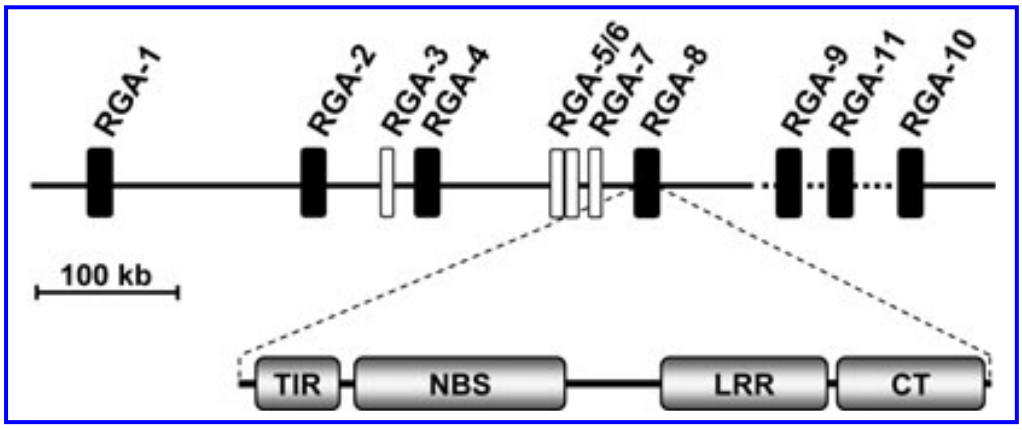
Grapevine genes for improved resistance to fungal pathogens to reduce chemical inputs in Australian vineyards



FINAL REPORT to
GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **CSP 06/04**

Principal Investigators: **Dr Ian Dry & Dr Mark Thomas**

Research Organisation: **CSIRO Plant Industry**

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Table of Contents

Abstract …… 2

Executive summary ……...…...3

Background ……..5

Project Aims & Performance targets …….……..…....7

Methods ……11

Results / Discussion ……14

Outcome / Conclusion ……42

Recommendations ……..……...43

Appendix 1: Communication ……....44

Appendix 2: Intellectual Property ……....46

Appendix 3: References ……..…..47

Appendix 4: Staff ……..…...49

Appendix 5: Supplementary Data …….……..…..50

Appendix 6: Budget reconciliation …….……..…..54

Appendix 7: Publications …….……..…..55

1. Abstract *(100 words max)*

This project has successfully cloned the powdery mildew resistance (*Run1*) and downy mildew resistance (*Rpv1*) genes from the wild North American grapevine *Muscadinia rotundifolia.* Introduction of these resistance genes into susceptible winegrape cultivars by genetic transformation produces transgenic vines with strong resistance to these fungal pathogens in laboratory-based assays. A unique group of "susceptibility" genes have also been identified which may pre-dispose grapevine to powdery mildew infection. Transgenic grapevines in which these "susceptibility genes" have been down-regulated are currently being generated and will be evaluated to determine if they show increased resistance to powdery mildew.

2. Executive summary

Powdery mildew caused by *Erysiphe necator* (syn. *Uncinula necator*) and downy mildew caused by *Plasmopora viticola* are the two most economically important grapevine diseases worldwide. Genetic resistance to powdery and downy mildew has been identified in many crop species, but the winegrape species *Vitis vinifera* displays no inherent resistance to these fungal pathogens. As a result, control of these diseases is entirely dependent on the widespread application of fungicides. It has recently been estimated that costs of disease management and yield losses to the Australian Wine Industry are of the order of 76 and 63 million AUD per year for powdery mildew and downy mildew respectively. There is also increasing world-wide pressure to reduce agrochemical use for the control plant pathogens on crops grown for human consumption. These problems could be overcome by the development of grapevine cultivars with enhanced genetic resistance to powdery and downy mildew.

In a previous GWRDC-funded CRCV project (CRV 99/14a), good progress was made towards the genetic mapping and sequencing of the *Run1* (resistance to *Uncinula necator*) / *Rpv1* (resistance to *Plasmopora viticola*) locus from the wild North American grapevine *Muscadinia rotundifolia.* That project identified four full-length resistance gene analog (RGAs), which show a high degree of similarity to fungal resistance genes isolated from other crop species. However, sequencing of certain regions of the *Run1*/*Rpv1* locus remained incomplete and evidence indicated that other RGA sequences were present.

The current project has successfully completed the cloning and sequencing of all seven major resistance gene analog sequences located at the *Run1*/*Rpv1* locus: *RGA-1*, *RGA-2*, *RGA-4*, *RGA-8*, *RGA-9* and *RGA-10*. In collaboration with our colleagues at INRA, Montpellier, France, over 300 transgenic grapevines were generated containing the individual RGA genes to determine which of these *RGA*s confer resistance to powdery and/or downy mildew. The testing of these transgenic vines is still ongoing, but based on the results obtained to date the following conclusions can be drawn:

- *RGA-10* is the major gene responsible for conferring powdery mildew resistance associated with the *Run1*/*Rpv1* locus. Three different susceptible *V. vinifera* cultivars transformed with the *RGA-10* gene show complete resistance to powdery mildew in *in-vitro* assays.
- Other genes at *Run1*/*Rpv1* locus ie. *RGA-2* and *RGA-4* may operate as minor powdery mildew resistance genes.
- *RGA-8* is the gene responsible for conferring downy mildew resistance associated with the *Run1*/*Rpv1* locus. Two different susceptible *V. vinifera* cultivars transformed with the *RGA-8* gene show significantly increased resistance to downy mildew in *in-vitro* assays.

RGA-10 (*Run1*) and RGA-8 (*Rpv1*) represent the first two resistance genes to be cloned from a wild grapevine species and shown to confer resistance when transferred to a susceptible *V. vinifera* cultivar. These results provide proof of concept that the natural genetic resistance of existing premium winegrape cultivars can be enhanced through the transfer of single resistance genes from wild grape species by genetic transformation

Although we currently have no knowledge of the existence of any powdery mildew isolates in France or Australia which are capable of breaking the resistance conferred by *Run1*, one can speculate that the potential deployment of these genes in vineyards, on a large scale, may increase the pressure for the selection of new mildew isolates which may reduce its effectiveness. This is especially true when dealing with a perennial crop, like grapevine, which can be expected to be in the field for at least 20 years. Thus, the second major aim of this project was to identify other unique sources of powdery mildew resistance which could be used to complement the action of *Run1*. This "pyramiding" of different powdery mildew resistance strategies within the same plant has been shown to be important in other crops in maintaining durable genetic resistance in the field.

This complementary approach focussed on the search, in grapevine, for the existence of a recently discovered group of powdery mildew "susceptibility" genes called *MLO*s. Research from other plant species has shown that specific members of the *MLO* gene family actually appear to pre-dispose plants to powdery mildew infection, as demonstrated by the fact that naturally occurring mutants which lack these *MLO* genes are much less susceptible (ie. more resistant) to this fungal pathogen..

This project undertook a functional characterisation of the seventeen member *VvMLO* gene family in grapevine in an attempt to identify those genes most likely to be involved in powdery mildew susceptibility. The research identified three genes *VvMLO3*, *VvMLO4* and *VvMLO17* which, based on a number of parameters including sequence similarity with *MLO* "susceptibility" genes from other plant species, transcriptional responses to powdery mildew infection and ability to complement Arabidopsis *mlo* mutants, are strong candidates for having a role in powdery mildew susceptibility in grapevine. To obtain definitive evidence for this, research was initiated to generate transgenic grapevines in which these candidate MLO genes were down-regulated or "silenced".

Gene-silencing constructs targeting these *VvMLO* candidate genes were initially evaluated in a rapid grapevine hairy root assay system and shown to be effective at silencing these *VvMLO* genes in grape cells. Transformation of these *VvMLO*-silencing constructs into powdery mildew susceptible grapevines has commenced and the first batch of transgenic grapevines successfully generated. Initial analysis of these transgenic lines indicates that some appear to show measurable decreases powdery mildew susceptibility, but further testing is required to confirm these preliminary results.

This project has made excellent progress in the identification of grapevine genes for improved resistance to fungal pathogens and demonstrates the potential of genetic transformation for enhancement of existing winegrape cultivars. Future research will seek to assess the performance of these vines in unsprayed vineyards and against a range of different fungal isolates. We will also endeavour to develop new 2nd generation transgenics with multiple resistance genes to ensure the durability of the resistance in the vineyard. Finally, using the information obtained from the sequencing of the *Run1*/*Rpv1* locus, we have designed new "perfect" genetic markers which will be used for marker-assisted selection of new disease-resistance cultivars by traditional breeding.

3. Background

Powdery mildew caused by *Erysiphe necator* (syn. *Uncinula necator*) and downy mildew caused by *Plasmopora viticola* are the two most economically important grapevine disease worldwide. Costs of disease management and yield losses in Australia are estimated at 76 million AUD and 63 million AUD per year for powdery mildew and downy mildew respectively (1). Control of these fungal pathogens is currently achieved by the widespread application of fungicides. However, there is world-wide pressure to reduce the use of "hard" chemicals to control plant pathogens on crops grown for human consumption. Indeed, the European Commission is currently considering a proposal to ban the use of a large number of agrochemicals in Europe by 2013 which are routinely used for the control of a number of fungal diseases, including powdery mildew in grapes (2). These problems could be overcome by the development of grapevine cultivars with enhanced genetic resistance to powdery and downy mildew.

In a previous GWRDC-funded CRCV project (CRV 99/14a), good progress was made towards the identification and isolation of natural genes for powdery mildew (*Run1*) and downy mildew (*Rpv1*) resistance from the wild North American grapevine *Muscadinia rotundifolia*. Genetic markers linked to the *Run1* and *Rpv1* genes were identified and used to define an introgressed region from *M. rotundifolia* comprising ~ 1 x 10^6 base pairs conferring resistance to grapevine powdery and downy mildew. Sequencing of this introgressed region identified a cluster of resistance gene-like sequences, designated resistance gene analogs (RGAs), which show homology with resistance genes isolated from other crop species. At the conclusion of project CRV99/14a, four full-length and four truncated (non-functional) RGAs had been identified. However, there was a region within the RGA cluster that had proved very difficult to clone leaving a "gap" in the genomic sequence. Furthermore, sequencing of genomic fragments cloned from this "gap" region indicated the presence of other RGA sequences which needed to be cloned and sequenced to determine if they are full-length or truncated genes.

Once all of the RGAs present at the *Run1/Rpv1* locus have been cloned, the next stage of the project requires each of the full-length RGA candidates be sub-cloned from the grapevine genomic DNA into suitable bacterial vectors in preparation for genetic transformation into susceptible *V. vinifera* cultivars (Fig. 1). Once generated, transgenic plant expressing individual RGA candidates will be tested for resistance to powdery mildew and downy mildew relative to untransformed controls. The results of these experiments will tell us how many of the RGA genes at the *Run1/Rpv1* locus contribute to the observed resistance. 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.

Although we currently have no knowledge of the existence of any powdery mildew isolates in France or Australia which are capable of breaking the resistance conferred by *Run1*, one can speculate that the potential deployment of this gene in vineyards, on a large scale, may increase the pressure for the selection of new powdery mildew isolates which may reduce the effectiveness of *Run1*. In order to improve the durability of this genetic resistance in the field, the second major component of this project is to identify other unique sources of powdery mildew resistance to complement the action of *Run1*. This "pyramiding" of different powdery mildew resistance genes within the same cultivar has been shown to be important in maintaining durable field resistance in other crops.

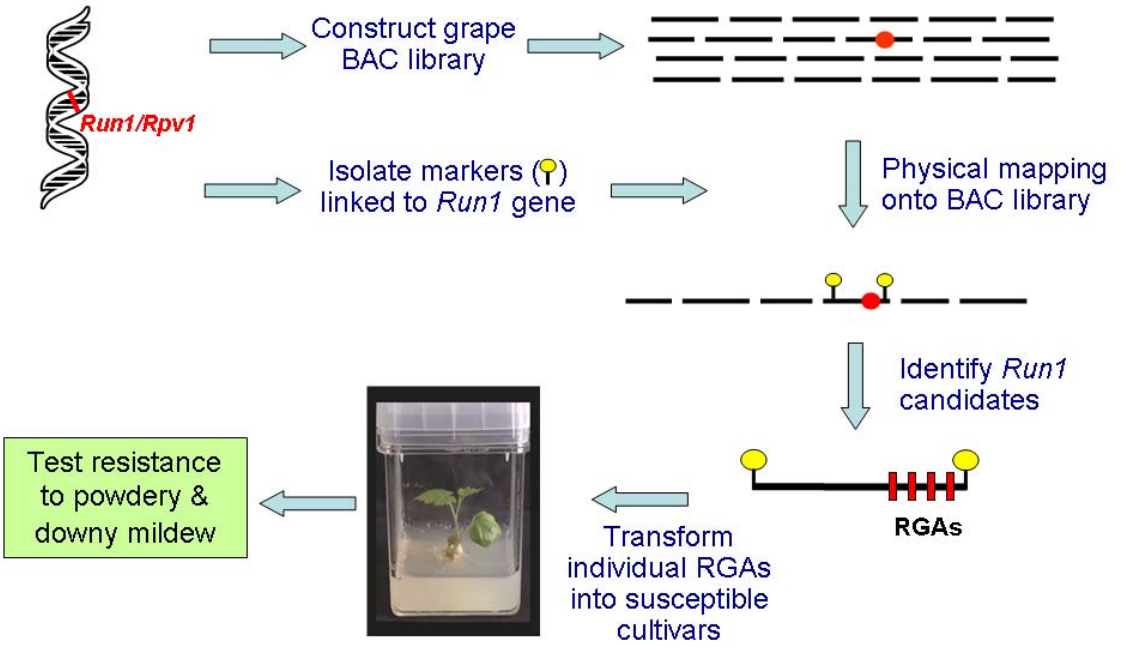


Figure 1. Strategy for map-based cloning and functional testing of powdery mildew (*Run1*) and downy mildew (*Rpv1*) resistance gene candidates from *M. rotundifolia*

In addition to resistance genes such as *Run1* which confer resistance to a fungal pathogen, recent research on a number of plant species indicates that plants may also contain "susceptibility" genes which predispose the plants to infection to powdery mildew. For example, barley plants carrying the normal or wild-type *MLO* gene are susceptible to barley powdery mildew, whereas plants carrying either a natural or induced recessive mutant *mlo* allele are fully resistant against nearly all known barley powdery mildew isolates (3). This gene has been used successfully for over two decades as a source of effective resistance against barley powdery mildew in most European spring barley varieties (4). Naturally occurring tomato and pea mutants have also been identified that show broad-spectrum resistance to powdery mildew due to loss of function of specific members of the *MLO* gene family (5, R. Panstruga, personal communication, 2009).

Plant species in which the *MLO* gene family has been studied to date ie. barley, wheat, rice and *Arabidopsis* are all found to contain large *MLO* gene families. However, only specific members of this gene family appear to be involved in regulating powdery mildew susceptibility. Prior to the commencement of this project we had been successful in isolating six partial length *MLO* homologs from grape, of which three (*VvMLO3, VvMLO6* and *VvMLO17*) showed close homology to the barley *HvMLO* and Arabidopsis *AtMLO2* genes involved in powdery mildew susceptibility. Thus, the second major aim of this project is to complete the cloning and sequencing of the *MLO* gene family in grapevine and then analyse their putative function by comparing sequence homology and expression patterns with *MLO* genes from other plant species. This information will then be used to select candidate *VvMLO* genes for suppression using gene-silencing techniques to see if this will result in grapevine cultivars with significantly reduced susceptibility to powdery mildew.

Project Aims:

- Complete the identification and cloning of all resistant gene candidates contained within the *M. rotundifolia* introgressed segment which confers resistance to powdery and downy mildew.

- Transform susceptible grapevine cultivars with individual resistance gene candidates and test the progeny for resistance to powdery mildew and downy mildew.

- Complete the identification, cloning and expression analysis of all members of the *MLO* gene family in grapevine

- Transform a susceptible grapevine cultivar with gene-silencing constructs targeting specific endogenous grape *MLO* gene(s) and test the transgenic progeny for reduced susceptibility to powdery mildew.

4. Performance targets

Outputs and Performance Targets in original application:

2006-07

Outputs	Performance Targets
1. New PDF	Postdoctoral Fellow appointed
2. Genomic clones isolated from "gap" region	Genomic library constructed, screened and hybridizing clones sequenced
3. Knowledge of resistance gene expression patterns in response to infection	Resistant germplasm inoculated with powdery & downy mildew, RNA extracted and R gene expression analysed
4. Binary vectors containing full length resistance gene candidates	Constructs for RGA-1, RGA-2, RGA-4, RGA-8 completed
5. Transgenic grapevines expressing resistance genes	Embyrogenic grapevine callus transformed with R gene constructs and transformed tissue selected
6. Full length clones of grapevine Mlo homologs	Cloning of grapevine homologs completed
7. Knowledge of Mlo expression patterns in grapevine tissues	RNA extracted from various grapevine tissues and Mlo gene expression analysis commenced
8. GWRDC Annual Report	Annual report submitted

2007-08

Outputs	Performance Targets
1. Knowledge of Mlo expression patterns in grapevine tissues	Mlo gene expression analysis completed
2. Binary vectors containing full length resistance gene candidates	Constructs prepared for any new full length R gene candidates identified in "gap" region
4. Binary vectors containing Mlo silencing constructs	Cloning of selected Mlo gene fragments into CSIRO RNAi hairpin vector completed
3. Transgenic grapevines expressing resistance genes	Embyrogenic grapevine callus transformed with R gene constructs and transformed tissue selected. Selected transgenic plants tested for transgene expression
4. Transgenic grapevines containing Mlo-silencing construct	Embyrogenic grapevine callus transformed with Mlo silencing construct and transformed tissue selected
5. GWRDC Annual Report	Annual report submitted

2008-09

Outputs	Performance Targets
1. Transgenic grapevines expressing resistance genes tested for powdery and downy mildew resistance	Continued transformation of embyrogenic grapevine callus transformed with R gene constructs if required. Testing of transgenic lines expressing R gene constructs resistance to powdery mildew and downy mildew commenced
2. Transgenic grapevines containing Mlo -silencing construct	Embyrogenic grapevine callus transformed with Mlo silencing construct and transformed tissue selected. Testing of transgenic lines with suppressed Mlo expression for decreased susceptibility to powdery mildew commenced
3. Preparation of publication for international journal	Paper prepared and submitted
4. GWRDC Annual Report	Annual report submitted

2009-10

Outputs	Performance Targets
1. Transgenic grapevines expressing RGA genes tested for powdery and downy mildew resistance	Testing of transgenic lines expressing R gene constructs for resistance to powdery mildew and downy mildew completed.
2. Transgenic grapevines containing Mlo -silencing construct tested for reduced susceptibility to powdery mildew	Testing of transgenic lines with suppressed Mlo expression for decreased susceptibility to powdery mildew completed
3. GWRDC final report	GWRDC final report submitted.

Variances reported 2007-2008

Staff changes

Claire Barker finished her 7 month PDF contract in January and a new PDF (Dr Angela Feechan) did not start until May. One of the Level 3 Technical Officers on the project, Karina Swann, resigned at the end of January. Permission was obtained from GWRDC to re-advertise the position as a Level 2 Technical Assistant and use the salary savings to extend the term of the position from 29 months to 36 months.

One of the Technical Officers employed on this project at its commencement (1/06/2006) resigned on 24/1/2007 to take up a new position in Melbourne. Her position was re-advertised as a Level 2 Technical Assistant for a 36 month term instead of Level 3 Technical Officer for a 29 month term. *Variation agreed by e-mail with John Harvey - 21/12/07*

Nicole Kempster was appointed to the position in April 2007. The time taken to start these new staff to the project has lead to a significant underspend in the 06/07 budget.

Lack of staff meant that completion some of Objectives for 2006/7 year were not completed. Objective 4: Constructs for RGA-1, 2 and 4 were completed but RGA-8 has not yet been tested for expression in tobacco. Objective 7: Mlo gene expression analysis was delayed until the 2007/2008 period.

Research

Due to unforseen technical issues relating to production of the RGA constructs for transformation into susceptible grapevine cultivars, there was a delay in anticipated progress of Output 5 within the initial six months of the project.

Genomic constructs for the first two full length RGA genes cloned (RGA-4 and RGA-2), encompassing 4 kb of upstream sequence containing the promoter, the RGA coding sequence and 2 kb of downstream sequence were cloned into binary vectors and transformation commenced in France and Adelaide. However, during the course of the initial transformation period, data obtained from other experiments in our lab, undertaken as part of Output 3, that indicated that the 3' untranslated region of the RGA-4 and RGA-2 transcripts actually contained two large introns (~ 1000 nt each) that had not been predicted in the original sequence analysis and, thus, had not been accounted for in the original transformation construct design. The presence of these two introns meant that the actual transcript end point of RGA-2 and RGA-4 was actually located a further ~120 nt downstream of the end of the 12 kb genomic fragment cloned into the binary transformation vectors. As this may potentially result in incorrect transcription of these RGA genes within the transgenic grapevines, it was decided to discard the original transformations and remake the original RGA2 and RGA 4 constructs. This has also necessitated replanning and reconstruction of the transformation vectors for RGA-1 and RGA-8 to take into account the potential presence of these unusually large introns. This resulted in a delay of approx 6 months in commencement of the transformation of RGA constructs into grapevine.

Revised Outputs and Performance Targets submitted in 14 May 2008 including project end date to 30 June 2010 are shown below. These were approved by GWRDC on 11th September 2008.

Revised Outputs and Performance Targets 2007-08

Outputs	Performance Targets
1. Knowledge of Mlo expression patterns in grapevine tissues	Mlo gene expression analysis completed including 10 new Mlo genes identified by release of grape genome sequence
2. Binary vectors containing full length resistance gene candidates	Completion of constructs to new full length R gene candidates identified in "gap" region and expression testing in tobacco
3. Transgenic grapevines expressing resistance genes	Embyrogenic grapevine callus transformed with R gene constructs and transformed tissue selected.
4. Transgenic grapevines containing Mlo-silencing construct	Embyrogenic grapevine callus transformed with selected Mlo silencing constructs
5. GWRDC Annual Report	Annual report submitted

Revised Outputs and Performance Targets 2008-09

	Performance Targets
1. Transgenic grapevines expressing resistance genes tested for powdery and downy mildew resistance	Continued transformation of embyrogenic grapevine callus transformed with R gene constructs. Selected transgenic plants tested for transgene expression. Commence testing of transgenic lines expressing R gene constructs for resistance to powdery mildew and downy mildew.
2. Transgenic grapevines containing Mlo -silencing construct	Embyrogenic grapevine callus transformed with Mlo silencing construct and transformed tissue selected. Testing of transgenic lines for suppressed Mlo expression.
3. Preparation of publication for international journal	Paper prepared and submitted
4. GWRDC Annual Report	Annual report submitted

Revised Outputs and Performance Targets 2009-10

Outputs	Performance Targets
1. Transgenic grapevines expressing RGA genes tested for powdery and downy mildew resistance	Testing of transgenic lines expressing R gene constructs for resistance to powdery mildew and downy mildew completed.
2. Transgenic grapevines containing Mlo -silencing construct tested for reduced susceptibility to powdery mildew	Testing of transgenic lines with suppressed Mlo expression for decreased susceptibility to powdery mildew completed.
3. GWRDC final report	GWRDC final report submitted.

5. Methods

5.1 Materials

Potted *Vitis vinifera* cv. Cabernet Sauvignon vines were grown in temperature-controlled glasshouses at Waite Campus (Adelaide, South Australia) maintained between 23-25ºC. *Erysiphe necator* (isolate APC1, kindly provided by Associate Professor Eileen Scott, University of Adelaide) was maintained on detached leaves of Cabernet Sauvignon using an 8-10 d rotation as previously described (6).

Arabidopsis thaliana (Col-0) plants were grown in chambers with 14 h light / 10 h dark light cycle, maintained at 24°C. *E. cichoracearum* was maintained on cucumber (*Cucumis sativus*) plants grown in chambers with a 16 h light / 8 h dark light cycle, maintained at 22°C. Cucumber leaves were gently brushed across *Arabidopsis* leaves to infect. The Arabidopsis *atmlo2,6,12* triple mutant was kindly provided by Dr Ralph Panstruga (Max-Planck-Institute for Plant Breeding Research, Department of Plant-Microbe Interactions, Köln).

Downy mildew (*P. viticola*) inoculum was collected from infected grapevines in the field and maintained on detached leaves incubated upside down on moist filter paper in large petri dishes at 22°C to facilitate sporulation. *P. viticola* sporangia were collected by placing sporulating leaves in a 50 ml Falcon tube containing 5 mL water and agitated to displace the spores. The spore solution was diluted to a concentration of 1 x 10^5 sporangia/ml before being sprayed on the abaxial surface of fully-expanded leaves or 1.5 cm diameter discs cut from these leaves. Discs were incubated for 6 days before assessing sporulation and the induction of programmed cell death.

Sequences of primers used in this study are listed in Appendix 5.

5.2 Visualisation of E. necator development on Grapevine and Arabidopsis

Detached leaf material was stained in trypan blue for an hour according to (7). Fungal structures were visualised using a Zeiss Axioscop 2 light microscope and photographed using a Lecia DFC 500 digital camera. Successful penetration was determined by the presence of a fungal haustorium in a clear epidermal cell and programmed cell death (PCD) scored when the penetrated epidermal cell was entirely blue.

5.3 Genomic library construction

Nuclei were prepared as described previously (8) from genomic DNA extracted from a resistant *V. vinifera* x *M. rotundifolia* BC5 progeny plant (designated R23). The genomic DNA was then either mechanically sheared and cloned into a pWEB::TNC™ cosmid vector (Epicentre, Madison, USA) or partially digested with *Sau*3AI and cloned into a λBlue STAR lambda vector (Novagen, Madison, USA) according to the individual manufacturer's protocols.

5.4 Grapevine RNA extraction and cDNA synthesis

Total RNA was isolated from grape tissues using the sodium perchlorate method as described in (9). Prior to reverse transcription, 100 µg of total RNA was DNase treated using an RNeasy Mini Kit (Qiagen) and an RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. DNase-treated RNA (2 µg) was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using the Oligo $(dT)_{20}$ primer according to the manufacturer's instructions. Before use in RT-PCR experiments, cDNA reactions were diluted 10-fold to 200 µL with 10 mM Tris-HCl, pH 7.6.

For RNA isolation from hairy root cultures, transformed root material was collected, frozen in liquid nitrogen and ground for RNA extraction. RNA was isolated with the Spectrum Plant Total RNA Kit (Sigma) and DNase treated as per the manufacturer's instructions. Total RNA (2ug) was used to synthesis cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit

(Roche) with the Oligo (dT)20 primer according to the company's instructions. Samples were diluted 10 fold with 10mM Tris-HCl pH 8 to a total volume of 200 ul before qRT-PCR.

5.5 Cloning of *VvMLO* cDNAs

A grape genomic library based on a partial *Mbo* I restriction digest was prepared in the bacteriophage vector Lambda Fix II (Stratagene) and approximately 250,000 plaques screened with radio-labelled cDNA fragments of *AtMLO1* (GenBank Accession No. Z95352), *AtMLO2* (AF369563), *AtMLO6* (AF369567) and *AtMLO12* (AF369573). From this screening, genomic fragments encompassing seven full-length and partial-length *VvMLO* homologs were identified and designated *VvMLO1*, *VvMLO3*, *VvMLO6*, *VvMLO7*, *VvMLO9*, *VvMLO13* and *VvMLO17*. Gene-specific primers were designed to these sequences to amplify full-length cDNA sequences from various *V. vinifera* cv. Chardonnay tissues. Where genomic clones covered the entire putative cDNA region (i.e. *VvMLO13* and *VvMLO17*) gene-specific forward and reverse primers were used to obtain full-length cDNA sequences. Where either the 5′- or 3′-end of the putative *VvMLO* cDNA sequence was absent from the genomic clone, RACE-PCR techniques (10) were employed to obtain full-length cDNA sequence. Gene sequences were submitted to GenBank and have been assigned accession numbers in the range EU812233-EU812239.

5.6 Plasmid constructs

5.6.1 Subcloning RGA genomic fragments into plant transformation vectors

The vectors pCAMBIA1300 (hygromycin selection) and pCAMBIA2300 (kanamycin selection) were modified by the insertion of a 35S:GFP5-ER expression cassette to generate the vectors pCLB1301 and pCLB2301 (Appendix 5). Further modifications were also made to these two vectors during the duration of this project to generate the plasmids pCLB1301NH and pCLB2301NK in which the 35S promoter controlling antibiotic selection was replaced with a NOS promoter, in an attempt to improve construct stability (Appendix 5). Genomic DNA of a the resistant BC5 progeny plant R23 was digested with restriction endonucleases and genomic fragments subcloned into pBS-SK cloning vectors before assembling in pCLB vectors.

5.6.2 *VvMLO* constructs

Hairpin (Hp) silencing constructs were designed to ~ 280 bp fragments in the 3' region of the target genes *VvMLO3*, *4* and *17* using the primer pairs: VvMLO3-Hp-F/R, VvMLO4-Hp-F/R, VvMLO17-Hp-F/R. The region selected had the least sequence homology to the other *VvMLO* genes to avoid silencing of other members of the *VvMLO* gene family. Hairpin fragments were amplified by PCR, cloned into pDONR221 (Invitrogen) and then transferred into the CSIRO destination vector pHellsgate12 (11) by Gateway cloning technology. An artificial microRNA silencing construct was generated as described in (12) targeting a 21mer sequence specific to *VvMLO17* (5'- ACCCCGCCCAATCCGAACCCA) using the primer pairs VvMLO17syn-miR-4F/R. The final cloning step for both *VvMLO* silencing constructs involved transfer of the silencing cassette into the pCLB1301NH binary vector to provide a GFP marker for selection of transgenic cells.

To generate *VvMLO* overexpression constructs, coding sequences for *VvMLO3*, *VvMLO4* and *VvMLO17* were cloned into the plant transformation vector pAM-PAT-GW (13) using gateway cloning which contains a 35S promoter for over-expression and the phosphinothricin acetyltransferase (PAT) gene for BASTA (glufosinate ammonium) resistance.

5.7 Promoter walking

Promoter-walking libraries were constructed from *Pml*I, *Pvu*II, *Sca*I, *Stu*I, *Swa*I, *Hinc*II and *EcoR*V restriction digests of genomic DNA from the resistant BC5 progeny plant R23 ligated to an adapter sequence according to (14). DNA regions upstream of the truncated RGA sequences were amplified using a nested PCR strategy. The first amplification reaction was performed using the forward adaptor primer AP1, corresponding to the adapter sequence, and a reverse gene-specific primer. For the second round of amplification, a second reverse nested adapter primer AP2, was used with a nested gene-specific primer.

5.8 Quantitative RT-PCR analysis

Expression analysis was carried out by real-time PCR analysis using a SYBR Green method on a Rotor-Gene 3000 thermal cycler (Corbett Research). Each 15 µl PCR reaction contained 330 nM of each primer, 2 µl of diluted cDNA and 1 x ABsolute™ QPCR SYBR® Green ROX Mix (Integrated Sciences). The thermal cycling conditions used were 95°C for 15 min followed by 40 cycles of: 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, followed by a melt cycle of 1°C increment per min from 65°C to 96°C. All primer pairs amplified a single product of the expected size and sequence, which was confirmed by melt-curve analysis, agarose gel electrophoresis and DNA sequencing. The expression of each target gene was calculated relative the expression of the housekeeping gene elongation factor 1 (*EF1*; GenBank Accession no. AF176496) as described (15) using Rotor-Gene 6.0 software (Corbett Research).

5.9 Grapevine transformation

Embryogenic callus initiation, maintenance media, culture conditions, *Agrobacterium*-mediated transformation and transgenic plant regeneration protocols were essentially similar to that previously described (16).

Grapevine hairy roots were generated from *V. vinifera* cv. Cabernet Sauvignon. *In vitro* plantlets were generated and maintained on shooting media (SM) media (16). Once plants reached 8 weeks of age, young, healthy leaves were cut off with intact petioles and placed on SM medium with waxy surface of leaf in contact with the medium and petiole facing upwards (~20 leaves per construct). To generate transformed hairy roots, a drop of *A. rhizogenes* (strain A4) culture carrying a *VvMLO*-silencing constructs was placed on the tip of the petiole. Control lines were generated by inoculating with A4 only. Petri dishes were then sealed and left to co-cultivate for 5 days in the dark at room temperature. After the co-cultivation, petioles with leaves attached were planted into SM plates containing 750 ug/ml timentin and incubated in the light at 28°C. Transformed roots were visualised for GFP with the Leica MZ12 microscope.

5.10 Arabidopsis transformation

Arabidopsis thaliana was transformed using the floral dip method (17). Seeds were collected and sown either onto half MS plates supplemented with either 15 µg/mL hygromycin B or 100 µg/mL kanamycin or selected on soil by spraying with a BASTA solution (125 mg/L) depending on the binary construct used in the transformation. Transformed lines were grown on soil and allowed to self pollinate to produce segregating F_2 seed.

5.11 Phylogenetic analysis

Protein sequence alignments were performed with ClustalW. The aligned sequences were used to generate an unrooted phylogenetic tree using the Phylip programs Seqboot, Protdist, Neighbor and Drawtree (18). Support for the branching arrangements was evaluated by bootstrap analysis using 100 replicates. Putative VvMLO protein sequences within the recently sequenced *V. vinifera* genotype PN40024 (19) were identified with the BLAT-Search tool of the Grape Genome Browser (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/) using the cloned Chardonnay *VvMLO* sequences.

6. Results / Discussion

6.1 *Run1*/*Rpv1* project

6.1.1 Completion of sequencing of the *M. rotundifolia* introgressed region

By the end of the previous GWRDC-funded CRCV project (CRV 99/14a), sequencing of the introgressed region from *M. rotundifolia* (representing ~ 950,000 bp), revealed the presence of a cluster or family of resistance gene analogues (*RGAs*) as shown in Fig. 2 comprising 4 full-length genes (*RGA-1*, *RGA-2*, *RGA-4* and *RGA-8*) and 4 truncated (non-functional) genes (RGA-3, RGA-5, RGA-6 & RGA-7). Two further partial-length RGA sequences (*RGA-x* & *RGA-y*) had also been identified, but had not been fully sequenced as they were located within a "gap" within the BAC contig. Attempts to clone genomic fragments from the "gap" region using a cosmid library had also only been partially successful suggesting this region may be composed of highly repeated DNA.

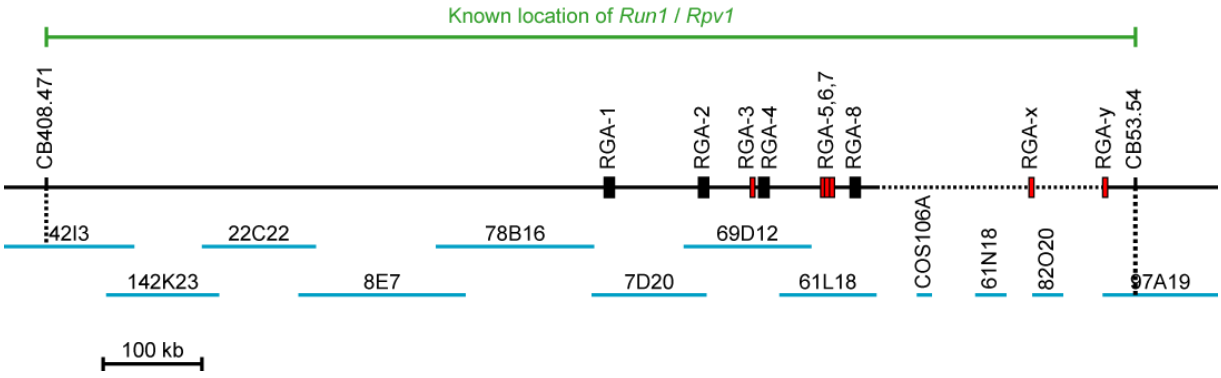


Figure 2. Map of the introgressed region from *M. rotundifolia* containing putative *Run1* / *Rpv1* resistance genes at completion of CRV 99/14a project (June 2006). The grapevine chromosome is shown as a solid black line and the "gap" region as a dotted line. Individual overlapping BAC and cosmid clones are shown in blue. Full length resistance gene analogue (RGA) coding sequences are shown as black boxes and partial length (incomplete) sequences shown in red. The relative locations of genomic fragments COS106A, 61N18 and 82O20 within the "gap" region are not known.

6.1.1.1 Cloning of *RGA-9*

A sequence defined as *RGA-x* encoding the C-terminal domain of an RGA-like protein, was found to be present at the 5' end of the small BAC clone 82O20 located within the gap" region (Fig. 2). To obtain the 5' end of RGA-x, a genomic lambda library constructed from the resistant BC5 progeny plant R23 was screened with a radio-labelled fragment encompassing a conserved region within RGA genes between primers GLP1-12 P1 and GLP1-12P3 (6). Positive clones were isolated and rescreened using *RGA-x* specific primers (CB45 & CB572) which led to the identification of a single 15kb genomic clone (9.1.2). Sequencing of clone 9.1.2 showed it to overlap with BAC 82O20 with 5.7 kb of new sequence at the 5'end of *RGA-x*. Analysis of the merged sequence between clone 9.1.2 and *RGA-x* revealed it to encode a full-length RGA protein sequence and was renamed *RGA-9*. Promoter walking techniques were employed with nested primers AJ70 and CB60 to obtain an additional 900 bp of 5' sequence resulting in a full-length clone of *RGA-9* including a promoter region of 1.3kb.

6.1.1.2 Cloning of *RGA-10*

A sequence defined as *RGA-y* encoding the C-terminal domain of an RGA-like protein, was present at the 5' end of BAC clone 97A19 located on the edge of the "gap" region (Fig. 2). An RGA generic primer was designed to a region immediately upstream of the predicted ATG start codon (RGA-ATG) that was highly conserved in all other *RGA* genes analysed. This primer was used in combination with an *RGA-y* specific primer (CB608R) to successfully amplify a 3.5kb fragment from genomic DNA isolated from resistant BC5 progeny plant R23. Sequencing of the cloned PCR fragment (608R1) showed it to overlap with the *RGA-y* sequence on BAC clone 97A19. Analysis of the merged sequence between clone 608R1 and *RGA-y* revealed it to encode a full-length RGA protein sequence and was renamed *RGA-10*. New *RGA-10* specific primers were designed (AJ68 and AJ69) and used to amplify 1 kb of the promoter region of *RGA-10* using the Promoter walking libraries.

6.1.1.3 Cloning of *RGA-11*

One of the clones (608R3) obtained during the cloning of *RGA-10* using primers RGA-ATG and CB608R was found to be a partial-length RGA-like sequence, but did not match any of the previously cloned RGA sequences. This RGA sequence was designated as *RGA-11*. Primer AJ84 was designed to this new sequence and in combination with primer CB181, a 5.6kb fragment was amplified from genomic DNA isolated from resistant BC5 progeny plant R23. To extend *RGA-11* in the 5' direction, a promoter walking strategy was employed. Two fragments were obtained in sequential steps using the nested primer combinations AJ93/94 and AJ110/111 extending the sequence of *RGA-11* to ~ 1.5kb of the promoter region. The 3'-end of *RGA-11* was obtained using an *RGA-11* specific primer (AJ112) in combination with the upstream primer RGA11F1 leading to the amplification of a 2.8kb fragment from the promoter walking library.

Analysis of the complete *RGA-11* sequence indicated that this gene encoded a full-length RGA protein sequence with a high degree of homology to the translated sequences of the other *RGA* genes present at the *Run1*/*Rpv1* locus. Mapping with co-dominant marker pair AJ80/AJ81 confirmed that *RGA-11* was located at the *Run1*/*Rpv1* locus, most probably within the "gap" region (data not shown). In summary, we have identified a total of 7 full-length RGA sequences present at the *Run1*/*Rpv1* locus which are possible candidates for conferring powdery mildew or downy mildew resistance (Fig. 3).

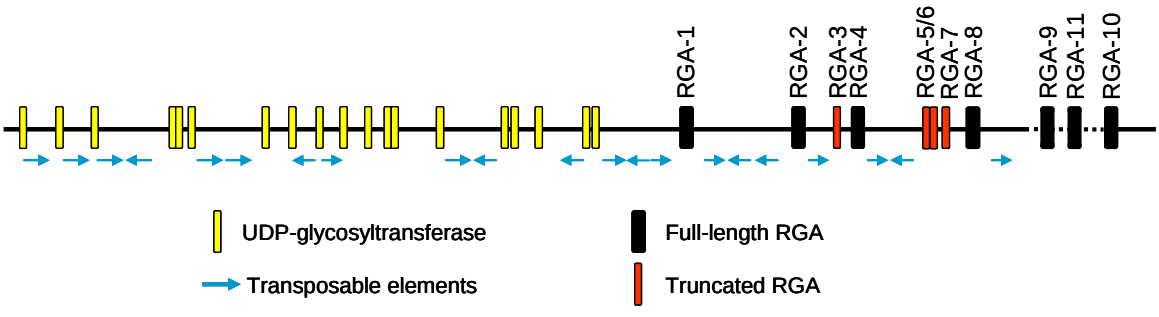


Figure 3. Location of RGA gene sequences within the introgressed region from *M. rotundifolia* containing putative *Run1* / *Rpv1* resistance genes.

6.1.2 Identification of repetitive genetic elements within the "gap" region

Despite the fact that our BAC library was predicted to contain 10-fold haploid genome coverage, it proved impossible to clone BAC fragments covering the entire "gap" region. The BAC library contained 49,920 clones generated using *Hind*III and 5,378 clones generated using *Bam*HI. Therefore, gaps in the library could have been caused by bias in restriction site distribution as might occur in centromeres, telomeres and highly repetitive regions. For this reason it has been suggested that BAC libraries should be generated from genomic DNA that has been digested with number of different restriction enzymes (20), or randomly sheared (21), to improve representation of problematic and repeat-rich regions at telomeres and in centromeric heterochromatin.

In an attempt to identify genomic fragments covering the "gap" region, two additional genomic libraries were constructed using the same powdery mildew-resistant plant that had been used for BAC library construction. The cosmid library contained 148,400 clones with an average insert size of 35.3 kb and the lambda library contained 395,600 clones with an average insert size of 12 kb. However, despite the fact that these two libraries contained approximately 11-fold and 10-fold genome coverage respectively, when the libraries were screened with BAC end sequences for which no overlapping BAC clones could be found, these sequences were also missing from the cosmid and lambda libraries. This suggested that the lack of representation of clones in this region might be caused by clone instability or lethality, rather than restriction enzyme bias. Indeed, cosmid clone Cos106A proved to be highly unstable and would spontaneously and unpredictably revert from a 40 kb insert to an approximately 15 kb insert in culture. Sequencing of this clone proved to be extremely difficult and assembly was hampered by the presence of numerous repeated sequences, including a partial tandem repeat 45S rDNA unit (Fig. 4). Eukaryotes generally have many copies of rDNA organised in tandem repeats (22) and it has been shown that BAC clones containing tandemly repeated DNA elements are not stable and can undergo drastic deletions during routine library maintenance and DNA preparation (23). The presence of these tandem repeat units within the genome may therefore explain at least some of the gaps observed in the contig assembly.

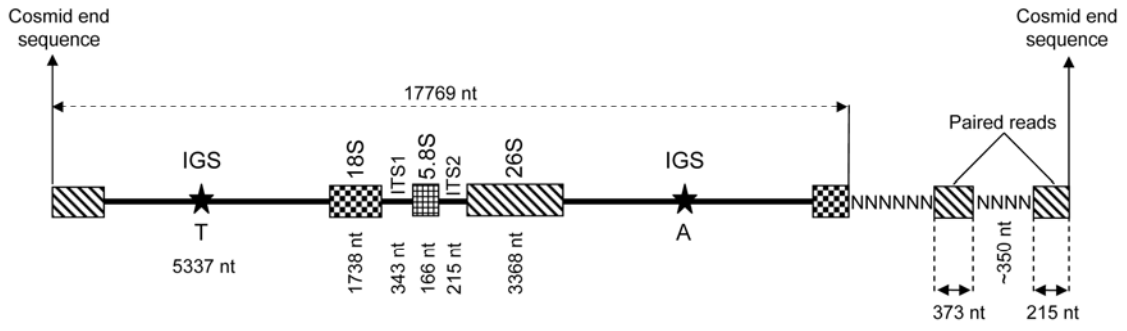


Figure 4. Sequence assembly of cosmid clone Cos106A. Sequence was analysed using PHRED, PHRAP and in-house Genoscope programs taking into account paired reads and a manual curation of the repeated sequences. The sequence obtained corresponded to a partial tandem repeat of a 45S rDNA unit. N's indicate where joining sequence could not be resolved. Abbreviations: IGS: InterGenic Spacer; ITS: Internally Transcribed Spacers.

6.1.3 Predicted structure of RGA proteins

The vast majority of *R* genes encode proteins that contain three main domains: a leucine-rich repeat (LRR) domain, a central nucleotide-binding site (NBS) domain and a variable domain at the N-terminus of the protein, consisting of either a coiled-coil (CC) domain or a domain that

has homology to the Toll/Interleukin-1 Receptor (TIR). With some exceptions, LRRs are mainly involved in recognition of pathogen-secreted effector molecules, the N-terminal domain determines signalling specificity and the NBS domain appears to function as a molecular switch (24).

Analysis of the predicted protein sequences of each of the full-length *RGA* genes at the *Run1/Rpv1* locus indicates that they are all of the TIR-NBS-LRR type (Fig. 5). Significantly, the only other powdery mildew resistance genes which have been cloned to date ie. the *Mla* locus from barley (25) and the *Pm3* locus from wheat (26) also encode NBS-LRR type proteins but possess a CC domain at the N-terminus instead of a TIR domain.

The RGA proteins are highly similar to each other in sequence with the only main difference being the number of LRR sequences within each protein (Fig. 5). This suggests that they may differ in terms of their specificity for detecting different pathogen isolates during infection. When the sequence of the proteins encoded by the RGA genes at the *Run1/Rpv1* locus are compared with the most closely related RGA-like proteins in the *V. vinifera* genome they are found to be more closely related to each other than to any RGAs from *V. vinifera*. This suggests that this family of *RGA* genes from *M. rotundifolia* have undergone selection during evolution to provide resistance to powdery mildew and downy mildew, unlike the related genes in *V. vinifera* which do not confer resistance to these important fungal pathogens

A.



B.

	Predicted protein size (aa)	No. of LRRs
RGA-1	1347	16
RGA-2	1086	10
RGA-4	1259	14
RGA-8	1399	20
RGA-9	1158	12
RGA-10	1332	16
RGA-11	1329	16

Figure 5. Structural features of RGA proteins at the *Run1/Rpv1* locus. (A) All RGAs contain four domains characteristic of TIR-NBS-LRR resistance genes: TIR, Toll/Interleukin-1 receptor domain; NBS, nucleotide-binding site domain; LRR, leucine-rich repeat domain; CT, C-terminal domain of undetermined function. (B) Predicted size and number of leucine-rich repeat domains of different RGA proteins.

6.1.4 Expression of candidate *RGA* genes in resistant leaves

Little is known about the regulation of plant resistance genes. Most resistance genes show a low level of constitutive expression in unchallenged plants because their primary function in resistance is to recognize signals signifying pathogen invasion. However, induction of resistance gene expression by pathogen infection has been reported for a very small number of genes including the barley powdery mildew *Mla* gene (27). We therefore examined the transcription of each of the cloned *RGA* genes in response to infection by powdery mildew (*E. necator*) and downy mildew (*P. viticola*) to see if we could gain any insight into which of these *RGAs* are likely to be involved in resistance.

Expression analysis indicated that all of the cloned *RGA* genes at the *Run1/Rpv1* locus are expressed to a low level in uninfected plants and that they showed little or no increase in expression following inoculation with powdery or downy mildew (Fig. 6). The exception to this was *RGA-10* which was significantly upregulated in response to both pathogens. While this points to a potential involvement of *RGA-10* in resistance, a role for the other RGAs cannot be ruled out because all appear to be expressed in resistant leaf tissue.

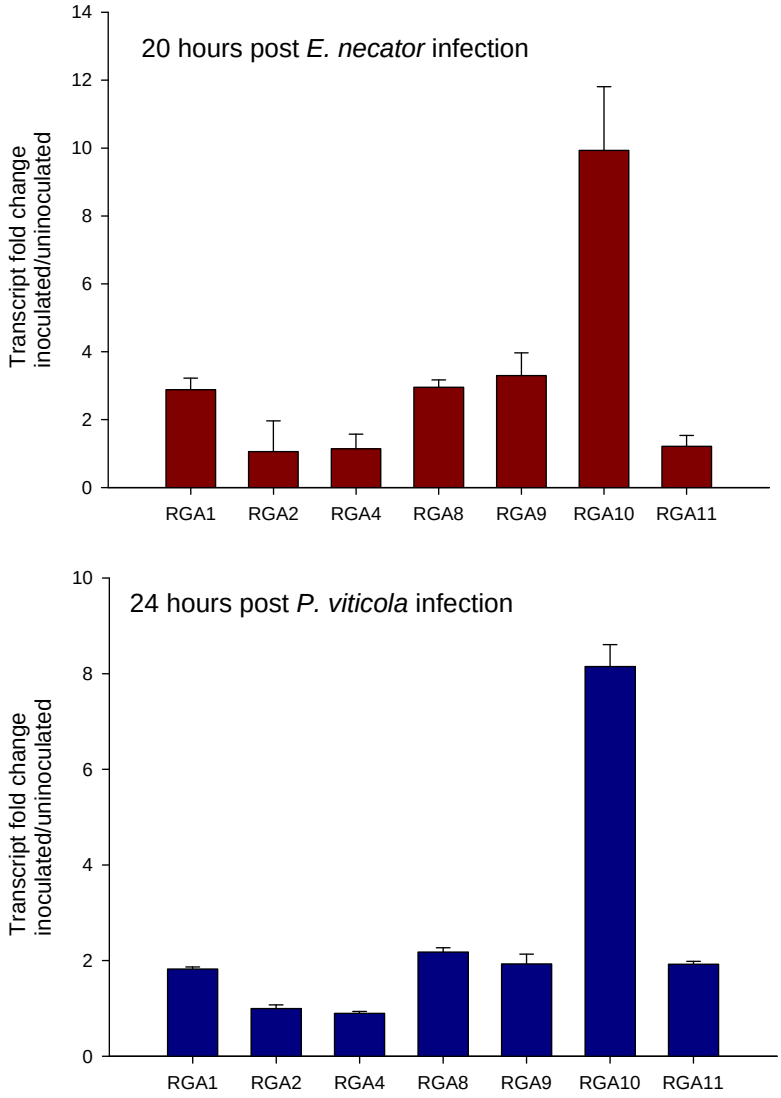


Figure 6. *RGA* transcript levels following powdery mildew and downy mildew infection. Quantitative RT-PCR analysis of *RGA* expression in *M .rotundifolia x V. vinifera* BC5 leaves 24 h post- *E. necator* inoculation and 20 h post *P. viticola* inoculation. Each data point is mean of three biological replicates.

6.1.5 Introduction of candidate *RGA* genes into susceptible grapevine cultivars

As the expression analysis (Fig. 6) indicated that all of the full-length RGA genes cloned from the *Run1/Rpv1* locus are expressed within resistant leaf tissue it was not possible to eliminate any of these genes as potential *Run1* or *Rpv1* candidates. It was therefore necessary to undertake the genetic transformation of each candidate *RGA* into a susceptible grapevine cultivar in order to properly assess the potential contribution of each gene to powdery mildew and downy mildew resistance

6.1.5.1 Design of *RGA* constructs for grapevine transformation

When evaluating resistance gene function in transgenic plants one important consideration is the selection of the promoter and terminator sequences that control the expression of the resistance gene in the transgenic plants. The use of commonly available highly active constitutive promoters, such as the plant viral 35S promoter, may lead to autoactivity of the resistance proteins resulting in cell death in the absence of the pathogen (28). It is also well known that the non-coding sequence regions (introns) within plant genes may regulate gene expression (29). With these considerations in mind, a decision was made to try and introduce the RGA genes into the susceptible grapevines as intact genomic fragments encompassing the entire promoter-RGA coding-terminator regions of each of the seven RGA candidates. In this way, the RGA genes transferred to susceptible *V. vinifera* cultivars would be as close as possible to the way they exist and operate in *M. rotundifolia*. However, due to the large size of the RGA genes, this meant that the size of the genomic fragments (~14 kb) used for grapevine transformation were significantly larger than commonly used for plant transformation experiments.

A decision was also made to utilize pCAMBIA binary vectors for grapevine transformation which are free of some of the intellectual property restrictions associated with the use of other plant transformation vectors. To improve the efficiency of selection of transgenic grapevine plants, the pCAMBIA vectors were further modified to include a GFP reporter gene generating the new vectors pCLB1301 (hygromycin selection) and pCLB2301 (kanamycin selection) (Appendix 5). These new vectors were verified for GFP activity by transient expression in grapevine cells and tobacco leaves before being used for stable grapevine transformation (data not shown). This meant that introduced RGA genomic fragments were subtended by both an antibiotic selection marker (kanamycin or hygromycin) and a GFP reporter gene. Therefore, only those transgenic lines that which were both antibiotic resistant and displayed GFP fluorescence were guaranteed to contain the entire introduced RGA genomic fragment and were selected for further analysis.

6.1.5.2 Overcoming problems associated with *RGA* construct stability

Genomic fragments (~14kb) containing *RGA-4* and *RGA-8* were the first to be successfully cloned into the new transformation vectors pCLB1301 and pCLB2301 and the transformation vectors were then introduced into *A. tumefaciens* strain EHA-105 in preparation for grapevine transformation. However, analysis of the transformed *Agrobacterium* cultures prior to grapevine transformation indicated that the *RGA* constructs were unstable in strain EHA-105 causing the introduced *RGA* genomic DNA to be deleted, most probably because of the large size of the genomic fragment being introduced. In order to try and overcome this problem, *RGA* transformation constructs were introduced into the recombination-repressed *A. tumefaciens* strain Agl1 which improved construct stability.

The constructs pCLB1301-RGA4 and pCLB1301-RGA8 were the first to be transformed into embryogenic callus of the susceptible *V. vinifera* cultivars Portan, Tempranillo and Maccabeo in the laboratory of our French collaborators in Montpellier, France. At the same time, the constructs pCLB2301-RGA4 and pCLB2301-RGA8 were transformed into the susceptible *V. vinifera* cultivars Shiraz in the CSIRO Adelaide laboratory.

No grapevine transgenic lines were obtained from transformation of either the kanamycin-based vectors pCLB2301-RGA4 and pCLB2301-RGA8 constructs into Shiraz (Table 1). In contrast, a total of 18 *RGA-4* and 20 *RGA-8* transgenic plantlets were successfully generated from transformation with the hygromycin-based vectors pCLB1301-RGA4 and pCLB1301-RGA8. However, on examination of GFP expression in these transgenics, only 7 *RGA-4* and 5 *RGA-8* lines were found to be positive. Thus, these were the only transgenic lines that contained the entire *RGA* genomic fragment, with the remaining plants showing significant deletions of the introduced *RGA* region making them unsuitable for evaluation of disease susceptibility. This indicated that despite the use of the *A. tumefaciens* strain Agl1, the RGA constructs remained unstable and susceptible to recombination during *Agrobacterium*-mediated grapevine transformation.

In an attempt to overcome this RGA stability problem, two different strategies were employed. The first strategy involved the use of smaller (~ 12kb) genomic fragments. The deleted version of the *RGA-4* construct (RGA4del) was found to be much more stable in transformation leading to the generation of over 40 GFP (+) transgenic lines (Table 1). However, this approach was not successful in generating any further RGA-8 transgenic lines.

The second strategy was based on more detailed analysis of *RGA-4* transgenic lines which had lost the RGA fragment. This analysis revealed that deletion of the RGA genomic fragment may be resulting from a recombination event between the two 35S promoter sequences within the pCLB vectors that are located on either side of the introduced RGA genomic fragment. Therefore a further two modified transformation vectors were constructed (pCLB1301NK & pCLB2301NK) in which one of the 35S promoter sequences was replaced with a NOS promoter. This strategy also appeared to overcome the inherent stability problems associated with the presence of large genomic RGA fragments.

6.1.5.3 Transgenic grapevines generated containing RGA constructs

Table 1 summarises the total number of transgenic lines successfully generated in the CSIRO PI (Adelaide) and INRA (Montpellier, France) laboratories containing each of the intact RGA genomic fragments in different susceptible *V. vinifera* cultivars including Shiraz, Portan, Tempranillo, Maccabeo and Carignan. Apart from *RGA-8*, over 20 independent transgenic lines have been established as *in-vitro* plantlets for each RGA construct, across at least two different susceptible *V. vinifera* cultivars, in the CSIRO PI Adelaide tissue culture facility. The final column of Table 1 also indicates how many of those *in-vitro* plantlets have been transferred to the glasshouse for assessing powdery mildew and downy mildew resistance. Due to the large number of constructs and cultivars involved, and the highly labour-intensive nature of grapevine transformation, transformations of certain RGA constructs into particular cultivars were not undertaken until the final year of the current project. Consequently, these transgenic lines have only recently been established *in-vitro* and were not sufficiently mature to move into the glasshouse for phenotyping before the end of the current project. These will be tested as part of the continuing GWRDC project (CSP 09/03).

Table 1. Summary of grapevine transgenic lines generated containing *Run1*/*Rpv1* candidate resistance genes (*RGAs*)

	Construct	Size inserted genomic fragment (nt)	Cultivar	No. GFP (+) lines generated *in-vitro*	No. lines transferred to glasshouse for phenotyping
RGA-1	pCLB1301-RGA1	11914	Shiraz	20	12
			Portan	21	#
			Temp.	21	#
RGA-2	pCLB2301-RGA2	12498	Shiraz	0	n/a
	pCLB1301-RGA2	12498	Temp.	40	16
			Macc.	29	10
RGA-4	pCLB2301-RGA4	14553	Shiraz	0	n/a
	pCLB1301-RGA4	14553	Portan	4	4
			Temp.	1	1
			Macc.	2	2
	pCLB1301-RGA4del	11925	Portan	24	8
			Temp.	20	4
RGA-8	pCLB2301-RGA8	14689	Shiraz	0	n/a
	pCLB1301-RGA8	14689	Portan	2	2
			Temp.	2	2
	pCLB1301-RGA8del	12306	Portan	0	n/a
RGA-9	pCLB2301NK-RGA9	13601	Shiraz	14	8
	pCLB1301NH-RGA9	13601	Portan	8	#
			Temp.	20	#
RGA-10	pCLB2310-RGA10	11525	Shiraz	3	2
	pCLB2301NK-RGA10	11525	Shiraz	5	2
	pCLB1301-RGA10	11525	Portan	25	13
			Temp.	28	4
			Carignan	3	#
			Macc.	1	#
RGA-11	pCLB1301-RGA11	11394	Shiraz	7	5
			Temp.	14	#

[#] indicates transgenic plants still *in-vitro*

6.1.6 Testing of grapevine transgenics for powdery and downy mildew resistance

6.1.6.1 Powdery mildew resistance assay

Resistance to grapevine powdery mildew in plants containing the *Run1* locus is characterised by the induction of programmed cell death (PCD) in a high percentage of cells which have been penetrated by the fungal feeding structure (haustorium). This is illustrated in Fig. 7A which shows that PCD is initiated in only <3% of penetrated cells in *V. vinifera* but over 50% of penetrated cells in *M. rotundifolia* or a resistant *V. vinifera* x *M. rotundifolia* BC5 plant. Cells which have undergone PCD can be readily visualised using the stain trypan blue (Fig. 7B). Thus the scoring of the proportion of penetrated cells which undergo PCD is a quantitative, reproducible measure of the level of resistance to powdery mildew and avoids the influence of other complicating factors such as % spore germination on measurements of powdery mildew susceptibility.

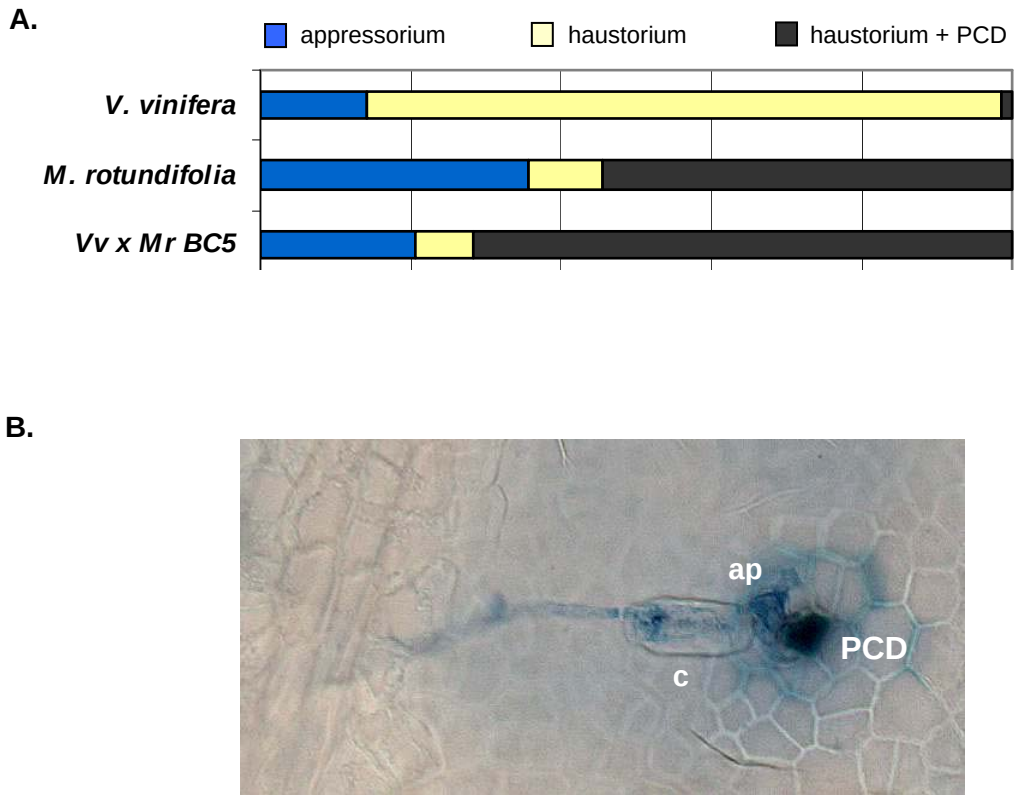
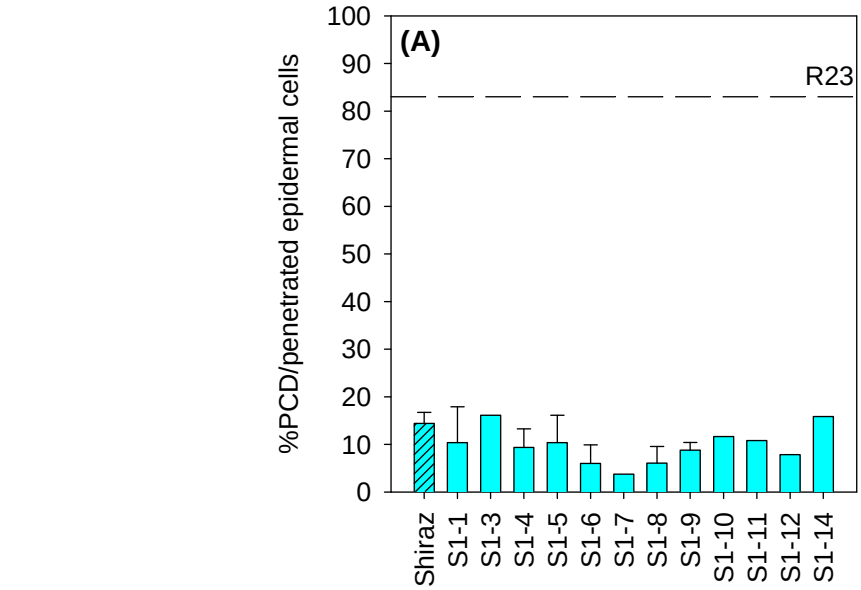
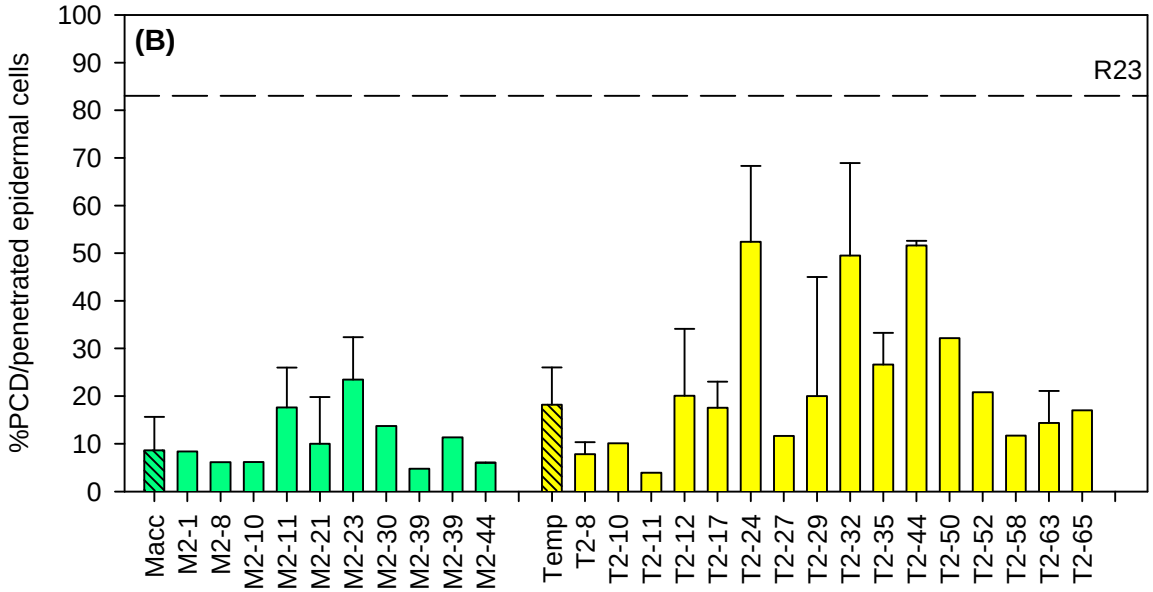


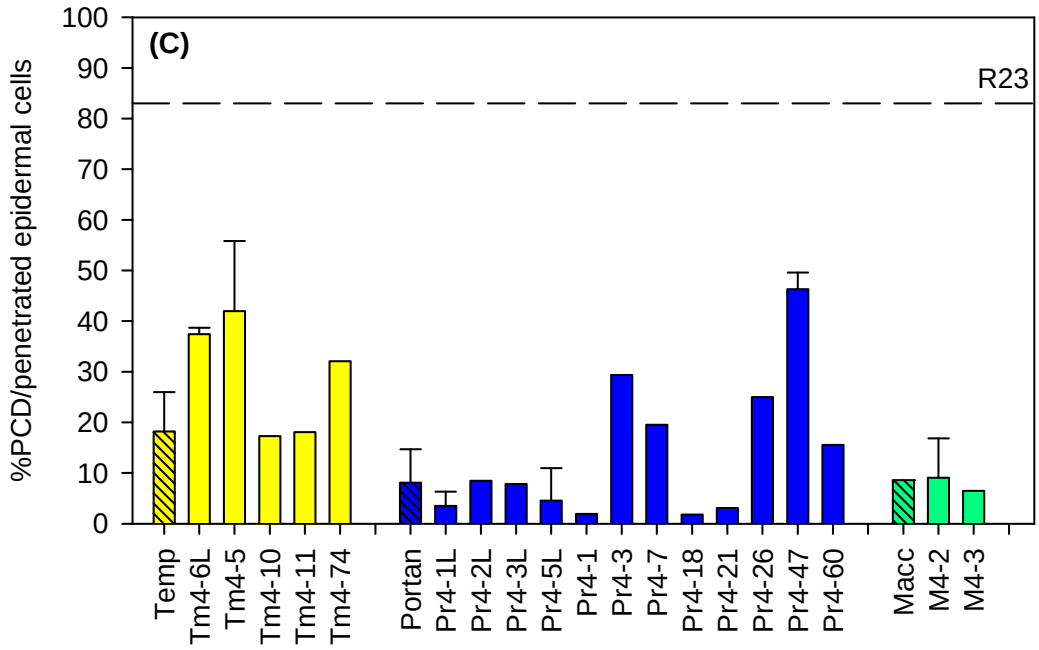
Figure 7. *Run1*-mediated resistance to grapevine powdery mildew. (A) Each germinated *E. necator* spore was scored 48 h post-inoculation in terms of whether it had formed an appressorium only, a haustorium within a penetrated cell or whether the penetrated cell containing a haustorium had undergone PCD. Each data point is based on triplicates with scoring of a minimum of 100 germinated conidia on each leaf. (B) Example of a penetrated epidermal cell which has undergone PCD as indicated by trypan blue staining. Trypan blue is a vital stain used to selectively colour dead cells blue. Conidium (c), appressorium (ap), penetrated cell which has undergone programmed cell death (PCD).

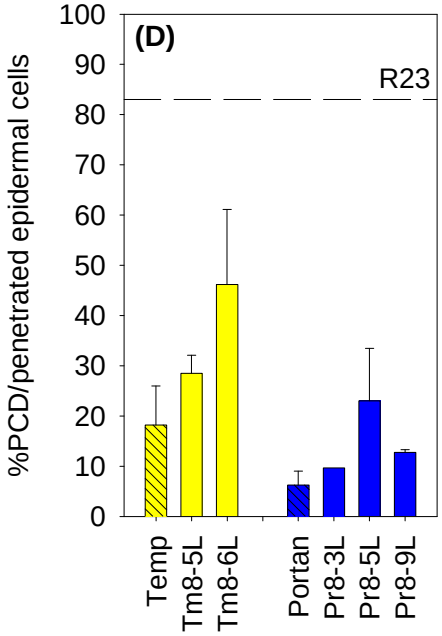
As transgenic lines were generated in Adelaide or received from our collaborators in Montpellier France they were first duplicated *in-vitro* and then one of the copies transferred to the glasshouse where they were allowed to acclimatise for 3-4 weeks before undergoing analysis. Before each transgenic line was assayed for powdery mildew resistance, it was first analysed for transgene expression by quantitative RT-PCR to ensure that the introduced RGA transgene was being expressed to levels approaching that observed in the resistant BC5 progeny plant R23.

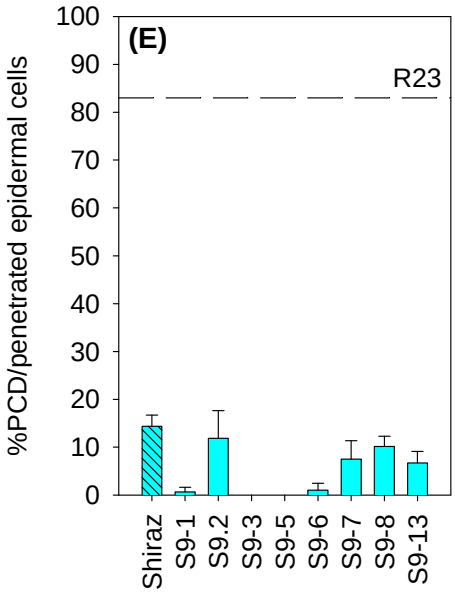
The graphs below (Fig. 8A-G) summarise the results obtained, to date, from assessing powdery mildew resistance in transgenic lines containing each one of the seven different RGA transgenes. In each graph, the levels of PCD/penetrated cell for each transgenic line is shown in comparison with the average results obtained under the same experimental conditions for (a) an untransformed susceptible control plant of the same *V. vinifera* cultivar (shaded bar) and (b) resistant BC5 R23 plant (dashed lines).






(C)
R23
%PCD/penetrated epidermal cells
100
90
80
70
60
50
40
30
20
10
0
Temp
Tm4-6L
Tm4-5
Tm4-10
Tm4-11
Tm4-74
Portan
Pr4-1L
Pr4-2L
Pr4-3L
Pr4-5L
Pr4-1
Pr4-3
Pr4-7
Pr4-18
Pr4-21
Pr4-26
Pr4-47
Pr4-60
Macc
M4-2
M4-3


(D)
R23
%PCD/penetrated epidermal cells
100
90
80
70
60
50
40
30
20
10
0
Temp
Tm8-5L
Tm8-6L
Portan
Pr8-3L
Pr8-5L
Pr8-9L


(E)
R23
%PCD/penetrated epidermal cells
100
90
80
70
60
50
40
30
20
10
0
Shiraz
S9-1
S9.2
S9-3
S9-5
S9-6
S9-7
S9-8
S9-13

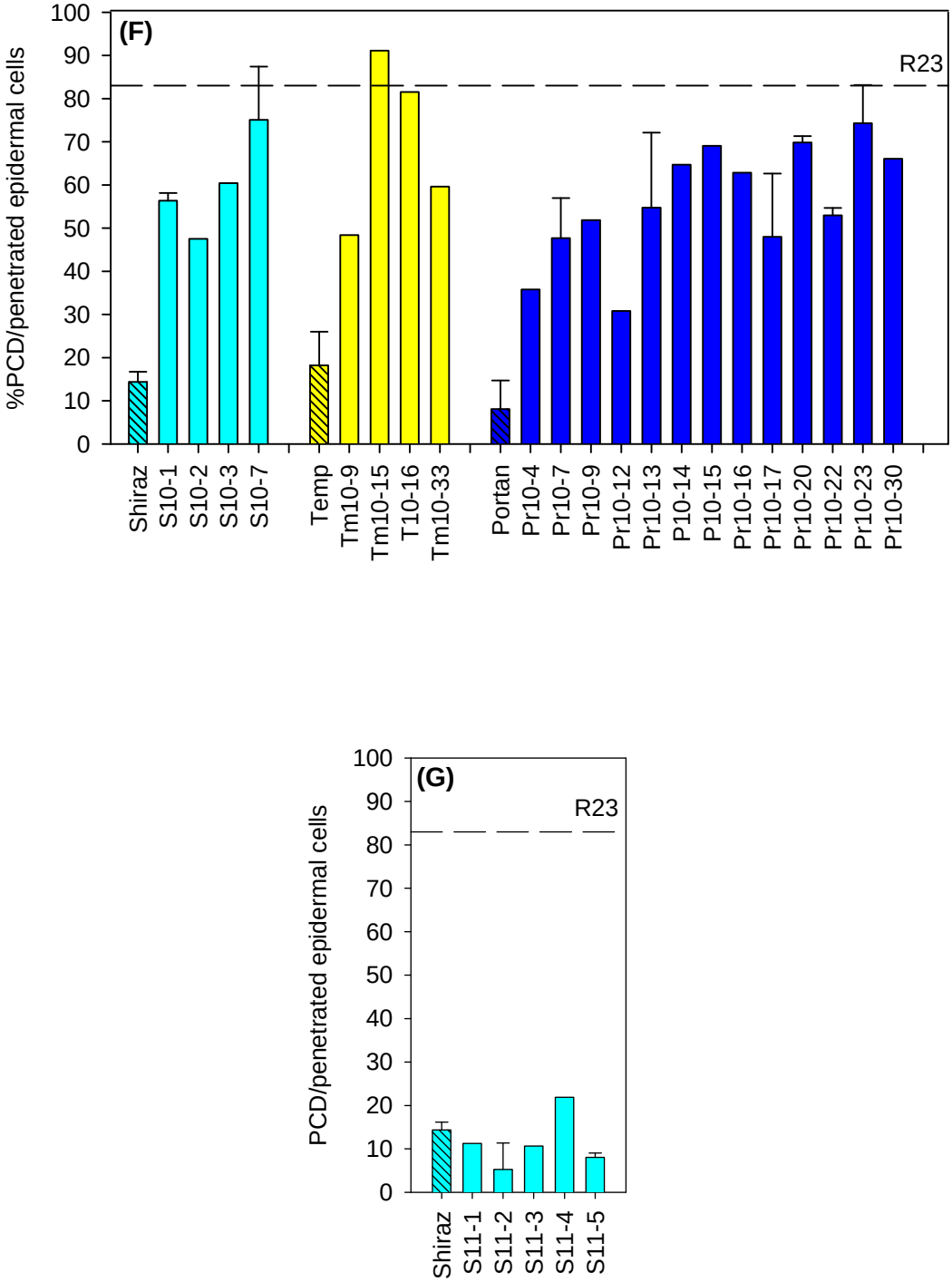


Figure 8. Effect of introduced RGA transgene on powdery mildew resistance of susceptible *V. vinifera* cultivars. Graphs show the frequency of initiation of PCD in epidermal cells penetrated by powdery mildew as scored using trypan blue stain. Rates for transgenic lines are shown in comparison to rates observed under the same experimental conditions for (a) untransformed control lines of the same *V. vinifera* cultivar (shaded box) and (b) R23 - a resistant *V. vinifera* x *M. rotundifolia* BC5 plant (dashed lines).

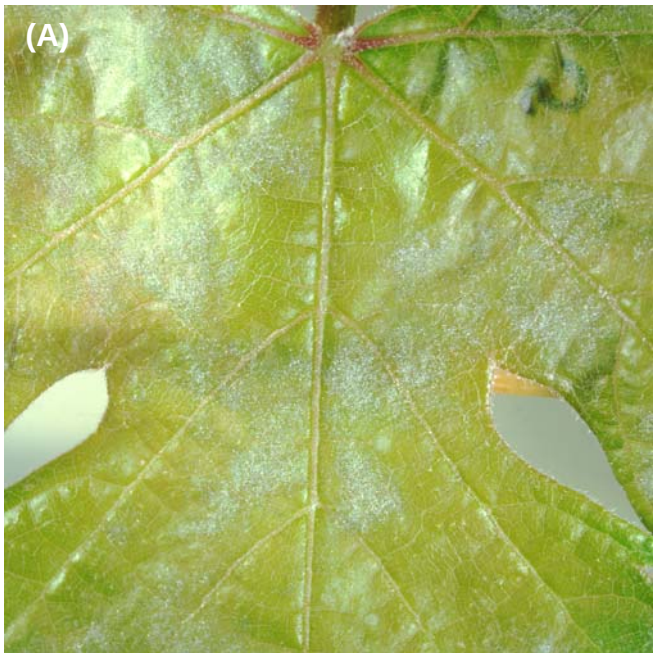
Although powdery mildew testing is still ongoing to analyse new transgenics and to increase the level of replication of those lines that have already been tested, the following general trends can be noted:

- Transgenic grapevines expressing *RGA-1*(Fig. 8A), *RGA-9* (Fig. 8E) and *RGA-11* (Fig. 8G) do not appear to show any significant increase in resistance (PCD/penetrated cell) to powdery mildew relative to untransformed Shiraz control vines. Transgenic lines of other cultivars (Portan & Tempranillo) have also recently been generated (Table 1) and will be used to confirm this finding when they are sufficiently developed for testing.
- The majority of transgenic grapevines expressing *RGA-2* (Fig. 8B), *RGA-4* (Fig. 8C) and *RGA-8* (Fig. 8D) show little or no increase in resistance to powdery mildew relative to untransformed vines. However, some individual lines expressing *RGA-2*, *RGA-4* or *RGA-8* do show significantly increased cell death following penetration. For example, the level of PCD/penetrated cell in transgenic line Pr4-47 was estimated at 46 ± 3 % compared to 8 ± 6 % for untransformed Portan. Similarly, the level of PCD/penetrated cell in transgenic lines Tm4-6L and Tm4-5 were 37 ± 1 % and 42 ± 14 % respectively compared to 18 ± 8 % for untransformed Tempranillo.
- Transgenic lines expressing *RGA-10* (Fig. 8F) show significant increases in resistance to powdery mildew. To date we have analysed 21 *RGA-10* transgenics in three different susceptible cultivars and found significant increases in PCD/penetrated cell in all lines examined compared to the untransformed controls. Indeed, the level of PCD/penetrated cell observed in certain transgenics of each cultivar ie. S10-7, Tm10-15 & Pr10-23 approaches or equals that observed in the R23 BC5 resistant line.

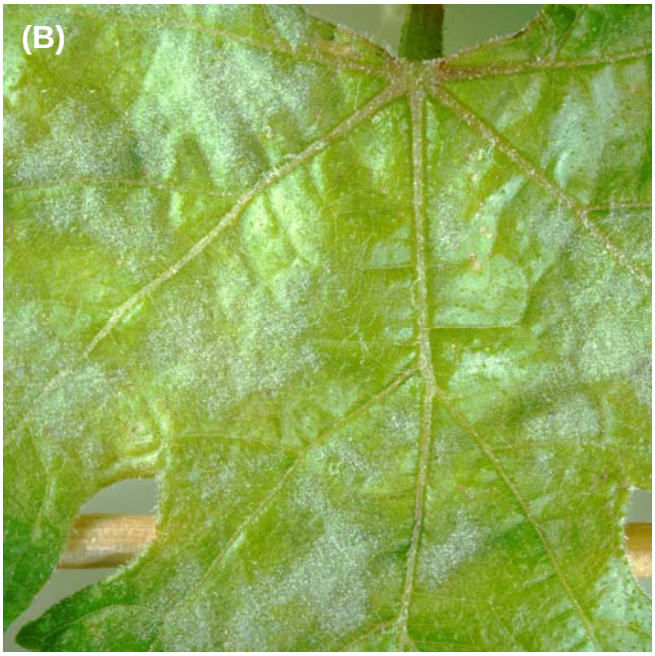
To test whether the increased level of PCD/penetrated cell observed in RGA-10 transgenic lines is sufficient to completely halt powdery mildew development, individual leaves were collected from vines in the glasshouse, inoculated with high level of powdery mildew spores and maintained under optimal conditions for powdery mildew growth. Eight days post-inoculation, leaves from both the untransformed Shiraz vine (Fig. 9A) and from a Shiraz *RGA-1* transgenic vine (S1-21; Fig. 9B) were covered with numerous sporulating colonies of powdery mildew whereas the Shiraz *RGA-10* transgenic vine (S10-7; Fig. 9C) was free of infection. Another comparison is also shown of powdery mildew development on leaves of an untransformed Portan vine compared with a Portan *RGA-10* transgenic vine inoculated at the same time. These higher magnification pictures clearly show the whereas sporulating conidiophores have developed on the untransformed Portan leaf (Fig. 9D), the development of powdery mildew spores on the leaf from the Pr10-23 transgenic vine (Fig. 9E) had been halted immediately following germination and penetration, with the penetrated cell undergoing PCD.

These results demonstrate that the RGA-10 gene confers complete resistance against powdery mildew in grapevine and that this *M. rotundifolia*-derived gene can be used to introduce effective powdery mildew resistance to a range of different *V. vinifera* susceptible cultivars. There is also some evidence to suggest that RGA-2, RGA-4 and RGA-8 may also make a minor contribution to the overall powdery mildew resistance conferred by the *Run1* locus.

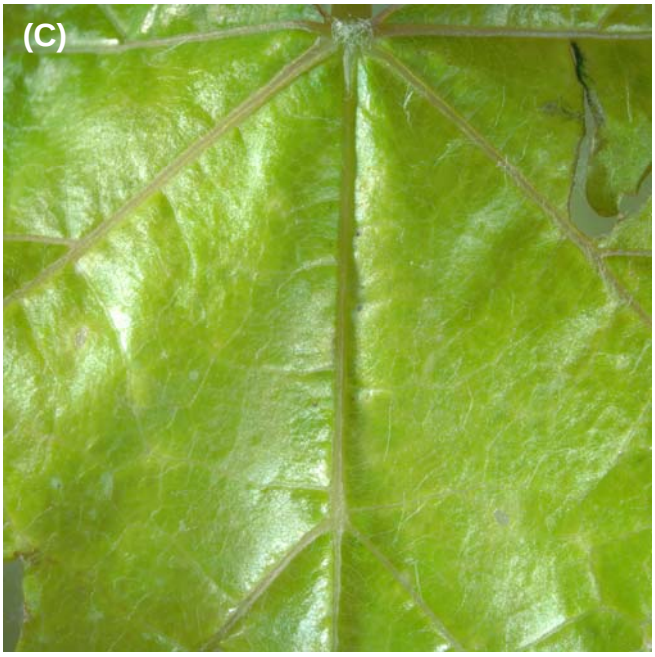
Shiraz (untransformed)



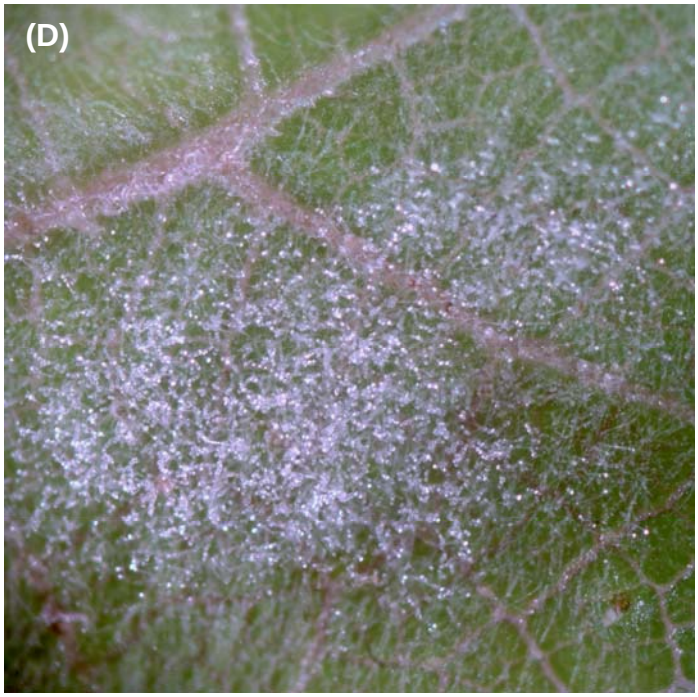
S1-21 (transgenic)



S10-7 (transgenic)



Portan (untransformed)



Pr10-23 (transgenic)

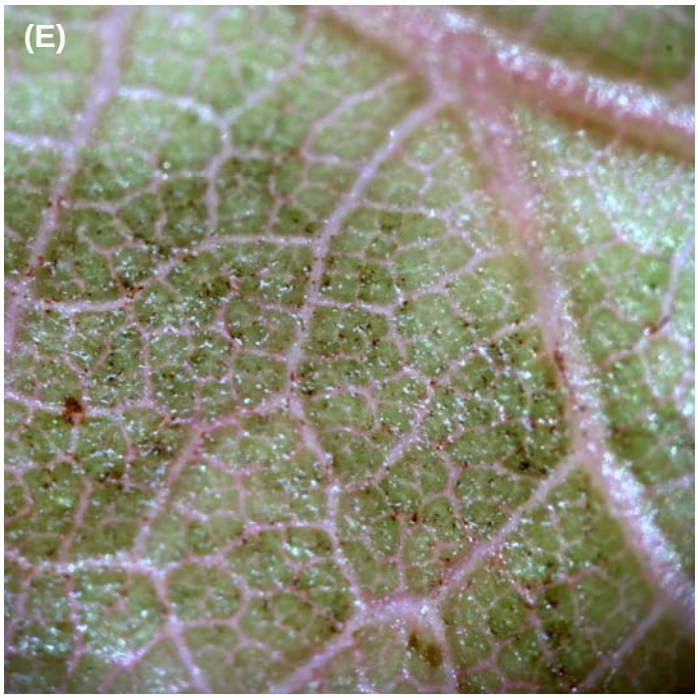


Figure 9. Comparison of powdery mildew development on susceptible and resistant (transgenic) *V. vinifera* leaves. Pictures were taken 8 days post-inoculation with *E. necator* spores. Panel E shows the numerous dark brown spots associated with an area of germinated spores which are epidermal cells which have undergone PCD following penetration by powdery mildew in the presence of the RGA-10 transgene.

6.1.6.2 Downy mildew resistance assay

Grapevine breeding lines containing the *Run1*/*Rpv1* locus not only show resistance to grapevine powdery mildew but also show significantly increased resistance to grapevine downy mildew. Unlike *Run1*-mediated resistance which can be considered as complete or "qualitative" resistance, the resistance conferred by *Rpv1* is classified as "quantitative" because low levels of sporulation are still observed under certain conditions (Fig. 10). The resistance conferred by the *Rpv1* locus is due to the induction of programmed cell death in mesophyll cells penetrated by the downy mildew fungus as indicated by the presence of dark necrotic lesions on the inoculated leaf containing the *Rpv1* locus.



Figure 10. Comparison of downy mildew sporulation on inoculated leaves of susceptible *V vinifera* cv. Cabernet Sauvignon (left) and resistant *V. vinifera* x *M. rotundifolia* BC5 plant (right). Note the formation of brown necrotic lesions on the leaf of the BC5 plant indicating where cells have undergone PCD following penetration of mesophyll cells by *P. viticola*.

One of the original objectives of this project was to test transgenic lines expressing RGA genes from the *Run1*/*Rpv1* locus for resistance to both powdery mildew and downy mildew. Due to unforseen delays in the generation of transgenic grapevines containing the different RGA constructs, the limited time available for disease resistance testing has been focussed mainly on powdery mildew resistance. However, a preliminary screening was undertaken on representatives of each of the different RGA transgenic groups to determine if any of the RGA genes confers increased resistance to downy mildew. Leaf discs (6 per leaf) were inoculated with *P. viticola* sporangia and incubated for 6 days prior to scoring. Discs were assessed for sporulation density and the presence/absence of necrotic lesions.

The results of the preliminary screen indicated that there was no apparent change in the downy mildew susceptibility of transgenic grapevines expressing *RGA-1*, *RGA-2*, *RGA-4*, *RGA-9*, *RGA-10* or *RGA-11*. However, all four transgenic grapevines generated expressing *RGA-8* (Table 1) were found to have significantly reduced susceptibility (ie. increased resistance) to downy mildew. This is clearly illustrated in Fig. 11A which compares downy mildew sporulation on leaf discs sampled from leaves of four Tempranillo vines: an untransformed control, a transgenic *RGA-4* vine (Tm4-6L) and two transgenic *RGA-8* vines (Tm8-5L & Tm8-6L). Sporulation rates were similar on the untransformed Tempranillo and transgenic Tm4-6L discs, but were reduced by >95% on leaf discs from both the Tm8-5L and Tm8-6L transgenic vines. The reduced sporulation on leaf discs of RGA-8 transgenics (Fig. 11) was also found to be associated with the appearance of necrotic lesions which are typical of the *Rpv1* phenotype (Fig. 10).

Similar results were also observed with Portan *RGA-8* transgenics (Fig. 11, panel B). In this case, downy mildew sporulation is compared between leaf discs sampled from Portan transgenic vines expressing either RGA-10 or RGA-8. Again it can be seen that the level of sporulation is significantly reduced in the Pr8-3L disc compared to the Pr10-23 disc and that this is also associated with the appearance of necrotic lesions. The results of Fig. 11B also demonstrate that the RGA-10 powdery mildew resistance gene does not confer resistance to downy mildew.

Although caution needs to be applied when drawing conclusions from results with only four transgenic lines, we believe there is sufficient evidence to suggest that RGA-8 confers strong resistance against grapevine downy mildew.

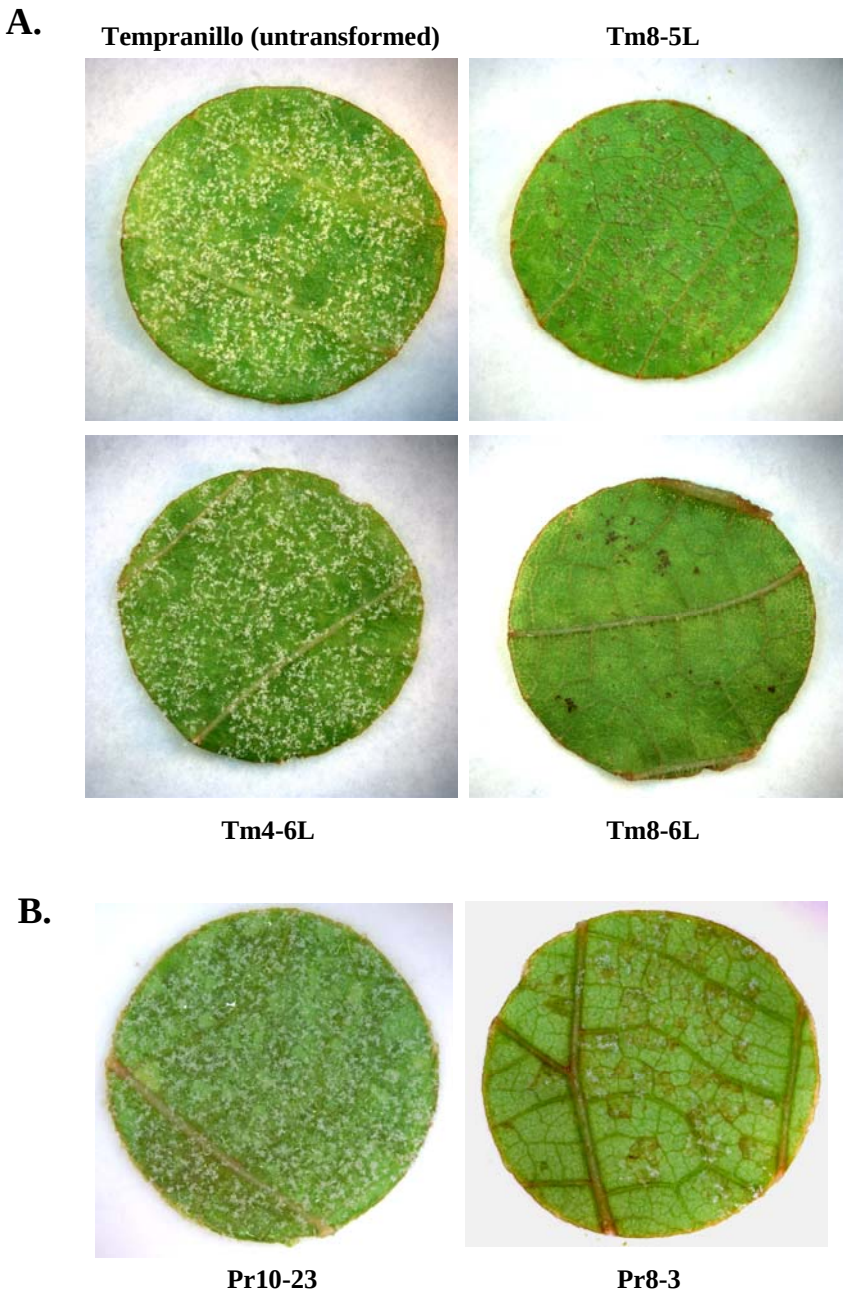


Figure 11. RGA-8 transgenics show significant increases in resistance to downy mildew. Leaf discs were inoculated with *P. viticola* sporangia (1 x 10^5 per ml) and incubated under humid conditions for 6 days. The individual discs shown are representative of the results obtained across the 6 discs tested from each plant.

6.2 Identification of grapevine *MLO* gene candidates involved in susceptibility to powdery mildew

6.2.1 Identification of *VvMLO* gene family members

Previous screening of a *V. vinifera* genomic library with probes derived from *Arabidopsis* sequences *AtMLO1*, *AtMLO2*, *AtMLO6* and *AtMLO12*, resulted in the isolation of several grapevine *MLO* genomic clones. Sequencing of these clones confirmed close sequence relatedness with *MLO* genes from other plant species and the respective genomic clones were designated *VvMLO1*, *VvMLO3*, *VvMLO6*, *VvMLO7*, *VvMLO9*, *VvMLO13* and *VvMLO17* based on original phage clone number.

In order to obtain full-length coding sequences for these *VvMLO* genes, specific primers (Appendix 5) were designed to the genomic sequences and used to successfully amplify full-length cDNAs from various *V. vinifera* cv. Chardonnay tissues using 3′ and 5′ RACE PCR. With the publication of the grapevine genome sequence in 2007 (19) a further ten putative full-length *MLO* gene sequences were identified within the grape genome in addition to the seven *VvMLO* genes we had already cloned from Chardonnay.

6.2.2 Phylogenetic analysis of the grape MLO family

Phylogenetic analysis was performed on the seventeen predicted *VvMLO* gene products together with a selection of known monocot and dicot MLO proteins. Six distinct clades were identified (Fig. 12). Clades IV and V contain the dicot and monocot MLOs previously described to be involved in powdery mildew susceptibility (30, 31). The grouping of VvMLO14 within clade IV is somewhat surprising considering that this clade was previously thought to comprise only monocot homologues (32). The monocot HvMLO from barley, which provides powdery mildew susceptibility, is orthologous to the wheat (TaMLOB-1) and rice (OsMLO2) MLO proteins. Both have been shown to complement powdery mildew resistance of barley *mlo* mutants (30). The largest grouping of grapevine MLO proteins, including VvMLO3, VvMLO4, VvMLO6, VvMLO9, VvMLO13 and VvMLO17, fall into clade V. This clade is significant in terms of putative function because this group also contains the *Arabidopsis* AtMLO2, AtMLO6, AtMLO12 and tomato (*S. lycospersicum)* SlMLO1 proteins that have been demonstrated to be required for powdery mildew susceptibility (5, 31).

6.2.3 Expression analysis of *VvMLOs* in grapevine tissues

6.2.3.1 Developmental expression

To further investigate the possible biological roles of the different members of the *VvMLO* gene family, expression analysis was conducted using different grapevine organs at different stages of development. The expression patterns of all of the *VvMLO* genes grouped in clades IV and V were investigated, together with at least one *VvMLO* gene from each additional clade.

The *VvMLO* genes were found to be expressed in all of the grape tissue types examined, but varied widely in the level of expression (Fig. 13). None of the *VvMLO* genes examined appeared to be tissue-specific and there were no clade-specific expression patterns. For example, of the genes in clade V, *VvMLO3* and *VvMLO4* were found to be expressed mainly in roots, *VvMLO6* in flowers and berries, *VvMLO9* in roots and old leaves and *VvMLO17* predominately in older leaves. A number of *VvMLO* genes such as *VvMLO1* and *VvMLO8* were expressed at relatively high levels in all tissues and stages of development. However, several of the *VvMLO* genes showed evidence of developmental regulation. For example, all of the *VvMLO*s analysed showed higher transcript levels in older leaves than in younger leaves with the exception of *VvMLO13* and *VvMLO12*. Furthermore, *VvMLO1*, *VvMLO4*, *VvMLO10* and *VvMLO17* were expressed at much higher levels in immature (pre-verasion) berries than in ripening (pos-tverasion) berries. Conversely, the transcript levels of *VvMLO6* and *VvMLO9* were higher in post-verasion berries.

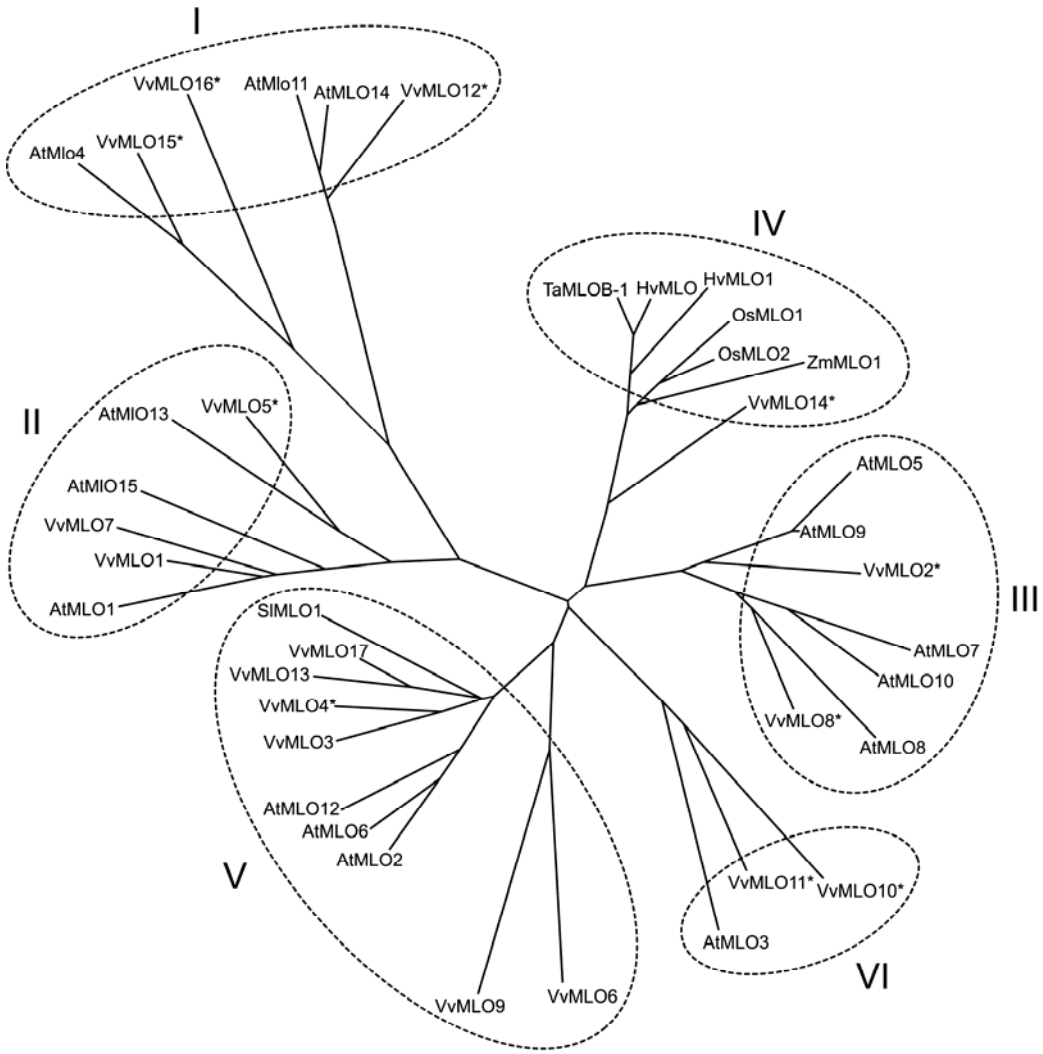


Figure 12. Phylogenetic relationship of MLO isoforms. A neighbour-joining phylogenetic tree of the amino acid sequences of MLO family members of grape (*V. vinifera* cv. Chardonnay and genotype PN40024), *Arabidopsis thaliana* and selected proteins from barley (*Hordeum vulgare*), rice (*Oryza sativa*), maize (*Zea mays*) and tomato (*Solanum lycopersicum*) is shown. The phylogenetic tree represents a consensus tree with branch lengths proportional to sequence distance. Numbers indicate bootstrap values (out of 100 replicates) that support the respective branch. VvMLO sequences identified from genome sequencing of genotype PN40024 (19) are indicated with an asterisk. Gene accession numbers of translated MLO proteins used in phylogenetic analysis: AtMLO1 (Z95352); AtMLO2-15 (AF369563-AF369576); HvMLO (Z83834); HvMLO2 (Z95496); OsMLO1 (Z95353); OsMLO2 (AF384030); SlMLO1 (AAX77013); TaMLOB-1 (AF361932); ZmMLO1 (NM_001112190).

These results are in agreement with observations regarding the diverse transcriptional regulation of the *Arabidopsis MLO* gene family, leading to the suggestion that this unique gene family functions in a diverse array of developmental and response processes in plants. Reports that MLOs are involved in pollen germination and root development in *Arabidopsis* also support this view (33).

6.2.3.2 Accumulation of *VvMLO* transcripts in response to powdery mildew infection

Transcript levels of barley *HvMLO* were previously found to increase rapidly within 6 h following inoculation with barley powdery mildew, which coincided with the period just prior to attempted fungal penetration (34). To establish the time course of *E. necator* penetration on grapevine leaves, infected leaves were microscopically examined 4, 8, 12, 24, 48 and 72 h post inoculation (Fig. 14A). Upon contact with an epidermal cell, *E. necator* conidiospores germinate and subsequently develop a lobed appresorium. A penetration peg then forms beneath the appresorium in an attempt to penetrate the underlying epidermal cell. Following successful penetration, the fungus forms a haustorial feeding structure. Figure 14A shows the levels of epidermal host cell penetration by individual germinated *E. necator* conidia, scored via the presence of a haustorium, on grapevine leaves. It can be seen that a small percentage (~5%) of germinated conidia penetrated grape epidermal cells within 8 h of *E. necator* inoculation. This increased to 10% penetration at 12 h and 55% at 24 h post-inoculation, at which point secondary fungal hyphae could also be observed.

The molecular processes required for the establishment of successful fungal penetration take place prior to haustorium formation and it has been proposed that MLO may be exploited as a docking molecule by the fungus during this time. We therefore examined the response of *VvMLO* genes to *E. necator* infection at 4, 8 and 20 h post-inoculation by qRT-PCR. A significant increase in the transcript levels of *VvMLO17* (~40 fold), *VvMLO3* (~12 fold) and *VvMLO4* (~15 fold) was observed in grape leaves within 8 h of *E. necator* inoculation (Fig. 14B). Transcript abundance of *VvMLO4* and *VvMLO17* then decreased and that of *VvMLO3* remained relatively constant during the period 8-20 h post-inoculation. *VvMLO9* was also found to exhibit increased transcript levels following *E. necator* inoculation, but the timing was delayed relative to the other powdery mildew-induced *VvMLO* genes. None of the other eight *VvMLO* genes examined were induced greater than 2-fold in response to powdery mildew inoculation.

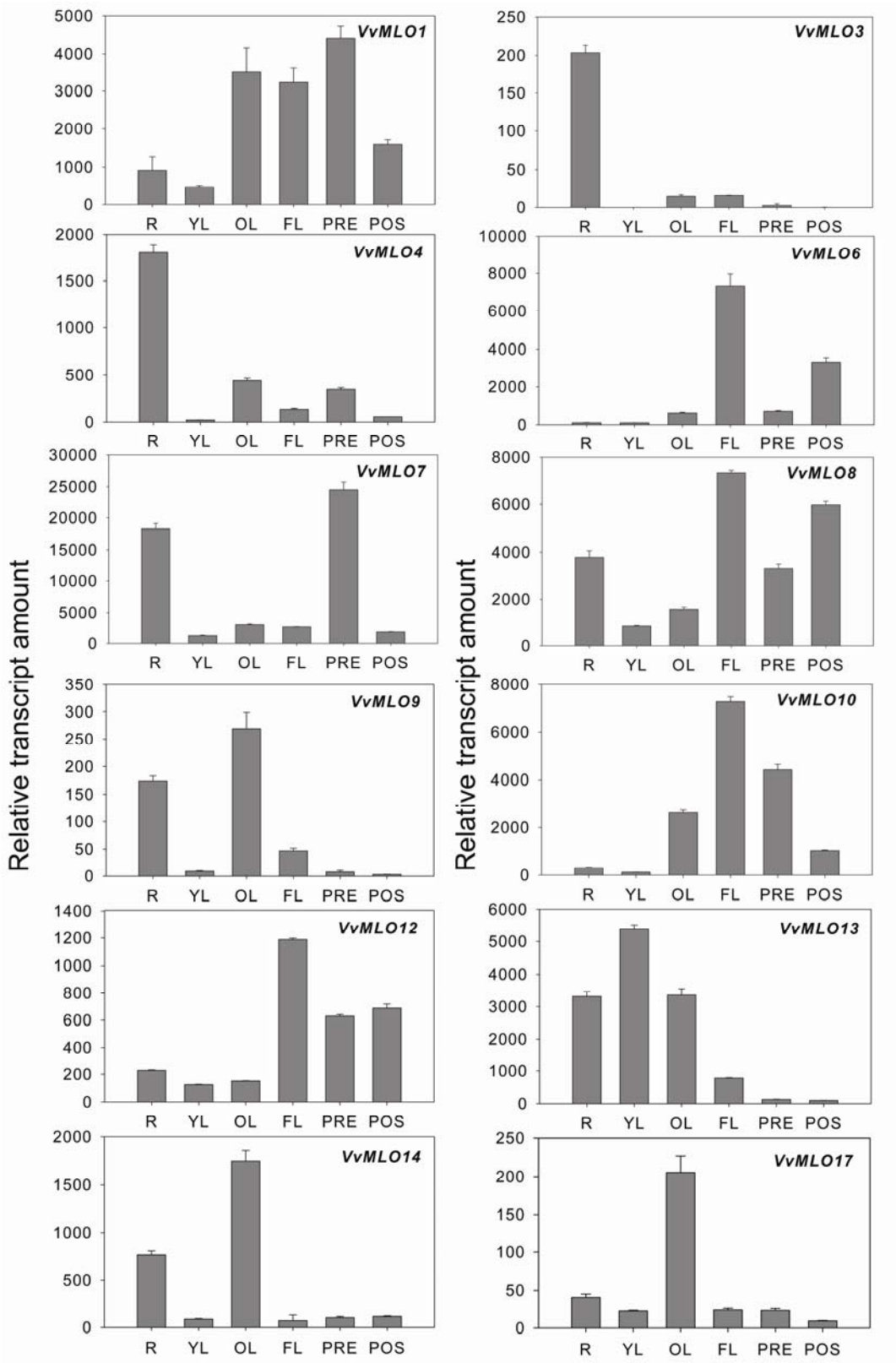


Figure 13. Quantitative RT-PCR analysis of *VvMLO* gene expression in different grapevine tissues. Transcript levels of *VvMLO* genes were normalised against the transcript levels of *EF1* in each cDNA sample. Relative normalised transcript levels are shown for roots (R), young leaves (YL), old leaves (OL), flowers (FL), pre-verasion berries (PRE) and post-verasion berries (POS). Means are totals calculated from triplicate technical qRT-PCR measurements within two biological replicates.

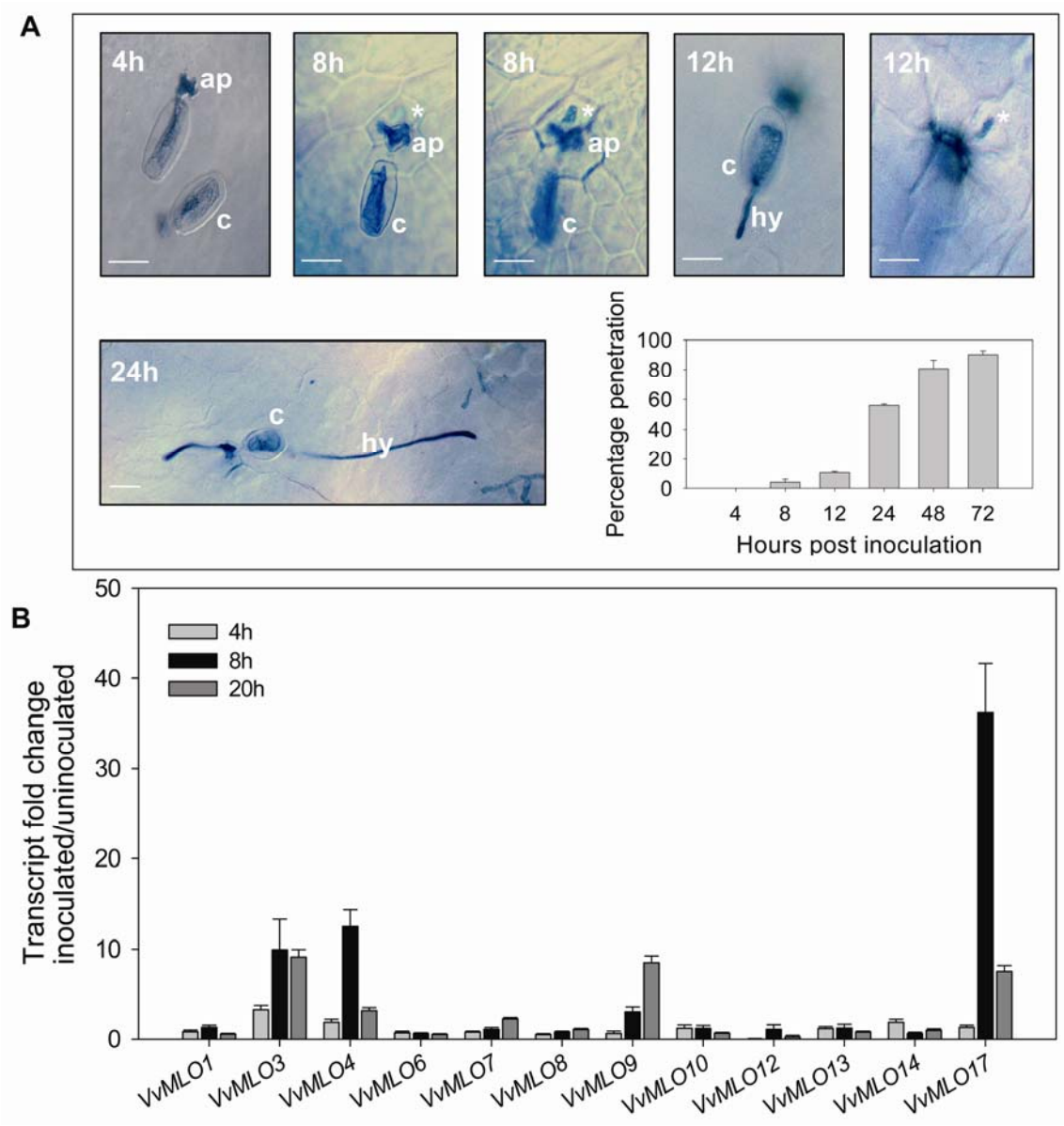


Figure 14 . Transcriptional response of grapevine *VvMLO* genes to powdery mildew infection. (A) Interaction phenotypes between grapevine and *E. necator* visualised with trypan blue at the indicated timepoints. Two panels are shown at 8 and 12 h: the first panel is focused at the epidermal cell surface and the second on the haustorium within the epidermal cell. Scale bars, 20 μm. Symbols: haustorium (*), appresorium (ap), conidium (c), hypha (hy). Time-course of powdery mildew (*E. necator)* infection on grapevine. Detached leaves were harvested at 4, 8, 12, 24, 48 and 72 h post-inoculation and stained with trypan blue. The incidence of epidermal host cell penetration by individual germinated conidia is shown, scored via the presence of a haustorium. Each data point represents the scoring of a minimum of 100 germinated conidia with two replicates. The mean is shown and error bars are standard errors. (B) Quantitative RT-PCR analysis of *VvMLO* gene expression in grape leaves response to powdery mildew infection. Detached leaves were heavily inoculated with *E. necator* spores and sampled at 4, 8 and 20 h post-inoculation. Control (un-inoculated) detached leaves were incubated and sampled as for the inoculated leaves. Transcript levels of individual *VvMLO* genes were normalised against the transcript levels of *EF1* in each cDNA sample. Results are plotted as a mean ratio of the normalised transcript level of each *VvMLO* gene in inoculated leaves versus the normalised transcript level of each *VvMLO* gene in control leaves. Means were calculated from triplicate technical qRT-PCR measurements within three biological replicates and the data shown is representative of the results obtained in the three independent experiments.

6.2.4 Functional characterisation of *VvMLO* genes in a model plant species

Three *VvMLO* genes (*VvMLO3*, *VvMLO4* and *VvMLO17*) have been identified that show similarities to *MLO* genes from other plant species implicated in powdery mildew susceptibility, both in terms of sequence homology (Fig. 12) and their transcriptional response to powdery mildew infection (Fig. 14B). In an attempt to gain further confirmatory evidence regarding the possible role of these three *VvMLO* genes in powdery mildew susceptibility in grapevine, they were each transformed into the *Arabidopsis AtMLO* triple mutant (*atmlo2,6,12*). Arabidopsis mutants which lack the function of *AtMLO2*, *AtMLO6* and *AtMLO12* are completely resistant to penetration by the Arabidopsis powdery mildew *E. cichoracearum* (33). Thus, our aim was to test whether *VvMLO3*, *VvMLO4* and/or *VvMLO17* could functionally complement the *atmlo2,6,12* mutant by restoring MLO function and in so doing restore susceptibility to *E. cichoracearum*. In other words, if the rate of *E. cichoracearum* penetration was increased in the *atmlo2,6,12* mutant in the presence of the wild-type VvMLO proteins this would strongly suggest they may play a similar role in increasing powdery mildew susceptibility in grapevine.

VvMLO3, *VvMLO4* and *VvMLO17* constructs were individually transformed into the *atmlo2,6,12* mutant and plants inoculated with the *E. cichoracearum*. *VvMLO3* was found to significantly increase the penetration rate of *E. cichoracearum* from 2.5% in the resistant *atmlo2,6,12* triple mutant background to between 7-13% in 4 of the 5 independent *VvMLO3-atmlo2,6,12* lines. *VvMLO4* was also found to significantly increase the penetration rate of *E. cichoracearum* from 2.5% in the resistant *atmlo2,6,12* triple mutant background to between 5-9% in the 3 out of 4 independent lines (Fig. 15). Surprisingly, *VvMLO17* was not found to complement the *atmlo2,6,12* triple mutant. These results provide good evidence that *VvMLO3* and *VvMLO4* are powdery mildew susceptibility genes as they partially restore the ability of *E. cichoracearum* to penetrate the Arabidopsis MLO triple mutant *atmlo2,6,12*. Although *VvMLO17* did not restore powdery mildew susceptibility in the Arabidopsis system it may still have a role in grapevine powdery mildew susceptibility since there may be limitations to transferring genes between different species to assess function.

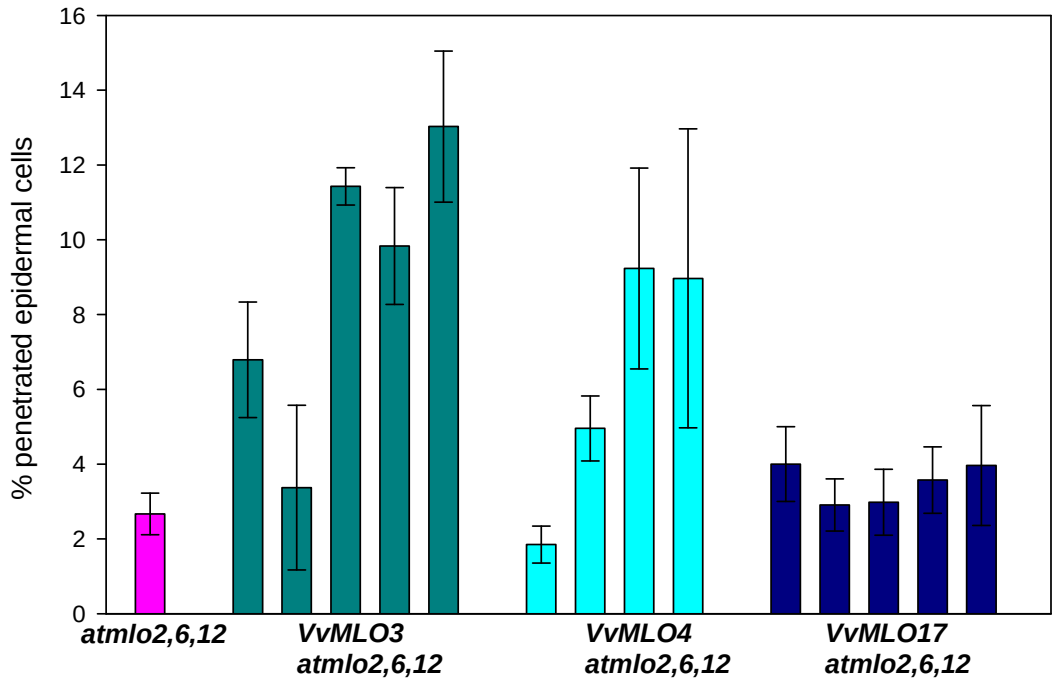


Figure 15. Over-expression of grapevine *VvMLO3* and *VvMLO4* genes in the Arabidopsis *atmlo2,6,12* mutant increases susceptibility to powdery mildew. The number of Arabidopsis powdery mildew (*E. cichoracearum*) penetrated epidermal cells were scored by the presence of a feeding structure (haustorium) stained with trypan blue. Each data point represents the scoring of a minimum of 100 germinated spores per leaf with 3 replicates on each independent Arabidopsis line.

6.2.5 Construction of *VvMLO* silencing constructs and testing in grapevine hairy root cultures

The use of MLO technology for generating powdery mildew resistant plants is currently covered by an existing patent (International Patent Publication Number WO 98/04586; Australian patent AU731487) managed by Plant Bioscience Limited, UK. An investigation of this patent by the CSIRO Plant Industry IP group indicated that the use of gene-silencing constructs of less than 300 contiguous nucleotides of the sequence of the grapevine *MLO* homologs would not be covered by the claimed subject matter of the patent.

Taking this advice into account, we designed two different types of gene-silencing constructs: hairpin (hp) RNA and artificial microRNA (amiRNA). Hairpin silencing constructs were designed to regions of ~ 280 nt in the 3'UTR sequence of *VvMLO3*, *VvMLO4* and *VvMLO17*. An amiRNA construct was designed to target a 21 nt sequence specific to *VvMLO17*.

Due to the large investment in time and resources required to carry out stable grapevine transformation a decision was made to first evaluate the effectiveness of these different *VvMLO* silencing constructs in a grapevine hairy root transformation system. Transgenic grapevine hairy roots can be generated within 2-3 months with much less input of resources compared to the ~ 1 yr required for the generation of transgenic grapevine plants. We had also previously demonstrated that *VvMLO3*, *VvMLO4* and *VvMLO17* were expressed in grapevine roots so that it would be feasible to demonstrate the efficacy of these constructs in this system.

For each *VvMLO* construct, a minimum of 8 independent transgenic hairy root lines were generated (based on the presence of the GFP marker gene) and analysed for gene silencing. The level of expression of each *VvMLO* gene was compared with the level in control transgenic hairy roots lacking the *VvMLO* silencing constructs (Fig. 16). When comparing the efficiency of silencing of *VvMLO17* expression using amiRNA vs hp constructs (Fig. 16A vs B), it can be seen that the hp construct was more effective. Each transgenic hairy root line tested showed a decrease in *VvMLO17* expression relative to control lines, but the two lowest levels were found in roots transformed with the hp*VvMLO17* construct (ie. 1.3 and 2.7 %) compared to the ami*VvMlo17* construct (i.e. 19 and 26 %). The hp*VvMlo3* and hp*VvMlo4* silencing constructs were also effective at producing highly silenced lines.

Thus, the results obtained with the hairy root system demonstrate that both the hp and ami silencing constructs are effective at significantly reducing *VvMLO* expression in grapevine cells. However, it also indicates that the efficiency of the silencing response is such that it will be necessary to generate a sufficiently large numbers of independent transgenic lines ie. 10-15 per construct to be sure of obtaining at least one line in which *VvMLO* expression is reduced sufficiently (ie. < 5%) to maximise the possibility of detecting a change in powdery mildew susceptibility.

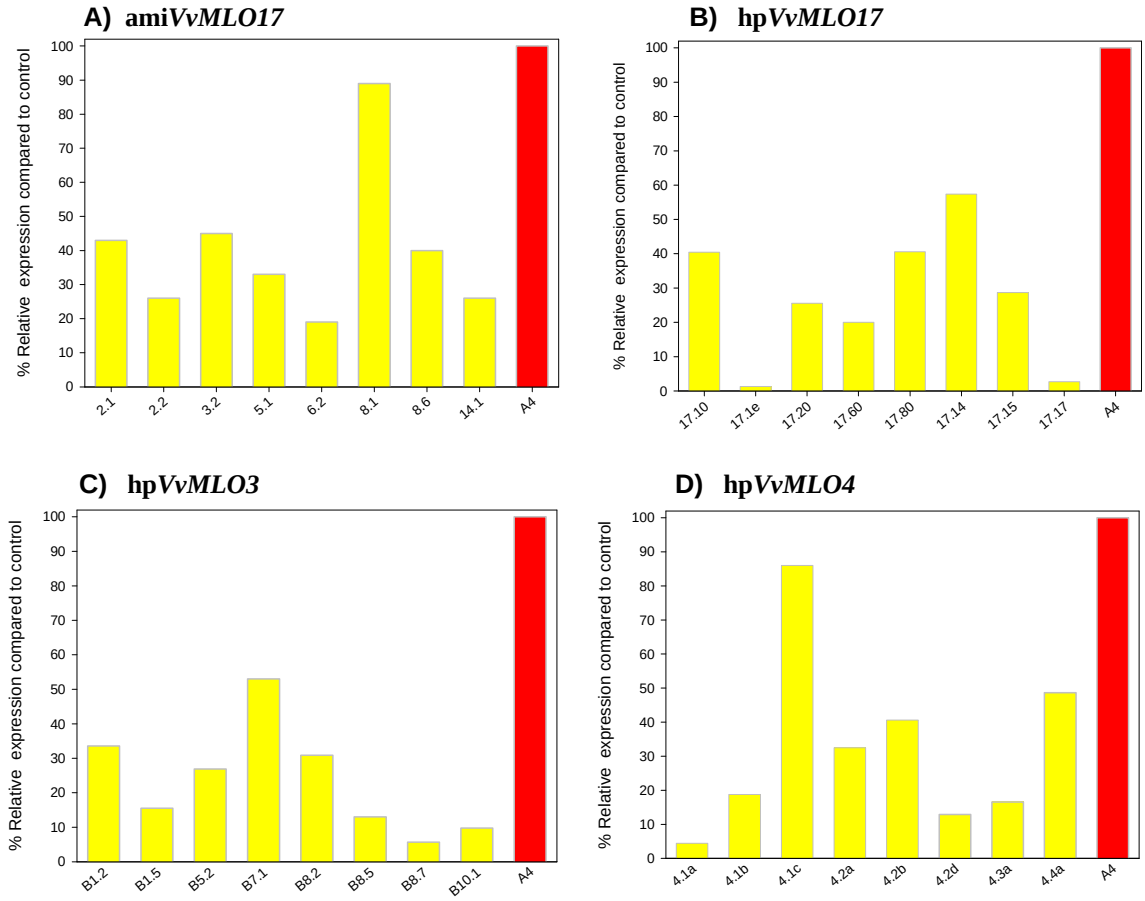


Figure 16. Evaluation of *VvMLO* silencing constructs in Cabernet Sauvignon hairy roots. Gene silencing is shown as percentage relative expression as compared to a control A4 line (red)

6.2.6 Transformation and phenotypic testing of *VvMLO* silencing constructs in grapevines

Having demonstrated that the *VvMLO* silencing constructs were effective at significantly reducing *VvMLO* expression in hairy roots, the next step was to transform these constructs into grapevine plants and evaluate whether this results in any reduction in powdery mildew susceptibility.

Previous research with *A. thaliana* has shown that as many as three *AtMLO* genes may contribute powdery mildew susceptibility in this plant species and that to achieve complete resistance it is necessary to silence all three genes within the one plant. If this were also the case for grapevine, it may be necessary to silence *VvMLO3*, *VvMLO4* and *VvMLO17* within the same grapevine to achieve maximum resistance to powdery mildew. With this possibility in mind, a decision was made to attempt to produce *VvMLO3, VvMLO4* and *VvMLO17*-silenced lines in the microvine genotype. The microvine has the advantage that it will produce flowers on vines in the glasshouse within 6 months as opposed to transgenics of normal *V. vinifera* cultivars which may take 2-3 years to flower in the glasshouse. The advantage of this early flowering character is that should *VvMLO*-silenced lines be identified, transgenic lines could be crossed to combine silencing constructs targeting the different *VvMLO* genes within the same plant, to determine if this enhances powdery mildew resistance above that observed with the silencing of a single *VvMLO* gene.

As part of GWRDC project (CSP06/01) a transformation system has been developed for the microvine through crossing with other *V. vinifera* cultivars. We have utilised some of these enhanced embryogenic microvine cell lines for transformation with *VvMLO*-silencing constructs. Table 2 summarises the number of transgenic microvine lines, generated with the different *VvMLO* silencing constructs, since initiating transformation experiments in October 2008. Unfortunately, transformation efficiency has been lower than expected and the number of transgenic lines currently available for analysis is lower than required to ensure the identification of highly silenced lines as discussed above.

Table 2. Summary of transgenic microvine lines generated to date containing various *VvMLO* silencing constructs.

Target gene	**Type of silencing construct**	**Transformation initiated**	**No. transgenics generated *in-vitro***	**No. transgenics transferred to glasshouse**	**No. transgenics assessed for *VvMLO* expression**	**No. transgenics assessed for powdery mildew susceptibility**
VvMLO3	hairpin	1/10/2008	10	10	7	5
VvMLO17	amiRNA	1/10/2008	4	4	2	2
VvMLO17	hairpin	5/11/2008	4	4	4	3
VvMLO4	hairpin	27/05/2009	10	4	-	-

Individual microvine transgenic lines have been duplicated *in-vitro* and replicate plants established in the glasshouse as they become available. Once the microvines reach sufficient maturity in the glasshouse, analysis of *VvMLO* expression and powdery mildew susceptibility has been undertaken.

Figure 17 shows the levels of *VvMLO3* and *VvMLO17* expression in microvine transgenic lines containing *VvMLO3*- or *VvMLO17*- silencing constructs compared to the expression of these genes in transgenic microvine controls. One technical issue that is yet to be resolved is the apparent large variation in gene expression data between replicate plants leading to large standard deviations in the data. Analysis is currently underway to determine the source of this large variation to increase the accuracy of these estimates in *VvMLO* gene expression. However, despite this variation, there do appear to be a number of transgenic microvine lines in which endogenous *VvMLO* expression is suppressed.

Although further microvine transformations will be required to increase the number of transgenic lines available for analysis, the results of a preliminary examination of powdery mildew susceptibility in five *VvMLO3*- and five *VvMLO17*-silenced microvine transgenics is shown in Fig. 18. In contrast to the cell death resistance response mediated by *Run1* (Fig. 7B) which is activated once the fungus penetrates the cell, *mlo*-mediated resistance is characterised by the failure of powdery mildew to successfully penetrate epidermal cells. Thus, in this assay, detached leaves of microvine transgenics were inoculated with powdery mildew, incubated for 48 h and then the number of successful penetrations per germinated spore measured.

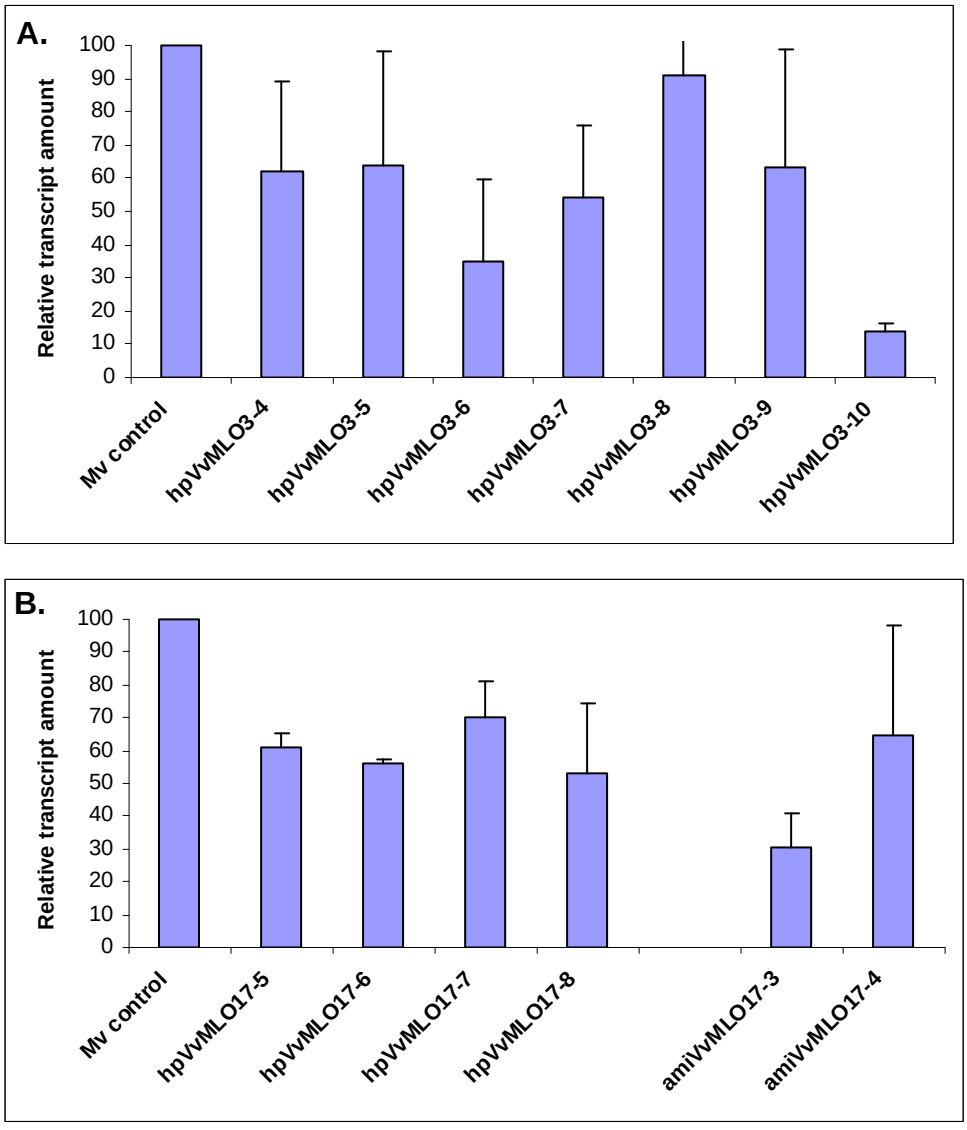


Figure 17. Expression of endogenous *VvMLO* genes in microvines transformed with MLO silencing constructs. (A) Comparison of *VvMLO3* expression in hpVvMLO3 transgenic lines compared to a transgenic microvine control (Mv control) set as 100%. (B) Comparison of *VvMLO17* expression in hpVvMLO17 and amiVvMLO17 transgenic lines compared to a transgenic microvine control (Mv control) set as 100%.

On microscopic examination of the powdery mildew-inoculated microvine leaves, two technical issues were identified, which we had not anticipated, which complicate the interpretation of the results obtained with transgenic microvines. The first of these issues is that the microvine leaves are extremely hairy which makes it very difficult to get sufficient numbers of powdery mildew spores onto the leaf surface to measure penetration efficiency. The second issue is that the rate of successful penetration of *E. necator* germinated spores that do land on the leaf surface of untransformed microvines (~ 50%; Fig. 18) is significantly lower than is normally observed on untransformed *V. vinifera* cultivars (~85%; see Fig. 7A). The reason for this is unclear, but may be a pleiotropic effect of the gene mutation responsible for the microvine dwarf phenotype. This property of microvines may potentially reduce the sensitivity of the powdery mildew penetration assay making it more difficult to identify transgenic microvines in which silencing of *VvMLO* candidate genes results in reduced powdery mildew susceptibility.

Despite these technical problems, the preliminary results shown in Fig. 18 suggest that certain of the *VvMLO3*- and *VvMLO17*-silenced lines generated to date, appear to show a reduced frequency of successful powdery mildew penetration (ie. reduced susceptibility) relative to the untransformed controls ie. hpVvMLO3-6, hpVvMLO17-6, amiVvMLO17-2 and amiVvMLO17-3. However, it should be noted that some of these lines have only been assayed once and these results require confirmation before any conclusions can be drawn.

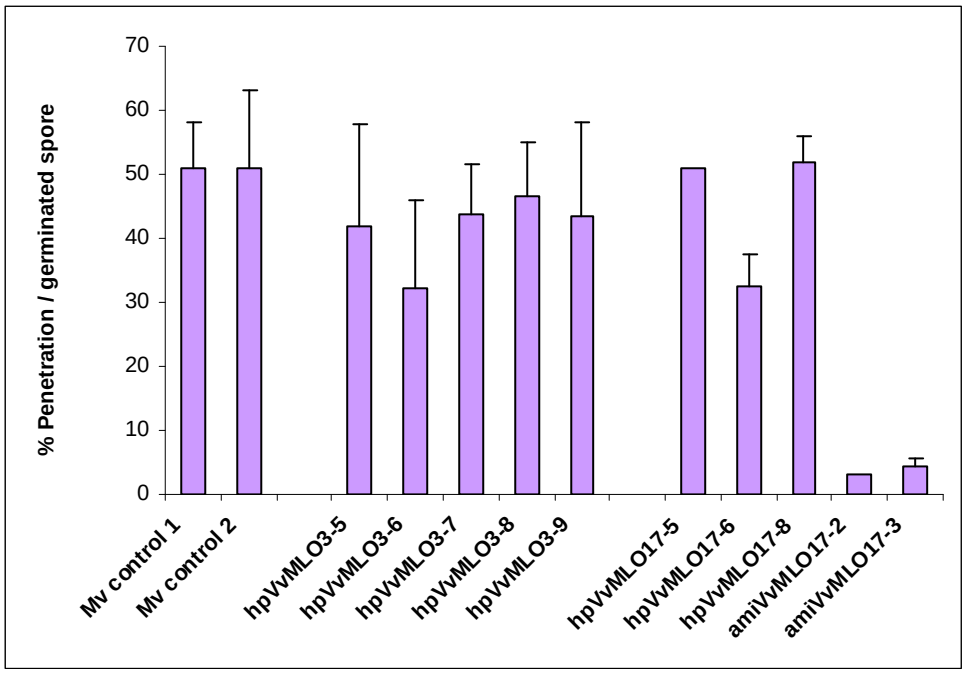


Figure 18. Susceptibility of *VvMLO*-silenced transgenic microvines to powdery mildew penetration. Detached leaves of microvine transgenics were inoculated with powdery mildew, incubated for 48 h and then the number of successful penetrations per germinated spore measured in comparison to two microvine transgenic controls.

7. Outcomes / Conclusions

This project has made excellent progress in the identification of grapevine genes for improved resistance to fungal pathogens. In the main, the specified performance targets have been met. However, due to a number of unforseen technical difficulties outlined in this report, some further analysis of transgenic plants remains to be completed. Despite this, the major outcomes/conclusions can be summarised as follows:

(A) Cloning and functional characterisation of powdery mildew (*Run1*) and downy mildew (*Rpv1*) resistance genes from *M. rotundifolia*

- Completion of the cloning and sequencing of all major resistance gene sequences (*RGAs*) at the *Run1*/*Rpv1* locus.
- Subcloning of genomic fragments containing each RGA into suitable plant transformation vectors.
- In collaboration with our colleagues at INRA, Montpellier, France, generation of over 300 transgenic grapevines across six different *V. vinifera* cultivars containing individual RGA genes.
- Identification of *RGA-10* as the major gene conferring powdery mildew resistance associated with the *Run1*/*Rpv1* locus. Demonstration that *RGA-10* transgenic lines of at least three different grape cultivars initiate programmed cell death in penetrated epidermal cells at levels approaching or equalling that observed in plants containing the entire *Run1*/*Rpv1* locus. The presence of *RGA-10* in susceptible *V. vinifera* cultivars effectively halts the development of powdery mildew in *in-vitro* assays.
- Preliminary evidence to suggest that other genes at *Run1*/*Rpv1* locus ie *RGA-2* and *RGA-4* may also make a minor contribution to powdery mildew resistance.
- Identification of *RGA-8* as the gene conferring downy mildew resistance associated with the *Run1*/*Rpv1* locus. Demonstration that the presence of *RGA-8* in susceptible cultivars of *V. vinifera* can reduce downy mildew sporulation by >95% in *in-vitro* assays.
- Sequencing of the *Run1*/*Rpv1* locus has enabled us to design new "perfect" genetic markers which can be used for highly accurate marker-assisted selection of new disease-resistance cultivars by traditional breeding.

(B) Identification of grapevine *MLO* gene candidates involved in susceptibility to powdery mildew

- Cloning and functional characterisation of the seventeen member *VvMLO* gene family.
- Identification of three members of the *VvMLO* gene family ie. *VvMLO3*, *VvMLO4* and *VvMLO17* that are good candidates for having a role in the susceptibility of grapevine to powdery mildew based on sequence and transcriptional response to powdery mildew infection.
- Demonstration that *VvMLO3* and *VvMLO4* partially complement the Arabidopsis MLO triple mutant *atmlo2,6,12* supporting their role in grapevine powdery mildew susceptibility.
- Design and construction of *VvMLO*-gene silencing constructs that are effective in significantly reducing endogenous *VvMLO* gene expression in a transgenic grapevine hairy root system.
- Generation of transgenic microvine plants containing *VvMLO*-silencing constructs, some of which, based on preliminary analysis, show measurable decreases in *VvMLO* gene expression and possibly powdery mildew susceptibility.

8. Recommendations

8.1 *Run1* / *Rpv1* - mediated resistance to powdery and downy mildew

- Downy mildew resistance conferred by *RGA-8* will need to be confirmed in a larger number of transgenic lines. As generation of individual transgenic lines expressing *RGA-8* has proved problematic, we propose to collect seed from RGA-8 transgenic vines and analysing the segregation of downy mildew resistance with the RGA-8 transgene in the progeny population.
- Having shown that *RGA-10* and *RGA-8* confer strong resistance to powdery and downy mildew respectively in transgenic *V. vinifera* cultivars in *in-vitro* assays, the next step is to assess the disease resistance of these vines in unsprayed vineyards. This is particularly important to enable us to assess whether these individual RGA genes also provide protection to berries from powdery and downy mildew infection. Such an experiment is very difficult to carry out under glasshouse conditions. As it is currently not possible to carry out GM field trials in South Australia, we have established a collaboration with Dr Lance-Cadle Davidson and Dr Bruce Reisch at Cornell University, New York State Agricultural Experimental Station where field testing of GM-vines is possible. We plan to send them our best performing transgenic vines for evaluation. This has the added benefit that RGA-10 and RGA-8 transgenic vines can be tested against a range of powdery and downy mildew isolates that are not available in Australia, thereby providing us with information about the likely durability of these genes in the field.
- Preliminary evidence indicates that other genes at *Run1*/*Rpv1* locus ie *RGA-2* and *RGA-4* may also make a minor contribution to powdery mildew resistance. Further analysis will be undertaken to assess powdery mildew resistance of the remaining 32 *RGA-4* and 43 *RGA-2* transgenic lines which are yet to be tested to determine the significance of these putative minor resistance genes.
- Having obtained proof of concept of the identity of the genes at the *Run1*/*Rpv1* locus that confer powdery and downy mildew resistance, immediate consideration must be given by CSIRO, INRA and GWRDC as to whether the partners wish to commercialise the use of these novel grapevine resistance genes (see Appendix 2).
- Due to ongoing uncertainties associated with the deployment of transgenic grapevines in the Australian wine industry, the introduction of the *Run1*/*Rpv1* locus into new grapevine varieties should be undertaken using marker-assisted selection. This will be part of the new GWRDC project (CSP 09/04): "Advanced grapevine genetics for varietal improvement".

8.2 *mlo*-mediated powdery mildew resistance in grapevine

- Continue transformations of *VvMLO3*-, *VvMLO4*- and *VvMLO17*-silencing constructs into the microvine genotype and evaluation of transgenics for powdery mildew penetration.
- Using seed collected from selected self-fertilized microvine transgenics, select for segregating normal "tall" progeny vines that contain the introduced *VvMLO*-silencing construct. Examination of powdery mildew penetration rates in non-dwarf vines will hopefully avoid some of the complicating factors associated with the dwarf phenotype. There is also the added advantage that one third of the non-dwarf progeny vines should be homozygous for the *VvMLO*-silencing construct which may lead to higher levels of VvMLO suppression and possibly reduced powdery mildew susceptibility.
- Initiate crosses between selected *VvMLO3*-, *VvMLO4*- and *VvMLO17*-silenced microvine transgenics microvine to combine the silencing constructs within the same grapevine to determine if this further enhances powdery mildew resistance.

Appendix 1: Communication

Scientific Publications

Feechan A, Jermakow AM, Torregrosa L, Panstruga R, Dry IB (2008) Identification of MLO gene candidates involved in susceptibility of grapevine to powdery mildew. *Functional Plant Biology* 12: 1255-1266

Feechan A, Jermakow AM, Dry IB (2009) Grapevine MLO candidates required for powdery mildew pathogenicity? *Plant Signaling & Behavior* 4: 1-2

Dry IB, Feechan A, Anderson C, Jermakow AM, Bouquet A, Adam-Blondon A-F, Thomas MR (2009) Molecular strategies to enhance the genetic resistance of grapevines to powdery mildew. *Aust. J. Grape and Wine Research* 16: 94-105

Anderson C, Choisne N, Adam-Blondon A-F and Dry IB. Positional Cloning of Disease Resistance Genes in Grapevine. In "*Encyclopaedia of Plant Genomics: Grapes*" (ed. C. Kolen) *in press*

Conference Presentations

Thomas M, Davies C, Dry IB, Walker A, Robinson S, Boss P, Clingeleffer P, Velasco R, Adam-Blondon A-F (2007) Unlocking the secrets of the grapevine genome Proceedings 13th Australian Wine Industry Technical Conference, Adelaide 28 July to 2 August 2007

Anderson C, Feechan A, Jermakow A, Bouquet A, Adam-Blondon A-F, Thomas M, Dry IB (2008) Isolation and functional characterisation of a cluster of TIR-NBS-LRR genes linked to powdery mildew resistance in grapevine. International Conference on Biotic Plant Interactions, Brisbane March 27-29, 2008

Feechan A, Tiburzi S and Dry IB (2008) Characterisation of resistance strategies to powdery mildew infection within the Vitaceae (grape) family. International Conference on Biotic Plant Interactions, Brisbane March 27-29, 2008

Dry IB, Feechan A, Anderson C, Jermakow A, Bouquet A, Adam-Blondon A-F, Thomas M. (2008) Molecular strategies to enhance the genetic resistance of grapevines to fungal pathogens. Keynote address at Eighth International Symposium on Grapevine Physiology and Biotechnology, Adelaide 23-28 November 2008

Feechan A, Tiburzi S and Dry IB (2008) Characterisation of resistance strategies to powdery mildew infection within the Vitaceae (grape) family. Eighth International Symposium on Grapevine Physiology and Biotechnology Adelaide 23-28 November, 2008

Jermakow A, Feechan A, Torregrosa L, Panstruga R and Dry IB. Identification of MLO gene candidates involved in susceptibility of grapevine to powdery mildew. Eighth International Symposium on Grapevine Physiology and Biotechnology, Adelaide 23-28 November, 2008

Feechan A, Pak H and Dry IB. Ontogenic resistance to powdery mildew is mediated via reduced penetration. Poster presented at 20th International Conference on Arabidopsis Research, Edinburgh Scotland 30th June - 4th July 2009

Feechan A, Anderson C, Jermakow A, Bouquet A, Adam-Blondon A-F, Thomas M and Dry IB (2009) Exploiting a non-host *Arabidopsis* mutant system to identify powdery mildew resistance genes in grapevine**.** Poster presented at XIV IS-MPMI Congress, Québec City, Canada. July 19-23, 2009

Industry Journal articles

Dry IB, Jermakow A, Feechan A (2009) Do cultivated grapevines contain genes that predispose them to powdery mildew infection? A potential new strategy to develop powdery mildew resistant grapevines. *Australian & New Zealand Grapegrower & Winemaker* **549**: 46-50

Dry IB (2010): Grapevine genes for improved resistance to fungal pathogens to reduce chemical inputs in Australian vineyards. Australia *& New Zealand Grapegrower & Winemaker* **553**: GWRDC R&D at Work: Viticulture value-add p2.

Brochures/Technical Guides

Dry IB "Beating powdery mildew in the lab and the vineyard". Article in CSIRO Plant Industry Newsletter Issue 26 - Winter 2009 available on CSIRO website for public distribution

Appendix 2: Intellectual Property

(A) *Run1* / *Rpv1*

Intellectual property for this component of the project is equally shared between CSIRO and INRA under an existing contractual agreement. As we have now obtained proof of concept of the identity of the genes at the *Run1*/*Rpv1* locus that confer powdery and downy mildew resistance, immediate consideration must be given by CSIRO, INRA and GWRDC as to whether the partners wish to commercialise the use of these novel grapevine resistance genes.

Our French partners, INRA, have already indicated to us that they do not favour patenting of the resistance gene sequences and support publication of the results as soon as possible to negate the possibility of any other groups patenting the same information and, in so doing, restricting the freedom of the Australian and French wine industries from deploying these resistance genes in their own genetic improvement programs.

An alternate view is that patenting of the powdery mildew (RGA-10) and downy mildew (RGA-8) resistance genes from *M. rotundifolia*, would enable us to generate disease-resistant transgenic versions of existing premium cultivars for the exclusive use of the Australian and French wine industries. However, any benefit may be offset by costs associated with lodging and maintaining worldwide patents and the presently high costs associated with complying with regulatory requirements associated release of GM crops.

A third alternative may be to consider patenting and licensing these genes for use in grapevines to be grown in developing countries such as China were there are major environmental and health issues relating to the overuse of agrochemicals but more freedom to deploy GM crops.

(B) *MLO*

It has not been possible to protect all information relating to the identity of the *VvMLO* genes which are likely to be involved in powdery mildew susceptibility due to a publication of grapevine *VvMLO* gene sequences by another research group in 2008 (35). Once we learnt that this information was to be published, we made a decision to publish similar data which we had also generated (see publication list). In this publication we speculated on which *VvMLO* candidates may be involved in powdery mildew susceptibility but, as yet, no other information has been published showing proof of concept for any of the suggested *VvMLO* candidates. We are hopeful that within the next 12-24 months that our experiments will ultimately provide this proof of concept in grapevine. However, even if we are able to obtain this proof, our ability to protect this information, should CSIRO and GWRDC decide to do so, will be determined by what disclosures are made by other research groups working in the same area.

It also needs to be remembered that the use of *MLO* technology for generating powdery mildew plants is currently covered by an existing patent owned by John Innes Centre and Keygene N.V. and managed by Plant Bioscience Limited (PBL). Based on advice from CSIRO PIant Industry's IP manager we believe the design of the silencing technology we are currently employing to evaluate *MLO* technology in grapevines falls outside of the coverage of the current patent. However, if the *MLO* technology was found to be effective in grapevine for reducing susceptibility to powdery mildew, further discussions would most likely need to take place between CSIRO, GWRDC and PBL regarding patenting the grapevine sequences under the existing PBL patent and licensing the *MLO* technology for use in grapevines in Australia.

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Appendix 4: Staff

Project Staff

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Dr Mark Thomas	CSIRO Plant Industry, Adelaide
Dr Claire Barker, Postdoctoral Fellow (1/7/06 - 31/1/07)	CSIRO Plant Industry, Adelaide
Dr Angela Feechan, Postdoctoral Fellow (1/05/07- 30/6/10)	CSIRO Plant Industry, Adelaide
Ange Jermakow, Technical Officer	CSIRO Plant Industry, Adelaide
Karina Swann, Technical assistant (30/7/06 - 24/1/07)	CSIRO Plant Industry, Adelaide
Nicole Kempster, Technical assistant (13/4/08 - 30/6/10)	CSIRO Plant Industry, Adelaide
Debra McDavid, Technical Officer - 0.5 time (1/7/06 - 30/6/09)	CSIRO Plant Industry, Adelaide

Collaborators

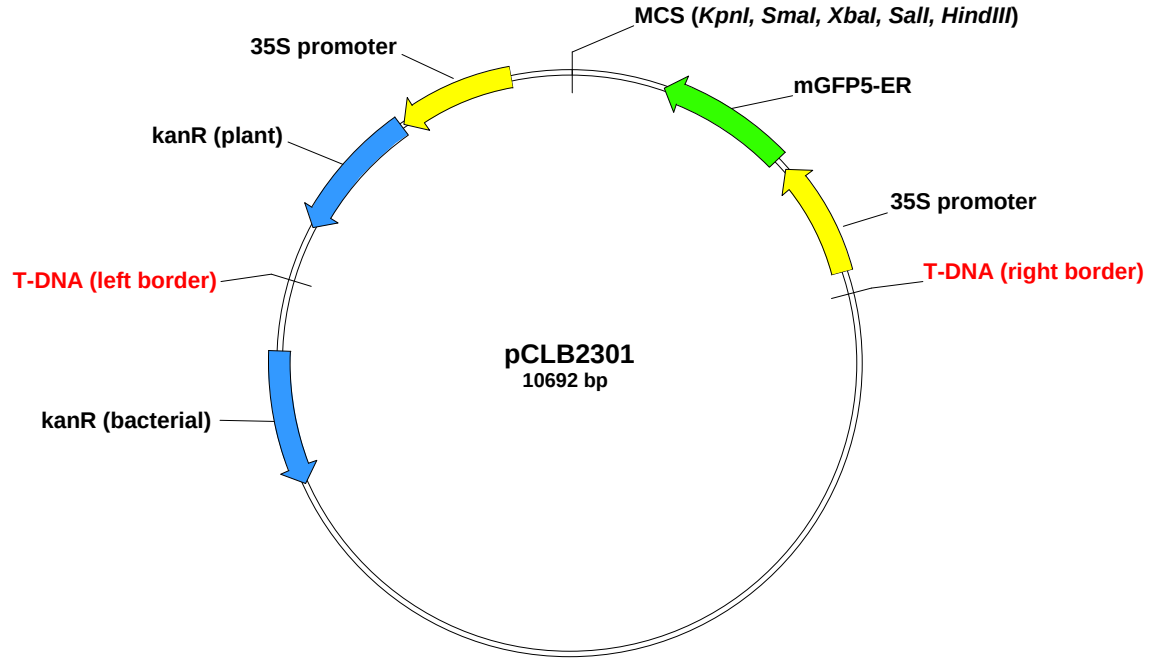
Dr Alain Bouquet
Dr Anne-Francoise Adam
Dr Laurent Torregrosa
Dr Bipna Shresta

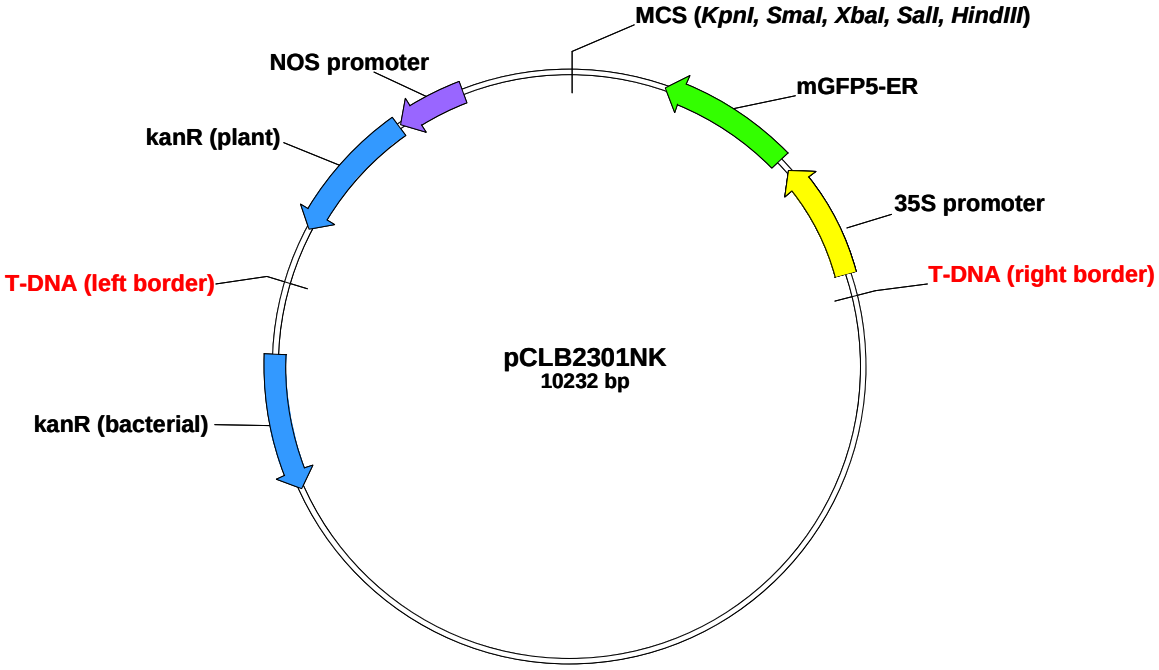
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INRA-URGV, Evry, France

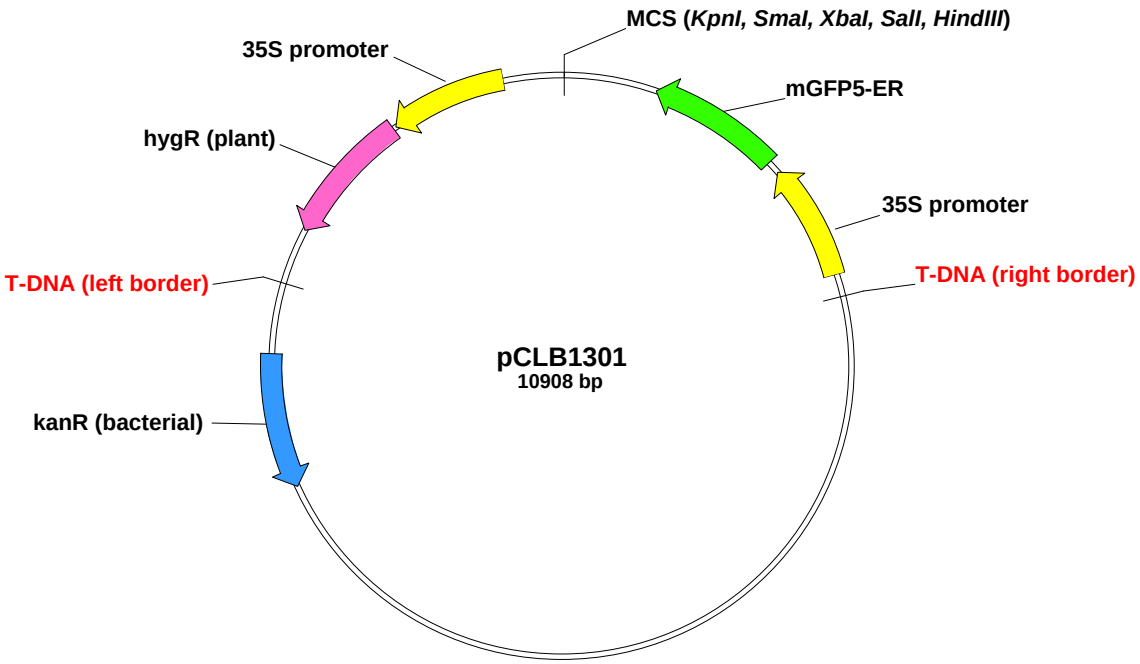
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Appendix 5: Supplementary data







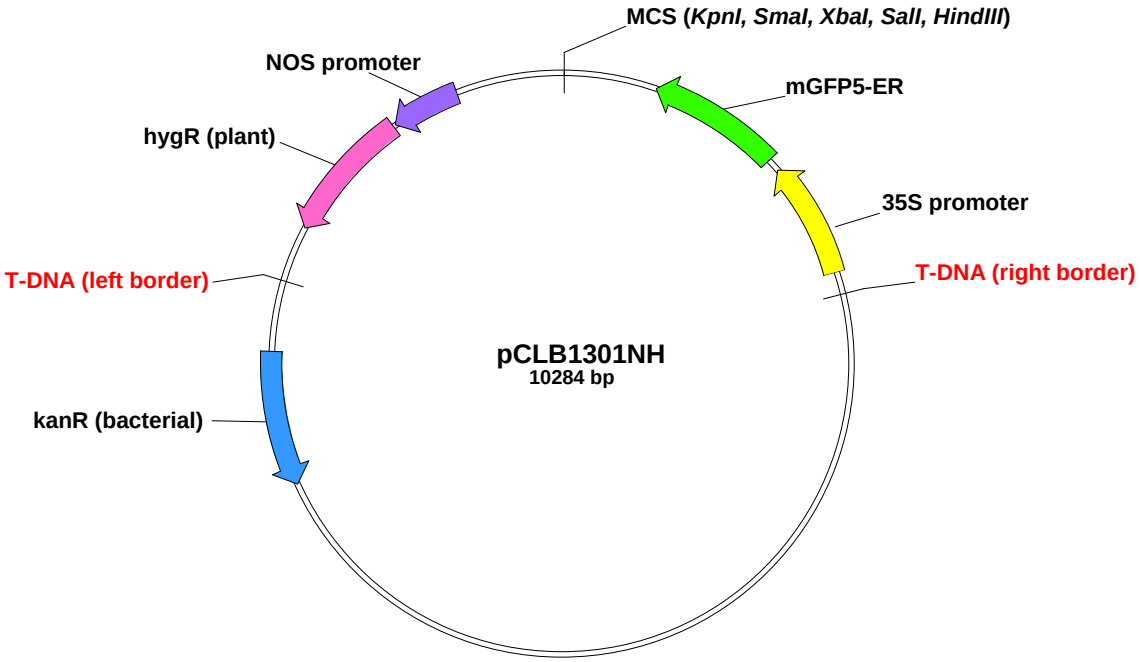


Figure 19. Maps of plant transformation vectors constructed as part of this project for grapevine transformation.

Table 1. Primers used to amplify full-length *VvMLO* genes from *V. vinifera* cv. Chardonnay cDNA

VvMLO gene	***3′*-RACE primer**	**Nested 5'-RACE primers**	**5'& 3'-gene-specific primers**
VvMLO1	GCAATTTTATGCATCTGTGACG	GCAACAAGAAGACAACCACAAAG TTAAAATCGTCTTCAAGGGCAC	
VvMLO3	TTATGGGATCCACCAACTTCAC	CTGCCCAAAGCCAATGTG ACAATAAAGTACGTGGAAAACAGC	
VvMLO4			TTGAGTTGTAAAGCCAGGAAATG CATCAGCCTCGTACCTGTGG
VvMLO6	ATGAAGAAATGGGAGTCCTGG	TCCGATGCTCACTCCAATATC GCTTTCCAAATGATGTCTGATG	
VvMLO7	AAGGATGAAGATCCTTGCTTC	TTCTCGGCAACCTCATGG GCTCCAGCTTAGTCCCCAC	
VvMLO9	CATTAACCACTTTCTTCTTGAAGATG		
VvMLO13	TGCTGCTTACAGTATTACAAACTCC		CCATGGCTGATGAACTTC CATCCTTCGCATCATCGAAG
VvMLO17	CCAGAGAGGTTTAGGTTTGCAAG		CCATGGCTGATGAACTTC TGAAGAAGCAGACAATCCAGAG

Table 2. Real-time primers used for gene expression analysis

Gene	**5'-qRT-PCR primer**	***3′*-qRT-PCR primer**	**Product size (bp)**
VvMLO1	CCCTTTATGCCATTGTCACC	CTGTTAGCGTTACCAGTCGTTG	130
VvMLO3	TGGGTCATCCGCATATCAATG	TGCCAGTTTCTCAGAGCTTTC	148
VvMLO4	GGATGCTATTCACCAACTGC	GAACTGGTACTCGACTGTTC	148
VvMLO6	TAAGGCTTGTCATGGGTGTG	TCAACGACTCCCTCAGTAAAGAC	124
VvMLO7	GCAGTTACAGTACCTTACCAC	CCGTTTTCAGCTTGGCTTTGT	129
VvMLO8	GTGTTATGGGCATCGTTTGTG	GAGCCATCTTTGTAAGAACAG	140
VvMLO9	TCAGTTCCTATGCGGTTATGTG	GTGCTGTGCCAGTGTCTGAG	123
VvMLO10	TGCTTCTTCAGACAGTTCTTTC	TACGACCTTGAAATCCTCGTC	150
VvMLO12	TGGTCTACATGCGGCTGATT	CCCATCCATGAATTGTCTCTC	150
VvMLO13	GGGTTGAAATCTTGCTATCATG	GAGCCACGGCTACTTTGTCA	172
VvMLO14	CAATCTTTGAGGAGCAAACCG	GGTTGCGTAGTAAATGCAAGG	152
VvMLO17	ACTCCCACTCTATGCCTTGG	ACACACCATTCGAGTGGC	143
RGA-1	CATTGGGAAGGGTAGCAAG	GTGGAATTTATCTGCACGATG	125
RGA-2	CAGTTTATTTCCGCCATGCTG	GCAATAACACCAGGATGAATGAA	98
RGA-4	CATCTTATGATACCGCAACTC	CAATTACACCAGCATGAATGAG	112
RGA-8	CATGTGTTTATCGTCCATCATG	CACCAGCATTCATGATAGCATA	127
RGA-9	CGTGCAGATGCTCGTGGC	CGCATACTACATCTCTCGGATA	139
RGA-10	CCTGAAGCGGAAATTCTCAG	TGCATGGAAATCACAAGCATCT	150
RGA-11	GACCTGATTATGATCCTGCATG	CACTCACACGAGCGTGAAG	115
EF1	CGGGCAAGAGATACCTCAAT	AGAGCCTCTCCCTCAAAAGG	147

Table 3. Primers used to construct gene-silencing vectors

VvMLO3-Hp1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATAGAGTGATGAAGATTGGATTG
VvMLO3-Hp1-R	GGGGACCACTTTGTACAAGAAAGCTGGGTGAAAGCATTGGCTTGATTTTGG
VvMLO4-Hp1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATCCCCGAGAAGGTCCAAC
VvMLO4-Hp1-R	GGGGACCACTTTGTACAAGAAAGCTGGGTGGGAAAATTCAATCTCCACGG
VvMLO17-Hp1-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGAAAAGGAGAAATTTGAATCCAG
VvMLO17-Hp1-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAAGAAAAGTATATTGAAATCACAAG
VvMLO17syn-miR-4 F	TATATGCTCTTCGAGAGGACCCCGCCCAAGACGAACCCTGGAGGGTTTAGCAGGGTGAAGTAAAG
VvMLO17syn-miR-4 R	TATATGCTCTTCGAGATGACCCCGCCCAATCCGAACCCAGAAGAGTGAAGCCATTAAAGGG

Table 4. Other primers used in this study

AJ68	AGAGGCCATCCAGAAAGATTGGCTTCC
AJ69	GCATGTCCCGGATCTTTCTTGTCTTTGTGG
AJ70	CCTCAAGATGGTGAGCAACTGAGGAGATTG
AJ80	GCTTCCCAAACAAACTGTAAC
AJ81	CGGCTGCCCAAATGATATACT
AJ84	ACTGGAAGGACAAGGTAGGT
AJ93	TTCCTTCAAGGCCGTCCTCCACCTACCTAC
AJ94	ATAACCGGCAAAGGCCTCTCCAAGCCTTCC
AJ110	TAGGCTTGGCTTCTCTTCAAGATGGTGAGC
AJ111	GGGAAGTGGAGAGGAACGTTAGGGACAGC
AJ116	CTCATGTGCTTGGTATAAGGCACTAAGTATC
AJ117	GGTGGAAACCTGAGGAGAAGTTAATCTCAAG
AJ118	AGGAAATGATCTAATTGACTTGAAGTAGGAGC
AJ119	AGTAGAGAAAGAAAGTGGTTTCGTTTGACTG
AP1	GGATCCTAATACGACTCACTATAGGGC
AP2	AATAGGGCTCGAGCGGC
CB45	GTATGCATGATTTGATACAAC
CB60	GTTGTTATGATGACTCTACTTCC
CB181	GTTGATAATGCACCTGGTG
CB572	CTCCTCATTATCTTTGAAAATAAC
GLP1-12P1	GGAATATTTACTTGGACATCG
GLP1-12P3	CATTTGAATTGGAGCATACTC

Appendix 6: Budget reconciliation

An End of Project Financial statement will be submitted as a separate document as discussed with Joanne Watson. This will ensure that all transactions have been captured at the close of FYE 2009-2010

Appendix 7: Scientific Publications / Industry Journal Articles

CSIRO PUBLISHING
www.publish.csiro.au/journals/fpb *Functional Plant Biology*, 2008, **35**, 1255–1266

Identification of grapevine *MLO* gene candidates involved in susceptibility to powdery mildew

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Abstract. The European cultivated grapevine, *Vitis vinifera* L., is a host for the powdery mildew pathogen *Erisyphe necator*, which is the most economically important fungal disease of viticulture. MLO proteins mediate powdery mildew susceptibility in the model plant species *Arabidopsis* and the crop plants barley and tomato. Seven *VvMLO* cDNA sequences were isolated from grapevine and were subsequently identified as part of a 17 member *VvMLO* gene family within the *V. vinifera* genome. Phylogenetic analysis of the 17 *VvMLO* genes in the grape genome indicated that the proteins they encode fall into six distinct clades. The expression of representative *VvMLO*s from each clade were analysed in a range of grape tissues, as well as in response to a range of biotic and abiotic factors. The *VvMLOs* investigated have unique, but overlapping tissue expression patterns. Expression analysis of *VvMLO* genes following *E. necator* infection identified four upregulated *VvMLO*s which are orthologous to the *Arabidopsis AtMLO2*, *AtMLO6* and *AtMLO12* and tomato *SlMLO1* genes required for powdery mildew susceptibility. This suggests a degree of functional redundancy between the proteins encoded by these genes in terms of susceptibility to powdery mildew, and, as such, represent potential targets for modification to generate powdery mildew resistant grapevines.

Additional keywords: *Erysiphe necator*, grapevine, MLO, powdery mildew.

Introduction

MLO proteins belong to a unique plant-based family of seven-transmembrane domain proteins (7TM) that contain a *C*-terminal calmodulin-binding domain (Büschges *et al*. 1997; Devoto *et al*. 1999; Kim *et al*. 2002*a*). Although the biological functions of MLO proteins remain largely unknown, some family members are thought to have a role in modulating host response to the phytopathogenic powdery mildew fungus.

In barley, homozygous *mlo* mutants possess broad spectrum resistance to the biotrophic powdery mildew pathogen *Blumeria graminis*. Although most known *mlo* alleles were obtained by mutagenesis, a natural barley allele, *mlo-11*, derives from Ethiopian landraces that were identified in 1937 (Jørgensen 1992; Piffanelli *et al*. 2004). *SlMLO1* is a natural loss-of-function mutation recently identified in tomato (*Solanum lycospersicum* L. var. *cerasiforme*) that provides resistance to the powdery mildew pathogen *Oidium neolycopersici* (Bai *et al*. 2008). In addition, a homozygous T-DNA insertion line in the *Arabidopsis AtMLO2* gene, shows significantly reduced susceptibility to *Golovinomyces orontii*. Two other closely related *Arabidopsis* genes, *AtMLO6* and *AtMLO12*, are also required to be mutated, along with *AtMLO2*, to achieve complete powdery mildew resistance (Consonni *et al*. 2006). Therefore, partial functional redundancy exists between these three *Arabidopsis* co-orthologues. These *MLO* genes are involved in powdery mildew susceptibility within the respective species (Jørgensen 1992; Consonni *et al*. 2006), and are induced in response to powdery mildew inoculation (Piffanelli *et al*. 2002; Chen *et al*. 2006; Bai *et al*. 2008).

In animals and fungi, 7TM domain proteins exist in sequence-diverse protein families (Devoto *et al*. 1999). Many of these function as G-protein coupled receptors (GCPR) that relay extracellular stimuli into downstream signalling events by the activation of heterotrimeric G-proteins. Despite the G-protein coupled signalling role of 7TM domain proteins in animals and fungi, MLO-mediated powdery mildew susceptibility in barley seems to be independent of heterotrimeric G-proteins (Kim *et al*. 2002*b*). Nevertheless, it is possible that heterotrimeric G-proteins are required for other, yet to be determined, MLO functions in plant cells (Chen *et al*. 2006).

Overexpression of barley *HvMLO* leads to powdery mildew super-susceptibility, allowing almost every attacked barley cell to be successfully colonised by the pathogen (Kim *et al*. 2002*b*). Thus, powdery mildew may exploit MLO proteins for pathogenesis. It is unlikely that the selection pressure for the presence of MLO proteins in plants is to mediate disease susceptibility, and since *mlo* mutants in *Arabidopsis* and barley have spontaneous cell death and accelerated leaf

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senescence phenotypes, the principal function of these MLO proteins might be related to plant development (Piffanelli *et al*. 2002; Consonni *et al*. 2006).

During fungal penetration MLO becomes polarised at the site of attempted pathogen infection (Bhat *et al*. 2005), where it negatively regulates components of penetration resistance including the function of a syntaxin PEN1/ROR2, a glycosyl hyrdolase (PEN2) and an ABC transporter (PEN3) (Collins *et al*. 2003; Lipka *et al*. 2005; Consonni *et al*. 2006; Stein *et al*. 2006). Syntaxins are members of the SNARE (soluble NSF attachment protein receptors) protein super-family that mediate membrane fusion events through the formation of four-helix SNARE bundles (Pratelli *et al*. 2004; Lipka *et al*. 2007). Large H_2O_2-containing membraneous compartments form in *mlo* barley in response to powdery attack. The incidence of these large membraneous compartments is reduced in the *ror2* background, in which penetration resistance is partially compromised (Collins *et al*. 2003). Experiments using split ubiquitin assays and fluorescence resonance energy transfer revealed a direct interaction between MLO and ROR2 (Panstruga 2005*b*). The actin cytoskeleton also undergoes polarised assembly at the site of pathogen attack (Kobayashi and Hakuno 2003; Shimada *et al*. 2006). It has, therefore, been proposed that MLO controls vesicle fusion events at the plasma membrane. Consistent with this hypothesis, Miklis *et al*. (2007) have shown that the barley MLO modulates actin-dependent and actin-independent defence pathways at the cell periphery. Genetic evidence suggests that PEN2 and PEN3 operate together in a penetration resistance pathway additional to that of PEN1 mediated vesicle trafficking, probably to deliver toxic compounds to the site of fungal attack (Consonni *et al*. 2006; Stein *et al*. 2006; Kwon *et al*. 2008).

Erisyphe necator infection of the cultivated grapevine, *Vitis vinifera* L., is a serious problem for viticulture worldwide (Barker *et al*. 2005). Control of this pathogen is currently achieved by the application of fungicides because the European winegrape species *V. vinifera* has no genetic resistance to *E. necator*, which arrived from North America in the 1840s. The existence of fungicide-resistant strains, and mounting pressure to reduce the use of agrochemicals, has increased the interest in the development of new grapevine cultivars with enhanced genetic resistance to powdery mildew. Thus, in an attempt to identify grapevine MLO homologues which are responsible for mediating susceptibility to *E. necator*, we used sequence information from the three *Arabidopsis* genes (*AtMLO2*, *AtMLO6* & *AtMLO12*) shown to be involved in powdery mildew susceptibility, to search for MLO genes in grapevine. This paper describes the identification of several *VvMLO* genes, which, based on sequence homology and transcriptional response to powdery mildew infection, are likely candidates for mediating powdery mildew susceptibility in grapevine.

Materials and methods

Plant cultivation and sampling

Leaves were sampled from potted *Vitis vinifera* L. cv. Chardonnay and cv. Cabernet Sauvignon vines grown in temperature-controlled glasshouses at Waite Campus, University of Adelaide, SA, Australia, maintained between 23 and 25°C. 'Young' leaves (YL) were sampled from nodes 1–4 with node 1 designated as the first expanding leaf below the growing tip and were less than 4 cm long measured along the central vein. 'old' leaves (OL) were sampled from nodes 9 and 10 and were fully expanded and ~10 cm in length. Cabernet Sauvignon flowers and berries were sampled from the Slate Creek vineyard (Willunga, SA). Flowering was defined as the date on which 50% of flowers within an individual bunch had undergone capfall. Pre-veraison and post-veraison berries were sampled 5 and 12 weeks post-flowering respectively. Roots were sampled from *in vitro*-grown plantlets.

For wounding experiments, the adaxial surface of glasshouse-grown Cabernet Sauvignon leaves were gently rubbed with fine sandpaper. Two control and two wounded leaves were sampled at 2, 4, 8 and 24 h post-wounding. Leaves were frozen in liquid nitrogen and stored at −80°C before RNA was extracted independently from each leaf. For treatments involving hydrogen peroxide (H_2O_2) and salicylic acid (SA), glasshouse-grown Chardonnay leaves were excised and the petioles submerged in distilled water containing either 5 mM H_2O_2 or 1 mM SA and incubated at 22°C under a 16 h light/8 h dark cycle. Control leaves were treated in the same way but without the addition of elicitors. Leaves were sampled at 6 and 24 h post-treatment.

Erysiphe necator *inoculation studies*

Erisyphe necator (isolate APC1, kindly provided by Associate Professor Eileen Scott, University of Adelaide, SA) was maintained on detached leaves of Cabernet Sauvignon using an 8–10 days rotation as previously described (Donald *et al*. 2002). For detached leaf time-course studies, young glossy leaves of similar developmental stage (~6 cm in diameter) were inoculated with *E. necator* conidia by gently tapping conidia from infected leaves (8 days post-inoculation) above the open dishes. The Petri dishes were sealed with Parafilm (American National Can, Chicago, IL, USA) and the leaves incubated at 25°C under a 16 h light/8 h dark cycle until sampled into liquid nitrogen and stored at −80°C before RNA was extracted independently from each leaf.

Cloning of VvMLO *cDNAs*

A grape genomic library based on a partial *Mbo*I restriction digest was prepared in the bacteriophage vector Lambda Fix II (Stratagene, La Jolla, CA, USA) and ~250 000 plaques screened with radio-labelled cDNA fragments of *AtMLO1* (GenBank Accession number Z95352), *AtMLO2* (AF369563), *AtMLO6* (AF369567) and *AtMLO12* (AF369573). From this screening, genomic fragments encompassing seven full-length and partial-length *VvMLO* homologues were identified and sequenced. These were designated *VvMLO1*, *VvMLO3*, *VvMLO6*, *VvMLO7*, *VvMLO9*, *VvMLO13* and *VvMLO17*. Gene-specific primers were designed to these sequences to amplify full-length cDNA sequences from various *V. vinifera* cv. Chardonnay tissues. Total RNA was isolated from *V. vinifera* cv. Chardonnay grape tissues using the method by Davies and Robinson (1996) and cDNA synthesised using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Where genomic clones

covered the entire putative cDNA region (i.e. *VvMLO13* and *VvMLO17*) gene-specific forward and reverse primers were used to obtain full-length cDNA sequences. Where either the 5′- or 3′-end of the putative *VvMLO* cDNA sequence was absent from the genomic clone, RACE-PCR techniques (Frohman *et al*. 1988) were employed to obtain full-length cDNA sequence. Gene sequences were submitted to GenBank and have been assigned accession numbers in the range EU812233–EU812239. Sequences of primers used in this study are listed in the Table S1 available from the online version of *Functional Plant Biology*.

Phylogenetic analysis

Protein sequence alignments were performed with ClustalW with default settings (Thompson *et al*. 1994). The aligned sequences were used to generate an unrooted phylogenetic tree using the Phylip programs Seqboot, Protdist, Neighbour and Drawtree (Felsenstein 1989). Support for the branching arrangements was evaluated by bootstrap analysis using 100 replicates. Putative VvMLO protein sequences within the recently sequenced *V. vinifera* genotype PN40024 (Jaillon *et al*. 2007) were identified with the BLAT-Search tool of the Grape Genome Browser (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/, accessed 26 August 2007) using the cloned Chardonnay *VvMLO* sequences. GenBank accession numbers of putative VvMLO sequences translated from the *V. vinifera* genotype PN40024 genome sequence are: VvMLO1 (CAO41068), VvMLO2 (CAO66267), VvMLO3 (CAO18135), VvMLO4 (CAO21819), VvMLO5 (CAO22254), VvMLO6 (CAO66388), VvMLO7 (CAO46388), VvMLO8 (CAO71699), VvMLO9 (CAN84002), VvMLO10 (CAO18134), VvMLO11 (CAO21818), VvMLO12 (CAO39251), VVMLO13 (CAO68971),VvMLO14 (CAO66265),VvMLO15 (CAO47031), VvMLO16 (CAO48195), VvMLO17 (CAO68972).

Quantitative RT-PCR analysis

Total RNA was isolated from grape tissues using a small-scale method based on the RNeasy plant mini-kit (Qiagen, GmbH, Hilden, Germany) but with a modified extraction buffer containing 0.2 M Tris–HCl pH 8.3, 5 M sodium perchlorate, 1% (w/v) SDS and 8.5% (w/v) insoluble PVPP (1 mL per 100 mg tissue). Genomic DNA contamination was removed using the RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. DNAse-treated total RNA (2 µg) was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) using the Oligo $(dT)_{20}$ primer according to the manufacturer's instructions. Before use in qRT-PCR experiments, cDNA reactions were diluted 10-fold to 200 µL with 10 mM Tris–HCl, pH 7.6.

Expression analysis was conducted by real-time PCR analysis using a SYBR Green method on a Rotor-Gene 3000 thermal cycler (Corbett Research, Mortlake, New South Wales, Australia). Each 15 µL PCR reaction contained 330 nM of each primer, 2 µL of diluted cDNA and 1× ABsolute QPCR SYBR Green ROX Mix (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions used were 95°C for 15 min followed by 40 cycles of: 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, followed by a melt cycle of 1°C increment per min from 65 to 96°C. All primer pairs (Table S1) amplified a single product of the expected size and sequence, which was confirmed by melt-curve analysis, agarose gel electrophoresis and DNA sequencing. The expression of each target gene was calculated relative to the expression of the housekeeping gene elongation factor 1 (*EF1*; GenBank Accession number AF176496) as described by Marchive *et al*. (2007) using Rotor-Gene 6.0 software (Corbett Research).

Microscopy

Detached leaf material was stained in trypan blue for an hour according to Koch and Slusarenko (1990). Fungal structures were visualised using a Zeiss (Göttingen, Germany) Axioscop 2 light microscope and photographed using a Lecia (Wetzlar, Germany) DFC 500 digital camera. Successful penetration was determined by the presence of a fungal haustorium.

Results

Identification of VvMLO gene family members

Screening of a *V. vinifera* genomic library with probes derived from *Arabidopsis* sequences *AtMLO1*, *AtMLO2*, *AtMLO6* and *AtMLO12*, resulted in the isolation of several grapevine *MLO* genomic clones. Sequencing of these clones confirmed close sequence relatedness with *MLO* genes from other plant species and the respective genomic clones were designated *VvMLO1*, *VvMLO3*, *VvMLO6*, *VvMLO7*, *VvMLO9*, *VvMLO13* and *VvMLO17* based on original phage clone number. Oligonucleotide primers were designed to the genomic sequences and used to successfully amplify full-length cDNAs from various *V. vinifera* cv. Chardonnay tissues. Subsequently, the draft genome sequence of the *V. vinifera* genotype PN40024 was published (Jaillon *et al*. 2007). Using the cloned Chardonnay *VvMLO* sequences, homology searches identified a further 10 putative full-length *MLO* gene sequences within the grape genome in addition to the seven *VvMLO* genes already cloned from Chardonnay. Comparison of the cloned Chardonnay *MLO* cDNA sequences with the predicted homologous cDNA sequences from the PN40024 genome sequence showed that five of them were 99–100% identical in both varieties. However, the predicted cDNA sequences for *VvMLO1* (GenBank Accession No. CAO41068) and *VvMLO13* (GenBank Accession No. CAO68971) from PN40024 were not found to match the cDNAs for the homologous genes cloned from Chardonnay with differences relating to exon/intron splicing sites (data not shown). These differences may relate to the different genetic backgrounds from which the sequences were derived. Alternatively, it may be that the predicted splicing sites for these two PN40024 *VvMLO* cDNAs are incorrect.

Phylogenetic analysis of the grape MLO family

Phylogenetic analysis was performed with the translated sequences of the seven *VvMLO* genes cloned from Chardonnay and the 10 other predicted *VvMLO* translation products from the PN40024 genome sequence together with a selection of known monocot and dicot MLO proteins. Six distinct clades were identified (Fig. 1). Previous analysis comparing MLO

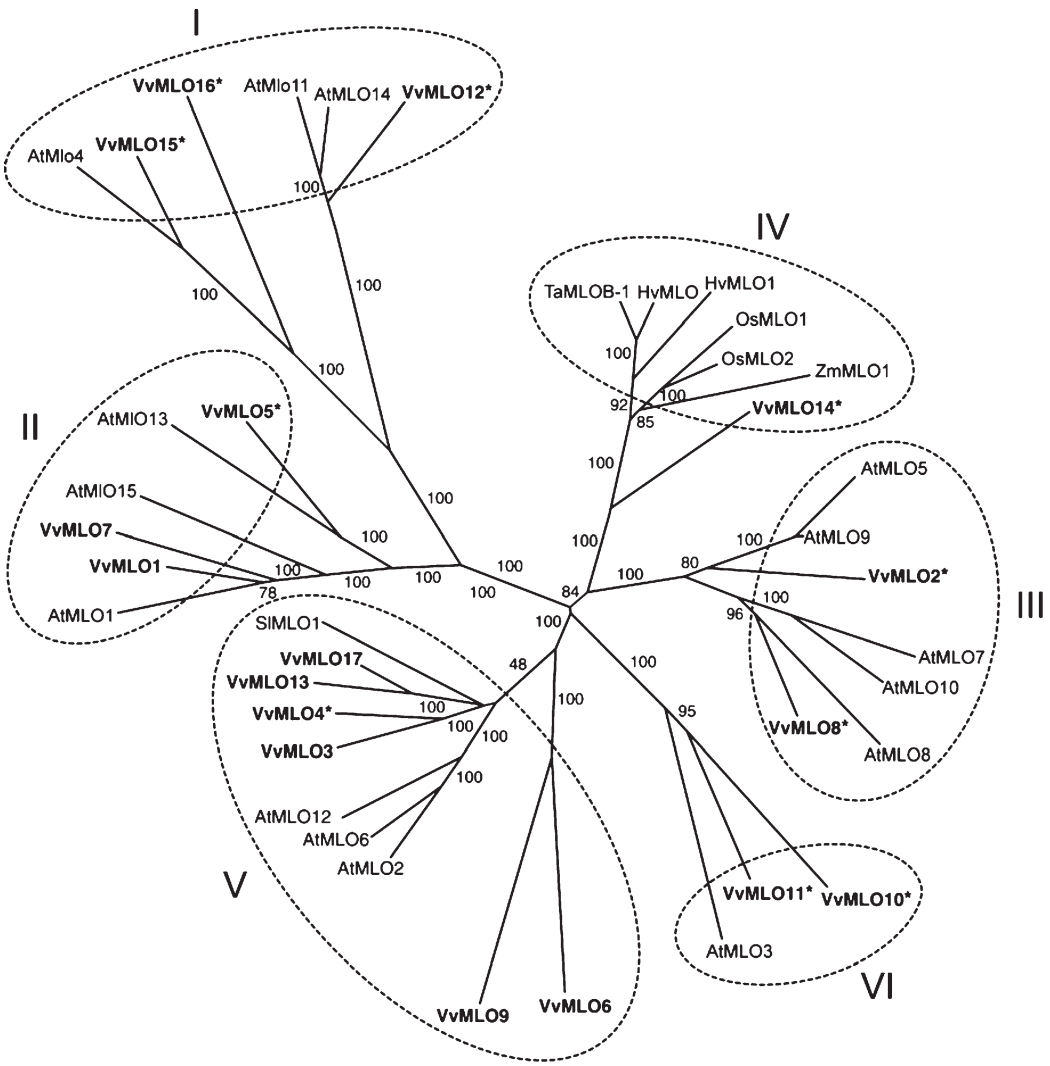


Fig. 1. Phylogenetic relationship of MLO isoforms. A neighbour-joining phylogenetic tree of the amino acid sequences of MLO family members of grape (*Vitis vinifera* cv. Chardonnay and genotype PN40024), *Arabidopsis thaliana* and selected proteins from barley (*Hordeum vulgare*), rice (*Oryza sativa*), maize (*Zea mays*) and tomato (*Solanum lycopersicum*) is shown. The phylogenetic tree represents a consensus tree with branch lengths proportional to sequence distance. Numbers indicate bootstrap values (from 100 replicates) that support the respective branch. VvMLO sequences identified from genome sequencing of genotype PN40024 (Jaillon *et al*. 2007) are indicated with an asterisk (*). Genbank numbers of translated MLO proteins used in phylogenetic analysis: AtMLO1 (Z95352); AtMLO2–15 (AF369563-AF369576); HvMLO (Z83834); HvMLO2 (Z95496); OsMLO1 (Z95353); OsMLO2 (AF384030); SlMLO1 (AAX77013); TaMLOB-1 (AF361932); ZmMLO1 (NM_001112190).

sequences from *Arabidopsis* with those from the monocots barley, rice, wheat and maize suggested the proteins grouped into only five clades since AtMLO3 was not considered as a separate clade, but as a single divergent lineage (Devoto *et al*. 2003). However, the clustering of AtMLO3 with VvMLO10 and VvMLO11 now suggests the existence of an additional clade (VI) to those described by Devoto *et al*. (2004). In contrast to the *Arabidopsis* MLO family, phylogenetic analysis indicated that grape MLO family members are distributed among all clades.

Clades IV and V contain the dicot and monocot MLOs previously described to be involved in powdery mildew susceptibility (Jørgensen 1992; Elliott *et al*. 2002; Kim *et al*. 2002*b*; Consonni *et al*. 2006). The grouping of VvMLO14 within clade IV is somewhat unusual considering that this clade was previously thought to comprise only monocot homologues (Devoto *et al*. 2003). The monocot HvMLO from barley, which provides powdery mildew susceptibility, is orthologous to the wheat (TaMLOB-1) and rice (OsMLO2) MLO proteins. Both complement powdery mildew resistance in barley *mlo* mutants driven by the constitutive maize ubiquitin promoter (Elliott *et al*. 2002). The largest grouping of grapevine MLO proteins including VvMLO3, VvMLO4, VvMLO6, VvMLO9, VvMLO13 and VvMLO17 fall into clade V. This clade is significant in terms of putative function because this group also contains the *Arabidopsis* AtMLO2, AtMLO6, AtMLO12 and tomato (*S. lycospersicum*) SlMLO1 proteins that are required

for powdery mildew susceptibility (Consonni *et al*. 2006; Bai *et al*. 2008).

Figure 2 illustrates a closer examination of the sequence conservation between the different members in clade V. All of the proteins show a high degree of conservation at the position of the predicted transmembrane (TM) domains (Devoto *et al*. 2003), which are hallmarks of this protein family. Proximal to TM domain 7 the sequence is less conserved apart from a stretch of ~10–15 amino acids with the potential to form an amphiphilic α-helix which has previously been shown to function as a calmodulin-binding domain (Kim *et al*. 2002*a*, 2002*b*; Bhat *et al*. 2005). Panstruga (2005*a*) also highlighted the presence of two other regions of sequence conservation within the *C*-terminus of several MLO orthologues with a proven or assumed roles in modulating powdery mildew infection. Peptide domain I is characterised by the presence of conserved

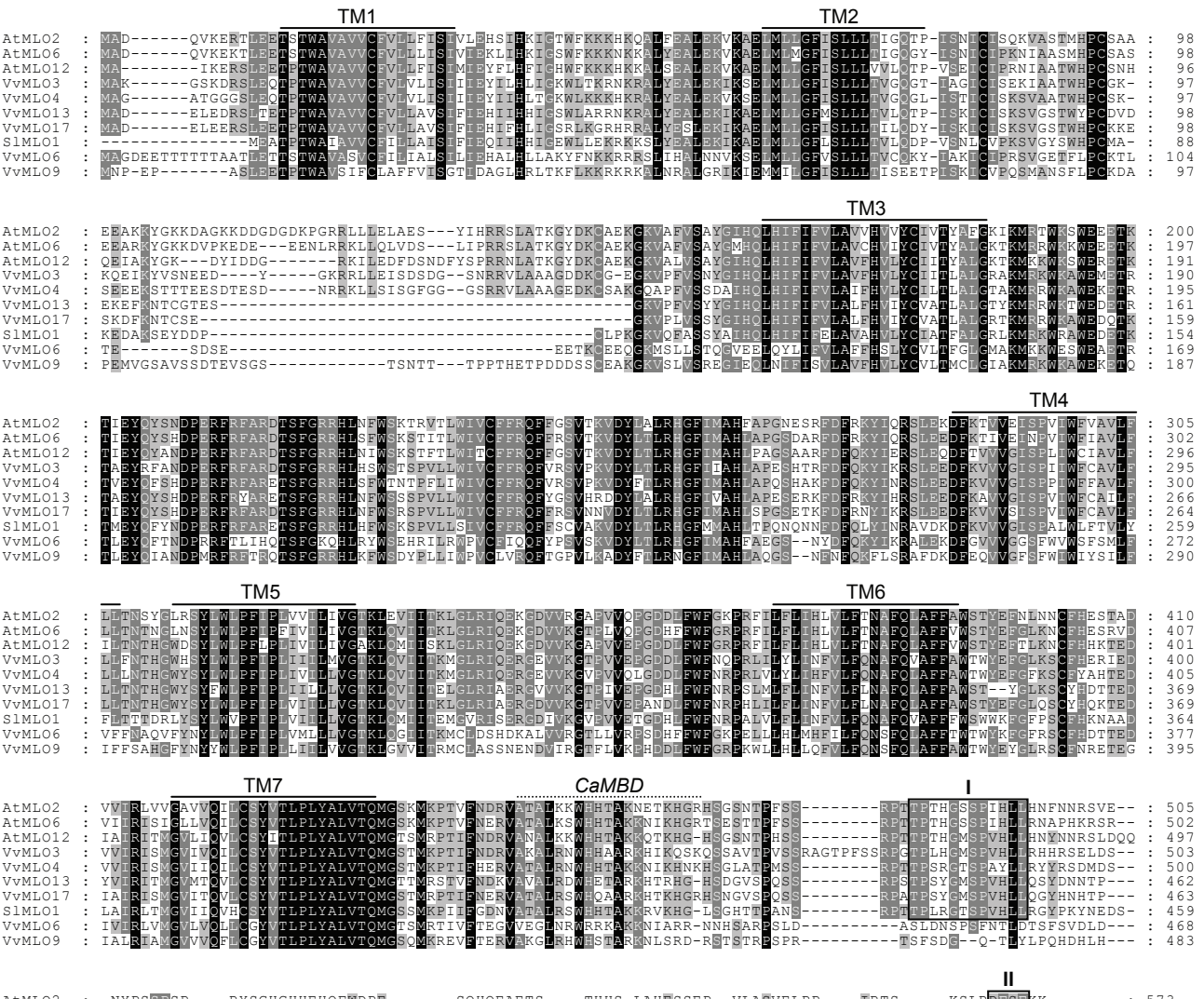


Fig. 2. Sequence alignment of VvMLO proteins in clade V (Fig. 1) with selected MLO proteins which have previously been shown to be involved in powdery mildew susceptibility in *Arabidopsis* (AtMLO2, AtMLO6 and AtMLO12; Consonni *et al*. 2006) and tomato (SlMLO1; Bai *et al*. 2008). The alignment was generated by CLUSTALW using default parameters. The positions of the seven transmembrane regions (TM1–7) inferred from the experimentally determined topology of HvMLO (Devoto *et al*. 1999) and the approximate position of the calmodulin-binding domain (CaMDB; Kim *et al*. 2002*b*) are indicated by lines above the sequences. Two conserved domains identified by Panstruga (2005*a*) within the highly polymorphic *C*-termini are highlighted in numbered boxes.

serine and threonine residues whereas peptide domain II contains the consensus motif (D/E-F-S/T-F) (Fig. 2). All of the MLO proteins within clade V, with the exception of VvMLO6 and VvMLO9 (which are phylogenetically more distantly related to the others; Fig. 1), contain these two conserved domains within the *C* terminus. This may indicate potential functional conservation between the MLO proteins that contain these two peptide domains.

Expression analysis of VvMLO*s in grapevine tissues*

To further investigate the possible biological roles of the different *VvMLO* genes, expression analysis was conducted using different grapevine organs at different stages of development. The expression patterns of all of the *VvMLO* genes grouped in clades IV and V were investigated, together with at least one *VvMLO* gene from each additional clade.

The *VvMLO* genes were expressed in all of the grape tissue types examined, but varied widely in the level of expression (Fig. 3). None of the *VvMLO* genes examined seemed to be tissue-specific and there were no clade-specific expression patterns. For example, of the genes in clade V, *VvMLO3* and *VvMLO4* were expressed mainly in roots, *VvMLO6* in flowers and berries, *VvMLO9* in roots and old leaves and *VvMLO17* predominately in older leaves (Fig. 3). Several *VvMLO* genes such as *VvMLO1* and *VvMLO8* were expressed at relatively high levels in all tissues and stages of development. However, several of the *VvMLO* genes showed evidence of developmental regulation. For example, all of the *VvMLO*s analysed showed higher transcript levels in older leaves than in younger leaves with the exception of *VvMLO13* (Fig. 3). Furthermore, *VvMLO1*, *VvMLO4*, *VvMLO7*, *VvMLO10* and *VvMLO17* were expressed at much higher levels in immature (preverasion) berries than in ripening (postverasion) berries. Conversely, the transcript levels of *VvMLO6* and *VvMLO8* were higher in postverasion berries (Fig. 3).

Accumulation of VvMLO *transcripts in response to* E. necator *infection*

Transcript levels of *HvMLO* were previously found to increase rapidly within 6 h following inoculation with barley powdery mildew, which coincided with the period just before attempted fungal penetration (Piffanelli *et al*. 2002). To establish the time course of *E. necator* penetration on grapevine leaves, infected leaves were microscopically examined 4, 8, 12, 24, 48 and 72 h post inoculation (Fig. 4*A*). Upon contact with an epidermal cell, *E. necator* conidiopspores germinate and subsequently develop a lobed appresorium (Fig. 4*A*). A penetration peg then forms beneath the appresorium in an attempt to penetrate the underlying epidermal cell. Following successful penetration, the fungus forms a haustorial feeding structure. Figure 4*A* shows the levels of epidermal host cell penetration by individual germinated *E. necator* conidia, scored via the presence of a haustorium, on grapevine leaves. A small percentage (~5%) of germinated conidia penetrated grape epidermal cells within 8 h of *E. necator* inoculation. This increased to 10% penetration at 12 h and 55% at 24 h post-inoculation, at which point secondary fungal hyphae was observed.

The molecular processes required for the establishment of successful fungal penetration take place before haustorium formation and it has been proposed that MLO may be exploited as a docking molecule by the fungus during this time (Panstruga 2005*b*). We, therefore, examined the response of *VvMLO* genes to *E. necator* infection at 4, 8 and 20 h post-inoculation by real time RT-PCR. A significant increase in the transcript levels of *VvMLO17* (~40 fold), *VvMLO3* (~12 fold) and *VvMLO4* (~15 fold) was observed in grape leaves within 8 h of *E. necator* inoculation (Fig. 4*B*). Transcript abundance of *VvMLO4* and *VvMLO17* then decreased and that of *VvMLO3* remained relatively constant during the period 8–20 h post-inoculation (Fig. 4*B*). *VvMLO9* was also found to exhibit increased transcript levels following *E. necator* inoculation, but the timing was delayed relative to the other powdery mildew-induced *VvMLO* genes (Fig. 4*B*). None of the other eight *VvMLO* genes examined were induced greater than 2-fold in response to powdery mildew inoculation (Fig. 4*B*).

VvMLO*s are induced by wounding and defence-signalling compounds*

Barley *HvMLO* and rice *OsMLO* have previously been shown to be induced not only by biotic factors, but also upon abiotic stress stimuli (Kim *et al*. 2002*a*; Piffanelli *et al*. 2002). To further characterise the *VvMLO* genes, transcript levels in *V. vinifera* leaves were investigated following wounding or treatment with the defence-signalling compounds H_2O_2 and SA. As observed with powdery mildew infection (Fig. 4*B*), only *VvMLO* genes within clade V (i.e. *VvMLO3*, *VvMLO4*, *VvMLO6* and *VvMLO17*) showed increased transcript abundance in response to wounding treatment (Fig. 5*A*). *VvMLO6* transcript levels peaked most rapidly 2 h post wounding treatment, whereas *VvMLO4* displayed the highest fold increase (~60 fold) and this was observed 6 h post wounding.

In barley, H_2O_2 is rapidly produced at the site of powdery mildew attack (Hückelhoven *et al*. 1999) and ultrastructural studies have shown the presence of H_2O_2 in small papillae beneath the fungal penetration peg (An *et al*. 2006). Mildew resistant barley *mlo* mutants exhibit a higher incidence of papillae containing H_2O_2 than susceptible wild-type barley (Hückelhoven *et al*. 1999; Piffanelli *et al*. 2002). Several *VvMLO* genes displayed small increases in transcript accumulation 6 h post H_2O_2 treatment (2–5-fold) but these were minor in comparison to the changes observed for *VvMLO8* and *VvMLO10*, which both showed over 50-fold higher transcript levels upon H_2O_2 treatment (Fig. 5*B*). We note that the kinetics of induction of these two *VvMLO* genes following H_2O_2 treatment was quite different with *VvMLO8* the transcript levels of showed a rapid increase within 6 h of treatment, whereas *VvMLO10* transcript levels were highest 24 h after treatment (Fig. 5*B*).

In *Arabidopsis*, SA plays a central role in plant defence against biotrophic pathogens (Thomma *et al*. 2001) and induces the expression of a range of pathogen defence genes in *V. vinifera* (Chong *et al*. 2008). Of the *VvMLO* genes tested *VvMLO4*, *VvMLO6* and *VvMLO10* displayed the most marked response to SA treatment (Fig. 5*C*). *VvMLO4* transcript abundance increased ~4 fold within 6 h of SA treatment and then decreased after 24 h, whereas the rise in transcript levels of *VvMLO6* and *VvMLO10* was slower but with greater fold changes 24 h after SA treatment.

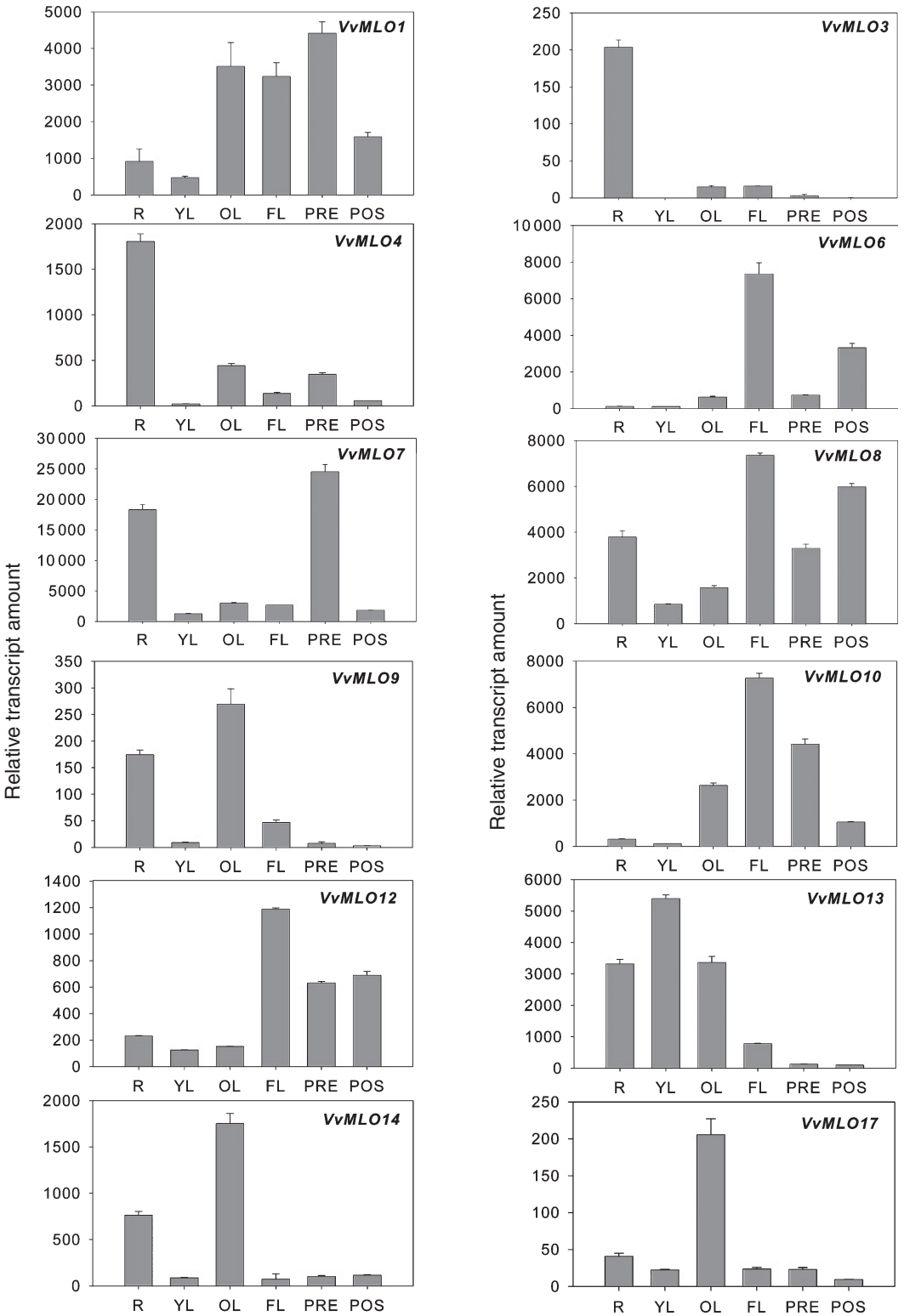


Fig. 3. Quantitative RT-PCR analysis of *VvMLO* gene expression in different grapevine tissues. Transcript levels of *VvMLO* genes were normalised against the transcript levels of *EF1* in each cDNA sample. Relative normalised transcript levels are shown for roots (R), young leaves (YL), old leaves (OL), flowers (FL), preverasion berries (PRE) and postverasion berries (POS). Means are totals calculated from triplicate technical qRT-PCR measurements within two biological replicates. Error bars show standard errors.

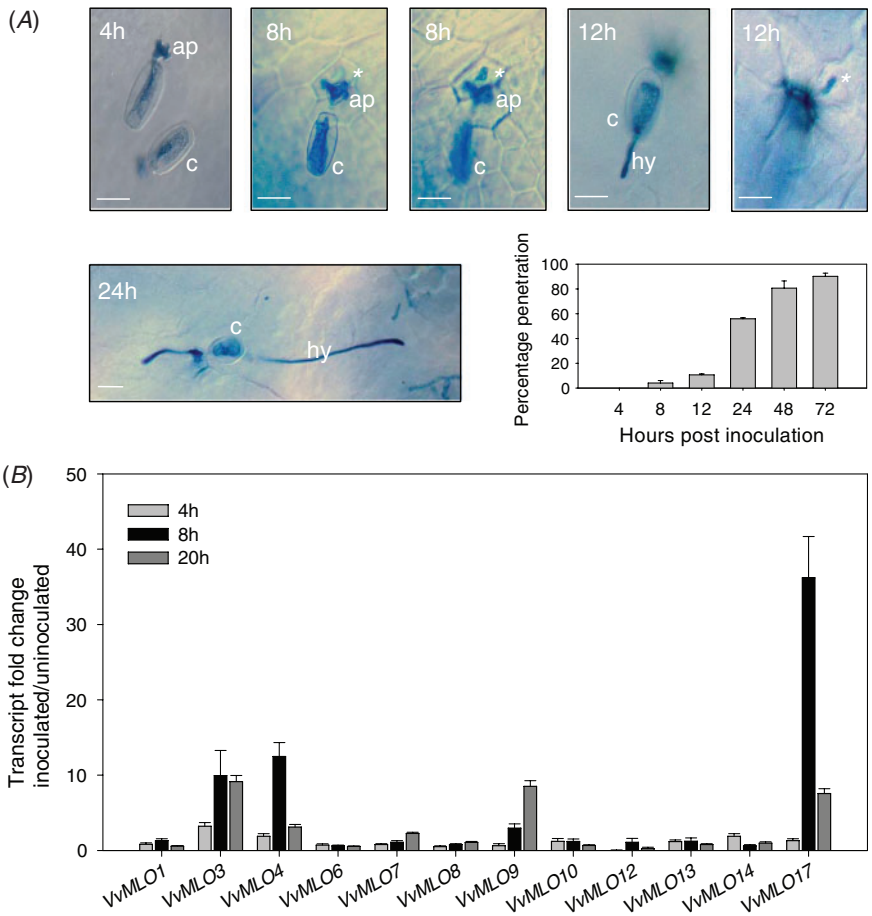


Fig. 4. Transcriptional response of grapevine *VvMLO* genes to powdery mildew infection. (*A*) Interaction phenotypes between grapevine and *Erisyphe necator* visualised with trypan blue at the indicated timepoints. Two panels are shown at 8 and 12 h: the first panel is focussed at the epidermal cell surface and the second on the haustorium within the epidermal cell. Scale bars = 20 µm. Symbols: *, haustorium; ap, appresorium; c, conidium; hy, hypha. Time-course of powdery mildew (*E. necator*) infection on grapevine. Detached leaves were harvested at 4, 8, 12, 24, 48 and 72 h post-inoculation and stained with trypan blue. The incidence of epidermal host cell penetration by individual germinated conidia is shown, scored via the presence of a haustorium. Each data point represents the scoring of a minimum of 100 germinated conidia with two replicates. The mean is shown and error bars are standard errors. (*B*) Quantitative RT-PCR analysis of *VvMLO* gene expression in grape leaves response to powdery mildew infection. Detached leaves were heavily inoculated with *E. necator* spores and sampled at 4, 8 and 20 h post-inoculation. Control (un-inoculated) detached leaves were incubated and sampled as for the inoculated leaves. Transcript levels of individual *VvMLO* genes were normalised against the transcript levels of *EF1* in each cDNA sample. Results are plotted as a mean ratio of the normalised transcript level of each *VvMLO* gene in inoculated leaves *v.* the normalised transcript level of each *VvMLO* gene in control leaves. Means are totals calculated from triplicate technical qRT-PCR measurements within three biological replicates and the data shown is representative of the results obtained in the three independent experiments. Error bars show standard errors.

Discussion

Analysis of the seven *MLO*-like cDNA sequences isolated from *V. vinifera* cv. Chardonnay, together with those identified from the genome sequence of the *V. vinifera* genotype PN40024 (Jaillon *et al*. 2007), has allowed the identification of a *MLO* gene family in grapevine comprising seventeen *VvMLO* members in total. This compares with a family of 15 *MLO* genes found in *Arabidopsis* (Devoto *et al*. 2003) and 12 in the monocotyledonous species *Oryza sativa* (Liu and Zhu 2008). Phylogenetic analysis of these dicot and monocot MLO proteins revealed that the proteins cluster into six clades, two of which (IV and V) contain members that have been implicated in powdery mildew susceptibility from monocots and dicots, respectively.

Each member of the *VvMLO* gene family was found to have a unique expression pattern within the different tissue types, including evidence of developmental regulation in grape leaves and berries (Fig. 3). The *VvMLO* genes also varied markedly in terms of their response to biotic and abiotic

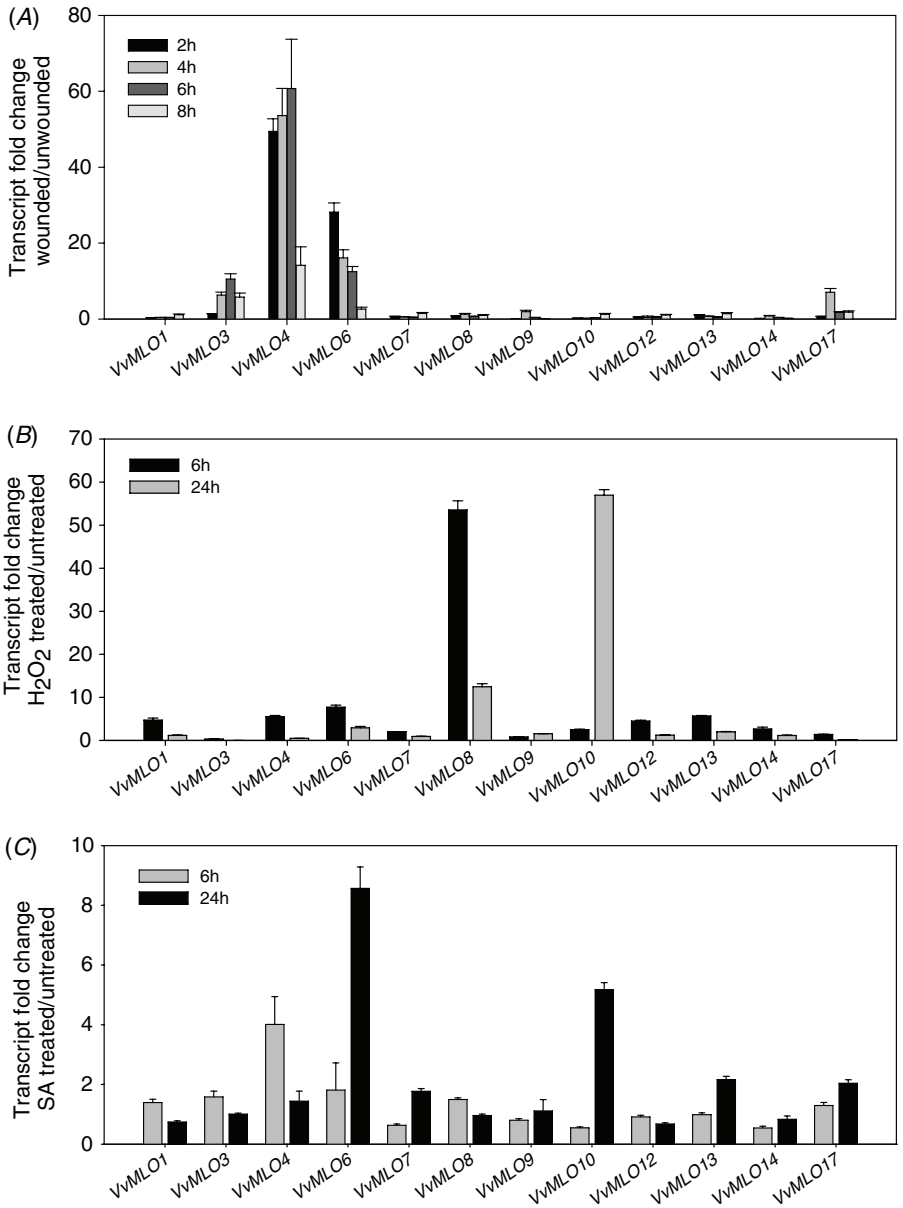


Fig. 5. Quantitative RT-PCR analysis of *VvMLO* expression in grapevine leaves in response to wounding and exogenous application of defence-signalling compounds. (*A*) Attached grapevine leaves were wounded by rubbing the adaxial surfaces with sandpaper and leaves collected at 2, 4, 8 and 24 h post-wounding. Control (unwounded) leaves were collected at the same time points. The petioles of detached leaves were placed into either (*B*) 5 mM H_2O_2, and (*C*) 1 mM SA and removed after 6 and 24 h. Detached control leaves were incubated in water and sampled at the same time points. Transcript levels of individual *VvMLO* genes were normalised against the transcript levels of *EF1* in each cDNA sample. Results are plotted as the mean ratio of the normalised transcript level of each *VvMLO* gene in treated leaves *v.* the normalised transcript level of each *VvMLO* gene in control leaves. Means are totals calculated from triplicate technical qRT-PCR measurements within two biological replicates. Error bars show standard errors.

stimuli (Figs 4, 5). These results are in agreement with observations regarding the diverse transcriptional regulation of the *Arabidopsis MLO* gene family, leading to the suggestion that this unique gene family functions in a diverse array of developmental and response processes in plants (Chen *et al*. 2006). Preliminary reports that MLOs are involved in pollen germination and root development in *Arabidopsis* also support this view (Panstruga 2005*b*; Chen *et al*. 2006).

Although no two members of the *VvMLO* gene family analysed showed the same expression pattern, there was some evidence of overlapping patterns in terms of tissue-specificity and response to certain biotic and abiotic stresses. For example,

phylogenetic analysis grouped *VvMLO1* and *VvMLO7* in clade I (Fig. 2) and both show relatively high expression levels in preverasion berries and developmental regulation during berry ripening. Furthermore, neither gene appeared to be responsive to any of the biotic and abiotic stresses treatments applied to grape leaves. Similarly, a subset of the *VvMLO* genes that cluster within clade V (i.e. *VvMLO3*, *VvMLO4* and *VvMLO17*) show overlapping responses both to powdery mildew infection and wounding (Figs 4, 5). The response of *VvMLO3*, *VvMLO4*, *VvMLO9* and *VvMLO17* to powdery mildew infection is particularly significant in light of the fact that they share clade V with the *Arabidopsis AtMLO2*, *AtMLO6* and *AtMLO12* and the tomato *SlMLO1* genes (Fig. 2), which have been demonstrated to be required for powdery mildew susceptibility (Consonni *et al*. 2006; Bai *et al*. 2008) and are induced by powdery mildew inoculation (Chen *et al*. 2006; Bai *et al*. 2008). This strongly suggests a common biological function for these proteins in modulating antifungal defence responses.

Further, VvMLO14 was found to cluster with monocot MLO proteins in clade IV, including HvMLO, TaMLOB-1 and OsMLO2, which have been demonstrated to have complementary functions in terms of powdery mildew susceptibility (Elliott *et al*. 2002). However, *VvMLO14* was not found to be responsive to *E. necator* inoculation, suggesting that this gene is unlikely to have a role in powdery mildew pathogenicity. The placement of *VvMLO14*, a dicot MLO, into a clade containing exclusively monocot MLO proteins, raises a question as to the accuracy of the published sequence for the predicted translation product of *VvMLO14* (GenBank Accession No: CAO66265). We note that two other predicted protein sequences (*VvMLO1* and *VvMLO13*) from the *V. vinifera* PN40024 genome showed significant divergence in protein sequence from the translated product of our cloned cDNA sequences isolated from *V. vinifera* cv. Chardonnay. Although it is possible these differences reflect differential gene splicing between the two grape cultivars it is more likely that the software tools used for cDNA prediction from genomic sequence are not always accurate and highlights the need to verify gene coding sequences from cDNA before assigning putative protein function.

The results from several studies indicate a degree of functional redundancy and co-function between phylogenetically closely related members of the *Arabidopsis* MLO family (Chen *et al*. 2006). For example, it has been observed that the mutants *Atmlo11* and *Atmlo14* show similar defects in root development (Chen *et al*. 2006), and *AtMLO9* and *AtMLO5*, which are both expressed highly in pollen, may function redundantly in pollen germination (Panstruga 2005*b*). Similarly, there is a documented degree of functional redundancy between *AtMLO2*, *AtMLO6* and *AtMLO12* as all three genes are required for full susceptibility to powdery mildew (Consonni *et al*. 2006). Moreover, *AtMLO2* and *AtMLO6* are believed to be the result of a large scale duplication event in the *Arabidopsis* genome, which may explain their functional redundancy (Devoto *et al*. 2003). The rapid induction of *VvMLO3*, *VvMLO4* and *VvMLO17* in response to *E. necator* suggests there is a degree of functional redundancy between the proteins encoded by these genes in terms of susceptibility to powdery mildew.

Some *MLO* genes associated with powdery mildew susceptibility in barley and *Arabidopsis* are also induced by wounding (Piffanelli *et al*. 2002; Chen *et al*. 2006). Wounding of grape leaves resulted in increased transcript levels of three of the powdery mildew-induced *VvMLO* genes (*VvMLO3*, *VvMLO4* and *VvMLO17*) together with *VvMLO6*, which is also clustered in clade V. We note that, whereas *VvMLO17* was the most strongly induced of the trio of genes in powdery mildew inoculated grape leaves (Fig. 3), it was the least responsive to wounding treatment (Fig. 4*A*). This shows some parallels to the relative responses of the functionally related *AtMLO2*, *AtMLO6*, *AtMLO12* group, as *AtMLO6* and *AtMLO12* appear to respond more significantly to wounding than *AtMLO2* (Chen *et al*. 2006), but *AtMLO2* is more important in terms of powdery mildew susceptibility (Consonni *et al*. 2006).

In *Arabidopsis*, defence against biotrophic pathogens is characterised by SA-induced responses (Thomma *et al*. 2001). Recent evidence suggests that SA could be also be important for disease resistance in *V. vinifera* since exogenous application of SA induces the expression of pathogen defence and signalling genes (Chong *et al*. 2008). SA also rapidly induces the expression of *OsMLO* (Kim *et al*. 2002*a*). However, unlike *OsMLO2*, this rice homologue was not able to functionally complement the barley *mlo* mutant and restore powdery susceptibility (Elliott *et al*. 2002). From the group of *VvMLO*s which were responsive to *E. necator*, only *VvMLO4* showed a 4-fold upregulation in response to SA. Therefore, although these defence signalling compounds are produced during powdery mildew infection (Hückelhoven *et al*. 1999; Vogel and Somerville 2000), it is unlikely that these are the signals which lead to the induction of the *E. necator* responsive genes *VvMLO3*, *VvMLO4*, *VvMLO9* and *VvMLO17*. Furthermore, the strong increase in transcript accumulation of *VvMLO17* upon inoculation with *E. necator*, and the much smaller rise in transcript levels following wounding, also suggests that tissue damage during penetration of the host cell wall by powdery mildew is not the main stimulus for this induction.

MLOs are calmodulin-binding proteins and calmodulin binding promotes the susceptibility of barley to powdery mildew (Kim *et al*. 2002*b*). Moreover, pharmacological studies suggest that the influx of Ca^{2+} ions is important for this MLO function (Kim *et al*. 2002*a*). Therefore Ca^{2+} may be a candidate signal for the induction of the *E. necator* responsive genes *VvMLO3*, *VvMLO4*, *VvMLO9* and *VvMLO17*, since plant cells generate a transient Ca^{2+} signal in response to pathogen attack (Jabs *et al*. 1997; Xu and Heath 1998).

Powdery mildew is the major fungal disease of grapevine cultivation worldwide (Donald *et al*. 2002). Barley *mlo* mutants have been successfully employed to control powdery mildew in the field for more than 25 years (Jørgensen 1992; Lyngkjaer *et al*. 2000). Our study has identified three *VvMLO* genes (*VvMLO3*, *VvMLO4* and *VvMLO17*) that show similarities to other *MLO* genes linked to powdery mildew susceptibility, both in terms of sequence homology of the translation products, and the transcriptional response to powdery mildew infection. Further functional studies will allow us to test the role of these VvMLO proteins in the susceptibility of grapevine to *E. necator*. The application of this knowledge will enable us to generate powdery mildew-resistant grapevines.

Note added in proof

During the processing of this manuscript, we became aware of a publication (Winterhagen *et al*. 2008) describing the up-regulation of specific *VvMLO* gene family members, in response to powdery mildew infection, consistent with the results presented in this paper.

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Article Addendum

Grapevine MLO candidates required for powdery mildew pathogenicity?

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Key words: MLO, powdery mildew, resistance, susceptibility, grapevine

MLOs belong to the largest family of seven-transmembrane (7TM) domain proteins found in plants. The Arabidopsis and rice genomes contain 15 and 12 MLO family members, respectively. Although the biological function of most MLO family members remains elusive, a select group of MLO proteins have been demonstrated to negatively regulate defence responses to the obligate biotrophic pathogen, powdery mildew, thereby acting as "susceptibility" genes. Recently we identified a family of 17 putative *VvMLO* genes in the genome of the cultivated winegrape species, *Vitis vinifera.* Expression analysis indicated that the *VvMLO* family members respond differently to biotic and abiotic stimuli. Infection of *V. vinifera* by grape powdery mildew (*Erysiphe necator*) specifically upregulates four *VvMLO* genes that are orthologous to the Arabidopsis and tomato MLOs previously demonstrated to be required for powdery mildew susceptibility. We postulate that one or more of these *E. necator* responsive *VvMLO*s may have a role in the powdery mildew susceptibility of grapevine.

The MLO family is only found in plants and its members are characterised by the presence of 7TM and C-terminal calmodulin-binding domains.[1-3] MLO proteins share the same topology and localisation within the plasma membrane as animal 7TM proteins.[2] Animal 7TM proteins commonly function as G-protein coupled receptors (GPCRs) that sense external stimuli and trigger downstream signalling through heterotrimeric G-proteins.[4] However, some 7TM proteins have been found to transmit signals independently of heterotrimeric G-proteins and plant MLOs appear to fall into that group.[3,5]

Despite the identification of large MLO families in different plant species, the function of MLO proteins in plants cells are still unknown. However, there is now a large body of evidence suggesting that certain members of the MLO family pre-dispose plants to powdery mildew infection and therefore act as "susceptibility" factors. For example, mutations in specific members of the MLO gene family from barley, tomato and Arabidopsis (either through natural genetic variation or targeted gene silencing) have been found to result in broad spectrum resistance to compatible powdery mildew isolates.[6-9] This function as a susceptibility factor is unlikely to be the primary role of MLOs, since there must be a selection pressure for retaining functional MLO genes in the plant genome. It has therefore been suggested that powdery mildew exploits MLOs to gain entry to their plant host.[10,11] This is similar to the exploitation of 7TM GPCRs in animals, by pathogens such as *Streptococcus pneumoniae*, to gain access to host cells.[12]

MLO proteins are localised in the plasma membrane, but during powdery mildew attack become polarised at the site of attempted powdery mildew ingress.[13] It is postulated that MLO negatively regulates two independent penetration resistance pathways.[14] The first involves the syntaxin PEN1/ROR2 which is thought to mediate vesicle trafficking.[15] Yeast two hybrid studies have provided evidence of a direct physical interaction between MLO and ROR2.[11] The second penetration resistance pathway involves a glycosyl hydrolase (PEN2) and an ABC transporter (PEN3) which may produce and secrete fungal toxins respectively.[16,17]

The cultivated grapevine, *V. vinifera*, is highly susceptible to the powdery mildew species *E. necator.* As a result, grape production worldwide is highly dependent on the use of fungicides to control this pathogen. The development of new grapevine cultivars with enhanced genetic resistance to powdery mildew would be of significant economic and environmental benefit. We therefore set out to identify and characterise the MLO gene family in grapevine with the aim of isolating potential candidates involved in powdery mildew susceptibility. Using the recently sequenced *V. vinifera* genome sequence,[18] we identified a family of 17 putative *VvMLO* genes.[19] Phylogenetic analysis of the translated products of the *VvMLO* gene family together with other known plant MLO sequences identified six *VvMLO* genes (*VvMLO3*, *VvMLO4*, *VvMLO6*, *VvMLO9*, *VvMLO13* and *VvMLO17*) which clustered within the same clade as the Arabidopsis and tomato *MLO* genes that have been demonstrated to be required for powdery mildew susceptibility.[6,7,19] Of these, three (*VvMLO3, VvMLO4* and *VvMLO17)* were found to be significantly induced in grape leaves within 8 h of *E. necator* inoculation, which coincided with

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the commencement of fungal penetration.[19] The transient transcriptional response of *VvMLO3*, *VvMLO4* and *VvMLO17* to *E. necator* penetration is consistent with that previously reported for *HvMLO* in response to barley powdery mildew (*Blumeria graminis*) infection.[20] *VvMLO9* was also induced, but not until 24 h post inoculation suggesting that this MLO may not be involved in the first stages of pathogen infection.[19] We have also subsequently shown these powdery mildew-induced VvMLO proteins are localised to the plasma membrane (Fig. 1). Thus, the similarities in both sequence homology, transcriptional regulation and cellular localization suggests that *VvMLO3*, *VvMLO4* and *VvMLO17* may have functionally similar roles to those *MLO* genes from barley, Arabidopsis and tomato which have been shown to modulate powdery mildew susceptibility.[6-9] It may also indicate some level of functional redundancy between these three *VvMLO* genes as is the case in Arabidopsis.[7]

It is not yet known which signalling molecules are responsible for the rapid induction of *VvMLOs* following powdery mildew infection. Despite the involvement of the signalling compounds H_2O_2 and salicylic acid in plant defence responses,[21,22] these compounds were found to induce a different subset of *VvMLO*s to those induced by *E. necator*.[19] On the other hand, wounding was found to induce the same subset of *VvMLO* transcripts (as well as *VvMLO6*), to those rapidly induced by *E. necator* infection, although the relative levels of induction differed.[19] Gene-silencing studies are now underway to establish which of the *E. necator* induced *VvMLO*s are required for powdery mildew susceptibility in grapevine. It will also be important to determine if silencing of *MLO* gene(s) results in any pleiotropic effects on grapevine growth and development, as has been observed in barley and Arabidopsis *mlo* mutants.[7,20,23] These include the spontaneous deposition of callose-containing cell wall appositions in pathogen-free conditions and the premature onset of leaf senescence. However, these were absent in powdery-mildew resistant tomato *mlo* mutants.[6] The appearance of similar pleiotropic effects on grapevine leaves is unlikely to cause significant issues for grape-growers because photosynthetic capacity is not normally a limiting factor to grape production. Indeed, it is likely that most growers would tolerate some loss in maximum cropping capacity in exchange for durable resistance to powdery mildew, provided there are no significant effects on grape berry quality.

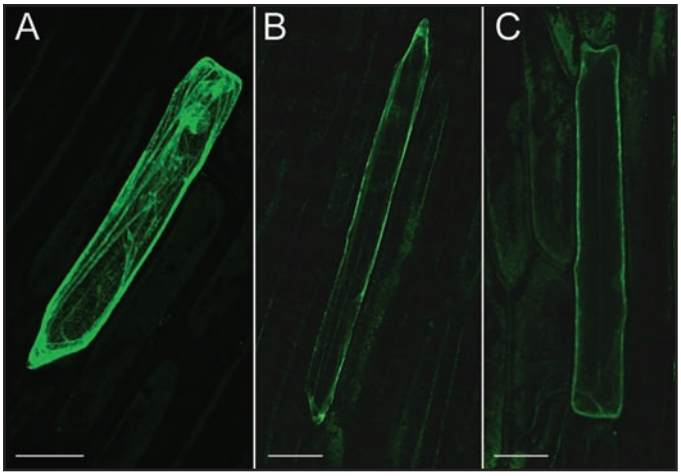


Figure 1. Plasma membrane localisation of VvMLO. Onion epidermal cells expressing (A) ER-targeted mGFP (pCAMBIA1301 with mGFP5-ER introduced from pBIN mGFP5-ER) (B) VvMLO4-GFP (C-terminal GFP fusion of VvMLO4 in pN'GFP) (C) VvMLO17-GFP (C-terminal GFP fusion of VvMLO17 in pGWB5). Scale bar 100 μM.

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Do cultivated grapevines contain genes that predispose them to powdery mildew infection?

A potential new strategy to develop powdery mildew resistant grapevines



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Grapevine powdery mildew, caused by the fungus *Erysiphe necator (syn. Uncinula necator),* was first introduced into French vineyards from North America in the 1840s before spreading to all major grape producing regions around the world. Powdery mildew infects all green grapevine tissues including leaves, stems and berries, and severe infection can debilitate vines, reduce net photosynthesis, retard ripening and degrade wine quality (Gadoury *et al.* 2001; Callonec *et al.* 2004; Summer *et al.* 2005). Even minor berry infections have been shown to be associated with elevated populations of spoilage micro-organisms, increased insect infestation and increased botrytis bunch rot at harvest (Gadoury *et al.* 2007).

The cultivated grapevine species, *Vitis vinifera*, which is native to the Eurasian region, has little or no genetic resistance to this fungal pathogen. As a result, modern grapevine cultivation relies heavily on the use of chemical fungicides such as sulphur and sterol biosynthesis inhibitors. Most grapegrowers apply between six to 10 fungicide sprays per season for powdery mildew control. In France alone, the cost of fungicides for powdery mildew control is estimated to be about €75 million per year ($A127.4m, $NZ154.3m) (A. Bouquet, personal communication), and this does not take into account the increasing fuel costs associated with the application of these fungicides.

Furthermore, fungal strains have been reported to have evolved resistance to a number of the commonly used fungicides (Savocchia *et al.* 2004). There is also now increasing pressure by legislators to restrict the use of certain agrichemicals because they consider them detrimental to the environment. The European Commission is considering a proposal to ban the use of a large number of agrichemicals by 2013 which are routinely used for the control of a number of fungal diseases in grapes including powdery mildew (Pesticides Safety Directorate 2008).

The introduction of effective genetic resistance into winegrape cultivars to reduce the dependence on chemical inputs therefore would be of significant economic and environmental benefit. We are currently utilising a number of different molecular strategies in our laboratory to try and generate new grapevine cultivars with increased natural resistance to powdery mildew.

New approaches to powdery mildew resistance

Much of the improvement in the yield and quality of agronomic crops during the past 100 years has been a result of the introduction of pathogen resistance (R) genes from wild species through classical breeding. These R genes generally act as early warning devices by detecting molecules released by the invading pathogen during colonisation, and then switching on host defence pathways.

Typically the R genes are found in wild plant species which have co-evolved with the pathogen in question. A good example of this is the existence of R genes that confer resistance to both powdery mildew and downy mildew in the wild North American grapevine *Muscadinia rotundifolia* because this grapevine species co-evolved with these pathogens in North America.

In contrast, the cultivated grapevine *V. vinifera*, which evolved in the absence of these pathogens, lacks similar R genes. A major focus of our laboratory during the past 10 years has been ongoing research to try and identify the powdery mildew and downy mildew R genes in *M. Rotundifolia*, and transfer them into the cultivated grapevine by genetic transformation or marker-assisted selection (Dry *et al.*, 2009).

Recently, however, the work of a number of different research groups throughout the world, working on a range of different plant species which are colonised by powdery mildew, has uncovered unexpected information which could provide an entirely new approach to the generation of powdery mildew resistant germplasm.

Plant pathologists now understand that for a plant species like *V. vinifera*, susceptibility to powdery mildew is not the norm.

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Grapevines are in fact actually highly resistant to colonisation by all other species of powdery mildew (>150) that successfully colonise a wide range of other plant species such as barley, peas, cucumber, apples, etc. For example, our own research has found that whereas inoculation of Cabernet Sauvignon with grapevine powdery mildew (*E. necator*) spores results in 90% of the germinated spores successfully penetrating leaf epidermal cells within 48 hours, inoculation with the cucurbit powdery mildew species (*E. cichoracearum*) under the same conditions results in rates of successful penetration of less than 15% (A. Feechan, unpublished).

Furthermore, we now understand from research on barley and the model plant species *Arabidopsis*, that the ability of a plant species to resist infection by the vast majority of powdery mildew species found in the environment is through the operation of a basal immune system, which enables plants to resist infection. The plant basal immune system can be likened to the human immune system which, provides ongoing protection from a myriad of infectious micro-organisms in the environment, unless the system fails, at which point infection results. In the case of powdery mildew, it would appear that individual 'adapted' powdery mildew species, which are able to infect specific plant hosts, have found a way to suppress the plant's basal immune system, enabling them to penetrate and colonise the plant cells. Although we still do not fully understand how adapted powdery mildew species are able to suppress basal immunity in the plant host, surprising evidence has been uncovered that the invading powdery mildew pathogen may use one of the plant's own genes to help it suppress the basal immune pathway and gain entry.

This extraordinary finding actually began with German expeditions to Ethiopia in 1937 to 1938 to collect new sources of barley germplasm. Barley lines collected on this expedition were found to exhibit unusually broad-spectrum resistance against all isolates of barley powdery mildew (*Blumeria graminis*; Jorgensen 1992). However, what was even more surprising was that this resistance gene, designated *Mlo* (powdery mildew resistance gene o), was inherited in a recessive manner. In other words, plants which contained the normal functional *Mlo* gene were susceptible to *B. graminis*, but plants lacking the functional *Mlo* gene were resistant. This represents completely the opposite case to the action of R genes, as described earlier, where inheritance of the R gene confers resistance. As such, *Mlo* should not be considered a resistance gene, rather a 'susceptibility' gene which predisposes the host plant species to infection by a specific powdery mildew species.

One characteristic of barley plants lacking a functional *Mlo* gene is that rates of successful penetration by *B. graminis* spores drop from 50-70% to below 1% , rendering these plants completely resistant to this pathogen (Figure 1).

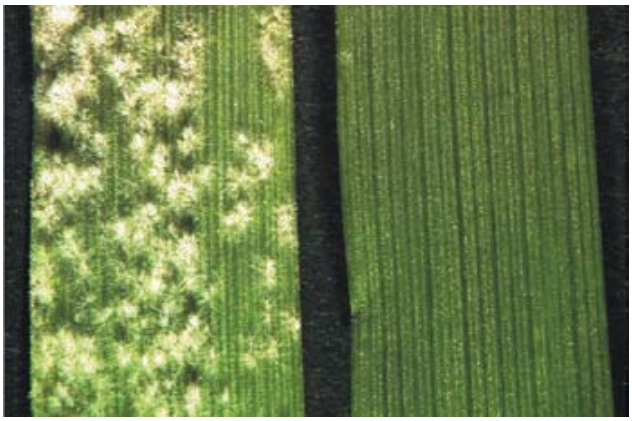


Figure 1. Comparison of powdery mildew susceptibility of wild-type and *mlo* mutant barley plants. Leaves of wild-type barley (left) and *mlo* mutant barley (right) were infected with a high density of barley powdery mildew (*B. graminis*) spores and incubated for six days. While wild-type plants support the growth of sporulating mildew colonies on the leaf surface, *mlo* mutant plants are immune to fungal attack and show no visible infection on the leaf surface.

Figure reproduced with permission from Panstruga R. and Shulze-Lefert P. (2003) Corruption of host seven-transmembrane proteins by pathogenic microbes: a common theme in animals and plants? Microbes and Infection 5: 429-437. Copyright Elsevier.



Figure 2. Comparison of the powdery mildew susceptibility of wild-type and *mlo* mutant tomato plants. Potted tomato plants of wild-type (left) and *mlo* mutant (right) were infected with tomato powdery mildew (*O. neolycopersici*) spores. After 17 days, the wild-type plant shows the growth of numerous powdery mildew colonies (white regions) on the leaves, while the *mlo* mutant tomato shows no signs of powdery mildew infection.

Image courtesy of Yuling Bai (Plant Sciences Group, Wageningen University, Holland)

Mlo resistance has been successfully deployed in the majority of European spring barley cultivars to provide broad-spectrum durable resistance to barley powdery mildew since 1979 (Peterhänsel and Lahaye, 2005). However, despite its success in barley cultivation, no other examples of *Mlo* resistant plant species had been reported until 2006 when a German group published research demonstrating that the targeted mutation of a single *Mlo* gene in the model plant *Arabidopsis* resulted in significantly reduced susceptibility to cucurbit powdery mildew (*Erysiphe orontii;* Consonni *et al*. 2006). More recently, Bai et al. (2008) reported the discovery of a naturally occurring tomato mutant collected from Ecuador that shows excellent resistance to tomato powdery mildew (*Oidium neolycopersici*; Figure 2). They demonstrated this resistance is due to the loss of function of a single *Mlo* gene in this tomato mutant.

The mechanism by which the *Mlo* susceptibility gene acts to interfere with or suppress basal immunity against powdery mildew is still unknown. However, independent of the mechanism involved, the research described earlier with barley, tomato and *Arabidopsis* suggests that it may be feasible to re-establish basal immunity in grapevine against *E. necator* by targeted mutation or silencing of selected *Mlo* gene(s) within the grape genome. We have initiated a research project to investigate this strategy.

Our first challenge was to try to determine which grape *Mlo* gene(s) to target because our analysis of the recently released *V. vinifera* genome sequence indicated there are 17 *Mlo* genes in grapevine (Feechan *et al*. 2008). In order to address this question, we compared the sequences of the 17 grape *Mlo* genes with the sequences of *Mlo* genes from barley, tomato and *Arabidopsis*. We discovered that four of the members of the grape *Mlo* gene family contained a unique sequence motif that is only found in *Mlo* genes from barley, tomato and *Arabidopsis* that have been demonstrated to act as powdery mildew susceptibility genes (Feechan *et al*. 2008). Further analysis indicated that three of these four candidate *Mlo* genes were activated in grapevines within eight hours of infection by *E. necator*, which is consistent with the response of barley *Mlo* to barley powdery mildew infection (Feechan et al. 2008).

Experiments are now underway in our laboratory to try to establish if selective silencing of any of these candidate grape *Mlo* genes individually, or in combination, leads to significant decreases in the susceptibility of grapevine to powdery mildew. The outcome of these ▸

experiments will have important implications for any future strategies to engineer powdery mildew resistance based on *Mlo* suppression.

If resistance in grapevine can be achieved through the silencing of a single *Mlo* gene, as in tomato, then it would be feasible to initiate a search for naturally occurring mutant alleles within *V. vinifera* germplasm collections that could be used in marker-assisted selection. If, on the other hand, complete powdery mildew resistance in grapevine is only achieved through the silencing of multiple grape *Mlo* genes, we will employ transgenic approaches using constructs designed to simultaneously silence multiple *Mlo* genes.

It will also be important to determine if silencing of *Mlo* gene(s) results in any negative effects on grapevine growth and development. *Mlo* mutants of barley and *Arabidopsis*, but not tomato, show a tendency to develop necrotic patches on older leaves (Peterhänsel et al. 1997; Consonni et al. 2006; Bai et al. 2008). However, the appearance of similar effects on grapevine leaves is unlikely to cause significant issues for grapegrowers because photosynthetic capacity is not normally a limiting factor to grape production. Indeed, it is likely that most growers may tolerate some loss in maximum cropping capacity in exchange for durable resistance to powdery mildew, provided there are no significant effects on grape berry quality.

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Molecular strategies to enhance the genetic resistance of grapevines to powdery mildew

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Abstract

The Eurasian winegrape *Vitis vinifera* has little or no genetic resistance to the major fungal pathogens, powdery mildew (*Erysiphe necator*) and downy mildew (*Plasmopora viticola*). These pathogens were first introduced into French vineyards from North America in the 1800s before spreading to all major grape producing regions of the world. As a result, grape production is highly dependent on the use of fungicides. With the increasing financial and environmental costs of chemical application and the emergence of fungicide-resistant strains, the introduction of natural genetic resistance against these fungal pathogens is a high priority for viticultural industries worldwide. We are utilising a number of different molecular approaches to increase our understanding of the basis of resistance to these important major fungal pathogens and to identify potential new sources of genetic resistance. This review will outline the progress and the potential of each of these different molecular strategies to the generation of fungal-resistant grapevine germplasm.

Abbreviations

BAC bacterial artificial chromosome; **BC** back-cross; **CC** coiled-coil; **CT** C-terminal; **LRR** leucine-rich repeat; **MAMP** microbe-associated molecular pattern; **NBS** nucleotide-binding site; **PEN** penetration; **RGA** resistance gene analogue; **RLK** receptor-like kinase; ***Rpv1*** Resistance to *Plasmopora viticola* 1; ***Run1*** Resistance to *Uncinula necator* 1; **TIR** Toll/Interleukin-1 receptor

Keywords: Erysiphe necator, *grapevine*, *MLO*, *powdery mildew*, *resistance*, *Run1*

Control of fungal diseases is a major problem for viticulture worldwide

The world wine industry is based on cultivation of the grape species, *Vitis vinifera*. However, this species is highly susceptible to a number of pathogens that can cause economically devastating diseases, including powdery mildew, caused by *Erysiphe necator* (syn. *Uncinula necator*), downy mildew (*Plasmopora viticola*) and Botrytis bunch rot (*Botrytis cinerea*). From a global perspective, powdery mildew is the most important of these three pathogens because it does not require specific humidity and temperature conditions for infection, as is the case with downy mildew and Botrytis infection. Consequently, although the severity of powdery mildew infection can vary from season to season, it is a constant threat to grapegrowers. Powdery mildew infects all green grapevine tissues including the leaves, stems and berries. Severe infection can debilitate vines, reduce net photosynthesis, retard ripening and degrade wine quality (Gadoury et al. 2001, Calonnec et al. 2004, Stummer et al. 2005). Even minor berry infections have been shown to be associated with elevated populations of spoilage microorganisms, increased infestation by insects and increased Botrytis bunch rot at harvest (Gadoury et al. 2007).

Modern grapevine cultivation relies heavily upon the use of chemical fungicides such as sulphur and sterol biosynthesis inhibitors to control this pathogen and most grapegrowers would apply between 6–10 fungicide sprays per season. In France alone, the cost of fungicides for powdery mildew control is around 75 million Euros per year (A. Bouquet, pers. comm., 2008) and this does not take into account the increasing fuel costs associated with the application of these fungicides. Furthermore, fungal strains have been reported that have evolved resistance to a number of the commonly used fungicides (Erickson and Wilcox 1997, Savocchia et al. 2004, Baudoin et al. 2008). There is also now increasing pressure by legislators to

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restrict the use of certain agrochemicals because they consider them to be detrimental to the environment and may pose a risk to human health. Indeed, the European Commission is currently considering a proposal to ban the use of a large number of agrochemicals in Europe by 2013 which are routinely used for the control of a number of fungal diseases in grapes including powdery mildew in grapes (Pesticides Safety Directorate 2008). Thus, the introduction of effective genetic resistance into winegrape cultivars to reduce the dependence of viticulture on chemical inputs would be of significant economic and environmental benefit.

Powdery mildews are obligate biotrophic pathogens, meaning that they require a living plant host for their growth and reproduction. Upon landing on the plant surface, powdery mildew spores (conidia) germinate to produce chains of elongated hyphal cells that grow across the surface of the plant. In order to obtain nutrients from the plant, the pathogen periodically produces a specialised structure called an appressorium that generates sufficient pressure to allow the fungus to insert a penetration peg through the cuticle and cell wall of a host epidermal cell. Once the cell wall has been breached, the pathogen invaginates the host cell membrane forming a multilobed structure called a 'haustorium' via which nutrients are obtained to allow the pathogen to reproduce and complete its life-cycle (Heintz and Blaich 1990, Glawe 2008).

The highly susceptible nature of *V. vinifera* to powdery mildew suggests that it lacks genetic mechanisms to protect itself from this pathogen. This is not surprising when one considers that *V. vinifera* is indigenous to Eurasia (This et al. 2006) whereas *E. necator* is endemic to North America and was only introduced into Europe in the 1840s, most probably on ornamental vines imported for use in European gardens. In evolutionary terms, therefore, *V. vinifera* has only been exposed to the *E. necator* pathogen for an extremely short period of time. In contrast, many grapevine species that are endemic to the USA display varying levels of resistance to powdery mildew (Pearson and Gadoury 1992) but these species lack the superior berry quality of the *V. vinifera* cultivars. Consequently, when attempts have been made to introduce powdery mildew resistance by conventional hybridisation between *V. vinifera* and North American *Vitis* sp., the resulting resistant hybrids have been generally unacceptable to growers and winemakers. The adoption of these 'French-American' hybrids has therefore been very limited throughout most viticultural regions of the world apart from eastern and mid-western USA (Pollefeys and Bousquet 2003). New molecular approaches are therefore required to introduce powdery mildew resistance into winegrape cultivars while maintaining wine quality.

Current model of plant resistance to pathogens

Bent and Mackey (2007) recently published an excellent review in which they proposed a revised four-part model to describe our current understanding of disease resistance mechanisms in plants (Figure 1). One of the key features of this model is that it helps describe the link, in both functional and evolutionary terms, between what we now understand are the two major mechanisms plants use in defence against pathogen attack.

The first of these is referred to as non-host resistance or basal immunity. This describes resistance which is effective across an entire plant species against all known isolates of a pathogen species. In cellular or molecular terms this encompasses both the preformed physical and chemical barriers that plant tissues have to pathogen invasion and the inducible immune response that is triggered by the recognition of a microbe-associated molecular pattern (MAMP) by an extracellular receptor-like kinase (RLK). MAMPs can be considered as defence elicitors that are evolutionarily stable, forming a core component of the microorganism that generally cannot be sacrificed or altered significantly without seriously impairing viability (Bent and Mackey 2007). In the case of powdery mildew, this is likely to include the chitin which makes up fungal cell walls (Bittel and Robatzek 2007). The recognition of a MAMP by the RLK triggers a basal immune response (also called MAMP-triggered immunity) which comprises a variety of defence responses including cytoskeleton rearrangements, callose deposition and the induction of antimicrobial compounds. Plant pathologists have long been aware of the existence of non-host resistance, but until recently much of the information we possessed was descriptive in nature because it was not amenable to classical genetic analysis. However, large-scale forward genetic screens on Arabidopsis have now identified a number of key genes designated *PENETRATION1 (PEN1)*, *PEN2* and *PEN3* which play major roles in the basal immune response against powdery mildews (Collins et al. 2003, Lipka et al. 2005, Stein et al. 2006). Of particular interest is *PEN1* which encodes a syntaxin involved in membrane fusion events at the plasma membrane. This protein has been shown to redistribute within cells, under powdery mildew attack, to focus beneath the point of attempted penetration (Assaad et al. 2004). Furthermore, PEN1 redistribution appears to be a unique response of plant cells to attempted penetration by powdery mildews and not by other fungal pathogens (Meyer et al. 2009). This suggests that PEN1 may have a role in trafficking of secretory vesicles to the plasma membrane containing cargo that is required for penetration resistance against powdery mildews.

In stage two of the model (Figure 1), certain species of the pathogen become 'adapted' to the host through the evolution of effector molecules that actively suppress the basal immune response and manipulate plant cell functions to facilitate infection. In the case of grapevines, *E. necator* appears to be the only powdery mildew species which has become adapted on this plant host. While there is a wealth of knowledge relating to the identity of the multiple effectors released by bacteria and oomycetes into plant cells during infection (Bent and Mackey 2007), to date there is only one report of two effectors identified from the cereal powdery mildew *Blumeria graminis f. sp. hordei* (Ridout et al. 2006).

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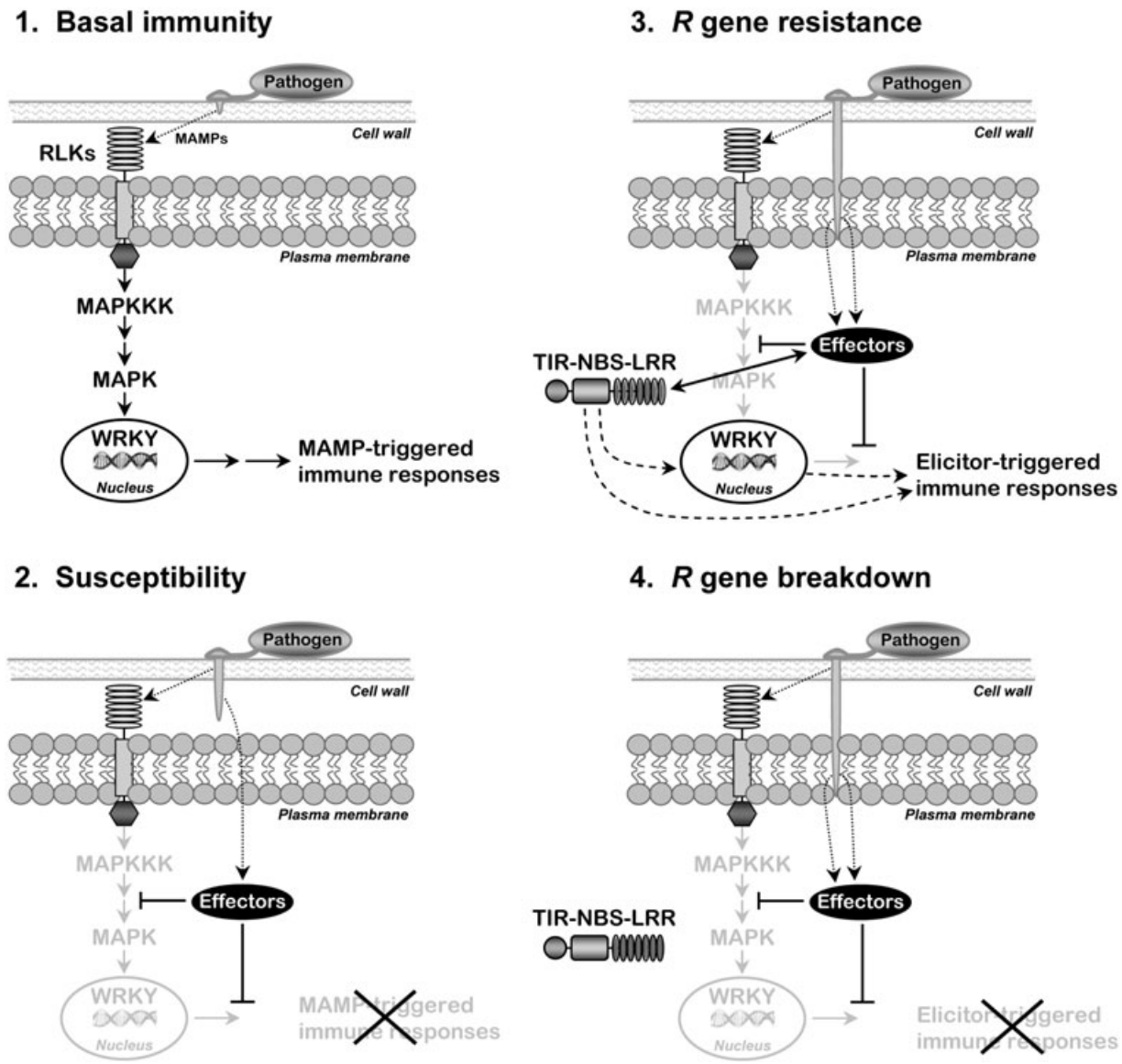


Figure 1. Model for the evolution of pathogen resistance in plants. This figure has been adapted from the model of Bent and Mackey (2007) using powdery mildew as the invading pathogen. Stage 1: Recognition of microbial-associated molecular patterns (MAMP; such as fungal chitin) by extracellular receptor-like kinases (RLKs) triggers basal immunity against non-adapted powdery mildew species, which includes signalling through MAP kinase cascades and transcriptional reprogramming mediated by plant WRKY transcription factors (proteins containing one or two highly conserved domains characterised by the heptapeptide WRYKGQK). Stage 2: Adapted powdery mildew species suppress basal immune responses possibly via release of effector molecules, allowing penetration of the cell wall. Stage 3: Plant resistance proteins (*R* gene products, such as TIR-NBS-LRR proteins: TIR, Toll/Interleukin-1 receptor domain; NBS, nucleotide-binding site domain; LRR, leucine-rich repeat domain) recognise effector activity and restore resistance through effector-triggered immune responses. Stage 4: Pathogen avoids *R* gene-mediated defences by modifying or eliminating the effector(s) that trigger those defences.

Stage three of the model (Figure 1) describes the initial stages of the plant-pathogen 'arms race', whereby plant hosts which are susceptible to adapted pathogens, evolve specific resistance (*R*) genes which enable the plant to detect the defence-suppressing effectors. This interaction initiates effector-triggered immune responses, thereby restoring resistance to the pathogen. Commonly, the result of defence activation involving *R* genes is the localised death of the infected cells, termed a hypersensitive response, which prevents the pathogen from obtaining nutrients and completing its life cycle (Mur et al. 2008). The vast majority of *R* genes encode proteins that contain leucine-rich repeats (LRRs), a central nucleotide-binding site (NBS) domain and a variable domain at the N-terminus, consisting of either a coiled-coil (CC) domain or a domain that has homology to the Toll/Interleukin-1 Receptor (TIR). With some exceptions, LRRs are mainly involved in recognition, the amino-terminal domain determines signalling specificity and the NBS domain appears to function as a molecular switch (Takken et al. 2006).

The final stage of the model describes a further evolution of the pathogen to avoid detection by the R protein by modifying or eliminating the effector(s) that triggers those defences (Figure 1). Clearly, any such changes in the pathogen effector must still be compatible with its role

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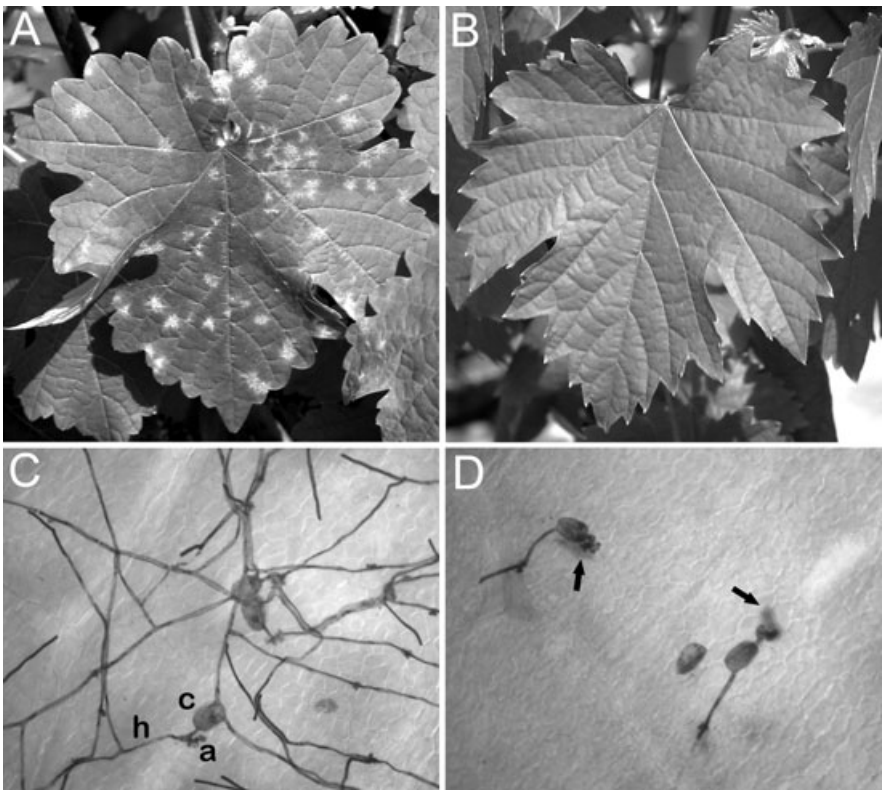


Figure 2. Powdery mildew development on leaves of susceptible (*V. vinifera* cv. Cabernet Sauvignon; panels A and C) and resistant (*Run1*-containing BC4 progeny (see text for details); panels B and D) grapevines. Panels A and B: glasshouse vines 2 weeks after inoculation with powdery mildew. Panels C and D: detached leaves sampled 48 h post-inoculation and stained with Coomassie Blue to visualise fungal structures. Arrows indicate individual epidermal cells penetrated by powdery mildew that have undergone programmed cell death in leaves containing the *Run1* locus. (c) conidium, (a) appressorium, (h) hypha.

as a virulence factor or the changes will not be maintained. This effectively returns the plant-pathogen interaction back to Stage 2, except that the pathogen has had to alter or lose an effector protein and resistance will only be re-established through the introduction of another *R* gene that recognises the modified effector.

The major message from this model is that plants have evolved two overlapping defence pathways to detect and restrict the growth of invading pathogens. In this short review, we will focus on the different molecular strategies that we are currently employing in our laboratory to develop resistance to grapevine powdery mildew by manipulating these two defence pathways in susceptible grapevine cultivars.

Identifying powdery mildew resistance genes in a wild North American grapevine

Genetic mapping and positional cloning

The wild grape species *Muscadinia rotundifolia*, a native to the southeastern USA, is highly resistant to a number of pathogens known to affect cultivated grapevines, including powdery mildew, downy mildew, phylloxera and nematodes (Olmo 1986). *M. rotundifolia* is taxonomically separated from Euvitis species by anatomical and morphological characteristics (Planchon 1887) and a difference in chromosome number (Vitis 2n = 38, Muscadinia 2n = 40; Branas 1932). Not surprisingly, therefore, the first attempts to produce inter-specific crosses between *M. rotundifolia* and *V. vinifera* in the mid 1800s, were of only limited success. However, in 1919, L.R. Detjen, a grape breeder from North Carolina was successful in producing the first authentic hybrids between *V. vinifera* and *M. rotundifolia* using the *V. vinifera* cultivar Malaga as the female parent (Detjen 1919). One of the progeny, NC6-15, was used as the resistant parent in a series of pseudo-backcrosses with *V. vinifera* cultivars that led to the identification of a single, dominant locus designated *Run1* (Resistance to *Uncinula necator* 1) that provided complete resistance to powdery mildew (Bouquet 1986, Pauquet et al. 2001). Analysis of the *Run1*-mediated resistance response indicates that it involves induction of programmed cell death, specifically within the penetrated epidermal cell, approximately 24–48 h following powdery mildew infection (Figure 2). Later studies with the same populations also identified a second resistance locus, designated *Rpv1* (for Resistance to *Plasmopora viticola* 1) that provided partial resistance to downy mildew (Merdinoglu et al. 2003). Interestingly, *Run1* and *Rpv1* were found to co-segregate making it feasible to identify both resistance genes in a single map-based cloning effort.

Three back-cross populations Mtp3294 (BC4:VRH3082-1-42 × Cabernet Sauvignon), Mtp3328 (BC4:VRH3082-1-49 × Marselan) and Mtp3322 (BC5: VRH3176-21-11 × Cabernet Sauvignon) were used in genetic mapping studies to identify markers linked to the resistance loci (Pauquet et al. 2001, Barker et al. 2005). Using a bulked segregant analysis approach (Michelmore et al. 1991), a number of amplified fragment length polymorphism (AFLP) markers (Pauquet et al. 2001) and resistance gene analogue markers (RGAs; Donald et al. 2002) were identified that are linked to the *Run*1/*Rpv1* locus. RGA markers are based on the knowledge obtained from the cloning of a large number of plant disease resistance genes from plants, which indicate a high degree of structural conservation independent of the pathogen they confer resistance to. Highly conserved motifs within the

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NBS domain of R proteins (Takken et al. 2006) have been used to design degenerate primers capable of amplifying novel resistance gene analogues (RGAs) many of which have been found to map to resistance loci in a range of plant species (Aarts et al. 1998, Collins et al. 1998, Shen et al. 1998). Di Gaspero and Cipriani (2002) used a similar approach to clone a set of RGA sequences from the grape species *Vitis amurensis* and *Vitis riparia* which are known to show resistance to downy mildew and demonstrated that one of these RGA clones, *rgVrip064*, was linked to downy mildew resistance in segregating hybrid populations. In all a total of 14 AFLP, RGA and simple sequence repeat (SSR) markers were identified that showed linkage to the *Run1*/*Rpv1* resistance locus in the Mtp3294 and Mtp3328 populations (Barker et al. 2005). Based on the closest flanking SSR markers, VMC4f3.1 and VMC8g9, *Run1*/*Rpv1* is located on chromosome 12.

These genetic markers were used to screen a bacterial artificial chromosome (BAC) library constructed using genomic DNA of a single powdery mildew-resistant plant from the Mtp3294 mapping population (Barker et al. 2005). Assembly of the contigs between VMC4f3.1 and VMC8g9 indicated that the physical distance between these markers was much greater than initially suggested by the genetic mapping, necessitating the identification of more recombinants to further narrow the genomic region containing the *Run1*/*Rpv1* locus. To achieve this, approximately 3300 new progeny of the Mtp3294 and Mtp3322 populations were screened for recombination between VMC4f3.1 and VMC8g9 and the recombinants phenotyped for powdery and downy mildew resistance (C. Anderson, unpublished). This genetic analysis narrowed the *Run1*/*Rpv*1 locus to a ~1 Mbp region of introgressed DNA. Sequencing of this region revealed the presence of a cluster of RGAs encoding TIR-NBS-LRR type resistance proteins. No other genes predicted to be involved in resistance against fungal pathogens were identified within this region.

In total, 11 RGAs have been identified at the *Run1*/*Rpv*1 locus (Figure 3). Seven of the RGAs encode full-length TIR-NBS-LRR resistance proteins; the remaining genes are truncated and lack either the TIR or the NBS-LRR domain. Three of the RGAs (RGA-9, RGA-10 and RGA-11) were originally recovered as only partial length genomic clones associated with one of a number of 'gap' regions within the *Run1* BAC library for which overlapping BAC clones could not be found. Full-length genomic clones of these RGAs were obtained by genome walking techniques. The full-length RGAs are highly homologous apart from a variable number of LRR domains. Expression analysis indicates that all of the full-length RGA candidates are expressed in powdery mildew-resistant progeny, indicating that any one of these genes are candidates for conferring powdery mildew (and/or downy mildew) resistance. Significantly, the only other powdery mildew resistance genes which have been cloned to date are the *Mla* locus from barley (Zhou et al. 2001) and the *Pm3* locus from wheat (Srichumpa et al. 2005) which also encode NBS-LRR type proteins but possess a CC domain at the N-terminus instead of a TIR domain.

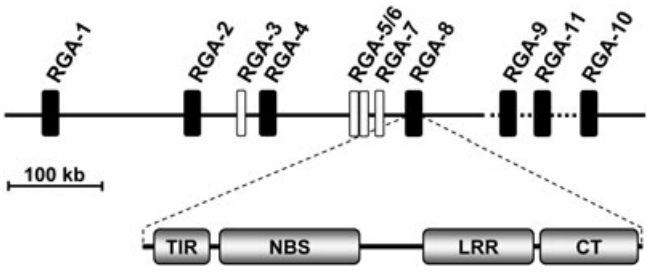


Figure 3. Arrangement and structure of resistance gene analogue (RGA) genes clustered at the *Run* locus. Black boxes represent full-length genes, open boxes are truncated genes. The dotted line represents regions of the genomic DNA for which overlapping clones could not be obtained from the *Run1* BAC library. Translation of the RGA genes at the *Run1* locus reveals the presence of four domains characteristic of TIR-NBS-LRR resistance genes: TIR, Toll/Interleukin-1 receptor domain; NBS, nucleotide-binding site domain; LRR, leucine-rich repeat domain; CT, C-terminal domain of undetermined function.

It is interesting to note that having screened over 5000 backcross progeny in the course of this mapping project, we have not identified any plants which segregate differently for powdery mildew (*Run1*) or downy mildew (*Rpv1*) resistance. This suggests that either both mildew resistances are encoded by the same resistance gene or different members of the same resistance gene cluster. An NBS-LRR resistance protein that confers resistance to two completely different pathogens, i.e. the ascomycete fungus *E. necator* and the oomycete *P. viticola*, would be highly unusual, but not unique. The *Mi* gene from tomato, for example, confers resistance against both root-knot nematodes and potato aphids (Rossi et al. 1998).

Functional testing of candidate resistance genes

NBS-LRR resistance genes show 'restricted taxonomic functionality' which generally means they are only functional within species of the same taxonomic family (Michelmore 2003). Furthermore, biotrophic pathogens such as *E. necator* and *P. viticola* are specifically adapted to the grapevine host and cannot infect other plant species. This means that whereas most genes isolated from grapevine can be functionally evaluated in readily transformable model species such as Arabidopsis, tobacco or tomato, candidate grapevine powdery mildew resistance genes are unlikely to reveal function unless experiments are carried out in transgenic grapevines, the generation of which remains a time-consuming and inefficient process. Some progress has been made in the development of transient assay systems in grapevine (Santos-Rosa et al. 2008), but whether these systems are efficient enough to enable testing of resistance to biotrophic pathogens, such as powdery mildew, remains to be seen.

Another consideration when evaluating *R* gene function in transgenic plants is the issue of promoter selection. The use of generic expression cassettes involving highly active constitutive promoters, such as 35S, may lead to autoactivity of the R proteins resulting in necrosis in the absence of the pathogen (Oldroyd and Staskawicz 1998, Tao et al. 2000, Bendahmane et al. 2002). This is thought

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to be either because of the titration of *trans*-acting repressors or an increase, beyond an activity threshold, of the steady-state level of R protein molecules, a proportion of which may exist in a spontaneously active state (Tao et al. 2000).

With these various considerations in mind, we are currently introducing individual genomic fragments (~12–14 kb) encompassing the endogenous promoter-RGA coding-endogenous terminator regions of each of the seven RGA candidates into powdery (and downy) mildew susceptible *V. vinifera* cultivars. This will enable us to determine not only which of the RGA candidates function as powdery and/or downy mildew resistance genes, but also test each of the candidate genes against different powdery and downy mildew isolates to determine if they are isolate-specific.

Considerations in the deployment of qualitative powdery mildew resistance genes in the vineyard

Durable resistance can be considered as resistance that remains effective when used in a large growing area, over a long period of time, under environments favourable to disease development (Leach et al. 2001). Perennial crops, like grapevines, provide particular challenges when considering the issue of durability. Most grapegrowers would have an expectation that individual vines would remain in the vineyard for at least 20 years and do not have the capacity to rapidly introduce new cultivars with different *R* genes, as is the case for annual crops such as cereals, should existing *R* genes fail.

Testing of *Run1* backcross lines against powdery mildew isolates in France and Australia has shown the *Run1* locus to be effective against all isolates tested to date. However, this may be because of the combined action of more than one *R* gene located at the *Run1* locus. The *M. rotundifolia* cultivar G52 from which the *Run1* locus was originally obtained is thought to have arisen from a cross between the two *M. rotundifolia* cultivars Thomas and Hope (Detjen 1919). Even if both cultivars have contributed different *R* genes to the *Run1* locus, it remains to be determined whether they would provide resistance to different powdery mildew isolates.

On the other hand, if it is determined that powdery mildew resistance at the *Run1* locus is only conferred by a single *R* gene then this has important implications for the deployment of this resistance gene within the vineyard. To date, there are few examples of single dominant *R* genes that have proven to be durable in the field. As outlined in Figure 1, interactions leading to host plant resistance involving these *R* genes can be lost through modification or elimination of pathogen effector(s) that trigger the resistance response. Even the barley *Rpg1* gene which had provided effective control for almost 50 years in North America against stem rust caused by *Puccinia graminis* f. sp. *tritici* was finally compromised by the evolution of a new virulent pathotype (Steffenson 1992).

One approach which may improve the durability of resistance in the field, is to stack or pyramid multiple *R* genes within a single cultivar such that, for a given pathogen, reproduction will be restricted even if individuals within the pathogen population are present that have lost, or modified, the interacting effector molecule for one of the *R* genes. At least two other sources of resistance (or at least reduced susceptibility) to grapevine powdery mildew have been reported that appear to map to a different locus to *Run1*. A major QTL originating from an undetermined North American *Vitis* species has been mapped to chromosome 15 (Akkurt et al. 2006, Welter et al. 2007). Hoffmann et al. (2008) also recently reported on a dominant locus for powdery mildew resistance (designated *REN1*) from the central Asian *V. vinifera* cultivar Kishmish vatkana which maps to a 10-cM region on chromosome 13. Interestingly, a family of NBS-LRR genes also maps to this same region raising the possibility that *REN1* is also an NBS-LRR gene. A number of groups have already commenced breeding programs to combine *Run1* resistance with these other powdery mildew resistance loci using marker assisted evaluation to follow the inheritance of both resistance loci (Eibach et al. 2007, Molnar et al. 2007)

The identification of dominant resistance to *E. necator* in *V. vinifera* cv. Kishmish vatkana, would appear to be at odds somewhat, with the model of evolution of plant resistance described earlier (Figure 1), which proposes that *R* gene mediated resistance is the result of a significant period of co-evolution between the host and an adapted pathogen. In addressing this conundrum, Hoffmann et al. (2008) proposed that wild *V. vinifera* populations in the Central Asia, unlike the clonally propagated cultivated grapevines in Europe, are undergoing sexual propagation, and may have evolved resistance since the arrival of *E. necator* from North America. An alternative explanation may be that *E. necator* has actually been in Central Asia for a much longer period than first thought. The existence of a large number of wild Chinese *Vitis* species which also show significant resistance to *E. necator* (Wan et al. 2007) also lends support for this hypothesis, although the actual mechanism of resistance in these Chinese species has not been reported.

An alternative strategy to powdery mildew resistance: re-establishing basal immunity against *E. necator*

According to the model outlined in Figure 1, powdery mildew species which have become adapted on particular plant hosts, are able to suppress basal immune responses to allow penetration, whereas non-adapted species are unable to do this and fail to enter the epidermal cell. For example, in the case of *V. vinifera* cv. Cabernet Sauvignon, inoculation with the adapted powdery mildew species *E. necator* results in rates of successful penetration (as determined by the presence of haustoria in epidermal cells) of over 90% within 48 h, whereas inoculation with the non-adapted cucurbit powdery mildew species *E. cichoracearum* under the same conditions, results in rates of successful penetration of <15% (A. Feechan, unpublished). It is still not understood how adapted powdery mildew species are able to suppress basal immunity to enable such high rates of successful penetration on host species. However, evidence obtained from studies on naturally

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occurring mutants, in which the basal immune response against a previously adapted powdery mildew species has been re-established, may offer some insights.

A role for MLO in suppressing basal immunity against powdery mildew?

German expeditions to Ethiopia in 1937–1938 collected seed samples of barley landraces which were found to exhibit broad-spectrum resistance against all isolates of barley powdery mildew (*Blumeria graminis f. sp. hordei*; Peterhänsel and Lahaye 2005). However, what was even more surprising was that this spontaneously occurring resistance allele, designated *mlo* (powdery mildew resistance gene o), was inherited in a recessive manner. The wild type *HvMLO* gene was cloned from barley in 1997 and found to encode a plant-specific 7 transmembrane domain protein that resides in the plasma membrane (Büschges et al. 1997, Devoto et al. 1999). Since then, a naturally occurring allele (*ol*-2), derived from a Central American tomato (*Solanum lycospersicum*) accession, that confers broad-spectrum and recessively inherited resistance to tomato powdery mildew (*Oidium neolycopersici*), has been demonstrated to be due to loss of function of the *SlMLO1* gene (Bai et al. 2008). Finally, a homozygous T-DNA insertion line in the Arabidopsis *AtMLO2* gene, has been shown to have significantly reduced susceptibility to *Erysiphe orontii* (Consonni et al. 2006), with mutation of two other closely related genes, *AtMLO6* and *AtMLO12* also required to achieve complete powdery mildew resistance.

One of the characteristics of the resistance mediated by *mlo* is the failure of the adapted powdery mildew species to successfully penetrate epidermal cells, which is reminiscent of the phenotype observed following inoculation with a non-adapted powdery mildew species. It has also been shown that *mlo* resistance in barley is dependent on the presence of *ROR2* (Collins et al. 2003), an orthologue of the *Arabidopsis PEN1* gene shown to be involved in basal immunity against non-adapted powdery mildews (see previous section). Furthermore, studies have revealed that, like the PEN1 and ROR2 proteins, wild-type MLO becomes polarised at the site of attempted powdery mildew ingress (Bhat et al. 2005). The shared histological and phytopathological characteristics, together with the conserved requirement for *PEN1*/*ROR2* in both basal immunity against non-adapted powdery mildew species, and MLO-mediated resistance against adapted powdery mildew species, strongly suggests a common mechanism of resistance (Humphry et al. 2006). In other words, mutation of the wild-type *MLO* gene effectively re-establishes basal immunity against the adapted powdery mildew species.

The mechanism by which wild-type MLO protein might interfere with or suppress PEN1-mediated basal immunity is yet to be determined. Meyer et al. (2009) have postulated that the co-localisation of MLO and PEN1/ROR2 within the plasma membrane, beneath the site of attempted penetration, might directly interfere or antagonise the PEN1/ROR2-mediated processes required for basal immunity against powdery mildew. If this were the case, it also implies that adapted powdery mildew species are able to hijack MLO to suppress basal immunity whereas the non-adapted species cannot.

Functional characterisation of the MLO gene family in grapevine

Independent of the mechanism involved, the work described previously with barley, tomato and Arabidopsis clearly demonstrates that it is feasible to re-establish basal immunity against an adapted powdery mildew species by targeted mutation or silencing of host *MLO* gene(s). However, the challenge is to determine which *MLO* gene(s) to target. For example, *A. thaliana* contains 15 *MLO* genes, but only three (*AtMLO2, AtMLO6 and AtMLO12*) appear to have a role in powdery mildew susceptibility (Consonni et al. 2006). Analysis of the recently released *V. vinifera* genome indicates there may be as many as 17 *VvMLO* genes in grapevine (Feechan et al. 2008, Winterhagen et al. 2008).

As a first step, we undertook phylogenetic analysis of the translated products of the *VvMLO* gene family together with other known plant MLO sequences. Of the 17 *VvMLO* genes analysed, six (*VvMLO3*, *VvMLO4*, *VvMLO6*, *VvMLO9*, *VvMLO13* and *VvMLO17*) clustered within the same clade as the Arabidopsis and tomato *MLO* genes that have been demonstrated to be required for powdery mildew susceptibility (Feechan et al. 2008). Of these six genes, three (*VvMLO3, VvMLO4 and VvMLO17)* were found to be induced significantly in grape leaves within 8 h of *E. necator* inoculation, which coincided with the commencement of fungal penetration (Figure 4). Transcript abundance of *VvMLO4* and *VvMLO17* then decreased, while *VvMLO3* expression remained relatively constant during the period 8–20 h post-inoculation. *VvMLO9* was also found to exhibit increased transcript levels following *E. necator* inoculation, but the timing was delayed relative to the other powdery mildew-induced *VvMLO* genes, whereas none of the other eight *VvMLO* genes examined were induced greater than twofold in response to powdery mildew inoculation. The transient transcriptional response of *VvMLO17*, *VvMLO3* and *VvMLO4* to *E. necator* penetration is consistent with that previously reported for *HvMLO* in response to *B. graminis* infection (Piffanelli et al. 2002).

The similarities in both sequence homology and transcriptional regulation suggests that *VvMLO3*, *VvMLO4* and *VvMLO17*, may have functionally similar roles to those *MLO* genes from barley, Arabidopsis and tomato (described previously) which have been shown to modulate powdery mildew susceptibility. It may also indicate some level of functional redundancy between these three *VvMLO* genes as is the case in Arabidopsis. Experiments are now underway in our laboratory to try and establish if selective silencing of any of these *VvMLO* genes individually, or in combination, has any impact on powdery mildew susceptibility. The outcome of these experiments will have important implications for any future strategies to engineer powdery mildew resistance based on *MLO* suppression. If resistance in grapevine can be achieved through the silencing of a single *VvMLO* gene, as in

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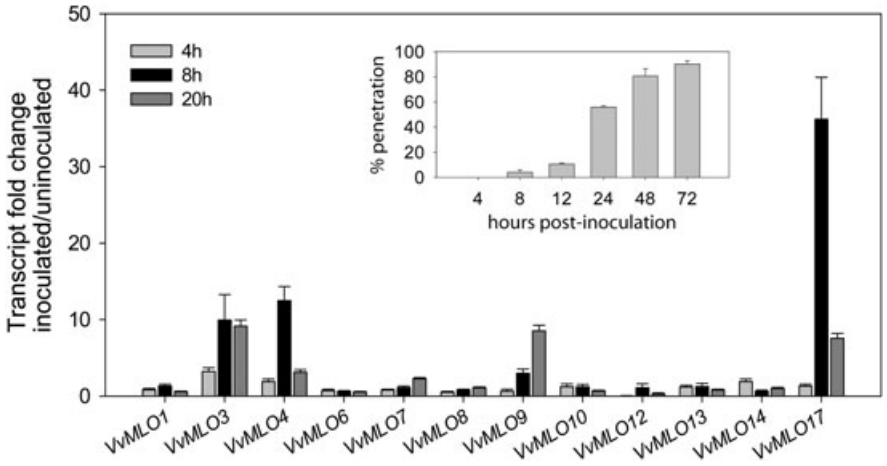


Figure 4. Transcriptional response of grapevine *VvMLO* genes to powdery mildew infection. Detached *V. vinifera* cv. Cabernet Sauvignon leaves were heavily inoculated with *E. necator* spores and sampled at 4, 8, 12, 24, 48 and 72 for scoring penetration efficiency and 4, 8 and 20 h for gene expression analysis. Inset: The incidence of epidermal host cell penetration by individual germinated conidia, scored via the presence of a haustorium. Each data point represents the scoring of a minimum of 100 germinated conidia with two replicates. The mean is shown and error bars are standard errors. Main graph: Quantitative RT-PCR analysis of *VvMLO* gene expression. Control (un-inoculated) detached leaves were incubated and sampled as for the inoculated leaves. Transcript levels of individual *VvMLO* genes were normalised against the transcript levels of *EF1* in each cDNA sample. Results are plotted as a mean ratio of the normalised transcript level of each *VvMLO* gene in inoculated leaves versus the normalised transcript level of each *VvMLO* gene in control leaves. Means are totals calculated from triplicate technical qRT-PCR measurements within three biological replicates and the data shown are representative of the results obtained in the three independent experiments. Error bars are standard errors. This figure has been modified from *Functional Plant Biology* 35(12) 1255–1266, http://www.publish.csiro.au/paper/FP08173.htm.

tomato (Bai et al. 2008), it would then be feasible to initiate a search for naturally occurring mutant alleles within *V. vinifera* germplasm collections that could be used in marker-assisted selection to generate progeny that are homozygous recessive at this locus. If, on the other hand, complete powdery mildew resistance in grapevine is only achieved through the silencing of more than one *VvMLO* gene, then this is realistically only amenable to transgenic approaches using constructs designed to simultaneously silence multiple *VvMLO* genes. Such an approach has already been shown to be feasible in Arabidopsis (Consonni et al. 2006).

Ontogenic resistance: the return of basal immunity?

Ontogenic or age-related resistance describes an increase in the ability of whole plants or plant tissues to resist pathogen infection as they age or mature. It is exhibited towards viral, bacterial, oomycete and fungal pathogens (Panter and Jones 2002, Develey-Rivière and Galiana 2007). Grapevines show ontogenic resistance to several fungal pathogens including powdery mildew (Gadoury et al. 2003), downy mildew (Kennelly et al. 2005), and black rot (Hoffman et al. 2002).

Delp (1954) originally reported field and laboratory observations that immature grapes were susceptible to infection, but that no new infections occurred once the concentration of soluble solids exceeded 8°Brix. Chellemi and Marois (1992) also reported that grapes became resistant to powdery mildew infection above 7°Brix. As the apparent decrease in powdery mildew susceptibility of grape berries post-veraison, coincided with a marked increase in the accumulation of pathogenesis-related proteins, it was postulated that these proteins may have a role in ontogenic resistance (Robinson et al. 1997, Tattersall et al. 1997). However, subsequent studies showed that ontogenic resistance to powdery mildew is actually expressed at a much earlier stage of berry development, with berries of several cultivars of *V. vinifera* and *V. labruscana* immune to infection within 3–5 weeks after bloom (Ficke et al. 2003, Gadoury et al. 2003).

Although the mechanism of ontogenic resistance in grape berries is not fully understood, it appears to operate at the level of the epidermal cell wall resulting in an inhibition of penetration (Ficke et al. 2003). This increased penetration resistance could not be correlated with cuticular or cell wall thickness or the production of antimicrobial phenolics, leading Ficke et al. (2004) to postulate that ontogenic resistance in grape berries is mediated by the development of either (i) a physical or biochemical barrier near the cuticle surface or (ii) an inducible system for the rapid synthesis of an antifungal compound.

It is interesting to note that the increased penetration resistance observed in developing grape berries against the adapted powdery mildew species *E. necator,* is comparable to the observed response of grape leaves to attempted penetration by a non-adapted powdery mildew species such as *E. cichoracearum* (see previous discussion). This raises the question as to whether the development of ontogenic resistance reflects a decrease in the capacity of *E. necator* to continue to suppress basal immunity. We are currently investigating components of the basal immunity pathway in grapevine in order to test this hypothesis.

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If we could determine the molecular/genetic basis of ontogenic resistance to powdery mildew in grape berries, it may be possible to use this information to manipulate broad-spectrum, durable resistance to powdery mildew in other highly susceptible grapevine tissues. Recent work by Gee et al. (2008) has identified a strategy via which the determination of the mechanism of ontogenic resistance might be achieved. They screened a diverse collection of *Vitis* species and interspecific hybrids for the development of ontogenic resistance in berries under field conditions. Of the 79 genotypes investigated, 50 showed little or no sign of disease when inoculated, irrespective of the stage of berry development at inoculation, 24 exhibited a significant gain of resistance as berries aged and four genotypes exhibited no statistically significant pattern. However, more importantly, one genotype (*V. rupestris* 'R-65-44') remained susceptible past the onset of ripening, over 1 month later than reported previously for *V. vinifera*. The discovery of significant genotypic variation in ontogenic resistance now offers the possibility of undertaking genetic studies to investigate the inheritance and molecular basis of this powdery mildew resistance character.

Final considerations

We are employing a number of molecular approaches in our quest to try and introduce significant and durable resistance to powdery mildew into *V. vinifera*. Of the three approaches described, integration of the *Run1* locus from *M. rotundifolia* into *V. vinifera* has clear potential for generating new powdery mildew-resistant winegrape cultivars. Our research is focussed on identifying which of the individual resistance gene candidates, located at the *Run1* locus, contributes to powdery (and downy) mildew resistance. Functional demonstration of mildew resistance mediated by a single *R* gene in a *V. vinifera* genetic background, would open up the possibility of introducing this gene into existing elite cultivars with minimal impacts on wine quality. However, as work with other pathosystems has shown, the use of single dominant resistance genes is not without potential problems in terms of durability in the field. It will be important to obtain information regarding the existence and distribution of any *E. necator* isolates, most probably within North America, which can overcome *Run1*-mediated resistance. Where possible, new winegrape cultivars should also contain resistance genes from multiple sources, to reduce the risk of generating new compatible isolates. However, this clearly adds another level of complexity in terms of plant breeding or genetic transformation.

While as yet unproven in grapevine, the *MLO* mutation approach offers some advantages over resistance based on *Run1*, because of its potential to deliver broad-spectrum, durable resistance to powdery mildew. If a member of the *VvMLO* gene family is demonstrated to act as a powdery mildew susceptibility gene in grapevine, the most likely mode of delivery is via genetic modification of existing cultivars. However, powdery mildew resistance obtained through *MLO* mutation may not come without a price. Barley and Arabidopsis *mlo* mutants have been found to show enhanced susceptibility to some hemibiotrophic and necrotrophic pathogens (Jarosch et al. 1999, Kumar et al. 2001, Consonni et al. 2006). As grapevines are exposed to a broad range of pathogens within the different regions of the world in which they are cultivated, deployment of grapevine MLO mutants, would need to be preceded by field trials designed to evaluate the impact of this mutation on susceptibility to pathogens other than powdery mildew. It also remains to be seen if silencing of *VvMLO* gene(s) will have any pleiotropic effects on grapevine growth and development. *MLO* mutants of barley and Arabidopsis display a range of developmentally regulated pleiotropic effects, including the spontaneous deposition of callose-containing cell wall appositions and the premature onset of leaf senescence (Peterhänsel et al. 1997, Piffanelli et al. 2002, Consonni et al. 2006). However, similar developmentally regulated pleiotropic effects were not observed on powdery mildew resistant tomato *mlo* mutants (Bai et al. 2008). The impaired leaf physiology of barley *mlo* plants results in some reduced grain yield compared with wild-type plants and this initially hampered the use this germplasm despite its broad-spectrum resistance to powdery mildew. The possible appearance of similar pleiotropic effects on grapevine leaves is unlikely to cause significant issues for grapegrowers because photosynthetic capacity is not normally a limiting factor in grape production. Indeed, it is likely that most growers would even tolerate some loss in maximum cropping capacity in exchange for durable resistance to powdery mildew. However, it will be important to confirm that the mutation of specific *VvMLO* gene(s) in grapevine does not lead to any deleterious effects on berry yield or quality.

An over-arching issue across all of these considerations is the continuing uncertainty regarding the adoption of transgenic vines in the major wine-making countries and whether this could be used by some countries as a trade barrier. In comparison with classical breeding techniques, genetic transformation offers the capacity to move resistance genes between different grapevine species that cannot be readily hybridised by traditional breeding techniques, or selectively silence host genes which pre-dispose plants to infection. It also retains the unique genetic make-up of our elite wine cultivars, which may be lost through the use of classical breeding techniques because of the high level of heterozygosity of the grape genome. Indeed, despite the best efforts of grapevine breeders, not one new powdery mildew-resistant cultivar has been developed, since the invasion of *E. necator* into Europe in the 1840s, that has been adopted to any significant extent by growers. However, with the development of genetic markers linked to both qualitative and quantitative disease resistance loci and the employment of marker assisted selection strategies, the breeding of new disease resistant winegrape cultivars may be about to undergo a renaissance.

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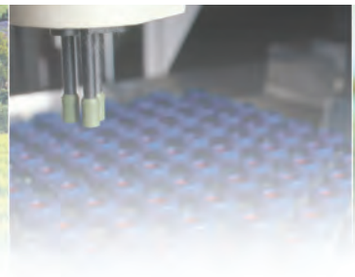
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Viticulture value-add

Increasing mildew resistance through genes

Some wild relatives of winegrapes are naturally resistant to mildews and do not require fungicide sprays. The introduction of effective genetic resistance against fungal pathogens into winegrape cultivars to reduce the dependence of chemical inputs in Australian vineyards would be of significant economic and environmental benefit.

Scientists at CSIRO Plant Industry are currently undertaking a Grape and Wine Research and Development Corporation funded project to investigate a number of different strategies to generate new grapevine cultivars with increased genetic resistance to powdery and downy mildew.

One of these approaches involves the identification of fungal resistance genes within the wild North American grape species Muscadinia rotundifolia. Grapevines containing these genes are completely resistant to powdery mildew.

Using sophisticated molecular techniques, the CSIRO team members in collaboration with scientists at the French National Institute for Agricultural Research (INRA) have successfully identified the region of the M. rotundifolia genome that provides resistance to these fungal pathogens. Subsequent cloning and sequencing of this region identified seven candidate genes that may confer resistance to powdery and/or downy mildew.

In order to determine which of these candidate genes is responsible for fungal resistance, each of the genes has been introduced separately into a range of susceptible winegrape cultivars, including Shiraz, by genetic transformation.

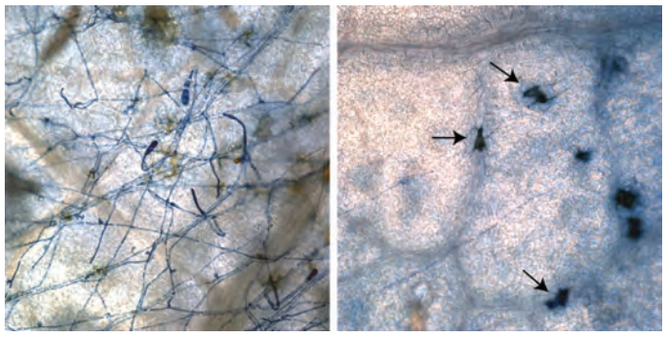
CSIRO Plant Industry senior researcher Dr Ian Dry is working with fellow senior researcher Dr Mark Thomas on the project and says the introduction of multiple candidate genes into different cultivars via genetic transformation has never before been attempted.

"This has involved a huge amount of work by both the CSIRO and INRA scientists over the past three years," Dr Dry said.

"Despite some technical setbacks along the way, new grapevines containing each of the candidate resistance genes have now been successfully generated and are currently undergoing evaluation at the CSIRO Plant Industry laboratory in Adelaide to determine if they show improved resistance to these important fungal diseases."

Although the testing is not yet complete, CSIRO scientists have preliminary evidence that at least one of the candidate genes tested so far provides good natural protection against powdery mildew infection. This is illustrated in the figure below, which shows that in transgenic Shiraz plants containing this resistance gene from M. rotundifolia, epidermal cells that have been penetrated by the powdery mildew fungus undergo a process called programmed cell death. This rapid death of the penetrated grapevine cell deprives the powdery mildew fungus of nutrients, causing its further development to be halted.

The identification of the M. rotundifolia resistance genes not only provides industry with the opportunity to develop new disease resistant versions of existing winegrape cultivars through genetic modification, but it will also facilitate the generation of completely new disease resistant winegrape cultivars through marker-assisted breeding techniques that have been used extensively in the development of disease resistant cultivars in many other crops such as wheat and barley.



This figure shows a comparison of the growth of powdery mildew fungus (stained blue) on the surface of leaves of a susceptible grapevine (left panel) and a transgenic Shiraz grapevine containing a candidate resistance gene from Muscadinia rotundifolia (right panel). The arrows in the right panel indicate examples of cells that have undergone cell death following penetration by the powdery mildew fungus.

vitiSnips

AWITC program released

The Australian Wine Industry Technical Conference (AWITC) is owned and conducted by the Australian grape and wine industry. It is held on a triennial basis for the benefit of the industry and is an important contributor to its excellence and competitive advantage.

First held in 1970, this is the premier technical wine conference held in Australia that will attract more than 1600 delegates.
The 14th AWITC and WineTech 2010 – the Australian Wine Industry Trade Exhibition – will be held on July 3-8, 2010 at the Adelaide Convention Centre. The first of the conference sessions will commence at 1pm on Sunday, July 4, and the last session will conclude at 6pm on Wednesday, July 7.

The eight formal sessions and three colloquia feature 35 Australian speakers and 18 international speakers from countries including China, Germany, Italy, New Zealand, Spain, the United States and United Kingdom.

The program covers a wide range of issues facing Australian grape and wine producers, from vineyard to the customer, and includes presentations on the future success of the industry, soil health, water, and the environment.

Program details are now available and can be found by visiting the AWITC website: www.awitc.com.au

Encyclopaedia of Plant Genomics

Genetics, Genomics and Breeding of Grapevine

Chapter 8: *Positional Cloning of Disease Resistance Genes in Grapevine*

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Introduction

The identification of plant genes by positional cloning is a lengthy process that requires a number of steps to be performed. Firstly, a mapping population must be constructed in which the gene of interest segregates in a genetically defined ratio. The mapping population can then be used to identify genetic markers linked to the locus of interest, enabling construction of a local genetic map. Conversion of the genetic map into a physical map requires a large-insert genomic library that can be used for chromosome walking or, as has become possible in recent years, the availability of a complete genome sequence. Finally, candidate genes identified in the interval between the genetic markers most closely flanking the locus of interest, must be tested by complementation to identify the functional gene.

Due to the considerable effort required, the positional cloning projects initiated in the late 20th century concentrated on highly important agricultural traits, such as those conferring disease resistance to agriculturally important plant pathogens. The first plant gene identified by positional cloning was the *Pto* gene of tomato (Martin et al. 1993) and its identification required the time-consuming development of introgressed genetic stocks and extensive marker development (Bortiri et al. 2006). *Pto* is a single dominant gene that confers resistance to the bacterial speck pathogen, *Pseudomonas syringae pv. tomato* and is an example of a gene that confers disease resistance in a "gene-for-gene" manner. Gene-for-gene resistance (Flor 1971) is a highly effective mechanism that relies on the direct, or indirect interaction, of the products encoded by a resistance gene in the plant and an avirulence gene in the pathogen. The interaction is highly specific and results in the activation of a suite of plant defence responses that culminate in localised cell death, called a hypersensitive response, at the site of attempted infection (Mur et al. 2008). This mechanism is most effective against biotrophic pathogens that require living tissue upon which to survive and gene-for-gene interactions have been described between plants and a wide variety of pathogen types, including bacteria, viruses, fungi, oomycetes, nematodes and aphids. Due to their strong effect and single gene basis, the isolation of plant resistance genes was a major goal of early positional cloning efforts.

Since the identification of *Pto*, over 50 plant R genes have been mapped and cloned from a range of plant species (Martin et al. 2003; van Ooijen et al. 2007) including woody perennials such as apple (Belfanti et al. 2004; Malnoy 2008). However, we now recognise that gene-for-gene resistance is the second of two distinct mechanisms that plants use to defend themselves against pathogens. The first layer of defences is termed basal immunity, or non-host resistance, and comprises both the preformed chemical and physical barriers that prevent pathogen penetration and the inducible immune response that can be triggered upon plant recognition of microbe-associated molecular patterns (MAMPs; reviewed in Bent and Mackey 2007). The genetic basis of resistance mediated by preformed chemical and physical barriers is likely to be complex, involving multiple loci. However, MAMP recognition is achieved by plant receptor-like kinase proteins (Chinchilla et al. 2006; Zipfel et al. 2006) that have a simple, single gene basis. Unlike avirulence (effector) proteins, which are only produced by certain races of the pathogen population, MAMPs are generic molecules forming a core component of the microorganism that cannot be sacrificed or altered significantly without seriously impairing viability such as the chitin that the chitin which makes up fungal cell walls (Bittel and Robatzek 2007). Receptor activation results in cytoskeletal rearrangements, callose deposition at the site of infection and the production of antimicrobial compounds which can be an effective form of defence against both biotrophic pathogens that require living tissue upon which to survive and necrotrophic pathogens that feed off dead plant cells. Identification of the genes controlling these two important plant defence mechanisms could prove useful in strategies to improve the disease resistance of crop plants.

The threat of disease is of great concern to grapevine growers throughout the world. The European grape species, *Vitis vinifera*, is the most widely cultivated grapevine and produces berries of excellent quality. However, this species is highly susceptible to all major grapevine pathogens, including powdery mildew, caused by the fungus *Erysiphe necator* (syn. *Uncinula necator*), downy mildew, caused by the oomycete *Plasmopara viticola,* Pierce's disease, caused by the bacterium *Xyllela fastidiosa*, and phylloxera, caused by the insect *Dactylosphaeria vitifolia* (reviewed in Riaz et al. 2007) with only one known exception (Hoffmann et al. 2008). These, and other grapevine pathogens, have caused the near disappearance of viticulture in some areas of the world (Galet 1996) are a major

barrier to the growth of European grapevine species in areas such as the South-Eastern USA, Central and South America. Where grapevine production has persisted, the threat of disease is so great that extensive use of chemical fungicides is required, particularly to control the powdery and downy mildews. For example, a study of fungicide use in EU member states (Phytowelt GmbH 2003) concluded that while grapevines accounted for only ~8% of total crop production area, they accounted for ~70% of all fungicides applied. This equates to costs of 150 and 75 million euros in France alone, for the chemicals used to control downy and powdery mildew, respectively, and does not take into account the increasing fuel costs associated with the application of these fungicides. Furthermore, fungal strains have been identified that have evolved resistance to a number of the commonly used fungicides (Erickson and Wilcox 1997; Savvocchia et al. 2004; Baudoin et al. 2008) and it is now considered that the widespread use of agrochemicals is both environmentally detrimental and may pose a risk to human health. Consequently, the identification of genes that confer resistance to these pathogens and their incorporation into *V. vinifera* would be of significant economic and environmental benefit.

The susceptible nature of *V. vinifera* might be explained by the fact that this species arose in Europe and Asia, whereas many of the major grapevine pathogens such as powdery mildew, downy mildew and phylloxera are endemic to North America and were only introduced into Europe in the mid 19th century (Boubals 1958, 1961, 1966). Therefore, *V. vinifera* has only been exposed to them for an extremely short period of time in evolutionary terms. In contrast, many grapevine species that are endemic to North America, display varying levels of resistance to these diseases. Although the potential exists to transfer resistance from these American relatives into *V. vinifera* cultivars, this has proven difficult to achieve by conventional breeding. One major constraint is that the North American grape species often have poor berry characteristics and, when hybrids have been generated, they lack the high berry quality of the original *V. vinifera* cultivar. The second constraint is that it is difficult to produce hybrids between *V. vinifera* and some of the most resistant American grapevines. For example, *Muscadinia rotundifolia*, a wild grapevine native to the south-eastern United States, is highly resistant to a number of pathogens known to affect cultivated grapevines, including powdery mildew,

downy mildew, phylloxera and nematodes (Olmo 1986) and, as such, is a rich source of potential disease resistance genes. However, *M. rotundifolia* is taxonomically separated from Euvitis species by anatomical and morphological characteristics (Planchon 1887) and a difference in chromosome number (*Vitis* 2n = 38, *Muscadinia* 2n = 40; Branas 1932) and when the first attempts to produce inter-specific crosses between *M. rotundifolia* and *V. vinifera* were performed in the mid 1800's they achieved only limited success. For these two reasons, the transfer of resistance genes into cultivated varieties by conventional breeding is still a major challenge for grapevine geneticists (Bisson et al. 2002). Consequently, a number of groups around the world are now interested in studying the North American grapevine species to determine the genetic basis of their disease resistance (Pauquet et al 2001; Akkurt et al. 2006; Welter et al. 2007; Wiedemann-Merdinoglu et al 2006 and chapter 6) as this could lead to novel control methods by generating genetically modified grapevine cultivars.

Positional cloning of the *Run1* and *Rpv1* genes

Development of mapping populations

In 1998, we initiated a project to isolate two important resistance genes from *M. rotundifolia* by positional cloning. Despite the problems inherent in using *M. rotundifolia* as a source of resistance in grapevine breeding programs, Detjen (1919) was successful in producing authentic hybrids using the *V. vinifera* cv. Malaga as the female parent. This paved the way for construction of modern mapping populations, which is a crucial first step in any positional cloning effort. One of the hybrids produced, NC6-15, was used as the resistant parent in a series of pseudo-backcrosses (Bouquet et al 1986; Fig. 1). At each back-cross step, a resistant individual was crossed with a different susceptible *V. vinifera* genotype to prevent in-breeding depression. This resulted in a number of populations from the fifth and sixth back-cross generations in which resistance to powdery and downy mildew segregated in a 1:1 ratio. Complete resistance to powdery mildew was controlled by a single, dominant locus named *Run*1 (for Resistance to *Uncinula necator* 1) and partial resistance to downy mildew was controlled by a major quantitative trait locus (QTL) named *Rpv*1 (for Resistance to *Plasmopora viticola* 1; Bouquet 1986; Pauquet et al. 2001; Merdinoglu et al. 2003; Fig. 2). Interestingly these two important

resistance loci are co-located in the *Muscadinia* genome making them highly valuable targets that could be identified in a single positional cloning effort.

Isolation of genetic markers linked to the Run1 and Rpv1 loci

At the time the *Run1/Rpv1* positional cloning project was conceived, limited genetic resources were available for grapevine and very few genetic markers existed. Therefore, we used three of our mapping populations, Mtp3294, Mtp3322 and Mtp3328 (Fig. 1), to identify novel markers that were linked to the resistance loci (Pauquet et al. 2001; Donald et al. 2002; Barker et al. 2005). A bulked segregant analysis approach (Michelmore et al. 1991) was initially used to identify amplified fragment length polymorphism (AFLP) markers that were linked to *Run1*. Pauquet et al. (2001) constructed bulks using the DNA of ten individuals from the Mtp3294 population known to be resistant to powdery mildew (heterozygous for *Run1*) and ten individuals known to be susceptible. Sixty four combinations of selective primers were then used to amplify fragments from the DNA pools and 19 polymorphic fragments were identified. To confirm linkage and determine marker order, each primer pair was used to amplify fragments from the genomic DNA of 157 individuals from the Mtp3294 population that had previously been challenged with powdery mildew. All 157 individuals from the Mtp3294 population were later also screened for resistance to downy mildew (*P. viticola*). *Rpv1* was found to co-segregate with *Run1* in all resistant progeny. This approach successfully identified fourteen AFLP markers that were closely linked to the *Run1*/*Rpv1* locus in VRH3082-1-42, of which 11 co-segregated with the resistance locus (Fig. 3).

To supplement the genetic map produced using AFLPs, a second class of markers was sought that are unique to the study of plant disease resistance genes. Despite the fact that they confer resistance to a wide range of pathogen types, comparison of the plant resistance genes that contribute to gene-for-gene resistance has shown that the majority encode proteins with a central nucleotide binding site (NBS) linked to a C-terminal leucine-rich repeat (LRR) domain (reviewed in Takken et al. 2006). Motifs within the NBS are highly conserved and have been used to design degenerate primers capable of amplifying novel resistance gene analogues (RGAs) from many plant species (Aarts et al. 1998;

Collins et al. 1998; Shen et al. 1998). Nested primers designed to four conserved amino acid motifs present in the NBS (GVGKTT (P-loop), L(I/V/L)VLDDV (kinase-2), GLPL and MHD) were used to amplify RGAs from the genome of a BC4 (VRH3082-1-42) resistant individual (Donald et al. 2002). Twenty eight unique grapevine RGA sequences were identified and were subdivided into 22 groups of sequences that showed nucleic acid sequence identity of 70% or greater. Representatives from each group were used in a bulked segregant analysis strategy to identify restriction fragment length polymorphisms (RFLPs) linked to the powdery mildew resistance locus. This led to the identification of three markers, MHD98, MHD145 and GLP1-12, for which linkage was subsequently confirmed by analysing the RFLP patterns produced by a selection of powdery mildew-resistant and powdery mildew-susceptible individuals from the Mtp3294 population. To facilitate screening of the entire Mtp3294 mapping population, GLP1-12 was subsequently converted into a Cleaved Amplified Polymorphic Sequence (CAPS) marker and MHD98 was converted into a dominant, PCR-based marker (Donald et al. 2002). The three markers were then examined in 167 individuals in the Mtp 3294 population, revealing that both GLP1-12 and MHD145 co-segregated with the *Run1*/*Rpv1* locus (Fig. 3). A similar approach was used by Di Gaspero and Cipriani (2002) to identify RGA sequences from the grape species *Vitis amurensis* and *Vitis riparia*. These species are known to display resistance to downy mildew and one of the RGA clones identified, *rgVrip064*, was linked to a downy mildew resistance locus segregating in a hybrid population.

Three further markers were added to the local genetic map surrounding *Run1* by searching for Simple Sequence Repeat (SSR) markers that showed linkage to the resistance locus in the Mtp 3294 population (Barker et al. 2005) from those generated by the Vitis Microsatellite Consortium coordinated by Agrogène, France. Together, the data obtained by analysis of ~160 individuals from the Mtp3294 population generated a genetic map for the VRH3082-1-42 parent in which 14 markers co-segregated with *Run1* (Fig. 3).

To further refine the genetic map surrounding the *Run1*/*Rpv1* locus, an additional 419 individuals from the Mtp3322 population and 416 individuals from the Mtp3328 population were then examined (Barker et al. 2005). Mtp3322 which was derived from a cross between the resistant parent, VRH3176-21-11 and the susceptible parent, *V. vinifera* cv. Cabernet Sauvignon and Mtp3328 which was derived from a cross between the resistant parent, VRH3082-1-49 and the susceptible parent, *V. vinifera* cv. Marselan (Fig. 1). To facilitate scoring of AFLP markers, EMhb1 and EMfd3 were cloned and converted into the dominant, PCR-based marker Shb1 or the RFLP marker Sfd3, respectively. Once recombinant plants had been identified, they were phenotyped for powdery and downy mildew resistance and additional markers were scored. Two plants were identified that were recombinant between *Run1*/*Rpv1* and VMC8g9 and four plants were recombinant between *Run1*/*Rpv1* and Shb1 (Table 1). This allowed a marker order to be assigned in which *Run1*/*Rpv1* was located between the SSR markers, VMC4f3.1 and VMC8g9, an interval that was considered sufficiently small to enable map-based cloning of the *Run1* and *Rpv1* loci.

Physical mapping of the *Run*1/*Rpv*1 locus

Generation of a Bacterial Artificial Chromosome library

As no genome sequence currently exists for *M. rotundifolia*, we chose to isolate the *Run1* and *Rpv1* genes using a chromosome walking approach. This required construction of a large-insert genomic library containing the allele of interest. In recent years, bacterial artificial chromosome (BAC) vectors have become the vehicle of choice for the generation of such libraries due to the large size of fragments that can be cloned and their increased stability and ease of manipulation compared to other vectors such as cosmids or yeast artificial chromosomes (Shizuya et al. 1992; Woo et al. 1994; Chapter 10). A BAC library using the genomic DNA of a single powdery mildew-resistant plant from the Mtp 3294 mapping population was generated (Barker et al. 2005). This individual was clonally propagated to generate sufficient leaf material for construction of the BAC library and megabase-sized DNA was extracted, digested with *Hind*III or *BamH*I and size-fractionated essentially as described by Peterson et al. (2000). The library was constructed in the pINDIGOBAC-5 vector (Epicentre,

Madison, WI) and individual clones were stored in 144 x 384-well plates (Barker et al. 2005). The library contained a total of 55,295 clones with an estimated average insert size of 93 kb. Less than 5 % of clones did not contain an insert and approximately 1.7 % of clones contained chloroplast-derived sequence as determined by hybridisation of clones arrayed on a filter with the grapevine RNA polymerase B gene. Given that the grapevine genome has been estimated at 475 Mb by flow cytometry (Lodhi and Reisch 1995) and 487 Mb by genome sequencing (Jaillon et al. 2007), the library contains around 10-fold haploid genome coverage.

Assembly of BAC contigs containing linked genetic markers

To initiate a physical map of the region containing the *Run1*/*Rpv1* locus, the BAC library was screened to identify clones containing three genetic markers that co-segregated 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 10-fold genome coverage (Barker et al. 2005). 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.

The individual plant chosen to generate the BAC library was heterozygous for *Run1* and *Rpv1*. Therefore, once clones containing genetic markers had been identified, they were grouped into "resistant" and "susceptible" contigs representing the chromosome containing *Run1*/*Rpv1* derived from the resistant parent, VRH3082-1-42, or the chromosome lacking *Run1*/*Rpv1* derived from the susceptible parent, *V. vinifera* cv. Cabernet Sauvignon, respectively. 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. These primers were also used to amplify PCR products from a selection of resistant and susceptible genotypes in the Mtp3294 population, enabling a large number of dominant and co-dominant markers to be identified that were linked to *Run1* and *Rpv1*. Primers that amplified products of identical size from both resistant and susceptible genotypes

were used to directly sequence BAC clones that contained the region of interest. Alignment of sequences enabled polymorphic nucleotides to be identified that could be utilised to generate CAPS markers. Primers were then used to isolate overlapping clones that could be used to extend BAC contigs (Fig. 4).

Assembly of BAC contigs containing resistance gene candidates

GLP1-12 and MHD145 were originally identified as RFLP markers (Donald et al. 2001). Therefore, BACs containing these sequences were examined by RFLP analysis, enabling them to be grouped into contigs representing the resistant or susceptible chromosomes (Barker et al. 2005). Out of 49 clones examined, only two overlapping BACs were identified that contained the 2.2 kb MHD145-hybridising *Eco*RI fragment characteristic of resistant plants. These BACs contained additional MHD145-hybridising fragments, suggesting that each clone might contain multiple copies of a related sequence. By comparing *Eco*RI digests and RFLP patterns, other MHD145-hybridising BACs could be aligned with those containing the fragments characteristic of the co-dominant marker and overlap between clones was confirmed by PCR amplification using primers designed from BAC end sequences. Three BACs containing MHD145-hybridising sequences were assembled into each of the resistant and susceptible contigs. Additional markers designed using BAC end sequence information confirmed that the two contigs were haplotypes and allowed overlapping BAC clones to be identified to extend the contigs.

Out of 43 GLP1-12-hybridising BACs examined, six contained the 1.6 kb *Eco*RI fragment characteristic of resistant plants. However, in this case the BAC clones did not directly overlap, suggesting that the hybridisation signals observed upon RFLP analysis of genomic DNA might be contributed by multiple members of a gene family (Barker et al. 2005). Five out of six BACs containing the 1.6 kb *Eco*RI fragment (4M9, 26C5, 61L18, 69D12 and 101P19; Fig. 4) were assembled into one contig using restriction fragment digest patterns and PCR products amplified using BAC end-derived primers (Barker et al. 2005). However, the sixth BAC containing the characteristic RFLP fragment (97A19; Fig. 4) did not overlap the main GLP1-12-containing contig, despite the fact

that primers designed using BAC end sequence from this clone (CB33.34) could be used as a PCR-based marker that co-segregated with *Run*1 and *Rpv*1. Therefore, both the main GLP1-12-containing contig and 97A19 were used to initiate chromosome walks.

Extension of the main GLP1-12-containing contig allowed identification of five overlapping BAC clones (7D20, 19G13, 87P7, 130N7 and 134D9) that contained additional GLP1-12-hybridising sequences. By RFLP analysis of PCR products amplified from all GLP1-12-hybridising BACs in the contig, Barker et al. (2005) showed that this region contained at least four members of a gene family. PCR-based markers designed from BAC end sequences were used to identify additional overlapping BACs. Although none of the BACs added to the contig beyond 87P7 contained GLP1-12-hybridising sequences, the contig eventually spanned the region between GLP1-12 and the SSR marker, VMC4f3.1, providing evidence that these two markers were adjacent in the genome.

No clones were present in our BAC library that could extend the main GLP1-12 contig beyond 61N18. Similarly, no clones were identified that overlapped the right-hand BAC end of 97A19. However, two overlapping BACs were identified using the PCR-based marker CB33.34 that had been designed from the left-hand BAC end sequence of 97A19. A further 16 clones were added to this contig, resulting in an assembly of 19 BAC clones that formed a contiguous region from 97A19 to 86E16. This region contained both the AFLP marker EMbd4, as determined using primers designed from the cloned AFLP sequence, and the RFLP marker Sfd3.

Genetic mapping using BAC-derived markers

A large number of new genetic markers were identified during the physical mapping process. Therefore, all Mtp3294, Mtp3322 and Mtp3328 progeny that had been identified as recombinant between VMC4f3.1 and Shb1 were re-tested using the panel of new BAC end sequence-derived markers. This identified recombination breakpoints in 3328-422 and 3328-174 between VMC8g9 and Shb1, which allowed the orientation of the VMC8g9 contig to be determined. The recombination breakpoint in 3322-42 was located between markers MHD145 and CB292.294, which allowed

orientation of the MHD145 contig and confirmed its placement between Sfd3 and VMC8g9 (Barker et al. 2005). The recombination breakpoint in 3294-R37 was located between CB314.315 and CB407.471 which, when taken together with the information obtained from 3322-42, suggests that *Run1* and *Rpv1* are located in the interval between CB314.315 and CB292.294 (Fig. 4).

Technical issues associated with the chromosome walking approach

Gaps in the BAC Library

Despite the fact that our BAC library was predicted to contain 10-fold haploid genome coverage, it proved impossible to join the three major contigs containing VMC4f3.1, GLP1-12, Sfd3 and MHD145 (Fig. 4). The BAC library contained 49,920 clones generated using *Hind*III and 5,378 clones generated using *Bam*HI. Therefore, gaps in the library could have been caused by bias in restriction site distribution as might occur in centromeres, telomeres and highly repetitive regions. For this reason it has been suggested that BAC libraries should be generated from genomic DNA that has been digested with number of different restriction enzymes (Wu et al. 2004; Suetsugu et al. 2007), or randomly sheared (Osoegawa 2007), to improve representation of problematic and repeat-rich regions at telomeres and in centromeric heterochromatin.

To supplement our BAC library we made two additional libraries using genomic DNA from the same powdery mildew-resistant plant that had been used for BAC library construction. For both libraries, nuclei were prepared according to Peterson et al. (2000). The genomic DNA was then either mechanically sheared and cloned into a pWEB::TNC™ cosmid vector (Epicentre, Madison, USA) or partially digested with *Sau*3AI and cloned into a λBlue STAR lambda vector (Novagen, Madison, USA) according to the individual manufacturer's protocols. The cosmid library contained 148,400 clones with an average insert size of 35.3 kb and the lambda library contained 395,600 clones with an average insert size of 12 kb. However, despite the fact that these two libraries contained approximately 11-fold and 10-fold genome coverage respectively, when the libraries were screened with BAC end sequences for which no overlapping BAC clones could be found, these sequences were also missing from the cosmid and lambda libraries. This suggested that the lack of representation of

clones in this region might be caused by clone instability or lethality, rather than restriction enzyme bias.

Evidence of clone instability was obtained when the gap in the GLP1-12 contig was examined in more detail. When the BAC library was screened with the BAC end marker CB55.56 (derived from clone 61L18) to find overlapping BACs, only one additional clone was identified in the library. This BAC clone, 82O20, contained an insert of 31.5 kb which was significantly smaller than the average BAC library insert size of 93 kb. Upon sequencing 82O20, it became apparent that while it contained large regions of homologous sequence to 61L18, these two clones did not overlap (Fig. 5). The BAC library was rescreened with a new marker designed to the end of 82O20 (CB63.64) and this identified BAC clone 61N18 which was also unusually short (31.4 kb) and which again did not overlap. A further marker (CB90.488) was designed to the end of 61N18. This marker did not identify any new clones in the BAC library, but a 40 kb clone (Cos106A) was identified in the cosmid library. Sequence comparison of BAC clones 61L18, 61N18, 82O20 and cosmid clone Cos106A indicated the presence of large regions of DNA sequence that is locally repeated in the *M. rotundifolia*-derived introgression (Fig. 5).

The small insert size of BAC clones 82O20 and 61N18 compared to the average insert size of our library also suggested that these clones contained sequences that have caused rearrangement and deletion during culture. Cosmid clone Cos106A also proved to be highly unstable and the estimated 40 kb insert in this clone would spontaneously and unpredictably revert to an approximately 15 kb insert in culture. Sequencing of this clone proved to be extremely difficult and assembly was hampered by the presence of numerous repeated sequences, including a partial tandem repeat 45S rDNA unit (Fig. 6). Eukaryotes generally have many copies of rDNA organised in tandem repeats (Lafontaine and Tollervey 2006) and it has been shown that BAC clones containing tandemly repeated DNA elements are not stable and can undergo drastic deletions during routine library maintenance and DNA preparation (Song et al. 2001). The presence of these tandem repeat units within the genome may therefore explain at least some of the gaps observed in the contig assembly.

Lack of Correlation between Physical and Genetic Distance

Our genetic mapping studies indicated that the *Run1/Rpv1 locus* is located in the interval between the SSR markers, VMC4f3.1 and VMC8g9. These two markers were placed just 0.6 cM apart in the local genetic map of VRH3082-1-42, the resistant parent of the Mtp3294 mapping population (Fig. 3; Barker et al. 2005), suggesting that either the markers were located physically close to one another in the grapevine genome, or that recombination rates were unusually low in this region. Riaz et al. (2004) and Adam-Blondon et al. (2004) have produced genome linkage maps for *V. vinifera* that span 1728 cM and 2200 cM, respectively. Given that the grapevine genome has been estimated at 475 - 487 Mb (Lodhi and Reisch 1995; Jaillon et al. 2007), this predicts that on average 1 cM is equivalent to 216-282 kb. Thus, the interval between VMC4f3.1 and VMC8g9 should be approximately 130-169 kb. However, as contigs spanning well over 1Mb were constructed, this is clearly not the case. Indeed, the actual estimated physical distance between VMC4f3.1 and VMC8g9, based on alignments of the sequenced BAC contigs with the current version of the Vitis genome sequence (improved from Jaillon et al. 2007) is approximately 5.4 Mbp (Choisne, unpublished results).

Reduced recombination frequencies have been observed around resistance loci in wheat, barley and poplar where resistance has been introduced via an introgressed region from a related species (Wei et al. 1999; Stirling et al. 2001; Neu et al. 2002). Therefore, the dramatically reduced recombination rate in the vicinity of *Run1* might be due to the presence of divergent DNA contributed by the introgression from *M. rotundifolia*. The implication of low recombination frequencies in this region for future isolation of the *Run1* gene is that large populations may be required in order to obtain sufficient recombinant individuals to delineate the resistance locus with suitable precision.

Fine mapping of the *Run*1/*Rpv*1 locus and functional evaluation of candidate resistance genes

As the physical distance between VMC4f3.1 and VMC8g9 was clearly much greater than the genetic distance had suggested, extended mapping populations were examined to identify rare recombinants that would allow the *Run1* and *Rpv1* loci to be delineated with greater precision. To achieve this, a further

3,300 progeny of the Mtp3294 and Mtp3322 populations (Fig. 1) were generated and screened for recombination between the markers 49MRP1.P2, CB69.70 and CB137.138 (Fig. 4) using a high-throughput multiplex PCR system (Anderson, in preparation). This identified 21 plants in which a recombination event was observed between the markers of interest. The reaction of these recombinant plants to powdery and downy mildew was determined, allowing the location of *Run*1 and *Rpv*1 to be localized to a ~ 1 Mb region of introgressed DNA. Sequencing of this region has revealed the presence of a cluster of 11 resistance gene analogues (RGAs) encoding TIR-NBS-LRR type resistance proteins which are very similar in structure to the *Mla* powdery mildew resistance genes isolated from barley (Zhou et al. 2001). Seven of the RGAs encode full-length proteins which are highly homologous apart from a variable number of leucine-rich repeat domains and real-time expression analysis indicates that all of the RGA candidates are expressed in resistant progeny. Full-length genomic clones including the encompassing the endogenous promoter and terminator regions of all seven RGA candidates are currently being transformed individually into powdery and downy mildew susceptible *V. vinifera* cultivars to determine which of the RGA candidates function as powdery or downy mildew resistance genes.

The future of positional cloning in grapevine

Manipulation of disease resistance is a major challenge to be met by grapevine geneticists (Bisson et al. 2002) and, as such, has been the first goal of fine mapping and map-based cloning approaches in grapevine (Table 2). In the future, we expect to see an increase in the application of positional cloning approaches for the identification of key genes involved in grapevine development (Fernandez et al. 2006) and adaptation to abiotic stress. Indeed, even in model species like Arabidopsis, where large collections of mutants are available which can be used to determine gene function, there are still many projects utilizing positional cloning techniques to gain an understanding of the genetic basis of trait variation (see as an example Loudet et al 2003; Mouille et al 2006).

Since the commencement of the *Run1*/*Rpv1* mapping project, the availability of genetic resources for grapevine has improved dramatically. Comprehensive genome linkage maps have been constructed (Adam-Blondon et al. 2004; Riaz et al. 2004) and a large number of SSR loci have been identified that are now the markers of choice for positional cloning projects due to their transferability between species. For example, Hoffmann et al. (2008) quickly localized a *V. vinifera* resistance gene to powdery mildew, *REN1*, in a 7.1 cM interval by screening 195 well scattered SSR markers by bulk segregant analysis. The release of the complete *V. vinifera* genome sequence (Velasco et al. 2007; Jallion et al. 2008) will also significantly increase the speed with which future genes of all types can be identified by positional cloning. However, as *V. vinifera* appears to be lacking resistance to many of the major pathogens, the published genome sequence can only act as a guide to the types of genes that may be present within the region of interest. Analysis of the *V. vinifera* genome sequence has revealed that many of the QTLs/genes for resistance which have been mapped to date to disease co-localise with clusters of NBS-LRR genes (Di Gaspero et al. 2007; Moroldo et al. 2008). However, identification of the alleles conferring resistance will still require sequencing of the genome in question.

This then leads to the question of the most cost-efficient way to obtain genomic sequence from the species containing the gene of interest ie. library construction / chromosome walking or whole genome sequencing? With the continued development of new, cost-efficient sequencing technologies, it may ultimately be quicker and cheaper to sequence an entire genome rather than construct a BAC library, assemble a contig and sequence individual clones, as we have done. Furthermore, as the new sequencing technologies do not require cloned DNA, this will reduce the risk of gaps occurring due to clone instability/lethality. Therefore it is possible that *Run*1 and *Rpv*1 may be the first and last grapevine genes isolated by chromosome walking. However, these new sequencing techniques are still not well suited to dealing with regions of highly repetitive DNA, which may still require construction of large insert libraries to resolve these regions.

Despite the tremendous improvements in genomic technologies over the last decade, the identification of unknown genes will still require significant resources and challenges in terms of the management of

large populations of perennial plants, accurate phenotyping of traits, the generation of recombinant genotypes within the region of interest and the issue of non-homogeneous recombination rates within the genome. Most projects aiming at the identification of disease resistance genes will involve generating integrated genetic and physical maps in non-*vinifera* species, which will also generate interesting information about genome evolution in the *Vitaceae* family.

Unlike most other genes isolated from grapevine which can be functionally evaluated in model plants such as Arabidopsis, tobacco or tomato which are readily transformable, NBS-LRR type resistance genes which make up the majority of resistance genes within a plant species, show "restricted taxonomic functionality" (Michelmore 2003) which means they are only function within species of the same taxonomic family. Furthermore, many biotrophic pathogens such as *E. necator* and *P. viticola* are specifically adapted to the grapevine host and will not infect model plant species. This means that functional evaluation of most cloned candidate resistance genes will require this evaluation to be undertaken in transgenic grapevines, the generation of which remains a time-consuming and inefficient process. The development of highly efficient transient assay systems involving agroinfiltration (Santos-Rosa et al. 2008) or particle bombardment will be crucial for the rapid evaluation of resistance gene candidates in grapevine.

Finally, an important decision for researchers to make in the future will be whether to devote time and energy into cloning and functionally characterising individual resistance genes which can be used for genetic transformation of existing cultivars or alternatively whether to concentrate on the development of tightly-linked markers for use in classical breeding approaches such as marker assisted selection. However, such a decision is more likely to be influenced by political and social issues associated with the adoption of transgenic vines for wine-making (discussed in Riaz et al. 2007; DeFrancesco 2008) rather than by the scientific merits of each approach.

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Table 1. Genotype of informative recombinant plants isolated from populations Mtp3294, Mtp3322 and Mtp3328. The presence of either the resistant (+) or susceptible (-) allele of each marker is shown.

		Marker								
Population	Plant	VMC1g3.2	MHD98	VMC4f3.1	GLP1-12	Run1/Rpv1	Sfd3	MHD145	VMC8G9	Shb1
3294	R11	+	-	-	-	-	-	-	-	-
3294	R102	+	-	-	-	-	-	-	-	-
3294	S100	+	-	-	-	-	-	-	-	-
3294	R149	+	+	-	-	-	-	-	-	-
3294	R5	-	-	+	+	+	+	+	+	+
3294	R104	-	-	+	+	+	+	+	+	+
3294	R37	-	-	-	+	+	+	+	+	+
3328	245	-	-	-	-	-	-	-	+	+
3322	42	-	-	-	-	-	-	-	+	+
3328	174	-	-	-	-	-	-	-	-	+
3322	422	-	-	-	-	-	-	-	-	+

Table 2. Fine mapping projects published so far in grapevine.

Locus	Trait	Mapping population	n° individuals	Marker types	Interval between the closest markers	Reference
Run1/ Rpv1	Resistance to powdery & downy mildew	See Fig. 1	996	AFLP CAPS RFLP PCR SSR	0.6 cM	Barker et al. 2005
Pdr1a	Resistance to Pierce disease	V. vinifera x (V. rupestris x V. arizonica)	425	SSR	0.6 cM	Riaz et al. 2008
Pdr1b	Resistance to Pierce disease	V. vinifera x (V. rupestris x V. arizonica)	361	SSR	0.4 cM	Riaz et al. 2008
REN1	Resistance to powdery mildew	V. vinifera x V. vinifera	310	SSR	7.1 cM; 0.9 to the closest marker	Hoffmann et al. 2008

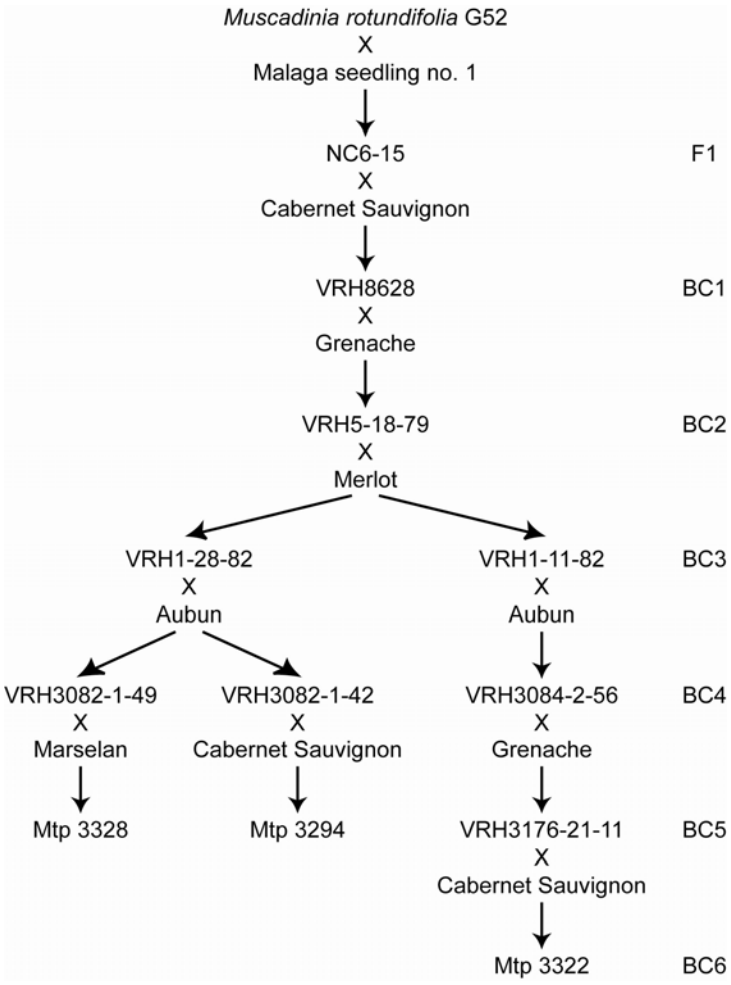


Figure 1. Parentage of mapping populations used to map the powdery and downy mildew resistance genes introgressed from *M. rotundifolia* cv. G52. BCn represents the pseudo-backcross step at which the individual was obtained.

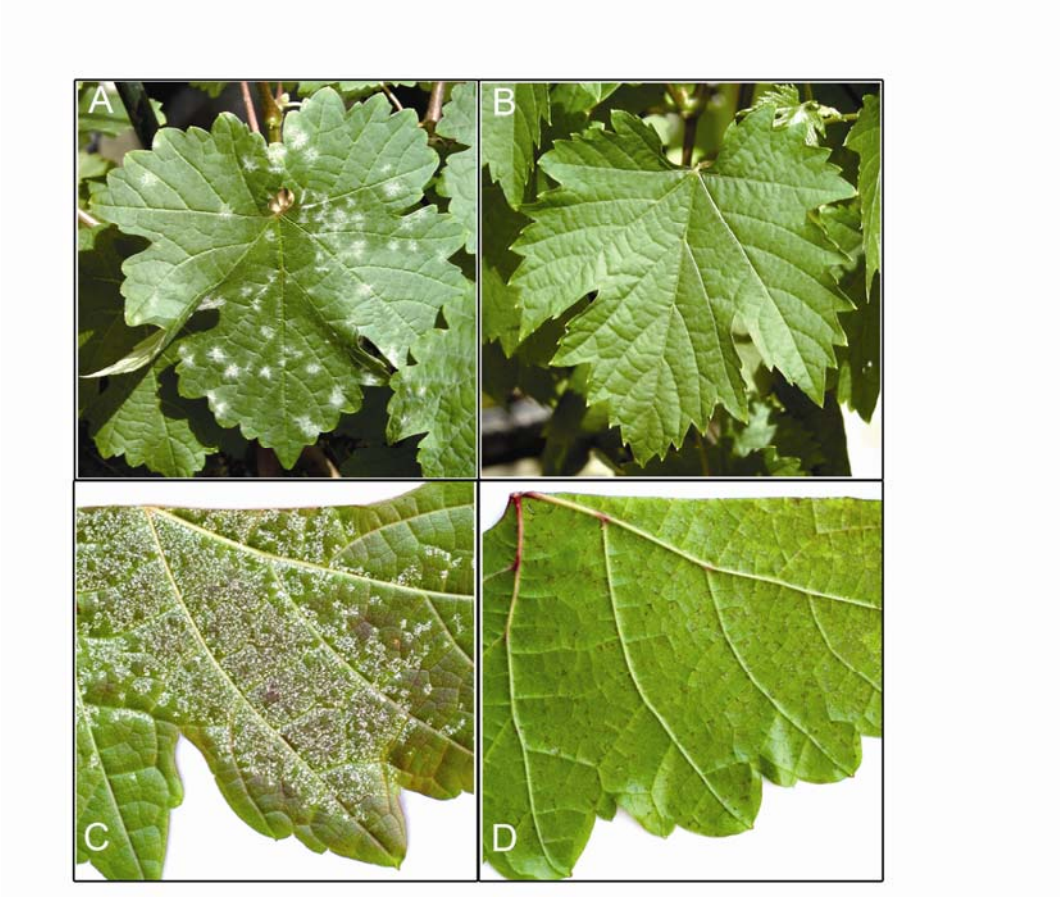


Figure 2. Comparison of powdery mildew (A, B) and downy mildew (C, D) susceptibility of leaves from *V. vinifera* cv. Cabernet sauvignon (A, C) and from *M. rotundifolia* x *V. vinifera* 4th generation back-cross (B, D). Powdery mildew growth is completely suppressed on the BC4 plant but a low level of downy mildew sporulation is supported on BC4 leaves. Patches of dead cells which have undergone programmed cell death in response to downy mildew infection are visible in panel D.

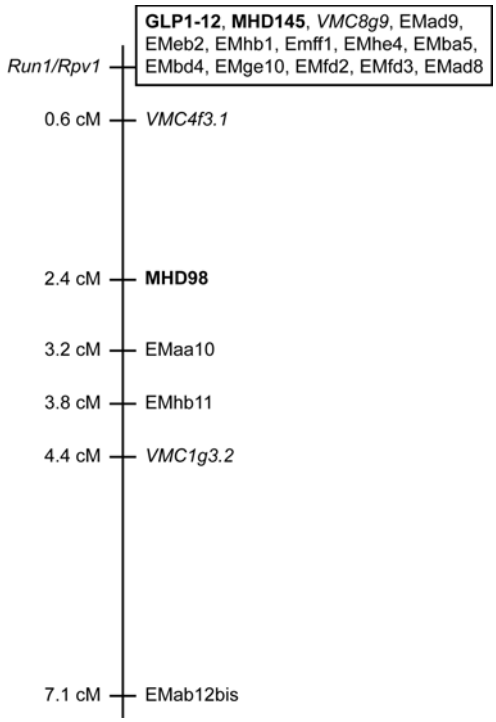


Figure 3. Genetic map of VRH3082-1-42 based on screening 160 individuals of Mtp3294 showing the location of SSR, AFLP and RGA markers linked to *Run*1/*Rpv*1 locus. RGA markers are shown in bold, SSR markers in italics.

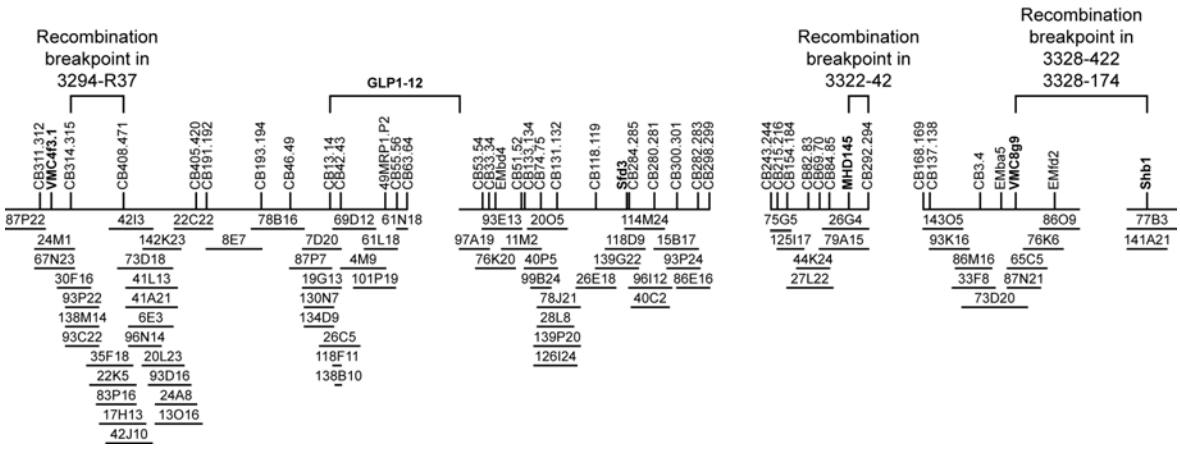


Figure 4. A physical map of the region surrounding the *Run1*/*Rpv1* locus. BAC clones were identified that contained genetic markers linked to *Run1*. Clones were assembled into contigs representing the resistant chromosome and extended by identification of overlapping BACs. Genetic markers used to initiate contigs are shown in bold and the location of multiple GLP1-12-hybridising sequences is bracketed. Unique genetic markers that facilitated contig assembly are shown and the approximate location of the recombination breakpoints found in four plants from the Mtp3294, Mtp3322 and Mtp3328 mapping populations are indicated.

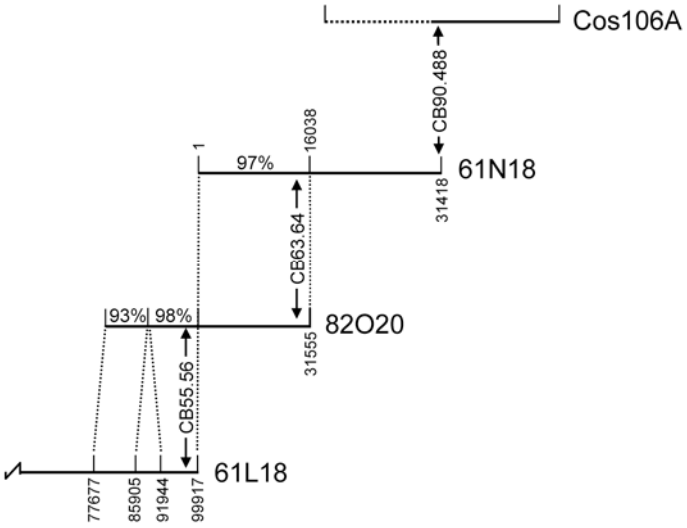


Figure 5. Homology of BAC and cosmid clones that contain repeated DNA. The identity and position of primers used to screen for overlapping clones (as outlined in the text) are shown by arrows. The percentage nucleotide identity and position of homologous regions within the clones are indicated. Only approximately half of Cos106A (solid line) could be successfully sequenced. Primers CB90.488 amplified a 1kb fragment from Cos106A that was 99% identical to the sequence from 61N18. BAC clones 61L18 and 61N18 were subsequently demonstrated to be adjacent within the genome as shown in Fig. 4.

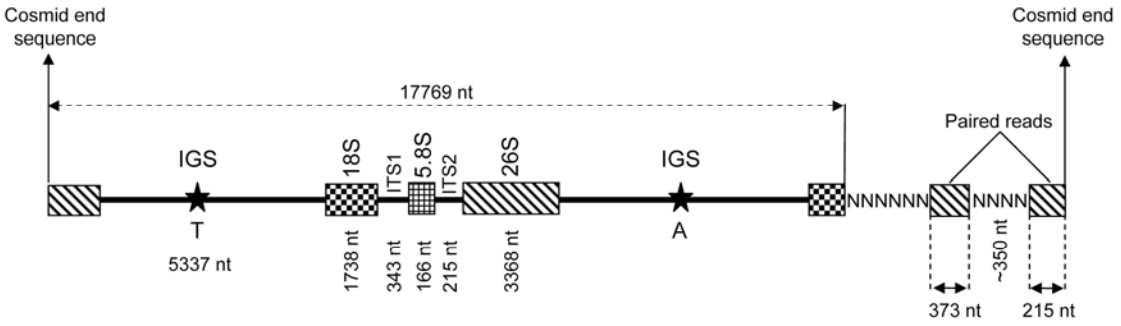


Figure 6. Sequence assembly of Cos106A using PHRED, PHRAP and in-house Genoscope programs taking into account paired reads and a manual curation of the repeated sequences. The sequence obtained corresponded to a partial tandem repeat of a 45S rDNA unit. N's indicate where joining sequence could not be resolved. Abbreviations: IGS: InterGenic Spacer; ITS: Internally Transcribed Spacers.