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Identification and marker-assisted selection of genes for reducing the susceptibility of new winegrape cultivars to fungal pathogens

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WINE AUSTRALIA

Project Number: **CSP 1301**

Principal Investigators: **Dr Ian Dry, Dr Harley Smith & Dr Mark Thomas**

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Author Details: Dr Ian Dry, Dr Harley Smith & Dr Mark Thomas
CSIRO Agriculture & Food

Postal address: Locked Bag 2, Glen Osmond, SA 5064, Australia

Phone: 08 8303 8632
Fax: 08 8303 8601
Email: ian.dry@csiro.au

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Table of Contents

1. Abstract

Worldwide winegrape production is highly dependent on the frequent use of fungicides which are costly for growers and have negative impacts on the environment. These issues could be minimised, or even eliminated, by the breeding of new winegrape varieties with reduced susceptibility to powdery mildew, downy mildew and botrytis. We have employed marker-assisted selection technology, in combination with the rapid flowering microvine mutant, to rapidly introgress powdery and downy mildew resistance genes from wild Chinese Vitis species into *V. vinifera* breeding lines which will be used to develop second generation mildew resistant premium winegrape varieties with increased durability of resistance in the field.

2. Executive summary

The two most economically important grapevine diseases worldwide are powdery mildew (PM) caused by the fungus *Erysiphe necator* (syn. *Uncinula necator*) and downy mildew (DM) caused by the oomycete *Plasmopora viticola.* The cultivated winegrape*, Vitis vinifera*, has little or no genetic resistance to these pathogens. As a result, control of these diseases is entirely dependent on the widespread application of fungicides. In addition to the economic cost of disease control, there is also increasing pressure to reduce agrochemical use for the control of plant pathogens on crops grown for human consumption.

This project has continued the research initiated in the previous Wine Australia project CSP 0904 *Advanced grapevine genetics for varietal improvement* in which the *RUN1/RPV1* locus from the wild North American grapevine *Muscadinia rotundifolia*, that confers strong resistance to both powdery and downy mildew, was successfully introgressed into premium winegrape varieties by marker-assisted selection. A major aim of this current project has been to develop germplasm resources and genetic markers that will facilitate the development of second generation mildew resistant winegrape varieties in which PM and DM resistance loci from different wild species have been combined to increase the durability of the resistance in the field.

To achieve this, we have targeted the introgression of mildew resistance loci from wild Chinese Vitis species. However, in order to remove any potentially deleterious quality traits that may also be introgressed from these wild species and to meet Australian quarantine requirements regarding the permitted release of hybrids generated from imported wild Vitis species, we have undertaken a backcrossing program to reduce the component of the genome from the wild species. To speed up this backcrossing program we have successfully employed marker-assisted selection (MAS) in combination with a unique rapid grapevine breeding system based on the *V. vinifera* Pinot Meunier microvine mutant.

We have successfully generated microvine lines containing the *REN4* PM resistance locus from *V. romanetii* and the *RPV12* DM resistance locus from *V. amurensis* and these two loci have further been combined within the same microvine breeding line. It is anticipated that these *REN4*/*RPV12* microvine lines will be used as parents in future crosses with selected first generation premium winegrape varieties containing the *RUN1*/*RPV1* locus to produce the second generation mildew resistant winegrape varieties with dual PM and DM resistance loci. As part of this project, we have also fine mapped the chromosomal position of the *REN4* and *RPV12* loci and this has led to the identification of tightly linked DNA markers that will be used to verify the inheritance of all four mildew resistance loci in the future selection of the second generation mildew resistant winegrape varieties.

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

Botrytis bunch rot also continues to be a major problem for winegrape production. This is especially the case in cool climate regions where there is a high chance of rain around harvest, which can lead to serious bunch rot infections epidemics. As there are no known single dominant resistance genes to *B. cinerea* in any known plant species we cannot use the same strategy for generating disease-resistant varieties as we have used for powdery and downy mildew. Winegrape varieties with tight bunches, such as Riesling and Chardonnay, are the most susceptible to botrytis bunch rot, most likely due to prolonged water retention within the bunch after rain events. Previous studies have shown that a reduction in bunch compactness will significantly reduce the incidence and severity of botrytis bunch rot in the vineyard.

Thus, the second major aim of this project has been to identify major quantitative trait loci (QTLs) responsible for regulating internode length during berry cluster development. This would enable the development of genetic markers that can be incorporated into our marker-assisted selection process to identify progeny that will have more open bunches. In this way, the second generation vines will not only have increased resistance to powdery and downy mildew but also reduced susceptibility to bunch rot.

Our results confirmed that there is a high genetic component for the heritability of rachis internode development in lateral branches which has a major influence on bunch architecture. However, with the mapping populations available to us, we were unable to identify major QTLs for bunch architecture. One possible explanation for this outcome is that the mapping populations we employed were too small i.e. ranging in size from 56 - 101 individuals. Using populations with > 1000 individuals would have a much higher rate of success, because internode development is likely to be controlled by many genes. CSIRO has approximately 500 varieties of table and wine grapes in its germplasm collection, which display significant variation in berry size, bunch architecture and fruit quality traits. Thus, a genome-wide association analysis may be more suitable approach to identifying loci and markers linked to bunch architecture than biparental mating.

Acknowledgments

The authors of this report would like to thank CSIRO staff Angelica Jermakow, Debra McDavid, Nayana Arunasiri and Maria Mrinak for valuable contributions to the project.

3. Background

Winegrapes are probably one of the few major crops in the world that have not undergone any significant genetic improvement for nearly 100 years. This is demonstrated by the fact that all of the major wine varieties grown in Australia (and for that matter most of the wine-growing regions of the world) were first mentioned in historical texts over 200 years ago (Robinson et al., 2012). The two major pathogens of cultivated grapevines today, grapevine powdery mildew (*Erysiphe necator* syn. *Uncinula necator*) and downy mildew (Plasmopora viticola) did not arrive in Europe from North America until after the 1850s (Campbell 2004). Consequently, none of our major winegrape varieties have any natural genetic resistance to these two pathogens, meaning that grape producers rely on the frequent use of agrochemicals to minimise the potentially devastating impact of these pathogens on grape yield and quality. It has been estimated that the costs of disease management and yield losses to the Australian wine industry (based on 2009 figures) are in the order of A$76 million and A$63 million per year for powdery mildew and downy mildew, respectively (Scholefield and Morison 2010). Not only does this translate into increased production costs for growers, but there is also the potential impact of these chemicals on the health of beneficial organisms in the vineyard (Gadino et al., 2011) and vineyard workers (Le Moal et al., 2014), as well as increased carbon emissions generated from their frequent application. Many of these issues could be minimised or completely overcome by the development of winegrape varieties with enhanced genetic resistance to powdery and downy mildew.

In a previous Wine Australia project CSP 0904 *Advanced grapevine genetics for varietal improvement* a BC5 breeding line (BC5:3294-R23) containing the *RUN1*/*RPV1* locus that confers strong resistance to powdery and downy mildew, was used as a parent in crosses with a number of premium white and red varieties. DNA marker analysis identified approximately 1200 seedlings containing the mildew resistance locus and these were planted in an unsprayed block in the Barossa Valley to evaluate resistance to naturally-occurring powdery and downy mildew infections, vine performance and wine style/quality. In terms of disease resistance, we are yet to observe any visual signs of powdery mildew or downy mildew infection on these vines, even though they have not been sprayed since they were planted over eight years ago.

Our ultimate aim is to select the 20 best mildew-resistant white and red varieties, based on consistent performance over a number of growing seasons in terms of good yields, good acid and pH at harvest and consistently high sensory scores as judged by commercial winemakers. Once these elite selections have been made, vines will be propagated for evaluation of performance and wine quality in other Australian wine-growing regions. The mildew-resistant winegrape varieties currently under evaluation in the Barossa Valley represent only the first generation of new premium mildew-resistant varieties selected for Australian conditions.

A major focus of this current project (CSP 1301) has been on the development of breeding resources to facilitate the production of generation premium winegrape varieties with further enhanced disease-resistance properties. This is a consequence of the fact that pathogens, such as powdery and downy mildew, are capable of undergoing rapid mutation in the field to produce new isolates that are capable of breaking the resistance conferred by single resistance genes such as *RUN1* and *RPV1*. This is commonly observed in broad-acre crops where large areas are sown to the same genotype, thereby creating a strong selection pressure for the evolution of pathogen isolates that are virulent on the resistant crop (McDonald and Linde 2002). Although such a situation is much less likely to occur within grapegrowing regions because of the diversity of grapevine varieties commonly grown within each region, we already have evidence of the evolution of isolates of both powdery mildew (Feechan et al., 2013) and downy mildew (Peressotti et al., 2010) that are capable of breaking the resistance conferred by loci from wild grapevine species. While this may not be catastrophic for a wheat farmer who can choose to plant one of a number of alternative wheat varieties, with different resistance genes, in the following season, it would be a major setback for a grapegrower who clearly does not have such flexibility.

One of the most effective ways to increase the durability of resistance genes in the field is to combine or 'pyramid' resistance genes, from different wild species, within the same plant (Mundt 2014). This is because major plant resistance proteins, such as RUN1 and RPV1, are activated by the recognition of specific proteins called 'effectors' that are secreted into the plant cell by the invading pathogen. Activation of the host resistance protein initiates a highly effective defence response within the plant cell that prevents further infection. Thus, if a mutation occurs in an effector that is normally recognised by the plant resistance protein, such that recognition can no longer take place, a defence response will not be initiated upon infection and the pathogen will be able to colonise the plant. Generally, resistance genes from different wild plant species have evolved to recognise different pathogen effector proteins. Thus, by combining resistance genes from different wild species, the likelihood of a single pathogen isolate simultaneously mutating both effectors which are recognised by the two different resistance proteins, is extremely low. While it has been recognised since the late 1800s that wild North American grapevine species represent an important source of resistance against a range of major grapevine pathogens, we are only now becoming aware that many wild Chinese grapevine species also contain major resistance loci that confer strong resistance against powdery mildew. As such, these resistance loci may represent good candidates for pyramiding with the *RUN1*/*RPV1* locus in our first generation mildew-resistant premium selections to produce second generation varieties with enhanced resistance durability.

However, introgressing resistance genes from wild Vitis species runs the risk of also introducing potentially deleterious quality traits often associated with non-vinifera genotypes. To remove any potentially negative genes the accepted strategy is to undertake a number of backcrosses with *V. vinifera* progeny to dilute out the contribution of the non-

vinifera genome (see Fig. 1). Using traditional breeding methods, each backcross generation is likely to take 2-3 years and a significant amount of cost and labor associated with establishing vines in the field. To overcome this bottleneck, we are currently employing marker-assisted selection (MAS) in combination with a rapid grapevine breeding system. This rapid breeding system uses a natural grapevine mutant known as a microvine (Chaib et al., 2010) that fruits rapidly, flowers prolifically and can be grown in a glasshouse making it possible to carry out breeding experiments all year round.

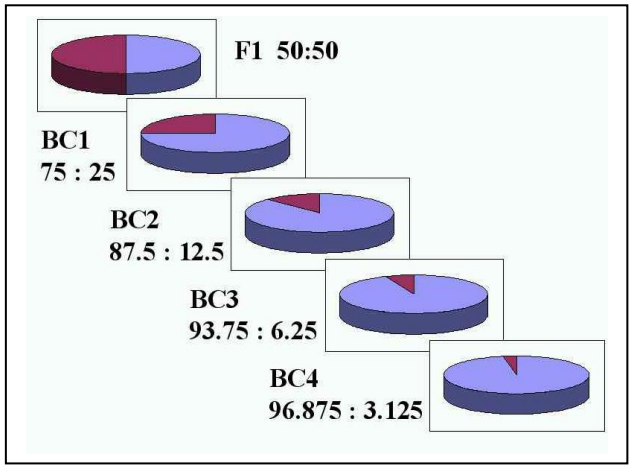

Figure 1. *The contribution of the donor parent genome is reduced by half with each generation of backcrossing. Percentages of recurrent parent (light purple) are expressed as a ratio to percentages of donor parent (dark red-purple). Image credit: David Francis, Ohio State University.*

Botrytis bunch rot, caused by the fungus *Botrytis cinerea*, continues to be a serious problem for winegrape growers, especially in cool climate regions. In many situations, *Botrytis* infection may be adequately controlled by the application of fungicides. However, if infection occurs close to harvest it may not be possible to apply fungicides because of the Maximum Residue Limits applicable in many markets, and spray penetration is always a problem with tight bunches.

As there are no known single dominant resistance genes to *B. cinerea* in any known plant species we cannot use the same strategy for generating disease-resistant varieties as we have used for powdery and downy mildew. However, there is good evidence to show that the susceptibility of different grape varieties to botrytis bunch rot, is closely correlated with bunch architecture and that reducing bunch compactness will significantly reduce the incidence and severity of botrytis bunch rot in the vineyard (Marois et al., 1986; Ferreira and Marais 1987; Vail and Marois, 1991; Percival et al., 1993; Smithyman et al., 1998; Vail

et al., 1998). This heightened susceptibility in tight bunches is most likely due to the combined effects of increased water retention and prolonged drying after rain events (Vail and Marois, 1991).

A number of studies have been carried out which demonstrate that a significant reduction in botrytis bunch rot infection can be achieved by physically or chemically modifying bunch compactness. Treatments have included the application of gibberellic acid at flowering (Weaver, 1962; Hopping, 1975; Ari et al., 1996), hand thinning (Barbetti 1980) and specific vine management systems (Zabadal and Dittmer 1998, Smithyman et al., 1998).

However, in many of these treatments, bunch compactness was reduced through decreases in fruit set. While this has significant benefits in terms of reduced susceptibility to bunch rot, it imposes a significant yield penalty. A preferable approach would be to use a genetic strategy to reduce bunch compactness in susceptible varieties by altering bunch architecture.

Thus, the second major focus of this project has been to identify major quantitative trait loci (QTLs) responsible for regulating internode length during berry cluster development. This would enable the development of genetic markers that can be incorporated into our marker-assisted selection process to identify progeny that will have more open bunches. In this way, the second generation vines will not only have increased resistance to powdery and downy mildew but also reduced susceptibility to bunch rot.

4. Project Aims and Performance targets

Project aims:

1. Development of *V. vinifera* breeding material containing new powdery and downy mildew resistance loci from genetic sources other than *Muscadinia rotundifolia*.
2. Development of genetic markers tightly linked to these new powdery and downy mildew resistance loci that can be used for marker-assisted selection of second generation mildew resistant vines.
3. Identification of quantitative trait loci and candidate genes responsible for regulating internode length during berry cluster development.
4. Development of genetic markers for marker-assisted selection to predict berry cluster architecture in future grapevine breeding programs.

5. Materials and Methods

Plant and fungal material

Potted *V. vinifera* cv. Cabernet Sauvignon vines were grown in glasshouses at Waite Campus maintained between 23-25 ˚C. To establish the VrBC2 population, seeds were surface sterilised by soaking in fresh 0.5M hydrogen peroxide solution overnight with gentle shaking (approximately 10 seeds per 8mL of peroxide). The next day seeds were thoroughly rinsed in sterile water (4 x washes) before treating with a 2.6M solution of gibberellic acid (GA). A 2 x seed volume of GA was added before gentle shaking overnight. Seeds were then thoroughly rinsed in sterile water (4 x washes) before transferring to sterile 50mL falcon tubes containing sterile cotton for long-term cold storage. Seeds were stored for a minimum of three weeks at 4 ˚C before germinating. Seeds were then germinated in petri dishes on moist filter paper in a growth room at 27 ˚C with 16 h light *Erysiphe necator* (isolate APC1) culture and inoculations were performed as previously described (Donald et al., 2002). *Plasmopora viticola* inoculum was collected from an experimental vineyard on the Waite Campus, Adelaide and maintained on discs cut from *V. vinifera* cv. Cabernet Sauvignon leaves.

For bunch architecture studies, immature inflorescences and mature bunches were isolated from two Pinot Meunier and Pinot Meunier microvine/*Vvgai* vines. For each vine, four immature inflorescences at stage 12 were harvested (Pearce and Coombe, 2004). The first and second internode along the main rachis was dissected for RNA extraction. Five mature bunches were isolated from Pinot Meunier and Pinot Meunier microvine/*Vvgai* and the berries were removed to quantify internode development.

Powdery mildew resistance screening

Staining of fungal structures was performed as described previously (Koch and Slusarenko, 1990). Visualisation and scoring for programmed cell death (PCD) for at least 100 germinated powdery mildew conidia per replicate were performed as described previously (Feechan et al., 2011).

Downy mildew resistance screening

Freshly cut leaf discs were placed onto moist filter paper in Petri dishes abaxial side up and inoculated with a 20 µL droplet of *P. viticola* suspension (5×10^4 sporangia/mL) overnight. After the droplet was removed, the leaf discs were maintained in a temperature-controlled incubator at 22-23 ˚C with a 16 h light / 8 h dark light cycle. A minimum of two replicates were performed for each individual of the mapping population and 10 discs for each replicate. Leaves were scored using the OIV 452 descriptor scale. A quantitative evaluation of sporulation was obtained six days after inoculation by measuring the number of

sporangia per disc for each plant with a Fuchs-Rosenthal Haemocytometer or a Beckman Z2 Coulter Counter.

Embryo rescue of microvine seedlings

Natural germination rates of seeds derived from crosses involving picovine/microvine parents are low and intervention is required to maximise seedling recovery. The embryo rescue method was adapted from Chatbanyong and Torregrosa (2015). Approximately 100 seeds were placed into a 50 mL falcon tube containing 0.5M hydrogen peroxide solution and left shaking overnight at 100 rpm. Next morning, seeds were rinsed four times with sterile water. Using a scalpel blade, seeds were split half along the ridge exposing the embryo. Seeds were then placed onto half-strength Murashige and Skoog (½MS) medium supplemented with activated charcoal (2.5 g/L) and antibiotics (200 mg/L) of Timentin and Cefotaxime sealed with parafilm, covered with aluminium foil and left in a growth room at 27 ˚C. After approximately three weeks, embryos were transferred to small tubs containing half strength MS for approximately three weeks. Plants destined for the glasshouse were subcultured onto rooting medium (Iocco et al., 2001) to initiate a more robust root system before transplanting into the glasshouse.

Identification of Single Nucleotide Polymorphisms (SNPs) by Genotype-by-sequencing analysis

Genotype-by-sequencing (GBS) was used to identify novel SNP genetic markers linked to the *REN4* and *RPV12* loci for MAS. DNA was first extracted from grape leaf tissue by Australian Genome Research Facility (AGRF), Adelaide and diluted to 10ng/µL and sent to the Cornell University Biotechnology Resource Centre. GBS analysis was undertaken on the following genotypes: a parental *REN4* line (VrBC2-2), microvine line 06C008V0003, 12 VrBC2 progeny lines, *V. vinifera* cv. Riesling and the *V. amurensis* parental line used for generating the VaF1 population. After DNA sample preparation, Illumina HiSeq 2000/2500 was used to sequence the genotypes. The raw sequence data was filtered and aligned to the grape reference genome - French-Italian Public Consortium (PN40024). Using the TASSEL= GBS pipeline, v3.0.166 (Glaubitz et al., 2014). SNPs are then identified from the aligned tags and scored based on depth of coverage and genotypic statistics.

As we already knew the approximate physical locations of *REN4* and *RPV12*, only SNPs in those regions were considered for the development of the SNP panel. Typically, we looked for SNPs that were heterozygous for the resistant plants and were not adjacent to other SNPs that could interfere with the design of the multiplex panel assays. Using the TASSEL files, we were able to manually locate the SNP from the 100 nucleotide sequence reads and align it to the grape reference genome using BlastN (NCBI) to get obtain additional genome sequence required for SNP panel design.

Selected SNPs and their surrounding sequences were submitted to AGRF for multiplex pipeline design which allows up to 50 SNPs to be tested in a single reaction. SNPs which fail to pass the design stage are omitted from the list and those that pass are used for initial testing on the supplied DNA. Some SNPs can also fail the initial testing and those that are left make up the SNP panel used for genotyping. Despite GBS identifying a SNP in a sequence that could be informative for that population, upon analysis a number are found to be homozygous for both resistant alleles which make that SNP marker unusable. Table 1 summarises the attrition rates of SNPs used for *Ren4* and *Rpv12* panels. SNP analysis was completed by AGRF, Brisbane using their Agena Bioscience MassARRAY platform.

Table 1. *Summary of SNP attrition from the initial SNPs submitted for panel design to those being informative for mapping the resistance loci.*

	***REN4* SNP panel**	***RPV12* SNP panel**
SNPs sent to AGRF	60	62
Passed design stage	54	59
Passed first analysis	47	57
Informative SNPs	32	22

Single Sequence Repeat (SSR) marker analysis of segregating populations

DNA extractions were carried out ~50 mg young leaf tissue by AGRF, Adelaide. SSR analysis was carried out by AGRF, Adelaide. Diluted DNA (10ng/µL) and fluorescently-labelled oligos were supplied for analysis.

Cleaved Amplified Polymorphic Sequences (CAPS) marker analysis

Genomic DNA for CAPS marker analysis was extracted from approx. 20 mg leaf tissue according to the method of Zhang et al. (1998). PCR reactions contained: gDNA 10ng/µL, 0.7 µM forward primer, 0.7 µM reverse primer, 1.25 units DNA Taq polymerase in a total volume of 15 µL. PCR cycling conditions were as follows: 95 ˚C for 1 minute, followed by 34 cycles of 95 ˚C for 15 s, 56 ˚C for 15 s, 72 ˚C for 25 s. Half of the reaction mixture was then digested for 2 h with the appropriate enzyme and then electrophoresed on 1.5 - 2% agarose gels to separate the digest products.

Histology

The first and second internode was dissected from R23, Grenache, Muscat Gordo Blanco, Exotic and Riesling at berry set. Internodes were isolated from six bunches for each genotype and immediately fixed in FAA (4% formaldehyde, 5% glacial acetic acid, 50% ethanol and 0.5% Triton X-100). After dehydration, the internode samples were embedded in Paraplast Plus®. Three samples were sectioned in a transverse to image the width of cells and cell length was examined in longitudinal sections in the remaining three samples. Internode sections were mounted on microscope slides and stained with Toluidine blue. Samples were imaged on a ZEISS Imager MI and cell number was determined using the Zen 2 Pro software. One-way ANOVA was used to examine the variance between all the varieties. Students T-test was used to examine differences between the parents from the crosses (R23 x Grenache, R23 x Muscat Gordo Blanco, R23 x Dunkelfelder and Exotic x Riesling).

Genetic mapping of bunch architecture traits.

For each F1-mapping population, GBS was used to identify SNPs for constructing genetic maps. DNA was extracted from the parents and F1-progeny genotypes for the R23 x Muscat Gordo Blanco (56 genotypes), R23 x Grenache mapping populations (65 genotypes) and R23 x Dunkelfelder (101 individuals). Sequencing and SNP calling was carried out at Cornell University Biotechnology Resource Centre as described above. SNPs were filtered by removing SNPs with missing data >20%, while retaining the remaining SNPs with an allele frequency between 40-60%. A pseudo-testcross mapping strategy (Grattapaglia and Sederoff, 1994) was used and genetic maps were constructed using R/onemap using the Kosambi function (Margarido et al., 2007). The total number of SNPs utilised for genetic map construction is displayed in Table 2 Genetic maps generated by R/onemap were used for mapping bunch architecture traits via R/qtl software (Browman et al., 2003). GBS was not performed with the Exotic x Riesling mapping population as it was deemed that more individuals were needed to successfully map bunch architecture traits.

Table 2 *GBS SNPs used in genetic map construction*

	Map 1		Map2	
Cross	**Parent**	**SNPs**	**Parent**	**SNPs**
R23 x Grenache	R23 (BC1)	4311	Grenache (BC2)	4242
R23 x Muscat GB	R23 (BC1)	4422	Muscat GB (BC2)	4384
R23 x Dunkelfelder	R23 (BC1)	2806	Dunkelfedler (BC2)	2749

BC1, backcross 1; BC2, backcross 2; Muscat GB, Muscat Gordo Blanco

Transcriptional analysis

RNA was extracted from dissected internodes from immature Pinot Meunier and Pinot Meunier microvine inflorescences using the Spectrum Plant Total RNA Kit (Sigma-Aldrich). RNA-Seq analysis was performed by the Australian Genome Research Facility Ltd. Single end reads were generated and aligned to the *Vitis vinifera* 12X genome. The EdgeR, DESeq and VOOM bioinformatic packages were used to identify differentially expressed genes using Galaxy.

For validation of RNA-Seq data, 10 differentially expressed genes and two gene standards were selected and primers designed (Appendix 5; Table 18). The SuperScript III cDNA synthesis system (Invitrogen) was used to generate single strand DNA templates for RT-qPCR. All cDNA samples were diluted 1:15 and RT-qPCR was performed suing the Roto-Gene RG3000 (Corbett Research).

6. Development of *V. vinifera* breeding material containing new powdery mildew resistance loci and identification of genetic markers tightly linked to these new resistance loci that can be used for marker-assisted selection

Introduction

A number of wild Chinese Vitis species have been reported to show strong resistance to powdery mildew, including *V. romanetii* (Wang et al., 1995; Wan et al., 2007). In a more recent study, Ramming et al. (2010) screened the progeny of *Vitis vinifera* × *V. romanetii* populations segregating for resistance to powdery mildew and determined resistance was conferred by a single, dominant locus designated *REN4*. In a previous Wine Australia project (CSP 0903) we established that *REN4* also conferred resistance to an Australian powdery mildew isolate. More importantly, we also demonstrated that grapevines containing the *REN4* locus are still resistant to the Musc4 powdery mildew isolate from North America that breaks *RUN1* resistance making it an excellent candidate for pyramiding with *RUN1* because it would extend the range of isolates to which these vines would be resistant and increase the durability of resistance in the vineyard. This chapter outlines the development of backcross breeding material containing the *REN4* locus and the identification of genetic markers to be used for the development of second generation disease-resistant winegrape varieties.

Results & Discussion

Generation of VrBC2 progeny

We previously outlined in the final report for Wine Australia project CSP 0903 that Dr David Ramming (USDA) had kindly undertaken a cross between the BC1 breeding line C87-14 (containing the *REN4* powdery mildew resistance locus) and *V. vinifera* cv. White Riesling (Fig. 3).

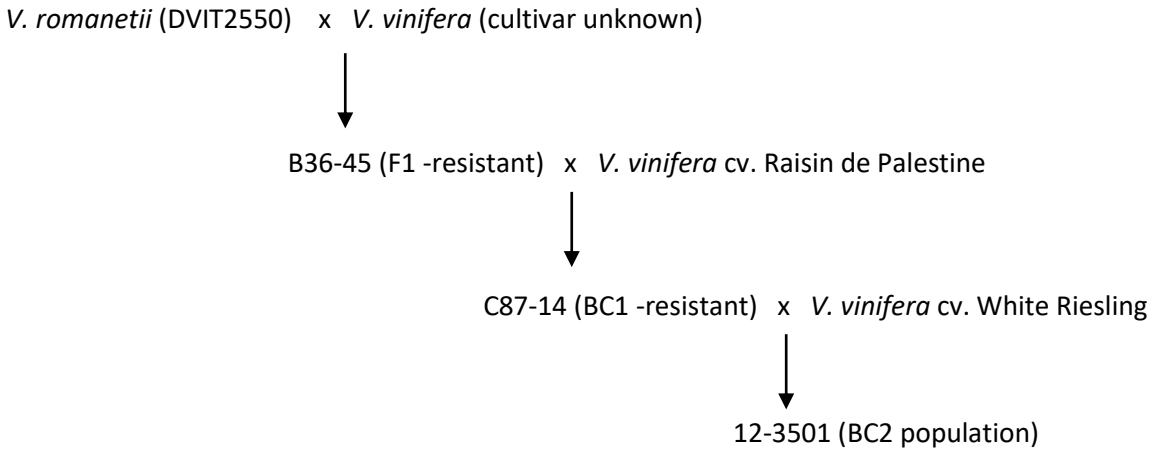

Figure 3. *Pedigree of V. romanetii BC2 population 12-3501 obtained from Dr David Ramming (USDA, California).*

The VrBC2 seed was imported and germinated in the CSIRO Agriculture & Food Quarantine glasshouse on the Waite Campus. A total of 75 seedlings were tested for powdery mildew resistance. Genomic DNA was extracted from each progeny plant and subjected to DNA typing analysis to confirm which progeny were the result of a backcross between C87-14 (BC1-*REN4* resistant) x *V. vinifera* cv. White Riesling. Approximately one third of the BC2 progeny were found to have resulted from self-pollination (Table 3).

Pollen Donor	**VrBC2 progeny**
White Riesling	38
Pinot Noir	1
Self-pollinated	36
Total	75

Table 3. *A summary of VrBC2 seed paternal origins. A total of 75 VrBC2 progeny were tested using the SSR markers VVMD7 and VVS2.*

When the selfed genotypes were excluded from the analysis, resistance was confirmed to be inherited as a single dominant locus (Fig. 4). Results of previous research on the *REN4* locus provided to us by Dr Lance Cadle-Davidson (USDA, Geneva) suggested that *REN4* was located on Chr 18. We therefore screened genomic DNA of the C87-14 (BC1-*REN4* resistant) and *V. vinifera* cv. White Riesling parents of the VrBC2 cross with a panel of 27 SSR markers located on Chr 18 (Doligez et al., 2006; Riaz et al., 2004). Ten of these markers were found to be polymorphic between the two parents and were subsequently used to screen the VrBC2 progeny (Fig. 5). The closest linked SSR marker to the *REN4* locus was found to be VMC7f2.

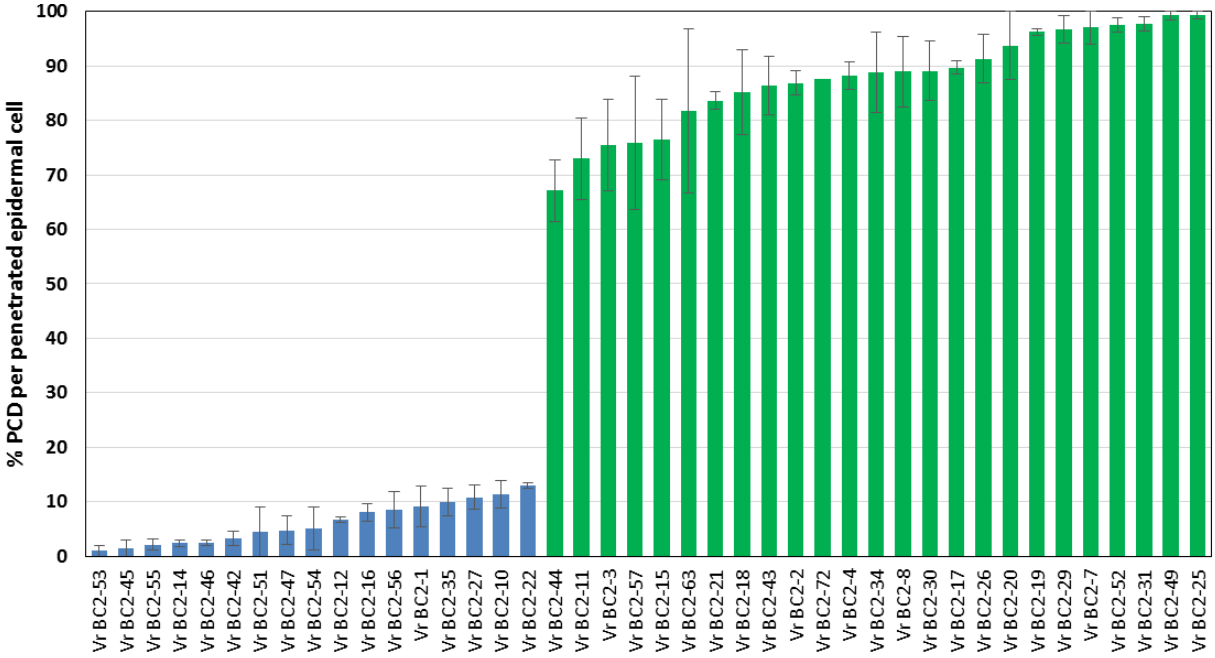

Figure 4. *Segregation of powdery mildew resistance in the VrBC2 progeny population. All data were collected 2 dpi and at least 100 germinated spores were scored following trypan blue staining for estimation of PCD. Each data point is the mean ±SE of at least two biological replicates.*

Selected VrBC2 lines (VrBC2-2, VrBC2-3 and VrBC2-8) that showed strong resistance to powdery mildew and were positive for the *REN4* locus, based on SSR marker VMC7f2, were clonally propagated and transported to the Plant Quarantine Facility, Knoxfield, Victoria, for post-entry quarantine evaluation. After about six months of evaluation, plants were certified disease-free by AQIS and transported back to Adelaide. All Knoxfield-released VrBC2 progeny were rechecked for powdery mildew resistance. VrBC2-2 was found to display the strongest resistance against powdery mildew and was selected as both a male and female parent in subsequent crosses with the susceptible *V. vinifera* picovine line 06C008V0003 to generate VrBC3 populations (Appendix 5; Fig. 35)

Phenotype PM (R/S)	Progeny	VMC8b5	VMC8f4.2	VVS54	SCU10	VVIN83	VVIM93	UDV117	VVIU04	VVIN16	VMC7f2
R	VrBC2-2	-	-	+	+	-	-	-	-	+	+
R	VrBC2-3	+	+	-	-	-	+	-	+	+	+
R	VrBC2-4	+	+	-	-	-	-	-	-	+	+
R	VrBC2-8	-	+	+	+	-	-	-	-	+	+
R	VrBC2-15	+	+	-	-	-	+	-	-	+	+
R	VrBC2-17	+	+	+	-	-	+	-	-	+	+
R	VrBC2-18	+	+	+	-	-	+	-	+	+	+
R	VrBC2-19	-	N/A	+	+	+	N/A	-	+	+	+
R	VrBC2-20	+	-	-	-	+	+	+	+	+	+
R	VrBC2-21	+	+	-	-	-	+	-	+	+	+
R	VrBC2-25	-	+	+	+	-	+	-	-	+	+
R	VrBC2-26	-	+	+	+	-	-	-	-	+	+
R	VrBC2-29	-	-	+	+	+	-	-	+	+	+
R	VrBC2-30	-	-	+	+	+	-	+	+	+	+
R	VrBC2-31	+	+	-	-	+	+	-	-	+	+
R	VrBC2-34	+	+	-	-	-	+	-	-	+	+
R	VrBC-2-43	-	-	-	+	+	-	+	-	-	-
R	VrBC-2-44	-	-	+	+	+	-	+	-	+	+
R	VrBC-2-52	-	-	+	+	+	-	-	+	+	+
R	VrBC-2-57	+	+	-	-	-	+	-	+	+	+
R	VrBC-2-63	+	+	+	-	-	+	-	+	+	+
R	VrBC-2-72	-	-	+	+	-	-	-	+	+	+
S	VrBC2-1	-	-	+	+	+	-	+	-	-	-
S	VrBC-2-10	-	-	+	-	+	-	+	-	-	-
S	VrBC2-12	-	-	-	-	+	-	+	-	-	-
S	VrBC2-14	+	-	+	-	+	+	+	-	-	-
S	VrBC2-16	-	-	-	+	+	-	+	-	-	-
S	VrBC2-22	+	+	-	-	-	+	-	+	-	-
S	VrBC2-27	+	+	-	-	-	+	+	-	-	-
S	VrBC2-35	-	-	+	+	+	-	+	-	-	-
S	VrBC-2-42	+	+	-	-	-	+	-	+	+	-
S	VrBC-2-45	+	+	-	-	-	+	+	-	-	-
S	VrBC-2-46	+	+	-	-	-	+	-	+	-	-
S	VrBC-2-47	+	-	-	+	+	-	+	-	-	-
S	VrBC-2-51	-	-	+	+	+	-	+	-	-	-
S	VrBC-2-53	+	-	-	+	+	-	+	-	-	-
S	VrBC-2-54	+	-	-	-	+	+	+	-	-	-
S	VrBC-2-55	+	-	-	+	+	-	+	-	-	-
S	VrBC-2-56	+	+	-	-	-	+	+	-	-	-

Figure 5. *Genetic mapping of the REN4 locus in VrBC2 progeny. Progeny were screened with SSR markers on Chr 18. The '+' symbol indicates a positive association and '-' a negative association with REN4-mediated powdery mildew resistance/susceptibility.*

Generation of VrBC3 microvine progeny

VrBC3 lines were successfully obtained using VrBC2-2 as both a male and a female parent. As a larger number of progeny (~25) were obtained using VrBC2-2 as a male parent, this population was analysed in more detail. Phenotyping confirmed the *REN4* locus was inherited as a single dominant locus (11R:14S). With the successful introgression of *REN4* into the microvine background, we were able to carry out a comparison of the resistance phenotype between *RUN1* and *REN4* in the same microvine genetic background. Figure 6 shows that the speed of onset of PCD is faster in *REN4* genotypes than in *RUN1* genotypes

with only 17% of *REN4* penetrated cells showing successful second hyphal formation growth compared to only 30% in *RUN1* penetrated cells.

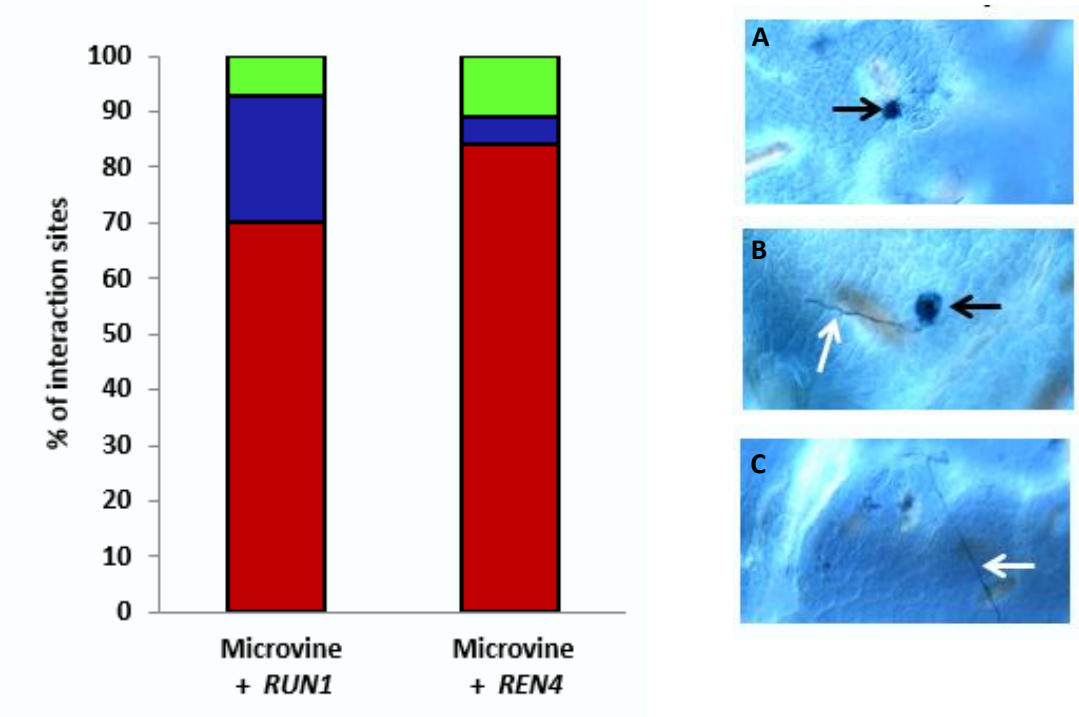

Figure 6. *Comparison of PCD induction and second hyphal formation following powdery mildew inoculation of microvines containing either the RUN1 or REN4 locus. Data were collected 1 dpi and each interaction site was scored for presence/absence of PCD and whether a second hyphae had been produced following penetration and grouped according to the outcome: Red (panel A) - rapid PCD with no second hyphae produced; Blue (panel B) - PCD but with second hyphae produced; Green (panel C) - no PCD with second hyphae*

SNP marker development using genotype-by-sequence (GBS) analysis

GBS is a multi-step process whereby genomic DNA is digested by restriction enzymes, barcode adapters ligated to the sticky ends, PCR performed and the resulting DNA products sequenced typically generating 100bp reads. The raw data are then filtered and aligned to the grape reference genome - French-Italian Public Consortium (PN40024). SNPs were then identified from the aligned tags and scored based on depth of coverage and genotype statistics.

In order to identify more genetic markers linked to the *REN4* locus for future MAS, genomic DNA samples of C87-14 parental line and several VrBC2 progeny were submitted for genotype-by-sequence (GBS) analysis at Cornell University. Approximately 60 putative SNPs were identified from the GBS analysis in and around the region of the *REN4* locus.

The SNPs and their associated sequences were submitted to the Australian Genome Research Facility (Brisbane) for multiplex assay development. Initial multiplex design and testing identified a total of 47 SNPs. Further genotype testing with progeny from the VrBC2 and VrBC3 populations confirmed that 24/47 SNPs were informative as genetic markers in these populations.

Genetic analysis of the VrBC3 population with these new SNP markers identified a number that were more tightly linked to the introgressed region containing the *REN4* locus. All powdery mildew resistant progeny in the VrBC3 population (Fig. 8, green columns) were found to be positive for SNP markers, Ren4_S18_31720383, Ren4_S18_32691612 & Ren4_S18_33103639. This defines a region of approximately 1.6 Mbp containing the *REN4* locus based on 12x Pinot genome sequence (Fig. 7).

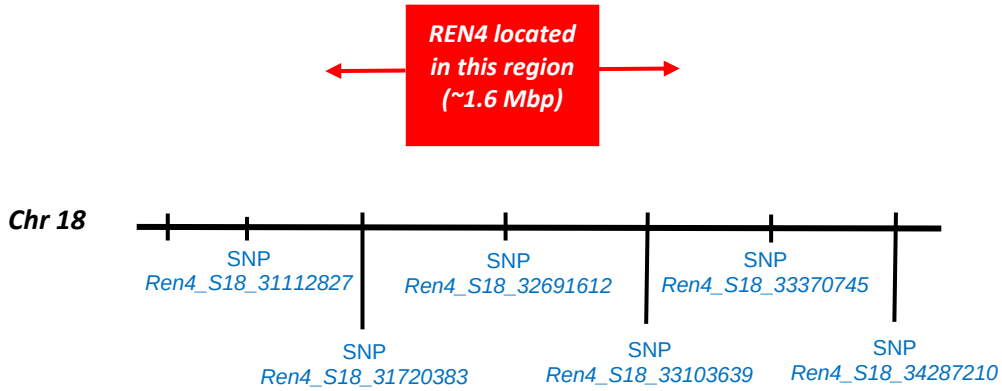

Figure 7. *Identification of new SNP markers for marker-assisted selection of the REN4 locus. New SNP markers subtending the region containing the REN4 locus were identified using genotype-by-sequence analysis. Only a selection of the new SNP markers linked to the REN4 locus is shown.*

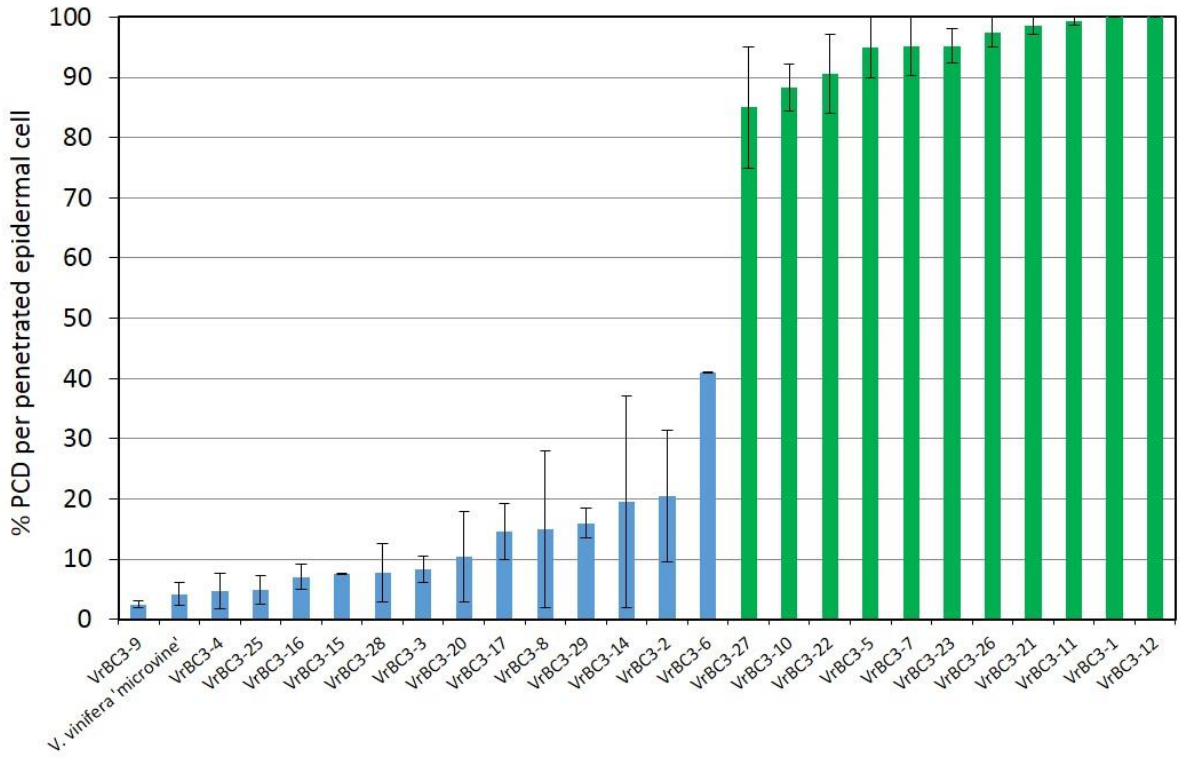

Figure 8. *Segregation of powdery mildew resistance in the VrBC3 progeny population. All data were collected 2 dpi and at least 100 germinated spores were scored following trypan blue staining for estimation of PCD. Each data point is the mean ±SE of at least two biological replicates. V. vinifera microvine is included to show the level of PCD observed in a normal microvine susceptible line.*

Generation of VrBC4 microvine progeny

VrBC4 populations were generated by using pollen collected from the two fastest flowering resistant VrBC3 hermaphroditic lines (VrBC3-1 & VrBC3-10) to pollinate the susceptible female *V. vinifera* picovine line 06C008V0003 (Appendix 5; Fig. 35). Using embryo rescue, 55 plants were generated from 150 seeds of VrBC3-10 pollen, but only three plants from 100 seeds pollinated with VrBC3-1 pollen. All progeny were tested for PM resistance/susceptibility (Fig. 9) and 23 plants were found to be resistant of which 13 were microvines and 10 were picovines. All VrBC4 progeny were screened with the SNP panel shown in Fig. 7. The markers confirmed the reliability of the SNP panel for predicting the presence of the *REN4* locus, but no new recombinants were identified to allow us to refine the location of the *REN4* locus within this 1.6 Mbp region.

To assess for berry flavour, five hermaphroditic microvine VrBC4 PM resistant plants were allowed to self and three female types were pollinated with picovine pollen to enable fruit set for analysis. Berries from all eight VrBC4 lines were found to be relatively neutral in flavour with some having a slight variation in green flavours (Table 4) indicating that they were all suitable to use as parents for the next cross with VaBC2 lines containing the *RPV12* downy mildew (DM) resistance locus described in Chapter 7.

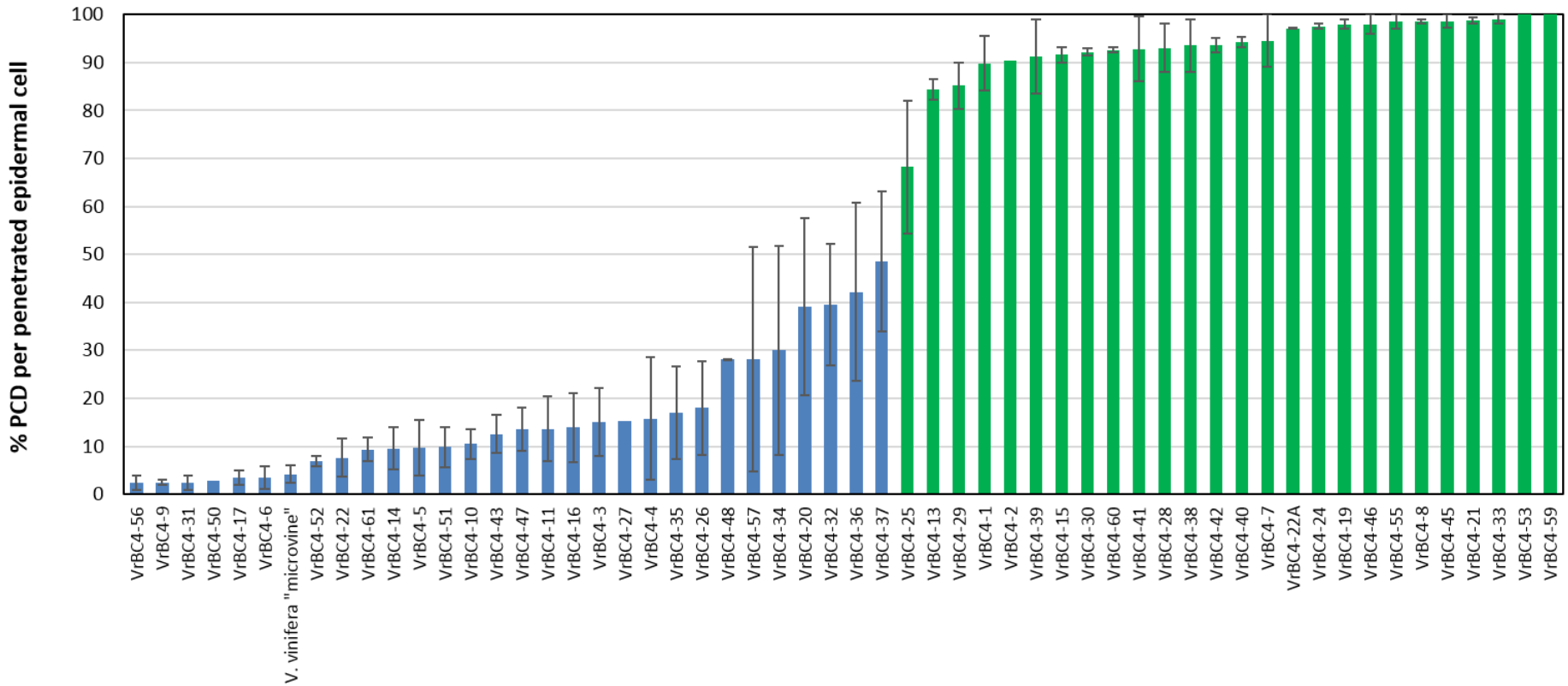

Figure 9. *Segregation of powdery mildew resistance in the VrBC4 progeny population. All data were collected 2 dpi and at least 100 germinated spores were scored following trypan blue staining for estimation of PCD. Each data point is the mean ±SE of at least two biological replicates. V. vinifera microvine is included to show the level of PCD observed in a normal susceptible line. Green columns indicate the presence of the REN4 locus as determined by SNP markers shown in Fig. 4, blue columns indicate genotypes lacking the REN4 locus.*

***Table 4**. Summary of sensory analysis of fruit produced by VrBC4 progeny tested for berry sensory analysis. Where possible bunches were picked at 22 Brix and flavour was determined from tasting 2-3 berries per bunch. H= Hermaphrodite, F= Female*

Plant	Flower Sex	Berry sensory profile
VrBC4-8	F	Neutral flavour
VrBC4-13	F	Neutral flavour
VrBC4-15	H	Slightly immature, green flavour but neutral
VrBC4-21	H	Green, neutral flavour
VrBC4-30	H	Juicy, neutral flavour
VrBC4-40	H	Juicy, neutral flavour
VrBC4-41	F	Neutral flavour
VrBC4-42	H	Green, neutral flavour

RUN1 x *REN4* testcross

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Permission to release disease-resistant lines derived from V. romanetii

The introduction and release of imported grapevine species (or hybrids derived from these species) in Australia is currently not permitted by the Department of Agriculture and Water Resources (DAWR), unless these species are listed on the Department's BICON database. The list of grapevine species that may be imported for release is very limited and does not include wild Chinese Vitis species such as *V. romanetii*. The major concern of DAWR relates to the potential 'weediness' of introduced grapevine species and represented a potential road-block in the deployment of new disease-resistant genotypes containing resistance loci introgressed from *V. romanetii*.

A submission was prepared for DAWR that outlined the fact that we were undertaking a series of backcrosses with this imported material that would reduce the genetic component of *V. romanetii* in the final selections to no more than 7% of the total genome. We argued that as the final disease-resistant varieties for field evaluation would be at least 93% *V. vinifera*, the chance of these new varieties becoming a weed problem was very low. DAWR considered our submission and issued an approval letter (Appendix 5) granting us permission to release from Quarantine any grapevine breeding lines with a genomic component of at least 93% *V. vinifera* (i.e. BC3 generation and above – see Fig. 1).

7. Development of *V. vinifera* breeding material containing new downy mildew resistance loci and identification of genetic markers tightly linked to these new resistance loci that can be used for marker-assisted selection

Introduction

In addition to the wild North American Muscadine grape species from which we previously identified the *RPV1* downy mildew resistance locus, a number of other wild North American species has been shown to confer resistance to downy mildew including *V. rupestris*, *V. labrusca and V. riparia* (Cadle-Davidson, 2008). However, each of these species is thought to confer resistance via the RPV3 locus (Di Gaspero et al., 2012) and this locus has been shown to be broken by DM isolates originating from Europe (Peressotti et al., 2010).

Cadle-Davidson (2008) also reported that the North American wild grape species *V. cinerea* was highly resistant to *P. viticola.* In the previous Wine Australia project CSP 0903 we investigated downy mildew resistance in *Vitis cinerea* (MF2-50) which is thought to have been derived by hybridisation of *Vitis cinerea* 'B41' x *Vitis cinerea cv*. *helleri* '7651'. *In-vitro* leaf disc assays confirmed that this particular accession displayed a high level of resistance to downy mildew and that this resistance did not appear to be mediated by PCD, suggesting a different mode of action to *RPV1* and therefore a good candidate for pyramiding with *RPV1*. Analysis of 97 individuals of an F1 population of a cross between *Vitis cinerea* (MF2-50) and the susceptible cultivar Riesling indicated that DM resistance inheritance displayed a continuous variation as a result of the contribution of multiple genes from the *V. cinerea* parent making it difficult to map downy mildew resistance QTLs in the F1 generation. As previously reported in CSP 0903 an attempt was made to separate out the different loci contributing to downy mildew resistance in *V. cinerea* (MF2-50) by crossing with a susceptible *V. vinifera* microvine. A downy mildew resistant VcF1 microvine line F35-4 was selected for further analysis in this project.

A number of wild Chinese Vitis species have not only been reported to show strong resistance to powdery mildew, but also to downy mildew including *V. pseudoreticulata*, *V. piazezkii* and *V. amurensis* (Wan *et al*., 2007). However, at the commencement of this project, the genetic basis of DM resistance had only been studied in any detail in *V. amurensis*. To date, two different loci have been identified in different accessions of *V. amurensis* which both confer strong resistance to DM - *RPV10* located on Chr 9 (Schwander et al., 2011) and *RPV12* on Chr14 (Venuti et al., 2013). Furthermore, *RPV12* was shown to confer resistance to DM isolates that are virulent on *RPV3* plants making it a good candidate for pyramiding with *RPV1*.

Thus the objectives of this component of the project were to introgress downy mildew resistance from *V. cinerea* and *V. amurensis* into the microvine and to investigate the inheritance of downy mildew resistance. Both Vitis species were available in public

germplasm collections which meant that it was possible to carry out crosses without first having to import germplasm from overseas and have it put through post-entry Quarantine clearance procedures.

Results and Discussion

Generation and phenotyping of DM resistance in segregating lines derived from V. cinerea

A BC1 population of 28 plants was derived from a cross between downy mildew resistant VcF1 microvine line F35-4 and the susceptible picovine line 06C008V0003. Phenotyping of this population indicated that downy mildew resistance segregated as a continuously variable quantitative trait ranging from high levels of resistance (low sporulation) in some progeny equivalent to that observed in resistant VcF1 parent F35-4 to fully susceptible compared to the microvine control (Fig. 12). These results suggested that segregation of downy mildew resistance from *V. cinerea* in the microvine background would be very difficult to map and inheritance may be unpredictable in subsequent backcross generations. A decision was therefore made to cease any further work on *V. cinerea*-derived populations and concentrate our efforts on mapping downy mildew resistance from *V. amurensis* accession in our germplasm collection.

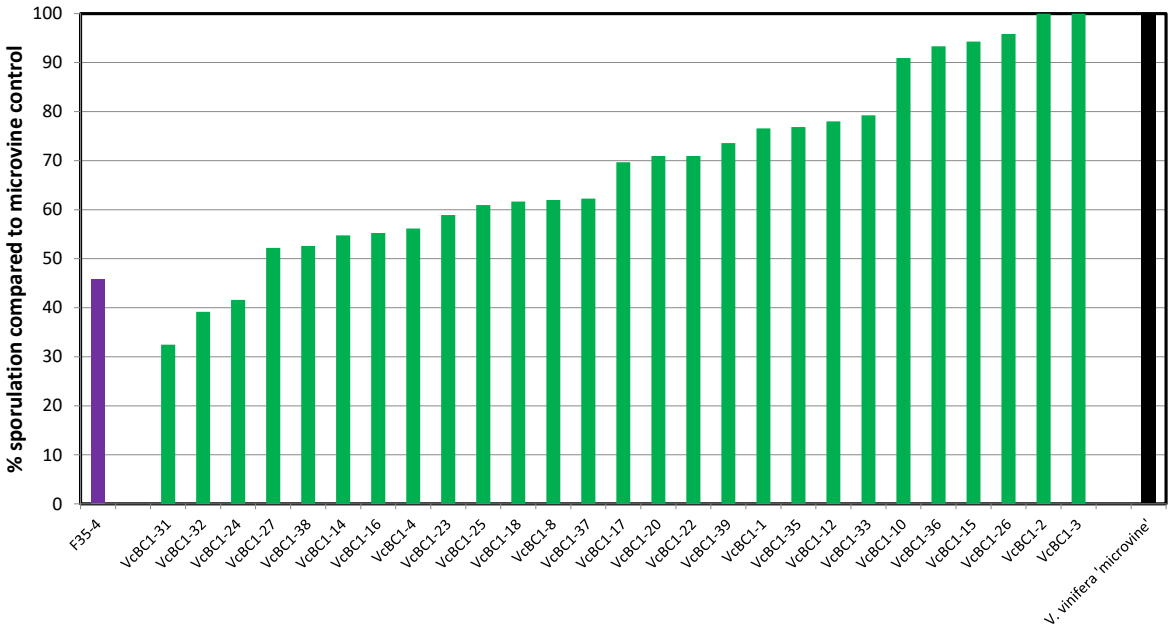

Figure 12. *Segregation of downy mildew resistance in the VcBC1 population. Leaf discs were inoculated with a sporangia and incubated in sealed petri dishes. After six days, the number of sporangia produced was counted using a Beckman Coulter Counter. Results are plotted as % sporulation relative to that observed with a susceptible V. vinifera microvine genotype. The relative level of sporulation observed with the F35-4 VcF1 resistant parent is also included.*

Mapping of the DM resistance locus from V. amurensis

A cross was successfully undertaken between *V. amurensis* x picovine line 06C008V0003. A total of 48 VaF1 progeny were recovered from 312 embryo-rescued seed and transferred to the glasshouse. Phenotyping of the 48 VaF1 progeny for downy mildew resistance showed there to be skewed segregation of R:S within the progeny population (Fig. 13). A total of 31/40 lines showed a significant reduction in the level of downy mildew sporulation (>79 - 98%) relative to the microvine susceptible control. This type of segregation pattern suggested that there may be two independently segregating downy mildew resistance loci inherited from the *V. amurensis* parent.

To investigate this further, the VaF1 progeny and parents were subjected to genetic marker analysis. The *RPV12* locus has been shown by to be located in the upper arm of Chr 14 (Venuti et al., 2013) whereas the *RPV10* DM resistance locus, identified in another *V. amurensis* accession, is located on Chr 9. Initial testing with SSR markers confirmed that the DM resistance observed in our VaF1 progeny was located on Chr14 suggesting it was conferred by *RPV12*.

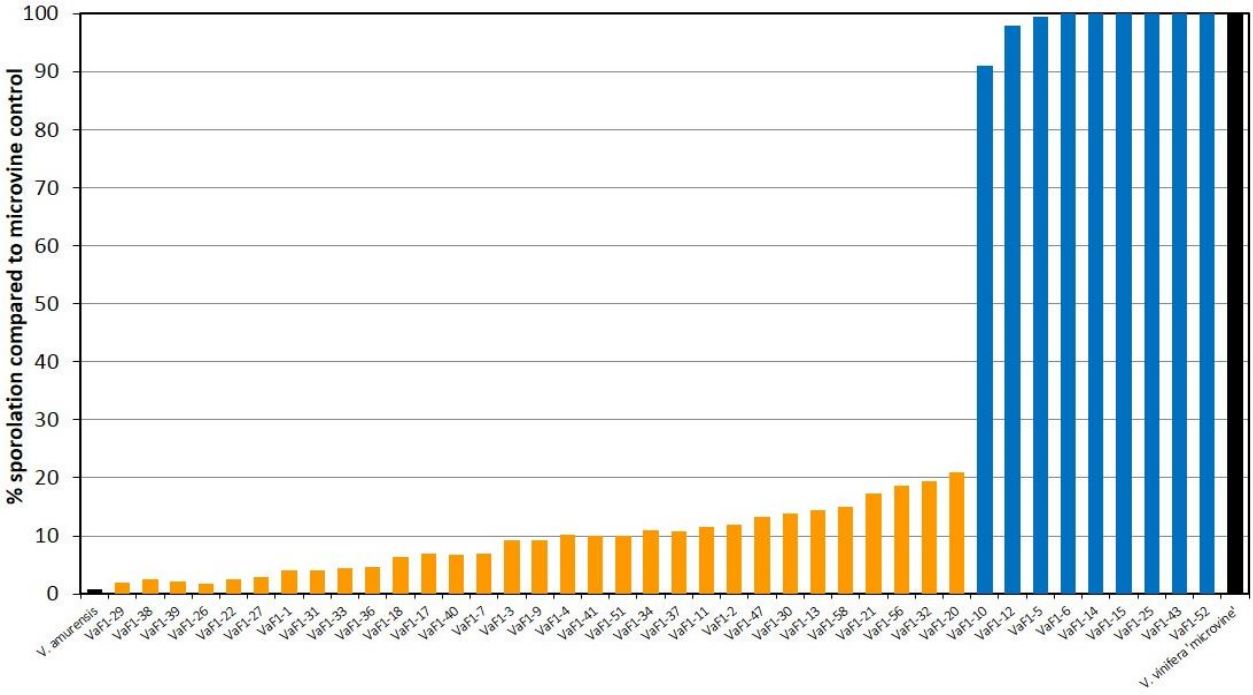

Figure 13. *Segregation of downy mildew resistance in the VaF1 progeny population. Leaf discs were inoculated with a sporangia and incubated in sealed petri dishes. After six days, the number of sporangia produced was counted using a Beckman Coulter Counter. Results are plotted as % sporulation relative to that observed with a susceptible V. vinifera microvine genotype. The relative level of sporulation observed with the V. amurensis parent is also included. Orange columns indicate progeny confirmed to contain the RPV12 locus using SSR genetic markers indicated in Fig. 5, blue columns indicate progeny lacking the RPV12 locus.*

To confirm this, we further analysed the VaF1 progeny with a number of *RPV12*-linked SSR markers (Venuti et al., 2013) including UDV014, UDV345, UDV370, VMC2H12 and VMC2C3 (Fig. 14). All *RPV12*-linked SSR markers tested were found to be tightly linked to DM resistance in our VaF1 progeny population, including three (UDV345, UDV370 and VMC2H12) which showed complete linkage. These results confirmed that DM resistance inherited from our *V. amurensis* parental line is conferred by the *RPV12* locus.

The other important outcome from the genetic marker analysis is that all of the VaF1 progeny that showed downy mildew resistance, were found to be positive for the *RPV12* locus (Fig. 13, orange columns) demonstrating that only one major downy mildew resistance gene has been inherited from our *V. amurensis* parental line. The variation in the quantitative level of resistance observed between the different *RPV12*-positive progeny (i.e. 79-98% reduction in sporulation compared to susceptible lines) may be due to the presence of another minor downy mildew resistance gene from *V. amurensis* parental line that is segregating independently of the *RPV12* locus.

The next step was to develop SNP markers that could be used for future high-throughput MAS of lines carrying the RPV12 locus. To do this we undertook genotype-by-sequence analysis on the parents of the VaF1 cross as described in Chapter 5 and successfully identified 36 SNP markers linked to the region containing the *RPV12* region. A selection of these RPV12-linked SNP markers is shown in Fig. 15.

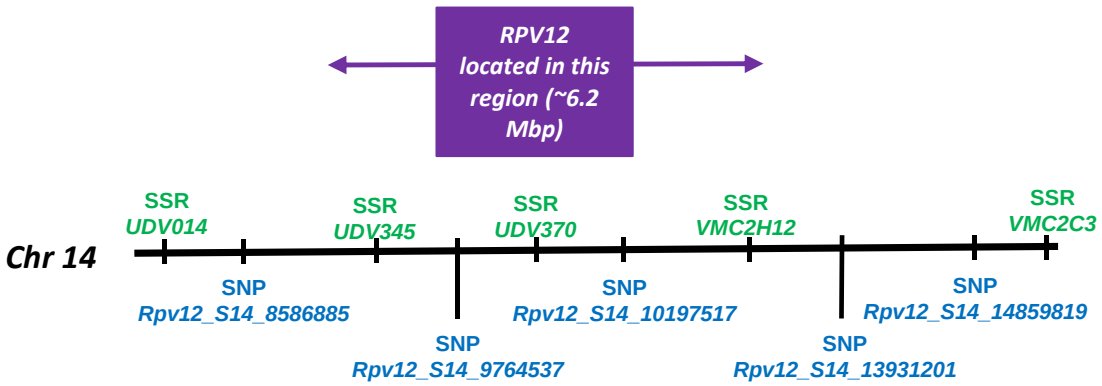

Figure 14. *Identification of genetic markers linked to the RPV12 locus for marker-assisted selection. The presence of the RPV12 locus in VaF1 progeny was confirmed using the SSR markers shown. A selection of the SNP markers identified in this study which are linked to the region containing the RPV12 locus are also shown. Numbers in brackets beneath the marker names indicate the number of VaF1 progeny out of the total number of progeny analysed that showed a recombination event between the RPV12 locus and the genetic marker.*

Phenotyping and marker analysis of VaBC1 microvine progeny

To generate the first backcross (VaBC1) population, pollen was collected from four different downy mildew resistant VaF1 microvine progeny (VaF1-3, -18, -40, -47) and used to pollinate the susceptible picovine line 06C008V0003 (Appendix 5; Fig. 36). The complementary cross using pollen from picovine line 06C008V0004 to pollinate female downy mildew-resistant VaF1 progeny (VaF1-32, -38, -56 & -59) was also undertaken and seed from this cross was kept as a back-up in case we observed of any issues with transmitting the *RPV12* locus through the pollen donor VaF1 plants.

VaBC1 seed was germinated by embryo rescue but the efficiency of germination of VaBC1 seed was found to be markedly lower than with other populations. In all, a total of 62 progeny were successfully established in the glasshouse from ~280 seed. Growth of the VaBC1 progeny in the glasshouse was also slower than expected.

Phenotyping of the VaBC1 population indicated that 32 were resistant to DM and 30 were susceptible (results not shown) indicating that resistance was segregating as a single dominant trait in the VaBC1 population. Of the 32 resistant VaBC1 progeny, 17 were picovines and 15 were microvines which were potential parents for the next cross to produce the VaBC2 progeny.

All 62 VaBC1 progeny were analysed using our GBS-derived *RPV12* SNP panel to identify any new recombination events within the genomic region containing the *RPV12* locus (Fig. 15). Genetic marker analysis revealed that four VaBC1 progeny (Va18BC1-18, Va18BC1-3, Va3BC1-19 & Va18BC1-19) had undergone recombination events within genomic region containing the RPV12 locus, refining the region to ~0.43 Mbp. Interestingly Va18BC1-18 and Va18BC1-3 appeared to have undergone double recombination events. To confirm these results, we designed a number of new CAPs and INDEL markers across this ~0.43 Mbp region and, together with previously published SSR markers (Venuti et al., 2013), rescreened a number of VaBC2 progeny including the four showing recombination events in this region. The recombination in Va3BC1-19 was mapped to a region between markers UDV360_9910299 and Rpv12_D_9949336. This further refined the *RPV12 locus* to a genomic region approx. 0.18Mbp in size with five markers showing perfect linkage to the *RPV12* locus.

Generation of VaBC2 microvine progeny

Five hermaphroditic resistant VaBC1 microvine lines were allowed to self-pollinate and produce fruit which was used for sensory analysis (Table 5).

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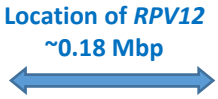
Figure 15. *Identification of new genetic markers linked to the RPV12 downy mildew resistance locus. The table contains a the combined mapping results obtained using a number of different marker types: blue - GBS derived RPV12 SNP panel; green - new SNP based CAPS markers; purple - GBS derived INDEL marker; orange - previously published SSR markers. The mapping results are only shown for a selection of the 62 VaBC1 progeny. Based on the identification of recombination events within progeny Va18BC1-18, Va18BC1-3, Va3BC1-19 and Va18BC1-19 we were able to reduce the size of the genomic region predicted to contain the RPV12 locus from 6.2 Mbp to 0.18 Mbp.*

Table 5. Summary *of sensory analysis of fruit produced by five downy mildew resistant hermaphroditic VaBC1* progeny. *Where possible bunches were picked at 22 Brix and sensory profile determined from tasting 2-3 berries per bunch*.

Plant	Berry colour / sensory profile
Va3BC1-1	Red - thick skinned, juicy, neutral flavours
Va3BC1-4	Red - thick skinned, low acid, red berry flavour
Va3BC1-21	Red - thick skinned, juicy, neutral flavours
Va40BC1-20	White - Neutral berry flavours
Va47BC1-1	Red - Neutral berry flavours

All five lines produced berries with a neutral flavour profile and were deemed as suitable parents for generating VaBC2 populations. Pollen was collected from each VaBC1 line and used to pollinate the susceptible picovine line 06C008V0003 (Appendix 5: Fig. 36). A total of 43 VaBC2 plants were established in the glasshouse and after phenotyping, 18 were found to be resistant to downy mildew (16 microvines) and 25 were susceptible (15 microvines). Genotyping of the VaBC2 population confirmed the presence of the *RPV12* locus but no new recombinants were identified (Fig. 16).



VaBC2 genotype	Phenotype	Rpv12_C_9773135	Rpv12_C_9870672	Rpv12_C_9884400	Rpv12_C_9909920	Rpv12_C_10079755	Rpv12_C_10118060
Va3-4BC2-1	R	+	+	+	+	+	+
Va3-4BC2-5	R	+	+	+	+	+	+
Va3-4BC2-21	R	+	+	+	+	+	+
Va3-21BC2-14	R	+	+	+	+	+	+
Va3-1BC2-9	S	-	-	-	-	-	-
Va3-4BC2-3	S	-	-	-	-	-	-
Va3-4BC2-10	S	-	-	-	-	-	-
Va3-4BC2-12	S	-	-	-	-	-	-

Figure 16*. Genotyping of VaBC2 population with CAPS markers. The mapping results are only shown for a selection of the 43 VaBC2 progeny.*

8. Combining of the powdery mildew and downy mildew resistance loci from different wild grapevine species within new microvine breeding lines

Introduction

Having successfully introgressed the *REN4* PM resistance locus from *V. romanetii* (Chapter 6) and the *RPV12* DM resistance locus from *V. amurensis* (Chapter 7) into the *V. vinifera* microvine the final aim of this project was to combine both resistance loci within the same microvine breeding line. These *REN4*/*RPV12* progeny could then be used in a subsequent project to generate microvine lines that are homozygous at the *REN4* and *RPV12* loci. Homozygous female microvine lines would then be used as parents in crosses with selected first generation mildew-resistant selections containing the *RUN1*/*RPV1* locus to produce second generation varieties with enhanced resistance durability in the vineyard. The generation of the *REN4*/*RPV12* progeny is described in this Chapter.

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Results and Discussion

Combining REN4 and RPV12 in the same microvine breeding lines

Table 6 shows the crosses that were undertaken to combine the *REN4* and *RPV12* loci using VrBC4 and VaBC2 parents. Crosses we carried out in both directions i.e. using both genotypes as either male or female parents. In total ~1300 seed were obtained from all crosses listed. Seeds were put through embryo rescue and seedlings were genotyped to identify those plants that had inherited both *REN4* and *RPV12*.

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Table 6. *Summary of crosses undertaken to combine the REN4 and RPV12 loci and number of seed obtained*

Cross name	Female parent	Male parent	Date of pollination	Number of seed collected
VrVa8-3-4-5	VrBC4-8	Va3-4BC2-5	23.12.16	45
VrVa13-3-4-1	VrBC4-13	Va3-4BC2-1	23.12.16	33
VrVa8-3-4-21	VrBC4-8	Va3-4BC2-21	19.01.17	51
VrVa8-3-21-9	VrBC4-8	Va3-21BC2-9	19.01.17	11
VrVa13-3-4-1	VrBC4-13	Va3-4BC2-1	25.01.17	66
VrVa41-3-4-21	VrBC4-41	Va3-4BC2-21	25.01.17	23
VaVr3-4-7-30	Va3-4BC2-7	VrBC4-30	25.01.17	8
VrVa8-3-4-21	VrBC4-8	Va3-4BC2-21	02.02.17	115
VrVa41-3-4-21	VrBC4-41	Va3-4BC2-21	09.02.17	47
VrVa13-3-4-5	VrBC4-13	Va3-4BC2-5	16.02.17	132
VrVa13-3-4-1	VrBC4-13	Va3-4BC2-1	23.02.17	18
VrVa13-3-4-5	VrBC4-13	Va3-4BC2-5	23.02.17	25
VrVa13-3-4-1	VrBC4-13	Va3-4BC2-1	09.03.17	4
VaVr3-1-15-30	Va3-1BC2-15	VrBC4-30	09.03.17	26
VrVa13-3-21-9	VrBC4-13	Va3-21BC2-9	09.03.17	58
VaVr3-4-16-15	Va3-4BC2-16	VrBC4-15	09.03.17	38
VaVr3-4-7-30	Va3-4BC2-7	VrBC4-30	09.03.17	20
VaVr3-1-17-42	Va3-1BC2-17	VrBC4-42	09.03.17	3
VrVa13-3-4-5	VrBC4-13	Va3-4BC2-5	17.03.17	16
VrVa41-3-4-21	VrBC4-41	Va3-4BC2-21	17.03.17	88
VrVa8-3-4-1	VrBC4-8	Va3-4BC2-1	17.03.17	30
VrVa41-3-4-21	VrBC4-41	Va3-4BC2-21	17.03.17	17
VaVr3-4-10-15	Va3-4BC2-10	VrBC4-15	23.03.17	5
VaVr3-1-15-30	Va3-1BC2-15	VrBC4-30	23.03.17	6
VrVa13-3-21-9	VrBC4-13	Va3-21BC2-9	23.03.17	57
VaVr3-4-7-30	Va3-4BC2-7	VrBC4-30	23.03.17	30
VrVa13-3-4-5	VrBC4-13	Va3-4BC2-5	03.04.17	37
VrVa41-3-4-21	VrBC4-41	Va3-4BC2-21	03.04.17	61
VrVa8-3-4-1	VrBC4-8	Va3-4BC2-1	03.04.17	13
VrVa3-4-11-42	Va3-4BC2-11	VrBC4-42	06.04.17	33
VaVr40-20-4-21	Va40-20BC2-4	VrBC4-21	06.04.17	62
VrVa8-3-4-5	VrBC4-8	Va3-4BC2-5	06.04.17	30
VrVa13-47-1-12	VrBC4-13	Va47-1BC2-12	04.05.17	70

Based on the predicted inheritance of unlinked dominant loci, we would expect 50% of the progeny to contain the *REN4* locus and 50% to inherit *RPV12*. Thus only 25% of the progeny would be expected to inherit both loci. In addition, as both parents were microvines we would also predict that only 50% of the *REN4*/*RPV12* progeny would be microvines, thereby reducing the expected number of progeny for final selection to approximately 12.5% of the total number of seedlings examined. A total of 533 seedlings was screened and 70 (13.1%) found to be *REN4/RPV12* microvines.

A subset of 30 *REN4/RPV12* microvine lines was selected for further growth to determine flower sex (Table 7) and to phenotype for PM and DM resistance to confirm the marker analysis. All plants were confirmed to be resistant to PM and DM (data not shown). Significantly, all lines displayed higher levels of DM resistance than grapevine lines containing *RPV12* alone. No sporulation was observed on DM-inoculated leaf discs of *REN4/RPV12* microvine lines whereas leaf discs from VaBC1/VaBC2 microvine progeny typically show a low level of sporulation (i.e. 3-6%) compared to leaf discs from Cabernet Sauvignon. This not only confirmed the presence of both DM resistance loci within these lines but demonstrated that the action of these two DM loci is complementary.

Generating homozygous REN4 grapevines.

The five hemaphroditic PM/DM resistant *VrBC4* lines listed in Table 4 were allowed to self-fertilise and ~900 seeds collected for embryo rescue. Once seedlings were established *in vitro*, leaf samples were taken for DNA analysis to determine which plants were homozygous for the *REN4* locus.

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

We showed in Chapter 6 (Fig. 7) that the speed of onset of the PCD resistance response is faster in grapevines containing *REN4* locus than the *RUN1* locus. We also examined whether grapevine lines that were homozygous at the *REN4* locus would show any further enhancement of the powdery mildew response. Figure 17 shows a comparison of the powdery mildew resistance response in the penetrated epidermal cells of VrBC4 microvine lines that are either homozygous or heterozygous at the *REN4* locus. It can be seen that the induction of PCD is indeed faster in the homozygous lines with 95-98% of epidermal cell showing 'rapid' PCD (induction of cell death is rapid enough to prevent second hyphae production) whereas only 90-93% of penetrated cells showed this phenotype in heterozygous lines.

Table 7. *Summary of crosses undertaken to combine the REN4 and RPV12 loci and number of seed obtained. H= Hermaphrodite, F= Female*

Progeny name	Flower sex	*RPV12* markers		*REN4* marker
		Rpv12_C_9870672	RPV12_C_9884400	Ren4_C_31720383
VrVa8-3-4-21_V035	H	+	+	+
VrVa13-3-4-5_V026	H	+	+	+
VrVa13-3-4-5_V035	H	+	+	+
VrVa13-3-4-5_V037	H	+	+	+
VrVa41-3-4-21_V005	H	+	+	+
VrVa41-3-4-21_V043	H	+	+	+
VrVa41-3-4-21_V044	H	+	+	+
VrVa41-3-4-21_V045	H	+	+	+
VrVa41-3-4-21_V050	H	+	+	+
VrVa8-3-4-1_V040	H	+	+	+
VrVa13-3-4-1_V062	H	+	+	+
VrVa41-3-4-21_V067	H	+	+	+
VrVa13-3-4-1_V054	H	+	+	+
VrVa13-3-4-1_V073	H	+	+	+
VrVa13-3-4-1_V077	H	+	+	+
VrVa13-47-1-12_V001	H	+	+	+
VrVa13-3-4-1_V059	H	+	+	+
VrVa13-3-4-1_V067	H	+	+	+
VrVa13-3-4-1_V066	H	+	+	+
VrVa41-3-21-14_V046	H	+	+	+
VrVa41-3-4-21_V088	H	+	+	+
VrVa13-47-1-12_V004	H	+	+	+
VrVa8-3-4-1_V019	F	+	+	+
VrVa8-3-4-1_V026	F	+	+	+
VrVa8-3-4-21_V006	F	+	+	+
VrVa13-3-4-1_V027	F	+	+	+
VrVa13-3-4-5_V016	F	+	+	+
VrVa13-3-4-5_V031	F	+	+	+
VrVa41-3-4-21_V014	F	+	+	+

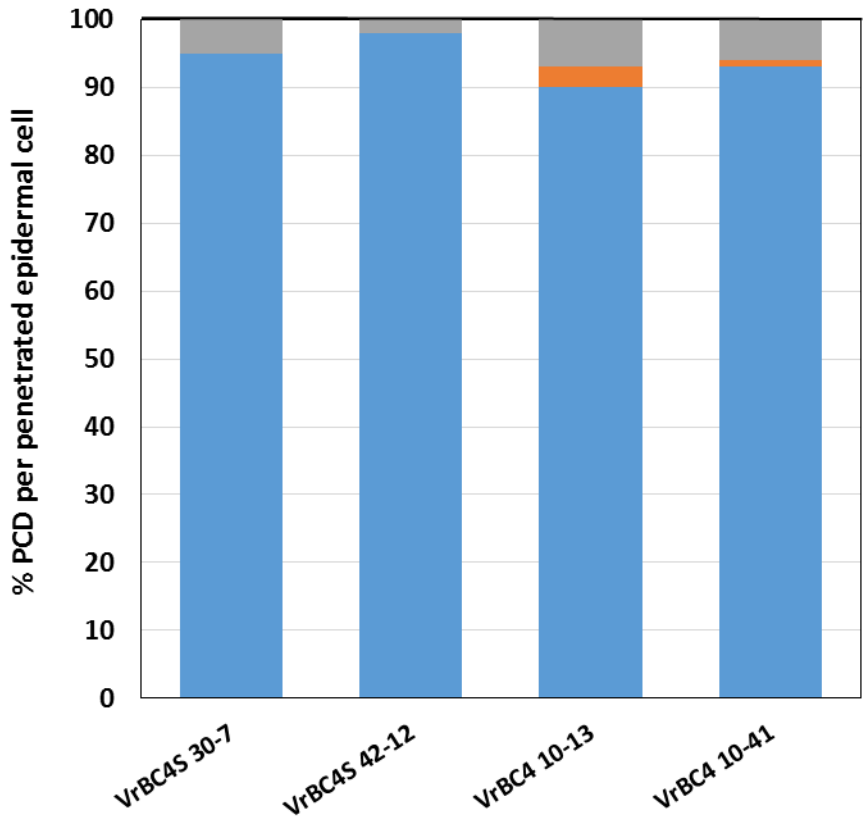

***Figure 17**. Comparison of speed of powdery mildew resistance response in epidermal cells of VrBC4 microvine lines that are homozygous or heterozygous at the REN4 locus. Homozygous progeny are indicated by VrBC4S notation. All data were collected 24 hpi and at least 100 germinated spores were scored following trypan blue staining for estimation of PCD. Blue indicates 'rapid' PCD i.e. PCD with no second hyphae production; Orange indicates PCD with second hyphae production; Grey indicates no PCD induction.*

9. Identification of quantitative trait loci involved in regulating rachis internode length during berry cluster development

Introduction

Previous studies have shown that inflorescences that produce compact bunches are more prone to botrytis bunch rot than bunches with an elongated or 'open' architecture. Overall, inflorescence or bunch architecture is determined by a number of factors including internode and pedicel development as well as flower number, branching pattern, fruit set and berry size (Harder and Prusinkiewicz, 2012). These determinants of inflorescence or bunch architecture appear to be under genetic control (Brown et al., 2006; Goosey and Sharrock, 2001). Therefore, the possibility exists to breed plants with favourable inflorescence architectures. Previous experimental studies carried out by our group have shown that a major contributing factor to differences between compact and elongated bunches is variability in rachis internode length due to differences in cell expansion and division (Sharvrukov et al., 2004). Thus, a major aim of this project was to identify key bunch architecture traits for genetic mapping in order to develop molecular markers that could be used to predict inflorescence or bunch architecture. These markers would be incorporated into the scion-breeding program for selecting new elite varieties with open inflorescences as a means to reduce the incidence of botrytis bunch rot.

Results

Phenotype variation for bunch architecture

To gain insight into the genetics of bunch architecture, four F1-mapping populations were selected for phenotype analysis, which included BC5:3294-R23 (abbreviated to R23 for the remainder of the report) x Grenache (63 individuals), R23 x Muscat Gordo Blanco (54 individuals), R23 x Dunkelfelder (101 individuals) and Exotic x Riesling (82 individuals). During the 2013/14 and 2014/15 seasons, F1 individuals and parents were phenotypically evaluated after berry set. For each inflorescence, we measured the length of the first and second internode as well as the distance from the first branch to the sixth branch (Rachis Internode Length (RAI)) (Fig. 18). Variation in internode development and berry set along the lateral branches is also a determinant for inflorescence architecture. To capture this trait, we measured the distance from the base of the branch to the node where the first berry set for the first and second as well as the fifth and six branches. Furthermore, the measurements for branches 1 and 2 (Basal Branch Width (BBW)) as well as branches 5 and 6 (Internal Branch Width (IBW)) were added together, as these sets of branches initiate in close proximity along the rachis. Therefore, the distance between the nodes of first berry set in the lateral branches determines how 'open' a bunch will be in the lateral dimension. The relationship between the three phenotypes evaluated was examined using Pearson's

correlation coefficient analysis. REML variance analysis was performed in R to estimate broad-sense heritability (Holland *et al.*, 2003).

Phenotype evaluation showed that the average size for Rachis Internode Length (RAI) was similar across all populations, 35.3 to 41.8 mm (Table 7). Moreover, the variation in range of RAI for individuals across the populations was between 13 to 79 mm in length. The average distance for Basal Branch Width (BBW) and Internal Branch Width (IBW) was between 26.1 to 32.9 mm and 15.8 to 12.2 mm, respectively (Table 7). Considerable variation for individuals across the populations ranged from 5-108 mm and 4-54 mm for BBW and IBW, respectively.

REML analysis was used to determine the heritability for RAI, BBW and IBW in each of the mapping populations. Results showed that the broad-sense heritability (h^2) had medium to high values (50-70%), indicating that a genetic component(s) controls bunch architecture (Table 8). Consistent with this result, the genotypic effect (g) for each trait in each population was always greater and significant to the genotype x season interaction (g x y) (Table 8). Therefore, there is a high probability that genetic determinants are involved in regulating inflorescence or bunch architecture.

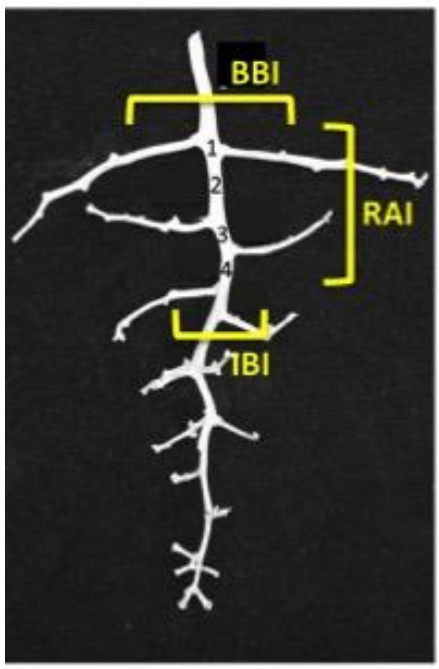

Figure 18. *Morphology of grape rachis showing where phenotypic measurements of rachis internode length were taken. RAI = Rachis Internode Length; BBW = Basal Branch Width; IBW = Internal Branch Width.*

Table 7. *Phenotype Cluster Trait Data*

Population: R23 x Gordo Blanco (54 genotypes plus parents)

Trait	Year	mean	std	range
RAI	2013-2014	40.9	11.0	13-75
	2014-2015	37.8	9.3	18-73
BBW	2013-2014	30.3	13.6	9-81
	2014-2015	26.6	13.3	5-70
IBW	2013-2014	20.6	8.0	7-45
	2014-2015	15.8	7.6	4-40

Population: R23 x Grenache (63 genotypes plus parents)

Trait	Year	mean	std	range
RAI	2013-2014	34.5	8.6	15-67
	2014-2015	35.3	7.2	21-57
BBW	2013-2014	30.5	13.7	9-81
	2014-2015	27.2	13.2	5-70
IBW	2013-2014	20.7	8.1	7-45
	2014-2015	16.2	7.5	4-40

Population: R23 x Dunkelfelder (101 plus parents)

Trait	Year	mean	std	range
RAI	2013-2014	36.3	9.23	15-71
	2014-2015	38.8	8.9	19-62
BBW	2013-2014	26.1	10.9	5-83
	2014-2015	31.0	11.4	10-67
IBW	2013-2014	15.9	6.07	5-43
	2014-2015	18.4	6.95	5-42

Population: Exotic x Riesling (82 plus parents)

Trait	Year	mean	std	range
RAI	2013-2014	41.8	11.7	16-79
	2014-2015	40.9	10.6	17-69
BBW	2013-2014	28.7	14.5	6-108
	2014-2015	32.9	15.2	7-82
IBW	2013-2014	17.1	8.66	5-44
	2014-2015	21.2	9.91	5-54

Table 8. *REML variance (%) for phenotypic variance and heritability*

Population: R23 x Gordo Blanco

Trait	g	g x y	h^2
RAI	48.9	19.5	0.64
BBW	96.8	27.3	0.70
IBW	33.4	13.7	0.70

Population: R23 x Grenache

Trait	g	g x y	h^2
RAI	29.1	8.42	0.63
BBW	97.7	27.5	0.69
IBW	33.8	13.6	0.71

Population: R23 x Dunkelfelder

Trait	g	g x y	h^2
RAI	32.0	17.9	0.56
BBW	47.8	28.4	0.56
IBW	18.7	10.9	0.61

Population: Exotic x Riesling

Trait	g	g x y	h^2
RAI	57.5	27.7	0.64
BBW	98.0	50.6	0.62
IBW	41.7	21.8	0.65

p-values for g and g x y in all populations <0.001

REML variance components of the genotype effect (g), genotype x season interaction (g x y) and the broad-sense heritability (h^2)

Phenotype = g + y + rep-in-year + g x y

Histological examination of R23, Grenache, Muscat Gordo Blanco, Riesling, Dunkelfelder and Exotic

Phenotypic analysis of bunch architecture in the R23 x Grenache, R23 x Muscat Gordo Blanco, R23 x Dunkelfelder and Exotic x Riesling F1 mapping populations indicate that bunch architecture is controlled in part by genetic factors. R23, Grenache and Muscat Gordo Blanco produce inflorescences with an intermediate bunch architecture phenotype. On the other hand, Exotic plants give rise to inflorescences that are highly 'open', while Riesling and Dunkelfelder produces 'compact' bunches. To better understand how the parents contribute to the genetic variation of bunch architecture displayed in the F1 individuals for each population, a histological examination was performed in the first and second internodes of the main rachis for each genotype after fruit set.

Evaluation of cell size in transverse sections through the cortex showed that there is little difference in cell size between all varieties (Fig. 18). However, in pairwise analyses, a significant difference in cell width was found between R23 and Grenache indicating that cell size is slightly smaller in Grenache compared to R23 (Fig. 18). Cell size determination in transverse sections through the parenchyma region of the cortex indicates that cell size is similar in all the varieties (data not shown). However, cell size was found to be slightly smaller in Exotic and R23 compared to Riesling and Grenache, respectively (Fig. 19). In addition, cell size was slightly larger in R23 compared to Muscat Gordo Blanco (Fig. 19).

Genetic map construction and QTL analysis

Two genetic maps, backcross 1 (BC1) and BC2, were generated from each F1 mapping population. Nineteen linkage groups were identified for BC1 and BC2 maps in the R23 x Grenache and R23 x Muscat Gordo Blanco mapping populations (Figs. 20-23). In many cases, SNPs that mapped to a specific chromosome were also linked, based on R/onemap analysis (data not shown). For R23 x Dunkelfelder, the BC1 map contained 19 linkage groups (Fig. 24). Linkage group 1.1 contained 14 SNPs that mapped to the end of chromosome 1 by GBS (Fig. 24). In addition, SNPs that mapped to chromosome 10 via GBS were subdivided into two linkage groups using R/onemap (Fig. 24). For the BC2 (Dunkelfelder) map, only eight SNPs mapped to chromosome 13 via GBS (data not shown). However, after genetic map construction, none of these SNPs formed a separate linkage group (Fig. 25). In addition, two linkage groups were formed for chromosome 15.

Using the Haley-Knot regression function in R/qtl, no QTLs with LOD values > 3 were detected for any of the measured traits in R23 x Grenache (Figs. 26 & 27) and R23 x Muscat Gordo Blanco populations (Figs. 28 & 29). Interval mapping was also applied and similar results were obtained (data not shown). QTL analysis was not performed for R23 x Dunkelfelder due to difficulties curating the genetic map. This was mainly due to the fact SNPs identified by genotyping-by-sequencing did not provide significant coverage for a number of chromosomes.

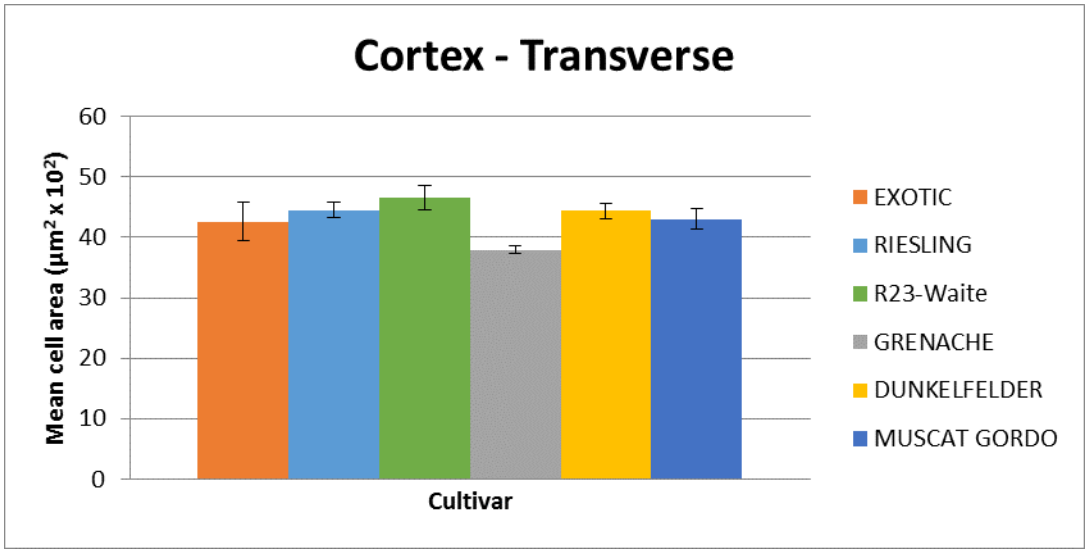

Figure 18. *Comparative analysis of mean cortex cell size in transverse sections of Exotic, Riesling, R23, Grenache, Dunkelfelder and Muscat Gordo Blanco. Mean values (± SE) were calculated based on all cell size measurements.*

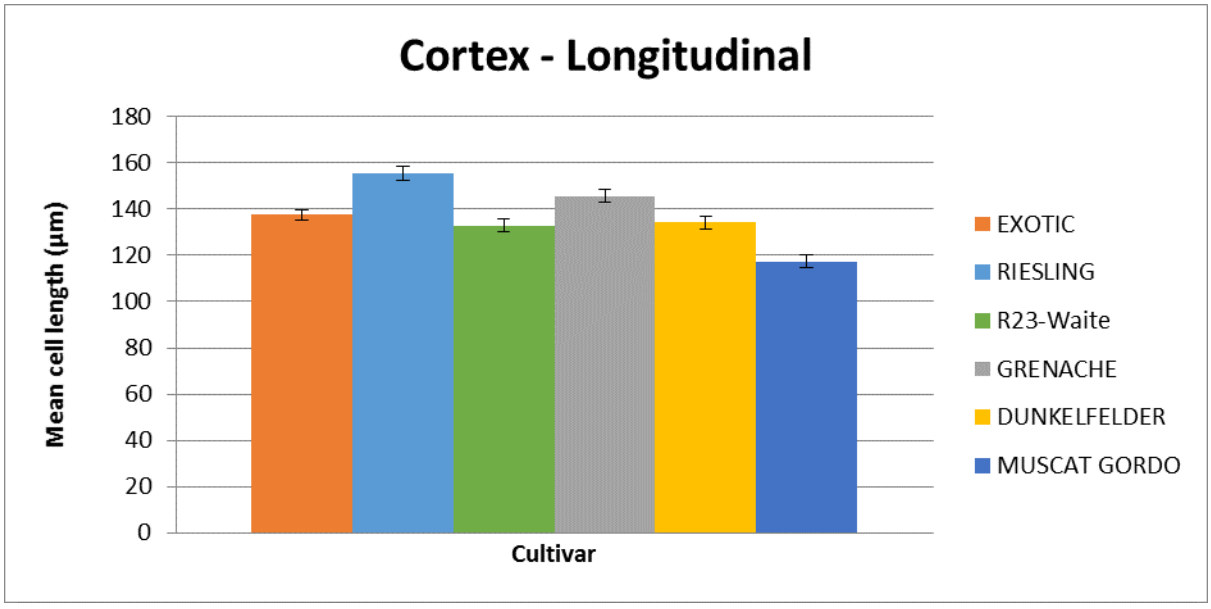

Figure 19. *Comparative analysis of mean cortex cell size in longitudinal sections of Exotic, Riesling, R23, Grenache, Dunkelfelder and Muscat Gordo Blanco. Mean values (± SE) were calculated based on all cell size measurements.*

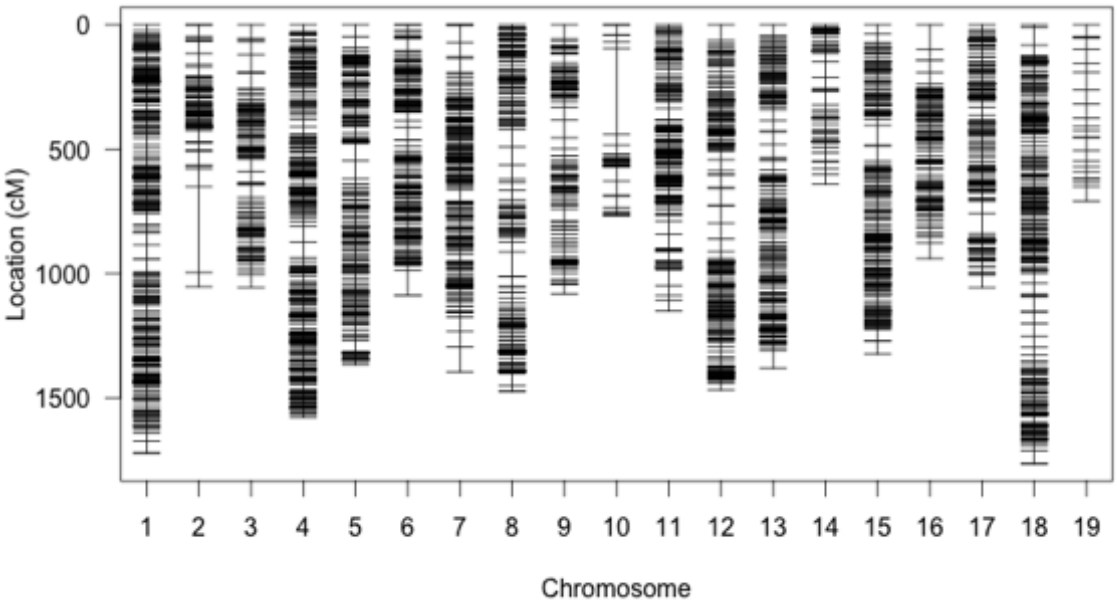

Figure 20. *Genetic map of BC1 (R23) for the R23 x Muscat Gordo Blanco F_1 mapping population. The genetic map was constructed with 4422 SNPs identified by genotype-by-sequence. Linkage groups or chromosomes are displayed on the x-axis, while the y-axis shows the size (cM).*

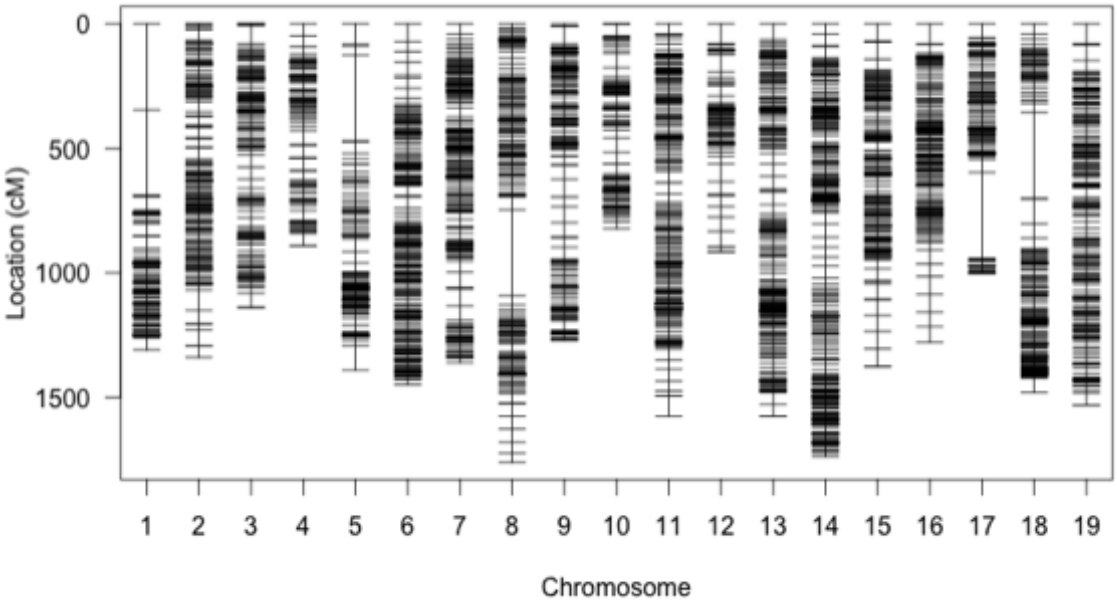

Figure 21. *Genetic map of BC2 (Muscat Gordo Blanco) for the R23 x Muscat Gordo Blanco F_1 mapping population. 4384 SNPs identified by genotype-by-sequence were used to construct the genetic map. Linkage groups or chromosomes are displayed on the x-axis, while the y-axis shows the size (cM).*

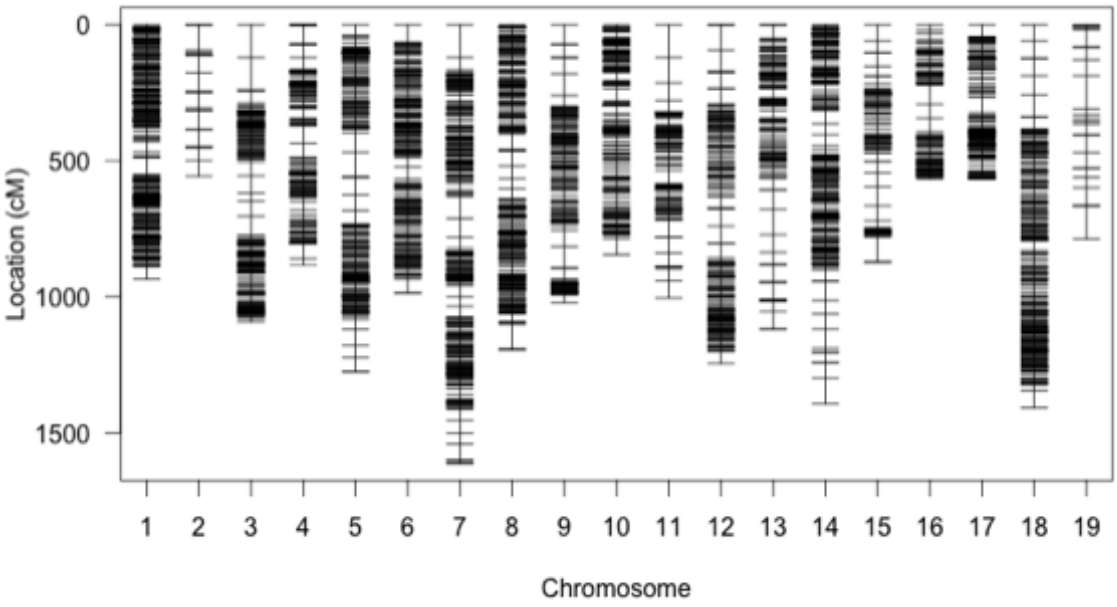

Figure 22. *Genetic map of BC1 (R23) for the R23 x Grenache F_1 mapping population. The genetic map was constructed with 4311 SNPs identified by genotype-by-sequence. Linkage groups or chromosomes are displayed on the x-axis, while the y-axis shows the size (cM).*

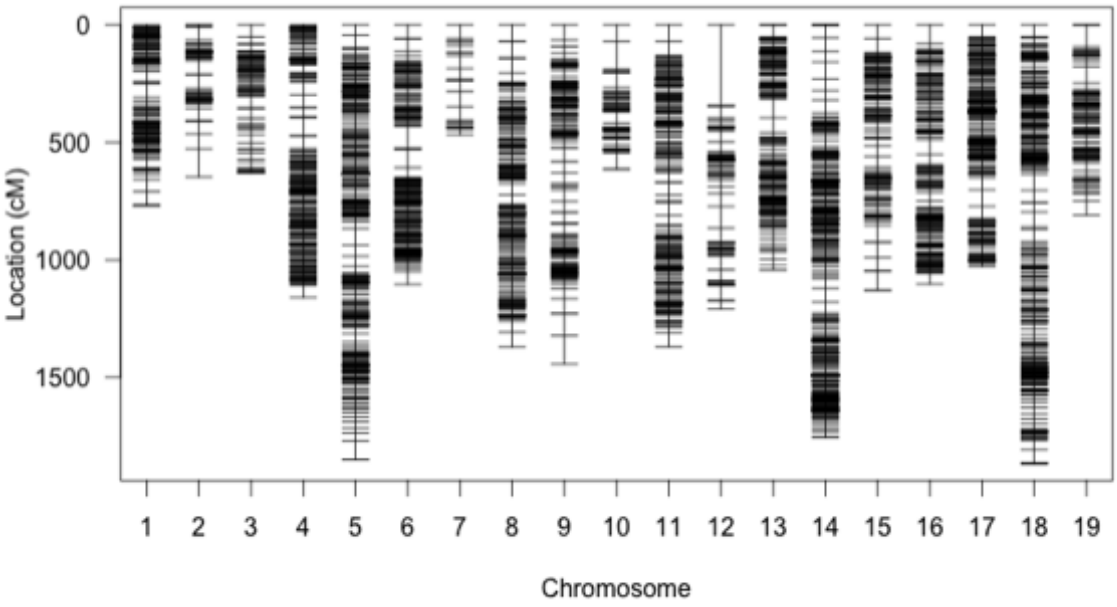

Figure 23. *Genetic map of BC2 (Grenache) R23 x Grenache the F_1 mapping population. 4242 SNPs identified by genotype-by-sequence were used to construct the genetic map. Linkage groups or chromosomes are displayed on the x-axis, while the y-axis shows the size (cM).*

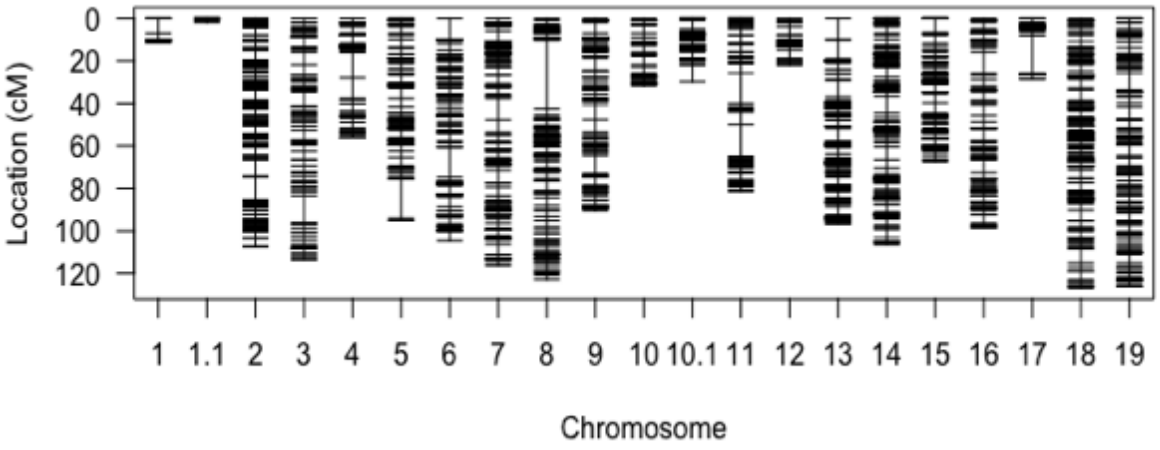

Figure 24. *Genetic map of BC1 (R23) for the R23 x Dunkelfelder F_1 mapping population. The genetic map was constructed with 2806 SNPs identified by genotype-by-sequence. Linkage groups or chromosomes are displayed on the x-axis, while the y-axis shows the size (cM).*

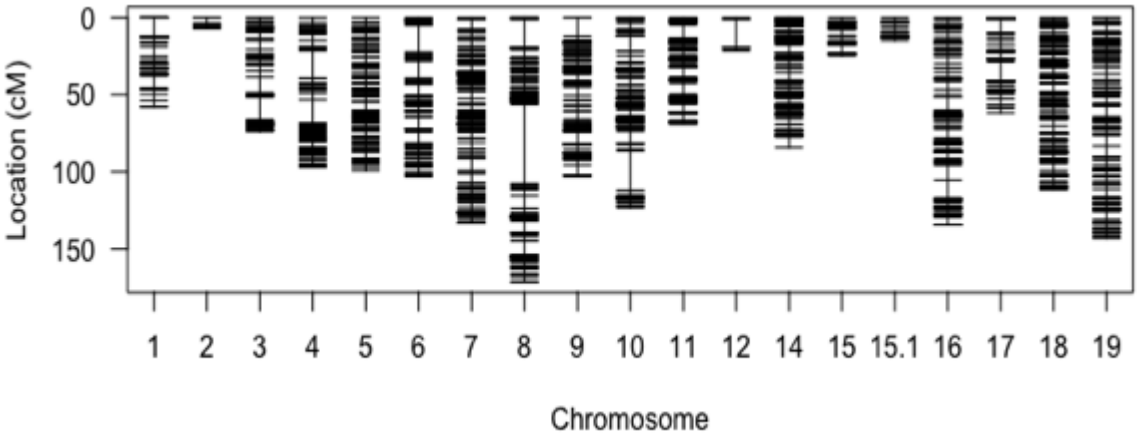

Figure 25. *Genetic map of BC2 (Dunkelfelder) for the R23 x Dunkelfelder F_1 mapping population. The genetic map was constructed with 2749 SNPs identified by genotype-by-sequence. Linkage groups or chromosomes are displayed on the x-axis, while the y-axis shows the size (cM).*

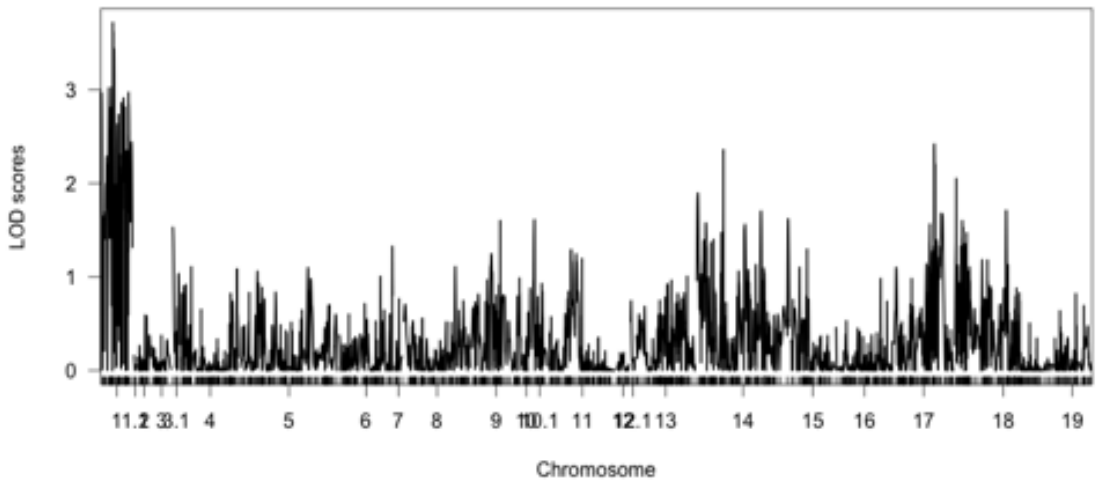

Figure 26. *QTL analysis using BC1 (R23) in R23 x Grenache F1 mapping population. Linkage groups or chromosomes are displayed on the x-axis, while the y-axis shows the LOD scores. LOD values < 3 are not significant.*

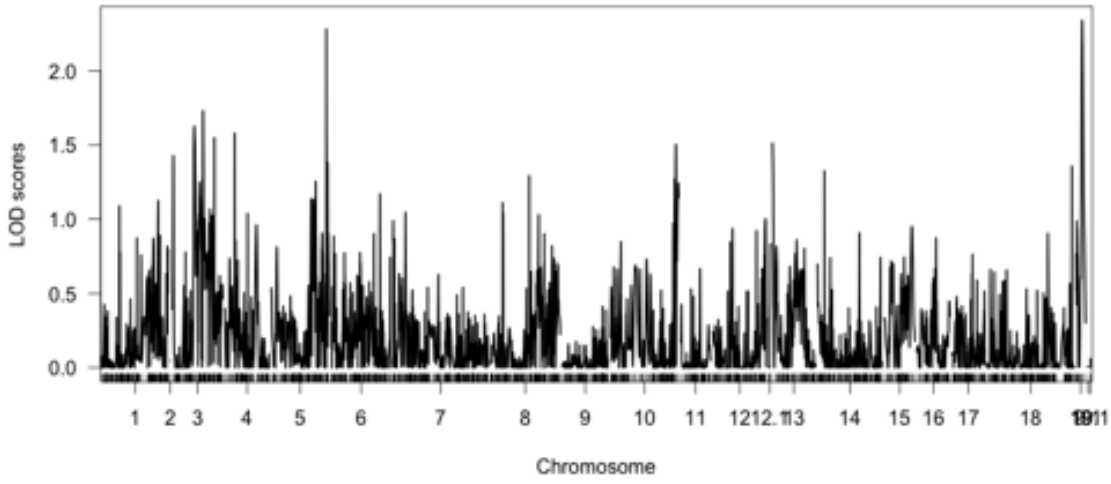

Figure 27. *QTL analysis using BC2 (Grenache) in R23 x Grenache F1 mapping population. Linkage groups or chromosomes are displayed on the x-axis, while the y-axis shows the LOD scores. LOD values < 3 are not significant.*

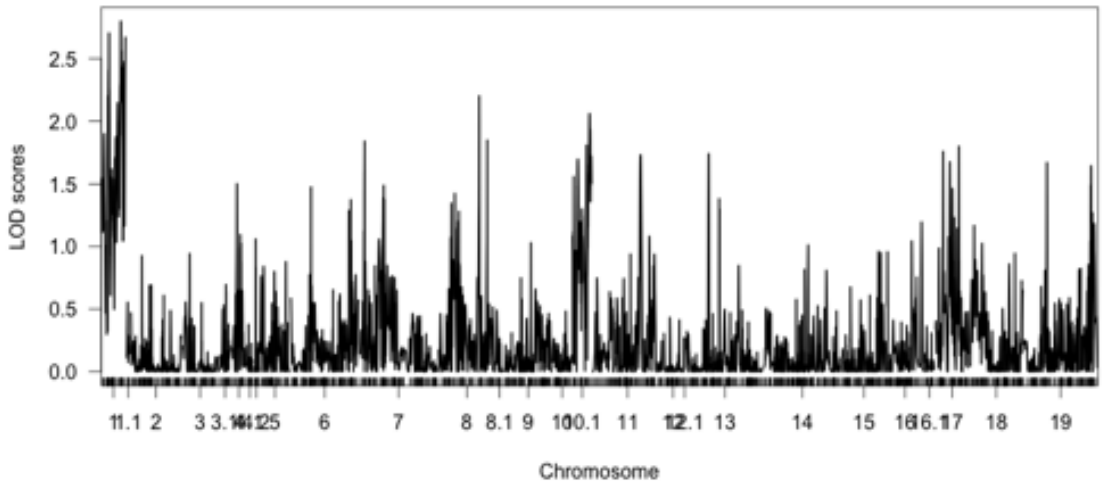

Figure 28. *QTL analysis using BC1 (R23) in R23 x Muscat Gordo Blanco F1 mapping population. Linkage groups or chromosomes are displayed on the x-axis, while the y-axis shows the LOD scores. LOD values < 3 are not significant.*

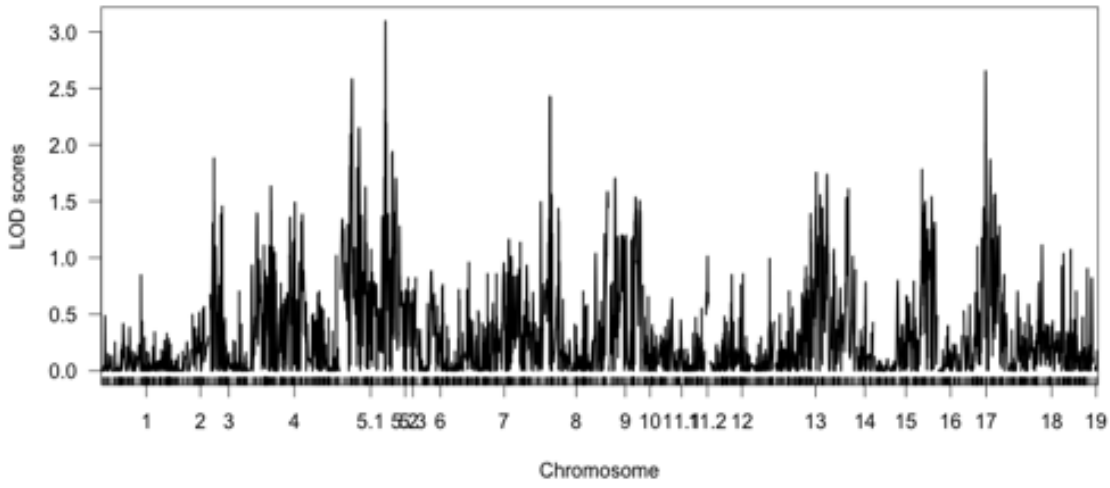

Figure 29. *QTL analysis using BC2 (Muscat Gordo Blanco) in R23 x Muscat Gordo Blanco F1 mapping population. Linkage groups or chromosomes are displayed on the x-axis, while the y-axis shows the LOD scores. LOD values < 3 are not significant.*

Discussion

Heritability studies showed that there is a high genetic component for rachis internode development and basal berry set in lateral branches, two traits critical for bunch architecture. Internode development is regulated by cell division and cell expansion (Kutschera and Niklas, 2013). Based on histological studies, it is difficult to pinpoint the cellular mechanism responsible for internode development in the R23 x Muscat Gordo Blanco and R23 x Grenache. However, given that there is little difference in cell size between Exotic and Riesling, as well as R23 and Dunkelfelder, it is likely that the duration of cell division is critical for determining the extent of rachis internode development in the subsequent F1 individuals.

A recent study examined the heritability and genetics of bunch architecture in a table grape mapping population derived from a Ruby Seedless x Sultanina cross (Correa et al., 2014). Twenty-three traits were evaluated in this mapping population and results showed that rachis length, rachis weight and shoulder length were major genetic factors that contributed to bunch architecture. Interval mapping show that bunch architecture QTLs are located on chromosome 5, 8, 9, 14, 17 and 18 (Correa et al., 2014). In our study, we attempted to identify bunch architecture QTLs in crosses between varieties that produce intermediate sized bunches (R23 x Muscat Gordo Blanco and R23 x Grenache) and intermediate x compact (R23 x Dunkelfelder). In our analysis, we were unable to identify QTLs for bunch architecture in R23 x Muscat Gordo Blanco and R23 x Grenache. Unfortunately, we were unable to effectively curate the BC1 (R23) and BC2 (Dunkelfelder) maps developed from the R23 x Dunkelfelder mapping population. In addition, we also performed single SNP association and none of the markers associated with bunch architecture in all three mapping populations (data not shown).

The lack of success for mapping bunch architecture loci is likely attributed to the size of the mapping populations, which consisted of 56, 65 and 101 individuals. Using populations with > 1000 individuals would have a much higher rate of success, because as results from our study in Chapter 10 indicates, internode development is likely to be controlled by many genes. CSIRO holds approximately 500 varieties of table and wine grapes in the germplasm collection, which display significant variation in berry size, bunch architecture and fruit quality traits. We recently performed an association analysis to map traits for seedlessness, berry size and bunch architecture in 94 genotyped individuals. Preliminary results indicate that markers on chromosome 1, 3, 6 and 8 associate with bunch architecture (data not shown). The ability to genotype additional varieties in the collection may help pinpoint the location of these and other favourable loci and the underlying genes responsible for regulating bunch architecture in grapevine. Indeed, genome-wide association analysis may be more suitable to identifying loci and markers linked to traits controlled by numerous genes than biparental mating.

10. Identification of candidate genes involved in regulating rachis internode length during berry cluster development

In addition to searching for genetic markers linked to bunch architecture using the un-biased approach of whole genome mapping studies outlined in Chapter 9, we also employed a candidate gene approach for marker identification. The candidate gene approach is based on the use of next-generation sequencing techniques to enable detailed analysis of the global gene expression pattern (i.e. transcriptome) within a selected tissue. For example, analysing the transcriptome of rachis internodes during the crucial stage of internode elongation that determines whether a bunch will have an open or compact architecture. Using bioinformatics software tools we can then compare the transcriptomes of rachis internodes that will give rise to open and compact bunches and identify any genes that are significantly differentially expressed between these different internode types. Such genes may represent candidates for involvement in the regulation of internode elongation and SNPs adjacent to the candidate genes may be used to develop markers for use in MAS of bunch architecture. The chromosomal location of these candidate genes on the grape genome can also be compared to the position of QTLs predicted to be involved in regulating rachis elongation (Chapter 9) to see if there is any overlap.

Hormones such as gibberellin (GA), auxin, cytokinin, brassinosteroid are key internal signalling molecules that promote growth as well as coordinate developmental processes in response to environmental stimuli (Kuppusamy et al., 2009; Vanstraelen and Benkova, 2012). The developmental pathways controlled by these growth-promoting hormones are interconnected in an elaborate network of intricate feedback systems. In addition, these hormones often converge at the transcription level to regulate key genes and pathways that regulate growth and development. As a result, comparative transcriptomic studies using hormone signalling mutants are key to identifying genes and pathways that regulate growth and developmental processes.

Gibberellin regulates many developmental processes such as flowering and germination as well as organ growth, which are mediated by distinct or overlapping phases of cell division and expansion (Claeys et al., 2014; Daviere and Achard, 2016). Mutants in GA biosynthesis or GA signalling result in plants with a dwarf or compact shoot phenotype. Given the role of GA in growth and development it is highly likely that differences in bunch architecture could be due, in part, to differences in GA-mediated growth. Numerous gene expression profiling experiments have been performed in Arabidopsis. Meta-analysis was recently performed to understand how GA regulates growth and results showed that only a few genes showed significant changes in multiple datasets (Claeys et al., 2014; Daviere and Achard, 2016). Therefore, the mechanism of how GA regulates growth and development is tissue dependent. To date, studies to understand how GA regulates growth have been focused on hypocotyl elongation and rosette growth as well as fruit development (Claeys et al., 2014;

Daviere and Achard, 2016). Therefore, there is a lack of understanding of how GA regulates internode growth during inflorescence development.

GA signalling is mediated in part through transcriptional regulation via DELLA proteins (Claeys et al., 2014; Daviere and Achard, 2016). There are five DELLA genes in Arabidopsis including Gibberellic Acid Insensitive (GAI). Gain of function alleles from deletions or mutations in the DELLA domain of GAI stabilise this protein allowing it to constitutively repress GA signalling (Daviere and Achard, 2013). Experimental evidence shows that the GID1 receptor binds to GA which facilitates the interaction with the DELLA domain of GAI, causing this repressor to be degraded. Furthermore, mutations or deletions in the DELLA domain of GAI prevent interaction with GID1-GA, allowing this DELLA protein to constitutively repress transcription in the presence of GA. Molecular and genetic studies performed with DELLA mutants, including GAI, indicate that DELLA proteins not only mediate GA responses but also function to integrate multiple hormone signalling pathways by directly interacting with auxin, brassinosteroid and cytokinin transcriptional regulators (Daviere and Achard, 2016). In grapevine, the *Vvgai1* gain-of-function allele in the *V. vinifera* Pinot Meunier microvine contains a point mutation in a conserved residue of the DELLA domain resulting in the conversion of a leucine to histidine (Boss and Thomas, 2002). As a result, it is highly likely that this mutation in *Vvgai1* reduces the ability of this DELLA protein to associate with the GID1-like receptors in the presence of GA. Therefore, the microvine is a potentially valuable tool to understand how GA regulates internode growth during inflorescence development in grapevine.

Results

Characterisation of Pinot Meunier microvine mutant

To better understand the role of GA in inflorescence internode development, internode lengths along the main rachis were measured and quantified in Pinot Meunier and the microvine mutant. Results showed that the length of the rachis internodes was significantly reduced in the microvine mutant compared to Pinot Meunier (Fig. 30, Table 9). These results demonstrate that GA is an essential regulator of inflorescence internode development in grapevine.

Figure 30. *(A) Grapevine bunches with berries, Pinot Meunier (left) and microvine mutant (right). (B) Berries were removed to display the architecture of the inflorescence, Pinot Meunier (left) and the microvine mutant (right).*

Table 9. *Rachis internode lengths in Pinot Meunier and microvine mutant bunches*

Genotype	1+2	3+4	5+6	Sum
Pinot Meunier	18.4 (±3.6)	10.8 (±3.5)	8.5 (±3.1)	37.7 (±5.8)
Microvine	8.44 (±1.6)	4.4 (±1.3)	3.4 (±0.82)	16.4 (±2.1)

1+2 = average sum of internode 1 and 2; 3+4 = average sum of internode 3 and 4

5+6 = average sum of internode 5 and 6; Sum = average sum of rachis length from internode 1 to 6

Identification of GA-regulated genes implicated in internode development

To determine the role of GA in internode growth, a transcriptome profiling experiment was performed comparing Pinot Meunier with the microvine mutant. Immature inflorescences and mature bunches isolated from two Pinot Meunier and microvine vines were used. For each vine, four immature inflorescences at stage 12 (Pearce and Coombe, 2004) were harvested. The first and second internode along the main rachis was dissected for RNA extraction. Five mature bunches were isolated from each genotype and the berries were removed to quantify internode development.

The first and second internodes were dissected from Pinot Meunier and the microvine immature inflorescences at stage 12. At this stage, histological analysis showed that there was no significant difference in cell size and length between the two samples indicating that the cells in the internodes had not undergone cell expansion (Fig. 31 & 32). After RNA extraction, RNA-Seq was performed and 363 differently expressed genes (DEGs) were identified with a log-fold change ≥1 and a FDR >0.05 using three bioinformatics approaches, EdgeR, DESeq2 and VOOM (Fig. 32). The 363 DEGs were annotated and the predictive function was assigned.

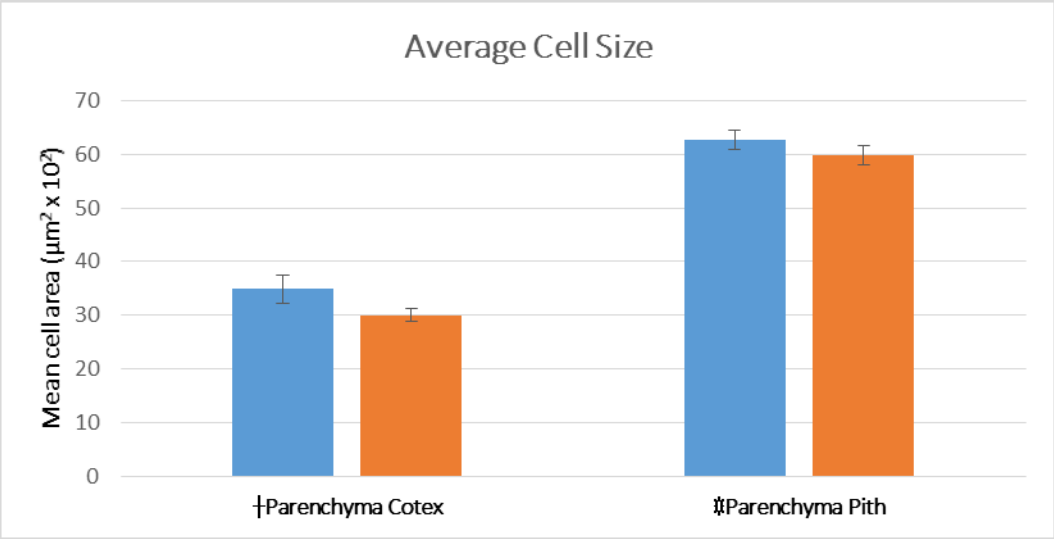

Figure 31. *Average parenchyma cell size (cortex and pith) in developing internodes (stage 12) of Pinot Meunier (red bar) and microvine mutant (blue). Cell size based on transverse sections through the internodes of each sample.*

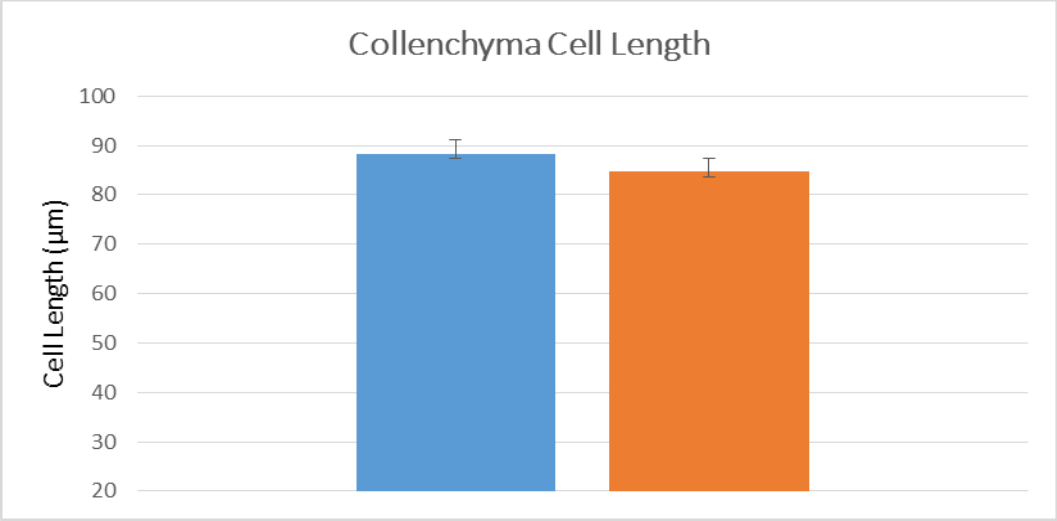

Figure 32: *The average length of collenchyma cells in developing internodes (stage 12) of Pinot Meunier (red bar) and microvine mutant (blue bar). Cell length was determined in longitudinal sections through developing internodes.*

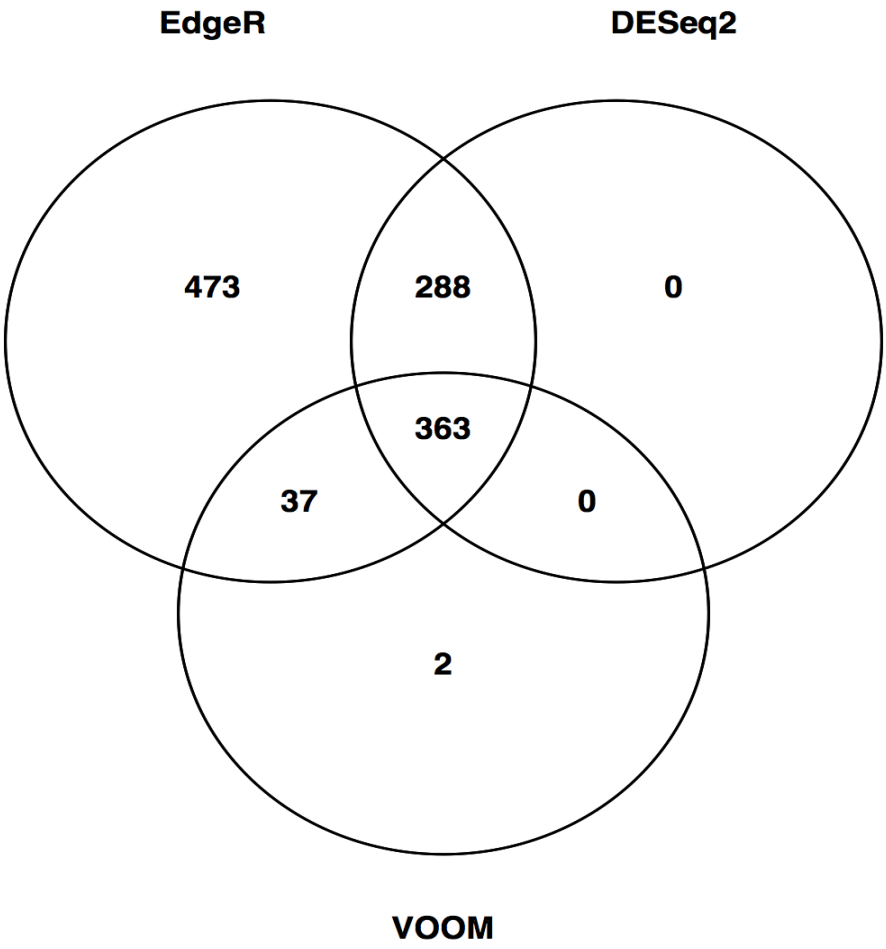

Figure 33. *Venn Diagram showing the overlapping differentially expressed genes identified using the bioinformatics tools EdgeR, DESeq2 and VOOM with a FDR <0.005 and log fold change ≥1.*

GAI hormone signalling and biosynthesis

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

Genes implicated in cell growth controlled by GAI

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

GAI regulates genes involved in cell expansion

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

GAI regulates a diverse array of developmental regulators

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Differentially expressed gene candidates that map to published QTLs for bunch architecture

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Table 10. *Differentially expressed genes annotated as proteins involved in hormone synthesis and signalling*

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

Table 11. *Differentially expressed genes annotated as proteins that function as potential growth regulators*

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

Table 12. *Differentially expressed genes functionally annotated to synthesize cell wall components*

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

Table 13. *Differentially expressed genes functionally annotated to modify the cell wall*

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

Table 14. *Differentially expressed genes functionally annotated to modify cell wall structural proteins*

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

Table 15. *Differentially expressed genes categorized as cytoskeleton proteins*

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

Table 16. *Differentially expressed genes that encode transcriptional regulators*

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

Table 17. *Differentially expressed genes that localize to QTLs that control bunch or inflorescence architecture*

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Discussion

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11. Outcomes / Conclusions

Development of germplasm resources and genetic markers to facilitate MAS of second generation mildew resistant winegrape varieties

- Successful introgression of the *REN4* powdery mildew resistance locus from the wild Chinese species *V. romanetii* into the *V. vinifera* microvine to the BC4 generation. *REN4*-mediated resistance was found to be more effective than *RUN1*-mediated resistance in the *V. vinifera* genetic background.
- Identification of genetic markers surrounding the *REN4* locus that can be used for MAS of second generation mildew resistant winegrape varieties containing both *REN4* and *RUN1* powdery mildew resistance loci.
- Successful introgression of the *RPV12* downy mildew resistance locus from the wild Chinese species *V. amurensis* into the *V. vinifera* microvine to the BC2 generation.
- Identification of genetic markers surrounding the *RPV12* locus that can be used for MAS of second generation mildew resistant winegrape varieties containing both *RPV12* and *RPV1* downy mildew resistance loci.
- Successful pyramiding of the *REN4* PM resistance locus from *V. romanetii* and the *RPV12* DM resistance locus from *V. amurensis* within the same microvine breeding lines.
- **This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.**

Identification of quantitative trait loci and candidate genes responsible for regulating internode length during berry cluster development.

- Genotype-by-sequence completed for R23 x Muscat Gordo Blanco (56 genotypes), R23 x Grenache (65 genotypes) and R23 x Dunkelfelder (101) mapping populations leading to the identification of >4000 SNPs, which were used to construct genetic maps for R23 x Muscat Gordo Blanco and R23 x Grenache populations.
- Phenotyping studies and heritability analysis completed on five F1 mapping populations segregating for bunch architecture. Results indicated a high probability that genetic determinants are involved in regulating rachis internode length. However, we were unable to identify major QTLs for rachis internode length. One possible explanation for this outcome is that the mapping populations we employed were too small i.e. ranging in size from 56 - 101 individuals. Using populations with > 1000 individuals would have a much higher rate of success, because internode development is likely to be controlled by many genes.

- Completed gene expression profiling (transcriptome) analysis of developing rachis internodes from wild-type Pinot Meunier (normal rachis internode development) and the Pinot Meunier microvine GA mutant (compressed rachis internode development). Identified and annotated over 360 genes that are differentially expressed between normal and compressed rachi. Results from this analysis suggest that GA-mediated grape rachis internode development is controlled by the interaction of a number of different processes including: (a) integration with auxin signalling, transport and metabolism (b) BR biosynthesis (c) cell wall synthesis and modification and (d) establishment of proper cell and tissue identities.

12. Recommendations

- Microvine breeding lines containing the *REN4*/*RPV12* mildew resistance loci should now be used as parents in future crosses with selected first generation mildew resistant winegrape varieties, containing the *RUN1*/*RPV1* locus, to produce second generation mildew resistant winegrape varieties with dual PM and DM resistance loci with enhanced resistance durability in the vineyard.
- Future crosses should include parents with a range of different fruit characters such as red-flesh and muscat because the progeny will not only be disease-resistant, but also offer the potential for the development of new wine styles. This should increase the likelihood of adoption of these new varieties by the Australian wine industry
- Microvines in which all four mildew resistance genes i.e. *RUN1*/*REN4*/*RPV1*/*RPV12* are combined in the homozygous state should be developed to increase the versatility of the scion breeding program. This would allow us to use a more diverse range of *V. vinifera* genotypes as parents in crosses with the microvine and ensure the F1 progeny will contain all four mildew resistance genes.
- It is clear that there is considerable genetic variation between different grapevine varieties in bunch architecture, through variation in rachis internode elongation and that this trait segregates in breeding populations. The major challenge is to identify key loci and genes that determine rachis internode elongation. We believe that our use of complementary strategies involving the use of both whole genome mapping studies on segregating populations, together with more targeted gene expression profiling studies on internodes undergoing different rates of elongation, will provide a knowledge base through which candidate genes involved in regulating rachis elongation can be identified in the future. These candidate genes may serve as possible genetic markers for the MAS of new winegrape varieties with more open bunches at maturity.
- While we were successful in identifying hormone and gene regulatory networks that regulate inflorescence internode development, we were unable to map QTLs for bunch architecture using the F1 mapping populations. This is most likely due to the fact there are numerous genetic loci that confer bunch architecture and screening larger F1 mapping populations (i.e. >1000 individuals) would be necessary to increase success of this mapping approach.
- CSIRO currently holds approximately 500 varieties of table and wine grapes in its germplasm collection, which display significant variation in berry size, bunch architecture and fruit quality traits. The possibility of using a genome-wide association analysis to identify loci and markers linked to bunch architecture should be investigated.

Appendix 1: Communication

Scientific Publications

- Ayliffe M, Periyannan SK, Feechan A, Dry I, Schumann U, Wang MB, Pryor A, Lagudah, E (2014) Simple Quantification of *In Planta* Fungal Biomass Paul Birch et al. (eds.), Plant-Pathogen Interactions: Methods and Protocols. Methods in Molecular Biology **1127**: 159-172 Springer Science + Business Media New York 2014
- Yin L,Li X, Xiang J, Qu J, Zhang Y, Dry IB*, Lu J*. (2015) Characterization of the secretome of *Plasmopara viticola* by *de novo* transcriptome analysis. *Physiological and Molecular Plant Pathology* **91**: 1-10.
- Feechan A, Kocsis M, Riaz S, Zhang W, Walker MA, Dry IB, Reisch B, Cadle-Davidson L (2015) Strategies for *RUN1* deployment using *RUN2* and *REN2* to manage grapevine powdery mildew informed by studies of race-specificity. *Phytopathology* **105**:1104-13
- Qiu W, Feechan A, Dry IB (2015) Current understanding of grapevine defense mechanisms against the biotrophic fungus (*Erysiphe necator*), the causal agent of powdery mildew disease. *Horticulture Research* **2,** 15020

Conference Presentations

- Dry IB, Feechan A, Thomas MR. 'New vines for new times'. Invited oral presentation at 15th Australian Wine Industry Technical Conference, Sydney, July 2013.
- Yin L, Feechan A, Lu J, Dry IB. 'Function characterization of the grapevine *MrRPV1* downy mildew resistance gene'. Poster presented at 10th International Congress of Plant Pathology, Beijing, August 2013.
- Dry IB. 'Cloning and functional characterisation of a powdery mildew resistance gene from a wild North American grapevine'. Oral presentation at 3rd International Powdery Mildew Workshop, Copenhagen, Denmark, 29-30th August 2013.
- Dry IB. 'New strategies for the generation of disease-resistant winegrapes'. Invited oral presentation at Verona Winter School in Biotechnology, Canazei, Italy, January 2017.

Conference Publications

- Dry IB (2014) Recent progress in understanding the genetics of pest and disease resistance in grapevine. *Acta Horticulturae* 1046: 27-34.
- Feechan A, Kabbara S, Dry IB (2014) Sources of penetration and PCD-mediated resistance to grapevine powdery mildew in the Vitaceae family. *Acta Horticulturae* 1046: 101-107.
- Dry IB, Feechan A, Thomas MR (2014) New vines for new times. Beames KS, Robinson EMC, Godden PW, Johnson DL (eds) Proceedings of the 15th Australian Wine Industry Technical Conference, 13-18 July 2013, Sydney, NSW. Australian Wine Industry Technical Conference Inc. Adelaide, SA. pp: 151-154.

Industry Journal articles / Interviews

- Dry IB, Thomas MR (2015) Disease resistance: Fast-tracking grape breeding for disease resistance. Wine & Viticulture Journal 30: 52-55.
- Interview leading to article entitled 'Finding the Balance' in Wine Business Monthly (Mar/Apr 2017) about the potential benefits of using GM technology to develop disease-resistant premium winegrape varieties.

Industry presentations

- Invited talk given by Dr Ian Dry on 'New vines for new times' at 15th Australian Wine Industry Technical Conference, July 2013, Sydney.
- Invited talk given by Dr Ian Dry on 'Opportunities with disease-resistant varieties' at the Australian Society of Viticulture and Oenology meeting in Mildura, July 2015.
- Invited talk given by Dr Ian Dry on 'The next generation of disease-resistant grapevines' at the 2nd Australian Cabernet Symposium, Coonawarra, October 2015.

Communications with general public

- The CSIRO website has a web page that is accessible to the general public and describes our use of marker-assisted selection to generate new disease-resistant winegrape varieties: https://www.csiro.au/en/Research/AF/Areas/Plant-Science/Grapes/grapevine-mildew

Appendix 2: Intellectual Property

CSIRO obtained the *V. romanetii* BC2 population 12-3501 containing the *REN4* resistance locus from Dr David Ramming (USDA/ARS/CDPG, Parlier, California) under a USDA MTA agreement. CSIRO and Wine Australia have complete freedom to use this material for the breeding of new disease-resistant winegrape varieties.

Appendix 3: References

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Appendix 4: Staff

Project Staff

Dr Ian Dry (25%)	CSIRO Agriculture & Food, Adelaide
Dr Mark Thomas (10%)	CSIRO Agriculture & Food, Adelaide
Dr Harley Smith (10%)	CSIRO Agriculture & Food, Adelaide
Ange Jermakow, Technical Officer (100%)	CSIRO Agriculture & Food, Adelaide
Nayana Arunasiri, Technical Officer (100%)	CSIRO Agriculture & Food, Adelaide
Debra McDavid, Technical Officer (20%)	CSIRO Agriculture & Food, Adelaide

Collaborators

- Dr Lance Cadle-Davidson - USDA-ARS Grape Genetics Res. Unit, Geneva, NY 14456, USA.
- Dr Bruce Reisch - Horticulture Dept, New York Agricultural Station, Cornell University, Geneva, NY 14456, USA.
- Dr David Ramming - USDA/ARS/CDPG, Parlier, California, 93648, USA.
- Dr Ling Yin - Guangxi Crop Genetic Improvement and Biotechnology Laboratory, Guangxi Academy of Agricultural Sciences, Nanning, China.

Appendix 5: Supplementary Data

This section of the final report is commercial in confidence and will not be available until after December 2018. If you have any questions please contact Wine Australia.

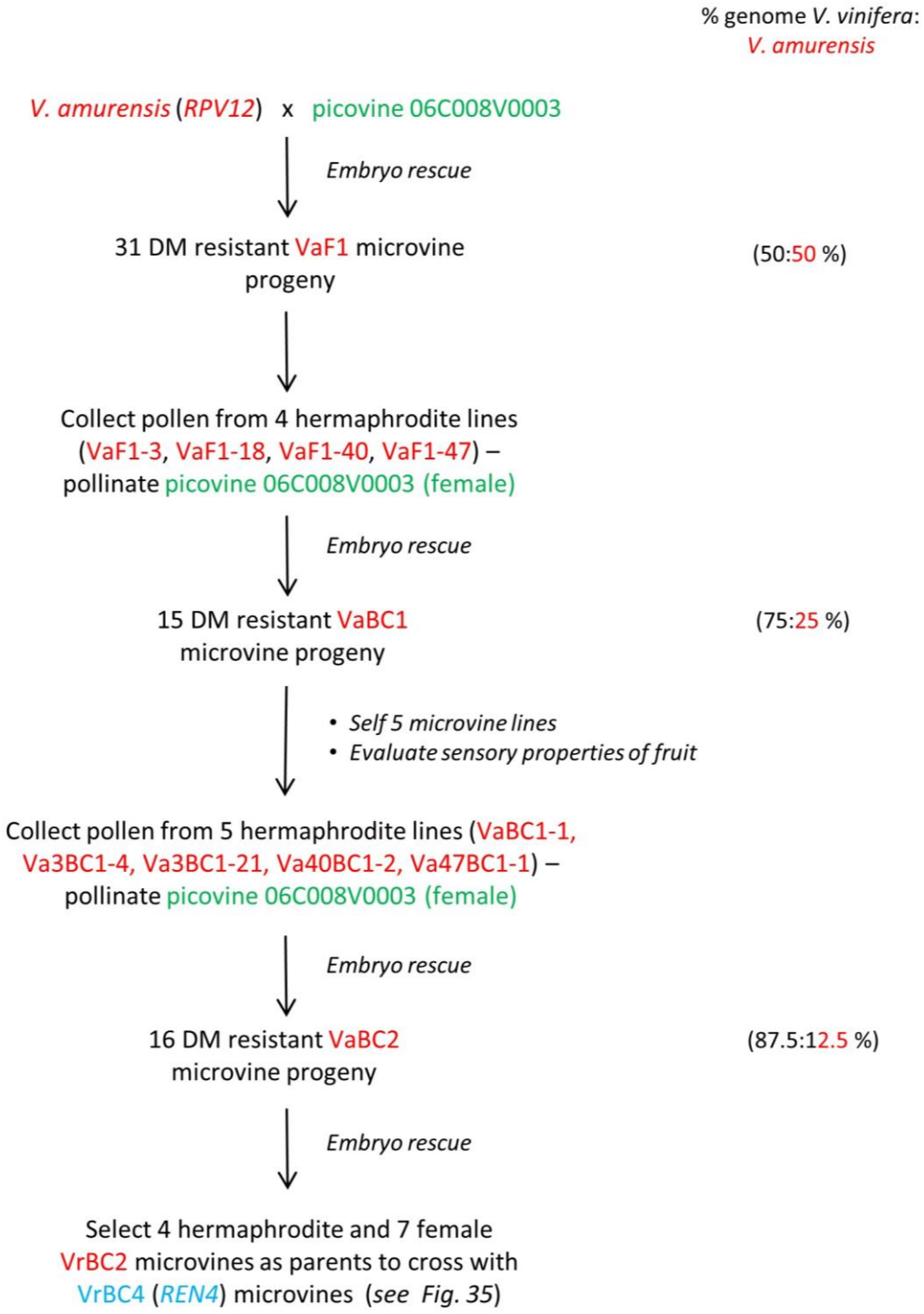

Figure 36. *RPV12* backcross breeding strategy

Australian Government
Department of Agriculture

Department of Agriculture Approval Letter

Release Approval for Quarantine Material

To: Dr Ian Dry	Email: Ian.Dry @csiro.au
QAP: CSIRO QAP GH 111D (#S1639; Class 6.11)	BA Ref: 2014/02536
Valid from: **7 May 2015**	
Import Permit Numbers: IP10019760, IP12013573, IP13000317, IP13014812	Approval letter reference number : **PIO-R-2015-01**
Approval to release: Specified Vitis vinifera hybrids	

Dear Ian,

The Department of Agriculture has completed the weed risk assessment for domestic *Vitis vinifera* plants that have been crossed with pollen of Chinese Vitis spp (*V. romanetti or V. piasezkii*).

The Department accepts that Vitis plants generated from BC3 seed is likely to contain approximately 93.75% *Vitis vinifera* and 6.25% (*V. romanetti or V. piasezkii*)

I note, this material has passed quarantine disease screening requirements.

Conditions of quarantine release:

1. Vitis hybrids (BC3) must be at least 93% Vitis vinifera and less 7% Chinese Vitis spp (V. romanetii or V. piasezkii).

2. Parent material of Chinese Vitis spp (V. romanetti or V. piasezkii) must either remain in quarantine at CSIRO for future breeding trials or be exported/destroyed.

NOTE: The Department reserves the right to revoke or amend this letter.

Yours sincerely

Luigi Paglia 7/5/15.

Luigi Paglia
Assistant Director
Plant Import Operations
Department of Agriculture
plantquar@agriculture.gov.au
Ph: (02) 6272 3917, Fax: (02) 6272 3745

Figure 37. *Dept. of Agriculture permission to release Vitis hybrids at BC3 generation*

Table 18. *Sequences of oligonucleotide primers used in this study*

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