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Identification of resistance gene analogs linked to a powdery mildew resistance locus in grapevine

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Abstract Oligonucleotide primers, designed to conserved regions of nucleotide binding site (NBS) motifs within previously cloned pathogen resistance genes, were used to amplify resistance gene analogs (RGAs) from grapevine. Twenty eight unique grapevine RGA sequences were identified and subdivided into 22 groups on the basis of nucleic acid sequence-identity of approximately 70% or greater. Representatives from each group were used in a bulked segregant analysis strategy to screen for restriction fragment length polymorphisms linked to the powdery mildew resistance locus, *Run*1, introgressed into *Vitis vinifera* L. from the wild grape species *Muscadinia rotundifolia*. Three RGA markers were found to be tightly linked to the *Run*1 locus. Of these markers, two (GLP1–12 and MHD145) cosegregated with the resistance phenotype in 167 progeny tested, whereas the third marker (MHD98) was mapped to a position 2.4 cM from the *Run*1 locus. The results demonstrate the usefulness of RGA sequences, when used in combination with bulked segregant analysis, to rapidly generate markers tightly linked to resistance loci in crop species.

Keywords Nucleotide binding site (NBS) · Leucine rich repeat (LRR) · *Uncinula necator* · *Vitis vinifera* · Disease resistance · Resistance gene analog (RGA)

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Introduction

Powdery mildew of grapevine, caused by *Uncinula necator*, is the most economically important fungal disease of grapes (*Vitis vinifera* L.) worldwide, causing reduced yield and loss of berry/wine quality. *U. necator* is an obligate biotroph and can infect all green tissues of the grapevine producing a distinctive whitish-grey, powdery appearance. These symptoms are due to the presence of hyphae producing conidiophores and conidia on the surface of the host tissue. The hyphae form lobed appressoria which produce a penetration peg that subsequently penetrates into the host epidermal cell and forms a haustorium via which the fungus absorbs nutrients from the host (Heintz and Blaich 1990).

There are no commercially grown cultivars of *V. vinifera* that are resistant to *U. necator.* Control of powdery mildew on grapevine is currently achieved by the widespread application of fungicides such as sulphur and, more recently, systemic de-methylation inhibitors. Whilst these chemicals are relatively effective, the cost to the grower and the environmental impact of the residues remain undesirable. Furthermore, isolates of *U. necator* resistant to the de-methylation inhibitors are now emerging, limiting the effectiveness of these sprays in controlling the disease (Erickson and Wilcox 1997). Thus, the isolation and incorporation of genes into *V. vinifera* for resistance to powdery mildew would be of significant economic and environmental benefit. Other *Vitis* species are known to have varying levels of resistance; however, attempts at producing powdery mildew-resistant cultivars through interspecific breeding have been of limited success due to the difficulty of selecting for quantitative traits controlling both resistance and fruit quality (Boubals 1961; Eibach et al. 1989).

The wild grape species *Muscadinia rotundifolia*, which originated from the south-east of the USA, is highly resistant to most pathogens of *V. vinifera* including *U. necator* (Olmo 1971). Unlike the case for the *Vitis* species discussed above, the resistance to powdery mildew derived from *M. rotundifolia* is thought to be con-

ferred by a dominant allele at a single locus designated *Run*1 (for Resistance to *Uncinula necator* 1) (Bouquet 1986; Pauquet et al. 2001). The *Run*1 locus from *M. rotundifolia* has been introduced into the *V. vinifera* genome using a pseudo-backcross strategy, whereby different *V. vinifera* genotypes were used at each backcross step (Bouquet 1986; Pauquet et al. 2001). Within the backcross population there are resistant individuals that are heterozygous for the *Run*1 gene and susceptible individuals that do not contain the gene. The development of techniques for map-based cloning of resistance genes, combined with the availability of a grapevine transformation system (Iocco et al. 2001), now offers the possibility of introducing the *Run*1 gene into a range of existing premium *V. vinifera* cultivars by genetic manipulation.

A new PCR-based strategy for the rapid generation of genetic markers linked to putative resistance loci has recently been developed. This approach is based on the observation that genes that confer resistance against a diverse range of pathogens, from a variety of plants, show a high degree of structural and amino-acid sequence conservation. In particular, the majority of cloned resistance (R) genes contain a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) domain (Meyers et al. 1999; Ellis et al. 2000; Young 2000). Two kinds of N termini have been identified in NBS-LRR resistance genes. The first type shows homology to a region in *Drosophila* Toll and the human Interleukin-1 Receptor (TIR-like), and the other contains a coiled-coil (CC) thought to be involved in protein-protein interactions (non-TIR-like) (Meyers et al. 1999; Pan et al. 2000a). The NBS sequences of R genes are characterised by the presence of up to seven conserved domains including the P-loop, kinase-2 and GLPL motifs (Meyers et al. 1999). The presence of these conserved domains has facilitated the cloning of resistance gene analogs (RGAs) from diverse species by PCR using degenerate oligonucleotide primers. NBS encoding sequences tend to be clustered in the genome and, in accordance with this, isolated RGAs are frequently genetically located at, or near, previously identified resistance loci (Yu et al. 1996; Kanazin et al. 1996; Aarts et al. 1998; Collins et al. 1998; Seah et al. 1998; Shen et al. 1998; Spielmeyer et al. 1998; Leister et al. 1999; Mago et al. 1999; Pan et al. 2000a). Thus, the identification of RGAs represents a potentially powerful strategy for the generation of markers for map-based cloning of resistance genes.

The work presented here describes the isolation and characterisation of resistance gene analogs (RGAs) from grapevine and the mapping of specific RGAs to the powdery mildew resistance (*Run*1) locus.

Materials and methods

Plant material

Segregating populations from a pseudo-backcrossing strategy, aimed at introgressing the *Run*1 locus from *M. rotundifolia* into *V. vinifera*, were generated as described by Pauquet et al. (2001). The BC5 population used in this analysis (population Mtp3294) was produced from a cross of the resistant BC4 individual (VRH3082-1-42) with the susceptible *V. vinifera* cv Cabernet Sauvignon.

Preparation of genomic DNA

Genomic DNA was prepared from leaf material essentially as described by Thomas et al. (1993) and Lodhi et al. (1994). For the Lodhi method a further extraction with an equal volume of phenol-chloroform (1:1) and precipitation with 0.5 vol of 4.5 M ammonium acetate and 0.6 vol of isopropanol was included. DNA was washed with 70% ethanol, dried in a rotary evaporator and resuspended in TE.

Powdery mildew detached leaf assay

U. necator (isolate Apc1 obtained from Dr. Eileen Scott, University of Adelaide) was maintained on in vitro plantlets of *V. vinifera* cv Cabernet Sauvignon. Young leaves (approximately 6-cm diameter) were collected from glasshouse-grown vines and surface-sterilised in a solution containing 50% (v/v) Milton solution (active ingredient 0.95% w/w sodium hypochlorite; Procter and Gamble, NSW, Australia) and 0.04% (v/v) Tween 20 for 3 min, and washed four times with sterile water. Leaves were placed on agar plates containing 1% agar (w/v) and 400 µl ml^{-1} of Pimaricin (Sigma Chemical Co., USA), allowed to dry, and spores from powdery mildew-infected detached in vitro leaves applied using a fine paintbrush. Plates were sealed with parafilm and incubated at 25°C under a 12-h light/dark cycle. Leaves were analysed for mildew infection after 7–14 days using a Zeiss Stemi 2000 microscope.

Oligonucleotide primers and PCR strategy

A number of primers were designed by Collins et al. (1998) to each of four conserved amino-acid motifs present in the NBS of cloned resistance proteins: GVGKTT (P-loop), L(I/V/L)VLDDV (kinase-2), GLPL and MHD (Fig. 1). All cloned PCR fragments were obtained from a semi-nested primer approach. PCR reactions were first performed with all possible combinations of primers based on two amino-acid motifs, i.e. P-loop/GLPL or P-loop/MHD. Individual reactions, with a common 3´ end primer, were then pooled and used as a template for nested PCR with all possible pairwise combinations of the 5´ nested primer with the same 3´ primer i.e. kinase-2/GLPL or kinase-2/MHD.

PCR conditions

First-round PCR reactions were carried out in a 20 µl volume containing 70 ng of genomic DNA, 10 mM of Tris-HCl (pH 9.0 at 25°C), 50 mM of KCl, 0.1% (v/v) Triton X-100, 2 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.25 µM of each primer and 0.5 units of *Taq* Polymerase (Promega, Madison, Wis.). The reaction mixture was subjected to thermal cycling in an Omn-E thermal cycler (Hybaid, Middlesex, UK) with the following program: 95°C for 2 min;

Fig. 1 Schematic model of the structure of nucleotide-binding site-leucine rich repeat (NBS-LRR) type resistance genes. Relative positions of the degenerate primers P-loop, kinase-2, GLPL and MHD, designed to conserved domains within the NBS, used for the amplification of RGAs from grapevine genomic DNA, are shown

40 cycles of 95°C for 30 s, 40°C for 30 s and 72°C for 2 min; 10 min at 72°C. Second-round nested PCR was carried out in 20-µl reaction volumes as described above but with the genomic DNA template replaced by 1 µl of first-round pooled PCR mix. Thermal cycling conditions were: 95°C for 2 min; 10 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 50 s; 25 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 50 s; 10 min at 72°C.

Cloning and analysis of PCR products

DNA products from the nested PCR reactions were fractionated on a 1.5% NuSieve GTG agarose gel (FMC Bioproducts, Rockland Me.). Fragments of the appropriate size were excised from the gel and ligated into pGem-T or pGem-T Easy Vector (Promega).

Inserts from recombinant clones were amplified by PCR directly from bacterial cultures, using T7 and SP6 sequencing primers. PCR reactions were carried out in a 20-µl volume as described above but with a 1–2 µl bacterial culture containing the DNA template. Thermal cycling conditions were: 95°C for 3 min; 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 2 min; 7 min at 72°C. Ten microlitres of unpurified PCR reaction product was digested in a 20-µl volume with *Rsa*I or *Sau*3AI for 2 h at 37°C and fractionated on a 2% agarose gel. pGem-T vectors do not contain *Rsa*I or *Sau*3AI sites within the region amplified by T7 and SP6 primers. Clones were organised into groups based on common digestion patterns and representatives of each group sequenced using an ABI Prism (Applied Biosystems, Foster City, Calif.) dye terminator sequencing system.

Sequence analysis

Identity of RGA clones was confirmed by comparisons of translated sequences with the non-redundant GenBank database using BLASTX (Gish and States 1993). Sequence comparisons were carried out with software programs in the GCG Wisconsin Package Ver. 8 (Devereux et al. 1984).

Bulked segregant and RFLP analysis

Genomic DNA bulks (4 µg total) generated from seven individual resistant (heterozygous for *Run*1) or susceptible BC5 progeny were digested separately with *Eco*RI, *Dra*I and *Rsa*I, electrophoresed on a 0.8% agarose gel and transferred to Hybond N^+ membrane (Amersham Pharmacia Biotech, Sydney, Australia) by alkaline blotting according to the manufacturer's instructions. Membranes were UV-crosslinked (UV Stratalinker 1800, Stratagene, San Diego, USA) and then rinsed in 2×SSC.

A representative clone from each RGA group was digested with either *Eco*RI or *Apa*I/*Sac*I to release the RGA DNA insert, and separated on a 1.5% agarose gel. The RGA DNA fragment was then excised from the gel and purified using a QIAquick Gel Extraction Kit (Qiagen). Radiolabeled (^{32}P) RGA probes were synthesised using a Gigaprime labeling kit (GeneWorks, Adelaide, Australia). Membranes were hybridised with RGA probes at 65°C overnight, washed twice with 2×SSC, 0.1% SDS at 65°C for 20 min and once with 0.1×SSC, 0.1% SDS at 65°C for 20 min. Autoradiography was carried out at –80°C with an intensifying screen.

Genomic DNA (4 µg) from individual BC5 progeny and representatives of BC2, BC3, BC4, Cabernet Sauvignon and *M. rotundifolia* were digested with *Eco*RI under conditions specified by the manufacturer (Boehringer). The digested samples were electrophoresed on a 0.8% agarose gel, transferred to a Hybond N+ membrane and hybridised with RGA probes as discussed in the previous section.

Generation of PCR markers

In order to convert the GLP1–12 and MHD98 RFLPs into PCR markers, 2 µg of genomic DNA from a resistant BC5 plant was digested with *Hin*dIII or *Eco*RI under the conditions recommended by the manufacturer (Promega), phenol/chloroform-extracted, precipitated and re-suspended in 1×TE. The DNA fragments were cloned into the corresponding sites of the pBluescript-SK^+ vector (Stratagene) in 10-µl ligations containing a 1:3 ratio of vector to insert, and incubated for 16 h at 16°C.

Genomic sequences 5′ and 3′ of the original cloned RGA sequences were obtained using an anchor-PCR technique. The *Hin*dIII ligation was used as a template for PCR with the primer GLP1–12P2R in combination with the vector specific primer, T3 (Promega). Similarly, the *Eco*RI ligation was used as a template for PCR with the primer MHD98P2 in combination with T3. PCR amplification was performed in a 25-µl reaction volume with 1 µl of ligation as template and 0.4 µM of each primer. The template was amplified using the program 95°C for 2 min; 25 cycles of 30 s at 95°C, 30 s 55°C, 2 min at 72°C; 10 min at 72°C. Subsequently, 1 µl of the PCR reaction was used in a nested PCR with GLP1–12P2Rn or MHD98P2n in combination with T3. The PCR conditions for the nested reaction were identical to the initial reaction. The resulting products, of approximately 1 kb (GLP1–12P2Rn) or 1.75 kb (MHD98P2n) in size, were cloned into pGEM-T Easy vector and sequenced as described above.

RGA specific primer sequences

GLP1–12P2R : 5′-TCT TCC CAA ATC AGA TTA CAG-3′,
GLP1–12P2Rn : 5′-CTT GCC TAT CGT GTT GTA GG-3′,
GLP1–12P1 : 5′-GGA ATA TTT ACT TGG ACA TCG-3′,
GLP1–12P3 : 5′-CAT TTG AAT TGG AGC ATA CTC-3′,
MHD98P2 : 5′-ACA AGA GTC CTC CAA GTG TTT-3′,
MHD98P2n : 5′-GGT AAT CCT TGG CAT TTC TTC-3′,
MHD98P5R : 5′-GAG GCT CTT AAA AGG GCA TAT-3′,
MHD98P12R : 5′-GTC TCT ATA GGC CCA TCT CCA TCT C-3′,
MHD98P12S : 5′-CCA TAC ACC CTA GAT TCA TCC ACC-3′.

Linkage analysis

The Kosambi map function in Map Manager QTXb08 (Meer et al. 2000) was used for linkage analysis. Segregation distortion was allowed for.

Results

Nested primers designed to four conserved amino-acid motifs present in the nucleotide binding site (NBS) of known resistance proteins, i.e. GVGKTT (P-loop), L(I/V/L)VLDDV (kinase-2), GLPL and MHD (Fig. 1), were used to amplify RGAs from the genome of a BC4 (VRH3082–1-42) resistant individual. PCR amplification with genomic DNA resulted in the production of DNA products of the predicted sizes based on previously published RGA sequences, i.e. P-loop/GLPL ~300 bp and P–loop/MHD ~600–650 bp (data not shown). Cloning and characterisation of these PCR products revealed that they were comprised of many different RGA sequences. These were grouped according to the restriction patterns obtained following digestion with four base-cutter enzymes. Representative clones of each group were sequenced and then compared to the GenBank database using BLASTX to confirm their identity. Of the

Fig. 2 Regions of amino acid similarity between previously isolated NBS-LRR resistance genes *N, L6, RPS2, I2C-1, Xa1* and *Mi* and the predicted products of the RGA sequences isolated from grapevine. Adapted from Collins et al. (1998). Similar residues are in **bold** and sequences between motifs are represented by *dots*. Motifs conserved between the sequences are shown above the previously isolated resistance genes. The consensus sequences used to design primers are indicated; *arrows* indicate the primer orientation

43 clones sequenced, 35 were predicted by BLASTX to encode RGA-like sequences, based on sequence homology with the NBS region of known resistance genes. Seven of the clones, which showed >97% nucleotide identity to other RGA clones, were not considered for further analysis because of difficulties in establishing whether these differences were real or an artifact of the two rounds of PCR amplification.

Translation of the remaining 28 distinct RGA clones revealed 22 to have a continuous open reading frame. Regions of amino-acid similarity between the predicted products of the grapevine RGAs and known NBS-LRR resistance proteins are shown in Fig. 2. The NBS motifs RNBS-B and RNBS-C were observed in all 22 translations. Clones amplified using a MHD primer (MHD30, 59, 98, 106, 145 and 148) also contained the GLPL and RNBS-D motifs (Fig. 2).

Association of RGAs with the powdery mildew resistance locus (*Run*1)

Initial screening of BC5 *Run*1 progeny was conducted using bulked segregant analysis (Michelmore et al. 1991) to identify any linkage between the isolated RGAs and the *Run*1 phenotype. For use in RFLP analysis, the RGA clones which showed nucleic acid sequence homology of approximately 70% or greater were grouped to minimise cross-hybridisation between closely related RGA sequences under conditions of high-stringency washing (i.e. 0.1×SSC, 65°C). A representative member of each of the 22 RGA groups (Fig. 3) was used as a probe in bulked segregant analysis with DNA bulked pools of seven resistant and seven susceptible individuals digested with *Eco*RI, *Dra*I and *Rsa*I. Analysis indicated that the RGAs GLP1–12, MHD98 and MHD145 were polymorphic in *Eco*RI digests between resistant and susceptible pools (data not shown).

These RGAs were then used to probe *Eco*RI digests of genomic DNA obtained from two sub-groups of eight resistant and eight susceptible BC5 progeny to confirm the genetic association (Fig. 4). It should be noted that the original *Muscadinia* parent used to generate the F1 progeny is now dead and could not be tested; however, another *M. rotundifolia* plant has been included in the blots in Fig. 4 for comparison. GLP1–12 hybridised to a single 1.6-kb fragment in the resistant BC2, BC3 and BC4 progeny, all of the resistant individuals from BC5 and the accession *M. rotundifolia*. There was no detectable hybridisation to any susceptible BC5 progeny analysed or to the susceptible *V. vinifera* parent Cabernet Sauvignon (Fig. 4).

When probed to genomic DNA digested with *Eco*RI, the RGA probe MHD145 hybridised to multiple fragments from both resistant and susceptible progeny, indicating that there are multiple copies of this, or closely related sequences, within the *V. vinifera* genome. One 2.2-kb hybridising fragment was identified to be polymorphic between resistant and susceptible individuals (Fig. 4). The polymorphism detected by MHD145 was

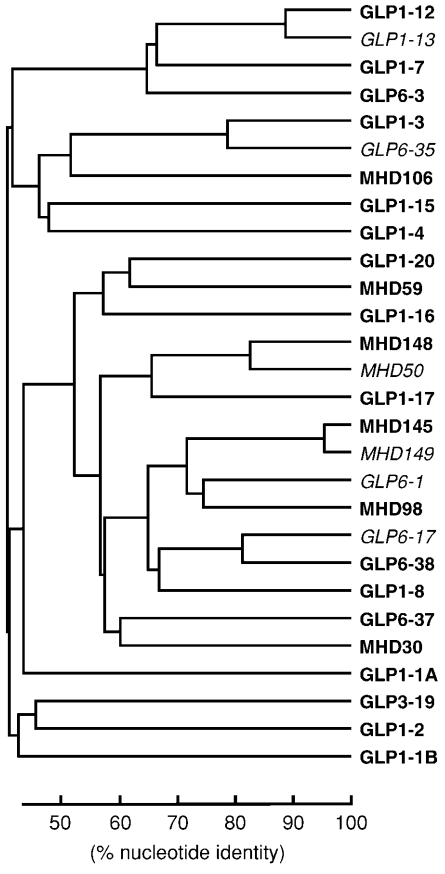

Fig. 3 Dendogram of the putative grapevine RGA sequences on the basis of nucleic acid similarity. Clustering relationships were plotted using the output from the multiple sequence alignment program PILEUP from the GCG Wisconsin Package. RGAs used for RFLP analysis are shown in *bold*

present in all resistant samples tested and absent in all susceptible samples tested.

MHD98, the third RGA marker identified as polymorphic by bulked segregant analysis, hybridised to 5-kb and 3.5-kb *Eco*RI fragments in resistant samples but only to the 3.5-kb band in all susceptible samples apart from recombinants for this marker, as in progeny number 4 (Fig. 4). Interestingly, the 5-kb MHD98 RFLP marker, which is linked to the resistance phenotype, was found to be absent from *M. rotundifolia* (Fig. 4) suggesting that it may have been introduced from one of the *V. vinifera* cultivars used during the pseudo-backcross breeding program.

Conversion of RFLP markers into PCR-based markers

To facilitate more-rapid screening of the remaining BC5 population, efforts were made to convert GLP1–12, MHD98 and MHD145 into PCR-based markers. Initially, PCR primers designed to each of the original RGA sequences were tested but were found to amplify PCR products of the same size from both resistant and susceptible samples in all three cases (data not shown). RGAs show high levels of conservation within the NBS regions to which the primers were designed and it is probable that they were amplifying a number of related sequences, including the polymorphic RGAs. It was therefore necessary to obtain further sequence information to enable conversion of the RGAs into PCR-based markers.

The GLP1–12 region was mapped by Southern blotting to identify restriction sites outside of the original sequence that could be used in cloning and sequencing. Restriction mapping identified a 1-kb *Hin*dIII restriction fragment, which hybridised to GLP1–12 in resistant BC5 samples and contained an internal *Eco*RI site (Fig. 5A). To determine whether this *Eco*RI site could be used to

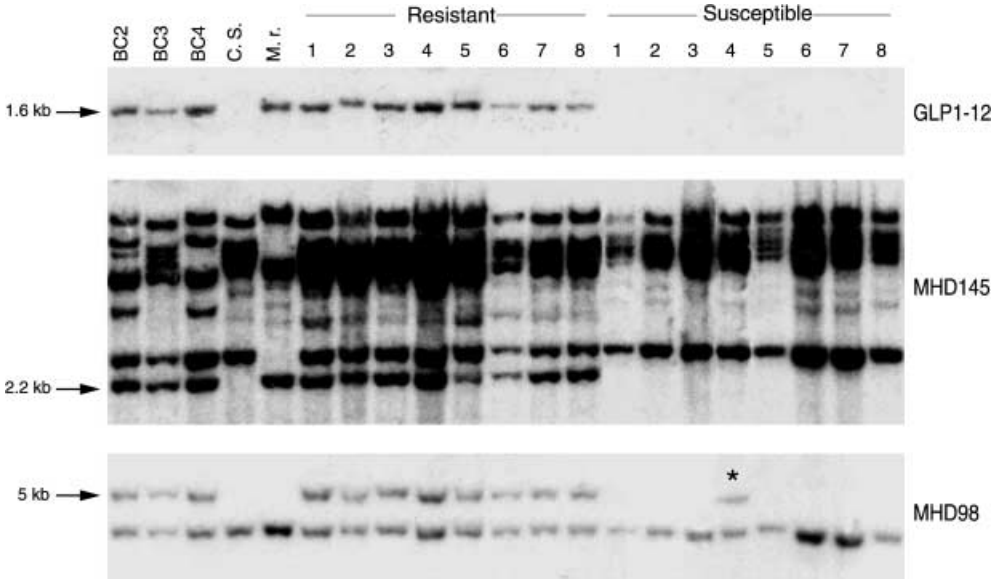

Fig. 4 Southern analysis of grapevine progeny segregating for powdery mildew resistance. Genomic DNA (4 µg/lane) was digested with *Eco*RI and hybridised with ^{32}P-labelled probes of the grapevine RGAs: GLP1–12, MHD145 and MHD98. Each of the probes identified an RFLP between plants resistant and susceptible to powdery mildew (RFLP indicated by *arrows*). Representative resistant progeny from the BC2, BC3 and BC4 populations as well as a Cabernet Sauvignon (*CS*) and *Muscadinia rotundifolia* (*Mr*) are shown, in addition to eight resistant and eight susceptible BC5 progeny. An example of a recombinant progeny for marker MHD98 is highlighted (*)

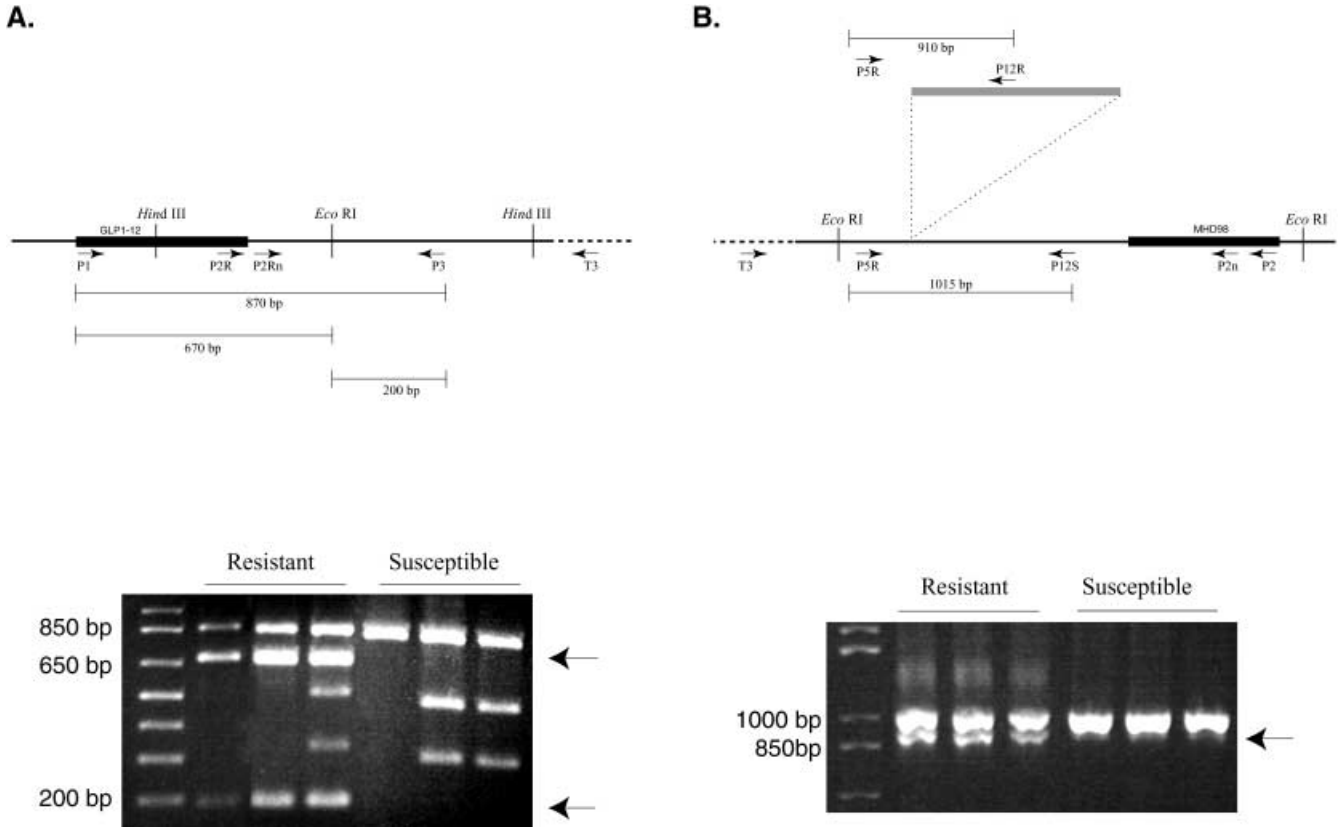

Fig. 5A, B Strategy for conversion of RGA RFLP markers into PCR-based markers. **A** Conversion of GLP1–12 into a CAPS marker. Map of RGA GLP1–12 showing the positions of restriction sites for enzymes *Hin*dIII and *Eco*RI in relation to the original RGA sequence (*bar*). The positions of primers GLP1–12P1, P2R, P2Rn, P3 and T3, and the size of PCR and restriction products are indicated. The lower panel shows polymorphism between resistant and susceptible samples detected by PCR with GLP1–12P1 and P3 followed by digestion with *Eco*RI. The *arrows* indicate the *Eco*RI digestion products (670 bp and 200 bp) produced in resistant samples and absent in susceptible samples. **B** Conversion of MHD98 into a SCAR marker. Map of RGA MHD98 showing the positions of restriction sites for the enzyme *Eco*RI in relation to the original sequence (*bar*). Positions of primers MHD98P5R, P12R, P12S, P2n, P2 and T3 and the sizes of PCR products are indicated. The lower panel shows polymorphism between resistant and susceptible samples detected by PCR with MHD98P5R, 12S and 12R. The *arrow* indicates the product (910 bp) amplified from resistant samples and not from susceptible samples

convert GLP1–12 into a cleaved amplified polymorphic sequence (CAPS) marker, the 1-kb *Hin*dIII fragment was cloned using an anchor-PCR technique described in the Materials and methods. The sequence obtained was used to design an additional primer, GLP1–12P3 (Fig. 5A). PCR-amplification of genomic DNA using the primers GLP1–12P3 and GLP1–12P1 (Fig. 5A) produced an 870-bp product from both resistant and susceptible samples (data not shown). Consistent with the Southern results discussed above, *Eco*RI digestion of the 870-bp PCR product produced two fragments of approximately 670 and 200 bp from resistant progeny, which were absent from susceptible progeny (Fig. 5A, lower panel). Several *Eco*RI restriction fragments of a different size were also produced after digestion of the P1/P3 PCR product which were not linked to the *Run*1 locus, indicating that the 870-bp PCR product was composed of more than one sequence.

Southern-blot restriction mapping of MHD98 suggested that the polymorphism shown in Fig. 4 was due to a DNA insertion upstream of the original MHD98 sequence (data not shown). The 3.5-kb *Eco*RI fragment to which MHD98 hybridised in all genomic DNA samples analysed (Fig. 4) was cloned by anchor-PCR and the additional sequence obtained was used to design the primer MHD98P5R (Fig. 5B). PCR with MHD98P5R and MHD98P2n (Fig. 5B) enabled amplification and sequencing of the region from both resistant and susceptible progeny and confirmed that the RFLP observed (Fig. 4) was due to a 1.5-kb insertion upstream of the original MHD98 sequence (Fig. 5B). Based on the sequence obtained, two additional primers were designed; one specific to the insertion (MHD98P12R; Fig. 5B) and one 3′ to the insertion site which was present in both the resistant and susceptible derived sequences (MHD98P12S; Fig. 5B). The primers MHD98P5R, MHD98P12R and MHD98P12S were then combined within the same PCR reaction to produce a sequence-characterised, amplified region (SCAR) marker with products of 1,015 bp and 910 bp amplified from resistant progeny and only the 1,015 bp product from susceptible progeny (Fig. 5B, lower panel) consistent with genetic linkage of the 1.5-kb DNA insertion to the *Run*1 locus.

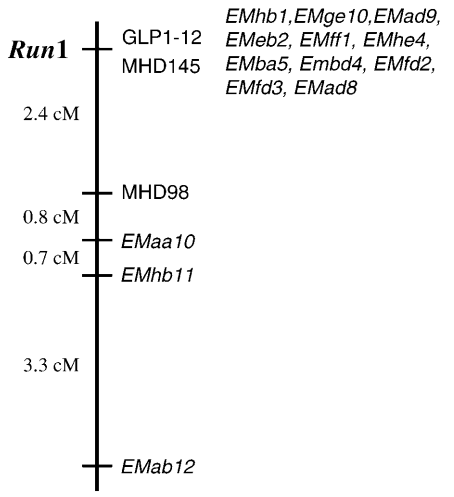

Fig. 6 Linkage map of the resistant genotype showing the relative positions of the powdery mildew resistance locus *Run*1 and RGA markers GLP1–12, MHD145 and MHD98. AFLP markers identified previously by Pauquet et al. (2001) are shown in *italics*. Genetic distances were calculated using the Kosambi map function and are shown in *centimorgans*

Attempts to convert the RGA marker MHD145 into a PCR-based marker were not successful due to amplification from the large number of closely related sequences present within the genome, which are not linked to resistance (see Fig. 4).

Mapping of the Run1 locus with RGA markers

A total of 167 BC5 progeny were screened with the GLP1–12, MHD145 and MHD98 RGA markers using either PCR or RFLP analysis. Segregation analysis confirmed that the RGA markers GLP1–12 and MHD145 cosegregated with the resistance phenotype in all BC5 progeny tested, indicating that these markers are tightly linked to the *Run*1 locus. In contrast, four out of the total 167 BC5 progeny were recombinant for marker MHD98, placing this marker approximately 2.4 cM from the *Run*1 locus (Fig. 6). Pauquet et al. (2001) have also recently identified amplified fragment length polymorphism (AFLP) markers linked to the *Run*1 locus in the same BC5 population and their positions are also shown in Fig. 6. Ten of the thirteen AFLP markers cosegregated with *Run*1, while the remaining three markers mapped on the same side of the *Run*1 locus at a genetic distance of 3.2–7.2 cM.

Discussion

There are at least 120 different NBS-LRR genes present in *Arabidopsis* (Initiative 2000). Our results indicate that large numbers of RGA-like sequences are also present in the grapevine genome. Using semi-nested PCR with primer combinations designed to four of the conserved motifs within the NBS region, we have been able to isolate 28 unique (i.e. <97% nucleic acid identity) grapevine RGA sequences, of which 22 could be translated into ORFs showing high homology to cloned NBS-LRR resistance gene sequences. This approach has previously been used to isolate RGAs from a broad range of crop species (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996; Aarts et al. 1998; Collins et al. 1998; Speulman et al. 1998; Mago et al. 1999; Pan et al. 2000a), and our work demonstrates that this technique is also applicable to a woody perennial such as grapevine.

Two major sub-classes of NBS-LRR proteins have been identified (Meyers et al. 1999). One class comprises sequences encoding an amino-terminal Toll/interleukin-1 receptor homology region (TIR). The second major group of NBS-LRR proteins lacks this TIR region and in many cases it has been replaced by a coiled-coil domain, of which the leucine zipper is an example (Pan et al. 2000b). Based on an analysis of over 400 NBS-encoding sequences of putative plant disease resistance genes, Meyers et al. (1999) observed that the RNBS-A and RNBS-D motifs within the NBS domain of these genes can be used to distinguish between these two types. Furthermore, they observed that the final residue in the kinase-2 motif can be used to predict the presence/absence of the TIR domain to more than 95% accuracy, with an aspartate residue (D) present in the TIR protein sub-class and a tryptophan residue (W) present in proteins lacking the N-terminal TIR domain. Analysis of the kinase-2 motifs of the grapevine RGA clones shown in Fig. 2 predict that 19 out of the 22 RGA clones obtained from grapevine using this PCR strategy are of the non-TIR class, with only GLP1–7, GLP1–12 and GLP6–3 predicted to have an N-terminal TIR region.

It is not known if the high proportion of non-TIR:TIR sequences observed in this study (i.e. 6:1) is a reflection of the true ratio of non-TIR:TIR sequences present in the grapevine genome or a consequence of the particular PCR strategy used. It is interesting to note that with the *Arabidopsis* genome now sequenced, the ratio of non TIR:TIR-type NBS sequences identified is 1:2 [49:100 (Initiative 2000)]. However, two separate studies involving the use of degenerate NBS primer PCR strategy to amplify RGA sequences from the *Arabidopsis* genome (Aarts et al. 1998; Speulman et al. 1998) resulted in the isolation of non-TIR:TIR-type sequences at ratios of 4:1 and 6:1. In contrast, the application of a similar strategy with soybean (Kanazin et al. 1996) identified predominantly TIR-type RGA sequences. Such differences may result from minor differences in the primers used in each study. Thus, the composition of RGA sequences obtained using a PCR-based strategy may not accurately reflect the composition of the genome being studied and may be biased towards one particular NBS-LRR gene family.

RGA markers are linked to a resistance locus in grapevine

Three of the RGAs isolated from the BC5 population were found to be linked to the powdery mildew resis-

tance locus, *Run*1, and these were more-extensively studied through segregation analysis. Two of the RFLPs identified by RGA probes, GLP1–12 and MHD145, co-segregated with the resistance phenotype in all 167 BC5 progeny analysed. Of particular interest is the marker GLP1–12, which was only present in resistant progeny, including the powdery mildew resistant accession *M. rotundifolia*. The absence of the GLP1–12 hybridising band in all susceptible individuals tested, including other *V. vinifera* parents used during the back-crossing strategy (data not shown), suggests that this marker is a *Muscadinia*-specific sequence located within the introgressed fragment. A total of 1,356 bp of genomic GLP1–12 sequence was obtained during conversion into a CAPS marker. A BLASTX search of the Genbank databases with the deduced amino-acid sequence of GLP1–12 revealed strong identity with several previously isolated resistance genes and RGAs including NL27 (*Solanacum tuberosum*), N (tobacco), N-like (*Arabidopsis*) and M (*Linum usitatissumum*). In addition, RNBS-A, Kinase-2 and RNBS-D sequences characteristic of TIR-like NBS-LRR genes were identified, supporting the hypothesis that GLP1–12 is a TIR-like NBS-LRR gene.

Previously, the closest marker to *Run*1, which could be genetically recombined with it, was the AFLP marker EMaa10 (Pauquet et al. 2001) which was recombinant in five BC5 progeny. MHD98 was found to be recombinant in four out of five of these progeny placing it closer to the *Run*1 locus on the genetic map (Fig. 6). Cloning and sequencing of the polymorphism detected by MHD98 indicated it was the result of an insertion of 1.5-kb within the N-terminal coding region of MHD98 (Fig. 5A). The sequence of the MHD98 1.5-kb insertion showed regions of significant nucleotide identity to a *Staphylococcus aureus* transposable element (>65% over a 105-nt overlap) and to *Melanoplus sanguinipes* entomopoxvirus (>60% over a 136-nt overlap) suggesting that the insertion is the result of a transposition event. The absence of this insertion in both *M. rotundifolia* and Cabernet Sauvignon (Fig. 4) suggests that this insertion may have been contributed by one of the other *V. vinifera* parents used in the backcross strategy. Indeed, the same polymorphism has been detected in the *V. vinifera* cultivar Riesling (data not shown).

The work presented here describes the isolation of markers linked to the powdery mildew resistance locus, *Run*1, which was introgressed into *V. vinifera* from *M. rotundifolia* (Bouquet 1986; Pauquet et al. 2001). Introgression of a gene from another species is frequently accompanied by inhibition of recombination, resulting in relatively large regions that appear to co-segregate with the introgressed gene (Wei et al. 1999; Chin et al. 2001). Therefore, despite the observed co-segregation of two of the grapevine RGAs with *Run*1 (Fig. 6) it is possible that these markers are not as close to the resistance locus as the data suggests. Analysis of an extended backcross population (Pauquet et al. 2001) is now being undertaken to search for additional recombinants which will aid in further fine-mapping the position of the co-segregating markers, relative to the *Run*1 locus. These markers will then be used to physically delimit the locus in a BAC library of genomic DNA isolated from one of the resistant BC5 individuals.

The powdery mildew-resistant *M. rotundifolia* accession is also resistant to many other important *V. vinifera* pathogens including downy mildew, *Botrytis cinerea*, nematodes and phylloxera (Olmo 1971; Bouquet 1983). Recently a number of RGAs were isolated from citrus, using PCR-based techniques similar to those employed in this study. Three of the RGA markers were found to be linked to the citrus tristeza virus resistance gene and the major gene responsible for citrus nematode resistance (Deng et al. 2000). A study in tomato produced similar results, with NBS sequences co-mapping to multiple regions of the genome that contained resistance genes for a variety of pathogens including *Verticillium* wilt and *Fusarium* (Pan et al. 2000b). It is likely, therefore, that the grapevine RGAs isolated in this study may also be useful in marker-assisted breeding or map-based cloning of other grapevine resistance genes from *M. rotundifolia*.

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