IMPROVED RESISTANCE TO ROOT PESTS

Phylloxera and associated gall on an in vitro grown transgenic grapevine root.
Blue staining (for GUS reporter gene expression) indicates promoter activity at the gall

FINAL REPORT to
GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **CRV 99/15**

Principal Investigator: **Dr Robyn van Heeswijck***
*Deceased September 5, 2003

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and Barry Rolfe (pjj430GH3:TAB10GUS; Australian National University). Through the provision of non-GWRDC and non-AWRI money, Peter Høj facilitated completion of draft manuscripts (Attachments 2 & 3) and this report by enabling extended employment for Tricia Franks and Steven Choimes.

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

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1. Abstract

There were two major aims of this project, which was jointly funded by GWRDC and CRCV2. The first aim was to identify and characterise grapevine genes involved in the plant's interaction with phylloxera and/or root-knot nematode. This information could be used to design strategies for engineering novel resistance to these pests in grapevine. The second aim of the project was to develop and use systems to rapidly assess candidate genes for preventing infestation of vine roots by phylloxera and/or root-knot nematode. Progress towards both goals was achieved despite premature termination of the project after 4, instead of 7 years.

2. Executive summary

This project was one of several that were jointly funded by GWRDC in the CRCV2 Program 3: Molecular Improvement of Grapevines. It had two principal aims, the first of which was to identify and characterise grapevine genes involved in the plant's interaction with phylloxera (*Daktulospaira vitifoliae*) and/or root-knot nematode (*Meloidogyne spp.*). This information could be used to design strategies for engineering novel resistance to these pests in grapevine. The second major aim of the project was to develop and use systems to rapidly assess candidate genes and promoters for use in preventing infestation of grapevine (*Vitis vinifera*) roots by phylloxera and/or root-knot nematode. Work which involved handling viable phylloxera was undertaken within a phylloxera quarantine district in partnership with Dr. Kevin Powell at the Department of Primary Industries, Primary Industries Research Victoria, Rutherglen Centre.

Investigation of grapevine-root pest interaction
Approaches to identifying grapevine genes that are involved in interactions of the plant with phylloxera and root-knot nematode had for their basis, the suggestion that there may be profound similarities between the general response of plants to parasitic nematodes and the grapevine response to infestation by phylloxera (Kellow, 2000). To initiate a non-targeted approach to identifying root pest responsive grapevine genes, a library of transcribed sequences that accumulate in early stage phylloxera galls was constructed. In future, experiments that compare the relative abundance of these sequences in different types of grapevine (infested or uninfested) tissues could be used to isolate those that are important in the interaction of grapevine with its root pests. A minor fraction (128) of the library sequences was contributed to the grapevine "expressed sequence tags" (ESTs) database compiled as part of the CRCV2 project 3.4: Genomics & Generic Technologies. These are the only root sequences in the Adelaide-based collection of expressed grapevine sequences.

Information from previous studies of the interaction of other plant species with root-knot nematode was used in targeted approaches to search for grapevine genes that are involved in the response of this species to root-knot nematode and/or phylloxera. Expressed sequences that may encode water transport molecules (aquaporins) were isolated from phylloxera galls and preliminary results provided some evidence for enhanced accumulation of the corresponding transcripts of two such sequences in root tissue compared with leaf and berry tissue. Accumulation of the sequences also appeared to be enhanced in phylloxera galls (but not root-knot nematode galls) compared with uninfested vine roots. If sequences such as these are absolutely required for initiation and/or maintenance of feeding sites, disruption of their expression could interfere with successful pest invasion and hence provide novel pest resistance. The same sequences can also provide a starting point for isolating promoters of gene expression that could be used to direct anti-root pest gene expression to precise locations in the plant – e.g. to the roots only, or to sites of root-pest feeding.

Development of assay systems
Field and glasshouse trials are typically used to determine the resistance status of grapevines with respect to their pests. *In vitro* conditions offer advantages in terms of time, space and labour requirements, as well as isolation from the variable effects of environmental factors such as soil type, climate and other interacting organisms. At the outset of the project, we already had reasonable expertise with *in vitro* co-culture of phylloxera and whole grapevine plants (Kellow et al., 2002). However, it was necessary to substantially develop similar *in vitro* systems for reliably discriminating grapevine genotypes that are resistant or susceptible to root-knot nematode, and this was done successfully by inoculating whole grapevine plants or rooted petioles with *Meloidogyne javanica* egg sacs. The systems would be generally applicable to assessing the interaction of different grapevine/root-knot nematode genotype combinations, as required by grapevine plant improvement programs.

We acquired expertise with *Agrobacterium rhizogenes*-mediated transformation of grapevine to produce transgenic hairy root cultures. This system provides an improved rate of gene transfer to grapevine roots compared with whole plant transformation using *A. tumefaciens*, and when combined with the *in vitro* co-culture systems described above, potentially provided a relatively

speedy experimental tool for assaying candidate anti-root pest genes and the activity of promoters of gene expression. Indeed, phylloxera and root-knot nematodes appeared to readily invade the transgenic grapevine hairy root cultures and the phylloxera life-cycle was reliably completed, however, development of root-knot nematodes to reproductive maturity may be impaired. Using co-culture of transgenic hairy roots with phylloxera, we found evidence for activation of an auxin responsive promoter at phylloxera feeding sites, implicating that hormone in the establishement/maintenance of phylloxera galls.

Identification of genes capable of disrupting root pest infestation, feeding and/or reproduction
A. rhizogenes-mediated transformation combined with *in vitro* co-culture was used in an attempt to assess the potential for engineered biosynthesis of a secondary metabolite, cyanogenic glucoside (dhurrin), to provide grapevine with protection from its root pests. Dhurrin biosynthesis has been engineered previously in arabidopsis using three genes from sorghum (*Sorghum biocolor*), and high levels of the compound were shown to coincide with acquired resistance to a flea beetle (Tattersall et al., 2001). Access to the three sorghum genes for use in grapevine came through collaboration with Prof. Birger Møller (Royal Veterinary and Agricultural University, Denmark) and we successfully used them to engineer dhurrin biosynthesis in grapevine hairy roots.

A dhurrin-positive grapevine hairy root line was tested and found to release cyanide upon maceration and can therefore be considered 'cyanogenic'. This indicates that an endogenous grapevine glucosidase is able to metabolise the engineered cyanogenic glucoside, which may be important for the expression of the anti-pest effects associated with cyanogenic glucosides. When immersed in extract prepared from this cyanogenic line, juvenile root-knot nematodes suffered temporary paralysis, which is consistent with the response of nematodes to cyanide exposure. After long-term *in vitro dual* co-culture of phylloxera or root-knot nematodes with one cyanogenic hairy root line compared with one acyanogenic hairy root line, the cyanogenic line had significantly more root axes but significantly fewer invading nematodes or phylloxera per root axis. These results may be indicating, on the one hand, that the cyanogenic roots have acquired improved protection from the root pests. Alternatively, the results may actually be reflecting limitations to the infestation capacity imposed by limiting concentrations of the initial inocula. Regardless, the levels of infestation of the cyanogenic line were not low enough to suggest that practical protection from the root pests had been acquired. However, since considerable variation in the amount of dhurrin that accumulated in the grapevine hairy root lines was observed, it is possible that the root pests were exposed to only low levels of the compound. Consequently, it is impossible yet to conclude that the trait is not useful for protecting grapevine from its root pests. Consistently high levels of dhurrin accumulation may be required for this to occur.

As an adjunct to this work, we built on a previous report of cyanogenesis in grapevine (*V. vinifera*) by Deibner (1967) by showing that the species is naturally polymorphic for the accumulation of cyanogenic glucosides (prunasin and sambunigrin) in leaves. Furthermore, one cultivar (Ruby Cabernet) was characterised for accumulation of cyanogenic glucosides in various tissues at three times during the growing season. Despite high levels in leaves, levels in roots were always negligible by comparison. This information is relevant to any future possible need to address issues relating to consumer acceptance of engineered cyanogenic glucoside biosynthesis in grapevine and also provides a basis for establishing a role for cyanogenic glucosides in the interactions of Ruby Cabernet with its pests and pathogens.

With its associated aims and potential outputs, the original project was designed assuming that a PhD student would be recruited and that the project would run for 7 years. Actually, work by a PhD student did not get underway and funding to the project was terminated after only 4 years. Despite this, reasonable progress towards the project's milestones was achieved, owing in substantial part to contributions that were made by several visitors (a Post Doctoral Research Fellow and 3 first class Honours students; Appendix 3) to Dr. Robyn van Heeswijck's laboratory at The University of Adelaide, Waite campus.

3. Background

Introduction
At present, rootstocks derived from American vine species provide an adequate solution to the problems associated with infestation of vineyards by the root pests: phylloxera (*Daktulosphaira vitifoliae*) or root-knot nematodes (*Meloidogyne* spp.). Some of the commonly used rootstocks have however proved not to be resistant to the full range of nematode pathogens present in vineyard soils, in particular the root lesion nematodes (*Pratylenchus* spp.), which are often associated with decline and replant problems. In addition, more recently bred rootstocks are often selected for traits other than phylloxera or nematode resistance and their resistance to root pests may be less than is desirable.

The long-term usefulness of natural rootstock resistances may also be limited by their specificity to particular races or species of nematodes, or biotypes of phylloxera. The identification of Biotype B phylloxera and the widespread demonstration of its debilitating effects on the rootstock AxR1 in California readily illustrates the danger of relying on one or a few narrow sources of natural resistance. The detection of root-knot nematode populations able to overcome the natural resistance of the rootstock Ramsey further suggests that these natural resistances cannot be assumed to be lasting in the face of continuous natural selection and/or adaptation of these root pests.

Based on all of the above, new genetic sources of resistance are sought to enhance the spectrum of root pest resistance of grapevines. The techniques of molecular biology and genetic engineering offer the potential for introduction of specific genes for nematode and/or phylloxera resistance into elite rootstock varieties, for example those bred specifically for salt tolerance. Alternatively, the root pest resistance might be introduced into *V. vinifera* itself, eliminating the need for rootstocks and resulting in `direct producers' which retain all the other characteristics of current commercial cultivars, with no compositional change to the grapes and wine produced therefrom.

Project Proposal
As described in the original application, it was proposed to use molecular biology and genetic engineering techniques to introduce phylloxera and/or nematode resistance into elite rootstock varieties, or into *V. vinifera* to synthesise root pest resistant `direct producers'.

The interactions between plants and nematodes, including mechanisms of resistance have been intensively studied for a number of years now. Some clear strategies for synthesis of nematode-resistant transgenic plants have emerged. In contrast, extremely little is known about the grapevine-phylloxera interaction, and there is a great need for strategic research into both the mechanism of root gall formation, and natural resistance, before such clear strategies against this pest can be formulated. For this reason, it was proposed to develop the capacity to introduce genes which may confer nematode resistance into grapevine rootstocks and *V. vinifera* cultivars in the first instance, with parallel development of the knowledge, tools and techniques which may confer phylloxera resistance. Initially, it was proposed to focus on resistance to root-knot and root lesion nematodes, which have been identified as common nematodes causing decline and replant problems in Australian viticultural systems (GWRDC project UA96/1).

Project Objectives
1. To increase our understanding of the biological processes involved in successful and unsuccessful infestation of grapevine by the root pests nematodes and phylloxera, including their feeding and reproduction.
2. To identify genes and gene products capable of disrupting infestation, feeding and/or reproduction by nematodes and phylloxera.
3. To isolate gene promoter(s) which confine gene expression to roots and/or are induced by root pest infestation, such that transgene products are not expressed in grape berries, and therefore are not contained in grape products.

4. To assess the resistance to root pests of transgenic grapevines containing above decribed genes and gene promoters.

Project Outcomes
1. Knowledge of biological processes involved in infestation of grapevine roots by root pests.
2. Genes capable of disrupting infestation, feeding and/or reproduction by root pests.
3. Gene promoters suitable for driving expression of root pest resistance genes.
4. Transgenic grapevines (rootstocks or *V.vinifera*) with enhanced resistance to root pests.

4. Project Aims and Performance Targets

Performance targets
The forecast outputs and performance targets appear here as they did in the original application. The capacity to meet these performance targets, however, was handicapped because work by a PhD candidate did not get underway, and the project was terminated prematurely after 4, instead of 7 years.

Output	Performance target
1. Description of cyanogenic glucoside products present in non-transgenic grapevine tissues	Measurement of levels of cyanogenic glucosides in a range of grapevine tissues by January 2001
2. Tissue culture and glasshouse based assays for root pest resistance	Assays for assessment of transgenic grapevines for resistance to phylloxera, root-knot nematodes and root lesion nematodes by July 2001
3. Protocol for transformation of grapevine roots	Production of hairy root cultures from *V.vinifera* by July 2002
4. Protocols for transformation of rootstocks	Transfer of transformation technologies developed for *V.vinifera* to at least one rootstock cultivar by July 2002
5. Transgenic grapevines producing natural products (CGs).	Transgenic roots and/or whole transgenic grapevines expressing the CG genes in tissue culture by July 2002
6. Information on the effect of CGs on grapevine resistance to nematodes and phylloxera	In vitro/glasshouse assessment of transgenic grapevines to phylloxera and nematodes by July 2003
7. PhD student lit. review & experimental plan	Completion of lit.review & experimental plan by January 2001
8. Grapevine cDNAs homologous to genes induced by root pests in other plant species	Characterisation of grapevine homologues to at least four tomato cDNAs induced by root knot nematode infestation by July 2001
9. Grapevine cDNAs induced by root pests	Isolation and characterisation of grapevine cDNAs induced by root pests by July 2003
10. PhD thesis	Completion of PhD thesis by January 2004
11. Root specific and/or root pest inducible gene promoters	Assessment of gene expression patterns of at least two gene promoters from other plant species, and two from grapevine by July 2004
12. Transgenic grapevines expressing other genes with potential anti-root pest activity	Transgenic roots and/or whole transgenic grapevines expressing other genes in tissue culture by July 2005
13. Transgenic grapevines expressing genes with anti-root pest activity using tissue specific gene promoters	Transgenic roots and/or whole transgenic grapevines expressing genes in a tissue specific manner in tissue culture by July 2006
14. Information on the levels of resistance to phylloxera and nematodes of transgenic grapevines expressing anti root pest genes in a tissue specific manner	In vitro/glasshouse assessment of transgenic grapevines expressing anti root pest genes in a tissue specific manner for resistance to phylloxera and nematodes by July 2006

Milestones

Originally, 14 milestones that correspond to the performance targets listed on the previous page were targeted for completion over 7 years. Amendments to the original milestones are listed below and accommodate the change in circumstances.

Milestone	Year 1	2	3	4	5	6	7
Appointment of a PhD student[1], Postdoctoral Scientist and part-time Research Assistant.	X						
Characterisation of natural levels of cyanogenic glucosides (CG) in grapevine tissues.	X	X					
Development of assays for root pest resistance.	X	X					
Development of grapevine root transformation protocol and application of transformation technologies to rootstocks.	X	X	X	X			
Transformation of grapevine with genes for anti-pest natural products (CGs) using constitutive gene promoters.	X	X	X				
In vitro/glasshouse assessment of CG transgenic grapevine roots for resistance to nematodes and phylloxera.			X	X			
Transformation of grapevine with other anti-root pest genes using constitutive gene promoters, and assessment of resistance[2].							
PhD student literature review and experimental plan[1].							
Isolation and characterisation of grapevine homologues to tomato cDNAs induced by nematodes[3,5].	X	X					
Isolation & characterisation of grapevine cDNAs induced by root pests[3,5].		X	X				
Write up PhD thesis[1].							
Isolation and characterisation of root specific and/or root pest inducible promoter(s) from grapevine or other species[4,5].							
Transformation of grapevine with gene(s) using promoters with specific expression patterns[5].							
Assessment of transgenic plants for gene expression patterns and resistance to phylloxera and nematodes[5].							

Explanation of amendments (May, 2003)

[1]The initially appointed PhD student withdrew candidature and, shortly after appointment, the second candidate transferred from the project because Dr. van Heeswijck retired due to ill health. Milestones obviously associated with a PhD student have been deleted.

[2]Work towards this milestone was originally targeted to commence at the end of year 2, but was delayed due to discussions about the current perceived level of consumer acceptance of transgenic grapevines (especially those containing genes from species other than grapevine) in a meeting of the Program 3 Industry Reference Group in June 2001.

[3]Two Honours students and a visiting Postdoctoral Scientist initiated work towards these milestones; however, progress was delayed due to the absence of a PhD candidate.

[4]Commencement of work on this task was linked to outcomes from milestones 9 & 10 and therefore was delayed – see footnote 3.

[5]These milestones have been deleted due to Dr. van Heeswijck's premature retirement at the end of year 3 and the premature termination of the project at the end of year 4.

5. Methods

5.1 Construction of a cDNA library from early stage phylloxera root galls

Messenger RNA extracted from early stage phylloxera galls (i.e. first signs of root swelling after phylloxera feeding site initiation) on Shiraz roots was used to construct a cDNA library of clones in the lambda phage vector uni-ZAP XR (Stratagene) using the ZAP-cDNA® Giga pack® III Gold Cloning Kit (Stratagene). The primary library, which consisted of about 4.6×10^4 clones, was amplified.

5.2 Degenerate PCR for isolation of TobRB7-like sequences from grapevine

The degenerate PCR reaction mix contained: 1 x PCR buffer (GibcoBRL), 0.2mM of each dNTP (GibcoBRL), 1.25 μM of each primer (Fig. 1), 4.5 mM $MgSO_4$ and 0.05 U μL^{-1} High Fidelity Platinum *Taq* DNA polymerase (GibcoBRL). Template was either 2 µL of first strand cDNA (stage 2 phylloxera galls) or 5 µL of excised pBluescript phagemid stock (isolated from the above described phylloxera gall cDNA library). PCR reactions were incubated as follows: denaturation at 94^oC for 10 min; then 25 cycles of denaturation at 94^oC for 1 min, annealing at 55^oC for 1 min and extension at 72^oC for 1 min followed by a final extension at 72^oC for 7 min. Products of degenerate PCR and RACE (rapid amplification of cDNA ends; FirstChoise™ RLM-RACE Kit, Ambion) were cloned into pCR®2.1-TOPO Invitrogen)

TIPforward: 5´-CAYKTKAAYCCKGC(inosine)GTKAC-3´
TIPreverse: 5´-GGBCCBRCCCAGTASAYCCA-3´

Fig. 1 Degenerate PCR primers customized from (Weig et al., 1997) for grapevine TIPs. Figure from Thieleke (2002).

5.3 Relative quantitative RT-PCR

DNAase treated RNA stock solutions were diluted to 40 ng μL^{-1} according to absorbance at 260 nm. For standardisation of stock solutions, RQRT-PCR was performed using the Superscript™ One-Step RT-PCR with Platinum® Taq system (Invitrogen). The reaction mix was: 1 x RT-PCR reaction mix; 0.2 μM of each primer (Vv18SF and Vv18SR; Fig. 2); 0.4 μL RT/Platinum *Taq* mix and 40 ng of total RNA template. Reactions were incubated as follows: 50^oC for 30 min; 94^oC for 2 min; 5 cycles of: 94^oC for 30 sec, 50^oC for 30 min and 72^oC for 30 sec; 72^oC for 10 min. Samples were verified to be free of genomic DNA contamination by substituting RT/Platinum *Taq* mix (Invitrogen) with 2 U *Taq* DNA polymerase (GibcoBRL) and amplifying 40 ng of standardised RNA template for 15 cycles. 10 µL of each RQRT-PCR reaction mix was electrophoresed through a 2% w/v agarose gel and the gel was Southern blotted and probed with a radiolabelled 18S rDNA fragment. Hybridisation of the probe was detected using a Storm 860 phosphorimager (Molecular Dynamics) and quantified using ImageQuantNT™ (Amersham) software and RNA solutions were equalised accordingly.

The reaction mixes for RQRT-PCR with gene specific primers (Fig. 2) were as described above except that $MgCl_2$ was adjusted to 1.2 mM and 2.0 mM for Vvmip15 and Vvmip 17, respectively and annealing temperatures for Vvmip15 and Vvmip17 were 53.6°C and 58°C, respectively. Amplification conditions were as described above, except that 30, 35 or 40 cycles were used.

Vv18SF: 5´-CAACAAACCCCGACTTCTG-3´
Vv18SR: 5´-TGTCACTACCTCCCCGTGTC-3´
Mip17F: 5´-GTTGGCTCCATTCTTGCATG-3´
Mip17R: 5´-GTTGTCCTTGAAGTCGCCG-3´
Mip15F: 5´-CAAGTTTCTCACTGGTGGAC-3´
Mip15R: 5´-GTTTGTCCAGTCCCAGCTC-3´

Fig. 2 PCR primers used for RQRT-PCR to determine the relative accumulation of transcripts from Vvmip15 and Vvmip17 in leaves, berries, roots as well as roots infested with phylloxera or root-knot nematode (Fig. 4). Primer sequence for amplification of (18S) rRNA (i.e. Vv18SF and Vv18SR) was provided by Ian Dry (CSIRO, Plant Industry). Figure adapted from Thieleke (2002).

5.4 Production of transgenic tissues

Hairy root transformation of grapevine

Methods for inoculating grapevine with *Agrobacterium rhizogenes* are described by (Bondar, 2001) and in Attachment 2. Hairy roots recovered from inoculation sites were cultured on a medium (LG_{o2}) which is almost identical to LG_o (Torregrosa & Bouquet, 1997), see Attachment 2.

Whole plant transformation of Schwarzmann rootstock

Embryogenic callus was initiated from immature anthers on TK´ medium (major elements (Nitsch & Nitsch, 1969); minor elements (Murashige & Skoog, 1962); vitamins (Gamborg et al., 1968); Fe-EDTA (Dalton et al., 1983); 60 g L^{-1} sucrose, 5 µM 2,4 D; 0.8 µM BAP, pH 5.7; 0.3% phytagel), which is a minor modification of that described by (Xue et al., 1999). The transforming *Agrobacterium* strain was AGL1 carrying a binary vector (pPLEXGGG), which contains in the T-DNA region: *gfp* and *nptII* under control of the CaMV35S and Sc1 constitutive promoters, respectively, as well as *gusA* behind the auxin responsive GH3 promoter from soybean. Two weeks following co-culture with *Agrobacterium*, the callus was separated into discreet lumps which were maintained separately thereafter on TK´ medium plus Timentin (1 mg mL^{-1}) and G418 (12.5 µg mL^{-1}) in order to promote recovery of unique transformants. GFP positive embryos were selected from plates and transferred to one or other of two germination media. Either hormone free GS1CA (Franks et al., 1998) plus Timentin (1 mg mL^{-1}) or MGC (Xue et al., 1999) slightly modified: (SM major elements (Iocco et al., 2001); minor elements (Murashige & Skoog, 1962); vitamins (Gamborg et al., 1968); Fe-EDTA (Dalton et al., 1983); 20 g L^{-1} sucrose, 4.6 g L^{-1} glycerol, 1 g L^{-1} casein hydrolysate, pH 5.8 and 0.8% agar) plus Timentin (1 mg mL^{-1}). Embryos that germinated (i.e. hypocotyls elongated) were transferred to shooting medium (Iocco et al., 2001) plus 10 µM BAP. When a shoot emerged it was transferred to rooting medium (Iocco et al., 2001).

Transfer of 3 sorghum genes for cyanogenic glucoside biosynthesis to Sultana hairy roots

Methods for gene transfer and characterisation of transgenic tissues are described in Attachment 2.

5.5 Characterisation of the interaction of grapevine with phylloxera and root-knot nematode

In vitro co-culture of whole plants and hairy roots with phylloxera and root-knot nematode

Whole plants were co-cultured with phylloxera as described by (Kellow et al., 2002) and hairy roots were co-cultured with phylloxera as described in Attachment 2. *In vitro* co-culture of grapevine plants with root-knot nematode (M. javanica) are described in Franks et al. (2003b) (Attachment 1). Hairy roots were co-cultured with root-knot nematode in 0.5 SM medium, essentially as described by Franks et al. (2003b) (Attachment 1).

Immersion of juvenile root-knot nematodes in transgenic hairy root extracts

To prepare extracts, hairy root tissue (about 120 mg) was ground in a mortar and pestle with 5 times volume of tap water. The slurry was transferred to a microfuge tube, mixed well by vortexing and incubated for 3 hours at room temperature. After incubation, the extract was mixed again by vortexing and the supernatant was recovered after centrifuging for 2 mins at 12,000 x*g*. This supernatant (i.e. hairy root extract) was used either immediately or the following day.

To hatch nematodes, root-knot nematode (*M. javanica*) egg masses (30) were first disrupted by treating for 5 minutes in 0.95 % w/w sodium hypochlorite equivalent to 1% w/v available chlorine (Milton ®), then washing 5 times in water using a Nalgene® vacuum filtration apparatus (2 µM pore size) as described in Attachment 1 (Franks et al., 2003b). The eggs (in 500 µL) were incubated overnight at 27°C in aliquots in the wells of a microtitre tray. Juvenile nematodes (J2s) that hatched were collected using a fine bore pasteur pipette. The nematodes (suspended in water) were mixed with an equal volume of extract (50 µL) in the wells of a microtitre plate and observed using a dissecting microscope (Nikon SM Z800).

6. Results and Discussion

Investigation of grapevine-root pest interaction

As described in the original application, characterisation of the molecular genetic response of grapevine to interactions with its root pests would provide resources for (i) isolating promoters of gene expression that are suitable for driving root pest-resistance genes and (ii) disrupting biological events required for infestation of grapevines by root pests to effect pest resistance, as described by Conkling et al. (1999).

6.1 Construction of a cDNA library from early stage phylloxera root galls

A cDNA library of transcribed sequences that accumulate in early stage phylloxera galls (i.e. first signs of root swelling after phylloxera feeding site initiation) on Shiraz roots was constructed (by visiting Postdoctoral Fellow, Harry Teicher). This library is stored in the Discipline of Wine and Horticulture at The University of Adelaide and remains a good resource from which grapevine genes that are expressed in roots and/or differentially regulated in the event of invasion by phylloxera may be recovered. 128 unique ESTs from the library are included in the Adelaide-based collection of expressed grapevine sequences (CRCV2 project 3.4: Genomics & Generic Technologies).

6.2 Identification of grapevine sequences with homology to genes that have been implicated in interactions with root-knot nematode in other plant species

Collaboration with Associate Professor David Bird (North Carolina State University, USA) brought to the project a subtracted library (of 196 tomato sequences) that had been produced from specialist feeding cells (giant cells) which occur within root-knot nematode galls (Wilson et al., 1994). The 196 sequences were used to screen nucleotide databases for sequence similarity and 76 were found to have homologues in other species. In particular, one of these sequences (clone DB#249) was confirmed to have homology with the complement of a tobacco sequence, Tob-RB7-5A, which is an aquaporin (water transport molecule) and a tonoplast intrinsic protein (TIP) of the larger group of major intrinsic proteins (MIPs). In tobacco, this sequence has also been implicated in giant cell formation in root-knot nematode interaction with tobacco (Opperman et al., 1994). Reduced stringency Southern analysis identified that at least one sequence in the grapevine genome has homology to DB#249 (Fig. 3).
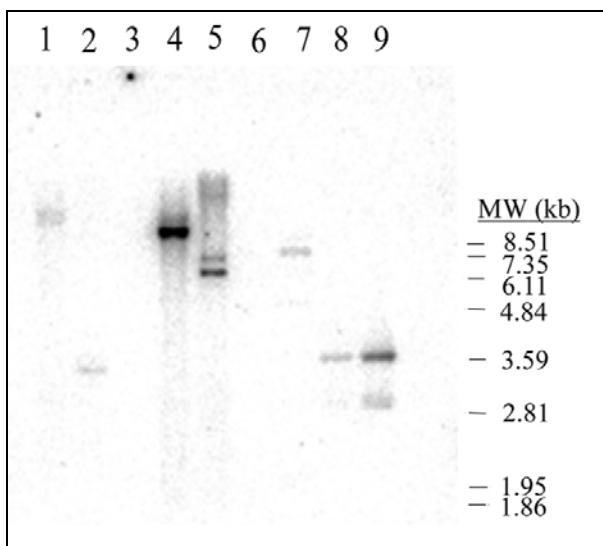

Fig. 3
Reduced stringency (60%) Southern analysis of grapevine genomic DNA with an aquaporin-like sequence (clone DB#249) that is up-regulated in giant cells in root-knot nematode galls on tomato roots.
Lanes 1 & 2 are grapevine genomic DNA digested with Eco RV and Bbu I, respectively. Lanes 4 & 5 are tomato genomic DNA digested with Eco RV and Bbu I, respectively. Lane 7 is the molecular weight marker and lanes 8 & 9 contain 1x and 5x genomic equivalents of the DB#249 clone, respectively. (Figure from Croser, 2000).

6.3 Isolation and characterisation of grapevine sequences with homology to genes that have been implicated in interactions with root-knot nematode from other plant species

A total of 15 partial Tob-RB7-like sequences were recovered from grapevine phylloxera gall cDNA using degenerate PCR with primers for major integral proteins (MIP) that were customised from (Weig et al., 1997) to accommodate the sequence specificities of 13 grapevine TIPs extracted from the local grapevine EST database or Genbank (Fig. 1). In particular, two of these sequences (Vvmip15 and VvmMIP17; Appendix 4) were recovered from early stage (stage 1) phylloxera gall cDNA using for template, excised pBluescript phagemid stock that had been prepared from the amplified lambda phage library (section 6.1). Like TobRB7, these sequences were both classified as TIPs by referring to the classification of Arabidopsis MIPs by (Johanson et al., 2001). Preliminary experiments with relative quantitative RT-PCR (RQRT-PCR), provided evidence that in grapevine, both of these sequences appear to accumulate in greater abundance in roots compared with leaves and berries and may also accumulate with greater abundance in phylloxera galls compared with uninfested roots (Fig. 4A & 4B). There was, however, no evidence for a relative increase in accumulation of either of the transcripts in *in vitro* grown root-knot nematode galls compared with unifested *in vitro* grown roots (Fig. 4B). To confirm these patterns of expression, these experiments would ideally be repeated in parallel with amplification of a standardising transcript (e.g 18S rRNA) to confirm that the amounts of template RNA are indeed equal for all tissue types.

Full-length sequence of Vvmip15 and Vvmip17 transcripts were recovered by cloning the 5´ and 3´ ends using RACE. For each 5´ or 3´ RACE, 6 clones were sequenced. For Vvmip15, length of the 5´ sequences was heterogeneous, with at least two different in-frame open reading frames differing in length by 144 bp (48 amino acids; Appendix 4) recovered. 3´ RACE sequences were quite similar in length. On the contrary, clones of 5´ sequence of Vvmip17 were all quite similar in length, whereas 3´ sequence was heterogeneous (Appendix 4).

Nominally complete sequence for each of the Vvmip15 and Vvmip17 transcripts were reconstructed by combining the longest, good quality 3´ and 5´ sequence for each case (Fig. 5). For Vvmip15, an alternative transcript was also generated by combining the shortest 5´ sequence with the longest 3´ sequence (Fig. 5A). Vvmip15 and Vvmip17 sequences were confirmed to encode MIPs by virtue of the presence of the diagnositic NPA region as well as a "loop E" signature sequence which is indicative of TIP-like sequence (Fig 5). A sequence similarity search of the Genbank Main database using the tblastn algorithm with both putative full-length versions of the Vvmip15 protein returned 66% identity to a transcript isolated from tobacco suspension culture and encoding a TIP (Genbank Acc. AJ237751). The sequence may therefore not have been isolated previously from grapevine. The Vvmip17 has, however, definitely been isolated from grapevine previously because two grapevine transcripts that encode amino acid sequences with 100% identity to the putative Vvmip17 protein were recovered from the Genbank main database. They are both putative TIPs: one from *Vitis vinifera* (Fort et al., unpublished; Genbank Acc. AJ289866) and the other from *V. berlandieri* x *V. rupestris* (Baiges et al., 2001; Genbank Acc. AF271661).

If more than one Vvmip15-like and Vvmip17-like sequence occurs in the genome, the recovered 3´ untranslated sequence will assist with identifying the bona fide coding regions for the respective sequences. Then, the 5´ sequence of Vvmip15 and Vvmip17 that have been recovered provide the starting points for isolating promoter sequence, immediately upstream of transcription initiation site. Such promoters would be useful for directing transgene expression to roots in preference to other tissues of the plant for various engineering applications. These promoters could also be modified (e.g. by deleting particular motifs) to more precisely confine transcription activation to the roots or even root pest feeding sites.
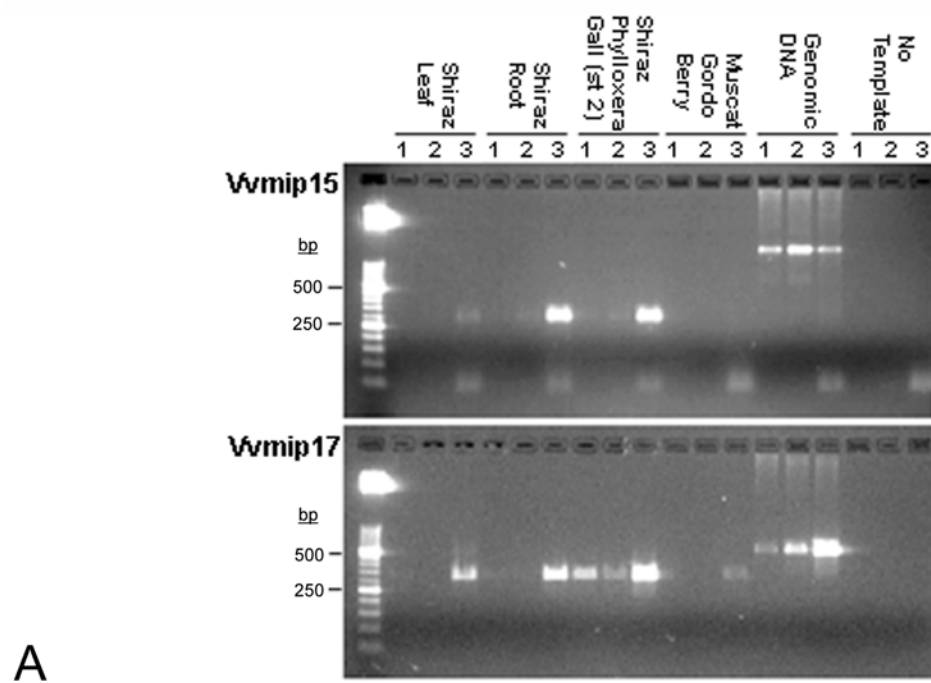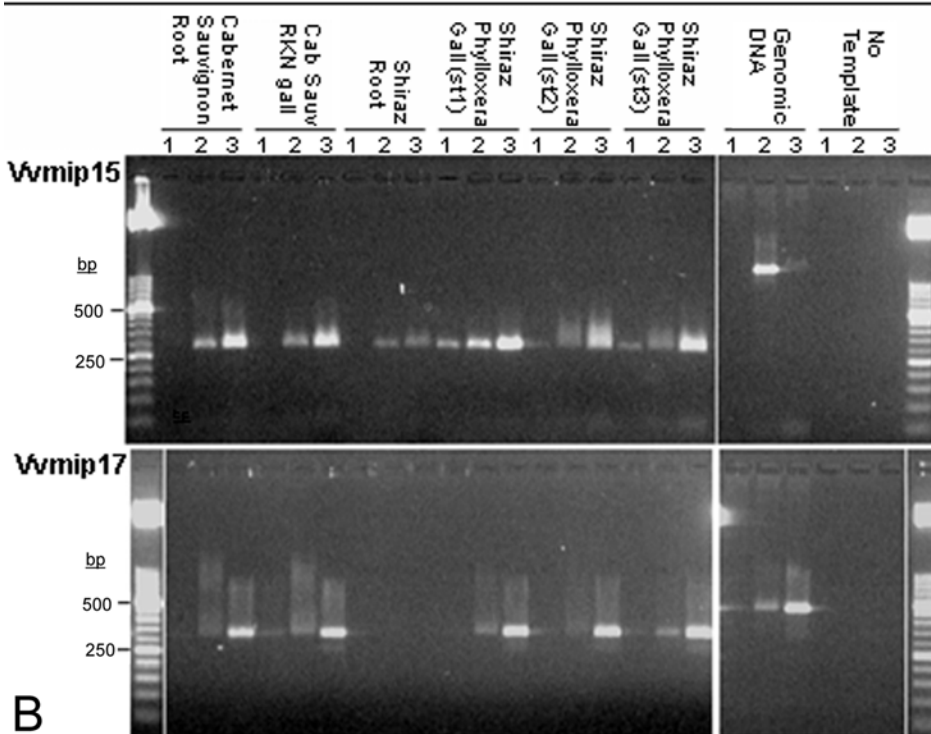
Fig 4 RQRT-PCR to examine the accumulation of transcripts of two putative TIPs using primers in Fig. 3. Phylloxera gall (st 1, st 2 or st 3) = early stage (st 1) galls; intermediate (st 2) and advanced (st 3) galls. Products were run on a 2% agarose gel and stained with ethidium bromide. Lanes labeled 1, 2 & 3 are PCR products after 30, 35 & 40 cycles of amplification, respectively. RNA samples were standardized by quantitating PCR amplification (after 5 cycles) of 18S rRNA using specific primers (Fig. 3). **A** Comparison of the amount of accumulated transcript in different types of tissues. **B** Comparison of amount of accumulated trancript in root tissues that were either uninfested or galled by phylloxera or root-knot nematode (adapted from Thieleke, 2002).

Vvmip15

```
   1  AAAAAAGTGTTAATTACAATGGCACACCTACTAATTTCCTGGAAAAATAAAGTGCCACCC
                        M  A  H  L  L  I  S  W  K  N  K  V  P  P

  61  TCCATAGAGGTGAAGGAGGAAGCTGATTCCTTATATAACTCTCCCATCTTCGATCCTGCC
       S  I  E  V  K  E  E  A  D  S  L  Y  N  S  P  I  F  D  P  A

 121  AAGCCAAAGCCTGAATCCACCACCCCCCATTCCTCTCTCAAAATGGCCAAGATGGCTCTC
       K  P  K  P  E  S  T  T  P  H  S  S  L  K  M  A  K  M  A  L

 181  GGCTCCGGCCGTGAGTTCGCCCAGCCCGACTGCATCCGAGCTCTCGTCATGGAGTTCATC
       G  S  G  R  E  F  A  Q  P  D  C  I  R  A  L  V  M  E  F  I

 241  GTCACCTTCCTGTTCGTCTTCGCGGGTGTCGGCTCCGCCATGGGCACCGAGAAGCTGAAG
       V  T  F  L  F  V  F  A  G  V  G  S  A  M  G  T  E  K  L  K
                                                  A

 301  GGAGATTCACTGGACTCCCTGTTTTTTGTGGCGATGGCACATGCGCTGGTGGTGGCGGTC
       G  D  S  L  D  S  L  F  F  V  A  M  A  H  A  L  V  V  A  V

 361  ATGGTATCGGCGGCGCTGCAGATCTCCGGTGGCCACGTGAACGCGGCGGTGACTCTTGGT
       M  V  S  A  A  L  Q  I  S  G  G  H  V  N  P  A  V  T  L  G
                                               L

 421  CTCTGCGTGGGTGGTCACATCACCGTTGTGAGATCCGTCCTGTACTTCATCGATCAGTGT
       L  C  V  G  G  H  I  T  V  V  R  S  V  L  Y  F  I  D  Q  C

 481  TTGGCTTCTACTGTAGCCTGTATTCTCCTCAAGTTTCTCACTGGTGGACGGGCAACTCCA
       L  A  S  T  V  A  C  I  L  L  K  F  L  T  G  G  R  A  T  P

 541  GTGCACACACTGGCGAGTGGAGTGGGGTGTCTACAAGGGGTAATGCTAGAATTTATCCTC
       V  H  T  L  A  S  G  V  G  C  L  Q  G  V  M  L  E  F  I  L

 601  ACGTTCTCGTTGTTGTTCACAGTCTATGCCAATATAGTATCAGCACAGAAGTCTGCCCAC
       T  F  S  L  L  F  T  V  Y  A  N  I  V  S  A  Q  K  S  A  H

 661  ATTGATGGGCTGGGCCCAATGATAACTGGGCTTGTGGTGGGGGCCAACGTCATGGCTGGT
       I  D  G  L  G  P  M  I  T  G  L  V  V  G  A  N  V  M  A  G

 721  GGGGCCTTTTCCGGGGCTTCCATGAACCCGGCCCGGTCCTTCGGGCCGGCTTTGGTGAGC
       G  A  F  S  G  A  S  M  N  P  A  R  S  F  G  P  A  L  V  S

 781  TGGGACTGGACAAACCACTGGGTGTACTGGGTTGGGCCACTGGTAGGTGGTGCGGTGGCT
       W  D  W  T  N  H  W  V  Y  W  V  G  P  L  V  G  G  A  V  A

 841  GGATTTGTGTATGAGAACTTTTTCATTAACAGACCCCATCTTCGTCTCCCAACAAGAGAC
       G  F  V  Y  E  N  F  F  I  N  R  P  H  L  R  L  P  T  R  D

 901  GAAGAAGAAGAAGGCTTCTAAGCTTTATATTCTATATTAGAAGCAGCCAAGTGGCGT
       E  E  E  E  G  F  *

 961  ATGATGAGATCTATGCCACTTTTTCTTTTGATTTCAGTCGTTTAGACTTCTTGTAATTCC
1021  ACCATGTTCATGGTTTCAGTGTGGCTCGGAGTGTTTTCTCTATTAATTTTTTTTTTTTTT
1081  TTTTTGTGGGTGTAATGGAAAGTCTGTGAGAAAGGACAACCACATAAATAAAACAGCGAG
1141  CCTTTTCACTTGTTTATTGAAAAAAAAAA
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Fig. 5A

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Vvmip17

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   1  AAAATCTCAGCCTTCTCCTATCCTCATCTCTTCATTCATCTTCAATAGTTGCTTCCAAAA
  61  AATGCCTAAAATAGCCTTCGGCCGCTTCGATGATTCCTTCAGTTTGGCCTCCTTCAAGGC
      M  P  K  I  A  F  G  R  F  D  D  S  F  S  L  A  S  F  K  A

 121  CTACCTTGCTGAGTTCCACTCCACCATACTCTTCGTCTTTGCTGGAGTCGGTTCAGTCAT
      Y  L  A  E  F  H  S  T  I  L  F  V  F  A  V  G  S  V  M  A

 181  GGCTTACAACAAGTTGACATCAGACGCTGCTCTTGACCCGGCGGGGCTGGTGGCGGTTGC
      Y  N  K  L  T  S  D  A  A  L  D  P  A  G  L  V  A  V  A

 241  TGTTGCCCATGGTTTCGCTCTCTTTGTTGCAGTCGCTATTAGCGCCAACATCTCCGGTGG
      A  H  G  F  A  L  F  V  A  V  A  I  S  A  N  I  S  G  G  H

 301  CCATGTTAACCCTGCGGTGACCTTCGGGCTGGTTGTTGGTGGTCAGATCACCATTCTCAC
      V  N  P  A  V  T  F  G  L  V  V  G  G  Q  I  T  I  L  T  G

 361  TGGCATCTTGTACTGGATTGcCCAGCTTGTtGGCTCCATTCTTGCATGTTTCcTACTCAA
      I  L  Y  W  I  A  Q  L  V  G  S  I  L  A  C  F  L  L  K  L

 421  ACTTGTCACAGGAGGCTTGACGACTCCCGTCCATAGTCTTGGAGCTGGGGTTGGAGTCAT
      V  T  G  G  L  T  T  P  V  H  S  L  G  A  G  V  G  V  I  D

 481  TGATGCTATTGTCTTCGAGATCGTGATCACTTTCGCTCTGGTCTACACCGTCTATGCAAC
      A  I  V  F  E  I  V  I  T  F  A  L  V  Y  T  V  Y  A  T  A

 541  GGCGGTTGACCCGAAGAAGGGCTCACTGGGCATCATTGCACCCATTGCCATAGGTCTTGT
      V  D  P  K  K  G  S  L  G  I  I  A  P  I  A  I  G  L  V  V

 601  TGTAGGTGCAAACATCCTGGCTGCAGGCCCATTCTCCGGTGGATCAATGAACCCCGCCCG
      G  A  N  I  L  A  A  G  P  F  S  G  G  S  M  N  P  A  R  S

 661  CTCCTTCGGCCCCGCCGTCGTCAGCGGCGACTTCAAGGACAACTGGATCTACTGGGTGGG
      F  G  P  A  V  V  S  G  D  F  K  D  N  W  I  Y  W  V  G  P

 721  ACCCCTAATTGGAGGTGGCATGGGAGGATCTGTCTATGCAATTATGTACATGGGCTCTGA
      L  I  G  G  G  M  G  G  S  V  Y  A  I  M  Y  M  G  S  D  H

 781  TCATCAACCACTAGCGTCCAGCGAATTCTAAGCTGAGTTTTTTTCAGGAACTCAATTGTT
      Q  P  L  A  S  S  E  F  *

 841  TGTTGTTGTCTCAACCCATTTCCCTTTGAAATAAAAAGGAGGAAGAGCAAGTTGTGCTAC
 901  TTCTTTCTTTTTGTACTTGTATTTTGTTCTTTGATCTTCTTCGATTCTCTCCCTTTTGGA
 961  TTTGTAAAGCCCTTGCTTTGGTTCAAGCTGTGTAAAAGATGCAATTATGCATATGAATGA
1021  GGTTGGTGATGTGCAGCCACTTTGCTTTCTGAAAAAAAAAAA
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Fig. 5B

Fig. 5 "Full-length" Vvmip15 and Vvmip17 cDNAs (generated by artificial fusions of sequences recovered by 5´ and 3´ RACE) with their respective deduced amino acid sequences. For Vvmip15, the two alternative cDNAs shown consist of alternative fusions of different 5´ and 3´sequences. The corresponding alternative translation starts are in green and two alternative amino acids are highlighted in bold. A single full-length sequence is shown for Vvmip17. For both Vvmip15 and Vvmip17, diagnostic NPA regions (blue) and Loop E signature sequence (red) are highlighted (Schäffner, 1998). Figure from Thieleke (2002).

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Development of assay systems

6.4 Hairy root transformation of *V. vinifera*, whole plant transformation of Schwarzmann rootstock

Hairy root transformation of grapevine has been reported previously (eg. Gribaudo et al., 1995; Torregrosa & Bouquet, 1997). Using a simple inoculation system (described by Bondar (2001) and in Attachment 2) in combination with the GFP reporter gene system, transgenic hairy roots were recovered from Cabernet Sauvignon and Sultana and maintained in culture.

For whole plant transformation of rootstocks, we worked with Schwarzmann (*V. rupestris* x *V. riparia*), which is differentially sensitive to different phylloxera biotypes (Kellow, 2000) and therefore provides the possibility of studying both susceptible and resistant interactions. Transformation protocols described by (Iocco et al., 2001) were modified slightly, as indicated in section 5.6, to recover five independent GFP positive plants.

6.5 *In vitro* systems for studying the interaction of root-knot nematode with grapevine

Vineyard isolates of root lesion nematode (*Pratylenchus sp.*) and root-knot nematode (*Meloidogyne sp.*) were established in *in vitro* culture on carrot discs and tomato hairy roots, respectively. However, resources were subsequently directed towards developing systems with root-knot nematode. Description of the *in vitro* systems for co-culture of grapevine with root-knot nematode (*M. javanica*) and applications of these systems to characterising aspects of the plant-pest interaction have been published (Franks et al., 2003b; Attachment 1).

6.6 Co-culture of transgenic grapevine roots with root pests
Co-culture with root-knot nematode

Mature egg-laying females were observed to develop infrequently on Cabernet Sauvignon hairy roots (results not shown) and the life-cycle generally did not progress to completion on Sultana hairy roots (see below in section 6.7). This may be a reflection of a departure of hairy roots from normality and/or suboptimal co-culture conditions. With further experimentation, it may be possible to define conditions for co-culture which support efficient completion of the nematode's lifecycle. For example, reduction of the concentration of nematode egg inoculum would result in reduced numbers of nematodes that initially penetrate the roots and may promote an increase in the rate of development of egg-laying females.

Co-culture with phylloxera

Transgenic grapevine material (whole plants and hairy root cultures) was successfully co-cultured with phylloxera. Using the system, reporter gene expression directed by the nominally constitutive CaMV35S and auxin responsive GH3 promoters was monitored in gall tissue after a long co-culture period. At this time, galls were at various stages of development, ranging from little to no swelling (insect present) to extensive swelling.

Evidence for enhanced CaMV35S promoter activity at phylloxera feeding sites

After co-culture with phylloxera of whole transgenic grapevine plants - *cvs* Shiraz and Sultana - transformed with constructs containing *35S:gusA* (pNTG+; Franks et al., 1998) and *35S:gfp* (pBINm-gfp5-ER; Haseloff et al., 1997), respectively, short incubation times for GUS staining and low backgroundl GFP fluorescence permitted resolution of phylloxera-induced expression. Compared with uninfested tissues, enhanced reporter gene expression was detected at newly established feeding sites as well as in established galls (Figs. 6 & 7). This demonstration of 35S promoter activity at the site of phylloxera feeding confirmed the suitability of the promoter for directing expression of anti-phylloxera genes, as described in section 6.7.

Phylloxera ingests GFP

Phylloxera feeding on transgenic roots expressing GFP fluoresced (Fig. 8) and as the phylloxera continued to feed and mature, they appeared to fluoresce with increasing intensity. This suggests that GFP - and presumably other nutrients - continuously accumulated in the gut, which is consistent with recent work showing that the anus is partially restricted and the posterior midgut
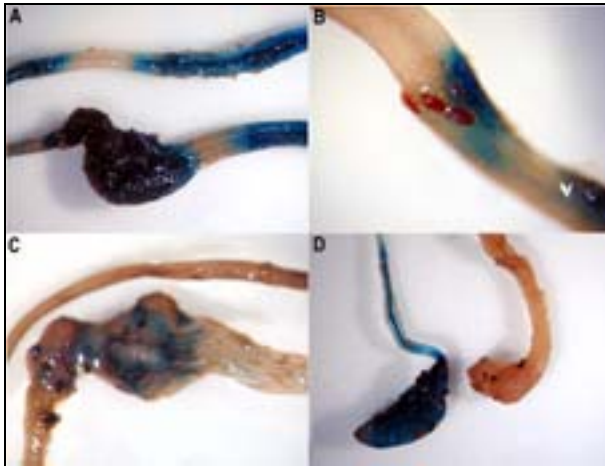
Fig. 6
CaMV35S directed *gus* expression in Shiraz roots with and without phylloxera. Root pieces were stained in 1mM X-Gluc for 6 hrs unless otherwise stated. **A** Transgenic line (54) un-inoculated (top) and with phylloxera gall (bottom). **B** Early stage feeding sites (line 161). **C** Line 161 un-inoculated (top) and with phylloxera gall (bottom). Staining in 1mM X-Gluc proceeded for only 30 min. **D** Galls on line 54 (left) and untransformed control. Figure adapted from Bondar (2001).
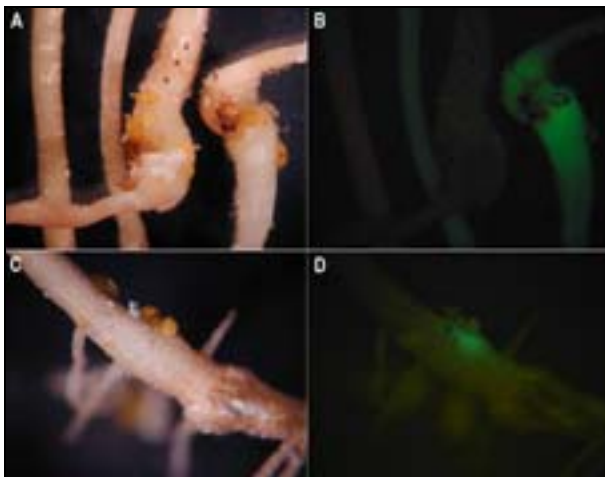

Fig. 7
CaMV35S directed *gfp* expression in phylloxera infested Sultana roots. Left hand panels were illuminated with white light and adjacent panels are the same field of view illuminated with UV light. **A&B** Galls on a transgenic root (line 2G3; far right) and an untransformed control root (inner right). The inner left-hand root was un-inoculated transgenic line 2G3 and the far left root was an un-inoculated, untransformed control root. **C&D** Localised green fluorescence at a phylloxera feeding site on a transgenic root (line 5B7). Figure adapted from Bondar (2001).
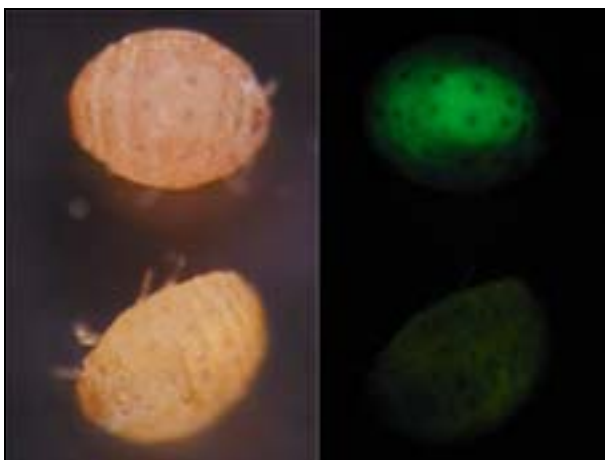

Fig. 8
Ingestion of GFP by phylloxera. The left hand panel was illuminated with white light and the right hand panel was illuminated with UV light. The two adult phylloxera were removed from similar sized galls: the top insect was removed from a transgenic root and the bottom insect was removed from an untransformed root. Figure from Bondar (2001).

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may be a site for absorption and act as a food reservoir (Kingston et al., 2003) and that the insect may not excrete honeydew through the anus (Anders, 1957).

<u>The GH3 promoter is responsive to auxin in grapevine and activated at phylloxera feeding sites</u>
For analysis of the auxin responsive GH3 promoter, whole transgenic grapevine plants (*cv* Schwarzmann; *V. rupestris* x *V. riparia*) or hairy roots cultures (*cv* Cabernet Sauvignon; *V. vinifera*) were generated using a construct (pPLEXGGG) which contains *GH3:gusA* and *35S:gfp*. The *GH3:gusA*-transformed hairy root lines and whole plants exhibited auxin inducible *gus* expression (Fig. 9). At times, phylloxera feeding and galls occurred coincidently with *gus* expression (Fig. 10 and Table 1), providing evidence for the involvement of auxin in the formation and/or maintenance of phylloxera feeding sites.
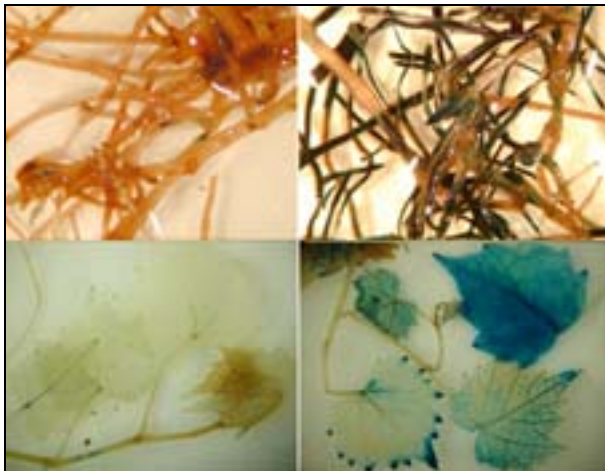

Fig. 9
GH3 directed *gus* expression in grapevine with and without treatment with auxin. Before GUS staining, hairy roots (top) and leaves/stems (bottom) were incubated overnight with (right hand panel) or without (left hand panel) 100 µM NAA.
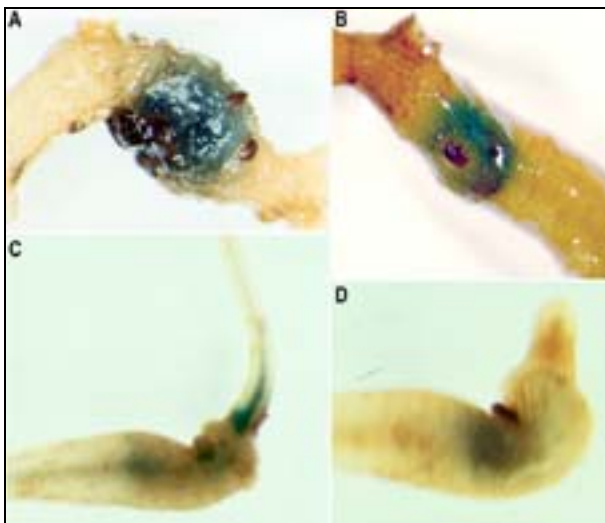

Fig. 10 GH3 directed *gus* expression in phylloxera galls. Root galls on a whole transgenic plant (**A**) or hairy root cultures (**B**,**C**,**D**). Panel **B** from Bondar (2001).

Table I *Incidence of GUS staining at phylloxera feeding sites on grapevine roots transformed with GH3::gus*

Transgenic Line	Total Galls	GUS Positive Galls/Feeding Sites	Other (i.e. non-gall) GUS Positive Regions
Hairy roots			
Control	49	0	0
16d	25	3	8
23b	53	0	2
30e	38	16	15
Plants			
Control	18	0	0
W3	46	4	3
Y6	26	0	2

Identification of genes capable of disrupting root pest infestation, feeding and/or reproduction
6.7 Engineered cyanogenic glucoside biosynthesis in grapevine roots
Transfer of three sorghum genes for cyanogenic glucoside biosynthesis to grapevine hairy roots and characterization of the transgenic lines has been documented in a draft manuscript for publication (Attachment 2). Levels of cyanogenic glucoside were highly variable between lines. Even the highest level that was ever measured (equivalent to about 100 mg HCN kg^{-1} fw) was still more than 3 fold less than that in both young leaves of naturally cyanogenic grapevine cultivars (Attachment 3) and in transgenic Arabidopsis that exhibited pest resistance (Tattersall et al., 2001).

Interaction of root-knot nematodes with grapevine roots engineered for cyanogenic glucoside biosynthesis
When juvenile (J2) root-knot nematodes were immersed in extract prepared from a transgenic line which accumulated cyanogenic glucosides and was 'cyanogenic', the nematodes suffered rapid onset paralysis which was characterized by a "C-shape" form and was temporary (Fig. 11). This response is consistent with previous observations of reversible immobilisation of juvenile root-knot nematodes immersed in cyanide solution (Robinson & Carter, 1986).
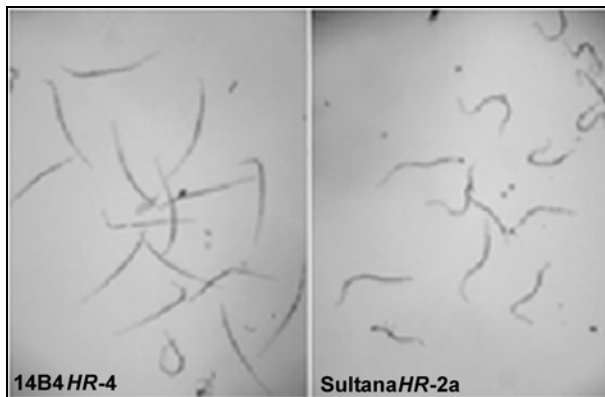

Fig. 11 Immersion of juvenile root-knot nematodes in extracts from cyanogenic (14B4*HR*-4) and acyanogenic (Sultana*HR*-2a) hairy root cultures. Photographs were taken about 5 minutes after mixing nematodes with the respective extracts.

Interactions between a cyanogenic (14B4*HR*-4) and an acyanogenic (Sultana*HR-2a)* transgenic hairy root line with root-knot nematode were also investigated in a preliminary experiment using long term *in vitro* co-culture (Table II). As discussed above, this experiment was compromised because the nematode's lifecycle was generally not completed on the Sultana control hairy root lines. However, a comparison of numbers of nematodes that penetrated the different hairy root lines was possible. For each of the life-cycle stages, no differences between the total nematodes in acyanogenic compared with cyanogenic hairy roots cultures were detected. Neither was there a difference between the total number of nematodes in the two transgenic lines for comparison. The two lines differed with respect to root proliferation, however, with the cyanogenic line having

on average, twice as many root tips as the acyanogenic line. When values for the total number of nematodes and the total number of root axes was expressed as a proportion, differences were significant, and the total number of nematodes per root axis was significantly less in the cyanogenic line compared with the cyanogenic line (Table II). This result could be interpreted to indicate that there is some reduction in the rate of penetration of the cyanogenic roots by nematodes. Alternatively, the result may actually be reflecting a limit on the total number of nematodes available for penetration that was imposed by the concentration of the initial inoculum.

Table II *Numbers of root-knot nematodes and numbers of root axes in hairy root cultures after 35 days co-culture (values are means of 10 replicates).*

Line	J2s	Intermediate Forms[1]	Males	Females	Total nemas	Root axes	Total nemas per root axis[2]
Sultana*HR*-2a	3.6	175.8	10.3	0.2	189.9	33.2[a]	6.0[a]
14B4*HR*-4	1.3	141.6	18.2	0.1	161.2	66.7[b]	2.7[b]

Data was analysed using log-linear modeling unless otherwise indicated.
Within a column, values with different letters are significantly different (testing at the 5% level).
[1]These nematodes were of development forms intermediate between J2s and mature males or mature females.
[2]Data was transformed by a power transformation (0.2) before analysis by General Analysis of Variance. Back-transformed means are presented, however conclusions on significant differences were made using the mean proportions from the power transformed data.

Interaction of phylloxera with grapevine roots engineered for cyanogenic glucoside biosynthesis
The same two acyanogenic and cyanogenic transgenic lines were also challenged with phylloxera and results are presented in Attachment 2. The numbers of nodosities, eggs, crawlers and total insects (including eggs) were significantly higher in co-cultures with cyanogenic roots compared with co-cultures with acyanogenic roots, but numbers of 2nd instar, 3rd instar, 4th instar, winged adults and apterous adults on cultures of the two lines were not significantly different. Similar to the nematode co-culture experiment, the cyanogenic hairy root line also had significantly more root axes compared with the acyanogenic line. When total insect data and total root axes data were combined and expressed as a proportion, there were significantly less insects per root axis in the cyanogenic line compared with the acyanogenic line. Similar to the nematode co-culture experiment, interpretation of these results is difficult. The results may either be indicative of a reduction in the rate of infestation of the roots by phylloxera or be relective of a limitation on the numbers of phylloxera available for infestation that was imposed by the concentration of the initial inoculum. At least it is possible to conclude that levels of infestation of this cyanogenic line by root-knot nematode or phylloxera are not low enough to suggest that practical protection from the root pests has been acquired. Consistently high levels of dhurrin accumulation may be required for this to occur and transgenic grapevine lines that accumulate higher levels of dhurrin must be generated before this can be tested. Such lines may be best produced using an alternative strategy such as a single-step transformation process that introduces all 3 sorghum genes for dhurrin biosynthesis at once.

6.8 Characterisation of the natural occurrence of cyanogenic glucosides in grapevine
This work is documented in a draft manuscript for publication (Attachment 3). In summary, two wine grape cultivars were found to naturally accumulate cyanogenic glucosides in leaves and when various tissues of one of these cultivars (Ruby Cabernet) were characterized for cyanogenic glucoside accumulation, the compounds were only detected at appreciable levels in the leaves. There are at least three implications of this information: (1) an understanding of the distribution of the trait amongst different cultivars and amongst different tissues of a cultivar, provides a basis for establishing a role of the trait in grapevine interactions with pests and pathogens; (2) absence of the trait from many cultivars, and indeed from roots of a cyanogenic cultivar, indicates that its introduction to grapevine may have some potential to impact on pest resistance (3) products of a grapevines engineered for cyanogenic glucoside biosynthesis may be more acceptable to consumers, knowing that the trait already occurs naturally in the species.

7. Outcomes/Conclusions

The project was well placed at the end of 3 years to pursue the remaining original objectives, however, notification during year 3 that the project would be terminated after 4, instead of the originally projected 7 years, meant that the original milestones required revision (Section 4, page 10). Efforts in the 4[th] and final year therefore concentrated on consolidating a few key aspects of the work that were already underway.

Short-term benefits to the industry could be derived from the *in vitro* systems for co-culturing grapevine with root-knot nematodes that were developed during the course of the project. The systems could well be used as alternatives for screening selections from grapevine improvement programs for relevant resistance, as discussed in Franks et al. (2003b) (Attachment 1).

For the much longer term, a solid framework of expertise and experimental tools for developing novel root pest resistance in grapevine was established.

8. Recommendations

There continues to be a strong international focus on developing engineered novel pest resistance in various plant species. If the Australian grape and wine industry chooses to revisit this possibility with respect to its important root pests (i.e. phylloxera and nematodes), outcomes of this project provide a starting point for reinitiating such research.

There are several candidate anti root-pest strategies that warrant investigation. For example, some novel anti-root pest genes may be derived from the resources (i.e. cDNA library of sequences that accumulate in phylloxera galls as well as partial and full-length TIP sequences) that were generated by this project. As discussed in section 6.7, cyanogenic glucoside biosythesis requires further investigation. Also, overexpression of glutamate decarboxylase leading to the enhanced accumulation of GABA (gamma-aminobutyrate) - which occurs naturally in plants and provides defense again invertebrate pests in tobacco (McLean et al., 2003) - may also be applicable to providing grapevine with novel resistance to its root pests (personal communication, Brent Kaiser, The University of Adelaide). Additionally, it is likely that RNAi (RNA interference)-mediated gene silencing will emerge as a strategy for engineering pest resistance by abolishing expression of the pest genes that are required for a successful interaction with its host. Root-knot nematode (for which large EST collections already exist) would be an immediate target using this strategy. For the case of phylloxera, the strategy would begin with characterization of expressed sequences from the insect.

9. Appendix 1: Communication

Results of the work have already been publicised in poster presentations at conferences (Franks et al., 2001; Franks et al., 2003a; Thieleke, 2003) in a technical report in a local industry publication (Franks et al., 2002) and in a research article published in an international refereed journal (Franks et al., 2003b; Attachment 1). Two draft manuscripts (Attachments 2 & 3) are also in preparation for submission to refereed journals.

In addition, aspects of the work have been documented in theses prepared by three students (Lucy Croser, André Bondar and Angelica Thieleke) who completed the research component of their final year of an Honours Degree of Bachelor of Biotechnology at the Flinders University of South Australia in Dr. Robyn van Heeswijck's laboratory. These students worked on projects that were very closely aligned with this one and each were awarded first class Honours. Results of work completed by Lucy Croser, André Bondar and Angelica Thieleke are summarised in part in this report in sections 6.2, 6.4/6.6 and 6.3, respectively. It is noteworthy that Angelica Thieleke was the South Australian recipient of the Ausbiotech 2003 National Student Excellence Awards for her research work.

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11. Appendix 3: Staff

Project Leader
Dr. Robyn van Heeswijck (The University of Adelaide) lead the project until her retirement in July 2002 when **Prof. Steve Tyerman** (The University of Adelaide) took over the role in an administrative capacity.

Other Investigators
Prof. Peter Høj (The Australian Wine Research Institute)
Dr. Kevin Powell (Department of Primary Industries, Primary Industries Research Victoria, Rutherglen Centre)
Dr. Simon Robinson (CSIRO, Division of Plant Industry)

Collaborators
Assoc. Prof. David McK. Bird (North Carolina State University, USA)
Prof. Birger Møller (Royal Veterinary and Agricultural University, Denmark)
Yoji Hayasaka (The Australian Wine Research Institute)
Pat Iocco (CSIRO, Division of Plant Industry)

Visiting Post Doctoral Research Fellow
Dr. Harry Teicher (Danish Research Foundation)

Personnel employed directly by this project
Post Doctoral Fellow: **Dr. Tricia Franks**, **Dr. Sandra Savocchia**
Research Assistant: **Ellen Marsh**, **Steven Choimes**
Casual Technical Assistance: **Dr. Kylie Nunan, Stephanie Edge, Dr. Merran Matthews, André Bondar, Dr. Sandra Savocchia, Catherine Gibson** (all at The University of Adelaide) and **Sarah Hetherington** and **Robert Bedirian** (both at Department of Primary Industries, Primary Industries Research Victoria, Rutherglen Centre)

Honours research students
Lucy Croser, **André Bondar**, **Angelica Thieleke** (all from Flinders University of South Australia)

12. Appendix 4: Additional data - Nucleotide sequence of two grapevine MIPs

Vvmip15

Multiple sequence alignment of 6 different 5′ RACE-PCR products with the partial *Vvmip15* (422 bp) sequence that was recovered originally by degenerate PCR. Shaded regions indicate areas of conservation: black shading represents complete identity of nucleotides and grey shading represents conserved nucleotides (from Thieleke, 2002).

```
5_15seq2  -----------------------------------------------------------
5_15seq1  -----------------------------------------------------------
5_15seq3  -----------------------------------------------------------
5_15seq4  -----------------------------------------------------------
5_15seq6  AAAAAAGTGTTAATTACAATGGCACACCTACTAATTTCCTGGAAAAATAAAGTGCCACCC
5_15seq5  AAAAAAGTGTTAATTACAATGGCACACCTACTAATTTCCTGGAAAAATAAAGTGCCACCC
Vvmip15   -----------------------------------------------------------
          1........10........20........30........40........50........

5_15seq2  -----------------------------------------------------------
5_15seq1  -----------------------------------------------------------
5_15seq3  -----------------------------------------------------------
5_15seq4  ------------------------------AAAATAACTCTCCCATCTTCGATCCTGCC
5_15seq6  TCCATAGAGGTGAAGGAGGAAGCTGATTCCTTATATAACTCTCCCATCTTCGATCCTGCC
5_15seq5  TCTATAGAGGTGAAGGAGGAAGCTGATTCCTTATATAACTCTCCCATCTTCGATCCTGCC
Vvmip15   -----------------------------------------------------------
          61.......70........80........90........100.......110........

5_15seq2  --AAAAAAGCCTGACTCCACCACCCCCCATTCCTCTCTCAACATGGCCAAGATGGCTCTC
5_15seq1  AAAAAAAAAGCCTGACTCCACCACCCCCCATTCCTCTCTCAACATGGCCAAGATGGCTCTC
5_15seq3  --AAAAAAGCCTGACTCCACCACCCCCCATTCCTCTCTCGACATGGCCAAGATGGCTCTC
5_15seq4  AAGCCCAAAGCCTGACTCCACCACCCCCCATTCCTCTCTCAACATGGCCAAGATGGCTCTC
5_15seq6  AAGCCAAAGCCTGAATCCACCACCCCCCATTCCTCTCTCAAAATGGCCAAGATGGCTCTC
5_15seq5  AAGCCAAAGCCTGAATCCACCACCCCCCATTCCTCTCTCAAAATGGGCAAGATGGCTCTC
Vvmip15   -----------------------------------------------------------
          121......130.......140.......150.......160.......170........

5_15seq2  GGCTCCGGCCGTGAGTTCGCCCAGCCCGACTGCATCCGAGCTCTCGTCATGGAGTTCATC
5_15seq1  GGCTCCGGCCGTGAGTTCGCCCAGCCCGACTGCATCCGAGCTCTCGTCATGGAGTTCATC
5_15seq3  GGCTCCGGCCGTGAGTTCGCCCAGCCCGACTGCATCCGAGCTCTCGTCATGGAGTTCATC
5_15seq4  GGCTCCGGCCGTGAGTTCGCCCAGCCCGACTGCATCCGAGCTCTCGTCATGGAGTTCATC
5_15seq6  GGCTCCGGCCGTGAGTTCGCCCAGCCCGACTGCATCCGAGCTCTCGTCATGGAGTTCATC
5_15seq5  GGCTCCGGCCGTGAGTTCGCCCAGCCCGACTGCATCCGAGCTCTCGTCATGGAGTTCATC
Vvmip15   -----------------------------------------------------------
          181......190.......200.......210.......220.......230........

5_15seq2  GTCACCTTCCTGTTCGTCTTCGCGGGTGTCGGCTCCGCCATGGCCACCGAGAAGCTGAAG
5_15seq1  GTCACCTTCCTGTTCGTCTTCGCGGGTGTCGGCTCCGCCATGGCCACCGAGAAGCTGAAG
5_15seq3  GTCACCTTCCTGTTCGTCTTCGCGGGTGTCGGCTCCGCCATGGCCACCGAGAAGCTGAAG
5_15seq4  GTCACCTTCCTGTTCGTCTTCGCGGGTGTCGGCTCCGCCATGGCCACCGAGAAGCTGAAG
5_15seq6  GTCACCTTCCTGTTCGTCTTCGCGGGTGTCGGGTCCGCCATGGGCACCGAGAAGCTGAAG
5_15seq5  GTCACCTTCCTGTTCGTCTTCGCGGGTGTCGGCTCCGCCATGGCCACCGAGAA-ATGAAG
Vvmip15   -----------------------------------------------------------
          241......250.......260.......270.......280.......290........

5_15seq2  CATGGTATCGGCGGCGCTGCAGATCTCCGGTGGCCACCTG-AACCCGGCGGTGAC TCTTG
5_15seq1  CATGGTATCGGCGGCGCTGCAGATCTCCGGTGGCCACCTG-AACCCGGCGGTGAC TCTTG
5_15seq3  CATGGTATCGGCGGCGCTGCACATCTCCGGTGGCCACCTG-AACCCGGCGGTGAC TCTTG
5_15seq4  CATGGTATCGGCGGCGCTGCAGATCTCCGTTGCCCACCTG-AACCCGGCGGTGAC TCTTG
5_15seq6  CATGTAATCGGCGGCACTCCAGATCTCCGGTGGCCACTGACGTGAAACGGCGGTGAC TCTTG
5_15seq5  CATGTTATCGGCGGCGCTGCAGATCTCCGGTGGCCACCTG-AACCCGGCGGTGAC TCTTG
Vvmip15   --------------------------------------------------------TCTTG
          361......370.......380.......390.......400.......410........

5_15seq2  GTCTCTGCGTGGGTGGTCACATCACCGTTGTGAGATCCGTCCTGTACTTCATCGATCAGT
5_15seq1  GTCTCTGCGTGGGTGGTCACATCACCGTTGTGAGATCCGTCCTGTACTTCATCGATCAGT
5_15seq3  GTCTCTGCGTGGGTGGTCACATCACCGTTGTGAGATCCGTCCTGTACTTCATCGATCAGT
5_15seq4  TTCTCTGCGTGGGTGGTCACATCACCGTTGTGAGATCCGTCCTGTACTTCATCGATCAGT
5_15seq6  GTCTCGCGTGGGTGGTCACATCACCGTTGTGAGATCCGTCCTGTACTTCATCGATCAGT
5_15seq5  TTCTCTGCGTGGGTGGTCACATCACCGTTGTGAGATCCGTCCTGTACTTCATCGATCAGT
Vvmip15   GTCTCTGCGTGGGTGGTCACATCACCGTTGTGAGATCCGTCCTGTACTTCATCGATCAGT
          421......430.......440.......450.......460.......470........

5_15seq2  GTTTGGCTTCTACTGTAGCCTGTATTCTCCTCAAGTTTCTCACTGGTGGACGGGCAACTC
5_15seq1  GTTTGGCTTCTACTGTAGCCTGTATTCTCCTCAAGTTTCTCACTGGTGGACGGGCAACTC
5_15seq3  GTTTGGCTTCTACTGTAGCCTGTATTCTCCTCAAGTTTCTCACTGGTGGACGGGCAACTC
5_15seq4  GTTCGGCTTCTACTGTAGCCTGTATTCTCCTCAAGTTTCTCACTGGTGGACGGGCAACTC
5_15seq6  GTTTGGCTTCTACTGTAGCCTGTATTCTCCTCAAGTTTCTCACTGGTGGACGGGCAACTC
5_15seq5  GTTTGGCTTCTACTGTAGCCTGTATTCTCCTCAAGTTTCTCACTGGTGGACGGGCAACTC
Vvmip15   GTTTGGCTTCTACTGTAGCCTGTATTCTCCTCAAGTTTCTCACTGGTGGACGGGCAACTC
          481......490.......500.......510.......520.......530........
```

```
5_15seq2   CAGTGCACACACTGGCGAGTGGAGTGGGGTGTCTACAAGGGGTAATGCTAGAATTTATCC
5_15seq1   CAGTGCACACACTGGCGAGTGGAGTGGGGTGTCTACAAGGGGTAATGCTAGAATTTATCC
5_15seq3   CAGTGCACACACTGGCGAGTGGAGTGGGGTGTCTACAAGGGGTAATGCTAGAATTTATCC
5_15seq4   CAGTGCACACACTGGCGAGTGGAGTGGGGTGTCTACAAGGGGTAATGCTAGAATTTATCC
5_15seq6   CAGTGCACACACTGGCGAGTGGAGTGGGGTGTCTACAAGGGGTAATGCTAGAATTTATCC
5_15seq5   CAGTGCACACACTGGCGAGTGGAGTGGGGTGTCTACAAGGGGTAATGCTAGAATTTATCC
Vvmip15    CAGTGCACACACTGGCGAGTGGAGTGGGGTGTCTACAAGGGGTAATGCTAGAATTTATCC
           541......550.......560.......570.......580......590.......

5_15seq2   TCACGTTCTCGTTGTTGTTCACAGTCTATGCCAATATAGTATCAGCACAGAAGTCTGCCC
5_15seq1   TCACGTTCTCGTTGTTGTTCACAGTCTATGCCAATATAGTATCAGCACAGAAGTCTGCCC
5_15seq3   TCACGTTCTCGTTGTTGTTCACAGTCTATGCCAATATAGTATCAGCACAGAAGTCTGCCC
5_15seq4   TCACGTTCTCGTTGTTGTTCACAGTCTATGCCAATATAGTATCAGCACAGAAGTCTGCCC
5_15seq6   TCACGTTCTCGTTGTTGTTCACAGTCTATGCCAATATAGTATCAGCACAGAAGTCTGCCC
5_15seq5   TCACGTTCTCGTTGTTGTTCACAGTCTATGCCAATATAGTATCAGCACAGAAGTCTGCCC
Vvmip15    TCACGTTCTCGTTGTTGTTCACAGTCTATGCCAATATAGTATCAGCACAGAAGTCTGCCC
           601......610.......620.......630.......640......650.......

5_15seq2   ACATTGATG---------------------------------------------------
5_15seq1   ACATTGATG---------------------------------------------------
5_15seq3   ACATTGATG---------------------------------------------------
5_15seq4   ACATTGATG---------------------------------------------------
5_15seq6   ACATTGATG---------------------------------------------------
5_15seq2   ------------------------------------------------------------
5_15seq1   ------------------------------------------------------------
5_15seq3   ------------------------------------------------------------
5_15seq4   ------------------------------------------------------------
5_15seq6   ------------------------------------------------------------
5_15seq5   ------------------------------------------------------------
Vvmip15    GTGGGGCCTTTTCCGGGGCTTCCATGAACCCGGCCCGGTCCTTCGGGCCGGCTTTGGTGA
           721......730.......740.......750.......760......770.......

5_15seq2   ----------------------
5_15seq1   ----------------------
5_15seq3   ----------------------
5_15seq4   ----------------------
5_15seq6   ----------------------
5_15seq5   ----------------------
Vvmip15    GCTGGGACTGGACAAACCAC
           781......790........
```

31

Vvmip17

Multiple sequence alignment of 6 3´ RACE-PCR products with the partial *Vvmip17* (422 bp) sequence that was recovered originally by degenerate PCR. Shaded regions indicate areas of conservation: black shading represents complete identity of nucleotides and grey shading represents conserved nucleotides (from Thieleke, 2002).

```
3_17seq2   TGTAGGTGCAAACATCCTGGCTGCAGGCCCATTCTCCGGTGGATCAATGA
3_17seq1   TGTAGGTGCAAACATCCTGGCTGCAGGCCCATTCTCCGGTGGATCAATGA
3_17seq4   TGTAGGTGCAAACATCCTGGCTGCAGGCCCATTCTCCGGTGGATCAATGA
3_17seq6   TGTAGGTGCAAACATCCTGGCTGCAGGCCCATTCTCCGGTGGATCAATGA
3_17seq5   AGTAGGTGCAAACATCCTGGCTGCAAGCCCATTCTCCGGTGGATCAATGA
3_17seq3   CGTAGGTGCAAACATCCTGGCTGCAGGCCCATTCTCCGGTGGATCAAAGA
Vvmip17    TGTAGGTGCAAACATCCTGGCTGCAGGCCCATTCTCCGGTGGATCAATGA
           301......310.......320.......330.......340........

3_17seq2   ACCCCGCCCGCTCCTTCGGCCCCGCCGTCGTCAGCGGCGACTTCAAGGAC
3_17seq1   AACCCGCCCGGTCCTTCGGCCCCG-CGTCGTCAGCGGCGACTTCAAGG--
3_17seq4   ACCCCGCCCGCTCCTTCGGCCCCGCCGTCGTCAGCGGCGACTTCAAGG--
3_17seq6   ACCCCGCCCGCTCCTTCGGCCCCGCCGTCGTCAGCGGCGACTTCAAGG--
3_17seq5   ACCCCGCCCGCTCCTTCGGGCCCGGCGTCGTCAGCGGTGACTTCAACG--
3_17seq3   ACCCCGCCCGCTCCTTCGGCCCCGCCGTCGTCAGAGCCGACTTCAAGG--
Vvmip17    ACCCCGCCCGCTCCTTCGGCCCCGCCGTCGTCAGCGGCGACTTCAAGG--
           351......360.......370.......380.......390........

3_17seq2   AACAACTGGATCTACTGGGTGGGACGCCTAATTGGAGGTGGCATGGGAGG
3_17seq1   -ACAACTGGATCTACTGGGTGGGACGCCTAATTGGAGGTGCCATGGGAGG
3_17seq4   -ACAACTGGATCTACTGGGTGGGACGCCTAATTGGAGGTGGCATGGGAGG
3_17seq6   -ACAACTGGATCTACTGGGTGGGACGCCTAATTGGAGGTGGCATGGGAGG
3_17seq5   -ACAACTGGATTTACTGGGTGGGACGCCTAATTGGAGCTGGCATGGGAGG
3_17seq3   -ACAACTGGATCTACTGGGTGGGACGCCTAATTGGAGGTGGCATGGGAGG
Vvmip17    -ACAACTGGATCTACTGGGTCGGCCG------------------------
           401......410.......420.......430.......440........

3_17seq2   ATCTGTCTATGCAATTATGTACATGGGCTCTGATCATCAACCACTAGCGT
3_17seq1   ATCTGTCTATGCAATTATGTACATGGGCTNTGATCATCAACCACTAGCGT
3_17seq4   ATCTGTCTATGCAATTATGTACATGGGCTCTGATCATCAACCACTAGCGT
3_17seq6   ATCTGTCTATGCAATTATGTACATGGGCTCTGATCATCAACCACTAGCGT
3_17seq5   ATCTGTCTATGCAATTATGTACATGGGCTCTGATCATCAACCACTAGCGT
3_17seq3   ATCTGTCTATGCAATTATGTACATGGGCTCTGATCATCAACCACTAGCGT
Vvmip17    --------------------------------------------------
           451......460.......470.......480.......490........

3_17seq2   CCAGCGAATTCTAAGCTGAGTTTTTTTTCAGG--AACTCAATTGTTTGTTG
3_17seq1   CCAGCGAATTTTAAGCTGAGTTTTTTTTCAGG--AACTCAATTGTTTGTTG
3_17seq4   CCAGCGAATTCTAAGCTGAGTTTTTTTTCAGG--AACTCAATTGTTTGTTG
3_17seq6   CCAGCGAATTCCAAGCTGAGTTTTTTTTCAGG--AACTCAATTGTTTGTTG
3_17seq5   CCAGCGAATTTTAAGTTGAGTTTTTTTTCAGG--AACTCACTTGTTTGTTG
3_17seq3   CCAGGGAATTCTAAGCTGAGTTTTTTTTCAGGGAACTCAGATTGTTTGTTG
Vvmip17    --------------------------------------------------
           501......510.......520.......530.......540........

3_17seq2   TTGTCTCAACCCATTTCCCTTTGAAATAA-AAAGGAGGAAGAGCAAGTTG
3_17seq1   TTGTCTCAACCCATTTCCCTTTGAAATAA-AAAGGAGGAAGAGCAAGTTG
3_17seq4   TTGTCTCAACCCATTTCCCTTTGAAATAA-AAAGGAGGAAGAGCAAGTTG
3_17seq6   TTGTCTCAACCCATTTCCCTTTGAAATAA-AAAGGAGGAAGAGCAAGTTG
3_17seq5   TTGTCTCAACCCATTTCCCTTTGAAATAA-AAAGGAGGAAGAGCAAGTTG
3_17seq3   TTGTCGCAACCCATTTCCCTTTGAAATAAGAAAGGAGGAAGAGCAAGGTG
Vvmip17    --------------------------------------------------
           551......560.......570.......580.......590........
```

```
3_17seq2  TGC TACTTCT TTC TT TTTGTACT TG TAT TT TGT TC TTTGA TCT TC TTCGA
3_17seq1  TGC TACTTCT TTC TT TTTGTACT TG TAT TT TGT TC TTTGA TCT TC TTCGA
3_17seq4  TGC TACTTCT TTC TT TTTGTACT TG TAT TT TGT TC TTTGA TCT TC TTCGA
3_17seq6  TGC TACTTCT TTC TT TTTGTACT TG TAT TT TGT TC TTTGA TCT TC TTCGA
3_17seq5  TCA TACTTCT TTC TT TTTGTACT TG TAT TT TGT TC TTTGA TCT TC TTCGA
3_17seq3  TGC TACTTCT TTC TT TTTGTACT TG TAT TT TGT TCGTTGA TCT TC TTCGA
Vvmip17   ------------------------------------------------------
          601......610.......620.......630.......640........


3_17seq2  TTC TC TCCCT TTTGGATT TG TAAAGCCC TTGCT TTGGT TCAAGCTGTGTA
3_17seq1  TTC TC TCCCT TTTGGATT TG TAAAGCCC TTGCT TTGGT TCAAGCTGTGTA
3_17seq4  TTC TC TCCCT TTTGGATT TG TAAAGCCC TTGCT TTGGT TCAAGCTGTGTA
3_17seq6  TTC TC TCCCT TTTGGATT TG TAAAGCCC TTGCT TTGGT TCAAGCTGTGTA
3_17seq5  TTC TC TCCCT TTTGGATT TG TAAAGCCC TTGCT TTGGT TCAAGCTGTGTA
3_17seq3  TTC TC TCCCT TTTGGATTGG TAAAGCCC TTGCT TTGGT TCAAGCTGTGTA
Vvmip17   ------------------------------------------------------
          651......660.......670.......680.......690........


3_17seq2  AAAGA TGCAA TTA TGCATATGAA TGAGG TTGGTGA TGTGCCAAAAAAAAA
3_17seq1  AAAGA TGCAA TTA TGCATATGAA TGAGG TTGGTGA TGTGCAAAAAAAAAA
3_17seq4  AAAGA TGCAA TTA TGCATATGAA TGAGG TTGGTGA TGTGCAGCCACTT TG
3_17seq6  AAAGA TGCAA TTA TGCATATGAA TGAGG TTGGTGAAAAAAAAAAAAA----
3_17seq5  AAAGA TGCAA TTA TGCATATGAA TGAGG TTGGTGA TGTGCAGCCACTT TG
3_17seq3  AAAGA TGCAA TTA TGCATATGAA TGAGG TTGGTGA TGTGCAGCCACTT TG
Vvmip17   ------------------------------------------------------
          701......710.......720.......730.......740........


3_17seq2  AAA----------------
3_17seq1  AA-----------------
3_17seq4  CTT TC TGAAAAAAAAAAAA
3_17seq6  -------------------
3_17seq5  CTT TCAAAAAAAAAAAA---
3_17seq3  CTT TCAAAAAAAAAAAA--
Vvmip17   -------------------
          751......760.......
```

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13. List of attachments

1: Published research article
Franks, T. K., Savocchia, S. and van Heeswijck, R. (2003). *In vitro* systems for studying the interaction of root-knot nematode with grapevine. *Nematology* **5**(2): 235-242.

2: Draft reseach article –CONFIDENTIAL- not for general distribution
Franks, T. K., Choimes, S., Marsh, E. Powell, K. S., Iocco, P. and van Heeswijck, R. Consequences of transferring three sorghum genes for secondary metabolite (cyanogenic glucoside) biosynthesis to grapevine hairy roots.

3: Draft research article –CONFIDENTIAL- not for general distribution
Franks, T K., Hayasaka, Y., Choimes, S. and van Heeswijck, R. Cyanogenic glucoside accumulation in tissues of grapevine (*Vitis vinifera* L.): polymorphism, characterization and patterns in development.