ISOLATION OF A MAJOR RESISTANCE GENE TO POWDERY MILDEW

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GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

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The Cooperative Research Centre for Viticulture is a joint venture between the following core participants, working with a wide range of supporting participants.
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Abstract

Good progress has been made towards the identification and isolation of natural genes for powdery mildew (Run1) and downy mildew (Rpvl) resistance from the wild North American grapevine Muscadinia rotundifolia. Up to four candidate genes have been identified which may confer powdery or downy resistance. Genetic markers linked to the Run1 and Rpvl genes have been identified and used to map-base clone up to four candidate resistance genes which may confer powdery or downy resistance in grapevine. The next stage involves testing the function of these individual resistance genes in susceptible winegrape cultivars. These genes could be introduced into existing cultivars, via genetic transformation techniques, resulting in the generation of premium winegrape cultivars with natural resistance to powdery mildew, without the negative impacts on wine quality associated with traditional breeding techniques.
Executive summary

Powdery mildew is the most economically important grapevine disease worldwide. Complete crop loss occurs in vineyards of unsprayed highly susceptible cultivars and frequent yield losses of 20% or more occur where fungicides are applied after the disease is well established. The effect of fungal infection of the fruit causes significant losses to both the wine and table grapes industries. Costs of disease management and yield losses in Australia are estimated at 5% the value of total grape production. Current control of the disease can only be achieved by the use of fungicides. However, powdery mildew strains resistant to DMI fungicides have recently been reported and there is world-wide pressure to reduce the use of "hard" chemicals to control plant pathogens on crops grown for human consumption. These problems could be overcome by the development of grapevine cultivars with enhanced resistance to powdery mildew.

Genetic resistance to powdery mildew has been identified in many crop species but the wine grape species *Vitis vinifera* displays no inherent resistance to this pathogen. Breeding experiments initiated at INRA, Montpellier, France have identified a single dominant gene (designated *Run1* for resistance to *Uncinula necator 1*) in the American wild grape species *Muscadinia rotundifolia* which confers complete resistance to powdery mildew. Cloning of this resistance gene would then enable the generation of mildew-resistant wine cultivars by genetic transformation.

The recent development of molecular techniques for the identification and cloning of plant resistance genes now offers the possibility to isolate and separate the *Run1* powdery mildew resistance gene from the other “negative” wine quality genes present in the Muscadine genome. This gene could then be introduced into existing *V. vinifera* cultivars, via genetic transformation techniques, resulting in the introduction of natural genetic resistance to powdery mildew into our premium winegrape cultivars, without the negative impacts on wine quality associated with traditional breeding techniques.

A collaboration was established between CSIRO Plant Industry and INRA in France for the molecular localisation and isolation of the *Run1* gene. The aim of this research project was to identify genetic markers tightly linked to *Run1* locus and establish the location of these markers on a physical map and clone *Run1* gene candidates. The strategy undertaken to achieve this goal involved two main components. The first involved construction of a large DNA insert library called a Bacterial Artificial Chromosome (BAC) Library which contains the entire grapevine genome digested into manageable fragments ranging in size from ~100,000-200,000 basepairs. Within this library are overlapping DNA fragments some of which will contain the *Run1* resistance gene. This goal was has successfully achieved with construction of a grapevine BAC library in our laboratory comprising 55,295 clones with an average insert size of approximately 120 kb, which represents 5x haploid genome copies.

The next step involved the screening of these DNA fragments to identify which contained *Run1* gene candidates. This was done using genetic markers, which are short specific DNA sequences which have been shown to be physically located close to the *Run1* gene by virtue of the fact that they consistently co-segregate with powdery mildew resistance in our *Run1* backcross breeding populations. Along with our French collaborators at INRA, we have identified a range of genetic markers including Amplified Fragment Length Polymorphisms (AFLPs), Resistance Gene Analogs (RGAs) and Microsatellites which are closely linked to the *Run1* resistance gene. This goal was has successfully achieved with construction of a grapevine BAC library in our laboratory comprising 55,295 clones with an average insert size of approximately 120 kb, which represents 5x haploid genome copies.
Computer analysis of the genomic sequence obtained indicates the presence of a single cluster or family of resistance genes that are likely candidates for the Run1 and Rpv1 genes. These genes have been designated resistance gene analogues (RGAs) and encode TIR-NBS-LRR type proteins very similar in structure to the Mla powdery mildew resistance genes isolated from barley. To date, 4 full-length and 4 truncated (non-functional) RGAs have been identified within the cluster. However, there is still a region within the introgressed fragment that we have unable to fully clone using our standard BAC library approach. An attempt to fill this “gap” through the construction of a separate cosmid library was also only partially successful. The presence of partial RGA-like sequences within this region indicates that it will be important to fill this gap in the future to determine if it contains any other potential full length RGA sequences that need to be functionally tested.

In conclusion, we have made excellent progress towards the identification and characterization of powdery mildew and downy mildew resistance genes from the wild American grapevine species M. rotundifolia. The introduction of these natural resistant genes into our current winegrape selections will provide the Australian Viticultural industry with significant competitive advantages not only in terms of reduced production costs and increased yields, but in terms of making a sustainable and quantifiable contribution to our image as a “clean & green” wine producer.

Research papers arising from the project accompanies this report.

This project was supported by the Commonwealth Cooperative Research Centres Program and conducted by the CRC for Viticulture (CRCV). Australian grapegrowers and winemakers are key stakeholders in the CRCV, contributing levies matched by the Commonwealth Government and invested by the Grape and Wine Research and Development Corporation in the Centre. The project was financially supported by the GWRDC, CRCV and CSIRO Plant Industry.
Background

Grapevine powdery mildew is caused by the biotrophic pathogen, *Uncinula necator*, and is the most serious fungal disease of grapevines worldwide. The pathogen infects the leaves and berries of susceptible plants, causing a reduction in yield and berry quality. The effect of fungal infection of the fruit causes significant losses to both the winegrapes industry. Costs of disease management and yield losses in Australia are estimated at 5% the value of total grape production. *U. necator* originated in North America and is a particular threat to the cultivated grapevine species, *Vitis vinifera*, which is European in origin and lacks natural resistance to the pathogen. Powdery mildew was introduced into Europe in the mid 19th century and caused devastating losses until adequate chemical control measures were introduced (Olmo 1986).

Modern grapevine cultivation relies heavily upon the use of chemical fungicides, such as sulphur and sterol biosynthesis inhibitors. However, chemical application is costly, fungal strains have evolved that are fungicide insensitive (Erickson and Wilcox 1997) and it is now considered that widespread agrochemical use is environmentally detrimental. Consequently, it is desirable to identify natural sources of resistance to *U. necator* that might be employed to increase the resistance of cultivated vines.

Genetic resistance to powdery mildew has been identified in many crop species but the wine grape species *V. vinifera* displays no inherent resistance to this pathogen. One source of powdery mildew resistance is *Muscadinia rotundifolia*, a wild grapevine species originating from South Eastern USA. *M. rotundifolia* is resistant to a number of pathogens known to affect cultivated grapevines including powdery mildew, downy mildew, phylloxera and nematodes and could, therefore, provide an important source of resistance to a number of economically significant diseases (Olmo 1986). Classical genetic studies determined that powdery mildew resistance is controlled by a single, dominant gene in *M. rotundifolia*, termed resistance to *Uncinula necator* (*Run1*) (Bouquet 1986). A collaboration has been established between CSIRO Plant Industry and INRA in France for the molecular localisation and isolation of the *Run1* gene. The aim of this research project is to collaborate with INRA in the identification of markers tightly linked to *Run1* locus and establish the location of these genetic markers on a physical map to enable map-based cloning of the *Run1* gene. Cloning of this resistance gene would then enable the generation of mildew-resistant wine cultivars by genetic transformation.

The strategy being undertaken to achieve this goal involves two main components as outlined in Fig. 1. The first involves construction of a large DNA insert library called a Bacterial Artificial Chromosome (BAC) Library which contains the entire grapevine genome digested into manageable fragments ranging in size from ~100,000-200,000 basepairs. Within this library are overlapping DNA fragments some of which will contain the *Run1* resistance gene. The next step involves the screening of these DNA fragments to identify which are likely candidates to contain the *Run1* gene. This is done using genetic markers, which are short specific DNA sequences which have been shown to be physically located close to the *Run1* gene by virtue of the fact that they consistently co-segregate with powdery mildew resistance in our *Run1* backcross breeding populations. Along with our French collaborators in Montpellier, we have identified a range of genetic markers including Amplified Fragment Length Polymorphisms (AFLPs), Resistance Gene Analogs (RGAs) and Microsatellites which are closely linked to the *Run1* gene (Pauquet et al. 2001; Donald et al. 2001). These genetic markers are currently being used in the Adelaide laboratory to identify candidate DNA fragments which are likely to contain the *Run1* gene.
Fig. 1 Strategy for map-based cloning and functional testing of the powdery mildew resistance gene candidates from *Muscadinia rotundifolia*

**Project Aims and Performance targets**

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<tr>
<td>Low density genetic linkage map</td>
<td>Generate low density localised linkage map around <em>Run 1</em> by end 2001</td>
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<td>BAC library construction</td>
<td>Produce BAC library of ~ 50,000 clones by 2002</td>
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<td>Physical mapping of <em>Run 1</em> locus on BACs</td>
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<td>High density genetic linkage map around <em>Run 1</em> locus</td>
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<td>Physical mapping of the <em>Run 1</em> locus onto clones in the BAC library</td>
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Methods

Plant Material

Three populations were used in mapping studies: VRH3294 (VRH3082-1-42 x *V. vinifera* cv. Cabernet Sauvignon; 161 individuals), VRH3322 (VRH3176-21-11 x *V. vinifera* cv. Cabernet Sauvignon; 419 individuals) and VRH3328 (*V. vinifera* cv. Marselan x VRH3082-1-49; 416 individuals). The *V. vinifera* cultivars Cabernet Sauvignon and Marselan are susceptible to powdery mildew, whereas all other parents are resistant heterozygotes that carry the *Run1* gene (Pauquet et al. 2001).

Powdery mildew detached leaf assay

*Uncinula 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 (approx 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 4 times with sterile water. Leaves were placed on agar plates containing 1 % agar (w/v) and 400 μl ml⁻¹ 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.

Visualisation of *E. necator* development

*E. necator* development was visualised on sections (1 cm²) of fresh tissue, placed on moistened filter paper and viewed under a Zeiss Epi-luminescence microscope, and on sections fixed overnight in FAA [50% (v/v) ethanol, 0.5% (v/v) glacial acetic acid, 1% (v/v) formaldehyde] and then destained in 50% (v/v) ethanol. Sections were stained in 0.6% (w/v) coomassie blue, rinsed in water and mounted on microscope slides in 50% (v/v) glycerol and viewed using a Zeiss Axioskop2 microscope and recorded using a Spot digital camera (Diagnostic Instruments, SciTech, Australia).

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 isopropanol was included. DNA was washed with 70% ethanol, dried in a rotary evaporator and resuspended in TE.

Cloning of RGA genetic markers linked to *Run1*

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 ie, 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 ie. kinase-2 / GLPLAL or kinase-2 / MHD.
First round PCR reactions were carried out in a 20 μL volume containing 70 ng genomic DNA, 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% (v/v) Triton X-100, 2 mM MgCl₂, 0.2 mM dNTPs, 0.25 μM of each primer and 0.5 unit Taq Polymerase (Promega, Madison, WI). 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; 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.

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 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, CA) 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 Run1) 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 manufacturers instructions. Membranes were UV-crosslinked and then rinsed in 2X SSC (UV Stratalinker 1800, Stratagene, San Diego, USA). 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 (³²P) RGA probes were synthesised using a Gigaprim label kit (GeneWorks, Adelaide, Australia). Membranes were hybridised with RGA probes at 65°C overnight, washed twice with 2X SSC, 0.1% SDS at 65°C for 20 min and once with 0.1X 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 Hind III or Eco RI under the conditions recommended by the manufacturer (Promega), phenol/chloroform extracted, precipitated and resuspended in 1 x 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, incubated for 16 hours at 16°C. Genomic sequences 5’ and 3’ of the original cloned RGA sequences were obtained using an anchor-PCR technique. The Hind III ligation was used as 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 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 sec at 95°C, 30 sec 55°C, 2 min at 72°C; 10 min at 72°C. Subsequently, 1 μl of the PCR reaction was used in 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.

Linkage analysis

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

SSR marker analysis

VMC1g3.2, VMC8g9 and VMC4f3.1 are simple sequence repeat markers that were developed by the Vitis Microsatellite Consortium coordinated by Agrogène, France. The VRH3322 and VRH3328 populations were screened with VMC8g9 and VMC4f3.1 as described by Adam-Blondon et al. (2004). The VRH3294 population was screened with VMC1g3.2, VMC4f3.1 and VMC8g9 essentially as described by Thomas et al. (1994).

BAC library construction

An individual U. necator resistant plant from the VRH3294 population (3294-R23) was clonally propagated to generate sufficient leaf material for construction of the BAC library. Megabase-sized plant DNA was extracted, digested with HindIII or BamHI and size fractionated essentially as described by Peterson et al. (2000). The library was constructed in pIndigoBAC-5 (Epicentre, Madison, WI) and individual clones were stored in 144 x 384-well plates. The library was gridded onto three 22.5 cm² Hybond N+ filters (Amersham Biosciences, Buckinghamshire, UK) using the Genetix Q-Bot (Genetix Ltd., Hampshire, UK) and screened by hybridisation using standard techniques (Sambrook et al. 1989). PCR products were used as templates to generate 32P labelled probes using the GIGAprime DNA labelling kit (Geneworks, Adelaide, Australia) or Rediprime II DNA labelling kit (Amersham Biosciences, Buckinghamshire, UK). Sequence from the chloroplast genome-encoded RNA polymerase B gene from grapevine was obtained by PCR using primers RPOB1 (5’-CTT CCG AAT TAT ATG TAT CCG CG-3’) and RPOB2 (5’-CGA TTC ATA TTT CGT CGA CCA AC-3’).

For PCR-based screening, 29 DNA superpools were constructed that contained equal volumes of BAC DNA extracted from 384-well plates after overnight culture. Products were initially amplified from the 29 superpools, then individually from DNAs extracted from the five plates that composed any superpool found to contain a clone of interest. Single plates were then replicated twice, enabling DNAs to be extracted from groups of four columns or four rows, respectively. PCR using row and column pool DNAs identified a group of 16 clones that were then individually tested by colony PCR to identify the clone of interest.
**BAC DNA sequencing**

BAC end sequences were obtained either by using the sub-cloning procedure of Yang and Mirkov (2000) or by direct sequencing. Approximately 25 μg BAC DNA was used for sequencing using BigDye terminator V3.1 chemistry (Applied Biosystems, Foster City, CA) in a 40 μl volume containing 0.5 μM sequencing primer. Thermal cycling was performed in a PTC-200 thermal cycler (MJ Research, Inc., Waltham, MA) using 80 cycles of: 94 °C for 30 sec, 55 °C for 30 sec, 68 °C for 4 min.

**Multiplex PCR screening for new recombinants**

Grapevine seed was germinated in 15 cm diameter Petri dishes on filter paper. After germination, seeds were transferred to plug trays that contained 12 x 8 cells (the same configuration as 96-well plates) and grown until the first true leaves had developed. At this time, cotyledon tissue was harvested and DNA was extracted using the Qiagen 96-well DNA extraction kit. Using this system a total of 3418 plants were screened from three different populations. This DNA was then used in a Multiplex PCR reaction.
**Results / Discussion**

Some of the results presented in this report were presented in an Interim final report submitted to GWRDC in November 2003 and have also been published in:


1. **Run1 resistance phenotype**

**Results**

Plants have evolved two major resistance strategies against invasion by powdery mildew. The first is classified as penetration resistance and involves the reinforcement of the cell wall to stop penetration of the powdery mildew infection peg. If the pathogen is able to avoid the penetration resistance it will form a feeding structure (haustorium) within the penetrated epidermal cell to enable it to obtain nutrition required for growth. Plants may also mount a second line of resistance based on the initiation of programmed cell death processes within the penetrated cell, resulting in a hypersensitive response. This process is normally mediated by a resistance gene capable of recognising a pathogenicity product excreted from the invading pathogen.

In order to determine the mode of action of the Run1 gene, detached leaves from a 4th generation backcross (*M. rotundifolia x V. vinifera*) resistant plant and a susceptible Cabernet sauvignon plant were inoculated with powdery mildew and samples taken 24 and 48 hours after inoculation. Leaves were cleared of pigment and fungal structures stained for visualisation under a light microscope. Figure 2 shows that there is little difference in growth of the powdery mildew pathogen on resistant or susceptible leaves after 24 hours. However, after 48 hours, powdery mildew growth has ceased on the resistant leaves but has spread rapidly on the susceptible leaf. It is also clear that the specific epidermal cells which were penetrated on the resistant leaf have undergone programmed cell death.

**Discussion**

Run1 resistance appears to involve an induction of programmed cell death in the specific epidermal cell containing the fungal haustorium within 24-48 hours after infection. This mode of action shows strong similarities to the resistance response observed in barley cultivars carrying the dominant barley powdery mildew resistance gene *Mla.*
Fig. 2 Powdery mildew development on leaves from susceptible (A, C) and resistant (B, D) Run1-backcross progeny plants. Arrows indicate the penetrated epidermal cells, which have undergone programmed cell death in the resistant leaves.

2. Identification of genetic markers linked to Run1

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. 2) 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).

<table>
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<tr>
<th>N-terminus</th>
<th>Nucleotide binding site</th>
<th>Leucine-rich repeat region</th>
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<tr>
<td>P-loop</td>
<td>kinase-2</td>
<td>GLPL</td>
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<td>MHD</td>
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Fig. 3 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.
Cloning and characterisation of these PCR products revealed 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 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. 4. 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. 4).

**Fig. 4** 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. 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.
Initial screening of BC5 Run1 progeny was conducted using bulked segregant analysis (Michelmore et al. 1991) to identify any linkage between the isolated RGAs and the Run1 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 (ie 0.1x SSC, 65°C). A representative member of each of the 22 RGA groups (Fig. 5) was used as a probe in bulked segregant analysis with DNA bulked pools of 7 resistant and 7 susceptible individuals digested with Eco RI, Dra I and Rsa I.

Fig. 5 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.
Analysis indicated that the RGAs GLP1-12, MHD98 and MHD145 were polymorphic in Eco RI digests between resistant and susceptible pools. These RGAs were then used to probe Eco RI digests of genomic DNA obtained from two sub-groups of 8 resistant and 8 susceptible BC5 progeny to confirm the genetic association (Fig. 6). 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. 6). 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. 6). The polymorphism detected by MHD145 was present in all resistant samples tested and absent in all susceptible samples tested.

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**Fig. 6** Southern analysis of grapevine progeny segregating for powdery mildew resistance. Genomic DNA (4 μg / lane) was digested with Eco RI and hybridised with 32P-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 (*).
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. 5). Interestingly, the 5 kb MHD98 RFLP marker, which is linked to the resistance phenotype, was found to be absent from M. rotundifolia (Fig. 5) suggesting that it may have been introduced from one of the V. vinifera cultivars used during the pseudo backcross breeding program. To facilitate more rapid screening of the remaining BC5 population, RFLP markers GLP1-12, MHD98 and MHD145 were converted into PCR-based markers as described in Donald et al. (2002).

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 (ie. <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 (Aarts et al. 1998; Collins et al. 1998; Kanazin et al. 1996; Leister et al. 1996; Mago et al. 1999; Pan et al. 2000a; Speulman et al. 1998; Yu et al. 1996), 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. 3 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 (ie. 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.

Three of the RGAs isolated from the BC5 population were found to be linked to the powdery mildew resistance locus, Run1, and these were more extensively studied through segregation analysis. Two of the RFLPs identified by RGA probes, GLP1-12 and MHD145, cosegregated 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 Muscadania 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 1356 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.

3. Generation of a coarse genetic linkage map around *Run1*

*Results*

The previous studies of Pauquet et al. (2001) and our initial mapping work (described in Donald et al. 2002 – see Appendix 4) indicated that *Run1* co-localised with 11 AFLP and two RGA-derived markers in a VRH3294 population of 160 plants derived from a cross between the resistant parent, VRH3082-1-42 and the susceptible parent, *V. vinifera* cv. Cabernet Sauvignon (Fig. 7). In an attempt to identify further markers that were linked to the *Run1* locus, simple sequence repeat (SSR) markers were examined to identify those that showed polymorphism between the resistant and susceptible parents of the VRH3294 population. Three such markers were identified (VMC1g3.2, VMC4f3.1, VMC8g9) and analysis of these markers in the VRH3294 population indicated that all three were genetically linked to *Run1* (Fig. 8). VMC8g9 completely co-segregated with *Run1* in this population, whereas VMC4f3.1 and VMC1g3.2 were located 0.6 cM or 4.4 cM away from the resistance locus, respectively.

To facilitate scoring of the AFLP markers identified by Pauquet et al. (2001), amplified products corresponding to EMfd3 and EMhb1 were cloned, sequenced and used as probes in RFLP experiments or as PCR-based markers. Sfd3 and Shb1 were markers derived from Emfd3 and Emhb1, respectively, and both completely co-segregated with *Run1* in a sample of 160 plants tested from the VRH3294 population (data not shown).

As a large number of markers co-segregated with *Run1* in the VRH3294 population, further recombinant plants were sought to enable markers in this region to be ordered. Two alternative populations were examined: VRH3322 which was derived from a cross between the resistant parent, VRH3176-21-11 (Pauquet et al. 2001) and the susceptible parent, *V. vinifera* cv. Cabernet Sauvignon and VRH3328 which was derived from a cross between the resistant parent, VRH3082-1-49 (Pauquet et al. 2001) and the susceptible parent, *V. vinifera* cv. Marselan (Fig. 7). Plants were initially scored using the PCR-based markers, GLP1-12, VMC4f3.1, VMC8g9 and Shb1. Once recombinant plants had been identified, they were tested for powdery mildew resistance and further markers were analysed. This resulted in the isolation of a number of recombinant plants, two of which showed recombination between VMC8g9 and *Run1* and four of which showed recombination between Shb1 and *Run1*. This allowed a marker order to be assigned in which VMC8g9 and Shb1 are located on the opposite side of *Run1* to VMC4f3.1 (Fig. 8). Thus, *Run1* is located in the interval defined by the closest flanking markers, VMC4f3.1 and VMC8g9.
Discussion

A number of different genetic markers were identified which are linked to the Run1 locus. Utilising the wealth of information now available through the construction of Vitis linkage maps, three SSR markers were identified that were genetically linked to the resistance locus. Run1 was located in the interval between the closest flanking SSR markers, VMC4f3.1 and VMC8g9 and, as such, is located in a region equivalent to linkage group 12 of the V. vinifera consensus maps of Riaz et al. (2004) and Adam-Blondon et al. (2004). Interestingly, our analysis of recombinant plants in the VRH3322, VRH3328 and VRH3294 populations indicated that, of the three SSR markers linked to Run1, VMC4f3.1 was located between VMC1g3.2 and VMC8g9. This order differs from the map published by Riaz et al. (2004), who place VMC1g3.2 between VMC4f3.1 and VMC8g9. The reason for this discrepancy is unknown. However, the identification of markers that flanked Run1 on both sides was of crucial importance, as it delimited the locus and allowed the production of a physical map spanning the locus to proceed following production of the BAC library.

Fig. 7 Breeding strategy used to generate Run1 backcross populations. Selected progeny containing the Run1 gene are shown in bold.
4. Preparation of the Run1 BAC library

Results

Generally, the fresh tissue method for preparation of high molecular weight (HMW) DNA (Peterson et al. 2000) resulted in the production of 10-15 nuclei-containing agarose plugs from each 100 g of starting tissue. The Hind III concentration producing the maximum amount of partially restricted DNA in the 150-400 kb range was 18-30 units per plug. In contrast, the optimal Bam HI concentration was 3-9 units per plug. For large-scale digestions a total of ten plugs were divided into three different concentrations within the optimal enzyme concentration range.

Following large-scale digestion and pulsed field gel electrophoresis (PFGE), the region containing DNA in the size range of 150-400 kb was excised from the gel and divided into three horizontal sections, as described by Peterson et al. (2000). Each of the three sections was treated to a second size selection, electro-elution and the DNA was concentrated using Centricon YM-100 columns. The total amount of DNA recovered from each section was estimated to range from 500-1200 ng, with 250 ng generally used in a ligation reaction.

Following transformation and plating, 10-20 recombinant clones from ligations of insert DNA from each of the three sections were selected for further analysis. The lower and middle sections always resulted in a higher ratio of white to blue colonies, a higher average insert size and less false positives than the higher section. DNA from the lower and middle sections was therefore used in the construction of the library. From clones randomly selected from the Hind III ligations that were used to construct the library the insert sizes ranged from 40 to 150 kb and at least 90% of the clones contained inserts greater than 100 kb in size (figure Z). The average insert size of the Hind III-derived clones was estimated at 115 kb. The insert size of the Bam HI-derived clones ranged from 20 to 140 kb with at least 50% of the clones containing inserts of 100 kb, or greater, in size (Fig. 9). The average insert size of the Bam HI clones was 85 kb (data not shown).
In total we generated a BAC library of 55,296 clones, 49,920 were Hind III-derived and the remaining 5,376 were Bam HI-derived. Less than 5% of the clones did not contain an insert as judged by random analysis of BACs sampled from each ligation preparation. In addition, to obtain an estimate of the representation of chloroplast DNA in the library one third of the library (represented on one filter) was screened with a portion of the grapevine RNA polymerase B chloroplast gene. Results from this screening indicated that approximately 1.7% of the cloned sequences were chloroplast-derived.

**Fig. 9** Analysis of Hind III derived BAC clones by pulsed field gel electrophoresis. Average insert size was approximately 120kb.

**Discussion**

Initial attempts at isolating HMW DNA from grapevine using the popular method of Zhang et al. (1995) yielded nuclei pellets, and nuclei containing plugs, that were dark brown in colour due to oxidisation of endogenous polyphenols. Although the DNA extracted using this method was of HMW, as seen by a large concentration of DNA around the 600 kb area following PFGE, the DNA was difficult to digest. Binding of oxidised polyphenols to DNA is thought to render it resistant to digestion (Katterman and Shattuck, 1983; Guillemaut and Maréchal-Drouard, 1992) and this is likely to account for difficulties in digesting the HMW DNA extracted using this method. In addition, the HMW DNA which could be digested was generally too small for BAC cloning (<100 kb), presumably due to digestion of the DNA by endogenous nucleases.

Subsequently, the method of Peterson et al (2000) was attempted as this method was designed for plant tissues, such as grapevine, that contain high levels of secondary compounds including polyphenols. This method involves the isolation of nuclei from fresh leaf tissue. The volumes of starting tissue and buffers used were scaled-down considerably from the published quantity (Peterson et al. 2000) indicating that the method can be successfully used for smaller scale preparations. Using the fresh tissue method enabled the isolation of nuclei pellets with significantly reduced browning. In addition, the amount of DNA degraded during the preparation was significantly decreased compared to what was seen after preparation using the
method of Zhang et al. (1995) suggesting that the activity of endogenous nucleases had been inhibited (result not shown).

From fresh leaf tissue of a powdery mildew resistant individual from the BC5 population (VRH 3294), we constructed a grapevine BAC library that meets the above criteria and is therefore suitable for physical mapping and map-based cloning. The grapevine genome is estimated to be approximately 511 Mb. The average DNA insert size of the Run1 BAC library is approximately 120 kb and taking into account false positives and chloroplast contamination, the library represents at least 13 haploid genome copies which gives a greater than 99 % probability that it contains any particular clone. The inclusion of 5,376 clones constructed using the Bam HI enzyme increases the chances that any particular sequence is present in the library as it avoids any bias that may result from using only one enzyme in library construction.

The quantity of Bam HI required to digest the maximum amount of DNA in the 150 - 400 kb range was consistently less than for Hind III. This suggests that either there were more Bam HI sites in the grapevine genome or, alternatively, that the Bam HI enzyme is more efficient under the reaction conditions used. The sizes of inserts recovered from positive BAC clones identified by library screening ranged from 100 to 145 kb, supporting the estimation of average insert sizes and suggesting that it may even be an underestimation. Alternatively, there may be a disproportionately low number of Hind III sites in genomic regions containing the target sequences.

1. Physical mapping of the Run1 locus

Results

The results obtained from screening the three plant populations enabled a genetic map to be proposed in which MHD145, GLP1-12 and Sfd3 completely co-segregated with Run1, with the closest flanking markers being VMC4f3.1 and VMC8g9 (Fig. 8). To convert the genetic map into a physical region containing the Run1 gene, the Run1 BAC library was screened, either by filter hybridisation or by PCR amplification of products from the DNAs of appropriately pooled BAC clones, to identify BACs that contained the resistant alleles of each of the five genetic markers most closely linked to Run1. A process of direct BAC sequencing was optimised to enable BAC end sequence to be obtained that could be used to identify further, overlapping BACs from the library. Repetition of this process allowed BAC contigs to be assembled surrounding each of the five genetic markers and linkage between the EMFD3 and GLP contigs was achieved. This process is described in more detail in Barker et al. (2005; see - Appendix 4). The resulting five contigs currently spanned over 2 Mb of DNA (Fig. 10)

During the course of building the BAC contigs, BAC end sequences were obtained that could be used to design primers to enable PCR identification of overlapping BACs. Some of these primers were also useful as genetic markers (Fig. 11), greatly increasing the number of markers available that were linked to Run1.
Fig. 10 A physical map of the region surrounding Run1. BAC clones were assembled into contigs representing the resistant or susceptible chromosome and extended by identification of overlapping BACs. Genetic markers used to initiate contigs are shown in bold and the location of multiple GLP1-12-hybridising sequences is bracketed. PCR products used to align BACs are shown and their presence in individual BAC clones is indicated by dotted lines. PCR products that could not be used as genetic markers, but were informative in contig assembly, are shown in parentheses. All other PCR products are dominant, co-dominant (*) or cleaved amplified polymorphic sequence (**) markers for powdery mildew resistance.

Fig. 11 Location of new genetic markers derived from BAC sequencing. The markers originally identified as AFLP, RGA or microsatellites are shown in red. All markers shown in black were derived from BAC sequences. Note that there is a gap in the vmc4f3.1/ GLP1-12/EmFd3 contig which has not yet been resolved.
Analysis of BAC contigs initiated from screening with genetic markers Sfd3, MHD145 and GLP1-12, indicated that the physical distance between these markers was much greater than initially suggested by genetic mapping. Therefore, more seed of populations VRH3294, VRH3322 (Fig. 7) and a new population involving a cross between VRH3161-6-4 x Cabernet sauvignon (designated VRH3264) were generated by our INRA collaborators and sent to CSIRO in 2003. These populations were then screened to find recombinants that would allow the marker order and position of Run1 to be more accurately determined. In order to enable screening of a large number of individuals we developed a rapid screening method based on large scale (96 samples per run) DNA extraction and multiplex PCR. The PCR screen involved the simultaneous amplification of three dominant markers (CB137.138, 49MRP1.P2 & CB69.70; see Fig. 11) and a control fragment (CB3.4). A second PCR reaction was also carried out on each plant to assess for the presence/absence of marker MHD98.

Analysis of these VRH3294, VRH3322 and VRH3264 populations enabled us to identify a large number of recombinant plants and the breakpoints in these plants were determined. Each of the recombinant lines was then evaluated for powdery and downy mildew resistance. We had previously observed that BC4 progeny not only showed total resistance to powdery mildew but also appeared to contain a major gene conferring resistance to downy mildew (*Plasmopora viticola*) designated Rpv1. The resistance to downy mildew is not complete, as in the case of Run1-mediated powdery mildew resistance, as a low level of sporulation is observed in detached leaf assays. However, it should be noted that detached leaf assays employ much higher levels of inoculum than are likely to occur in the field and recent observations from France, suggest that the level of resistance conferred by Rpv1 is sufficient to give good field resistance to downy mildew (Alain Bouquet, personal communication).

In the search for further recombinants, a collaboration was also established with Dr Didier Merdinoglu at INRA Colmar in France who is working with other *M. rotundifolia* x *V. vinifera* mapping populations designated 40525 and 41524. All 40525 and 41524 plants were first tested for powdery and downy mildew resistance in Colmar and then screened with genetic markers linked to Run1 supplied by CSIRO. No plants were identified in which powdery mildew resistance was separated from downy mildew resistance, however a number of plants with recombination events within the introgressed region were identified.

The full range of recombinant plants obtained from the screening of the VRH3294, VRH3322, VRH3264, 40525 and 41524 populations are summarised in Fig. 12, together with the results of disease testing for these individual recombinant progeny.

**Discussion**

Over 50 BACs known to form contigs surrounding each of the five original genetic markers were isolated with each contig spanning an estimated 270-720 kb. BAC end sequences obtained during contig assembly were used to generate over 25 new genetic markers in the vicinity of Run1, vastly increasing the density of markers in this region. Mapping of the recombination breakpoints in progeny from 5 different segregating populations greatly decreased the size of the region in which Run1 and Rpv1 are thought to be located. The next step in the process was to sequence the region thought to contain Run1 and Rpv1 to identify any potential resistance gene analogues within this linked region.
Fig. 12 Summary of genetic marker distribution and disease susceptibility in *M. rotundifolia* x *V. vinifera* progeny populations. Resistance (R) or susceptibility (S) to detached leaves to powdery mildew (PM) or downy mildew (DM) is shown. The blue box indicates the region predicted to contain *Run1* and *Rpv1* resistance genes based on the marker and phenotypic data.

2. Sequencing of the putative *Run1* region and identification of *Run1* and *Rpv1* resistance gene candidates

*Results & Discussion*

Twelve individual BACs covering the region of interest as defined in Fig. 13 were sent to Genoscope, France where they were sequenced by shot-gun cloning each BAC into ~5 kb fragments and sequencing the ends of each fragment in the sub-clone library. Sufficient sub-clones were end sequenced until a 10X coverage of the BAC was achieved. Raw sequence data was then made available to CSIRO PI for Bioinformatic analysis to identify all potential gene coding sequences. These potential coding sequences were then compared with known gene sequences on the NCBI GenBank database to determine the putative identity of these unknown grapevine gene sequences. To date 11 BAC clones have been fully sequenced (representing ~950,000 bp) and 7 fully analysed using Bioinformatic analysis software tools to identify potential gene sequences.

Analysis of the BAC sequence obtained to date has revealed that much of the introgressed region from *M. rotundifolia* is composed of non-coding DNA. Of the genes that are present we have identified the presence of a large family of genes with strong homology to glycosyltransferases and a large number of transposable elements. However, of significance to this project is the identification of a single cluster or family of resistance gene analogues (RGAs) as shown in Fig. 13. Closer analysis of the RGA sequences reveals that the RGA family contains 4 full-length genes (*RGA-1*, *RGA-2*, *RGA-4* and *RGA-8*) and 4 truncated or non-functional genes (*RGA-3*, *RGA-5*, *RGA-6* & *RGA-7*). Two further partial length RGA sequences have not yet been fully characterized as they are located within the gap in our contig. Attempts to clone genomic fragments from the “gap” region using a cosmid library have only been partially successful suggesting this region may be composed of highly repeated DNA.
Reverse transcriptase-PCR analysis of RGA expression in Run1 resistant grapevine progeny has confirmed that all of the full-length coding sequences ie. RGA-1, RGA-2, RGA-4 and RGA-8 are expressed in resistant plants. Significantly, translation of these RGA gene sequences show them to encode TIR-NBS-LRR proteins which are very similar to that of the *Mla* powdery mildew resistance genes isolated from barley (Halterman et al. 2001). Based on this information, members of this RGA family represent our strongest candidates for the Run1 and Rpv1 resistance genes.
Outcome / Conclusion

This CRCV2 project has made excellent progress in the localisation and identification of candidates for the Run1 powdery mildew resistance gene and the Rpv1 downy mildew resistance gene from *M. rotundifolia*. This is highlighted by the following major achievements:

- Development of in-house technology to construct a high molecular weight bacterial artificial chromosome (BAC) library from a selected BC5 resistant progeny plant containing approximately 55,000 clones, with average insert size of approximately 120 kb, which represents 5x haploid genome copies (Barker et al. 2005)
- Isolation of over 30 genetic markers (RGAs, AFLPs and microsatellites) closely linked to the Run1 locus (Pauquet et al. 2001; Donald et al. 2002) and use of these genetic markers to screen the BAC library to identify BAC clones linked to the Run1 locus (Barker et al. 2005)
- Development of a high-throughput screening technique to enable screening of a further 4000 segregating seedlings from seed supplied by INRA. This enabled us to delimit the region containing the Run1 locus to approx 1 x 10^6 base pairs.
- Confirmation that Rpv1, a major gene conferring resistance to downy mildew, is also co-located within this same region of DNA introgressed from *M. rotundifolia*.
- Isolation of 12 BAC & cosmid clones that form a nearly contiguous region spanning the region containing the Run1 / Rpv1 locus (Fig. 13). Eleven BAC clones have now been fully sequenced (~950,000 bp) and 7 fully analysed using Bioinformatic analysis software tools to identify potential gene sequences.
- Computer analysis of the BAC sequence obtained, so far, suggests the presence of only one cluster of genes that are likely candidates for the Run1 and Rpv1 genes. These genes have been designated resistance gene analogues (RGAs) and encode TIR-NBS-LRR type proteins very similar in structure to the *Mla* powdery mildew resistance genes isolated from barley. To date, 4 full-length RGA genes and 4 truncated (non-functional) genes have been identified (Fig. 14).

It is important to recognise that the cloning of a resistance gene from grapevine has never been previously attempted, unlike the situation in many other crop species. As such it is a particularly challenging and difficult project with the potential for unforeseen delays and problems. For example, one of the unforeseen delays in the project has been caused by the presence of a region of highly repetitive DNA within the introgressed region that we have had difficulty cloning and sequencing using our BAC library approach resulting in a “gap”. The presence of partial RGA-like sequences within this “gap” region (RGA-x & RGA-y) necessitates the development of new techniques to try and fill this gap to determine if it contains any other potential full length RGA sequences that need to be functionally tested. Another unforeseen delay in the expected progress of this project has been due to the lack of genetic recombination surrounding the Run1 locus. Despite screening over 5000 individual grape progeny plants, the region containing the Run1 and Rpv1 genes could only be narrowed down to approx 1 x 10^6 base pairs. In contrast, the barley *Mla* powdery mildew resistance gene was successfully localised to a region approx 4 times smaller (Wei et al. 1999). This has meant that we have had to map, sequence and analyse approximately 1 million bp of the grapevine genomic sequence to look for all potential resistance gene candidates in the region containing Run1 and Rpv1. We estimate that both of these factors combined have probably delayed our original milestone forecasts at the beginning of the Run1 CRCV project by approximately 2 years. These unforeseen delays in identifying the resistance gene candidates has meant that it was impossible for us to meet the final milestone of our project involving transformation of these candidates into susceptible grapevine cultivars. However, we are now in a position to initiate this next phase of the project.
In conclusion, we have made good progress towards the identification and characterization of natural resistance genes from the wild American grapevine species *M. rotundifolia*. The introduction of these natural resistant genes into our current winegrape selections, will provide the Australian Viticultural industry with significant competitive advantages not only in terms of reduced production costs and increased yields, but in terms of making a sustainable and quantifiable contribution to our image as a “clean & green” wine producer.

**Recommendations**

The next stage of the project requires each of the full-length RGA candidates be sub-cloned from the grapevine genomic DNA into suitable vectors in preparation for transformation into susceptible *V. vinifera* cultivars. However, there is still a region within the introgressed fragment that we have not been able to fully clone and sequence using our BAC library approach (see Fig. 1). An attempt to fill this “gap” through the construction of a separate cosmid library was also only partially successful. The presence of partial RGA-like sequences within this region (RGA-x & RGA-y) indicates that it will be important to fill this gap to determine if it contains any other potential full length RGA sequences that need to be functionally tested.

Once generated, transgenic plantlets expressing individual RGA candidates will need to be tested for resistance to powdery mildew and downy mildew relative to untransformed controls. The generation of these transgenic lines containing individual RGA candidates will also provide the possibility to evaluate each of these RGA gene candidates against a range of different powdery mildew isolates to enable us to determine both the degree, phenotype and race-specificity (if any) of the resistance conferred by each RGA in the absence of the other alleles.

Work is now underway to sub-clone the RGA candidates into binary expression vectors for transformation into susceptible *V. vinifera* cultivars. Constructs are first being tested by transient expression in tobacco by agroinfiltration to check for expression of the constructs under the control of the native grapevine promoter. These experiments will also provide confirmation of the predicted splicing regions in the coding region.

One of the important questions to be resolved regarding the efficacy of the *Run1* and *Rpv1* genes in the field is the possible existence and geographic distribution of powdery mildew and downy mildew isolates which are capable of breaking the genetic resistance. The most likely location for such isolates would be North America. However, it would be impossible to import North American mildew isolates into Australia to do this type of research because of the risks of accidental release. Therefore, in order to address this question, a collaboration has been established with Dr David Gadoury at Dept of Plant Pathology, Cornell University and Dr Lance Cadle-Davidson from the USDA Grape Genetics Research Unit, both located at the New York Agricultural Experimental Station, Geneva, New York State, with the aim of establishing and genotyping a collection of North American powdery and downy mildew isolates. These isolates could then be used to evaluate the pathogen specificity of *Run1* / *Rpv1* transgenic progeny sent to New York Agricultural Experimental Station. Funding is currently being sought from the USDA National Research Initiatives Program to support this research.
Appendix 1: Communication

Scientific Publications


Conference Presentations


Industry Journal articles

Article published in National GrapeGrowers Magazine, Sept 2001 "Search for a powdery mildew resistance gene" outlining research in CRCV Project 3.1.1


“Running towards resistance” – Article in CSIRO Plant Industry newsletter: Issue 6, Winter 2004

Improved grapevine performance and fruit quality through gene technology. Do you know the latest – Edition 3 July 2005 Winetac

Brochures/Technical Guides

Appendix 2: Intellectual Property

Intellectual property for this project is equally shared between CSIRO and INRA under an existing contractual agreement. Commercialisation will most likely be through registration of new grapevine cultivars via PVR. Consideration will also be given to patenting the Run1 gene once identified and then licensing its use third parties for the introduction into other grapevine cultivars.
Appendix 3: References


Erickson EO and Wilcox WF (1997) Distributions of sensitivities to three sterol demethylation inhibitor fungicides among populations of Uncinula necator sensitive and resistant to triadimefon. Phytopathology 87: 784-791.


Appendix 4: Staff

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Appendix 5:

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Appendix 6: Budget reconciliation
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Identification of resistance gene analogs linked to a powdery mildew resistance locus in grapevine

Abstract Oligonucleotide primers, designed to conserve 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, Run1, introgressed into Vitis vinifera L. from the wild grape species Muscadinia rotundifolia. Three RGA markers were found to be tightly linked to the Run1 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 Run1 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)

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 Run1 (for Resistance to Uncinula necator 1) (Bouquet 1986; Pauquet et al. 2001). The Run1 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 Run1 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 Run1 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 (Run1) locus.

### Materials and methods

**Plant material**

Segregating populations from a pseudo-backcrossing strategy, aimed at introgressing the Run1 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 re-suspended 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 MgCl2, 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 Omni-E thermal cycler (Hybaid, Middlesex, UK) with the following program: 95°C for 2 min;
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 (FMIC 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 with either EcoRI or SacII for 2 h at 37°C and fractionated on a 2% agarose gel. pGem-T vectors do not contain Rsal or SacII 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 terminating 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 Runl) or susceptible BC3 progeny were digested separately with EcoRI, DraI and Rsal, electrophoresed on a 0.8% agarose gel and transferred to Hybond N+ membranes (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 EcoRI or ApaI/SacI 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 [32P] RGA probes were synthesised using a Gigaprim 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 ~8°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 EcoRI 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 described 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 HindIII or EcoRI 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 HindIII ligation was used as a template for PCR with the primer GLP1–12PR in combination with the vector specific primer, T3 (Promega). Similarly, the EcoRI 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 GLP1–12PRn or MHD98P2n in combination with T3. PCR conditions for the nested reaction were identical to the initial reaction. The resulting products, of approximately 1 kb (GLP1–12PRn) 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′,
GLP1–12PRn : 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 ATT TGG AGA AGC ATC-3′,
MHD98P2 : 5′-ACA AGA GTT CTC CAA GTG TTT-3′,
MHD98P2n : 5′-GGT AAT CCT TGG CAT TTC TCT-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
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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 (Run1)

Initial screening of BC5 Run1 progeny was conducted using bulked segregant analysis (Michelmore et al. 1991) to identify any linkage between the isolated RGAs and the Run1 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 EcoRI, Dral and Rsal. Analysis indicated that the RGAs GLP1–12, MHD98 and MHD145 were polymorphic in EcoRI digests between resistant and susceptible pools (data not shown).

These RGAs were then used to probe EcoRI 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 EcoRI, 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 Dendrogram 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.

Fig. 4 Southern analysis of grapevine progeny segregating for powdery mildew resistance. Genomic DNA (4 µg/lane) was digested with EcoRI and hybridised with 32P-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 (*).

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 HindIII restriction fragment, which hybridised to GLP1–12 in resistant BC5 samples and contained an internal EcoRI site (Fig. 5A). To determine whether this EcoRI site could be used to 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 EcoRI 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.
convert GLP1–12 into a cleaved amplified polymorphic sequence (CAPS) marker, the 1-kb HindIII 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, EcoRI 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 EcoRI restriction fragments of a different size were also produced after digestion of the P1/P3 PCR product which were not linked to the Run1 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 EcoRI 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 Run1 locus.
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 Run1 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 Run1 locus (Fig. 6). Pauquet et al. (2001) have also recently identified amplified fragment length polymorphism (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.

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 resistance locus Run1.
tance locus, Run1, 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 Musca
dinia-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 (Solanum tuberosum), N (tobacco), N-like (Arabidopsis) and M (Linum usitatissimum). 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 Run1, 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 Run1 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, Run1, which was introgressed into V. vinifera from M. rotundifolia (Bouquet 1986; Pauquet et al. 2001). Introduction 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 Run1 (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 Run1 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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Use of molecular techniques for the transfer of powdery mildew resistance from a wild American grapevine into elite winegrape cultivars

Powdery mildew of grapevine, caused by *Uncinula necator*, is the most economically significant fungal disease of grapes (*V. vinifera* L.) worldwide, causing reduced yield and loss of berry/wine quality. While it is difficult to assess the true cost of this pathogen to the Australian viticultural industry, it is estimated that the combined costs of disease management and yield losses are in the order of 5% of total value of winegrape production, which represented a loss of ~$80m in 2002.

There are no commercially-grown cultivars of *V. vinifera* that are resistant to *U. necator* and control of this pathogen is currently achieved by the widespread application of fungicides, such as sulphur and, more recently, systemic demethylation inhibitors. Whilst these chemicals are relatively effective, the cost to the grower and the environmental impact of the residues remains undesirable. Furthermore, isolates of *U. necator* resistant to the demethylation 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.

Powdery mildew is thought to have originated on wild vines in the eastern and central United States. The disease was introduced into Europe in the 1840s causing serious crop losses up until the introduction by French growers of sulphur applications in 1855. Early researchers noted that American vines were much less susceptible to powdery mildew than European vines. This led to importations of American germplasm for breeding programs in an attempt to introduce genetic resistance into the French varieties. However, most French-American hybrids have not proved successful for the production of premium wine because of the undesirable flavour compounds that are also introduced from the American germplasm during the breeding process.

The wild American grape species *Muscadinia rotundifolia*, a native to the south-eastern United States, is highly resistant to most pathogens of *Vitis vinifera* including powdery mildew (Olmo, 1971). French scientists working at INRA in Montpellier have carried out crosses between *M. rotundifolia* and *V. vinifera* to study the genetic basis of the powdery mildew resistance from this American grape species. From this work they concluded that resistance to powdery mildew derived from *M. rotundifolia* is conferred by a single locus which they designated *Run1* (for Resistance to *Uncinula necator* 1) (Bouquet, 1986; Pauquet et al., 2001). The *vinifera* x *rotundifolia* crosses obtained from their work have been growing in France for 40 years and have shown no signs of susceptibility to powdery mildew over that period.
In 1997, CSIRO Plant Industry established a research collaboration with INRA to determine the identity of the Run1 gene. The Run1 germplasm was first imported into our quarantine glasshouse and confirmed to be resistant to all Australian powdery mildew isolates tested (Figure 1).

*U. necator* is an obligate biotroph, which means that it derives its nutrition from living host cells. As the fungal hyphae grows across the surface of the leaf or berry tissue, it forms a lobed structure called an appressorium under which a penetration peg is formed. This peg penetrates through the cuticle and into the host epidermal cell and forms a feeding structure called a haustorium via which the fungus absorbs nutrients from the host (Heintz and Blaich, 1990). Some plants have evolved an efficient system to restrict the growth of biotrophic pathogens, such as powdery mildew, which involves triggering the death of the host cell in which the haustorium is located, thereby depriving the fungus of the nutrients it requires for further growth and development. This programmed cell death (also known as a hypersensitive response) has been shown to be activated by a host resistance gene following recognition of a specific protein excreted by the invading fungal pathogen (Hammond-Kosack and Jones, 1997). Our investigations indicate that the resistance response controlled by the Run1 gene also involves induction of programmed cell death, specifically within the penetrated epidermal cell, approximately 36-48 hours following powdery mildew infection (Figure 2).

The recent development of molecular techniques for the identification and cloning of plant resistance genes now offers the possibility to isolate and separate the Run1 powdery mildew resistance gene from the other “negative” wine quality genes present in the Muscadine genome. This gene could then be introduced into existing *V. vinifera* cultivars, via genetic transformation (Iocco et al., 2001), resulting in the generation of premium winegrape cultivars with natural genetic resistance to powdery mildew, but without the negative impacts on wine quality associated with traditional breeding techniques.

The strategy being undertaken to achieve this goal involves two main components as outlined in Figure 3. The first involves construction of a large DNA insert library called a Bacterial Artificial Chromosome (BAC) Library which contains the entire grapevine genome digested into manageable fragments ranging in size from ~100,000-200,000 basepairs. Within this library are overlapping DNA fragments some of which will contain the Run1 resistance gene from the other “negative” wine quality genes present in the Muscadine genome. This gene could then be introduced into existing *V. vinifera* cultivars, via genetic transformation (Iocco et al., 2001), resulting in the generation of premium winegrape cultivars with natural genetic resistance to powdery mildew, but without the negative impacts on wine quality associated with traditional breeding techniques.

The next step involves the screening of these DNA fragments to identify which are likely candidates to contain the Run1 gene. This is done using genetic markers, which are short specific DNA sequences that have been shown to be physically located close to the Run1 gene by virtue of the fact that they consistently co-segregate with powdery mildew resistance in our Run1 backcross breeding populations. Along with our French collaborators in Montpellier, we have identified a range of genetic markers, including Amplified Fragment Length Polymorphisms (AFLPs), Resistance Gene Analogs (RGAs) and Microsatellites, which are closely linked to the Run1 gene (Pauquet et al., 2001; Donald et al., 2001). These genetic markers are currently being used in our Adelaide laboratory to identify candidate DNA fragments that are likely to contain the Run1 gene.

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**Fig. 1.** Comparison of powdery mildew susceptibility of *V. vinifera* cv. Cabernet sauvignon (A) with fourth generation progeny plant from *V. vinifera* × *M. rotundifolia* backcross (B) containing Run1 powdery mildew resistance gene. Both plants were inoculated at the same time with the same powdery mildew inoculum.

**Fig. 2.** Powdery mildew development on leaves from susceptible (A, C) and resistant (B, D) Run1-backcross progeny plants. Spores were inoculated onto detached leaves and incubated at 25°C. Leaf samples were taken at 24hr (A, B) and 48hr (C, D) post-inoculation, fixed and stained with Coomassie blue to visualise fungal structures. Arrows indicate the penetrated epidermal cells, which have undergone programmed cell death in the resistant leaves.
Based on the work of research groups who have successfully cloned powdery mildew genes from other crops, it is likely that the Run1 gene will be located within a cluster of closely-related resistance-type genes (Wei et al., 1999; Halterman et al., 2001), making it difficult to determine, by sequence alone, which gene confers powdery mildew resistance. It will be necessary, therefore, to carry out a functional analysis of the various Run1 candidates within the resistance cluster, by transforming each gene into a susceptible V. vinifera cultivar and testing each of the transgenic lines for resistance to powdery mildew (Figure 3). Only after this functional analysis has been undertaken can the identity of the DNA fragment containing the Run1 gene be confirmed.

Another significant outcome of this research is the discovery that the Run1 grapevine material also shows strong resistance to another important grapevine pathogen, downy mildew. Our initial studies indicate that the downy mildew resistance gene appears to be located in the vicinity of the Run1 gene. We are hopeful, therefore, that future research will also enable us to identify a gene conferring natural genetic resistance to downy mildew and that this gene could ultimately be introduced, along with Run1, into the genetic makeup of our elite wine cultivars. The introduction of these natural resistance genes into our current vinegrape selections will provide the Australian viticulture industry with significant competitive advantages, not only in terms of lowered production costs and increased yields, but also in terms of enhancing our image as a ‘clean & green’ wine producer through a reduction in the use of chemical inputs in the vineyard.

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References


Abstract Resistance to grapevine powdery mildew is controlled by Run1, a single dominant gene present in the wild grapevine species, Muscadinia rotundifolia, but absent from the cultivated species, Vitis vinifera. Run1 has been introgressed into V. vinifera using a pseudo-backcross strategy, and genetic markers have previously been identified that are linked to the resistance locus. Here we describe the construction of comprehensive genetic and physical maps spanning the resistance locus that will enable future positional cloning of the resistance gene. Physical mapping was performed using a bacterial artificial chromosome (BAC) library constructed using genomic DNA extracted from a resistant V. vinifera individual carrying Run1 within an introgression. BAC contig assembly has enabled 20 new genetic markers to be identified that are closely linked to Run1, and the position of the resistance locus has been refined, locating the gene between the simple sequence repeat (SSR) marker, VMC43.1, and the BAC end sequence-derived marker, CB292.294. This region contains two multigene families of resistance gene analogues (RGA). A comparison of physical and genetic mapping data indicates that recombination is severely repressed in the vicinity of Run1, possibly due to divergent sequence contained within the introgressed fragment from M. rotundifolia that carries the Run1 gene.

Introduction

Grapevine powdery mildew is caused by the biotrophic pathogen, Uncinula necator, and is the most serious fungal disease of grapevines worldwide. The pathogen infects the leaves and berries of susceptible plants, causing a reduction in yield and berry quality. U. necator originated in North America and is a particular threat to cultivated grapevines as most are of the species Vitis vinifera, which is European in origin and lacks natural resistance to the pathogen. Powdery mildew was introduced into Europe in the mid-19th century and caused devastating losses until adequate chemical control measures were introduced (Olmo 1986). Modern grapevine cultivation relies heavily upon the use of chemical fungicides, such as sulphur and sterol biosynthesis inhibitors, to control the pathogen. However, chemical application is costly, fungal strains have evolved that are fungicide-insensitive (Pearson 1980; Erickson and Wilcox 1997) and it is now considered that widespread agrochemical use is environmentally detrimental. Consequently, it is desirable to identify natural sources of resistance to U. necator that might be employed to increase the resistance of cultivated vines.

One source of powdery mildew resistance is Muscadinia rotundifolia, a wild grapevine species originating from southeastern USA. M. rotundifolia is resistant to a number of pathogens known to affect cultivated grapevines, including powdery mildew, downy mildew, phylloxera and nematodes and could, therefore, provide an important source of resistance to a number of economically significant diseases (Olmo 1986). Classical genetic
studies determined that powdery mildew resistance is controlled by a single, dominant gene in *M. rotundifolia*, termed resistance to *U. necator* 1 (*Run1*) (Bouquet 1986; Pauquet et al. 2001), and a number of genetic markers have been identified that are linked to the resistance locus (Pauquet et al. 2001; Donald et al. 2002). Interestingly, the GLP1-12 and MHD145 markers identified by Donald et al. (2002) that co-segregate with *Run1* are sequences that show homology to many other plant resistance genes. The majority of plant resistance genes identified to date encode modular proteins with a central nucleotide binding site (NBS) linked to a C-terminal leucine-rich repeat (LRR) domain (Belkhadir et al. 2004). Conserved motifs within the NBS have been used to design degenerate primers capable of amplifying novel resistance gene analogue (RGA) sequences in many plants (Leister et al. 1996; Aarts et al. 1998; Collins et al. 1998) and, in the case of GLP1-12 and MHD145, the products amplified by Donald et al. (2002) were used as probes to identify restriction fragment length polymorphisms (RFLPs) linked to *Run1*. GLP1-12 is a dominant RFLP marker that hybridizes a 1.6-kb *Eco*RI fragment found only in the genome of resistant plants, whereas MHD145 is a co-dominant RFLP marker that hybridizes a 2.7-kb *Eco*RI fragment from the genome of susceptible plants or a 2.2-kb fragment from resistant plants (Donald et al. 2002). However, of the 17 amplified fragment length polymorphism (AFLP) and RGA-derived markers identified in previous studies, 13 co-segregate with the resistance locus in the population of approximately 160 plants tested and the remaining four adjacent markers were all located on one side of *Run1* (Pauquet et al. 2001; Donald et al. 2002). Therefore, an improved genetic map is required to define the location of the resistance locus before positional cloning can proceed.

The second requirement for successful positional cloning is the availability of a large-insert DNA library containing the gene of interest that is suitable for chromosome walking. In recent years, bacterial artificial chromosome (BAC) vectors have become the vehicle of choice for the generation of large-insert libraries due to their increased stability and ease of manipulation in comparison to previously used vectors such as cosmids or yeast artificial chromosomes (Shizuya et al. 1992; Woo et al. 1994). We describe here the construction of a BAC library suitable for positional cloning of *Run1* and the generation of comprehensive genetic and physical maps spanning the resistance locus.

### Materials and methods

Plant material and evaluation of resistance to powdery mildew

Three populations of *Vitis vinifera* were used in the mapping studies: Mtp3294 (VRH3082-1-42 × *V. vinifera* cv. Cabernet Sauvignon; 161 individuals), Mtp3322 (VRH3176-21-11 × *V. vinifera* cv. Cabernet Sauvignon; 419 individuals) and Mtp3328 (*V. vinifera* cv. Marselan × VRH3082-1-49; 416 individuals). The *V. vinifera* cultivars Cabernet Sauvignon and Marselan are susceptible to powdery mildew, whereas all other parents are resistant heterozygotes that carry the *Run1* gene (Pauquet et al. 2001). Resistance to powdery mildew in the Mtp3294 population has been described by Donald et al. (2002). The Mtp3322 and Mtp3328 populations were tested according to Pauquet et al. (2001).

#### Simple sequence repeat marker analysis

VMC1g3.2, VMC8g9 and VMC4f3.1 are simple sequence repeat (SSR) markers that were developed by the *Vitis* Microsatellite Consortium coordinated by Agrogène, France. The Mtp3322 and Mtp3328 populations were screened with VMC8g9 and VMC4f3.1 as described by Adam-Blondon et al. (2004). The Mtp3294 population was screened with VMC1g3.2, VMC4f3.1 and VMC8g9 essentially as described by Thomas et al. (1994).

MHD145 and GLP1-12 marker analysis

Primers GLP1-12P1 and GLP1-12P3, the analysis of MHD145 and GLP1-12 as RFLP markers and the analysis of GLP1-12 as a PCR-based marker have been previously described (Donald et al. 2002).

#### BAC library construction

An individual *Uncinula necator*-resistant plant from the Mtp3294 population (3294-R23) was clonally propagated to generate sufficient leaf material for construction of the BAC library. Megabase-sized plant DNA was extracted, digested with *Hind*III or *Bam*HI and size-fractionated essentially as described by Peterson et al. (2000). The library was constructed in pIndigoBAC-5 (Epigenome, Madison, Wis.), and individual clones were stored in 144 × 384-well plates.

The library was gridded onto three 22.5-cm² Hybond N+ filters (Amersham Biosciences, Buckinghamshire, UK) using the Genetix Q-Bot (Genetix, Hampshire, UK) and screened by hybridisation using standard techniques (Sambrook and Russell 2001). The PCR products were used as templates to generate [³²P]-labelled probes using the GIGAprime DNA labelling kit (Geneworks, Adelaide, Australia) or Rediprime II DNA labelling kit (Amersham Biosciences). The sequence of the chloroplast genome-encoded RNA polymerase B gene from grapevine was obtained by PCR using primers RPOB1 (5'-CTT CCG AAT TAT ATG TAT CCG CG-3') and RPOB2 (5'-CGA TTC ATA TTT CGT CGA CCA AC-3').
For PCR-based screening, we constructed 29 DNA superpools that contained equal volumes of BAC DNA extracted from 384-well plates following overnight culture. Products were initially amplified from the 29 superpools, then individually from DNAs extracted from the five plates that composed any superpool found to contain a clone of interest. Single plates were then replicated twice, enabling DNAs to be extracted from groups of four columns or four rows, respectively. The PCR using row- and column-pooled DNAs identified a group of 16 clones that were then individually tested by colony PCR to identify the clone of interest.

BAC DNA sequencing

The BAC end sequences were obtained either by using the sub-cloning procedure of Yang and Mirkov (2000) or by direct sequencing. Approximately 25 μg BAC DNA was used for sequencing using BigDye terminator V3.1 chemistry (Applied Biosystems, Foster City, Calif.) in a 40-μl volume containing 0.5 μM sequencing primer. Thermal cycling was performed in a PTC-200 (MJ Research, Waltham, Mass.) using 80 cycles of: 94°C for 30 s, 55°C for 30 s, 68°C for 4 min.

Results

Genetic mapping

The previous studies of Pauquet et al. (2001) and Donald et al. (2002) indicated that Run1 co-localised with 11 AFLP and two RGA-derived markers in a Mtp3294 population of 160 plants derived from a cross between the resistant parent, VRH3082-1-42 and the susceptible parent, V. vinifera cv. Cabernet Sauvignon. In an attempt to identify further markers that were linked to the Run1 locus, we examined SSR markers to identify those that showed polymorphism between the resistant and susceptible parents of the Mtp3294 population. Three such markers were identified (VMC1g3.2, VMC4f3.1 and VMC8g9), and analysis of these markers in the Mtp3294 population indicated that all three were genetically linked to Run1 (Fig. 1). Thus, Run1 is located in the interval defined by the closest flanking markers, VMC4f3.1 and VMC8g9.

Generation of BAC library

To facilitate positional cloning of the Run1 gene, a BAC library was generated using the genomic DNA of a resistant individual from the Mtp3294 population (3294-R23) as the DNA source. In total, the 3294-R23 BAC library contained 55,295 clones, of which 49,920 were HindIII-derived and 5,376 were BamHI-derived. An analysis of 38 randomly selected clones indicated that insert sizes ranged between 40 kb and 160 kb, with an average insert size of 93 kb. Less than 5% of the clones did not contain an insert, and approximately 1.7% of the clones contained chloroplast-derived DNA, as determined by screening one-third of the library with the grapevine RNA polymerase B chloroplast gene (data not shown).

As a large number of markers co-segregated with Run1 in the Mtp3294 population, we sought additional recombinant plants to enable markers in this region to be ordered. Two alternative populations were examined: Mtp3322, which was derived from a cross between the resistant parent, VRH3176-21-11 (Pauquet et al. 2001), and the susceptible parent, V. vinifera cv. Cabernet Sauvignon, and Mtp3328, which was derived from a cross between the resistant parent, VRH3082-1-49 (Pauquet et al. 2001), and the susceptible parent, V. vinifera cv. Marsevan. Plants were initially scored using the PCR-based markers GLP1-12, VMC4f3.1, VMC8g9 and Shb1. Once recombinant plants had been identified, they were tested for powdery mildew resistance, and additional markers were analysed. This resulted in the isolation of a number of recombinant plants, two of which showed recombination between VMC8g9 and Run1 and four of which showed recombination between Shb1 and Run1. This allowed a marker order to be assigned in which VMC8g9 and Shb1 are located on the opposite side of Run1 to VMC4f3.1 (Fig. 1). Thus, Run1 is located in the interval defined by the closest flanking markers, VMC4f3.1 and VMC8g9.

Table 1

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Fig. 1 Genotype of informative recombinant plants isolated from the Vitis vinifera populations Mtp3294, Mtp3322 and Mtp3328. The presence of either the resistant (+) or susceptible (−) allele of each marker is shown. Markers shown in bold are RGA markers previously described by Donald et al. (2002).
not shown). Taking into account average insert size, percentage of empty clones, percentage of clones containing chloroplast DNA and a genome size of 511 Mb (Thomas et al. 1993), the library is calculated to contain 9.4-fold coverage of the haploid grapevine genome.

Assembly of BAC contigs containing genetic markers

To initiate a physical map of the region containing Run1, we screened the 3294-R23 BAC library to identify clones containing three genetic markers that co-segregate with resistance (GLP1-12, MHD145 and Sfd3) and two flanking markers (VMC8g9 and VMC4f3.1). For VMC4f3.1, VMC8g9 and Sfd3, between seven and nine clones were isolated from the library, close to the expected representation of a single-copy sequence in a library of predicted 9.4-fold haploid genome coverage. In contrast, over 60 clones were identified that contained MHD145-hybridising sequences, and over 50 hybridised the GLP1-12 probe. This indicated that GLP1-12 and MHD145 might represent repeated elements, or gene families, present within the grapevine genome.

Once clones containing genetic markers had been identified, they were assessed to determine the allele present. The BACs were then grouped into “resistant” and “susceptible” contigs representing the chromosome containing Run1 derived from the resistant parent, VRH3082-1-42, or the chromosome lacking Run1 derived from the susceptible parent, V. vinifera cv. Cabernet Sauvignon, respectively. A comparison of restriction digest patterns was used to determine overlap between BAC clones, and alignments were confirmed by PCR using primers designed from BAC end sequences. To determine if primers designed using BAC end sequences could be used as PCR-based markers for powdery mildew resistance, we amplified products from a selection of resistant and susceptible genotypes from the Mtp3294 population. This enabled a large number of dominant and co-dominant markers to be identified. Primers that amplified products of identical size from both resistant and susceptible genotypes were used to directly sequence BAC clones assigned to contigs representing each chromosome that were known to contain the region of interest. Alignment of sequences enabled polymorphic nucleotides to be identified that could be utilised to generate cleaved amplified polymorphic sequence (CAPS) markers. Primers were then used to isolate overlapping clones that could be used to extend BAC contigs (Fig. 2).

For all three single-copy genetic markers (VMC4f3.1, Sfd3 and VMC8g9), BAC contigs were assembled that represented the resistant and susceptible chromosome surrounding the marker. The haplotypic nature of corresponding contigs was confirmed in each case as BAC end sequences could be used to generate new co-dominant markers. The largest BAC contig assembled was initiated from Sfd3, and the minimum tiling path consisted of five clones representing the resistant chromosome. The contig was estimated to span over 400 kb, but could not be extended beyond CB298.299 as 86E16 was the only clone in the library that contained this sequence.

Assembly of BAC contigs containing RGA families

BACs containing MHD145-hybridising and GLP1-12-hybridising sequences were examined by RFLP analysis, which enabled them to be grouped into contigs representing the resistant or susceptible chromosomes (Fig. 2). Of 49 clones examined, only two overlapping BACs were identified that contained the 2.2-kb MHD145-hybridising EcoRI fragment characteristic of resistant plants, and of 43 GLP1-12-hybridising BACs examined, five contained the 1.6-kb EcoRI fragment characteristic of resistant plants.

The two overlapping BACs that contained the 2.2-kb MHD145-hybridising EcoRI fragment contained additional fragments that hybridised the probe, indicating that more than one homologous sequence was present.

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Fig. 2 A physical map of the region surrounding Run1. The BAC clones were identified that contained genetic markers linked to Run1. Clones were assembled into contigs representing the resistant or susceptible chromosome and extended by the identification of overlapping BACs. Genetic markers used to initiate contigs are shown in **bold**, and the location of multiple GLP1-12-hybridising sequences is shown by **square brackets**. PCR products used to align BACs are shown, and their presence in individual BAC clones is indicated by **dotted lines**. The PCR products that could not be used as genetic markers, but were informative in contig assembly, are shown in **parenthesis**. All other PCR products are dominant, co-dominant (**single asterisk**) or CAPS (**two asterisks**) markers for powdery mildew resistance.
By comparing EcoRI digests and RFLP patterns, other MHD145-hybridising BACs could be aligned with those containing the fragments characteristic of the co-dominant marker (Fig. 3), and overlap between clones was confirmed by PCR amplification using primers designed to BAC end sequences. Three BACs containing MHD145-hybridising sequences were assembled into each of the resistant and susceptible contigs (Fig. 2), and additional co-dominant markers designed using BAC end sequence information confirmed that the two contigs were haplotypes. The resistant contig contained three MHD-145 hybridising sequences, and the susceptible contig contained six, as determined by RFLP analysis (Fig. 3).

In contrast, the five clones identified that contained the 1.6-kb EcoRI fragment that hybridised the GLP1-12 probe did not completely overlap, suggesting that the hybridisation signals observed upon RFLP analysis of genomic DNA might be contributed by multiple members of a gene family. Three BACs containing the characteristic 1.6-kb EcoRI fragment formed the minimum tiling path through the resistant contig (26C5, 4M9 and 101P19), and a further GLP1-12-hybridising BAC (87P7) could be added to the contig using the dominant marker, CB13.14, to confirm overlap with 26C5 (Figs. 2, 4a). Two BAC clones that did not contain GLP1-12-hybridising sequences (82O20 and 61N18) were also added to the contig using the BAC end-derived markers, CB37.38 and CB63.64 (Figs. 2, 4a). However, the contig could not be extended beyond CB90.91 due to a lack of additional clones present in the library that contained this dominant marker sequence.

To confirm that multiple GLP1-12-hybridising sequences were present in the BAC contig, products were amplified from GLP1-12-containing BACs using primers GLP1-12P1 and GLP1-12P3. Following RFLP analysis (Fig. 4), PCR products were amplified from GLP1-12-containing BACs using primers GLP1-12P1 and GLP1-12P3. Following RFLP analysis, four clones displaying distinct restriction patterns are shown (numbered 1–4). Approximate sizes are given in base pairs.

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**Fig. 3** MHD145 hybridises to a multigene family of RGAs. BAC DNAs containing MHD145-hybridising sequences were digested with EcoRI, and DNA fragments were separated by agarose gel electrophoresis. Ethidium bromide-stained DNA fragments were visualised using a UV transilluminator (left) before preparation of a Southern blot (right). Approximate sizes are given in kilobase pairs, and the location of the 2.2-kb EcoRI fragment that cosegregates with powdery mildew resistance upon RFLP analysis of genomic DNA is indicated by an asterisk.

**Fig. 4** GLP1-12 hybridises to a multigene family of RGAs. A BAC DNAs containing GLP1-12-hybridising sequences were digested with EcoRI, and the fragments were separated by agarose gel electrophoresis. Ethidium bromide-stained DNA fragments were visualised using a UV transilluminator (left) before preparation of a Southern blot (right). Approximate sizes are given in kilobase pairs, and the location of the 1.6-kb EcoRI fragment that cosegregates with powdery mildew resistance upon RFLP analysis of genomic DNA is indicated by an asterisk. B PCR products were amplified from BAC DNAs containing GLP1-12-hybridising sequences using primers GLP1-12P1 and GLP1-12P3. Products were cloned and used as templates for RFLP analysis. Four clones displaying distinct restriction patterns are shown (numbered 1–4). Approximate sizes are given in base pairs.
analysis of individually cloned products, we were able to identify four distinct family members (Fig. 4b).

Genetic mapping using BAC-derived markers

All Mtp3322, Mtp3294 and Mtp3328 recombinant plants were rescreened with BAC-derived genetic markers obtained as a result of physical mapping. This allowed the recombination breakpoint in 3322-42 to be located within the MHD145 contig as between the BAC-derived markers, CB292.294 and CB69.70 (Fig. 5). These two markers were generated using sequence data obtained from the two ends of a single BAC clone, 79A15, which also contains MHD145 (Fig. 2). As 3322-42 possesses the resistant allele of CB292.294 (Fig. 5) but the susceptible alleles of MHD145 (Fig. 1) and CB69.70 (Fig. 5), the recombination breakpoint is located in an interval predicted to be less than 100 kb, between markers MHD145 and CB292.294. Significantly, the identification of the recombination breakpoint in 3322-42 defines the position and orientation of the MHD145 contig with respect to other genetic markers, placing MHD145 between VMC8g9 and GLP1-12/Sfd3 (Fig. 6). It also allows the position of Run1 to be refined, placing the resistance locus between VMC4f3.1 and CB292.294.

**Discussion**

**Genetic and physical mapping of the Run1 locus**

Utilising the wealth of information now available through the construction of *Vitis* linkage maps, we identified three SSR markers that were genetically linked to the resistance locus. Run1 was located in the interval between the closest flanking SSR markers, VMC4f3.1 and VMC8g9 and, as such, is located in a region equivalent to linkage group 12 of the *V. vinifera* consensus maps of Riaz et al. (2004) and Adam-Blondon et al. (2004). Interestingly, our analysis of recombinant plants in the Mtp3322, Mtp3328 and Mtp3294 populations indicated that, of the three SSR markers linked to Run1, VMC4f3.1 was located between VMC1g3.2 and VMC8g9. This order differs from the map published by Riaz et al. (2004), who place VMC1g3.2 between VMC4f3.1 and VMC8g9. The reason for this discrepancy is unknown. However, the identification of markers that flanked Run1 on both sides was of crucial importance, as it delimited the locus and allowed the production of a physical map spanning the locus to proceed. The production of a comprehensive BAC library suitable for chromosome walking allowed contigs containing five genetic markers that co-localised or flanked the Run1 locus to be constructed. Interestingly, for four

![Fig. 5](image-url)  
**Fig. 5** Analysis of recombinant plants using BAC-derived markers identifies the recombination breakpoint in 3322-42. Two resistant and two susceptible, non-recombinant genotypes from the Mtp3294 population were analysed using BAC-derived markers along with the recombinant genotypes 3294-R37, 3328-245 and 3322-42. For CB314.315, *Alu*I-digested products are shown; for CB3.4, *Taq*I-digested products are shown. All other markers are dominant PCR-based markers for resistance. Approximate product sizes are indicated in base pairs.

![Fig. 6](image-url)  
**Fig. 6** A schematic representation of marker order as determined by a combination of genetic and physical mapping. As a result of contig assembly, 20 new BAC-derived genetic markers have been identified that are closely linked to Run1. The location of these markers is shown with respect to previously characterised genetic markers. An *arrow* indicates the predicted location of Run1, and an *asterisk* marks the recombination breakpoint in 3322-42 that defines the position and orientation of the MHD145 contig with respect to other markers. Markers shown in *bold* are RGA markers previously described by Donald et al. (2002). Markers belonging to BAC contigs that cannot be orientated by available recombinant plant data are grouped and *boxed*. 
out of five of these markers, contigs representing homologous regions could be assembled that were derived either from the resistant chromosome that carried Run1 or from the susceptible chromosome contributed by V. vinifera cv. Cabernet Sauvignon. The haplotypic nature of all four pairs of contigs was confirmed by the discovery of numerous co-dominant markers designed using BAC end sequences, indicating that the introgression from M. rotundifolia that carries the Run1 gene shows co-linearity with the corresponding region of the V. vinifera genome. The only contig for which an equivalent region representing the susceptible chromosome could not be constructed was initiated from the RGA-marker, GLP1-12. GLP1-12 is a dominant marker for resistance, as were all six new genetic markers designed using BAC end sequences from the GLP1-12 contig. Consequently, these markers could not be used to identify BAC clones representing the susceptible chromosome. The inability to identify co-dominant markers might indicate significant sequence divergence in this region between the V. vinifera and M. rotundifolia chromosomes.

Although our BAC library was theoretically comprehensive, contig extension revealed that in at least two locations there are gaps in the coverage of the library. The extension of contigs beyond markers CB90.91 and CB298.299 was impossible due to a lack of overlapping clones. Two restriction enzymes were used to digest high-molecular-weight genomic DNA to produce BAC clones. However, the majority of clones (91.3%) were generated as a result of HindIII digestion. The existence of gaps in the library might represent bias in the distribution of HindIII sites in the grapevine genome, as over-representation or under-representation of HindIII sites would lead to the production of HindIII fragments too small or too large for cloning, respectively. This problem may be overcome by using high-molecular weight DNA digested with a number of different restriction enzymes, ensuring that the library constructed is completely representative of the genome.

Two different families of RGAs co-segregate with Run1

The 20 BAC-derived genetic markers that resulted from physical mapping were used to refine the location of Run1 to within an interval between markers CB292.294 and VMC4f3.1. This region contains two families of RGA sequences, both of which are currently candidates for containing the Run1 gene. The MHD145 probe hybridises to at least three homologous genes present on the resistant chromosome and six on the susceptible. The multigenic nature of this locus supports the previous observations of Donald et al. (2002), who noted that multiple restriction fragments hybridised MHD145 in RFLP studies. In contrast, Donald et al. (2002) observed only one RFLP fragment that hybridised GLP1-12 in mapping studies, whereas our physical mapping data now suggests that this locus is also multigenic, comprising at least four members on the resistant chromosome.

Many disease resistance genes are members of multigene families, and these vary in size, both in terms of number of homologues present and the region they span. One of the largest RGA families is the Dm3 cluster found in lettuce that confers resistance to the downy mildew pathogen, Bremia lactucae. This locus contains 24 NBS-LRR encoding genes in a region spanning 3.5 Mb, with an average intergenic distance of 145 kb (Meyers et al. 1998). In contrast, the tomato I2 locus contains seven NBS-LRR encoding genes in a region of 90 kb, with an intergenic distance of 8–10 kb (Simons et al. 1998), the potato Gpa2 locus contains four NBS-LRR encoding genes in a region of 115 kb (van der Vossen et al. 2000) and the tomato Ml locus contains three NBS-LRR encoding genes in a region of 52 kb (Milligan et al. 1998). Consequently, the multigenic nature of the MHD145 and GLP1-12-hybridising sequences linked to Run1 is not unusual for an RGA locus. The introgression from M. rotundifolia is associated with repressed recombination

The pronounced clustering of markers identified by Pauquet et al. (2001), Donald et al. (2002) and in the current study suggests that either these markers are physically close to Run1 or that recombination rates are unusually low in the vicinity of the resistance locus. Two lines of evidence now suggest that the latter is true. First, both Riaz et al. (2004) and Adam-Blondon et al. (2004) used the SSR markers, VMC4f3.1 and VMC8g9, during the construction of linkages maps spanning the V. vinifera genome and placed these two markers 12.85 cM or 10.9 cM apart in consensus maps, respectively. In the Mtp3294 mapping population studied, VMC8g9 and VMC4f3.1 were placed 0.6 cM apart in the map of the resistant female parent and 14.1 cM apart in the susceptible male map (Adam-Blondon unpublished), indicating at least an 18- to 23-fold reduction in recombination frequency in genotypes heterozygous for powdery mildew resistance. Second, based on the genome size of grapevine being between 475 Mb (Lodhi and Reisch 1995) and 511 Mb (Thomas et al. 1993) and the total linkage map of V. vinifera being between 1,728 cM (Riaz et al. 2004) and 2,200 cM (Adam-Blondon et al. 2004), on average 1 cM should be equivalent to approximately 216–296 kb. However, we have assembled three contigs that represent the resistant chromosome surrounding genetic markers GLP1-12, Sfd3 and MHD145 that together span over 1 Mb, but have not yet observed physical linkage between contigs, despite complete co-segregation of these markers in over 900 plants studied from three mapping populations.

Reduced recombination frequencies have been observed around resistance loci in wheat, barley and poplar where resistance has been introduced via an introgressed
The generation of comprehensive genetic and physical maps spanning the Run1 locus has greatly improved our ability to localise this agriculturally significant disease resistance gene and will provide an excellent foundation for future map-based cloning efforts. Once identified, Run1 will provide a natural means to improve the resistance of cultivated grapevines to *U. necator* worldwide without the need for extensive agrochemical use.

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