Resistance Gene Analogs from *Vitis cinerea*, *Vitis rupestris*, and *Vitis* Hybrid Horizon

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Abstract: Resistance gene analogs (RGAs) characterized by the presence of nucleotide-binding sites (NBS) were cloned from *Vitis cinerea*, *V. rupestris*, and *V.* hybrid Horizon. Two degenerate PCR primer pairs were designed from conserved regions of NBS motifs within known resistance (*R*) genes and used for PCR amplification of putative RGAs. A total of 122 putative RGA sequences were cloned from all three genotypes by P-loop/GLPLAL-1 primers. Based on nucleic acid sequence-identity of 90% or greater, RGA clones were subdivided into eight, four, and seven groups for *V. cinerea*, *V. rupestris*, and Horizon, respectively. All of these clones showed similarity of nucleotide sequences to other known *R* genes or NBS-type nucleotide sequences, and seven clones showed high similarity. Thirty sequences were cloned from *V. cinerea* by P-loop/Rev loop and subdivided into four sequence groups, none of which were similar to nucleotide sequences of other *R* genes. Nineteen representative RGA clones were classified into 13 TIR- (*Drosophila Toll* and mammalian *Interleukin-1 Receptors*) NBS-leucine rich repeat (LRR)-like genes and six non-TIR-NBS-LRR-like genes based primarily on nucleotide sequences of kinase-2 motifs and phylogenetic analysis with known TIR or non-TIR proteins. Twenty-three sequence tagged site (STS) and three cleaved amplified polymorphic sequence (CAPS) markers developed from RGAs were checked for segregation among 179 seedlings from Horizon x Ill. 547-1, and 18 showed goodness-of-fit using a chi-square test. Marker stkVa011 correlated with segregation for downy mildew resistance in this population. These STS markers are currently being investigated for their potential in molecular breeding for disease resistance.

Key words: resistance gene analog, disease resistance, nucleotide-binding site, marker-assisted selection

Grapevines (*Vitis* spp.) are one of the most widely grown fruit crops in the world. The most widely cultivated grape species (*V. vinifera*) is highly susceptible to many diseases caused by fungi, bacteria, and viruses. However, many North American grape species such as *V. riparia*, *V. rupestris,* and *V. rotundifolia* are reported as highly resistant to several important diseases of *V. vinifera* (Eibach et al. 1989, Alleweldt et al. 1990).

Grape downy mildew, caused by the oomycete *Plasmopara viticola,* is one of the most economically important grape diseases worldwide. The disease can rapidly affect entire vineyards, destroying 50 to 75% of a crop in one season (Muller et al. 1934). Most European grapes (*V. vinifera*) are highly susceptible to downy mildew, and young leaves and fruits are particularly susceptible (Kennelly et al. 2005). Application of fungicides is the most common tactic for control of grape downy mildew (Lafon and Clerjeau 1988). Although effective, chemical control is expensive and may have environmental consequences. Downy mildew resistant grape cultivars are a desirable alternative.

The development of molecular markers linked to disease-resistance genes could provide a valuable tool for breeding programs using marker-assisted selection (MAS) or for map-based cloning efforts. Cloned *R* genes from a number of plant species have been shown to confer resistance to individual diseases caused by viruses, bacteria, fungi, oomycetes, or nematodes (Hammond-Kosack and Jones 2000, Taler et al. 2004). Indeed, some *R* genes encode proteins that act in the signaling process by interacting with pathogen avirulence (*Avr*) gene products, or, in other cases, *R* genes encode proteins involved in race-specific recognition or act as general elicitors (Dangl and Jones 2001). *R* genes have been previously cloned from plants such as tobacco (*N*), flax (*L6*), rice (*Xa21*), tomato (*Cf*), and *Arabidopsis* (*RPS2* and *RPM1*) (Bent et al. 1994, Whitham et al. 1994, Song et al. 1995, Dixon et al. 1998). The major class of *R* genes in plants is characterized by the presence of NBS-LRR domains. LRRs and NBS domains have a role in both cell surface recognition and intracellular signaling (Parker et al. 1997). The se-

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quences of NBS domains in general are highly divergent among members; however, short motifs such as the P-loop, kinase-2, and RNBS (resistance nucleotide binding site) are conserved in both dicots and monocots (Meyers et al. 1999, Ellis et al. 2000).

The sequence conservation of specific domains (e.g., the P-loop and GLPL domains) has facilitated the use of degenerate oligonucleotide primers to amplify and clone RGA sequences from genomic DNA of diverse species, including *Brassica*, *Hordeum*, *Arabidopsis*, *Beta,* and *Helianthus* (Aarts et al. 1998, Joyeux et al. 1999, Gedil et al. 2001, Hunger et al. 2003). RGA sequences have been developed as molecular markers using the resistance gene analog polymorphism (RGAP) technique, which has allowed identification of markers linked to disease resistance genes in plants such as barley, rice, sunflower, and wheat (Toojinda et al. 2000, Gedil et al. 2001, Yan et al. 2003). Because of the high probability of finding clustered RGAs in the plant genome, molecular markers developed from RGAs have great potential for co-localization with alleles for disease resistance on linkage maps. Six and 11 markers from RGA primers were co-segregating and tightly linked, respectively, to the *YR5* locus conferring stripe rust resistance in wheat (Yan et al. 2003). RGAs cloned from *V. amurensis* and *V. riparia* by degenerate primers of the P-loop and GLPL domains revealed 12 RGA groups with at least 40% identity to known *R* genes such as *Arabidopsis RPS5* and tobacco *N* (Di Gaspero and Cipriani 2002); the major groups of RGAs cloned were used to distinguish three disease-resistant varieties and six susceptible grape varieties. Also, 45 STS markers were developed from RGA sequences that showed polymorphism among 20 *Vitis* (Di Gaspero and Cipriani 2003). RGAs have also been isolated from *V. rotundifolia* and converted to 20 RGAP markers co-segregating with the resistance to *Uncinula necator* 1 (*Run*1) locus that controls powdery mildew resistance (Baker et al. 2005).

Previous work in our laboratory used the interspecific hybrid population, Horizon (Seyval x Schuyler) x Illinois 547-1 [Ill. 547-1] (*V. rupestris* x *V. cinerea*), to identify quantitative trait loci (QTL) and to study the inheritance of powdery mildew resistance. Ill. 547-1 has been shown previously to be highly resistant to several fungal diseases, including downy mildew and powdery mildew (Dalbo et al. 2000). One hundred fifty-three markers were mapped onto Horizon covering 1199 cM, whereas the Ill. 547-1 map had 179 markers covering 1470 cM (Dalbo et al. 2000). A single marker (CS25b) was associate with a major QTL (LOD score 6.56) from Ill. 547-1, which accounted for 41% of the phenotypic variation for powdery mildew resistance. Interestingly, the allele of this marker associated with resistance was also present in *V. cinerea* B9, a parent of Ill. 547-1 (Dalbo et al. 2001).

In this study we report the cloning of RGA sequences from the disease-resistant genotypes *V. cinerea* B9, *V. rupestris* B38, and Horizon. These sequences were classified based on variation in the NBS domain. These RGA sequences were then used to develop molecular markers for placement on the Horizon x Ill. 547-1 genetic map, thus facilitating future identification and cloning of disease resistance genes.

Materials and Methods

Plant materials and DNA extraction. Grapevine genotypes *V. cinerea* B9, *V. rupestris* B38, and Horizon (Seyval x Schuyler) were used as template for PCR-based cloning of NBS sequences. DNA was extracted from 2 g of young leaves using an existing method (Lodhi et al. 1994) with the following modification to the CTAB (cetyltrimethylammonium bromide) extraction buffer: 3% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, pH 8.0, 0.1 M Tris HCl, pH 8.0, 2% (w/v) polyvinylpolypyrrolidone, and 0.2% (v/v) ß-mercaptoethanol. DNA pellets were dissolved in sterile water, and, after RNase A treatment, DNA concentrations were calculated from absorbance values at 260 nm using a spectrophotometer.

Amplification and cloning of NBS-LRR genes by degenerate primers. Two oligonucleotide primers, P-loop (5'GGIGGIGTIGGIAAIACIAC3') and GLPLAL-1 (5' IAGIGCIAGIGGIAGICC 3') were modified (Hunger et al. 2003), having been designed from the most conserved domains within the NBS P-loop and GLPL motifs from the *N, RPS2,* and *L6* and the *N, RPS2, RPM1, L6* genes, respectively. The other degenerate primer, Rev loop (5'GTIGTITTICCIACICCICC3') (Hunger et al. 2003), was derived from the *N, RPS2,* and *L6* genes. The P-loop/GLPLAL-1 and P-loop/Rev loop primer pairs were used to amplify RGA fragments from *V. cinerea* B9 (Figure 1). NBS regions of *V. rupestris* B38 and Horizon were amplified using only the P-loop/GLPLAL-1 primer pair. PCR amplifications were performed in a reaction volume of 20 µL containing 1x PCR buffer, 0.1 mM dNTPs, 2.5 mM $MgCl_2$, 2 µM of each primer and 1 unit of Taq DNA polymerase (Promega, Madison, WI). The initial step of the amplification reaction was denaturation at 95°C for 4 min; followed by 35 cycles of 95°C for 45 sec, 50°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min.

Cloning and sequencing of RGAs. PCR products for cloning were fractionated on 0.8% agarose gel and stained with 3x SYBR Green (Applied Biosystems, Foster City, CA). Fragments of the expected size were excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (Promega). The PCR products were then cloned into the pGEM-T Easy plasmid vector (Promega) and transformed into competent *Escherichia coli* Top-10 cells (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The transformation reaction was plated on selective media containing 20% (w/v) X-gal/2% (w/v) IPTG for blue/white screening of plasmids with inserts. Plasmid DNA from single white colonies was examined by *Eco*RI restriction analysis to determine the size of the inserted fragments.

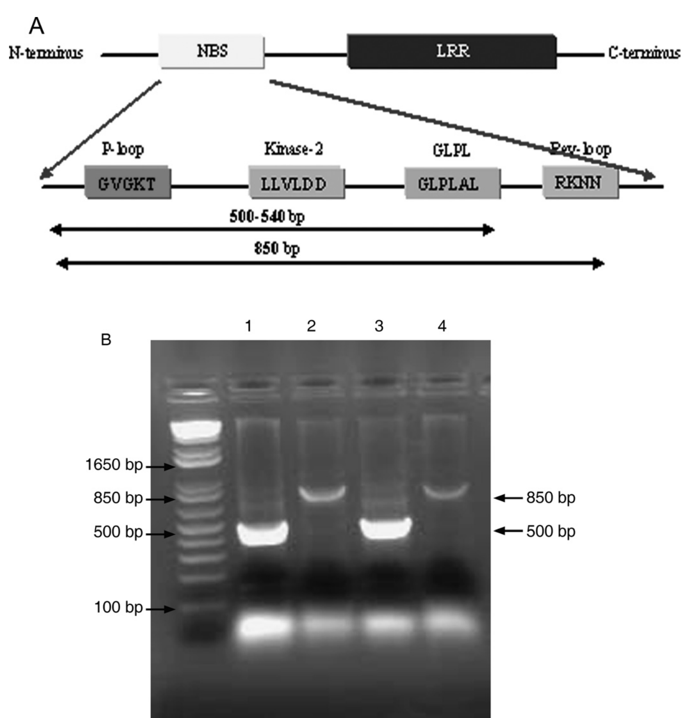

Figure 1 Model of the structure of NBS-LRR type resistance genes (**A**). PCR products amplified with two degenerate primer pairs from *V. cinerea* B9 (**B**). The expected sizes of amplified DNA bands are 500 bp for the P-loop/GLPLAL-1 primer pair (samples 1, 3) and 850 bp for the P-loop/Rev loop primer pair (samples 2, 4). Marker DNA (1 kb) is shown in the lane on the left.

Plasmid DNA harboring inserts were sequenced using the Applied Biosystems Automated 3730 DNA Analyzer at the Biotechnology Resource Center (Cornell University). Sequencher 4.2 software (Genecodes Corp., Ann Arbor, MI) was used to select representative clones based on 90% minimum overlap and 90% minimum identity of nucleotide sequence.

Sequence analysis. Identification of clones showing significant homology to known RGA sequences and resistance proteins in GenBank was performed by Nucleotide-Nucleotide Basic local alignment search tool (BLASTN) and translated query vs. Protein database (BLASTX) software (www.ncbi.nlm.nih.gov/Blast). Nucleotide sequences of RGAs cloned from the P-loop/GLPLAL-1 primer pair were translated into amino acid sequences by Translate software (http://bio.lundberg.gu.se/edu/translat.html). Amino acid sequences from *R* genes already classified as TIR or non-TIR proteins were searched using GenBank Entrez (www.ncbi.nlm.nih.gov). In addition, selected TIR-NBS-LRR genes (*L6* and *M* from flax, *N* from tobacco and *RPS4* from *Arabidopsis)* and non-TIR-NBS-LRR genes (*RPS2* and *RPS5* from *Arabidopsis*, *Xa1* from *Oryza sativa,* and *I2* from tomato) were added to the alignment. The alignment of *Vitis* RGA clones, along with known TIR and non-TIR amino acid sequences, was performed by ClustalW-XXL software (http://clustalW.genome.jp). Motif structures in RGA clones were analyzed by MEME software (http://meme.nbcr.net). A phylogenetic tree of *Vitis* RGA clones, known TIR, and non-TIR amino acid sequences was constructed with Phylip software, version 3.6 (http://evolution.genetics.washington.edu/phylip.html).

RGA-STS marker. PCR primers specific to the 19 cloned *Vitis* RGAs were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/primer3_www.cgi). Nine additional RGA-STS primers (Di Gaspero and Cipriani 2003) were also used (Table 1). PCR amplifications were performed in a reaction volume of 20 µL containing 1x PCR buffer, 0.1 mM dNTPs, 2.5 mM $MgCl_2$, 2 µM of each primer and 1 unit of Taq DNA polymerase (Promega). The initial step of the amplification reaction was denaturation at 94°C for 1 min, followed by 25 cycles of 92°C for 50 sec, 46 to 58°C (variable by primer) for 50 sec and 72°C for 1 min, and a final extension at 72°C for 10 min. Amplified DNA fragments were cut by 1 U restriction enzyme and incubated for 3 hr at the appropriate temperature. The corresponding restriction enzymes were selected using Sequencher 4.2 software. CAPS analyses were performed on 2% agarose gels and stained with SYBR Green. Single-strand conformation polymorphism (SSCP) analyses were performed using 2% glycerol and 8% polyacrylamide gels at 4°C and 12-13 W. Gels were stained with silver nitrate.

***Plasmopara viticola* inoculation.** Sporangia of *P. viticola* were harvested from sporulating leaves of *Vitis* hybrid cv. Delaware into double distilled water using a spray bottle. The collected sporangial suspensions were counted in a hemacytometer and adjusted to 10^5 sporangia per mL. Leaves from nodes 5, 6, and 7 (node 1 being the first expanded leaf) of 179 Horizon x Ill. 547-1 seedlings were used for inoculation. The inoculated leaves were placed abaxial surface up on moist filter paper in Petri plates. The sporangial suspensions were sprayed onto the abaxial leaf surface. Petri dishes were held at 22°C, 18-hr photoperiod for 8 days. A second set of Petri dishes were incubated for 10 days. Infected leaves were placed in 5 mL double distilled water per 50-mL tube and then shaken for 3 min. The total number of spores produced per leaf was determined by counting the number of spores in 5 µL under a microscope. Length and width of infected leaves were measured. The area of 10 different leaves was measured using a leaf area meter. Regression analysis was then used to convert leaf length and width to leaf area, and the number of spores per leaf was converted to number of spores/25 cm^2 leaf area. Resistance levels were based on spore production. The six-point disease resistance classification was defined as: 0 = 0 to 5 spores/25 cm^2, highly resistant; 1 = >5 to 10 spores/25 cm^2, resistant; 2 = >10 to 15 spores/25 cm^2, moderate or intermediate; 3 = >15 to 25 spores/25 cm^2, moderately susceptible; 4 = >25 to 40 spores/25 cm^2, susceptible; and 5 = >40 spores/25 cm^2, highly susceptible.

Results

Cloning RGA sequences. Genomic DNA of *V. cinerea* B9, a genotype resistant to multiple diseases includ-

Table 1 Specific primers, annealing temperatures, and sizes of PCR product for RGA-STS markers.

Name[a]	Forward primer (5'—3')	Reverse primer (5'—3')	Temp.	Size	Enzyme
rgVrip064[b]	GACTACTATTGCCAAGGCTGTTT	AATCTACTGCTTGGTAGGAGAG	58	467	*EcoR*I
rgVamu085[b]	GACGACCCTCTTGACCAGGAT	TGAGAATTTATAGTGTCTTCTCCTACA	58	435	*Sau* 3AI
stkVa011[b]	GAAGGCACTTTGAGCAATGG	AACCATTCGGGAGCCAAG	57	479	*EcoR*V
rgVrip145[b]	GCCAGACTTGCTTATAACGATGA	CGCACTTTTCCACAATCTTCTT	58	475	*Alu* I
GLPL6-1[b]	GCATATGCTACAAACTCCATTCA	CAATTTCTTCTAGTTCTGGGATG	58	206	*Hinf* I
rgVrip158[b]	CCAGTTGATATACAGGGACGATG	GATCCTTGTATCAAGCAATCTCA	58	463	*MnI* I
rgVamu100[b]	CATCAATATGATGGTAGTAGCTTTCTT	GAGCTTAGACACCTCTTTATCACACT	58	164	
rgVamu092[b]	AACTCACATCAATTTGAGAGTAGAATC	TGATTTGAGAGGTCAACATAGTCA	58	431	*Alu* I
rgVamu111[b]	ACCAGAGAGTGGTGGGACAC	CCTTTTATCTTGTAAATACTGCCTGA	58	194	
rgVcin109	GGAAGACGACAATTGCCAAA	GCATCGACTCCAAGCACAT	56	358	*Alu* I
rgVcin111	ATGGTGTCATGAAGGGAAAAA	AGACCAAACCAACCATGCTC	57	164	*Xba* I
rgVcin123	GATGGGATGGAGTCAAAGGA	CACTCACTCCATGGCACATT	58	217	*aTaq* I
rgVcin125	GTCCAGGAAACCGTTCTCAA	CCTTGGTCCGAAACAAAGAA	54	304	*Hinf* I
rgVcin127	GATGGGATGGAGTCAAAGGA	GGGGAGGCCTTTAGCATAAT	54	352	*MnI* I
rgVcin139	TGACGTGGATGATTTGATGC	GGGGAGGCCTTTAGCATAAT	58	259	*Alu* I
rgVcin165	CATGGTATCCTGAGGGGAAA	GGAGGCCATCAGCATAATCT	58	361	*Mae* II
rgVrup103	CATGGTATCCTGAGGGGAAA	GGCCATCAGCGTAATCTATGA	56	358	*Rsa* I
rgVrup119	GGTGCAAATGCTCACAGAGA	CTCCCAAACAAGGTCCAAGA	58	383	*EcoR* I
rgVrup124	AATGGAGGCTCGTTTTGAGA	GCCGATGTGTTCTCTCTTCC	58	323	*MnI* I
rgVrup126	GGTCCAGGAAACCATTCTCA	CCTTGGTCCGAAACAAAGAA	54	304	*Hinf* I
rgVhyb101	GGGGTGGGGAAGACAACTAT	CCTACTTCTTGGACCAAACCA	50	306	*Aci* I
rgVhyb102	CACAAATGCAATTTGCCCTA	GCTGGAGGAGGTTGGTGTTA	58	329	*EcoR* I
rgVhyb110	ATCCAGGGTTGAGTTTGACG	CAATGCCCTTAGCTCCCATA	58	317	*Dpn* II
rgVhyb121	AATTCGATTGAGGGCAAGTG	GTGAGGATGAAAGGGCAGAA	58	347	*Nco* I
rgVhyb127	TGATCGTGGTGTGCTTCAAT	TTCCGTAGCTTGCTTGTGTG	54	310	*Nco* I
rgVhyb149	GATTGGTTTGGTCGAGGAAG	CGGCAGACCTTGAGGATAAA	46	212	*EcoR* I

[a]Primers named according to the RGA cloned.
[b]Markers developed by Di Gaspero and Cipriani (2003).

ing downy mildew, was amplified using two degenerate primer pairs, P-loop/GLPLAL-1 and P-loop/Rev loop, producing ~500-bp and 850-bp PCR products, respectively (Figure 1). Complete nucleotide sequences were obtained from 78 of 100 clones. Based on 90% minimum overlap and 90% minimum identity, 48 clones from P-loop/GLPLAL-1 primers were subdivided into eight unique groups. In contrast, 52 clones from P-loop/Rev loop primers were sequenced but unambiguous nucleotide sequences were obtained for only 30 clones, which were subdivided into four representative groups.

With the finding that clones generated from *V. cinerea* B9 using the P-loop/GLPLAL-1 oligonucleotide primer pair were highly conserved at the NBS domain, this primer set was considered to have more potential for marker development than the P-loop/Rev loop primers. Therefore, only P-loop/GLPLAL-1 primers were used to clone RGA sequences from *V. rupestris* B38 and Horizon. Twenty-seven and 47 sequences were cloned from *V. rupestris* B38 and Horizon, respectively. RGAs cloned from *V. rupestris* B38 were separated into four unique groups, while 47 clones from Horizon were subdivided into seven unique groups based on 90% identity or greater.

Sequence analysis of RGA clones. Nucleotide sequences from 8 of the 12 unique groups from *V. cinerea* B9 had significant BLASTN hits to RGAs in GenBank (Table 2). All of these were generated with the P-loop/GLPLAL-1 primer pair, whereas the four RGA clones from P-loop/Rev loop primers were not similar to any RGA clones in GenBank. Seven showed similarity to RGA clones that were isolated from *V. amurensis* (Di Gaspero and Cipriani 2003). Moreover, nucleotide sequences of rgVcin109, rgVcin125, rgVcin139, and rgVcin165 were nearly or completely similar to RGA clones from *V. amurensis* (E-value = 0 and (bit) value = 724, 894, 876, and 718, respectively). Only one clone (rgVcin152) showed sequence similarity to a NBS-LRR like gene from a non-*Vitis* species (*Oryza sativa)*.

Similarly, BLASTX analysis showed that 10 of 12 representative clones from *V. cinerea* B9 had amino acid sequence similarity to resistance protein candidates in GenBank (Table 2). As with the nucleotide sequences,

Table 2 Results of the search for similarity between *Vitis* RGA sequences with nucleotide and amino acid GenBank accessions carried out using the programs BLASTN and BLASTX.

Genotype/ primer pairs	RGAs (n) /unique clones	Representative clone/ number cloned in group	GenBank nucleotide accession showing highest similarity					
			Nucleotide	(bit) Value	E value[a]	Amino acid	(bit) Value	E value[a]
***V. cinerea* B9**	48/8							
P-loop/GLPLAL-1		rgVcin109/3 (DQ885292)	*Vitis amurensis* isolate rgVamu090 gi/38045679/gb/AY427105.1/	724	0.0	Resistance-protein candidate (*V. amurensis*)	267	1e-70
		rgVcin111/5 (DQ885293)	*Vitis amurensis* isolate rgVamu092	502	1e-139	Resistance-protein candidate (*V. amurensis*)	254	6e-67
		rgVcin123/6 (DQ885294)	*Vitis amurensis* isolate rgVamu090	86	1e-13	NBS-type resistance protein (*Gossypium barbadense*)	115	8e-25
		rgVcin125/4 (DQ885295)	*Vitis amurensis* isolate rgVamu151 gi/38045730/gb/AY427133.1/	894	0.0	Resistance protein candidate (*V. amurensis*)	306	2e-82
		rgVcin127/13 (DQ885296)	*Vitis amurensis* isolate rgVamu092	80	7e-12	Resistance-protein candidate (*V. amurensis*)	127	2e-82
		rgVcin139/6 (DQ885297)	*Vitis amurensis* isolate rgVamu053 gi/38045673/gb/AY427102.1/	876	0.0	Resistance-protein candidate (*V. amurensis*)	272	3e-72
		rgVcin152/8 (DQ885298)	*Oryza sativa* clone sk98 NBS-LRR-like gene	74	4e-10	Disease resistance-like protein 585-8 (*Mentha longifolia*)	118	8e-26
		rgVcin165/3 (DQ885299)	*Vitis amurensis* isolate rgVamu094 gi/38045681/gb/AY427106.1/	718	0.0	Resistance-protein candidate (*V. amurensis*)	295	5e-79
P-loop/Rev loop	30/4	rgVcin209/4	No significant similarity found			No significant similarity found		
		rgVcin210/6	No significant similarity found			No significant similarity found		
		rgVcin254/13	No significant similarity found			RCa10.6 NBS type resistance protein (*Manihot esculenta*)	140	5e-32
		rgVcin269/7	No significant similarity found			Resistance-protein candidate (*V. amurensis*)	52	3e-05
***V. rupestris* B38**	**27/4**							
P-loop/GLPLAL-1		rgVrup103/4 (DQ885300)	*Vitis amurensis* isolate rgVamu094	712	0.0	Resistance-protein candidate (*V. amurensis*)	292	3e-78
		rgVrup119/6 (DQ885301)	*Oryza sativa* clone sk98 NBS-LRR-like gene	87.7	3e-14	Putative disease-resistance gene analog (*Malus prunifolia*)	143	3e-33
		rgVrup124/10 (DQ885302)	*Vitis riparia* isolate rgVrip148	446	3e-122	Putative disease-resistance gene analog (*Arabidopsis thaliana*)	228	1e-58
		rgVrup126/7 (DQ885303)	*Vitis amurensis* isolate rgVamu151	900	0.0	Resistance protein candidate (*V. amurensis*)	195	6e-49
Horizon	47/7							
P-loop/GLPLAL-1		rgVhyb101/11 (DQ885304)	*Vitis amurensis* isolate rgVamu050 gi/38045671/gb/AY427101.1/	860	0.0	Resistance protein candidate (*V. amurensis*)	248	4e-63
		rgVhyb102/9 (DQ885305)	*Oryza sativa* clone sk98 NBS-LRR-like gene	65.9	1e-07	probable methyeletrahydrofolate red	65	2e-04
		rgVhyb110/4 (DQ885306)	*Vitis riparia* isolate rgVrip068	628	4e-177	Resistance protein candidate (*V. riparia*)	248	6e-65
		rgVhyb121/3 (DQ885307)	*Vitis amurensis* isolate rgVamu035	261	9e-69	Resistance protein candidate (*V. amurensis*)	261	9e-69
		rgVhyb127/7 (DQ885308)	*Vitis riparia* isolate rgVrip004	173	3e-42	Resistance protein candidate (*V. amurensis*)	173	2e-42
		rgVhyb139/5 (DQ885309)	*Oryza sativa* clone sk50 NBS-LRR-like gene	78.9	8e-12	NBS/LRR resistance protein-like (*Theobroma cacao*)	43.1	0.004
		rgVhyb149/8 (DQ885310)	*Malus prunifolia* putative disease	67.9	3e-08	Resistance protein candidate (*V. amurensis*)	53.5	3e-06

[a]Expected (E) value refers to the number of matches expected by chance alone. The lower the E value, the more strongly supported the match.

amino acid sequences of most RGA clones were similar to resistance protein candidates from *V. amurensis*. Two exceptions among those derived from the P-loop/GL-PLAL-1 primers were rgVcin123 and rgVcin152, which showed high similarity with resistance protein candidates and NBS-type resistance proteins from *Gossypium barbadense* and *Mentha longifolia*, respectively. In addition, rgVcin254 and rgVcin269 from P-loop/Rev loop primers

were similar to resistance protein candidates from *Manihot esculenta* and *V. amurensis*, respectively; however, these were not as strongly matched as the RGA clones from P-loop/GLPLAL-1 primers (Table 2).

Three of four RGA sequences from *V. rupestris* B38 and one of seven sequences from Horizon showed high similarity to the same RGA GenBank accessions as those from *V. cinerea* B9 (Table 2). Some RGA sequences, including rgVrup103, rgVrup126, and rgVhyb101, with E-values = 0, had near or complete similarity to RGA clones isolated from *V. amurensis* (Di Gaspero and Cipriani 2003). RGA amino acid sequences from *V. rupestris* B38 and Horizon were similar to resistance protein candidates from *V. amurensis* and *V. riparia*, as well as putative disease resistance proteins from *Malus prunifolia*, *Theobroma cacao,* and *Arabidopsis thaliana* (Table 2).

NBS-LRR domain. Nineteen RGAs amplified by P-loop/GLPLAL-1 primers were analyzed for the presence of conserved amino acid motifs. As expected, P-loop and GLPL motifs were present in the first seven and last six amino acids of all RGAs cloned, except rgVhyb121. Those amino acids correspond to oligonucleotide primers derived from P-loop and GLPL motifs (modified from Hunger et al. 2003). RNBS-A, kinase-2, RNBS-B, and RNBS-C motifs also appeared in all RGAs cloned (Figure 2). The RNBS-A motifs could not be identified by MEME analysis because they were diffuse and poorly conserved. However, these motifs were found and verified by visual inspection of alignments. As previously suggested, kinase-2 is useful to distinguish between TIR or non-TIR proteins (Meyer et al. 1999). The presence of tryptophan in the kinase-2 motif is predictive of non-TIR proteins (e.g., *RPS2*, *RPS5, I2,* and *Xa1*). On the other hand, *L6* and *N* from flax and *M* from tobacco have aspartic acid in the kinase-2 motif, which is typical of TIR proteins (Figure 2). The amino acid sequence of the kinase-2 motif classified rgVcin125, rgVcin152, rgVrup119, rgVrup126, and rgVhyb110 as well as *RPS2*, *RPS5*, *I2,* and *Xa1* as non-TIR proteins. On the other hand, *L6, N*, rgVcin109, rgVcin139, rgVcin165, rgVrup103, and rgVhyb101 were classified as TIR proteins (Figure 3).

Using the amino acid change in the kinase-2 motif to classify proteins, 9 out of 19 RGAs (rgVcin111, rgVcin123, rgVcin127, rgVrup124, rgVhyb102, rgVhyb121, rgVhyb127, rgVhyb139, and rgVhyb149) could not be classified as either TIR or non-TIR types, possibly because of incomplete amino acid sequences, especially tryptophan and aspartic acid, in conserved domains of these clones. Therefore, phylogenetic analysis was used to verify the overall sequence similarity to other *R* genes representative of the two subclasses. The unclassified RGA, rgVhyb124, can be found in the major branch along with *Xa1,* and *I2,* as well as *Vitis* non-TIR proteins, suggesting that this clone is more closely related to non-TIR than to TIR proteins (Figure 3). In addition, rgVcin111, rgVcin123, rgVcin127, rgVhyb102, rgVhyb121, rgVhyb127, rgVhyb139, and rgVhyb149 clustered in the same branch with *RPS4, M*, *L6,* and *N,* the known TIR proteins (Figure 3). Therefore, these clones are likely more closely related to TIR than to non-TIR proteins. Even though the rgVcin152 appeared in the same branch with other TIR proteins, it was still classified as a non-TIR protein since tryptophan appeared in the kinase-2 motif. In total, 19 RGA clones were classified into 13 TIR-NBS-LRRs-like genes and 6 non-TIR-NBS-LRR-like genes.

***P. viticola* resistance analysis.** A segregating seedling population developed by the grape breeding program at Cornell University, Geneva, New York, was tested for downy mildew resistance. The parents, Horizon and Ill. 547-1, were reported to be moderately and highly resistant to downy mildew, respectively (Dalbo et al. 2000). A detached leaf assay confirmed this observation. Horizon and Ill. 547-1 had 15.1 and 2.2 spores/25 cm^2, respectively. The seedling population segregated for resistance, with the number of downy mildew spores/25 cm^2 ranging from 2.2 to 122.1 (data not shown). Eighty-seven seedlings, or 48.6%, grouped into the intermediate classification (2 and 3). Resistant (classes 0 and 1) and susceptible individuals (classes 4 and 5), comprised 32.4 and 19.0% of the population, respectively.

RGA-STS marker. Twenty-three STS and three CAPS primer pairs were used to amplify the parents and 179 progeny of the Horizon x Ill. 547-1 population. The 17 STS markers developed from the RGA nucleotide sequences from the present study (from *V. cinerea*, *V. rupestris,* and Horizon) produced polymorphic markers among 179 progenies. Surprisingly, at least 4 to 5 primer pairs from two clones, rgVcin152 and rgVhyb139, were tested but PCR products could not be amplified. Nine (six STS and three CAPS) of these 26 markers were developed by Di Gaspero and Cipriani (2003) based on RGA sequences from *V. amurensis* and *V. riparia*. From segregation analyses, eight STS markers (rgVamu085, GLPL6-1, rgVcin125, rgVcin127, rgVcin139, rgVrup126, rgVhyb102, and rgVhyb110) were present in the male (Ill. 547-1) but absent in the female parent (Horizon). Five STS markers (rgVamu100, rgVrup119, rgVhyb101, rgVhyb121, and rgVhyb127) were present in the female and absent in the male parent. The rest of the STS markers were either present or absent in both parents. The chi-square goodness-of-fit statistic was use to check conformity of marker segregation with the expected ratio. A 1:1 segregation ratio could not be rejected for all markers present only in the male or female parent except rgVamu085, rgVamu100, rgVrup126, and rgVhyb102. Also, markers showing a 3:1 segregation ratio, such as rgVrip064, rgVrip145, stkVa011, rgVamu092, rgVcin109, rgVcin111, rgVcin123, rgVrup103, and rgVrup124, will be useful for mapping to both Horizon and Ill. 547-1 linkage maps.

Based on correlation analysis among CAPS and STS markers, three groups of markers emerged (Table 3). Some of the markers in each group were designed from closely related nucleotide sequences (Di Gaspero et al. 2007). As described above, some of markers were de-

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                          P-loop                  RNBS-A
rgVcin139     GGVGKTTITKAVYN-----DISCQFDGSSFLNNVRERSKD--NALQLQQELLHGSLKGKS
rgVhyb101     GGVGKTTIAKAVYN-----DISYQFDGSSFLNNVRERSKD--NALQLQQELLG-ILKGKS
rgVcin165     GGVGKTTIAKAIYN-----EISNQYDGRSFLRNIRERSKG--DILQLQQELLHGILRG-K
rgVrup103     GGVGKTTIAKAIYN-----EISHQYDGSSFLINIRERSKG--DILQLQQELLHGILRGKN
rgVcin109     GGVGKTTIAKVVYN-----NISHQFESRIFLENVRERSKDQSSLLQLQKELLNGVVKGKN
N             GGVGKTTIARAIFTLLGRMDSSYQFDGACFLKDIKENKRG---MHSLQNALLSELLREKA
L6            GGIGKTTTAKAVYN-----KISSCFDCCCFIDNIRETQEKDGVVVLQKKVSEILRIDSGS
M             GGIGKTTLAKAVYN-----KISSHFDRCCFVDNVRAMQEQKDGIFILQKKLVSEILRMDS
rgVcin125     GGVGKTTLLKRIDN-----DFLQTGYEVDVVIWVVVSQQGNVEKVQETVLNKLEIAEYKW
rgVrup126     GGVGKTTLLKRINN-----EFLQTCYEVDVVIWVVVSQQG-VKKVQETILNKLEIAEYKW
rgVhyb110     GGVGKTTLLNRVNN-----EFLKSRVEFDAVIWVIVSRPANVEKVQQVLFNKLEIPSNNW
RPS5          GGVGKTTLLTKINN-----KFSKIDDRFDVVIWVVVSRSSTVRKIQRDIAEKVGLGGMEW
RPS2          GGVGKTTLMQSINN-----ELITKGHQYDVLIWVQMSREFGECTIQQAVGARLGLS---W
I2            -GVGKTTLAKAVYN-----NEK-LKDRFDLKAWICVSEPYDASRITKGLLEEIGSSNLMV
Xa1           GGIGKTTLAQLVCK-----DLV-IKSQFNVKIWVYVSDKFDVVKITRQILDHVSNQSHEG
rgVrup124     GGVGKTTLAQVVFN------DREMEARFERRMWVSVTGTPNEKRILRSMLRNLGDMNVGD
rgVrup119     GGVGKTTMVKQVGAN---------AHRDGLFQRVAMAVISQNPDLRKIQAQIADMLNLKL
rgVcin152     GGWGRRLLGQTYIMMG-------EFAHFEKRMWVCVSEFD----VKRLIKEIITSATHGK
RPS4          -GIGKTTLLKELYK----------TWQGKFSRHALIDQIRVKSKHLELDRLPQMLLGELS
rgVhyb149     GGVGKTTFLEDGKG-----------------------------------FRLIRVSNYNK
rgVhyb102     GGVGKTTISPMAQM---------------QFALSRECQNLKRSHLLHQNKNINNIFNKRP
rgVcin123     GGVGKTTYSQSCLS------------------NLSIGHLPCYRSLQKLWFASITETTSRY
rgVcin111     GGVGKTTTCQSCIY---------LTSIENLSKCRKIQRLLKSTSITKRTSWCHEGKKKNK
rgVhyb127     GGVGKTTIAKVCLQ--------------------YVRSITPLSKCEREIRSWCASITRKT
rgVcin127     GGVGKTTIAVCFIIQ-------------------SLVNLRALASLQILEKSPKTVVCFHY
rgVhyb121     GGKFDGQVGGRHTLWQ-----PSPPALVYLDENLWCLLLPWSETEQKLPPMGGNSLQCSW
rgVhyb139     GGVGKTTGITRHSR-------------------------------AHCQTTHFGHVLLGD
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                                          GLPL
rgVcin139     IMLKASPSSITKIR--
rgVhyb101     VVYAGLPLAL------
rgVcin165     IIDYADGLPLAL----
rgVrup103     IIDYADGLPLAL----
rgVcin109     EVNYVNGLPLAL----
N             VVNYAKGLPLAL----
L6            VVDTTAGLPLTL----
M             IVSTTGGLPLTL----
rgVcin125     FVKECKGLPLAL----
rgVrup126     KSAASPSPSITSEFA-
rgVhyb110     ----------------
RPS5          VARKCRGLPLAL----
RPS2          IVSKCGGLPLAL----
I2            IAKKCKGLPLAL----
Xa1           IASELKGNPLAA----
rgVrup124     IVHKCGGLPLAL----
rgVrup119     IVKECGGLPLAL----
rgVcin152     IVCRPPPRPQSLVNRP
RPS4          FVHYARGHPLAL----
rgVhyb149     ILKVCRLPSFIIA---
rgVhyb102     ----------------
rgVcin123     TLCRPPPHPQSLVNSR
rgVcin111     LNSRPPAC--------
rgVhyb127     GLCRPPPSSQSL----
rgVcin127     YIMLKASPPL------
rgVhyb121     SRGWFSRRPQSLV---
rgVhyb139     QPSAVGTLPL------
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                                        Kinase-2
rgVcin139     LKVSNMDEGIQMIKRSLSSKRVLVVFDDVDD-LMQIENLAEEHIWFG----------PRS
rgVhyb101     LKVSNMDEG-IMIKRSLCSKRVLVVFDDVDD-LMQLN--LAEHSWFG----------PRS
rgVcin165     FFINNVDEGISMIKRCLTSNRVLVIFYDVDE-LKQLEYLVEEKDWFH----------AKS
rgVrup103     FKINNVDEGISMIKRCLTSNRVLVIFYDMDE-LKQLEYLAEEKDWFH----------AKS
rgVcin109     LEISNVHEGIDVIRNRFNSKKVLLILDDVDN-LKQLKFLAGGHGWFG----------PRS
N             NYN-NEEDGKHQMASRLRSKKVLIVLDDIDNKDHYLEYLAGDLDWFG----------NGS
L6            VGFNNDSGGRKTIKERVSRFKILVVLDDDEK--FKFEDLG-SPKDFI----------SQS
M             VGFTNDSGGRKMIKERVSKSKILVLDDVDEK--FKFEDILGCPKDFD----------SGT
rgVcin125     KDRSVHE-RAEEIFSVLQTKKFVLLLDDIWKQLDLLEVGIPPLNDQK-----------KS
rgVrup126     KDRSVHE-RAEEIISVLQTKKFVLLLDDIWKQLDLLEVGIPPLNDQN-----------KS
rgVhyb110     EGRSEDE-RKEAIFNVLKMKKIVVLLDDIWELLDLFAVGIPPINDEN-----------KS
RPS5          SEKNDNQ-IAVDIHNVLRRRKFVLLLDDIWEKVNLKAVGVPYPSKDN-----------GC
RPS2          DEKETGENRALKIYRALRQKRFLLLLDDVWEEIDLKTG-VPRPDREN-----------KC
I2            DNTLNQL--QIKLKESLKGKKFLIVLDDVWNDKYIEWDDLRNPFAPG---------EIGS
Xa1           ISNLDTL--QQDLEEQMKSKKFLIVLDDVWEIRTDDWKKLLAPLRPNDQVNSSQEEATGN
rgVrup124     DCGELLR----KINQYLLGKRFLLVMDDVGENTNTWWRKISDGLPKG----------NGS
rgVrup119     EEESEAGRAARLRERIMRGKSVLIILDDIWRRIDLSEIGIPSTGSDLD--------ACKS
rgVcin152     CDDLPMDELARLLINVLDDKKFLLILDDVWSKNRDKWLELKALLDGG----------AKG
RPS4          KLNHPHVDNLKDPYSQLHERKVLVVLDDVSKREQIDALREILDWIKEG--------KEGS
rgVhyb149     NFFLNLRKRIIKDSHLRLDSVQKRFLLFSTMMIQEYWNAIGNRDWFG----------RGS
rgVhyb102     LTNYKGSKSQKICKNSKQITMDIVIVYQADQEITALYLKNISVILLS----------LNK
rgVcin123     FDGMESKDKQCRMNQCANGQTSLKGSYYSRGFESITILSWKCLVWYW-------------
rgVcin111     CSDCDKKQVSLKKGSSYSCRQFEAITILSWRAWLVWSKNHHNLRSTLFK-------CAWS
rgVhyb127     SLYSNGEKFEVKYRNQDNEASLGKGSYCSCRLSKTIKIFCSSMVSSR-----------KH
rgVcin127     RNNFVIFWDGVKGASMKESMCWTDFTLKRFLLFLMTWMIINNPLEMLIG-----------
rgVhyb121     IPSFGTLVIALIPSSTYLDDSEEEYQLWIDPAAPICHLTKAQISLSS-----------GH
rgVhyb139     CLRRRPCMSVLMHRSYCRKDDFLFHQTGGNHFSCFIIKTIAGKTEFCS---------KIR
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                 RNBS-B                     RNBS-C
rgVcin139     RIITTTRHKHFLTQYGV----IESYEVPKLHDAEAIELFSWWAFK-QNLPNEIYKNLSYR
rgVhyb101     RIIITTRHKRFL-QYGV----KESYEVQKLHDEIELSLA----FP-QNLPSEIYR--NYR
rgVcin165     TIIITTRDKHVLAQYGA----DIPYEVSKLNKEEATELFSLWALK-QNHPQEVYKNLSYN
rgVrup103     TIIITTRDKHVLAQYGA----DIPYEVSTLNKEEATELFSLWAFK-QNHPQEVYKNLSYN
rgVcin109     RIIITSRDQHCLNVLGV----DASYEVKALNYEESIQLFCQHAFQ-QNIPKSDYVDLSNH
N             RIIITTRDKHLIEKN------DIIYEVTALPDHESIQLFKQHAFG-KEVPNENFEKLSLE
L6            RFIITSRSMRVLGTLNENQ--CKLYEVGSMSKPRSLELFSKHAFK-KNTPPSYYETLAND
M             RFIITSRNQNVLSRLNENQ--CKLYEVGSMSEQHSLELFSKHAFK-KNTPPSDYETLAND
rgVcin125     KVIFTTRFSTVCHDMGAKS---IEVECLAWEEAFSLFRTKVGED--TLDSHPDIQKLAEI
rgVrup126     KVIFTTRFSTVCHDMGAKA---LKLSAWHGRKLFLCFGPREKTP--ILIQIYKRLRRFLS
rgVhyb110     KVVFTTRFSTVCRDMGAKG---IEVCLEEAFALFQAYVGKDT------------------
RPS5          KVAFTTRSRDVCGRMGVDD--PMEVSCLQPEESWDLFQMKVGKN--TLGSHPDIPGLARK
RPS2          KVMFTTRSIALCNNMGAEY--KLRVEFLEKKHAWELFCSKVWRK--DLLESSSIRRLAEI
I2            KIIVTTRKESVAEMMGSRP---IIMEILSSEFSWPLFKRHAFEKR-DPKEHPELEEVGKH
Xa1           MIILTTRIQSIAKSLGTVQ--SIKLEALKDDDIWSLFKVHAFGND-KHDSSPGLQVLGKQ
rgVrup124     SIIITTRTKEVATMMGVEEERTHRPKVLSKDDSWLLFRNVAFAANGGICTSSELENIGRE
rgVrup119     KILLTTRLENVCHVMESQAK--VPLNILSEQDSWTLFGRKAG----RVVDSPDFHNVAQK
rgVcin152     SKIIVTR-DKLVASMGTCP----MYELKGLSDEECLSLFITCAFD---DRDKYPRLVGKD
RPS4          RVVIATSDMSLTNGLVDDT---YMVQNLNHRDSLQLFHYHAFIDDQANPQKKDFMKLSEG
rgVhyb149     RIIITTRDKCLLFSHGVNY---YEVEKFYRDEAYDSIIHHSLTHELPTAFLGAFKIIDIF
rgVhyb102     YLYVNIRIYQLTTPLTPTS----SSKILDGSPPRSQSLVNSRPP----------------
rgVcin123     KNCYNNRTICMCHGVSEIY-------EAKELPEEALQLFSQYAFKSKKSRIRLYEPLQCT
rgVcin111     RCIISLRHDTRSLSNFSVN-----------MPLNTTFLKVTMTSQIMYIMKASPSPLQSL
rgVhyb127     NHCDHEKTLPRCTVIFIIG---------GISTQASYGTLLLECLSTTPSKRELCGPLSYI
rgVcin127     LVLELLQLEINCMCMEVKY--------MRLRNNRKLFNFSVNMLS----KEKVKIITSLM
rgVhyb121     IFLLPFHPHSYLPIYLSQS-----PIYKELAEPFPHWLVWTQSPKSLHQIQPWFGAHCSW
rgVhyb139     CLGILCPTARLEFPLELTE--------QPSQPPDQSYVCLRMVCEIIKCGPTFDRSMGAF
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Figure 2 Multiple alignments of representative amino acid sequences of 19 RGA clones and eight known R-genes based on ClustalW analysis. The P-loop and GLPL motifs corresponding to primer sequences are shown in the first seven and the last six amino acids. Aspartic acid (D) and tryptophan (W; underline) in the kinase-2 motif are characteristic of TIR and non-TIR proteins, respectively.

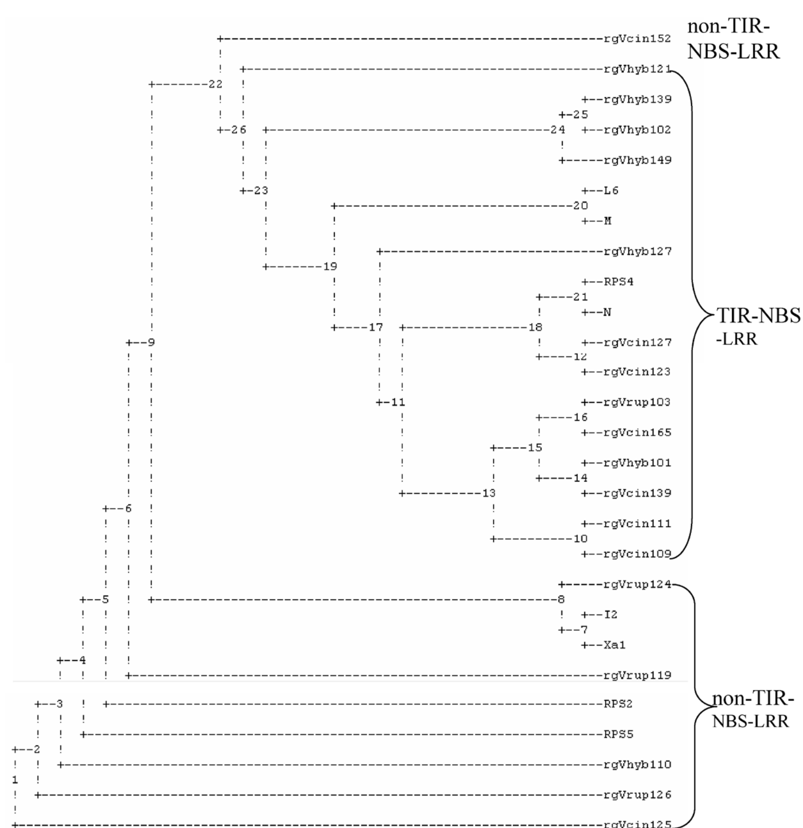

Figure 3 Phylogenetic tree of 19 *Vitis* RGA sequences and eight known TIR or non-TIR proteins.

veloped by Di Gaspero and Cipriani (2003), and these have already been located on the *Vitis* map. For example, rgVamu092 is located on linkage group 13, rgVrip064 on linkage group 18, and rgVamu085 on linkage group 19 (Di Gaspero et al. 2007); linkage groups are numbered according to set standards (Riaz et al. 2004, Doligez et al. 2006). We therefore suggest that markers with a significant correlation of their segregation in the same populations are located on the same linkage group (Table 3). Interestingly, we found a significant correlation between segregation for downy mildew resistance and segregation of three markers, rgVamu085, stkVa011, and rgVcin165 (r = -0.173, 0.152, and 0.151, respectively), suggesting that each of these markers may co-segregate with a gene controlling downy mildew resistance in grape. Further work is needed to confirm this possibility.

Discussion

Nineteen unique groups of RGA sequences from *V. cinerea* B9, *V. rupestris* B38, and Horizon have been cloned from P-loop/GLPLAL-1 PCR, and four unique groups were derived from *V. cinerea* B9 by P-loop/Rev loop PCR. The P-loop/Rev loop primers produced sequence data with a low percent match with known RGAs in GenBank. These data suggest that the P-loop/GLPAL-1 primer pairs used are highly conserved at the NBS domain and have greater potential for cloning RGAs from grape, compared with P-loop/Rev loop primers.

Most of the RGA clones from the three grape genotypes used here displayed high similarity with RGA clones from *V. amurensis*, especially seven RGA clones that showed complete similarity to RGAs cloned from *V. amurensis*. We also found similarity with RGAs cloned from *V. riparia*. Interestingly, *V. amurensis*, *V. cinerea*, *V. riparia,* and *V. rupestris* are reported as being highly resistant to downy mildew and powdery mildew (Eibach et al. 1989, Alleweldt et al. 1990). Moreover, none of the RGA clones identified showed similarity to sequences from susceptible species such as *V. vinifera*. There is evidence that RGA sequences from *V. amurensis* and *V. riparia* had a high probability of linkage with disease resistance genes in *Vitis* germplasm (Di Gaspero and Cipriani 2002). Since these RGAs were found in several grape species that are resistant to diseases such as downy mildew and powdery mildew, they could possibly be linked to or be candidate genes for disease resistance (Di Gaspero and Cipriani 2002). The RGA sequences we identified in resistant genotypes may also be linked to disease-resistance loci in *Vitis*, but further segregation studies will be needed for verification.

***R* gene evolution.** Plants respond to pathogens by direct and/or indirect interaction between plant *R* genes and *Avr* genes in the pathogen (Dangl and Jones 2001).

Table 3 STS markers on three expected linkage groups and correlation analysis for marker segregation within each group.

STS marker	Expected LGs	Correlation within group	
		Marker	r^b
rgVamu092[a]	13	rgVamu092 -rgVcin109	0.248**
rgVcin109[a]		rgVamu092 -rgVcin165	0.163*
rgVcin123		rgVcin109 -rgVcin165	0.240**
rgVcin165		rgVcin109 -rgVhyb149	0.258**
rgVhyb121		rgVcin109 -rgVhyb121	0.288**
rgVhyb149		rgVhyb121 -rgVamu092	0.174*
		rgVhyb121 -rgVcin165	0.261**
		rgVhyb121 -rgVcin123	0.266**
		rgVcin123 -rgVamu092	0.188*
rgVrip064[a]	18	rgVrip064 -rgVhyb101	-0.128**
rgVcin139[a]		rgVcin139 -rgVhyb101	0.315**
rgVhyb101[a]			
rgVamu085[a]	19	rgVamu085 -rgVcin125	-0.254**
rgVcin111		rgVamu085 -rgVhyb110	-0.301**
rgVcin125[a]		rgVcin125 -rgVhyb110	0.604**
rgVrup126		rgVcin125 -rgVcin111	-0.201*
rgVhyb110[a]		rgVhyb110 -rgVrup126	0.282**

[a]Closely related original nucleotide sequence in the group.
[b]* and ** indicate significance at $p < 0.05$ and 0.01, respectively.

Gene-for-gene interactions may increase resistance to pathogens if *R* genes increase the ability to recognize the pathogen. The LRR and TIR/non-TIR have a role in pathogen recognition; therefore unbalanced selection was needed to recognize rare *Avr* gene products in pathogen populations (Zhou et al. 2004). Mechanisms such as chromosome breaking, rearrangement, preexisting divergent duplication, unequal crossing over, gene conversion, and diversifying selection have been proposed to generate diversity in LRRs and TIR/non-TIR domains (Ellis et al. 2000, Richter and Ronald 2000).

Diversifying selection relies upon genomic instability mediated by unequal crossing over in meiosis (Richter and Ronald 2000). Interestingly, diversifying selection has been found in non-TIR-NBS-LRR rather than TIR-NBS-LRR genes. As shown in rice, there is no significant increase in the number of similar or almost identical genes within non-TIR-NBS-LRRs, whereas copies of similar TIR-NBS-LRR genes were duplicated (Zhou et al. 2004). Their data support the hypothesis that non-TIR-NBS-LRR genes are more highly variable than TIR-NBS-LRRs. The difference in diversity between TIR and non-TIR domains was also present in the NBS-LRR domains of the three grape genotypes in the present study. The DNA sequences among the TIR-NBS-LRR group displayed high similarity, ranging from 73.5 to 97.4% (for example, rgVcin111/rgVcin123, *L6*/*M,* and rgVcin165/rgVrup103 have 73.5, 78.2, and 97.4% identity, respectively). On the other hand, the amino acid sequence in the non-TIR-NBS-LRR group showed more variation within groups, with similarities ranging from 43 to 97.4% (for example, *RPS*2/*RPS*5, rgVhyb110/rgVrup119, and rgVhyb110/rgVcin125 have 43, 57.5, 77.1% identity, respectively). The shorter branch lengths among TIR-NBS-LRR groups as compared with non-TIR-NBS-LRR groups support the hypothesis that these proteins are more highly conserved (Figure 3). Therefore, in terms of gene diversification, non-TIR-NBS-LRRs might be more adaptively responsive and selection acting on this fluidity could lead to the more rapid development of pathogen recognition.

Potential of *Vitis* RGAs as molecular markers. Molecular markers based on RGAs have been developed from conserved domains of diverse plant species. The RGA sequences cloned in the present study from highly conserved domains of three disease-resistant grape genotypes will hopefully be useful for the development of markers linked to disease resistance. Because of their complete association with resistance-like genes, RGA markers may have the potential to improve the efficacy of MAS for disease-resistance traits. At least, three markers developed from RGA sequences (rgVamu085, stkVa011, and rgVcin165) are candidates for co-segregation with downy mildew resistance loci because of their significant correlations of marker segregation with disease resistance in a segregating population. However, rgVamu085 and rgVcin165 have a nonnormal segregation ratio. Therefore, stkVa011 seems to be the best candidate to co-segregate with a downy mildew resistance locus. Many of our RGA-STS markers are expected to map to linkage groups 13, 18, and 19. Interestingly, RGA-STS markers from Di Gaspero and Cipriani (2003) also show high numbers on these three linkage groups from Cabernet Sauvignon, Bianca, and Chardonnay maps (Di Gaspero et al. 2007), suggesting that RGAs cluster on linkage groups 13, 18, and 19 of the *Vitis* genome. However, further proof is required through final map placement of the RGA-STS markers identified here. Future work will also be able to determine whether RGAs in grapevine are responsible for disease resistance and to relate each RGA to the disease it affects.

Conclusions

Downy mildew may cause severe losses in yield and reductions in fruit quality of susceptible grape varieties. *Vitis vinifera* is the primary scion variety grown around the world and is highly susceptible to downy mildew. *R* genes are accessible in American and Asiatic species of *Vitis*, which hybridize readily with *V. vinifera*, and have been used extensively in breeding programs to create resistant cultivars. To improve the efficiency of grapevine breeding, an important goal is to locate molecular markers linked to alleles responsible for disease resistance. Genetic maps have been created using numerous crosses involving *Vitis* species, and quantitative trait loci analyses have been used to locate markers with strong associations to disease resistance. Breeders are actively trying to incorporate various molecular markers into their programs.

RGAs should have very good potential for use as molecular markers for disease resistance traits because of their known associations with disease resistance in plants. We have shown that RGA sequences derived from three downy mildew resistant genotypes have a high degree of similarity with RGAs cloned from *V. amurensis* and *V. riparia*, two species that harbor downy mildew resistance. There is a possibility that the RGA sequences characterized in the present work may confer functional resistance to downy mildew, but that will require further work to confirm. The precise linkage map locations of the most promising markers identified from *V. cinerea*, *V. rupestris,* and Horizon will need to be identified in future work. Indications at this time are that some of the RGAs we have identified are located on linkage groups already known to be associated with resistance loci.

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