRCAIKYA
23_Ri_4-12 KQILSGVKA----KTYC--PGLLRPKEALLVLFRQFVFRKI--NPSTEYRRFSRHAIELA
46_Ri_4-40 KQILSGVNA----KTYC--PGLLRPKEALLVLFRQFVFRKI--NPSTEYRRFSRHAIELA
40_Ri_4-3  QQILSGVNA----ITYC--PGLLRPKEA-LVLFRKFAFRTI--DPTTEYRRLSRYAIEFA
52_Ri_4-57 EQILSAVDA----SKYF--PSLLRPREA-LILFRKIAFRTI--DPSTEYRRLSRHAIEFA
32_Ri_4-20 SRILNGFE------IYK--AELLIDGNA-GKLFSQYAFKTN--KPSGEYDHLSSRAVEYA
49_Ri_4-53 KQSLSGVH-----ELYE--PKHLSHDEA-HQLFMKYAFRKN--QPTGDYNHLSRRAINYA
50_Ri_4-54 KQSLSGVN-----EFFE--PKALSGDEA-YELFMKHAFNTK--QLTGDYNHLSRRAINYA
58_Ri_4-62 KQSLSGVH-----ELYE--PKHLSHDEA-HELFMKYAFRKN--QPTRDYNHLSRRVIEYA
28_Ri_4-17 KQSLSGVP-----ELYK--PEKLSGEEA-DELFMKHAFRKN--QPTEDYNHLSWRAREYA
33_Ri_4-22 RSLLSESK------MYD--PDFMEKKEA-LELFRKYA------KPSTQYDHLLSHAINYA
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GLPL

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           ------
20_Ri_4-1  KGLPLALKE-
34_Ri_4-23 KGLPLRLQSL
17_Ri_23-1 KGLPFALKD-
57_Ri_4-61 KGLPLALKN-
16_Ri_19-9 GGLPL-----
44_Ri_4-37 AGFPLALKV-
60_Ri_4-8_ AGLPL-----
21_Ri_4-10 GGLPLAFKD-
19_Ri_23-9 EGLPFALKI-
36_Ri_4-25 EGLPFALKK-
29_Ri_4-18 EGLPLALKI-
61_Ri_4-9  NGLPLALKV-
18_Ri_23-6 ----------
45_Ri_4-4  ----------
55_Ri_4-6  HGLPLALKE-
39_Ri_4-29 QGLPFALKN-
22_Ri_4-11 QGLPFALKI-
56_Ri_4-60 QGLPFALKD-
31_Ri_4-2  QGLPFAFKE-
35_Ri_4-24 QGLPLALKN-
43_Ri_4-36 QGLPFAFKK-
48_Ri_4-5  QGLPLALKV-
54_Ri_4-59 QGLPLALKM-
41_Ri_4-33 QGLPLALKD-
37_Ri_4-26 HGLPLAFKV-
38_Ri_4-28 ----------
42_Ri_4-34 ----------
47_Ri_4-41 QGLPFALKN-

23_Ri_4-12 QGLPLALKN-
46_Ri_4-40 QGLPLALKV-
40_Ri_4-3  QGLPFALKE-
52_Ri_4-57 QGLPLALKI-
32_Ri_4-20 QGLPLALKI-
49_Ri_4-53 RGLPLALKE-
50_Ri_4-54 QGLPLALKK-
58_Ri_4-62 QGLPFALKV-
28_Ri_4-17 KGLPLALKN-
33_Ri_4-22 QGLPLALKI-
```

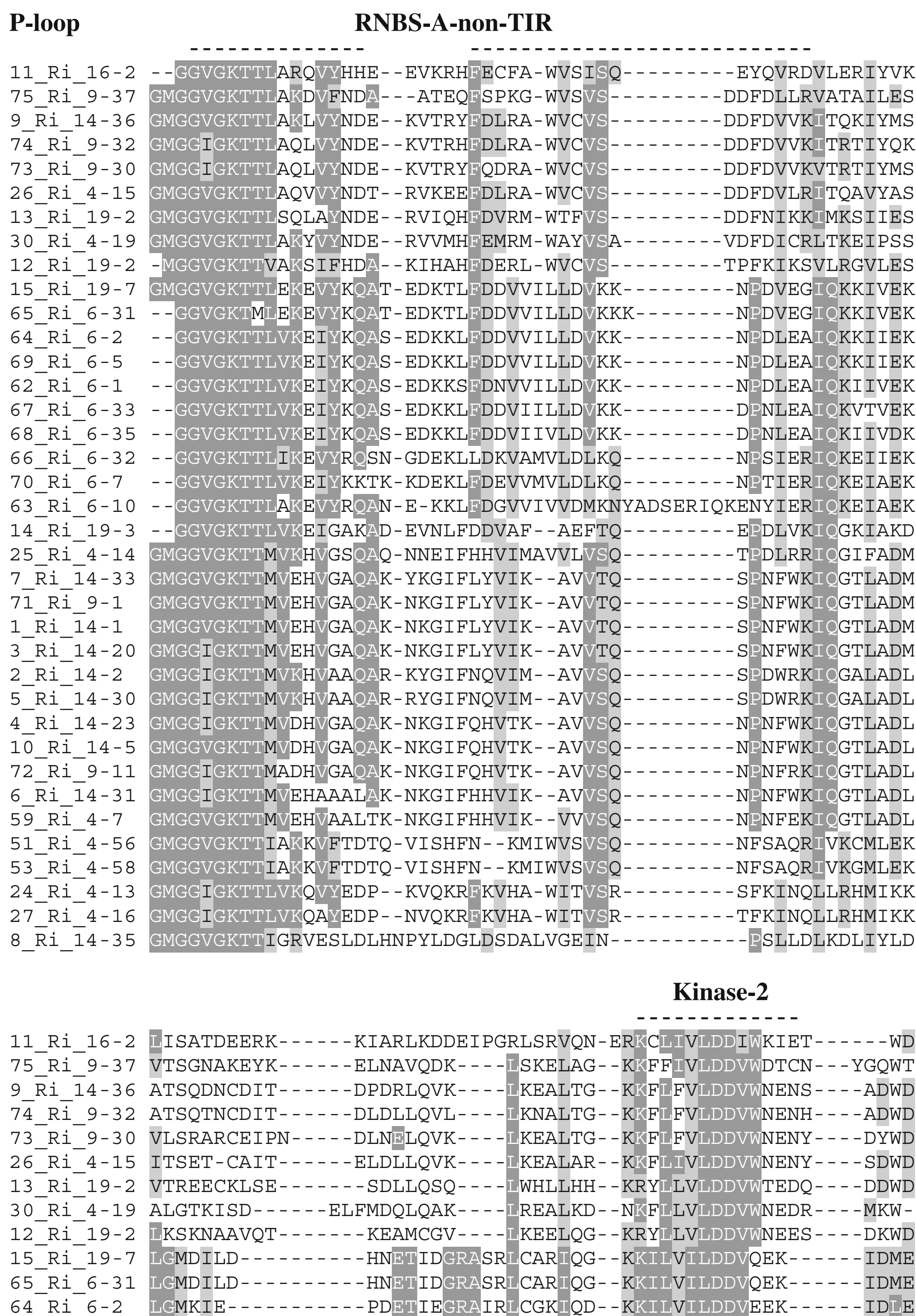

Fig. 3 Alignment of conceptually translated *Rubus* non-TIR sequences. The RNBS-A-non-TIR motif is characteristic of plant non-TIR-NBS-LRR RGAs. Identical residues with 50% or greater frequency in a column have a *dark grey background*. Similar residues with 50% or greater frequency have a *light grey background*

TIR sequences clustered separately from the non-TIR sequences with a 75.8% bootstrap value (Fig. 4a and b). Within the TIR sequences, there were 11 distinct clusters with bootstrap values of more than 50% containing sequences from multiple genera (Fig. 4a). There were also 11 such clusters among the non-TIR sequences. Nine of these multigenus clusters have a bootstrap value of more than 75%, and seven have a bootstrap value of more than 90%. Four of the mapped *Rubus* sequences clustered near the TIR I group, while three others were spread between the non-TIR groups. Only one mapped sequence is in a terminal multiple-genus group; 67_Ri_6-33, in non-TIR group VIII. Among all the *Rubus* sequences isolated in this study, there were representatives among groups TIR I, TIR III, TIR V, TIR X, and TIR XI and non-TIR II, non-TIR IV, non-TIR VII, non-TIR VIII (10 of the 11 members), non-TIR IX, and non-TIR XI (13 of the 14 members; Fig. 4b). A more detailed illustration with color coding for each genus is provided in Supplemental Fig. S3.

Fig. 3 (continued)

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69_Ri_6-5   LGMKIE-----------PDETIEGRAIRLCGKIQD--KKILVILDDVEEK------IDLE
62_Ri_6-1   LDMEIL-----------QTETKEGRARRLCGKIQD--KKILVILDDVEEK------IDLE
67_Ri_6-33  LGMEIL-----------PNETKDGRASRLCARIQD--KKILVILDDVEEE------INLE
68_Ri_6-35  LGMEIK-----------QNETKEGRASRLCGRIQD--KKVLVILDDVQEK------IDLE
66_Ri_6-32  LGLDLH-----------EIETLAGRALHLCNKIKD--KKILVILDDVWEY------INLE
70_Ri_6-7   LGLNLQ-----------EIETPATRALHLRNRMKG--KKTLVLLDDVWEN------IDLE
63_Ri_6-10  LNIDIR-----------ECLTEKGRARHLWDKLKD--KKILVILDDVWEK------IELE
14_Ri_19-3  LGLEFT------------PD--DDRAAKLRERLSGGTKRVLVILDNVWTDNSSPDQLTLW
25_Ri_4-14  LGLKFE------------EETETGRANRLMTKIES-GNKILIILDDIWD------RINLS
7_Ri_14-33  LGVNLA------------GETETGRAVSLNKEIMR-REKILIILDDIWE------MIDLS
71_Ri_9-1   LGVNLA------------GETETGRAVSLNKEIMR-REKILIILDDIWE------MIDLS
1_Ri_14-1   LGVNLA------------GETETGRAVSLNKEIMR-REKILIILDDIWE------MIDLS
3_Ri_14-20  LGVNLA------------GETETGRAVSLNKEIMR-REKILIILDDIWE------MIDLS
2_Ri_14-2   LGVKLE------------EETEIGRAATLSKEIMR-RNKILIILDDIWK------GLDLS
5_Ri_14-30  LGVKLE------------EETEIGRAATLSKEIMR-RNKILIILDDIWK------GLDLS
4_Ri_14-23  LGVKLA------------GETETGRAASLNKEIMR-REKILIILDNVWN------RVELS
10_Ri_14-5  LGVKLA------------GETETGRAASLNKEIMR-REKTLIILDNVWN------RVELS
72_Ri_9-11  LGVKLA------------GETETGRAASLNKEIMR-REKILIILDNVWN------RVELS
6_Ri_14-31  LGVKLA------------GETETGRAASLNKEIMR-REKILIILDNVWN------RVELS
59_Ri_4-7   LGVKLA------------DETEAGRATSLNKAIMR-REKILIILDDVWS------RIELS
51_Ri_4-56  ANMQAPDVSE-------SDDMFTR----LKQGLDD--QDYLIVMDDVWPKPN----LEIF
53_Ri_4-58  ANMQAPDVSE-------SDDMFTR----LKQGLDD--QDYLIVMDDVWPKPN----LEIF
24_Ri_4-13  IFKVIRKPVPEDEE--VENMDDNQLRERIKKLLQN--SRYLIVLDDLWHIPD------WE
27_Ri_4-16  IFKVIRKPVPEDEE--VENMDDNQLRERIKKLLQN--SRYLIVLDDLWHIPD------WE
8_Ri_14-35  VSMNN------------FGGIQLP---SFIGSLEK--LKYLNLSGASFGG---------

                             RNBS-B                     RNBS-C
                             --------              ----------------
11_Ri_16-2  R--LKAAFECDDESKSKILLTTRK--------------KEVALYPDVNCFAHPP-----
75_Ri_9-37  T--LQSSFRVGAA-GSKIIVTTRDANVARMMGD---TNPYKLGSISQDDCWKIFEHHALL
9_Ri_14-36  F--LRGPFKYGAC-RSKIIVTTRNEGVASVMGT---LQTHTLPVISDEDCWLLFAKHAFE
74_Ri_9-32  V--LRQPFQSGGC-GSKIIVTTRNEGVASVMGT---LQTHPLPVISDEDCWWLFAKHAFE
73_Ri_9-30  S--LRRPFESGAC-GSKIIVTTRNEGVASMMCT---LQTHHLQDISDEDCWLLFAKHAFE
26_Ri_4-15  R--LRRPFGIGAC-GSNILVTTRNEAVAAVMGT---LPTYHLKHISEEDGWLLFAKHAFK
13_Ri_19-2  K--LRPLFRGGVD-GCKIIVTSRSKKVPFMMDSP--TSTYHLKGLMEVDCWALFKQRAFG
30_Ri_4-19  ----------------IPFCTQGT-----------------------------------
12_Ri_19-2  D-LRNCLLRGTDTRGSKLIVTTRKDKVGKIVETLL-PRPDLEK-LSVEDCWRIMKDKSMG
15_Ri_19-7  VVGLPRL-----A-TCKILLTCRTREVLSIDKMCA-EKVFQFDILGKEEYIVEFVWEDGR
65_Ri_6-31  VVGLPRL-----A-TCKILLTCRTREVLSIDKMCA-EKVFQFDILGKEEYIVEFVWEDGR
64_Ri_6-2   AVGLPRL-----P-TCKVLLTFRTRQVF--DEMRA-DKVVQLDLLGKEDSWNLFVKMAGD
69_Ri_6-5   AVGLPRL-----P-TCKILLTFRTRQVF--DEMCV-DKVFRLDLLDKQETWILFVKMAGD
62_Ri_6-1   AVGLPRL-----P-TCKILLTFRTRQVF--DETRA-DKVIQLDLLDKEDSWNLFVKMAGD
67_Ri_6-33  AVGLPRL-----P-TCKILLTCRTRQVF--DEMRV-QKVFQLDLLGKEDTWNLFVKMAGD
68_Ri_6-35  AVGLPRQ-----P-TCKILLTCRTPQVF--DEMRV--KVFRLDLLGKEDTWNLFVKMAGD
66_Ri_6-32  DVGLPRM-----S-TLKILLTSRTKKVLS-RDMGT-QKEFHLDVLDKKETWSLFHKKAGD
70_Ri_6-7   AVGLPRM-----P-TLKILLTSRSKIVLS-RDMGT-QKEFHIDLLGQEETWSLFQKMAGD
63_Ri_6-10  DLGIP-------Q-TCNILFTSRNREVLY-SKMGT-QKEFLLGVLGDEESWRLFEKMAGA
14_Ri_19-3  EVGIPISRDPK---SCKVLVSSREQDIFK--EMKT-KKNFPI------------------
25_Ri_4-14  CIGIPSYNELQRC-NSKVVLTTRRLHVCH--TMET-QAKIPLDILSE-DSWNLFTKKARI
7_Ri_14-33  SIGIPNYKDLQNC-NSKVLLTTRIQHVCH--TMKS-QEKIALNILSQEDSWTLFVKNARR
71_Ri_9-1   SIGIPNYKDLRNC-NSKVLLTTRIQHVCH--TMKS-QEKIALNILSQEDSWTLFVKNARR
1_Ri_14-1   SIGIPNYKDLQNC-NSKVLLTTRIQHVCH--TMKS-QEKIALNILSQEDSWTLFVKNARR
3_Ri_14-20  SIGIPNYKDLQNC-NSKVLLTTRIQHVCH--TMKS-QEKIALNILSQEDSWTLFVKNARR
2_Ri_14-2   RIGLPSYEELQNC-NSKVLLTTRIRNVCH--VMKC-QEKITLNILSKQDSWTLFVRNAGC
5_Ri_14-30  RIGLPSYEELQNC-NSKVLLTTRIRNVCH--VMKC-QEKITLNILSKQDSWTLFVRNAGC
4_Ri_14-23  RIGVPGYKKLQTC-NSKVIITTRIKNTCT--SMHT-QEKIHLSVLSEKDSWSLFANTTGM
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Discussion

Improvement of disease resistance is one of the main priorities in plant breeding. Markers developed through molecular techniques can be used for marker-assisted selection and eventually facilitate the map-based cloning of genes involved in the response to pests or other stresses. PCR approaches utilizing degenerate primers based on conserved NBS motifs from known disease-resistance genes have led to the cloning and identification of RGAs from many plant species (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996; Aarts et al. 1998; Leister et al. 1998; Timmerman-Vaughan et al. 2000; Donald et al. 2002; Gaspero and Cipriani 2002; Tan et al. 2003; Zhang et al. 2003; Baldi et al.

Fig. 3 (continued)

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10_Ri_14-5  RIGVPGYKKLQTC-NSKVIITTRIKNTCT--SMHT-QEKIHLSVLSEKDSWSLFANTTGM
72_Ri_9-11  RIGVPGYKKLQTC-NSKVIITTRIKNTCT--SMHT-QEKIHLSVLSEKDSWSLFANTTGM
6_Ri_14-31  RIGVPGYKKLQTC-NSKVILTTRIKNTCT--AMHT-QEKIHLSVLSEKDSWSLFANTTGM
59_Ri_4-7   RIGVPGYKKLQTC-NSKVILTTRMKNTCT--SMHT-QVKILLGVLSEKDSWSLFADTTGM
51_Ri_4-56  WTDLCNILPTKVGKSSCIVITTRYKDIARGMVDQD-SQILQPSTLNEMDSWSLFCRFAFR
53_Ri_4-58  WTDLCNILPTKVGKSSCIVITTRYKDIARGMVEQD-SQILQPSTLNEVDSWSLFSRFAFR
24_Ri_4-13  TINHAMPNNNHGS---RVMLTTRHVYVASASCLGNPDMLYHLEPLSPEDSWTLLCRRTFQ
27_Ri_4-16  TINHAMPNNNHGS---RVMLTTRHAYVASASCLGNPDMLYRLEPLSPEDSWTLLCRKTFQ
8_Ri_14-35  --VIPPDLGNLSR---LLYLDLSNNAIES----------DLRWLPSVSSLRFLNLGGAN
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GPLAL

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                                          --------
11_Ri_16-2  ---------------------------------
75_Ri_9-37  NG-----TPQNVDLLKKKVILKCNGLPLVART--
9_Ri_14-36  NKRV--GAYPNLVVIGRKIVKKCKGLPLAAKS--
74_Ri_9-32  NKMV--SAYPNLEVIGRKIVKKCKGLPLAAKS--
73_Ri_9-30  NKSV--SAYPNLEVIGRKIVKKCKGLPLAAKS--
26_Ri_4-15  NAHALGTEHPDLANIGRKIVKKCNGLPLALKE--
13_Ri_19-2  RGEE--ENYPNLCLIGKQIAKKCGGVPL------
30_Ri_4-19  ---------------------------------
12_Ri_19-2  SAPI----TEDQTKIGRRIATKCGGLPL------
15_Ri_19-7  RCTQWFMVGYDLRDVAIQVAEKCGGLPL------
65_Ri_6-31  RCTQWFMVGYDLRDVAIQVAEKCGGLPL------
64_Ri_6-2   VINQ----NRGIRDVAIKVAERCGGLPL------
69_Ri_6-5   VINQ----NGGIRDVAIKVAESCGGLPL------
62_Ri_6-1   VINQ----NGAIRDVAIKVAERCGGLPL------
67_Ri_6-33  VIHQ----KSGIRDVAIKVAERCGGVPL------
68_Ri_6-35  VINQ----NGGIRDVAIKVAERCGGLPL------
66_Ri_6-32  IVKK-----TDIQTVAIQVAEKCGGLPL------
70_Ri_6-7   IVEK-----PDIQTVATKVAEKCGGLPL------
63_Ri_6-10  VVKD-----ERKREIAIHVSNKCGGVPL------
14_Ri_19-3  ----------------------AGLPLALKE--
25_Ri_4-14  SFQK----SSDFYDVARKVARECAGLPLALKI--
7_Ri_14-33  SF-E----PTNFKDVARKVARECSCFSDSSHS--
71_Ri_9-1   SF-E----PTNFKDVARKVARECSCLSYSTHS--
1_Ri_14-1   SF-E----PTNFKDVARKVAGECSGLSDSTHS--
3_Ri_14-20  SF-E----PTNFKDVARKVARECSGLTDTSHS--
2_Ri_14-2   PF-E----SSTFEDVARRVAGECCGFTNSSHS--
5_Ri_14-30  PF-E----SSTFEDVARRVAGECCSLSYSSHS--
4_Ri_14-23  SFDE----SSELYNVARKVSNECSCFSYTSHS--
10_Ri_14-5  SFDE----SSELYNVARKVSNECSCFSNTSHS--
72_Ri_9-11  SFDE----SSESYNVARKVSNECSCLSDTSHS--
6_Ri_14-31  SFDE----SSELYNVARKLSNECSGLS-------
59_Ri_4-7   SFDE----SSELYNVARKLSNECSGLPFALKD--
51_Ri_4-56  STDGK-SPDELFEKEGKVIVKRCGGLPLALKM--
53_Ri_4-58  STDGK-CPDEWFEKEGKVIVKRCGGLPFALKD--
24_Ri_4-13  G----NSCLPNLEEICRSILRKCGGLPLALKI--
27_Ri_4-16  G----NSCLPNLEEICRSILRKCGGLPLALKD--
8_Ri_14-35  FTKAAPYWLPTVNMLPSLVELHLPGCCFSDSSHS
```

2004; Xu et al. 2005, 2007). In this study, we have isolated 75 genomic fragments that revealed 50–87% similarity to NBS-LRR genes from other species, thus providing the first sampling of RGA sequences from red raspberry.

The percent nucleotide identity between the *Rubus* sequences identified ranged from 9% (18_Ri_23-6, 74_Ri_9-32) to 100% (42_Ri_4-34, 38_Ri_4-28). The majority of sequences were widely divergent from each other. Only 20 pairs of sequences out of 5,550 pairwise comparisons showed more than 90% sequence identity between them. Probably there are many other RGA sequences in red raspberry, as only 75 amplified fragments based on seven primer combinations were analyzed. In fact, the NBS-LRR class of resistance genes has been shown to be very large in plants. For example, the genome of the model plant species *A. thaliana* is estimated to contain approximately 200 genes that encode related NBS motifs (Meyers et al. 1999). Red raspberry is diploid ($2n=2x=14$) and has a small genome (0.58 pg/2C, 280 Mbp/1C), which compares favorably to *A. thaliana* with a genome size of 0.30 pg/2C, 145 Mbp/1C (Arumuganathan and Earle 1991), and so would be expected to proportionately have approximately 386 genes encoding NBS motifs.

It is likely that the RGA sequences with identity of more than 90% (20 pairs) have arisen from a recent duplication of a common ancestor gene. Unequal crossing-over is

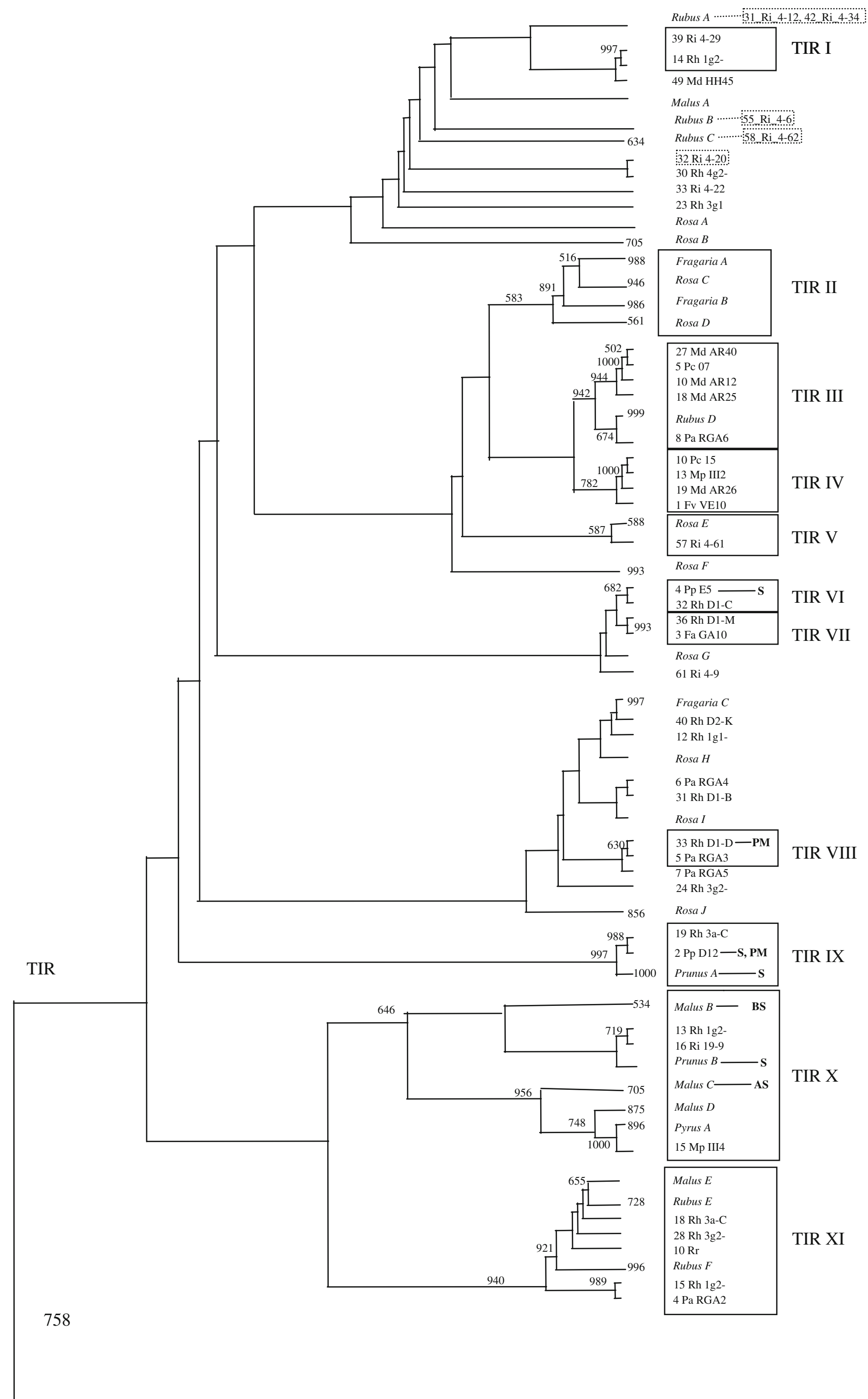

Fig. 4 Majority-rule consensus of 1,000 bootstrap replicates of a neighbor-joining cluster of Rosaceae RGA fragments (amino acid). Bootstrap confidence for branches more than 60% is reported. To conserve space, terminal clusters composed of sequences from a single genus are assigned a *letter*. Membership in these clusters is indicated in Supplemental Table S1. Individual sequences that do not group with others from the same genus are *labeled with a number*, *taxonomic abbreviation*, and as much of the *original published ID* as possible. Taxonomic abbreviations are: *Fc*, *Fragaria chiloensis*; *Fv*, *F. vesca*; *Fa*, *F. ananassa*; *Mb*, *Malus baccata*; *Mp*, *M. prunifolia*; *Md*, *M.* × *domestica*; *Pa*, *Prunus armeniaca*; *Pp*, *P. persica*; *Pc*, *Pyrus communis*; *Rh*, *Rosa* hybrid cultivar; *Rr*, *R. roxburghii*; *Ri*, *Rubus idaeus*. Terminal clusters of sequences derived from more than one genus with a bootstrap of more than 50% are assigned a *Roman numeral*. Mapped *Rubus* sequences are indicated with *dashed lines* and *boxes*. Sequences localized in a genome near resistance genes or QTLs are *labeled*: *AS* apple scab, *BS* bacterial spot, *N* nematode, *PM* powdery mildew, *PRR Phytophthora* root rot, *S* sharka. **a** TIR Motif sequences. The original published classifications for these sequences are indicated in Supplemental Table S1. **b** non-TIR Motif sequences. The original published classifications for these sequences are indicated in Supplemental Table S1. These include non-TIR designations such as CC-NBS-LRR Malus G, and LZ

believed to be one mechanism through which this type of diversity is created in RGA clusters in plant genomes (Meyers et al. 1998). Alternatively, point mutations and/or small insertion/deletions in the regions between the conserved motifs may account for the genetic divergence between fragments of very high homology. Two main hypotheses have been proposed to explain the evolution of *R* genes: the first suggesting that it is a result of slow evolving process (Michelmore and Meyers 1998; Stahl et al. 1999), while the second suggests a rapid evolution (Leister et al. 1998; McDowell et al. 1998). A greater degree of divergence was observed among the TIR-type

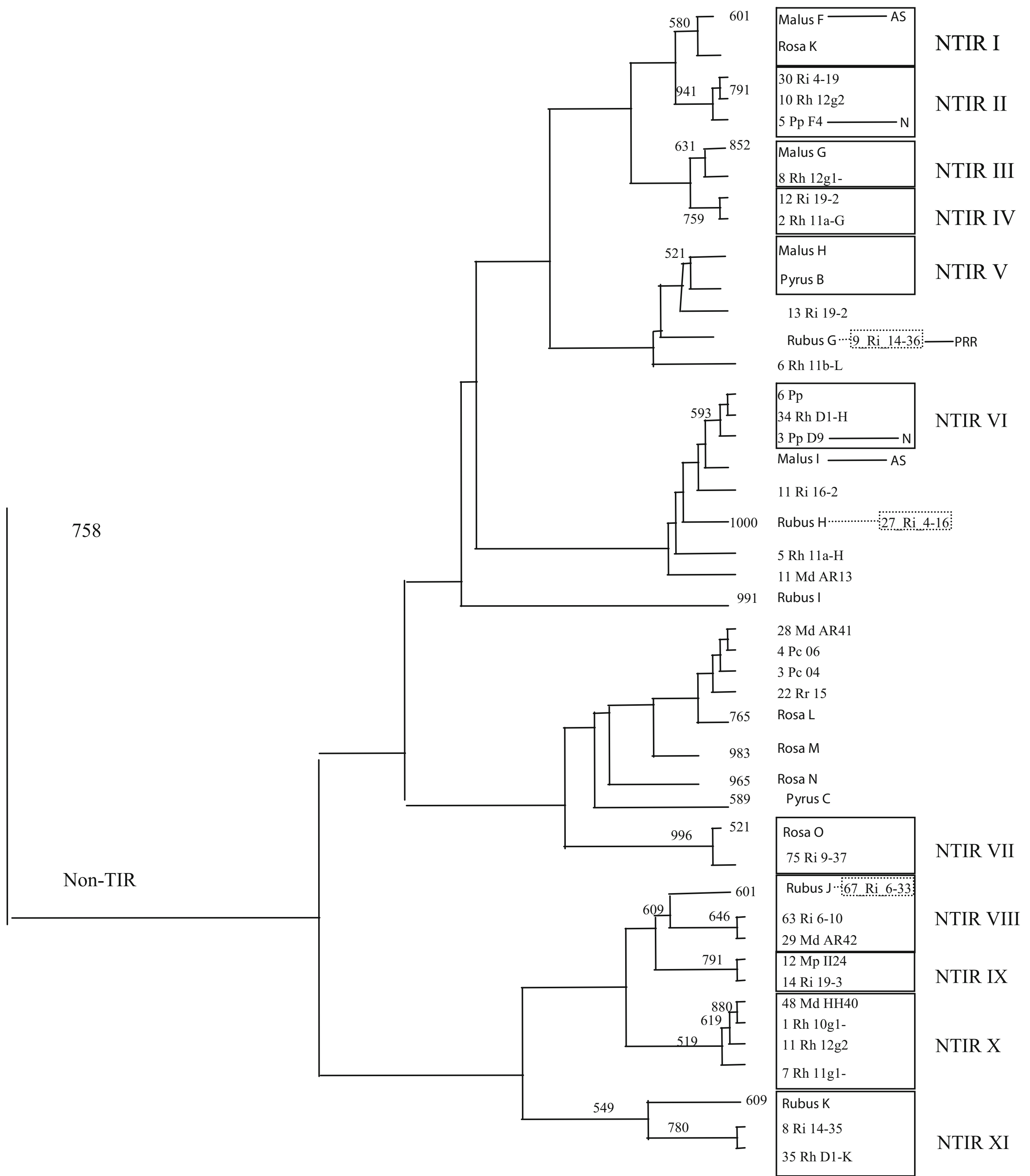

Fig. 4 (continued)

RGAs compared to the non-TIR-type RGAs suggesting that the former have been evolving more rapidly than the latter type. However, the full set of NBS-LRR sequences in red raspberry must be characterized to properly answer the question of which mechanism is responsible for the evolution of RGAs in this plant. Interestingly, evidence of functional resistance identified in other related plants clustered in TIR groups IX and X (four cases each, Fig. 4a). TIR X,

in particular, has the most genera represented (5), and the most sequences (41). The evidence of both spread and number of sequences as well as conservation of function implies that the TIR X group may represent the ancestral Rosaceous TIR R gene from which additional RGA sequences evolved. Among the small TIR IX group, three of the four sequences are implicated in functional resistance. These two groups provide an intriguing target for further studies on R gene evolution and a likely source of functional polymorphism.

This study identifies 22 clusters with a bootstrap greater than 50% and more than one genus represented (Fig. 4a and b). A number of these clusters contain NBS-LRR sequences mapped near resistance QTL and loci from other species (Fig. 4a and b, Supplemental Table S1). Among the TIR type sequences, there were four such clusters. TIR IV, TIR VIII, TIR IX, and TIR X contain sequences linked to sharka (plum pox; Lalli et al. 2005), powdery mildew (Lalli et al. 2005), apple scab (Baldi et al. 2004), and bacterial spot resistance (Lee and Lee 2003). Three of the non-TIR type clusters (NTIR I, NTIR II, NTIR VI) contained sequences linked to root-knot nematode (Lalli et al. 2005) and apple scab (Baldi et al. 2004) resistance. None of 22 clusters identified here contained a mapped *Rubus* sequence; however, one *Rubus* sequence, located near a QTL for PRR resistance (Fig. 1), grouped with the larger clade containing clusters NTIR I to NTIR V (Fig. 4b).

The sequence relationships of RGA fragments share patterns with several previous studies that had a more limited taxonomic scope among the Rosaceae. Twenty of our 22 clusters (11 TIR, 9 non-TIR) corresponded to NBS-LRR clusters published in other studies (Supplemental Table S4). Six of the TIR clusters and three of the non-TIR clusters were corroborated by more than one additional study.

There was only one discrepancy between the sequence designation indicated by our neighbor-joining tree and those already published. Specifically, Baldi et al. (2004) designated ARGH 22 as a TIR sequence, while our analysis showed the same sequence (20_Md) to belong to the NTIR group (Supplemental Table S1). Only one "cluster" (really a paraphyletic group) designated by Hattendorf and Debener (2007) does not correspond with the sequence relationships found in this study; the "Rose–Pyrus" non-TIR group has members scattered among many of our non-TIR groups: NTIR IV, NBIR VII, and several other places in between (2_Rh_11a-G, 6_Rh_11b-L, 5_Rh_11a-H, 3_Pc_04, 4_Rh_11a-G).

The overall agreement of all 14 studies of Rosaceae NBS-LRR sequences (including this one) indicates that these groupings are relatively robust. Each study used a different subset of data, and a variety of methods were used for analysis. Hattendorf and Debener (2007) shares seven clusters with this study, even though the clustering method used was very different (parsimony). Figure 1 of Xu et al. (2007) shares 15 clusters with this study, although it contains only 83 Rosaceae sequences and does not include *R. hybrid or Rubus sequences.*

Given the overall similarity of sequence fragments from multiple genera (as supported by bootstraps of more than 90%), it seems probable that a significant proliferation of the RGA family occurred before the Rosaceae evolved into the different species present today. Large clusters of sequences, all from the same species, imply gene duplication after speciation (Xu et al. 2007). This pattern is seen for *Rubus*, *Malus*, *Fragaria*, and *Rosa*, but not for *Prunus* or *Pyrus*. However, it is difficult to compare the proliferation of particular NBS-LRR sequence types between specific Rosaceae using public data, as different primers and cloning procedures were used in each study. In this work, certain primer combinations tended to isolate related sequences. For example, the *Rubus* J group contains many products from the N6 primer and the *Rubus* K group many N4 products (Supplemental Table S1). Large clusters of similar sequences in *Prunus* or *Pyrus* might exist, but not have been detected, due to the way sequences were isolated. It would require strict control in methodology to generate the data necessary to address patterns of sequence proliferation between individual species.

RGAs are widely distributed in plant genomes and often organized in clusters (Kanazin et al. 1996; Meyers et al. 1999). In this study, eight RGA sequences were mapped in a previously developed red raspberry genetic map (Pattison et al. 2007) covering six out of the seven linkage groups. Clustering RGAs was not observed, probably due to the small amount of sequences mapped. Previous studies indicate that some RGAs might be genetically located at or near known resistance loci (Kanazin et al. 1996; Yu et al. 1996; Collins et al. 1998; Donald et al. 2002; Radwan et al. 2004). For example, two RGAs were found in close linkage to the nematode resistance locus Gro1 in potato (Leister et al. 1996), three RGA markers were linked to the citrus tristeza virus and nematode resistance in *Citrus* (Deng et al. 2000), and four RGA-derived markers (three RFLP markers and one STS marker) were found to be associated with CRPM1, a major R locus contributing to powdery mildew resistance in chestnut rose (Xu et al. 2005). Lalli et al. (2005) identified a number of RGAs near resistance QTL and other loci (Supplemental Table S1). In this study, one RGA marker (9_Ri_14-36) was mapped within the QTL for resistance to PRR (Pattison et al. 2007) on LG1 and might prove useful for marker-assisted selection. RGA marker(s) could not be co-localized with the second QTL for resistance to PRR. This result is not surprising because (1) a small portion of theoretically possible NBS-containing

sequences from red raspberry have been identified, (2) the methods used did not allow the mapping of all the RGAs analyzed, and (3) not all QTL for resistance are necessarily associated with RGA sequences.

To date, few studies have been conducted toward the cloning and characterization of disease-resistance-related genes in red raspberry. Locating and mapping additional NBS-LRR homologues as well as the analyses of the RGAs mapped so far will help to accelerate the identification of genomic regions containing functional resistance genes and facilitate the long process of map-based cloning. This will lead to a better understanding of disease resistance in red raspberry and other plants and hopefully to the development of improved cultivars for commercial production that require fewer pesticides.

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