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Resistance gene analogs are candidate markers for disease-resistance genes in grape (*Vitis* spp.)

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Abstract A set of NBS-containing sequences was isolated from genomic DNA of two grape species (*Vitis amurensis* and *Vitis riparia*) and characterised in a panel of *Vitis* genotypes carrying different levels of resistance against downy mildew and other diseases. A PCR-mediated approach made use of degenerate primers designed on conserved regions encoding known *R*-genes, and provided the source for cloning grape analogous sequences. Cloned sequences were digested with ten endonucleases and 29 out of 71 putative recombinant clones, which showed unique restriction patterns, were sequenced. Using a threshold value of 40% identity, at least 12 grape NBS-sequences had a high overall similarity with known *R*-genes, such as the *Arabidopsis* gene *RPS5* and the tobacco gene *N*. The presence of internal conserved motifs provided evidence that sequences isolated from grape may belong to the NBS-LRR gene family. A cluster analysis based on the deduced amino acid sequence and carried out on grape NBS-sequences, together with several analogous domains of known *R*-genes, classified grape sequences into three major groups. A grape sequence of each group was used as a probe on Southern blots with digested genomic DNA from resistant and susceptible grapes. One of the NBS-containing probes showed a clear-cut separation between resistant species and susceptible varieties. This evidence makes the probe a candidate marker for disease resistance genes in *Vitis* germplasm.

Keywords Grapevine · RGA · NBS-LRR · DNA markers · Pathosystems

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Introduction

In recent years, several molecular approaches have been used to investigate resistance against biotic diseases in grape, and these have provided some insight on the mechanisms involved in host-pathogen interactions. These studies concerned harmful fungal diseases such as downy mildew, caused by *Plasmopara viticola*, powdery mildew, caused by *Oidium tuckeri*, and grey mold, caused by *Botrytis cinerea*.

De novo synthesised physical barriers, erected in tissues surrounding the infection site, are thought to contribute to resistance, only after infections have taken place. Callose deposit only appears 5 days after experimental inoculation and could be found in tolerant as well as in susceptible genotypes (Kortekamp et al. 1997). The presence of lignin and suberin in strengthened cell walls appears as a defence shield only at a late stage of infection (Dai et al. 1995). A broad spectrum of elicited chemicals are synthesised early in fungal infection, including some pathogenesis-related (PR) proteins and phytoalexins (Renault et al. 1996). Even earlier expression and increased activity of chitinases (Busam et al. 1997), β-1,3-glucanases (Reuveni 1998; Renault et al. 2000) and peroxidases (Kortekamp et al. 1998) were observed in non-compatible interactions that led to a hypersensitive response to *P. viticola* and *B. cinerea*. Jacobs et al. (1999) isolated cDNA clones encoding different PR proteins, including chitinases, β-1,3-glucanases and thaumatin-like proteins, in grapevine infected by *O. tuckeri*. Chitinase and β-1,3-glucanase genes have already been transferred to some genetically engineered cultivars of *Vitis vinifera*, such as Chardonnay, Merlot, Neo Muscat and Riesling (Harst et al. 2000; Kikkert et al. 2000; Yamamoto et al. 2000). Beside the production of these PR proteins that are also commonly found in other plant pathosystems, the infected grapes produce a group of secondary metabolites with antifungal activities known as phytoalexins and are chemically classified as low-molecular-weight phenolic or stilbenic compounds: i.e. *trans*- and *cis*-resveratrol (3,5,4′-thihydroxystibene), resveratrol glycosides

(piceid, piceatannol or astringin, polydatin), resveratrol trimers and tetramers (viniferins) and pterostilbene (Langcake 1981; Dercks and Creasy 1989). The genes encoding phytoalexins are widespread among *Vitis* species and varieties, even in the susceptible *V. vinifera*, but disease-resistant genotypes have greater speed and magnitude of expression of this defence machinery when compared to susceptible ones. Some genes encoding the key enzymes of stilbenes biosynthesis, including phenylalanine ammonia-lyase (*pal*), chalcone synthase (*chs*) and stilbene synthase (*stsy*), have been cloned from grapevine DNA (Sparvoli et al. 1994). Wiese et al. (1994) demonstrated that two types of *stsy* genes are involved in resistance, the first one accounting for a quick response, the other for a delayed but more stable response. Two full-size *stsy* genes (*Vst1* and *Vst2*) showed high identity in their coding regions, but substantial differences in promoter regions, likely to be responsible for different expression patterns (Melchior and Kindl 1991). The transfer of grape *stsy* genes in tobacco, rice, barley and wheat increased resistance to the pathogens of those species (reviewed in Bavaresco and Fregoni 2001).

In spite of many studies on post-invasion physiological mechanisms involved in the defence responses, information is still lacking about the early recognition of the invading pathogens that act as a trigger for the cascade of reactions involved in the defence system.

A gene-for-gene mechanism is postulated to occur in specific recognition of the invading haustoria of *P. viticola*. Resistant *Vitis* genotypes can recognise specific elicitors released by the pathogen which initially activate the plant defence mechanisms (Langcake 1981). Other fungal pathogens, such as *O. tuckeri* and *B. cinerea*, may elicit a defence system that could resemble such a model.

A particular class of proteins was demonstrated to play a crucial role in host-pathogen interaction for many plant pathosystems (Ellis et al. 2000). These proteins contain functional domains, such as a nucleotide binding site (NBS), a leucine-rich repeat region (LRR), either a *Drosophila* Toll/mammalian Interleukin-1 receptor (TIR) or a leucine-zipper/coiled-coil motif (cc) and several kinase domains, which allow a specific recognition of the pathogen through a protein-protein interaction. This is thought to initiate the signal transduction for the activation of the defence mechanisms (Hammond-Kosack and Jones 1997).

NBS-LRR resistance genes have been cloned from different species, such as tobacco (Whitham et al. 1994), *Arabidopsis* (Bent et al. 1994; Parker et al. 1997), flax (Lawrence et al. 1995) and tomato (Milligan et al. 1998). In such species, these genes confer resistance to a wide range of diseases caused by viruses, bacteria, fungi and nematodes. Since some domains are widely conserved, degenerate PCR-primers have been designed in previous studies to amplify analogous sequences in other plant species. NBS-sequences were obtained by a PCR-mediated approach and provided candidate resistance genes (or Resistance Gene Analogs, RGAs) in several crops such as soybean (Kanazin et al. 1996; Yu et al. 1996), potato (Leister et al. 1996), maize (Collins et al. 1998), lettuce (Shen et al. 1998), *Brassica* (Joyeux et al. 1999) and *Citrus* (Deng et al. 2000). In these species, genetic analysis demonstrated that RGAs are abundantly repeated through the genome in high copy number, organised in clusters and closely linked to known resistance loci. Some 200 NBS-coding genes were estimated to be present in the *Arabidopsis thaliana* genome, representing nearly 1% of all coding genes. Data on physical mapping demonstrated that NBS-coding sequences are clustered, ranging from 2 to 18 adjacent sequences, with an average of 4.9 sequences per cluster (Meyers et al. 1999). In *Arabidopsis*, genetic mapping provided evidence that the positions of RGAs correspond to 21 known disease resistance loci (Speulman et al. 1998) and they co-segregate with fungal disease-linked markers (Aarts et al. 1998).

Since NBS-LRR sequences are linked to, or possibly part of, the resistance genes and are well conserved in the plant kingdom, they provide a molecular tool for genetic mapping and positional cloning of resistance genes in grape. The aims of this work were the characterisation of RGAs in grape and the development of a set of markers based on NBS-sequences suitable for discriminating resistant and susceptible genotypes.

Materials and methods

Plant material and DNA extraction

Leaf samples of the *Vitis* genotypes reported in Table 1 were collected from the germplasm repository held at the Experimental Farm of the University of Udine, Italy.

Genomic DNAs were extracted from 1 g of young leaves using a cetyltrimethylammonium bromide (CTAB) method (Doyle and

Table 1 *Vitis* genotypes used for cloning grape NBS-sequences and for RFLP analysis with NBS-based probes

Genotype	Type of accession	Origin	Type of response to fungal diseases
V. amurensis	Wild species	East Asia	Resistant to downy mildew
V. riparia	Wild species	North America	Resistant to downy mildew
V. cinerea	Wild species	North America	Resistant to downy and powdery mildew
V. vinifera cv Cabernet Sauvignon	Cultivated grapevine	South Europe	Susceptible to several fungal diseases
V. vinifera cv Chardonnay	Cultivated grapevine	South Europe	Susceptible to several fungal diseases
V. vinifera cv Merlot	Cultivated grapevine	South Europe	Susceptible to several fungal diseases
Vitis hybrid cv Regent	Complex hybrid	Breeding	Tolerant/resistant to downy mildew
Vitis hybrid cv Seyval	Complex hybrid	Breeding	Tolerant/resistant to downy mildew
Vitis hybrid cv Bianca	Complex hybrid	Breeding	Tolerant/resistant to downy mildew

Doyle 1990) with the following modifications: 2.5% CTAB, 1 mM of Tris-HCl (pH = 8.0) and 1% polyvinylpolypyrrolidone were mixed in the extraction buffer, and purification from polysaccharides was performed by means of a selective precipitation with 2-butoxyethanol. Genomic DNAs of *Vitis amurensis* and *Vitis riparia* were used as a template for PCR-based cloning of grape NBS-sequences. Genomic DNAs of all *Vitis* genotypes listed in Table 1 were used in Southern blots for RFLP analysis.

Amplification and cloning of NBS-like sequences from genomic DNA using degenerate primers

The following four pairs of degenerate primers were based on the results of previous studies in order to amplify DNA sequences spanning the most conserved domains of the nucleotide binding site: *NBS-F1*/*NBS-R1* (Yu et al. 1996), *RG1*/*RG2* (Aarts et al. 1998), *LM637*/*LM638* (Kanazin et al. 1996) and primers from Shen et al. (1998) with some modifications (*P-loop*: 5′GAATTC-GGNGTNGGNAARACNAC; *GLPL*: 5′GTCGACARNGCNAR-NGGNARHCC).

All primer pairs were tested for the amplification of DNA from *V. vinifera* cv Merlot, following the PCR conditions reported in the original experiments.

The primer pair *RG1*/*RG2* was retained as the oligonucleotides of choice for NBS priming in grape on the basis of the clearest, size-specific amplified band produced from grape genomic DNA.

PCR-amplifications for cloning were performed in a 50-μl reaction volume containing 200 μM of each dNTP, 0.5 μM of each *RG1*/*RG2* primer, 2.5 U of Expand High Fidelity polymerase (Roche) with the buffer solution supplied by the manufacturer, 2.5 mM of $MgCl_2$ and 50 ng of template DNA. The PCR templates were genomic DNAs of *V. amurensis* and *V. riparia*. The initial denaturation step was 95 °C for 5 min, followed by 30 thermal cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, with the final extension step increased to 10 min. PCR products were electrophoresed on a 2% agarose gel and the target 530-bp band was selectively recovered onto a DEAE cellulose membrane (Schleicher and Schuell, NA-45), eluted and purified by means of ethanol precipitation. Samples were cloned in TOPO-TA vector (Invitrogen) and transformed in competent *Escherichia coli*, following the manufacturer's instructions. A total of 71 putative recombinant colonies were obtained, 57 from *V. amurensis* (individually named as *rgVamuxxx*) and 14 from *V. riparia* (individually named as *rgVripxxx*, where *xxx* is the number of the clone).

Screening and sequencing a multigene NBS family

Each recombinant colony was amplified by streaking a sub-culture onto selective solid medium. A small amount of sub-cultured bacteria was re-suspended in 200 μl of dH_2O, boiled for 2 min and centrifuged at 13,000 g for 1 min. Ten microliters of the supernatant containing the released plasmid DNA were used as a template for PCR amplification in a 100-μl reaction volume containing 10 mM of Tris–HCl (pH = 9.0), 50 mM of KCl, 1.5 mM of $MgCl_2$, 200 μM of each dNTP, 0.2 μM each of forward and reverse M13 universal primers and 2.5 U of *Taq* polymerase (Pharmacia). The initial denaturation step was 95 °C for 5 min, followed by 25 thermal cycles of 95 °C for 50 s, 56 °C for 50 s and 72 °C for 1 min, with the final extension step increased to 7 min.

A 12.5-μl aliquot of the PCR products was electrophoresed on a 2% high resolution agarose gel (MetaPhor, FMC Bioproducts) and stained with ethidium bromide at 0.5 μg/ml in order to verify the length of the cloned fragment. The remaining 90 μl were submitted to RFLP analysis with the aim of selecting unique, non-redundant clones. Seven aliquots containing 12.5 μl each of the PCR-amplified fragment from each clone were digested overnight with 0.5 U of the endonucleases *Taq*I, *Sau*3AI, *Tsp*509I (Biolabs), *Alu*I, *Mse*I/*Msp*I, *Sal*I/*Pvu*II and *Hae*II/*Hind*III (Pharmacia) at the temperature recommended by the manufacturer's instructions. The restriction fragments were separated on a 2% MetaPhor gel.

All cloned fragments that revealed a unique restriction pattern were retained and the corresponding sub-cultured colonies were processed for sequencing of the recombinant plasmid DNA.

Sequence analysis

A total of 24 out of 29 sequenced clones gave DNA sequence information with a high level of accuracy and were submitted to sequence analysis. Nucleotide sequences were translated and the corresponding amino acid sequences were aligned with CLUSTALX software (Thompson et al. 1997). Amino acid sequences from resistance genes cloned from other plant species (gene *N* from tobacco, genes *M* and *L6* from flax, genes *RPS2*, *RPS4*, *RPP5* from *Arabidopsis*) were added to the set of NBS-sequences isolated from grape. Cluster analysis was carried out by the CLUSTALX package based on the Neighbor-joining method (Saitou and Nei 1987) and drawn by TREEVIEW (Page 1996). Searches of amino acid sequence similarities with GenBank accessions were performed by BLASTP software. Pairwise comparisons of grape NBS-sequences to each other were performed using the FASTA algorithm (GCG Wisconsin package) with a gap creation penalty = 12 and a gap extension penalty = 2.

RFLP markers generated by RGA probes tested on resistant and susceptible grape genotypes

Three downy mildew resistant species (*V. amurensis*, *V. riparia* and *Vitis cinerea*), three susceptible *V. vinifera* cultivars (Cabernet Sauvignon, Chardonnay and Merlot) and three inter-specific hybrids classified as tolerant/resistant to the disease (Seyval, Regent and Bianca) were screened with RGA-based probes. A set of complete digestions was performed using 7 μg of each genomic DNA and 0.5–1.0 U/μg of the following endonucleases: *Taq*I and *Dde*I at 65 °C and 37 °C, respectively. Digested DNAs were electrophoresed on a 0.8% agarose 22 cm-long gel for at least 6 h at 3.5 V/cm and blotted onto uncharged nylon membranes. The membranes were hybridised at 68 °C with the following RGA-based probes labelled with digoxygenin: *rgVamu012*, *rgVamu026* and *rgVrip064*. The stringency wash was 0.2 × SSC, 0.1% SDS at 60 °C for 20 min repeated twice. Chemi-luminescent detection was performed using Anti-Digoxygenin-AP, Fab fragments and CSPD (Roche) according to the manufacturer's instructions.

Results and discussion

Isolation and characterisation of grape NBS-sequences

Three out of four primer pairs produced a major band of the expected size according to the source species from which the primers were designed and other species reported in the literature (Fig. 1). In each pair, forward primers were designed to anneal to the sequence encoding the *kinase-1a* or *P-loop* domain that is thought to interact with the γ-phosphate of ATP or GTP in some phosphorylation reactions. Reverse primers were designed in the antisense direction corresponding to the coding sequences of an amino acid domain, known as *GLPL* or domain 5 region (Lawrence et al. 1995), which in the *Arabidopsis RPS2* protein is postulated to reside in the trans-membrane region (*LM637*, *RG2*) or to a ‘SSIIITTR’ coding sequence laid closely downstream to a *kinase-3a* domain (*NBS-R1*).

The *NBS-F1*/*NBS-R1* primers amplified two further strong bands, 900 and 1000-bp long, respectively, beside

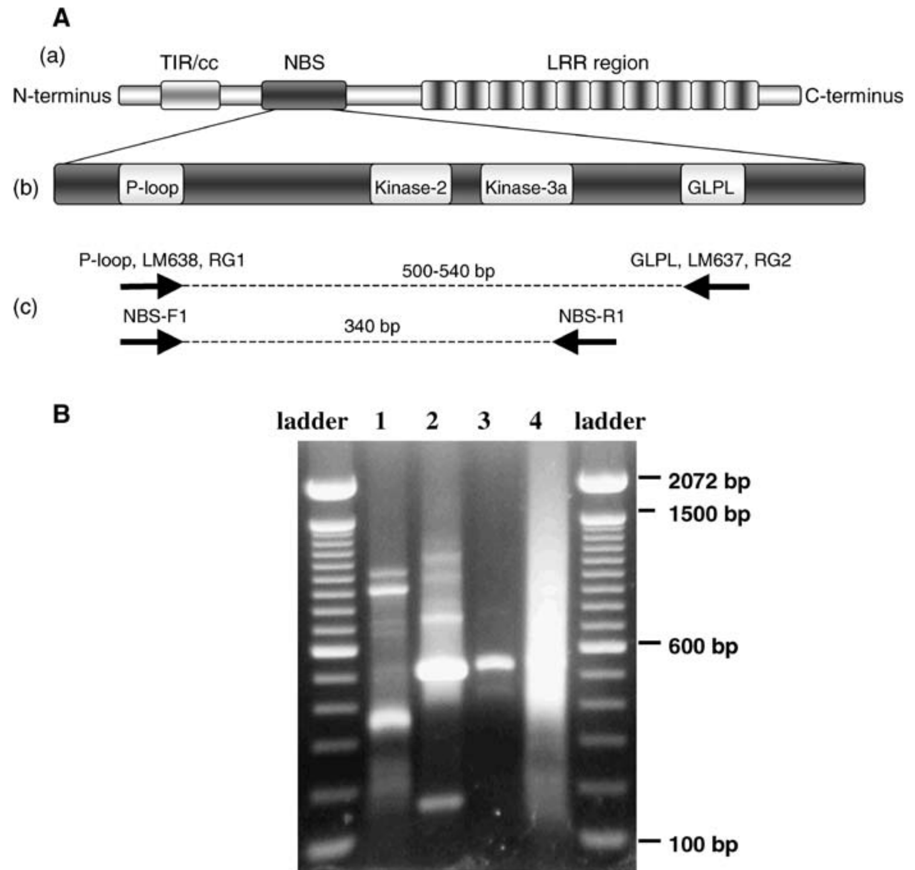

Fig. 1A, B Model of the NBS-LRR type of *R*-genes with either a TIR or a coiled coil (*cc*) N-terminal domain and heterologous amplification in grape. **A** Relative positions of (*a*) putative functional features of NBS-LRR resistance proteins, (*b*) the most conserved domains within the NBS, and (*c*) degenerate primers obtained from previous studies (see text for details). **B** PCR products amplified with four pairs of degenerate primers from template DNA of *V. vinifera* cv Merlot. The primer pairs used for PCR and the expected size of the amplified bands are respectively: *lane1 NBS-F1/NBS-R1*, 340 bp; *lane2 LM637/LM638*, 500 bp; *lane3 RG1/RG2*, 530 bp; *lane4 P-Loop/GLPL*, 540 bp. The 100-bp marker was loaded in the external lanes

the 340-bp target fragment, so that the resulting pattern was similar to that already shown in soybean (Yu et al. 1996). *LM637/LM638* primers produced at least five scorable bands of unexpected size in a slight smear, thus making the target fragments hard to be cloned. *P-loop/GLPL* primers modified from the original work of Shen et al. (1998) produced a smear, most likely due to a too high level of degeneracy in the oligonucleotides used. *RG1/RG2* primers amplified a clear, unique and size-specific major band. The length of the amplified band was about 530-bp long in both *V. amurensis* and *V. riparia* (data not shown), a size similar to that observed in tobacco, flax and *Arabidopsis*. An additional, very faint 800-bp long band, almost unscorable when stained with ethidium bromide, was recovered among the cloned fragments, and its length was similar to that reported in *Arabidopsis* by Aarts et al. (1998). The primer pair *RG1/RG2* was retained as the oligonucleotides of choice for NBS priming in grape and used to generate the PCR-products to be cloned.

A total of 60 size-specific fragments were identified from 71 putative recombinant clones, 53 of which were 530-bp long and the remaining seven about 800-bp. Since the longer fragments isolated in *Arabidopsis* contained introns (Aarts et al. 1998), only the 530-bp-long sequences were further characterised. As expected for a multi-sequence family, the 530-bp inserts resulted in a pool of electromorphs of the same size but different in nucleotide sequences. Based on the restriction pattern, 29 out of the 53 clones showed unique patterns (data not shown) and were sequenced. This high number of unique inserts recovered from a limited number of clones screened could be considered as indirect evidence for the high number of NBS-containing sequences spread along the grape genome.

A single endonuclease with a 4-bp recognition site, such as *Sau*3AI, discriminated at least 14 classes of inserts on the agarose gel. Among other endonucleases, *Tsp*509I, *Taq*I and a combination of *Hae*II/*Hin*dIII allowed us to discriminate 13, 9 and 9 classes of fragments, respectively, while lower levels of polymorphism were detected with the remaining endonucleases (*Alu*I, *Mse*I/*Msp*I, *Sal*I/*Pvu*II). The high number of RFLP patterns identified in the restriction experiment suggests a very high polymorphism for these fragments at the nucleotide sequence level.

Relationships between grape NBS-like sequences and other plant disease resistance genes

Several NBS-sequences identified in grape matched the amino acid sequences of comparable regions of disease-resistance genes published in GenBank (Table 2). Only grape NBS-like sequences showing more than 30% identity with other plant *R*-genes, and supported by a E value lower that e^{-05}, are being discussed here. The expected (E) value refers to the number of matches expected by chance

Table 2 Results of the search for similarity between grape NBS-sequences and GenBank accessions carried out using the BLASTP algorithm

Grape NBS	GenBank protein accession showing highest similarity	%[a]	E value[b]	Other GenBank protein accessions showing high similarity	%[a]	E value[b]
rgVrip003	T1F9.21, *Arabidopsis thaliana*	49	4e-46	Similar to RPS2 disease resistance protein, *Arabidopsis thaliana*	50	9e-46
rgVrip004	TMV resistance protein N, *Arabidopsis thaliana*	63	1e-04			
rgVamu006	unknown, *Cicer arietinum*	44	2e-29	Resistance protein LM6, *Glycine max*	41	2e-28
rgVamu010[c]	Resistance-like protein KNBS3, *Glycine max*	44	4e-10	Flax rust resistance protein L6, *Linum usitatissimum*	37	6e-05
rgVamu012[c]	Putative resistance gene homologue, *Cucumis melo*	45	5e-36	TMV resistance protein N, *Nicotiana glutinosa*	46	2e-30
rgVamu014[c]	NBS-kinase protein Z2, *Solanum tuberosum*	43	3e-15	Flax rust resistance protein M, *Linum usitatissimum*	31	3e-12
rgVamu018[c]	T1F9.21, *Arabidopsis thaliana*	53	1e-48	Resistance to *Pseudomonas syringae* protein 5, *Arabidopsis thaliana*	51	3e-43
rgVamu025	Putative resistance gene homologue, *Cucumis melo*	41	7e-29	Resistance protein LM6, *Glycine max*	41	8e-28
rgVamu026[c]	T1F9.21 *Arabidopsis thaliana*	48	5e-45	Resistance to *Pseudomonas syringae* protein 5, *Arabidopsis thaliana*	46	3e-40
rgVamu028	unknown, *Cicer arietinum*	45	6e-33			
rgVamu029[c]	Contains similarity to NBS/LRR disease resistance protein, *Arabidopsis thaliana*	51	3e-41	Resistance to *Pseudomonas syringae* protein 5, *Arabidopsis thaliana*	45	1e-35
rgVamu031	AC018721 hypothetical protein, *Arabidopsis thaliana*	28	9.1			
rgVamu032	Putative resistance gene homologue, *Cucumis melo*	41	2e-32			
rgVamu035[c]	Contains similarity to NBS/LRR disease resistance protein, *Arabidopsis thaliana*	51	4e-47	Resistance to *Pseudomonas syringae* protein 5, *Arabidopsis thaliana*	51	7e-47
rgVamu037[c]	Disease resistance-like protein, *Glycine max*	68	5e-10	Flax rust resistance protein L6, *Linum usitatissimum*	54	3e-08
rgVamu038[c]	Putative resistance gene homologue, *Cucumis melo*	43	8e-33	TMV resistance protein N, *Nicotiana glutinosa*	41	3e-27
rgVamu044	NBS-kinase protein Z2, *Solanum tuberosum*	43	3e-13	Putative disease resistance protein SB5, *Phaseolus vulgaris*	43	2e-11
rgVamu045	Disease resistance-like protein, *Glycine max*	70	1e-05			
rgVamu050[c]	unknown, *Cicer arietinum*	44	4e-30	TMV resistance protein N, *Nicotiana glutinosa*	43	3e-25
rgVamu053[c]	Resistance protein LM6, *Glycine max*	42	7e-34	TMV resistance protein N, *Nicotiana glutinosa*	44	7e-31
rgVamu058	A sunflower resistance gene homolog, *Helianthus annuus*	46	2e-30	Avr9/Cf-9 rapidly elicited protein 4, *Nicotiana tabacum*	44	2e-24
rgVrip064[c]	A sunflower resistance gene homolog, *Helianthus annuus*	44	2e-29	TMV resistance protein N, *Nicotiana glutinosa*	41	4e-25
rgVrip068	NBS/LRR disease resistance protein, *Arabidopsis thaliana*	64	0.059			
rgVrip070	A sunflower resistance gene homolog, *Helianthus annuus*	44	2e-28	Resistance protein LM6, *Glycine max*	39	5e-26

[a] Percentage of amino acid identity
[b] The expected (E) value refers to the number of matches expected by chance alone. The lower the E value, the more strongly supported the match is
[c] Only grape NBS-like sequences showing high similarities to other plant *R*-genes supported by a E value lower than e^{-05} were discussed in the text

alone. The lower the E value, the more strongly supported the match is. When adopting these criteria, the highest level of the most strongly supported identity was found between clones *rgVamu018*, *rgVamu035* and the gene *RPS5* of *Arabidopsis* (51% identity, E value $3e^{-43}$ and $7e^{-47}$, respectively). Among the others, clone *rgVamu012* shared lower identity with tobacco gene *N* (46%, E value $2e^{-30}$), followed by clones *rgVamu053* (44%, E value

Table 3 Amino acid identities (%) of grape NBS-sequences when compared to each other and to known resistance genes of other plant species. Pairwise comparison was performed using the FASTA algorithm with a gap creation penalty = 12 and a gap extension penalty = 2. * No sensible values were obtained using the above parameters of FASTA. Columns and/or rows without any sensible value were not included in the matrix

	003	006	010	012	014	018	025	026	028	029	031	032	035	037	038	044	045	050	053	058	064	070	N	L6	RPS4	RPP5	M	RPS2
rg*Vrip003*	#	27,7	*	*	*	55,9	27,0	86,9	28,8	83,0	*	26,0	56,2	*	28,8	*	*	30,9	28,7	28,7	30,6	29,5	*	*	*	*	*	*
rg*Vrip004*		*	29,7	*	29,7	*	*	*	*	*	21,9	*	*	*	*	29,7	38,2	*	*	*	*	*	*	*	*	*	*	*
rg*Vamu006*		#	*	53,4	*	26,1	97,1	28,3	91,9	26,1	*	95,9	27,4	*	91,9	*	*	91,9	98,8	98,3	95,4	95,9	46,1	42,1	33,0	31,8	40,7	27,8
rg*Vamu010*			#	*	42,6	*	*	*	38,1	*	32,0	*	*	39,4	*	42,6	*	*	*	*	*	*	*	*	22,9	*	*	*
rg*Vamu012*				#	*	26,8	54,1	30,8	54,0	31,9		53,2	27,0	*	54,4	*	*	54,0	55,1	55,1	55,1	54,4	50,8	41,1	37,9	39,9	40,8	*
rg*Vamu014*					#	*	*	*	*	*	47,5	*	*	37,6	100	*	*	*	*	*	*	*	*	*	*	*	*	25,1
rg*Vamu018*						#	23,7	60,7	28,6	58,4	*	25,4	60,7	*	28,9	*	*	28,3	27,5	27,5	27,8	25,7	27,2	28,9	21,7	26,1	28,6	41,6
rg*Vamu025*							#	29,0	91,2	27,9	*	97,6	26,1	*	91,2	*	*	91,2	98,2	97,6	94,7	95,3	45,8	42,0	34,5	32,0	39,9	26,7
rg*Vamu026*								#	29,8	92,1	*	28,4	55,4	*	29,6	*	*	29,4	30,1	29,6	28,9	27,4	28,8	29,8	26,2	25,7	25,9	40,4
rg*Vamu028*									#	28,7	*	90,6	29,3	31,1	97,1	*	*	97,7	93,1	93,1	91,4	91,8	45,6	41,7	34,6	33,1	39,1	28,7
rg*Vamu029*										#	*	27,4	54,8	*	29,6	*	*	28,7	29,5	29,0	29,6	27,7	27,2	27,9	24,6	25,8	24,9	38,2
rg*Vamu031*											#	*	*	36,0		47,5	*	*	*	*	*	*	*	*	*	*	*	*
rg*Vamu032*												#	25,6	*	90,6	*	*	90,6	97,1	96,5	93,5	94,1	45,2	42,0	33,9	32,6	39,9	24,7
rg*Vamu035*													#	*	28,7	*	*	28,7	27,1	27,1	26,8	*	27,7	23,8	26,6	24,5	25,3	38,4
rg*Vamu037*														#	30,5	37,6	*	30,5	*	*	*	*	*	*	*	*	*	*
rg*Vamu038*															#	*	*	97,7	93,1	93,1	91,4	91,8	45,1	42,2	35,2	33,1	39,7	28,2
rg*Vamu050*																		#	93,1	93,1	91,4	91,8	46,2	41,7	33,5	33,1	39,1	28,7
rg*Vamu053*																			#	99,4	96,6	97,1	47,3	43,3	35,7	33,7	41,3	27,8
rg*Vamu058*																				#	96,6	*	47,3	43,3	35,7	33,7	41,3	27,8
rg*Vrip064*																					#	99,4	46,7	42,2	34,6	32,6	40,8	28,2
rg*Vrip070*																						#	45,8	41,4	32,8	31,4	39,9	26,7
gene *N*																							#	38,0	35,7	41,1	36,6	26,5
gene *L6*																								#	31,0	31,7	80,7	26,9
gene *RPS4*																									#	26,7	29,7	25,1
gene *RPP5*																										#	31,7	*

```
                                 P-loop                                                                                  kinase-2           kinase-3a
rgVamu018                  1 GMGGVGKTTLLKKINNDFLT-----TSSDFDVVIWVVVSK----PSNIEKIQEVIWNKLQIPRDIWEFRSSKEEKAVEILRVLKTKKFVLLLDDIW-ERLDLLEMGVPRPDARNK---SK 107
rgVamu035                  1 GMGGVGKTTLLTRINNELLK-----TRLEFDAVIWVTVSR----PANVEKVQRVLFNKVEIPQDKWEGRS-EDERAEEIFNVLKTKKFVLLLDDIW-ERLDLSKVGIPPLNPQDK---LK 106
rgVamu026                  1 GMGGVGKTTLLRKINNEYFG-----KSNDFDVVIWIVVSK----PISIEKIQEVILKKLSTPEHNWKSSS-KEERTAEIFKLLKAKNFVILLDDMW-ERLDLLEVGIPDLSDQTK---SR 106
rgVamu029                  1 GMGGVGKTTLLRKINNEYFA-----KRNDFDVVIWIVVSK----PISIEKIQEVILKKLSTPEHNWKSSS-KEEKTAEIFKLLKAKNFVILLDDTW-ERLDLLEVGIPDLSDQTK---SR 106
rgVrip003                  1 GMGGVGKTTLLRKINNEYFG-----KRNDFDVVIWIVVSK----PINIGNIQDVILNKLPTPEHKWKNRS-KEEKAAEICKLLKAKNFVILLDDMW-ERLDLFEVGIPHLGDQTK---SK 106
rgVamu006                  1 GMGGVGKTTIAKAVYNDIS------YQFDGSSFLNNVRERS---KDNALQLQQELLHGILKGKS--LKVSNMDEGIQMIKRSLSSKRVLVVFDDVD-DLMQIENLAEEHSWFGPR---CX 105
rgVamu053                  1 GMGGVGKTTIAKAVYNDIS------YQFDGSSFLNNVRERS---KDNALQLQQELLHGILKGKS--LKVSNMDEGIQMIKRSLSSKRVLVVFDDVD-DLMQIENLAEEHSWFGPR---SR 105
rgVamu025                  1 GMGGVGKTTIAKAVYNDIS------YQFDGSSFLNNVRERS---KDNALQLQQELLHGIZKGKS--PKVSNMDEGIQMIKRSLSSKRVLVVFDDVD-DLMQIENLAEEHSWFGPR---SR 105
rgVamu032                  1 GMGGVGKTTIAKAVYNDIS------YQFDGSSFLNNVRERS---KDNALQLQQELLHGILKGKS--PKVSNMDEGIQMIKRSLSSKRVLVVFDDVD-DLMQIENLAEEHSWFGPT---SR 105
rgVamu058                  1 GMGGVGKTTIAKAVYNDIS------YQFDGSSFLNNVRERS---KDNALQLQQELLHGILKGKS--LKVSNMDEGIQMIKRSLSSKRVLVVFDDVD-DLMQTENLAEEHSWFGPR---SR 105
rgVrip064                  1 GMGGVGKTTIAKAVYNDIS------YRFDGSSFLNNVRERS---KDNALQLQQELLHGSLKGKS--LKVSNMDEGIQMIKRSLSSKRVLVVFDDVD-DLMQLENLAEERSWFGPR---SR 105
rgVrip070                  1 GMGGVGKTTIAKAVYNDIS------YRFDGSSFLNNVRERS---KDNALQLQQELLHGSLKGKS--LKVSNMDEGIQMIKRSLSSKRVLVVFDDVD-DLMQLENLAEEHSWFGPR---SR 105
rgVamu038                  1 GMGGVGKTTIAKAVYNDIS------YLFDGSSFLNNVRERS---KDNALQLQQELLHGILKGKS--LKVSNMDEGIQMIKRSLCSKRVLVVFDDVD-DLMQLENLAEEHSWFGPR---SR 105
rgVamu050                  1 GMGGVGKTTIAKAVYNDIS------YQFDGSSFLNNVRERP---KDNALQLQQELLHGTLKGKS--LKVSNMDEGIQMIKRSLCSKRVLVVFDDVD-DLMQLENLAEEHSWFGPR---SR 105
rgVamu028                  1 GMGGVGKTTIAKAVYNDIS------YQFDGSSFLNNVRERS---KDNALQLQQELLHGTLKGKS--LKVSNMDEGIQMIKRSLCSKRVLVVFDDVD-DLMQLENLAEEHSWFGPR---SR 105
rgVamu014                  1 GMGGVGKTTIAKAVYNDIS------YQFDGSSFLNNVIERS---KDNALQLQQELLHGSLKGKS--LKVSNMDEGIQMIKRSYCSTRVLVVFYDVD-DLMQLENLAEEHSCLVQE---VG 105
rgVamu044                  1 GMGGVGKTTIAKAVYNDIS------YQFDGSSFLNNVIERS---KDNALQLQQELLHGSLKGKS--LKVSNMDEGIQMIKRSYCSTRVLVVFYDVD-DLMQLENLAEEHSCLVQE---VG 105
rgVamu012                  1 GMGGVGKTTIARAVYEQIA------KQFKACCFLSNVRKDSE--KRGLVKLQEELLSRLLEEGK--ISISTVDIGLAFIKTRLCFKRVLIVLDDVD-KPQQLEYLAGKHDWFGPG---SR 106
TOBACCO gene N             1 GMGGVGKTTIARAIFDTLLGRMDSSYQFDGACFLKDIKEN----KRGMHSLQNALLSELLRE-K--ANYNNEEDGKHQMASRLRSKKVLIVLDDIDNKDHYLEYLAGDLDWFGNG---SR 110
FLAX gene M                1 GMGGIGKTTTAKAVYNKIS------SHFDRCCFVDNVRAMQE-QKDGIFILQKKLVSEILRMDS--VGFTNDSGGRKMIKERVSKSKILVVLDDVD-EKFKFEDILGCPKDFDSG---TR 107
FLAX gene L6               1 GMGGIGKTTTAKAVYNKIS------SCFDCCCFIDNIRETQE--KDGVVVLQKKLVSEILRIDSGSVGFNNDSGGRKTIKERVSRFKILVVLDDVD-EKFKFEDMLGSPKDFISQ---SR 108
ARABIDOPSIS gene RPS2      1 GPGGVGKTTLMQSINNELIT-----KGHQYDVLIWVQMSR----EFGECTIQQAVGARLGLSWD--E-KETGENRALKIYRALRQKRFLLLLDDVW-EEIDLEKTGVPRPDRENK---CK 104
ARABIDOPSIS gene RPP5      1 GQSGIGKSTIGRALFSQLS------SQFHHRAFLTYKSTSGSDVSGMKLSWQKELLSEILGQKD------IKIEHFGVVEQRLNHKKVLILLDDVD-NLEFLKTLVGKAEWFGSG---SR 104
ARABIDOPSIS gene RPS4      1 GMPGIGKTTLLKELYKTWQ------GKFSRHALIDQIRVKS--KHLELDRLPQMLLGELSKLNH------PHVDNLKDPYSQLHERKVLVVLDDVS-KREQIDALREILDWIKEGKEGSR 107
rgVamu010                  1 GMGGVGKTTIAKIVYNEIQ------YQFTGASFLQDVRETFN--KGYQLQLQQQLLHDTVGN-DV--KFSNINKGVNIIKSRHFAQKRFLLZLMMWIGCSNZSQWLEVLNGLVQE---VQ 106
rgVamu037                  1 GMGGVGKTTIARAVYEQIA------KQFKACCFLSNVRKDSE--KRGLVKLQEEFFLGXWRKGKZ---AZALSILDLLLZRQGCALKGFLLFLMMLISHNNZNTZLESMIGLVQE---VE 106
rgVamu031                  1 GMGGVGR-PLSPRLFIMIS-----HINLMVVVFLTMLEKDP---KTMHFNYNKNYFMVSZREKV---QKZAIWMKEFRZZRGVSALKGFLLFLMTWMIZCKZKTWQKSIVGLVQE---VG 106
rgVamu045                  1 GMGGVGKTTIARAIYEQIY------TQFEGFYFLANVREGSQ--KTWPGSFTSGTSLKNIEGWKS-KCRNFQYKNZFYKGZTPLZEGSYCSZZCGZLGTVGIFSRKPRLVWFRKZ----- 106
rgVrip004                  1 GMGGVGKTTITKYICNEIS------YEFESISFLENVREKSK--LLLFASITGTTSZQCSKAEEZNKIEQYSZRNQYDKEZGRLKKSSYCSZZCRLFTPIKCLSWKLZLVWFGKZ----- 107
rgVrip068                  1 GMGGWENYPFEQDQQZVPQIQG-WVZRSDLGDCVQTSKCR----EGSASSFQZIGDSZZQLGRZ----KZ-GRKERSNIQCLEDEENCRLIRZYMG--ASZSLCSGDSSCNZWKZ---VQ 105
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                                                                           GLPL-hydrophobic domain 5
rgVamu018                108 IVFTTRSQDVCHQMKA--HKSIEVACLSSEAAWTLFQKEVGEETLS---HPHILRLAKTVAEECKGFPFAFKVLG 178
rgVamu035                107 MVLTTRSKDVCQDMEV--TESIEVNCLPWEDAFALFQTKVGADTINS--HPDIPKLAEMVAKECCGFPLALKVLG 178
rgVamu026                107 VVLTTRSERVCDEMEV--HKRMRVECLTPDEAFSLFCDKVGENILNS--HPDIKRLAKIVVEECNGLPLAFKVLG 178
rgVamu029                107 IVLTTRSERVCDEMEV--HKRMRVECLTPGEAFSLFCDKVGENILXS--HPDINLLAQIVVEECCZLPLPLKVLG 178
rgVrip003                107 VILTTRSERVCDEMEV--HKRMRVKCLTPDEAFSLFRDKVGENILNS--HPEIKRLAKIVVEECKGLPLALKVL- 177
rgVamu006                106 IIITTRHKHFLTQYGV--KESYEVPKLHDAEAIELFSWWAFKQNLP---NEIYKNLSYQVVDYAKGLPLALKV-- 174
rgVamu053                106 IIITTRHKHFLTQYGV--KESYEVPKLHDAEAIELFSWWAFKQNLP---NEIYKNLSYQVVDYAKGLPLALKVLG 176
rgVamu025                106 IIITTRHKHFLTQYGV--KESYEVPILHDAEAIELFSWWAFKQNLP---NEIYKNLSYQVVDYAKGLPLA----- 171
rgVamu032                106 IIIXTRHKHFLTQYGV--KESYEVPILHDAEAIELFSWWAFKQNLP---XEIYKNLSYQVVDYAKGLPLA----- 171
rgVamu058                106 IIITTRHKHFLTQYGV--KESYEVPKLHDAEAIELFSWWAFKQNLP---NEIYKNLSYQVVDYAKGLPLALKVLG 176
rgVrip064                106 IIITTRHKHFLTQYGV--QESYEVPKLHDAEAIELFSWWAFKQNLP---NEIYKNLSYQAVDYAKGLPLALKVLG 176
rgVrip070                106 IIITTRHKHFLTQYGV--QESYEVPKLHDAEAIELFSWWAFKQNLP---NEIYKNLSYQAVDYAKGLPLA----- 171
rgVamu038                106 IIITTRHKRFLTQYGV--KESYEVQKLHDEEAIELFSLWAFPQNLP---SEIYKNLSYRVVDYARGLPFALKVLG 176
rgVamu050                106 IIITTRHKRFLTQYGV--KESYEVQKLHDEEAIELFSLWAFPQNLP---SEIYKNLSYRVVDYAKGLPFALKVLG 176
rgVamu028                106 IIITTRHKRFLTQYGV--KESYEVQKLHDEEAIELFSLWAFPQNLP---SEIYKNLSYRVVDYATGFPLALKVLG 176
rgVamu014                106 SSZQLDINVFZPNMES--KNHMKFKNCMMKKLLSCLVCGLFHKIFL---AKFIKISPTGZZIMLKVSPSPSRSS- 175
rgVamu044                106 SSZQLDINVFZPNMES--KNHMKFKNCMMKKLLSCLVCGLFHKIFL---AKFIKISPTGZZIMLKVSPSPSRSS- 175
rgVamu012                107 IIITTRDVHLLNKVGV--NGVYEVVHLNNXDAITLFSRHAFKEDHP---TEDYIELSNYAVXYAKGLPLALKVLG 177
TOBACCO gene N           111 IIITTRDKHLIEKN----DIIYEVTALPDHESIQLFKQHAFGKEVP---NENFEKLSLEVVNYAKGLPLALKVWG 179
FLAX gene M              108 FIITSRNQNVLSRLNENQCKLYEVGSMSEQHSLELFSKHAFKKNTP---PSDYETLANDIVSTTGGLPLTLKVTG 180
FLAX gene L6             109 FIITSRSMRVLGTLNENQCKLYEVGSMSKPRSLELFSKHAFKKNTP---PSYYETLANDVVSTTGGLPLTLKVTG 181
ARABIDOPSIS gene RPS2    105 VMFTTRSIALCNNMGA--EYKLRVEFLEKKHAWELFCSKVWRKDLLE--SSSIRRLAEIIVSKCGGLPLALITLG 176
ARABIDOPSIS gene RPP5    105 IIVITQDRQLLKAHEI--DLVYEVKLPSQGLALKMISQYAFGKDSP---PDDFKELAFEVAELVGSLPLGLSVLG 177
ARABIDOPSIS gene RPS4    108 VVIATSDMSLTNGLV---DDTYMVQNLNHRDSLQLFHYHAFIDDQANPQKKDFMKLSEGFVHYARGHPLALKVLG 180
rgVamu010                107 LSLQPETNICWLSMEZ-----LYHIRLQDYIMRKLFNSSANMPLNKTFLRKIMZTSQIAWFNMLKVSPSPSKSS- 176
rgVamu037                107 SLZQQEMCICLIKLES-----MEYMKLCIZITTMLLRSLVDMPLKRTIPQKIIZSSPXMQFLMQRVSPSPSRSS- 176
rgVamu031                107 SSZQLDTNIFZPNMES-----KNHMKFRNYMMQKLLSCLVGGLSNKIFLMKFIKISPTGZZIMLKVSPSPSRS-- 175
rgVamu045                107 NYYNKQRKAFAYYTWS--QHYLZSZGIRGZZSFSTLSSVCLZTQPP---WRRLYAAMLQINTLZQRSPLRPQSPR 177
rgVrip004                108 NHHNHERZACTMCASS--TCIIRGQGIEKQRSKSTLZLECLZNKSP---NKKLCISLRTCSWLZKVSPSPSKSS- 177
rgVrip068                106 GGIYNPIFNCRPRYGS---ZGHZSEVLSMGGSICSVSDICRRRRZYS--HPHIPKLAEIAAKECDGLPLALKV-- 174
```

key domains in tobacco gene *N*:
P-loop: N-terminal aa position 1-9 GMGGVGKTT
kinase-2: aa position 82-89 LIVLDDID
kinase-3a: aa position 105-110 FGNGSR
GLPL: C-terminal aa position 170-177 GLPLALKV

Fig. 2 ClustalX multiple alignment of deduced amino acid sequences of grape NBS-sequences and analogous domains in known *R*-genes. The conserved domains are *shaded* as well as the reference gene *N* of tobacco for which the amino acid position of the key domains is reported

7e–31), *rgVamu050* (43%, E value 3e–25), *rgVamu038* (41%, E value 3e–08) and *rgVrip064* (41% E, value 4e–25). Clones *rgVamu037* and clone *rgVamu010* were 54% (E value 3e–08) and 37% (E value 6e–05), respectively, identical to flax gene *L6*. Clone *rgVamu014* was 31% (E value 3e–12) identical to flax gene *M*. Even if the level of amino acid identity is considered moderate, at least in principle, the extent of identity at the NBS-region between genes *N* and *L6* (38.0%), *N* and *RPP5* (41.1%), *L6* and *RPS2* (26.9%) provides a comparison that could account for the values found for grape NBS-sequences and other plant disease-resistance genes (Table 3). Moreover, PCR-derived NBS-sequences identified in other plant species with a similar approach showed a comparable range of identities with published disease-resistance genes: 30–45% in *Phaseolus vulgaris* (Rivkin et al. 1999), 31–56% in *Brassica oleracea* (Vicente and King 2001), 30–39% in soybean (Graham et al. 2000), 18–48% in *Citrus* (Deng et al. 2000) and 19–44% in lettuce (Shen et al. 1998).

When using a more stringent threshold value of 40% identity, irrespective of the E value, at least 12 grape NBS-sequences showed a high similarity with known *R*-genes, and the presence of internal conserved motifs provided further evidence that these grape sequences may belong to the NBS-LRR resistance gene family.

Multiple sequence alignment of the deduced amino acid sequences of grape NBS and the NBS-domains of other plant *R*-genes (Fig. 2) showed a very high conservation of the key domains. The sites of the *P-loop*, *kinase-2*, *kinase-3a* and *GLPL* are present in almost all sequences isolated from grape.

The phenetic analysis of the sequences based on the N-J method (Fig. 3) led us to identify at least two well-defined groups of related sequences. A group of 18 grape sequences formed a major cluster with the *N*, *L6* and *M* genes, and a further six grape sequences were grouped with the *RPS2*, *RPS4* and *RPP5* genes in a second major cluster.

tobacco gene N
rgVamu31
rgVamu006
rgVamu053
rgVamu058
rgVamu025
rgVamu032
rgVrip064
rgVrip070
rgVamu028
rgVamu038
rgVamu050
grape-NBS1
rgVamu045
rgVrip004
rgVamu012
rgVamu010
rgVamu037
grape-NBS2
flax gene M
flax gene L6
rgVamu018
rgVamu035
rgVamu026
rgVamu029
grape-NBS3
rgVrip068
Arabidopsis gene RPS2
Arabidopsis gene RPS4
Arabidopsis gene RPP5
0.1

Fig. 3 Phenetic tree based on the Neighbor-joining method of the deduced amino acid sequences of grape-NBS and analogous domains in known resistance genes. The three main groups of grape NBS-like sequences, identified as described in the text, are shown in *shaded boxes*. The sequences used as probes for RFLP-analysis are *underlined*. The *R*-genes coming from other plants are reported in a *black background*. The scale bar in the bottom left corner displays a distance corresponding to 0.1 amino acid substitutions per site

Among the sequences of the first major cluster, two main sub-groups were identified. The first (*grape-NBS1*), included ten sequences which share more than 90% identity and at least 45% identity with the gene *N*; the second group (*grape-NBS2*), encompassing three sequences with a high level of sequence heterogeneity related to genes *L6* and *M*. Within the second major cluster (*grape-NBS3*), five sequences showed inter-sequence identities ranging from 55% to 87% and one of those sequences shared 40% identity with the gene *RPS2*.

Development of RFLP markers for disease-resistance genes

Three grape NBS-sequences, each one representative of the major groups identified above, were used as probes for the detection of candidate RFLP-markers of disease resistance genes in *Vitis*. The probes were based on the *rgVrip064* sequence for the group *grape-NBS1*, *rgVamu012* for the group *grape-NBS2* and *rgVamu026* for the group *grape-NBS3*. The pool of *Vitis* genotypes screened includ-

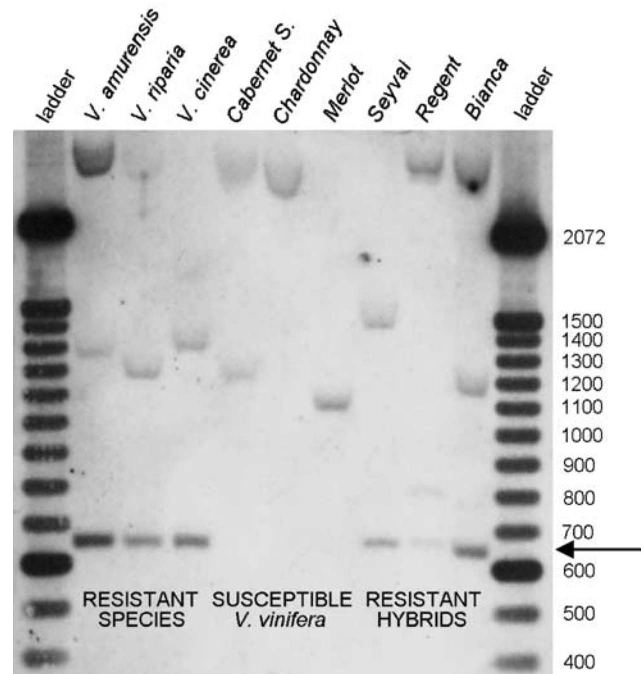

Fig. 4 Chemi-luminescent detection with the *rgVrip064* probe of Southern blots of genomic DNAs digested with the restriction enzyme *Taq*I. *Lanes 1 to 9* were loaded with *V. amurensis*, *V. riparia*, *V. cinerea*, *V. vinifera* cv Cabernet Sauvignon, *V. vinifera* cv Chardonnay, *V. vinifera* cv Merlot, *V*. hybrid cv Seyval, *V*. hybrid cv Regent, *V*. hybrid cv Bianca; external lanes were loaded with a 100-bp marker. The *arrow* points to a 650-bp polymorphic band that is present in resistant species as well as in tolerant/resistant hybrids and absent in susceptible cultivars of *V. vinifera*

ed three wild species known for their resistance to downy mildew (Staudt and Kassermeyer 1995). Of these, *V. cinerea* also carries resistance to powdery mildew (Staudt 1997). The panel also included three *V. vinifera* cultivars susceptible to both diseases, and three inter-specific hybrids Seyval (with blood of *V. vinifera* 50%, *Vitis rupestris* 37%, *Vitis lincecumii* 13%), Regent (*V. vinifera* 80.06%, *Vitis labrusca* 1.76%, *V. rupestris* 14.32%, *Vitis berlandieri* 1.56%, *V. riparia* 0.98%, *V. lincecumii* 1.32%) and Bianca (*V. vinifera* 78.09%, *V. labrusca* 1.56%, *V. rupestris* 14.58%, *V. berlandieri* 3.13%, *V. lincecumii* 2.64%).

A pair of restriction enzymes (*Taq*I and *Dde*I) in combination with the probe *rgVrip064* showed a clear polymorphism between the three classes of *Vitis* accessions. The *rgVrip064* probe detected a 650-bp long band in *Taq*I-digested DNAs (Fig. 4) and a 2000-bp long band in *Dde*I-digested DNAs (data not shown) that were found in all resistant wild species as well as in all the putative tolerant hybrids and absent in all the susceptible cultivars of *V. vinifera*.

Conclusions

Our results demonstrate that conserved domains of resistance genes cloned from a wide range of plant *taxa* can be used to isolate analogous sequences in grape.

These RGAs isolated in grape appear to be part of a multi-gene family, since 29 out of the 60 size-specific clones isolated from two species (*V. amurensis* and *V. riparia*) showed unique restriction patterns. Although we have no evidence from genetic mapping that these sequences are linked to disease resistance genes, we have shown that probes obtained from grape NBS-sequences, when blotted to genomic DNA of different species and genotypes, gave a hybridisation signal only in disease-resistant species and tolerant/resistant hybrids. The signal was lacking in susceptible varieties of the cultivated European grapevine. This evidence makes RGAs isolated in *Vitis* the candidate markers of disease resistance genes in grape and related *taxa*. The screening of a significant number of *Vitis* genotypes and/or the analysis of the hybridisation signal in a population segregating for resistance traits will be the next task of this research.

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