

Characterisation of the germin-like
gene family in *Vitis vinifera*:
A possible role in defence against
Erysiphe necator

by

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A thesis submitted for the degree of
Doctor of Philosophy

at

The University of Adelaide
School of Agriculture, Food and Wine
Discipline of Plant and Pest Science

in collaboration with

CSIRO Plant Industry,
Horticulture Unit

Urrbrae, Adelaide
January, 2006

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Abstract

The grapevine powdery mildew pathogen, *Erysiphe necator* causes extensive economic losses, not only through lost grape production, but also due to costs associated with fungicide application. Economic and environmental impacts, and the emerging resistance to fungicides, have increased the need to develop new molecular strategies to confer resistance of grapevine to this obligate fungal biotroph.

Germin-like proteins (GLPs) have a proposed role in the defence response of cereals against powdery mildew (Wei *et al.* 1998). Epidermal-specific powdery mildew-inducible barley *GLP*, *HvGLP4*, accumulates to greater levels in cells with cell wall appositions (papillae) effective in preventing penetration (Gjetting *et al.* 2004). *HvGLP4* exhibits superoxide dismutase (SOD) activity, catalysing the conversion of superoxide to hydrogen peroxide which is postulated to be involved in the production of papillae (Christensen *et al.* 2004). Manipulation of levels of some GLPs in cereals has been shown to significantly reduce powdery mildew infection (Schweizer *et al.* 1999a), offering a new strategy to reduce susceptibility to this fungal pathogen. A similar approach may provide strategies to reduce susceptibility of grapevine to *E. necator*.

This thesis presents an investigation into the grapevine *GLP* genes with emphasis on the potential role of powdery mildew-inducible grapevine *GLP* in the defence against *E. necator* infection. Seven novel cDNA clones of the *GLP* gene family in grapevine have been cloned and characterised. RT-PCR and Northern analysis revealed that the isolated

GLP genes exhibit diverse patterns of expression in response to a variety of abiotic and biotic stresses (including challenge by *E. necator*, *Plasmopara viticola* and *Botrytis cinerea*, and wounding treatment), suggesting a diversity of roles for each of the *GLP* family members.

Significantly, expression analysis has revealed that one of the grapevine *GLP* genes, *VvGLP3*, is specifically induced by powdery mildew infection and expression appears to be localised to the infected epidermal cells. To further characterise the potential role of *VvGLP3* in papillae formation, two main strategies have been employed. First, the *VvGLP3* promoter region was isolated and used to express the *GUS* reporter gene in *Arabidopsis thaliana* and was shown to control epidermal-specific, powdery mildew-induced expression in response to *Erysiphe cichoracearum*. An *A. thaliana* *GLP* gene (*AtGLP9*) that is induced by powdery mildew pathogen, *E. cichoracearum*, was also identified. The inducible expression of this gene was localised to the mesophyll cells, directly beneath the infected epidermal cells.

Second, recombinant *VvGLP3* was expressed in *A. thaliana*, and the partially purified recombinant protein was demonstrated to have SOD activity. The subcellular localisation of *VvGLP3* was determined using a *VvGLP3:GFP* fusion construct, transiently expressed in onion cells. Examination by confocal microscopy indicated that the recombinant protein was targeted to the cell wall.

Collectively, this work has provided insight into the diverse nature of the *GLP* family in *V. vinifera*. Furthermore, the data suggests that *VvGLP3* is probably involved in the

defence response against *E. necator*, and highlights potential molecular strategies for the use of *VvGLP3* to reduce susceptibility of grapevine to this fungal pathogen.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Dale Godfrey

January 2006

Acknowledgments

I wish to thank my supervisors, Dr Ian Dry and Dr Amanda Able. Ian, thank you for giving me the opportunity to work with you. It has been a fantastic experience for me. Thanks for your enthusiasm, inspiration for and commitment to the project, it would not have been possible without you.

Amanda, your encouragement, motivation and reassurance has made it possible for this thesis to come to fruition. Thanks for the fun times and great company at conferences, and for throwing a good party.

Thanks to Dr Mandy Walker and Elizabeth Lee for passing on their Arabidopsis knowledge. I really enjoyed sharing great meals with you both, at the pub, Mandarin House and in your homes. Thank you for your friendship.

To my fellow PhD students Susan Wheeler, Luke Selth, Matt Hayes and Alan Little, thanks for your friendship, tolerance of my regular dramas, tearoom crosswords and Friday afternoon beers.

Thanks to Muhammad Saeed, Mark Goetz, Pat Iocco, Tamzin Donald and Claire Barker for offering your advice and assistance in the lab.

To Terri King, Jamus Stonor and Karina Swann, thanks for your technical assistance and advice.

Thanks also to my colleagues at CSIRO Plant Industry and The University of Adelaide for providing a fantastic work and social environment during my PhD studies.

The Confocal Laser Scanning Microscopy expertise of Ghafar Sarvestani at the Institute for Medical and Veterinary Science is gratefully acknowledged.

I also wish to acknowledge the Australasian Plant Pathology Society and the Australian Federation of University Women for travel funding.

I thank my mum and dad, Angela and Rex Godfrey, for their support and encouragement.

Finally, I wish to thank my partner Leon Linden for your constant support, love, devotion and for enduring the emotional rollercoaster of a girl doing a PhD. If we can make it through this we can survive anything.

Abbreviations

AAP	abridged anchor primer
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
bp	base pair(s)
cDNA	complementary DNA
cm	centimetre(s)
cv.	cultivar
d	day(s)
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediamine- <i>tetra</i> -acetic acid
EST	expressed sequence tag
FD	Faraday(s)
g	gram(s)
<i>g</i>	relative centrifugal force
GFP	green fluorescent protein
h	hour(s)
hai	hours after inoculation
IPTG	isopropyl- β -D-galactoside
kb	kilobase pairs
kDa	kilo Dalton
L	litre(s)
LB	Luria broth
M	molar
mRNA	messenger RNA
min	minute(s)

MOPS	3-N-Morpholinopropanesulfonic acid
nt	nucleotide(s)
ORF	open reading frame
PEG	polyethylene glycol
PCR	polymerase chain reaction
PVPP	polyvinylpolypyrrolidone
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcription-PCR
s	second(s)
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSC	sodium chloride, sodium citrate
TBE	Tris-borate-EDTA
Tris	Tris(hydroxymethyl)aminomethane
U	unit(s)
UTR	untranslated region
V	volt(s)
v/v	volume per volume
WT	wild-type
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

Chapter 1. Introduction

1.1 Powdery mildew disease of grapevine

Powdery mildew of grapevines caused by the fungus *Erysiphe necator* (syn. *Uncinula necator*) is one of the most economically important diseases of grapevines worldwide. *E. necator* is one of the 650 species of powdery mildew belonging to 10 genera within five tribes of the sole family Erysiphaceae in the Order Erysiphales (Braun *et al.* 2002). *E. necator* is thought to have originated on wild vines in eastern and central USA (Roy and Ramming 1990). The most commonly cultivated species of grapevine, *Vitis vinifera*, is highly susceptible to *E. necator* infection, resulting in a characteristic whitish-grey, powdery appearance on the surface of host tissue (Fig. 1.1A). This distinctive symptom of the disease is the result of superficial hyphae producing conidiophores and conidia on the surface of host tissue.

Although Schweinitz first described powdery mildew of grapevines in North America in 1834 (Kapoor 1967), it was not until the epidemics in Europe, from 1847 to 1854, that the first significant economic losses to grapevine powdery mildew occurred. By 1855, crop losses were significantly reduced by the widespread application of sulphur as a fungicide (Yarwood 1978). Today, the disease is present in all vineyards throughout the world (Delye *et al.* 1997).

Most of the economic loss results from the effects of fungal infection on the fruit (Fig. 1.1B). Uneven maturation, swelling and cracking of the fruit results in fruit rot, which reduces yield (Buit and Lafon 1978) and wine quality (Ough and Berg 1979). Diseased grapes exhibit modified anthocyanin and sugar content, and wine produced from infected fruit exhibits modified anthocyanin, tannin and compounds affecting varietal aroma content (Calonnec *et al.* 2004). Although crop damage and loss of crop quality results primarily from colonisation of the fruit, leaf infection is an important source of inoculum (Doster and Schnathorst 1985b).

Control of powdery mildew in commercial vineyards is generally based on the use of conventional fungicides. Sulphur and demethylation-inhibiting fungicides (DMIs) are commonly used. While these chemical fungicides are relatively effective, the cost to the grower and the environmental impact of pesticide residues remains undesirable. Furthermore, isolates of *E. necator* showing reduced sensitivity to DMIs are now emerging, limiting the effectiveness of these sprays in controlling the disease (Gubler *et al.* 1996, Erickson and Wilcox 1997, Savocchia *et al.* 2004). However, the ability to genetically modify grapevines (Franks *et al.* 1998) allows the potential introduction of novel gene constructs to reduce susceptibility of favourable wine grape cultivars against powdery mildew.

A



B

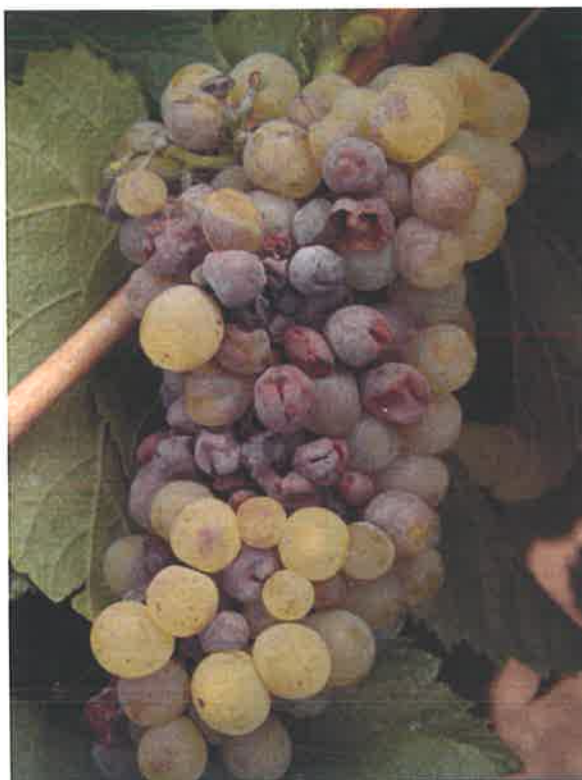


Figure 1.1. Powdery mildew infected grapevine. (A) Powdery mildew colonies on the adaxial surface of an infected *V. vinifera* cv. Cabernet Sauvignon leaf. (B) Mature *V. vinifera* cv. Chardonnay bunch showing damage caused by early season powdery mildew infection.

1.2 The infection process

Powdery mildew fungi are true Ascomycete fungi. They are obligate biotrophic pathogens that only invade host epidermal cells. Powdery mildew disease is spread asexually by wind-borne conidia. Infection is a complex process initiated almost instantaneously after conidia come into contact with host tissue (Green *et al.* 2002). As infection of *Hordeum vulgare* (barley) with the powdery mildew pathogen, *Blumeria graminis* f.sp. *hordei*, is well understood at the morphological and cytological levels, the following review of the infection process focuses on this interaction.

1.2.1 *B. graminis* infection of barley

Within seconds of settling on the host tissue the conidium releases an extracellular matrix (Carver *et al.* 1999) containing several proteins including a non-specific esterase and cutinase capable of degrading the host cuticle (Nicholson *et al.* 1988, Kunoh *et al.* 1990, Pascholati *et al.* 1992). The emergence of the primary germ tube (Fig. 1.2A), 1 to 2 h post inoculation, is the first visible morphological differentiation of the conidium. The primary germ tube develops a penetration peg that penetrates the cell cuticle and partially penetrates the cell wall (Kunoh *et al.* 1978). A small papilla is deposited within 5 h after inoculation (Bushnell and Zeyen 1976; Fig. 1.2B).

Papillae are host cell wall appositions, deposited between the plasmalemma and the cell wall at the sites of attempted penetration, that are thought to play an important role, both mechanically and chemically, in preventing fungal penetration. Although the primary germ tube inevitably fails to penetrate the cell wall (Kunoh *et al.* 1978; Fig. 1.2B), it is a pre-requisite for appressorial germ tube elongation. Various functions for the primary

germ tube have been proposed including attachment of the germinating conidium to the host surface, access to host water, and recognition of host surface characteristics (Carver and Bushnell 1983, Carver and Ingerson 1987).

Subsequently, the appressorial germ tube emerges around 5 h after inoculation (Fig. 1.2B). Elongation and swelling of the appressorial germ tube results in the formation of an appressorium (Fig. 1.2C). The appressorium develops a penetration peg (Fig. 1.2D) that challenges the host by attempting to penetrate the host cuticle and epidermal cell wall (10 to 12 h post inoculation). Penetration is facilitated by a combination of cell wall degrading enzymes from the fungus (cutinases and cellulases) and a high turgor pressure in the appressorium, thereby forcing the infection peg through the cell wall and any papillae that may have formed under the penetration peg (Pryce-Jones *et al.* 1999). The heterogeneous appearance of mature papillae suggests they vary in composition (Aist 1976) and while callose is a major component of papillae, thionins, phenolic autofluorogens, silicon, and Ca^{2+} have also been detected (Zeyen *et al.* 1983, Kunoh *et al.* 1986, Smart *et al.* 1986). Various proteins with hydrolytic properties also accumulate in maturing papillae (Takahashi *et al.* 1985).

Following successful penetration of the epidermal cell wall and papilla, haustorium formation is initiated. The penetration peg reaches the plasma membrane (14 to 16 h post inoculation) and the extrahaustorial membrane, a specialised plant membrane that encloses the haustorium, is formed (Bracker 1968). Within the epidermal cell, the haustorium enlarges and the extrahaustorial matrix is completely sealed from the outside by a 'collar' (Fig. 1.2E). The collar is probably derived from the penetrated papilla and

forms a tight seal with the hole made by the penetration peg in the epidermal cell wall, thus permitting the host epidermal cell to maintain its integrity (Bracker 1968). Finger-like protrusions (or lobes) grow out from the haustorial body allowing the absorption of nutrients, minerals and water from the host (Green *et al.* 2002). The energy gained from the host is utilised to generate superficial hyphae growing out from the appressorial germ tube at around one day after inoculation (Fig. 1.2F). Subsequently, secondary and tertiary haustoria are formed, followed by the development of conidiophores and a mature sporulating colony within a week post inoculation leading to the whitish-grey powdery appearance of infected leaves.

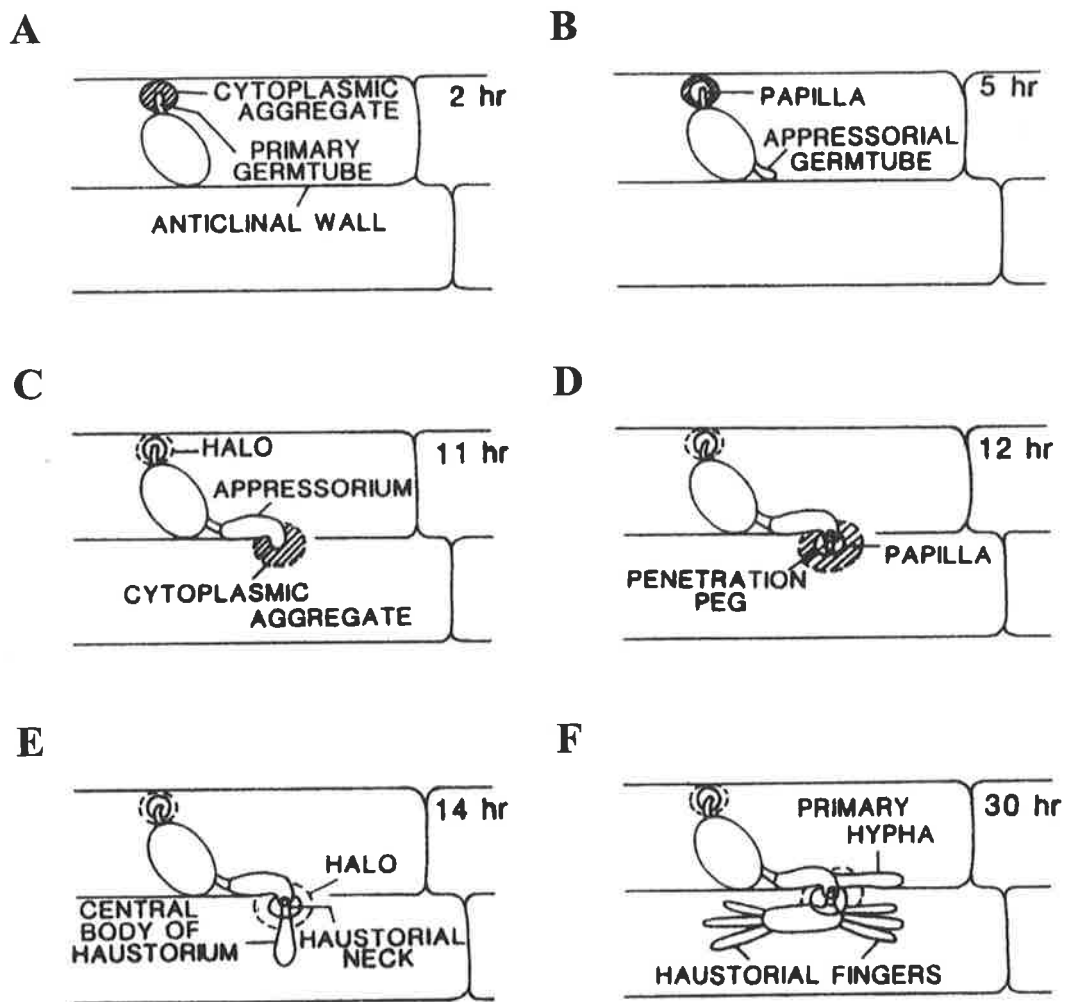


Figure 1.2. Primary infection structures of *B. graminis* and host responses. (A) 2 h, (B) 5 h, (C) 11 h, (D) 12 h, (E) 14 h and (F) 30 h after inoculation with *B. graminis*. From Aist and Bushnell (1991).

1.2.2 *E. necator* infection of grapevine

E. necator can infect all green tissue of grapevine and both surfaces of leaves are liable to infection (Built and Lafon 1978). Similar to that observed for the well-studied *B. graminis* on barley (see Section 1.2.1), the *E. necator* infection process on grapevine leaves is characterised by a distinct sequence of differentiation events following a regular time course (Leinhos *et al.* 1997). Upon germination, a germ tube emerges from

the conidium and elongates, but unlike *B. graminis* conidia, *E. necator* conidia only produce a single germ tube. The elongated germ tube subsequently differentiates to form a lobed appressorium from which a penetration peg then emerges and attempts to penetrate the host epidermal cell. Papillae are also produced beneath *E. necator* appressoria during attempted *E. necator* penetration in grapevine (Heintz and Blauch 1990). If penetration is successful, a haustorium is produced in the host epidermal cell.

1.3 Host resistance to powdery mildew

While the study of the molecular basis of host resistance to grapevine powdery mildew is relatively new, much can be learnt from data regarding the genetic basis of resistance to powdery mildew in barley and the model plant, *Arabidopsis thaliana*. Complete host powdery mildew resistance in these plant species is monogenic and can be dominantly or recessively inherited (Hückelhoven 2005). In barley, two major resistance mechanisms to powdery mildew have been identified. Inheritance of recessive *mlo* alleles at the *Mlo* locus confers non-race specific penetration resistance against all tested isolates of powdery mildew (Jørgensen 1994). In contrast, the *Mla* genes confer dominant, race-specific resistance against specific isolates of barley powdery mildew, as determined by the product of a requisite avirulence (*Avr*) gene during invasion by the pathogen (Jørgensen 1994). In this case, following attempted penetration, resistance is mediated by the hypersensitive response (HR), a form of controlled cell death resulting in elimination of the pathogen (Boyd *et al.* 1995). More recently, a new class of powdery mildew resistance gene, *RPW8*, has been identified in *A. thaliana* that also acts post-penetration and is associated with a HR but is effective against a wide range of powdery mildew isolates.

A brief overview of each of these powdery mildew resistance genes is given below. More detailed information regarding the structure and proposed mechanism of action of these genes can be found in review articles by Collins *et al.* (2002), Panstruga and Schulze-Lefert (2002), Hückelhoven and Kogel (2003), Schulze-Lefert (2004), Hückelhoven (2005) and Panstruga (2005).

1.3.1 Barley *mlo*

The penetration resistance conferred by *mlo* has been durable in the field for approximately 20 years (Baker *et al.* 1998). *Mlo* encodes a putative cell membrane receptor that consists of seven trans-membrane domains. Single amino acid deletions, substitutions and frame shifts result in mutated and recessively inherited *mlo* alleles that confer epidermal cell penetration resistance to *B. graminis* (Buschges *et al.* 1997). Freialdenhoven *et al.* (1996) observed that papillae of *mlo* resistant plants are penetrated less frequently (0.5%) than those of *Mlo* susceptible plants (70%). Spontaneous papillae formation can also be observed in healthy *mlo* resistant plants unchallenged with pathogens (Wolter *et al.* 1993). Although *mlo* confers resistance to powdery mildew, it is not effective against barley leaf rust, stripe rust, stem rust, scald or take-all (Jørgensen 1977). However, *Mlo* mutations enhance susceptibility to two necrotrophic fungal pathogens, rice blast pathogen *Magnaporthe grisea* (Jarosch *et al.* 1999) and barley spot blot pathogen *Bipolaris sorokiniana* (Kumar *et al.* 2001). Spontaneous necrotic lesions are also observed in healthy un-inoculated *mlo* resistant plants just prior to the onset of leaf senescence suggesting that *Mlo* may also function as a negative regulator of leaf senescence (Wolter *et al.* 1993).

The action of two genes, *ROR1* and *ROR2* are also required for *mlo* resistance (Freialdenhoven *et al.* 1996). Mutations in either gene undermine the penetration resistance of *mlo* barley to powdery mildew. While *ROR1* is not yet characterised, *ROR2*, and the *A. thaliana* ortholog *PEN1*, encode plasma-membrane-localised synaptome-associated protein receptor (SNARE) syntaxins (Collins *et al.* 2003). *ROR2* interacts with SNAP34, a soluble NSF (N-ethylmaleimide-sensitive fusion protein) attachment protein, possibly building a binary SNARE complex for vesicle-mediated exocytosis. Subsequently, roles for *ROR2* and *PEN1* in local vesicle dynamics have been proposed (Collins *et al.* 2003). Fusions of *ROR2* and *PEN1* to yellow and green fluorescent protein respectively, demonstrated that *ROR2* and *PEN1* accumulate at the site of papilla formation in response to *B. graminis* inoculation (Assaad *et al.* 2004, Bhat *et al.* 2005). Other components of the defence response that are critical for *mlo* resistance remain unidentified.

When and how the penetration peg is arrested is unknown, as are the components of papillae essential to preventing penetration. It is also not clear as to how the *Mlo* gene regulates or influences papillae formation, but it is thought to act as a negative regulator of a defence response. In cells where *Mlo* function is absent the defence responses are induced earlier or in a different manner to that of susceptible cells. The transcripts of various PR genes, including a peroxidase and a chitinase, accumulate at greater levels in *mlo* resistant than *Mlo* susceptible plants (Peterhansel *et al.* 1997). A novel phenolic conjugate, *p*-coumaroyl-hydroxyagmatine (*p*-CHA) with phytoalexin activity is produced in significantly greater quantities in *mlo* resistant plants than *Mlo* susceptible plants challenged with powdery mildew (von Ropenack *et al.* 1998). As *p*-CHA has

antifungal activity towards *B. graminis* it may play a role in stopping early development, subsequently preventing penetration from occurring.

Additionally, Hückelhoven *et al.* (1999) observed that hydrogen peroxide (H_2O_2) accumulation within and surrounding papillae, was much greater in resistant *mlo* barley plants, than in susceptible *Mlo* plants following inoculation with powdery mildew. Success of papillae preventing penetration in *mlo* resistant genotypes may be a result of enhanced H_2O_2 driving greater or faster cell wall cross-linking reactions (Hückelhoven *et al.* 1999). H_2O_2 may also act as a direct antimicrobial compound or signalling molecule thus contributing to *mlo* resistance in mechanisms other than papillae strengthening (Lamb and Dixon 1997). It is possible that MLO may act by stimulating antioxidant machinery, or limiting reactive oxygen species (ROS) accumulation by regulating the enzymes that synthesise ROS.

More recently, it has been demonstrated that the cytoplasmic C-terminal of MLO binds calmodulins and MLO function is enhanced, but not dependent, on calmodulin binding (Kim *et al.* 2002). As calmodulins regulate the activity of a number of proteins by binding them in a Ca^{2+} -dependent manner and an increase in cytoplasmic Ca^{2+} is a known plant cell response to pathogens, increases in cytoplasmic Ca^{2+} may also enhance defence suppression in barley inoculated with powdery mildew (Collins *et al.* 2002).

1.3.2 Barley *Mla* / *Mlg*

Compared to the durability of non-race specific resistance, race-specific resistance is usually only short lived in the field as virulent pathogens can evolve with either loss or

modification of avirulence genes. Most of the cereal R genes isolated to date encode proteins containing a central domain with a nucleotide binding site (NBS), binding either ATP or GTP, and a carboxyterminal domain consisting of a series of leucine-rich repeat residues (LRR) (Ayliffe and Lagudah 2004). Within the cereal NBS-LRR proteins two subgroups exist: one with proteins containing an amino terminus with homology to the *Drosophila* Toll protein and mammalian interleukin-1-receptor (TIR domain) and the other with proteins containing a coiled-coil (CC) domain at the amino terminus. Race-specific resistance is usually associated with recognition of a specific pathogen by the plant via a NBS-LRR protein leading to a HR. The HR is triggered after penetration of the cell wall and papillae, and is often considered as a backup defence that occurs if penetration resistance has failed (Heitefuss 2001). H_2O_2 accumulates during the HR (Hückelhoven *et al.* 1999, Trujillo *et al.* 2004) and is thought to contribute to the HR in two ways. Firstly, H_2O_2 may act as a signal for programmed cell death and secondly, H_2O_2 might be fungitoxic and prevent the fungus from suppressing cell death (Hückelhoven 2005). Currently some eighty five genes have been identified as being responsible for race-specific resistance of *H. vulgare* to *B. graminis*, and have been studied extensively in cultivars with resistance genes at the *Mla* and the *Mlg* loci (Boyd *et al.* 1995, Hückelhoven and Kogel 1998, Hückelhoven *et al.* 2000).

In *H. vulgare* over thirty different *Mla* alleles (*Mla1* to *Mla32*) that each confer resistance to a specific *B. graminis* isolate (Jørgensen 1994) have been characterised. Although there are variations in the resistance phenotypes for the *Mla* alleles, a consistent feature is the HR. This restricts further growth of the biotrophic pathogen by preventing formation of the haustorium, which is required for nutrient uptake. Many, but

not all *Mla* genes, require at least one additional gene, *Rar1* to be fully functional. In *Mla* resistance, *rar1* mutants exhibit loss of the HR and reduction in *PR* gene transcript accumulation. In addition to *Rar1*, *Mla6* also requires *Sgt1* to function. *Rar1* encodes a zinc-binding protein (Shirasu *et al.* 1999) and *Sgt1* encodes a subunit of the SCF ubiquitin ligase complex (Azevedo *et al.* 2002). Mutants in *Mla6*, *Rar1* and *Sgt1* lose the ability to undergo hypersensitive cell death (Freialdenhoven *et al.* 1994). *Mlg* resistance is characterised by both the formation of papillae and a HR of the attacked epidermal cells. In contrast to *Mla*, *Mlg* resistance results from prevention of fungal penetration. Loss of *Rar1* in *Mlg* resistance does not result in compatibility or loss of HR, but does lead to an increase in cell wall penetration (Peterhansel *et al.* 1997).

1.3.3 *A. thaliana* RPW8

RPW8 mediates broad-spectrum resistance across a wide range of powdery mildew species that infect *A. thaliana* (Xiao *et al.* 2001). Although the *RPW8* locus was initially identified as a single dominant gene mediating resistance to *Erysiphe cichoracearum* (Xiao *et al.* 1997), subsequent cloning of *RPW8* revealed that either of two closely linked genes (*RPW8.1* and *RPW8.2*) is sufficient to mediate resistance. The *RPW8* genes also confer resistance to powdery mildew in transgenic tobacco, indicating these genes also function in other plant families (Xiao *et al.* 2003). As the *RPW8* genes do not confer resistance in *A. thaliana* to other plant pathogens, including the fungus *Peronospora parasitica*, the cauliflower mosaic virus, and the bacterium *Pseudomonas syringae*, they represent R-genes conferring specific resistance to a range of powdery mildew species (Xiao *et al.* 2001). The predicted amino acid sequences of the *RPW8* genes, which share ~45% identity, lack similarity to known classes of R proteins.

Although *RPW8*-mediated resistance is conferred by a novel class of R proteins, characteristic resistance responses including oxidative bursts at sites of attempted infection and the HR are consistent with R gene-mediated resistance (Jones 2001).

1.3.4 Powdery mildew resistance in grapevine

1.3.4.1 Penetration resistance in developing berries

All above ground tissues of the cultivated grapevine species *V. vinifera* are highly susceptible to powdery mildew infection (Built and Lafon 1978). Susceptibility of leaves and berries may differ for specific cultivars, although generally they correspond (Doster and Schnathorst 1985a). Susceptibility decreases with leaf age (Doster and Schnathorst 1985b). Similarly, berries from a range of different *V. vinifera* cultivars have been shown to exhibit ontogenic resistance to powdery mildew infection (Pearson and Gadoury 1992). Pre-veraison berries were reported to be susceptible to infection until Total Soluble Solid levels (°Brix) reach 8% (Delp 1954, Chellemi and Marois 1992). As the expression of genes encoding a chitinase (Robinson *et al.* 1997) and a thaumatin-like protein (Tattersall *et al.* 1997) correlated with the onset of sugar accumulation, the proteins encoded by these genes were implicated in the defence response of grapevine to powdery mildew. However, more recent research has shown that berries became resistant to powdery mildew infection much earlier in development, within 2 to 4 weeks after bloom, long before °Brix levels reach 8%, discounting a role for these genes in berry ontogenic resistance (Gadoury *et al.* 2001, Gadoury *et al.* 2003).

Using microscopic analyses on powdery mildew-inoculated berries at different stages of development, Ficke *et al.* (2003) determined that ontogenic resistance in developing

grape berries is characterised by failed *E. necator* penetration. Conidial attachment, germination and appressorium formation were not influenced by berry age, but penetration and haustorium formation were reduced significantly in older berries where ontogenic resistance is acting. The absence of visible penetration pores at sites of attempted penetration suggests that berry ontogenic resistance acts early in the infection (Ficke *et al.* 2003), thereby preventing *E. necator* penetration.

Ficke *et al.* (2004) further investigated potential host barriers, which may be responsible for the development of ontogenic resistance in developing grape berries. Cuticle and epidermal cell wall thickness, polyphenolic accumulation and papillae formation were discounted as possible contributors to berry ontogenic resistance. In fact, *E. necator* was prevented from penetrating prior to papillae formation, suggesting that the mechanism responsible for grape berry ontogenic resistance may involve cuticle or cell wall modification that prevents fungal penetration.

1.3.4.2 HR-mediated resistance in wild grapevines

Within the different European and US *Vitis* species, susceptibility to powdery mildew varies significantly (Pearson and Gadoury 1992). There are sources of quantitative genetic resistance to powdery mildew in the *Vitis* species *V. aestivalis*, *V. berlandieri*, *V. labrusca*, *V. riparia* and *V. rupestris* and *V. cinerea*. However, attempts at producing powdery mildew-resistant cultivars through breeding have been of limited success due to the difficulty of selecting for quantitative traits controlling both resistance and fruit quality (Boubals 1961, Eibach *et al.* 1989).

Furthermore, Boubals (1961) recognised that species within other genera of the Vitaceae family also vary in susceptibility to powdery mildew. The wild grape species *Muscadinia rotundifolia*, which originates from the south-east of the USA, is completely resistant to infection by *E. necator* (Olmo 1971). The resistance derived from *M. rotundifolia* is thought to be conferred by a dominant allele at a single locus designated *Run1* (Bouquet 1986, Pauquet *et al.* 2001) and has been introduced into the *V. vinifera* genome using a pseudo-backcross strategy (Bouquet 1986, Pauquet *et al.* 2001). Phenotypically, *Run1*-mediated resistance to *E. necator* resembles that conferred by the barley powdery mildew resistance gene *Mla12*, with induction of a specific hypersensitive response, within infected epidermal cells, following formation of the first fungal haustorium (Dr Claire Barker, CSIRO Plant Industry, personal communication). Donald *et al.* (2002) reported the isolation of genetic markers tightly linked to *Run1* and these have been used to fine-map the location of the *Run1* locus in a bacterial artificial chromosome (BAC) library (Barker *et al.* 2005) for eventual map-based cloning of this grapevine powdery mildew resistance gene.

1.4 Germin and germin-like proteins (GLPs)

Barley host defence responses to powdery mildew attack begin prior to direct fungal penetration activity, suggesting that elicitors originating from the germinating conidia exist (Schweizer *et al.* 2000). Numerous barley defence-related genes are induced in response to pathogen attack. These genes encode for proteins such as β -1,3-glucanase, chitinase, specific peroxidases, phenylpropanoid biosynthesis proteins, regulatory proteins and H₂O₂ metabolism-related proteins (Collinge *et al.* 2002). Studies on defence-related gene induction in grapevine in response to powdery mildew are limited.

Jacobs *et al.* (1999) observed the induction of a chitinase, *VvChi3*, a glucanase *VvGlub* and a thaumatin-like protein, *VvTL2*, in leaves and berries infected with powdery mildew.

Defence-related proteins are thought to be involved in defence via their antifungal or antimicrobial activities, or by their ability to build barriers against pathogen ingress (Collinge *et al.* 2002). However, the same defence-related transcripts generally accumulate in both compatible and incompatible interactions (Gregersen *et al.* 1997). An exception is a barley germin-like protein gene, *HvGLP4* (Wei *et al.* 1998, Christensen *et al.* 2004, Gjetting *et al.* 2004), which appears to accumulate specifically in cells with papillae effective in preventing penetration.

1.4.1 GLP characteristics and classification

The first member of the GLP family to be identified, germin, was isolated from germinating wheat embryos in a search for germination-specific proteins (Thompson and Lane 1980). Early studies suggested this protein was specific to seed germination in monocotyledonous species. However, more recent studies have also identified sequences with homology to germin in various tissues of dicotyledonous species. The term germin-like protein (GLP) has been used to include sequences sharing homology to germin regardless of their tissue of origin (Heintzen *et al.* 1994, Ono *et al.* 1996, Carter *et al.* 1998, Vallelian-Bindschedler *et al.* 1998). All GLP polypeptides have three highly conserved oligopeptides termed germin boxes A, B, and C (Fig. 1.3A) and are composed of approximately 220 amino acids (Bernier and Berna 2001). The region spanning germin boxes B to C comprises a cupin domain; named on the basis of

conserved key amino acid residues involved in forming the core β -barrel structure of the three-dimensional protein (Gane *et al.* 1998, Requena and Bornemann 1999). Barley germin is a homohexameric protein organised as a trimer of dimers (Woo *et al.* 2000).

Figure 1.3B shows the architecture of germin, a manganese-containing, homohexameric glycoprotein (Woo *et al.* 2000). The highly conserved histidine and the glutamate residues within boxes B and C (Fig. 1.3A) are involved in manganese binding (Requena and Bornemann 1999). Two conserved cysteine residues, one in box A, and the other between boxes A and B (Fig. 1.3A) are thought to stabilise the structure by forming disulfide bonds (Woo *et al.* 2000) resulting in a protein that is extremely resistant to proteases, heat, extreme pHs and sodium dodecyl sulfate (SDS) (Grzelczak and Lane 1984).

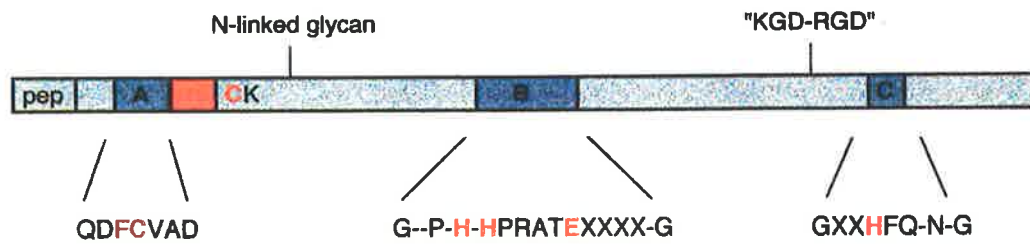
Most GLP genes encode a signal peptide and GLPs studied at the protein level are associated with the extracellular matrix (Lane *et al.* 1992, Heintzen *et al.* 1994, Vallelia-Bindschedler *et al.* 1998, Ohmiya *et al.* 1998). A number of GLPs have been demonstrated to have oxalate oxidase (OXO) or superoxide dismutase (SOD) activity. OXO catalyses the conversion of oxalic acid to carbon dioxide and H_2O_2 (Lane 1994), and SOD catalyses the dismutation of superoxide and its protonated form ($\text{HO}_2^-/\text{O}_2^-$) to H_2O_2 (Sutherland 1991). A GLP from barley also functions as an ADP glucose pyrophosphatase/phosphodiesterase (AGPPase), catalysing the hydrolytic breakdown of several NDP-monosaccharides (Rodriguez-Lopez 2001). However, enzyme activity and function for the majority of GLPs has not yet been determined.

The GLPs are a considerably heterogeneous group of proteins, with amino acid identities ranging from as low as 25 to almost 100% (Bernier and Berna 2001). Carter and Thornburg (2000) proposed that GLPs could be subdivided into five clades: true germin subfamily, gymnosperm subfamily and GLP subfamilies 1, 2 and 3. The true germin subfamily includes cereal germans with demonstrated OXO activity (Dumas *et al.* 1993, Lane *et al.* 1993). Some GLPs, within these subfamilies, have SOD activity (Carter and Thornburg 2000), but to date none have been demonstrated to have OXO activity. The enzyme activity and function for the majority of the GLPs has not yet been determined. Recently Nakata *et al.* (2004) identified a further 2 clades: bryophyte subfamily1 and 2. The Bryophyte subfamily 2 includes the *Barbula unguiculata* BuGLP and *Physcomitrella patens* PpGLP6, both GLPs with Mn-SOD activity (Yamahara *et al.* 1999, Nakata *et al.* 2004).

Germans and GLPs have recently been included in the cupin superfamily, along with a range of proteins including spherulins, vicilin-like and legumin-like seed storage globulins, and sucrose-binding proteins from bacteria, archaea and eukaryotes (Dunwell 1998). Cupins are recognised by an overall low sequence homology and the presence of either single or multiple cupin domains (Dunwell and Gane 1998). The tertiary structure of the cupin domain is thought to be advantageous owing to the flexibility of the active site within the β -barrel (Dunwell *et al.* 2000). Although a wide range of activities exists within members of the cupin superfamily, many of them are associated with cell wall synthesis and various stress responses (Dunwell *et al.* 2000).

GLPs are expressed in various organs and developmental stages in plants, and in response to a number of abiotic and biotic stresses (Bernier and Berna 2001). Thirty-two GLP genes have been identified in *A. thaliana* (Carter *et al.* 1998, Carter and Thornburg 1999), fourteen GLP genes have been described from *H. vulgare* (Wu *et al.* 2000, Druka *et al.* 2002), and at least eight different GLP genes are expressed in rice (Membre and Bernier 1998). The following section comprises a review of GLPs, with emphasis on the proposed role of GLPs in defence against powdery mildew.

A



B

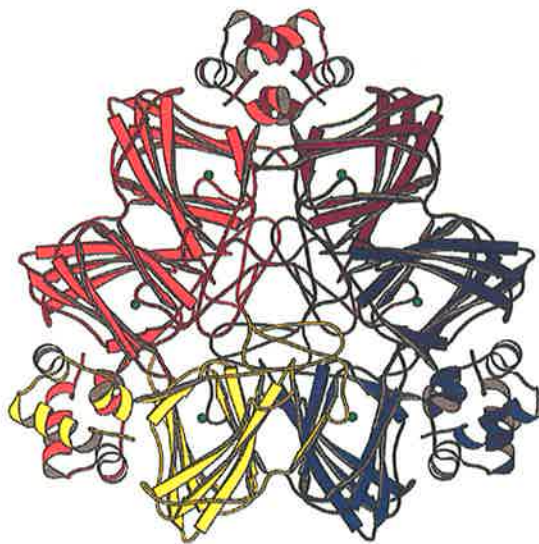


Figure 1.3. Structure of germin and GLPs. **(A)** Schematic representation of germins and GLPs. Light blue indicates moderate sequence conservation, dark blue (boxes A, B and C) indicates high sequence conservation and red represents a highly variable region. The conserved amino acids defining boxes A, B and C are shown. The two cysteines that form an internal disulphide bridge and the four amino acids involved in the binding of a metal ion are shown in red. Pep = Signal peptide; X = any amino acid. From Bernier and Berna (2001). **(B)** Architecture of the germin hexamer. Germin is a homohexamer, with six manganese ions indicated (green spheres). From Woo *et al.* (2000).

1.4.2 GLP function

1.4.2.1 Developmentally regulated expression

1.4.2.1.1 Germination

Following imbibition, successful wheat germination involves the emergence of the radicle from the grain tissues. This process occurs via the expansion of cells in the root primordia. Within the shoot, elongation of the coleoptile and the leaf primordia also occurs. However, the coleorhiza, epiblast and scutellum are terminally differentiated and do not experience growth during germination. Significantly, initial germin protein accumulation occurs specifically within these terminally differentiated tissues (Caliskan and Cuming 1998). At later stages of germination, germin protein accumulates in other organs, but is localised specifically to cells and tissues whose enlargement is restricted, including vascular tissue and root cells that have progressed through the zone of elongation (Caliskan and Cuming 1998). As germin is a bifunctional enzyme with both OXO and SOD activities (Dumas *et al.* 1993, Lane *et al.* 1993, Woo *et al.* 2000), germin-derived H₂O₂ may be involved in lignification and peroxidase-mediated cell wall cross-linking reactions in terminally differentiated cells and tissues (Fry 1986) preventing cell expansion and restricting further growth (Caliskan and Cuming 1998).

1.4.2.1.2 Somatic embryogenesis

Wheat germin transcripts also accumulate during synthetic auxin-induced embryogenic callus formation (Caliskan *et al.* 2004). Callus formation involves the suppression of zygotic embryo germination, loss of cellular specification and the initiation of cellular proliferation. Some cells within the callus retain the ability to undergo reorganisation to form somatic embryos. Upon removal of the synthetic auxin, somatic embryogenesis is

initiated. Significantly, germin transcript accumulation during embryogenic callus formation occurs specifically in cell masses that undergo somatic embryogenesis. H_2O_2 generated by GLP (with OXO activity) may prevent cellular expansion, via peroxidase-mediated cross-linking of cell wall components, thereby maintaining these cell masses in an embryogenically competent state (Caliskan *et al.* 2004).

GLPs from *Pinus caribae* cultures have also been shown to be present in embryogenic cultures, but absent from non-embryogenic cultures. These GLPs, which do not exhibit OXO activity, represent the first markers of somatic embryogenesis in this species (Domon *et al.* 1995, Neutelings *et al.* 1998).

1.4.2.1.3 Floral induction

GLPs from the long day plant *Sinapis alba* (Heintzen *et al.* 1994), the short day plant *Pharbitis nil* (Ono *et al.* 1993, Ono *et al.* 1996) and *A. thaliana* (Staiger *et al.* 1999) exhibit circadian patterns of expression. *PhGLP* transcript accumulates in cotyledons and leaves during dark treatment, but if short exposure to light interrupts the dark treatment, accumulation is reduced (Ono *et al.* 1996). Conversely, transcript accumulation of *Atger3*, expressed in cotyledons, young leaves and all floral organs, except petals and anthers, and *SaGLP*, expressed in leaves and petioles, peaks at the end of the light period (Heintzen *et al.* 1994, Staiger *et al.* 1999). It has been suggested that these GLPs may function in signalling during photoperiodic induction of flowering.

1.4.2.1.4 Nectar production

Nectarin I is the most abundant protein in ornamental tobacco floral nectar, representing approximately 50% of the total protein. Carter *et al.* (1999) observed *Nicotiana plumbaginifolia* GLP, *Nectarin I*, transcript accumulated in the nectary and at low levels in the ovary, but not in leaves, stems, roots or other floral organs. *Nectarin I* was also expressed developmentally with transcript only accumulating while the nectary was actively secreting nectar. Using *Nectarin I* promoter reporter studies Carter and Thornburg (2003) demonstrated 15 to 100 fold higher levels of reporter gene expression in the nectary than any other organ, and promoter deletions identified multiple elements responsible for nectary-specific expression. Nectarin I protein exhibits SOD activity and H₂O₂ was shown to accumulate at high levels, up to 4 mM, in ornamental tobacco nectar (Carter and Thornburg 2000). At this concentration H₂O₂ is toxic to microorganisms, suggesting nectar may be involved in protecting the gynoecium and ovules from pathogen attack (Carter and Thornburg 2004). Elevated H₂O₂ levels in the nectar may act to limit microbial growth, thereby reducing toxin accumulation and microbial sugar metabolism, and maintaining palatability for potential pollinators.

1.4.2.1.5 Cotton fibre development

A *Gossypium hirsutum* (cotton) GLP, *GhGLP1*, is expressed specifically in cotton fibres, but not in roots, cotyledons, hypocotyls, ungerminated pollen, or leaves of various developmental stages (Kim and Triplett 2004). Cotton fibre development involves four overlapping stages: fibre cell initiation, elongation, secondary wall synthesis, and maturation (Basra and Malik 1984). Interestingly, *GhGLP1* transcript accumulates at highest levels in elongating fibres, 4 to 14 days post-anthesis (DPA), and

is reduced significantly during subsequent secondary wall synthesis. Kim *et al.* (2004) used confocal immunofluorescent microscopy to localise GhGLP1 to cytoplasmic vesicles during fibre cell initiation (2 to 4 DPA) and to the apoplast of older fibres (7 to 10 DPA). Intriguingly, although GhGLP1 is conserved in all three germin boxes (Kim and Triplett 2004), it does not exhibit any of the enzyme activities (OXO, SOD and AGPPase) previously reported for GLPs (Kim *et al.* 2004). A role for GhGLP1 in fibre elongation and cell wall expansion has been proposed (Kim *et al.* 2004).

1.4.2.2 Expression in response to abiotic stress

1.4.2.2.1 Salt

The first evidence for a role for GLPs in response to abiotic stress was provided by studies of salt stress in barley (Hurkman *et al.* 1989, Hurkman *et al.* 1991, Hurkman *et al.* 1994, Hurkman and Tanaka 1996a). Barley germin transcript is upregulated during the growth of germinating barley seedlings in the presence of NaCl. In control seedlings, germin mRNA accumulates at maximum levels 2 d post imbibition, whereas when seedlings are grown in the presence of 200 mM NaCl, the levels of germin transcript remain high for an additional day (Hurkman and Tanaka 1996a). A role for GLPs in salt stress is also supported by the altered expression of a GLP in the halophyte *Mesembryanthemum crystallinum* in response to salt stress. Intriguingly, *M. crystallinum* GLP decreased when roots were subjected to 6 h of salt stress (Michalowski and Bohnert 1992).

Yamahara *et al.* (1999) identified a moss (*B. unguiculata*) GLP, *BuGLP*, which was salt-inducible and encoded a protein with extracellular Mn-SOD activity. During

unstressed conditions, *BuGLP* was not expressed during the stationary growth phase and *BuGLP* protein was bound to the cell wall. *BuGLP* transcripts accumulated in cells during the logarithmic growth phase, suggesting a role for *BuGLP* in cell wall synthesis during cell propagation via SOD-generated H_2O_2 (Nakata *et al.* 2002). Addition of NaCl to the cells resulted in the release of *BuGLP* protein from the cell wall and induction of *BuGLP*. The dissociation of *BuGLP* from the cell wall in response to salt stress may lead to the generation of H_2O_2 for signalling, and the eventual induction of *BuGLP* expression via H_2O_2 related signal transduction (Nakata *et al.* 2002).

1.4.2.2 Wounding

In addition to salt stress, GLPs are also induced in response to wounding. Wheat germin (*gf-2.8*) is induced in response to wounding (Berna and Bernier 1999) and in ryegrass, wounding leads to accumulation of GLP transcripts, increase in OXO activity and H_2O_2 accumulation (Le Deunff *et al.* 2004). Wound-induced H_2O_2 is thought to be important in cell wall cross-linking during wound healing.

1.4.2.3 Role of GLPs in plant-microbe interactions

1.4.2.3.1 Nodulation

Symbiotic *Rhizobium* spp. soil bacteria are capable of colonising compatible leguminous plants and forming nitrogen-fixing root nodules. These nodules enable the bacterial conversion of atmospheric dinitrogen to ammonia for assimilation by the plant (Vance 1990). Swart *et al.* (1994) identified a cell wall protein from *Pisum sativum* that was implicated in the attachment of the Rhizobiaceae to the plant cell wall, a process mediated by the bacterial protein rhicadhesin. Significantly, this rhicadhesin receptor

was later recognised to be GLP (Dunwell 1998). An RGD (Arg-Gly-Asp) tripeptide in the GLP rhicadhesin receptor is thought to mediate the recognition between rhicadhesin and the *P. sativum* root (Swart *et al.* 1994). In animals, proteins containing RGD tripeptides, including fibronectin and vitronectin, are extracellular adhesion proteins that interact with integrins. These proteins are thought to exchange information between the inside and the outside of the animal cells (Ruoslahti and Pierschbacher 1987). Interestingly, RGD-like tripeptides are present in over half the germin-like proteins, but are absent in the true germins (Bernier and Berna 2001).

1.4.2.3.2 Arbuscular mycorrhiza symbiosis

Many plants have the ability to supplement their limited root phosphate supply by associating with arbuscular mycorrhiza (AM) fungi (Smith and Read 1997). Fungal hyphae colonise the root cortex of a host plant and form branched intracellular structures known as arbuscules within cells of the inner cortex. During the symbiotic relationship, the host plant supplies carbohydrates to the fungal partner, and the fungus transfers phosphorous to its host (Harrison *et al.* 2002). A *Medicago truncatula* GLP, *MtGLP1*, is strongly induced in roots colonised by the arbuscular mycorrhiza (AM) fungus *Glomus intraradices* (Doll *et al.* 2003). Significantly, *MtGLP1* is not expressed in healthy non-infected roots, nor is it induced after infection with either the oomycete root pathogen *Aphanomyces euteiches* or with Rhizobia, suggesting that *MtGLP1* is induced by AM specifically. Using *in situ* hybridisation Doll *et al.* (2003) were able to localise *MtGLP1* to arbuscule-containing cells. The enzyme activity of *MtGLP1* has not been determined although if it were to possess either SOD or OXO activity, it may account for the H₂O₂ that accumulates in roots colonised with *G. intraradices* (Salzer *et al.* 1999).

1.4.2.3.3 Virus infection

A GLP protein from *Capsicum annuum*, *CaGLP1*, was found to accumulate specifically in the incompatible, but not the compatible, interaction with Tobacco Mosaic Virus (TMV) or *Xanthomonas campestris* (Park *et al.* 2004). This suggests that *CaGLP1* is involved in the specific defence response between resistant plants and avirulent pathogens. *CaGLP1* transcript also accumulated in plants treated with the growth regulators salicylic acid (SA) and ethylene (released from ethephon), but was not induced by wounding, NaCl or methyl jasmonate (MeJA), suggesting that SA and ethylene may play a fundamental role in the induction of *CaGLP1*.

1.4.2.3.4 Powdery mildew

Although a role for germin in pathogen response was one of the earliest suggested (Lane *et al.* 1986, Lane 1994), the connection was not established until the identification of germin as an OXO (Dumas *et al.* 1993) and studies had been undertaken on the interaction of powdery mildew with barley (Wei 1994, Dumas *et al.* 1995, Zhang *et al.* 1995, Wei *et al.* 1998) and wheat (Hurkman and Tanaka 1996b). A preliminary study of defence-related gene expression identified a cDNA clone, from barley leaves infected with *B. graminis*, which shared homology to germin (Wei 1994). Subsequently, Dumas *et al.* (1995) and Zhang *et al.* (1995) independently observed the involvement of GLPs in the defence response of *H. vulgare* seedlings subjected to *B. graminis* infection.

More recent work has demonstrated that there are two distinct forms of barley GLP induced during *B. graminis* infection. One form, HvGLP1 (formerly HvOxOa), exhibits OXO activity and is located exclusively within the cell walls of leaf mesophyll tissue

(Zhou *et al.* 1998). A second form, HvGLP4 (formerly HvOxOLP), exhibits SOD activity, but not OXO activity, and has been detected exclusively within the epidermis (Wei *et al.* 1998, Christensen *et al.* 2004).

The mesophyll-specific barley GLP transcript, *HvGLP1*, accumulates in leaf mesophyll tissue 6 h after inoculation with *B. graminis*, with HvGLP1 protein detected within mesophyll cell walls 15 to 24 h after inoculation. OXO activity was also restricted to the leaf mesophyll, and was detected within 24 h after inoculation (Zhou *et al.* 1998). H₂O₂ has also been observed to accumulate in barley leaves infected with *B. graminis*. The H₂O₂ accumulates in the attachment zones between epidermal cells undergoing HR and subjacent mesophyll cells (Thordal-Christensen *et al.* 1997). H₂O₂ observed in the attachment zones appears to be produced by the HvGLP1 protein. Acidification of the apoplast, resulting from H⁺ATPase activity of the challenged epidermal cell, has been proposed to activate the HvGLP1 protein (Zhou *et al.* 2000).

The epidermal-specific barley transcript, *HvGLP4*, accumulates in leaf epidermal tissue 3 h after inoculation with *B. graminis*, with maxima at 6 and 24 h, followed by a rapid decline. In barley, papillae formed in host epidermal cell walls, in response to the formation of the *B. graminis* primary and appressorial germ tubes, 3 to 4 h and 10 to 18 h post inoculation respectively. Hence, the spatial and temporal expression of *HvGLP4* suggests a role for this protein in papillae formation (Wei *et al.* 1998). H₂O₂ accumulation has been observed in the papillae adjacent to the primary and appressorial germ tubes (Thordal-Christensen *et al.* 1997). HO₂[•]/O₂^{•-} produced by NADPH oxidase or peroxidases in response to pathogen attack (Bolwell and Wojtaszek 1997) may

potentially be dismutated to H_2O_2 by HvGLP4 accounting for this accumulation. This H_2O_2 could be involved in cross-linking cell wall components during papillae formation, function as a messenger to activate other defence genes (either in the same cell, neighbouring epidermal cells or mesophyll cells), or act directly as an anti-microbial compound (Wei *et al.* 1998).

Further support for the role of *HvGLP4* in defence against powdery mildew has been obtained recently using a novel technique for generating transcript profiles of single barley cells (Gjetting *et al.* 2004). Following *B. graminis* inoculation, contents of single barley epidermal cells (uninoculated control cells, penetrated haustorium-containing cells and resistant papilla-containing cells) were sampled using microcapillaries. After cDNA synthesis and PCR amplification, the sample was hybridised to dot-blots spotted with various genes, including *HvGLP4*. Significantly, accumulation of *HvGLP4* transcript, was specifically greater in cells with papillae effective in preventing penetration compared to uninoculated control cells and haustorium-containing infected cells, strongly supporting the case for GLP involvement in papillae-based defence.

1.4.2.4 Reduced susceptibility in transgenic plants over-expressing GLP

1.4.2.4.1 Powdery mildew

Although expression data and enzyme activities are known for a number of GLPs, functional data from transgenic plants over-expressing or silencing these genes is limited. Significantly, Schweizer *et al.* (1999a) and Christensen *et al.* (2004) used a transient expression system, based on particle bombardment of wheat leaves (Schweizer *et al.* 1999b), to functionally test the role of GLPs in defence against powdery mildew.

The system relies on co-expression of the GUS reporter gene with the test gene in single epidermal cells. GUS-expressing cells are scored for resistance phenotype, as indicated by the presence or absence of haustoria within the transformed cells. Significantly, over-expression of wheat germin *gf-2.8* (OXO activity), wheat TaGLP2a (no OXO activity) and two engineered germin *gf-2.8* genes (without OXO activity) all reduced the penetration efficiency of *B. graminis*. Furthermore, using the same system, silencing *HvGLP4* increased *B. graminis* penetration efficiency (Christensen *et al.* 2004). These studies demonstrate that GLPs can reduce susceptibility to powdery mildew attack, which is not related to a specific enzyme activity.

1.4.2.4.2 White mould

Transgenic plants constitutively expressing wheat germin (*gf-2.8*) also have reduced susceptibility to another fungal pathogen, *Sclerotinia sclerotiorum* (Donaldson *et al.* 2001, Hu *et al.* 2003). Unlike powdery mildew, *S. sclerotiorum* is a necrotrophic pathogen that infects hundreds of species worldwide including a number of important crop species (soybean, bean, sunflower and canola). Oxalic acid is the major pathogenicity factor of *S. sclerotiorum* (Godoy *et al.* 1990), the causal agent of white mould. The fungus secretes oxalic acid, leading to the formation of lesions in advance of the invading fungal hyphae (Lumsden and Dow 1973) and disease severity is correlated with the level of oxalic acid in the infected tissue (Noyes and Hancock 1981). Cessna *et al.* (2000) demonstrated oxalic acid suppression of the oxidative burst in host plants, thus identifying a role for oxalic acid in disrupting a major plant defence pathway. In addition, oxalic acid also lowers extracellular pH, allowing optimum activity of fungal enzymes capable of degrading the plant cell wall (Marciano *et al.* 1983).

Soybean (*Glycine max*) genotypes displaying partial field resistance to *S. sclerotiorum* have been correlated with laboratory resistance to oxalic acid (Wegulo *et al.* 1998). Donaldson *et al.* (2001) expressed wheat germin (*gf-2.8*) with OXO activity, capable of degrading oxalic acid, under the control of Cauliflower Mosaic Virus (CaMV) 35S promoter in soybean. The transgenic product of wheat germin (*gf-2.8*) exhibited OXO activity and significantly, the transgenic plants, inoculated with *S. sclerotiorum*, exhibited reduced disease progression and lesion length, indicating wheat germin reduced susceptibility to this pathogen. The mechanisms reducing susceptibility have yet to be elucidated, but may result from either the removal of oxalate, possibly acting as a plant toxin or suppressing the oxidative burst, or the production of H₂O₂-triggering defence responses (Donaldson *et al.* 2001).

Likewise, Hu *et al.* (2003) demonstrated transgenic sunflower (*Helianthus annuus*), constitutively expressing wheat germin (*gf-2.8*), also exhibited to reduced susceptibility to *S. sclerotiorum* infection. The transgenic plants were further characterised and healthy un-inoculated plants displayed elevated levels of H₂O₂, SA and defence related gene transcripts compared with wild type plants. Two groups of transgenic plants were identified; the first expressed higher OXO activity, the other lower OXO activity. Lines expressing higher OXO activity also accumulated H₂O₂ at a higher level and developed HR-like lesions in the absence of the pathogen, that were not observed on the lines expressing lower OXO activity. Thus, defence-related gene expression and HR-like cell death may involve separate signalling pathways, or the H₂O₂ thresholds for these responses are different (Hu *et al.* 2003). The reduced susceptibility to *S. sclerotiorum* may result from multiple mechanisms; metabolism of endogenous substrates to generate

H₂O₂ thus triggering defence related gene expression and sensitivity to subsequent pathogen attack, degradation of fungal oxalic acid thereby counteracting the suppression of the oxidative burst, or H₂O₂ may act as a direct antifungal compound on the invading pathogen.

1.4.2.4.3 Canker

Hybrid poplar trees are highly susceptible to the canker fungal pathogen *Septoria musiva*. Infection results in leaf necrotic lesions, premature defoliation and stem and bark cankering. It is unclear if *S. musiva* requires oxalic acid for pathogenicity, but a role for OXO in reducing the pH at infection sites has been proposed (Shimada *et al.* 1994). Liang *et al.* (2001) expressed wheat germin (*gf-2.8*) under the control of CaMV 35S promoter in the hybrid poplar, *Populus × euramericana* ('Ogy') to test this possibility. Transgenic plants exhibited constitutive OXO activity, undetected in wild type plants, and transgenic leaf discs were more tolerant to oxalic acid. Notably, transgenic leaf discs also displayed to reduced susceptibility to *S. musiva*. However, the mechanism responsible for reduced susceptibility is unclear (Liang *et al.* 2001).

1.5 Project aims

GLPs have been shown to constitute a relatively large family of proteins with potentially disparate activities, and roles in plant responses to abiotic and biotic stress. In cereals, expression of certain members of the GLP family is specifically upregulated in response to powdery mildew infection (Wei 1994, Dumas *et al.* 1995, Zhang *et al.* 1995, Wei *et al.* 1998) and is associated with successful defence against powdery mildew (Gjetting *et al.* 2004). Finally, manipulation of levels of some GLPs in cereals significantly reduces

powdery mildew infection (Schweizer *et al.* 1999a, Christensen *et al.* 2004), offering a new strategy to reduce susceptibility to this fungal pathogen.

The aim of this research project is to characterise the GLP gene family in *V. vinifera* and to investigate their potential role in defence against powdery mildew infection. This information may then be used to design molecular strategies to reduce susceptibility of grapevine cultivars to powdery mildew using genetic manipulation technologies.

The project has the following primary objectives:

- Isolation and characterisation of the GLP gene family in grapevine
- Spatial and temporal localisation of GLP gene expression in grapevine leaves and berries in response to powdery mildew attack
- Isolation and functional characterisation of promoter sequences controlling powdery mildew-inducible GLP expression in grapevine
- Localisation of the cellular targeting of grapevine GLP gene products
- Functional characterisation of grapevine GLP enzyme activity using recombinant protein

Chapter 2. Isolation and characterisation of *GLP* genes and their expression patterns

2.1 Introduction

Specific GLPs have been implicated in the defence response of barley against powdery mildew disease (Dumas *et al.* 1995, Zhang *et al.* 1995, Wei *et al.* 1998, Zhou *et al.* 1998, Christensen *et al.* 2004). Powdery mildew-inducible barley *GLP*, *HvGLP4*, transcript accumulates in leaf epidermal tissue 3 h post-inoculation with *B. graminis*, with maxima at 6 and 24 h, followed by a rapid decline (Wei *et al.* 1998). The temporal expression and localisation of *HvGLP4* transcript in the epidermis suggests a role in papillae formation in response to emergence of primary and appressorial germ tubes, 3 to 4 h and 10 to 18 h post inoculation, respectively. *HvGLP4* exhibits SOD activity (Christensen *et al.* 2004) and has been proposed (Wei *et al.* 1998) to catalyse the accumulation of H₂O₂ observed in the papillae adjacent to the primary and appressorial germ tubes (Thordal-Christensen *et al.* 1997).

The most commonly cultivated species of grapevine, *V. vinifera*, is susceptible to the powdery mildew species *E. necator*. Including *V. vinifera*, there are 14 genera and some 700 species in the Vitaceae family worldwide. Boubals (1961) recognised that certain members of this family, including *M. rotundifolia*, *Parthenocissus tricuspidata* and *Cissus antarctica*, exhibited enhanced penetration resistance to powdery mildew. To

investigate the basis of this resistance, the development of *E. necator* was observed on these species.

At the commencement of this project no GLPs from grapevine were present in the public databases. Dr Ian Dry (CSIRO, Plant Industry, South Australia) provided two *V. vinifera* partial length genomic clones with sequence homology to GLPs from other species. In the presented research, grapevine GLP genes were cloned and characterised. Grapevine GLPs, potentially involved in the defence response to *E. necator*, were identified by their temporal and spatial expression in response to this pathogen. Expression of the GLP gene family in grapevine has also been characterised in a range of grapevine tissues and at different developmental stages and in response to a range of biotic (*Botrytis cinerea* and *Plasmopara viticola* infection) and abiotic conditions (wounding). In an attempt to identify signalling pathways involved in the induction of powdery mildew-inducible grapevine GLP, expression in response to various plant hormone and growth regulator treatments was also observed.

2.2 Materials and methods

2.2.1 Materials

Buffers and solutions, as summarised in Table 2.1, were prepared with nanopure water and autoclaved where appropriate. All chemicals were analytical or molecular biology grade.

Table 2.1 Solutions (and their composition) used in this research.

Solution	Composition
10× agarose gel loading dye (DNA)	78% glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 10 mM EDTA
10× agarose gel loading dye (RNA)	50% (v/v) glycerol, 1 mM EDTA, 0.4% (w/v) bromophenol blue
10× agarose gel loading buffer (RNA)	70% (v/v) deionised formamide, 10% (v/v) formaldehyde, 6% (v/v) agarose-gel loading dye (RNA), 14% (v/v) MOPS/EDTA buffer
Hybridisation buffer	0.5 M NaHPO ₄ (pH 7.2), 0.25 M NaCl, 7% (w/v) SDS, 1 mM EDTA
LB (liquid growth media)	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0
LB agar (solid growth media)	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.2% (w/v) bacto-agar, pH 7.0
10× MOPS/EDTA buffer	200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0
RNA extraction buffer	5 M sodium perchlorate, 0.3 M Tris-HCl (pH 8.3), 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 8.5% (w/v) PVPP, 2% (w/v) PEG 4000
RNA extraction buffer (modified)	5 M sodium perchlorate, 0.3 M Tris-HCl (pH 8.3), 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol
10× SSC	1.5 M NaCl, 0.15 M trisodium citrate
TBE buffer	90 mM Tris-HCl, 90 mM borate, 2 mM EDTA, (pH 8.3)
TE buffer (pH 7.6)	10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0)

2.2.2 Plant materials and growth conditions

Roots, leaves, flowers and berries of *V. vinifera* (L.) were sampled from vineyards and potted glasshouse vines grown at the Waite Campus (Adelaide, South Australia, latitude 34° 56' south, longitude 138° 36' east). Dormant Chardonnay cuttings were obtained from the Riverland Vine Improvement Committee (Monash, South Australia) and stored at 4°C before use. The base of each cutting was dipped in Clonex Hormone Rooting Gel

(Growth Technology, Western Australia) and root growth was encouraged by incubating the base of the cuttings in a 25°C heat-bed containing perlite for 4 weeks. During this period, shoot development was suppressed by maintaining the heat-bed in a 4°C cold-room in the dark. Green shoot cuttings of *P. tricuspidata* (Siebold & Zucc.) and *C. antarctica* (Vent.) were sampled from the Adelaide Botanic Gardens (Adelaide, South Australia) for propagation. *M. rotundifolia* (Michx.) green shoot cuttings were sampled from vineyard vines grown at the Waite Campus (Adelaide, South Australia). Each green shoot cutting consisted of two nodes. The basal node was wounded with secateurs and dipped in Clonex Hormone Rooting Gel (Growth Technology) to promote root growth. The cuttings were planted in perlite and housed in a mist chamber for several weeks to prevent desiccation. Rooted cuttings were planted in 20 cm plastic pots containing a potting mix of sand:composted pine bark (1:2 v/v) with 1 g L⁻¹ ferrous sulphate and 4 g L⁻¹ Osmocote slow release fertiliser (Yates, Australia). Plants were maintained in a temperature-controlled glasshouse at the Waite Campus (Adelaide, South Australia) with a minimum air temperature of 23°C and a maximum temperature of 25°C. Natural powdery mildew infections were controlled in the glasshouse using a sulphur vaporiser.

2.2.3 Pathogen culture and maintenance

2.2.3.1 *E. necator*

E. necator (isolate APC1, kindly provided by Associate Professor Eileen Scott, University of Adelaide) was maintained on a detached leaf *in vitro* system using an 8 d rotation. Young glossy leaves, typically 5 to 10 cm in diameter, were collected from potted glasshouse grown vines, grown as per Section 2.2.2, and kept on ice, prior to

sterilisation. Leaves were sterilised for 3 min in 50% Milton Solution [active ingredient 0.95% (v/v) sodium hypochlorite (Procter and Gamble, NSW, Australia)] containing 0.01% (v/v) Tween 20 with gentle agitation, and washed four times with sterile water. Leaves were then placed on petri dishes containing 1% (w/v) bacto-agar (pH 7.0) and 400 $\mu\text{L mL}^{-1}$ of the antifungal antibiotic Pimaricin (Sigma, USA). The petiole was submerged in the agar to prevent desiccation. The petri dishes were left open in the laminar flow hood for 45 min to allow the leaves to dry. Leaves were inoculated by gently tapping conidia from infected leaves (8 d post inoculation) above the open dishes. The petri dishes were sealed with Parafilm® and incubated at 24.5°C under a 16 h light / 8 h dark cycle. Leaves were examined for sporulation after 8 d using a Zeiss Stemi 2000 microscope. Infected leaves were used to maintain the isolate as described above and for inoculation studies as per Sections 2.2.6.2.

2.2.3.2 *P. viticola*

Field isolates of *P. viticola* (kindly provided by Lee Bartlett, Horticulture Department, South Australia Research and Development Institute, South Australia) were maintained on glasshouse-grown attached grapevine leaves, grown as per Section 2.2.2, on a 7 to 10 d rotation. Infected leaves (7 to 10 d post inoculation) were sampled and incubated upside down on moist filter paper in large petri dishes overnight at 22°C under a 12 h light / 12 h dark cycle. The following day a leaf exhibiting sporulating lesions was placed in a 50 mL tube containing 5 mL water and agitated to displace the conidia. The leaf was removed and the process repeated with new leaves until the water was visibly cloudy. A haemocytometer was used to estimate the spore concentration, which was diluted to 1- $\times 10^6$ spores mL^{-1} accordingly. The spore solution was sprayed on the

abaxial leaf surface of attached leaves of glasshouse-grown vines. The leaves were enclosed in plastic bags overnight to maintain humidity and plants were maintained in a temperature-controlled glasshouse with a minimum air temperature of 23°C and a maximum temperature of 25°C. Infected leaves (7 to 10 d post inoculation) were used to maintain the isolate as described above and for inoculation studies as per Section 2.2.6.2.

2.2.3.3 *B. cinerea*

Field isolates of *B. cinerea* (kindly provided by Dr. Mary Cole, Department of Management, Monash University, Victoria, Australia) were cultured on 3.9% (w/v) Potato Dextrose Agar (PDA) and incubated in the dark at 25°C for 5 d. To obtain a source of active mycelium, infected PDA was cut into 0.5 cm² plugs and excess PDA removed. Infected PDA was used to maintain the isolate as described above and for inoculation studies as per Section 2.2.6.2.

2.2.4 Visualisation of *E. necator* development

E. necator development was visualised on sections (1 cm²) of fresh tissue, placed on moistened filter paper and viewed under a Zeiss Epiluminescence microscope, and on sections fixed overnight in FAA [50% (v/v) ethanol, 0.5% (v/v) glacial acetic acid, 1% (v/v) formaldehyde]. Fixed sections were boiled for 1 min in 95% (v/v) ethanol and immediately dehydrated in ethanol (20 min each in 90%, 80%, 70% and 50% (v/v) ethanol). Sections were stained in 0.6% (w/v) coomassie blue, rinsed in water and mounted on microscope slides in 50% (v/v) glycerol and viewed using a Zeiss

Axioskop2 microscope and recorded using a Spot digital camera (Diagnostic Instruments, SciTech, Australia).

2.2.5 Isolation of GLP genes

2.2.5.1 Total RNA extraction and gel electrophoresis

Total RNA was isolated from grape tissues using a sodium perchlorate method (Rezaian and Krake 1987) with modifications. Plant material (0.5 to 4 g fresh weight), was ground to a powder in liquid nitrogen with a pre-cooled mortar and pestle. Tissue samples were extracted in 20 mL of RNA extraction buffer (Table 2.1) and homogenised by shaking at 200 rpm at room temperature for 30 min. The homogenate was then centrifuged at 80 g for 10 min at 4°C through a crude separation apparatus consisting of a 20 mL disposable syringe plugged with approximately 2 cm³ of silane-treated glass wool wrapped in a double layer of Miracloth and secured into a 250 mL centrifuge tube (Corning, Acton, USA). A further 15 mL of modified extraction buffer (Table 2.1) was added to the crude separation apparatus and centrifuged at 80 g for 10 min at 4°C. The combined eluate was then centrifuged at 4,000 g for 10 min at 4°C to enable pelleting of insoluble particulates. The supernatant was retained, mixed with 2.5 vol of cold 100% (v/v) ethanol and stored at -20°C for a minimum of 1 h. The precipitate was pelleted via centrifugation at 10,000 g for 20 min at 4°C and the resulting pellet was dried under vacuum and resuspended in 1 mL of TE Buffer (Table 2.1) containing 0.2% (v/v) β -mercaptoethanol. The suspension was extracted three times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and once with an equal volume of chloroform:isoamyl alcohol (24:1 v/v). The upper aqueous phase was retained, mixed with 2.5 vol of cold 100% (v/v) ethanol and 0.1 vol of 3 M sodium

acetate (pH 5.2) and then stored at -20°C overnight to precipitate RNA. The RNA precipitate was pelleted at 23,000 g for 10 min at 4°C, dried under vacuum, rinsed with cold 70% (v/v) ethanol, resuspended in 50 to 200 µL of cold sterile water, and stored at -80°C. The concentration of the RNA was determined spectrophotometrically at 260nm where 1 absorbance unit (A_{260}) is equal to 40 µg mL⁻¹ RNA.

RNA electrophoresis was undertaken in horizontal minigel tanks (EasyCast Electrophoresis Systems, OWL Scientific Inc., Cambridge, UK). Agarose (1.2% w/v) gels were prepared in TBE buffer (Table 2.1) and contained 0.5 µg mL⁻¹ ethidium bromide. Gel tanks, trays, and combs were treated with 0.2 M NaOH (for approximately 2 h) prior to use, and samples were adjusted to 2x RNA loading dye (Table 2.1) before loading. Gels were electrophoresed at approximately 100 V in TBE buffer before being visualised and photographed using a short wavelength UV transilluminator (NovaLine Gel Documentation System, Lambda Physik, Germany).

2.2.5.2 cDNA synthesis

Total RNA was extracted (Section 2.2.5.1) from grape tissues and synthesis of first strand cDNA from total RNA was performed with a Preamplification System (Invitrogen, Australia) according to manufacturer's instructions using either oligo(dT)-adapter B26 primer (Appendix 1) or gene-specific primers.

2.2.5.3 DNA amplification by PCR

2.2.5.3.1 RACE-PCR

Full-length *GLP* gene sequences were obtained using rapid amplification of cDNA ends-PCR (RACE) (Frohman *et al.* 1988). For amplification of 3' cDNA ends (3' RACE) target cDNA (Section 2.2.5.2), synthesised using the oligo(dT) adapter B26 primer (Appendix 1), was amplified using the B26 primer and a gene-specific forward 3' RACE primer (see Section 2.3.2.1.1). PCR reactions (20 μ L) contained 2 μ L first strand cDNA, 60 mM Tris-SO₄ (pH 8.9), 18 mM (NH₄)₂SO₄, 200 μ M dNTPs, 2 mM MgSO₄, 0.5 μ M 3' RACE gene-specific primer, 0.5 μ M B26 primer and 0.5 U PLATINUM *Taq* High Fidelity DNA polymerase (Invitrogen, Australia). Reaction conditions for PCR amplification were 2 min denaturing at 94°C, 30 cycles of 30 s denaturing at 94°C, 30 s annealing at 55°C, and 1 min of elongation at 68°C, and a final extension for 10 min at 68°C.

For the amplification of the 5' cDNA ends, a nested 5' RACE strategy was followed using the 5' RACE System Protocol (Invitrogen). First strand cDNA (Section 2.2.5.2) was synthesised from total RNA using a gene-specific reverse 5' RACE initial primer (see Section 2.3.2.1.1) and purified using the QIAquick PCR purification kit protocol (Qiagen, Victoria, Australia) according to the manufacturer's instructions. A homopolymeric tail was added to the 3' end of the purified first strand cDNA using terminal transferase. Tailed cDNA was then amplified using the complementary homopolymer-containing Abridged Anchor Primer (AAP; Appendix 1) and a gene-specific reverse 5' RACE nested primer (see Section 2.3.2.1.1). PCR reactions (20 μ L) contained 2 μ L first strand cDNA, 60 mM Tris-SO₄ (pH 8.9), 18 mM (NH₄)₂SO₄, 200

μM dNTPs, 2 mM MgSO_4 , 0.5 μM 5' RACE gene specific primer, 0.5 μM AAP primer and 0.5 U PLATINUM *Taq* High Fidelity DNA polymerase (Invitrogen). PCR conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 68°C for 1 min, and a final extension for 10 min at 68°C. Where required a second nested amplification was performed using the Abridged Universal Amplification Primer (AUAP; Appendix 1), which contained the same adapter sequence as AAP, and a gene-specific reverse 5' RACE nested primer (see Section 2.3.2.1.1). PCR products obtained from 3' and 5' RACE were separated on ethidium bromide-stained 0.8 to 2.0% (w/v) agarose gels. Electrophoresis of DNA was essentially the same as that described for RNA (Section 2.2.5.1) except samples were mixed with a DNA loading dye before loading (Table 2.1).

2.2.5.3.2 Amplification of partial length *P. tricuspidata* GLPs

First strand cDNA was synthesised (Section 2.2.5.2) using the oligo(dT) adapter B26 primer (Appendix 1) from total RNA (Section 2.2.5.1) extracted from uninfected *P. tricuspidata* leaves and leaves 24 h post inoculation with *E. necator*. PCR amplification of *P. tricuspidata* GLPs was undertaken using *V. vinifera* forward and reverse primer pairs (see Section 2.3.2.2). PCR reactions (20 μL) contained 0.8 μL cDNA, 60 mM Tris- SO_4 (pH 8.9), 18 mM $(\text{NH}_4)_2\text{SO}_4$, 200 μM dNTPs, 2 mM MgSO_4 , 0.5 μM forward primer, 0.5 μM reverse primer and 0.5 U PLATINUM *Taq* High Fidelity DNA polymerase (Invitrogen). PCR conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 68°C for 1 min, with a final extension for 10 min at 68°C.

2.2.5.4 Cloning of PCR products

2.2.5.4.1 Purification of DNA from agarose gel slices

PCR products were visualised on ethidium bromide-stained agarose gels as per Section 2.2.5.3.1 and DNA bands of expected size were excised and purified using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions

2.2.5.4.2 DNA Ligation

Purified fragments were ligated into pGEM[®]-T Easy (Promega). Reactions contained insert and vector DNA, 1x reaction buffer, 1 U of T4 DNA ligase (Promega) and sterile nanopure water to a final volume of 10 μ L. After mixing the reaction was incubated at 16°C overnight and stored at -20°C.

2.2.5.4.3 Transformation of bacteria

Electro-competent cells were prepared by inoculating 500 mL LB media with 5 mL overnight culture of *E. coli* XL1-Blue cells. Cells were grown at 37°C with shaking to an optical density (OD₆₀₀) of 0.5, chilled on ice for 10 min and centrifuged at 5,000 g for 15 min at 4°C. The cells were resuspended in 500 mL of sterile ice-cold water and centrifuged at 5,000 g for 15 min at 4°C, then resuspended in 250 mL sterile ice-cold water and centrifuged again. The cells were then resuspended in 10 mL of sterile ice-cold 10% glycerol, transferred to a sterile 30 mL tube, centrifuged at 5,000 g for 15 min at 4°C and finally resuspended in 2 mL of ice-cold glycerol. Aliquots of 40 μ L were placed into ice-cold microfuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C until required.

Electro-competent *E. coli* XL1-Blue cells (Stratagene, U.S.A) were transformed by electroporation using a Gene-Pulser apparatus (Bio-Rad, Hercules, CA). Ligation reactions (0.5 μL) were mixed with 40 μL of cells and transferred to ice-cold electroporation cuvettes (1mm path length; Invitrogen, Groningen, The Netherlands). Cuvettes were given a single pulse (1.8 kV, 125 μFD , 200 Ohms) and cells were immediately resuspended in 1 mL of LB media (Table 2.1) prior to incubation at 37°C for 1 h to allow expression of antibiotic-resistance genes. Aliquots (90 and 900 μL) of the transformed cells were spread on 1.2% LB agar (Table 2.1) containing 100 $\mu\text{g mL}^{-1}$ ampicillin, 25 $\mu\text{g mL}^{-1}$ IPTG, and 40 $\mu\text{g mL}^{-1}$ X-gal, and incubated overnight at 37°C to allow blue-white selection of positive clones.

2.2.5.4.4 Confirmation of transformation

Single bacterial colonies were used to inoculate LB (Table 2.1) liquid cultures containing 100 $\mu\text{g mL}^{-1}$ ampicillin. Cultures were incubated at 37°C overnight with shaking (200 rpm). Mini-preparations of plasmid DNA were performed on 2 mL overnight cultures using a boiling method according to Holmes and Quigley (1981). DNA was digested with various restriction endonucleases from Roche Diagnostics (New South Wales, Australia) using appropriate buffers supplied by the manufacturer. DNA was electrophoresed on 0.8 to 2.0% (w/v) agarose gels as per Section 2.2.5.3.1.

2.2.5.5 Sequencing of GLP clones

Mini-preparation of plasmid DNA was performed on 5 mL overnight culture using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. DNA was prepared for sequencing using the ABI PRISM® BigDye™ Terminator Cycle

Sequencing Ready Reaction Kit (PE Applied Biosystems, Norwalk, CT), according to the manufacturer's instructions. Each reaction consisted of 8 μ L of Terminator Ready Reaction Mix, 400 ng of template DNA, 3.2 pmol of either T7 or SP6 sequencing primer (Appendix 1), and nanopure water to a final volume of 20 μ L. The components were mixed, spun down in a microcentrifuge and PCR amplified for 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. The DNA was precipitated by adding 80 μ L of 75% (v/v) isopropanol to the reaction, incubating for 20 min at room temperature, followed by centrifugation at 16,000 g for 20 min. The supernatant was then discarded and another 250 μ L of 75% (v/v) isopropanol was added and the samples were centrifuged for a further 5 min at 16,000 g. The samples were then drained, dried under vacuum for 15 min, and sent to the Institute of Medical and Veterinary Science (Adelaide, South Australia) for sequencing.

2.2.5.6 Sequence analysis

Sequences were analysed using BLAST searches within the databases maintained by the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the GCG WebANGIS Wisconsin software package operated by the Australian National Genomic Information Service (<http://www.angis.su.oz.au/>). Alignments were displayed by the GenDoc program (www.psc.edu/biomed/genedoc). To identify putative signal peptides the deduced amino acid sequences were analysed using PSORT Prediction software (<http://psort.nibb.ac.jp/>). N-glycosylation sites were predicted using NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/>).

To examine the relationship between the *V. vinifera* GLPs and GLPs from other plant species, deduced amino acid sequences were aligned using ClustalW with default settings (Thompson *et al.* 1994) at the ANGIS BioManager server (<http://biomanager.angis.org.au/>). The aligned sequences were used to build a Neighbor Joining unrooted tree with Phylip. The distance matrix for the multiple sequence alignment was calculated using Protdist (Felsenstein 1989). The NCBI RefSeq/GenBank protein ID of the sequences used for the analysis in this study were AtGLP, BAA78563; AtGLP1, AAB51584; AtGLP2a, AAB51570; AtGLP3a, AAB51566; AtGLP6, AAB51572; AtGLP8, AAB51585; AtGLP9, NP_193199; BuGLP, BAC53790; GhGLP1, AAF21988; HvGLP1, CAA74595; HvGLP2, CAA75907; HvGLP3, AAG00426; HvGLP4, CAA63659; McGLP, AAA33030; Nectarin I, AAF03355; PcGER1, AAC99473; PnGLP, BAA08266; PpABP19, AAD00295; PpABP20, AAB51240; PpGLP6, BAD86502; PrGLP, AAC05146; PsGER1, CAB65369; SaGLP, CAA59257; TaGER2.8, AAA34270; TaGER3.8, AAA34271.

2.2.6 Collection of material for expression analysis

2.2.6.1 Expression in various grapevine tissues

Leaves, flowers and berries were sampled from healthy *V. vinifera* vineyard and glasshouse-grown vines (see Section 2.2.2). Roots were harvested from 20 cuttings propagated inside a 25°C heat-bed in a 2°C cold-room (see Section 2.2.2). Flowers were collected at 50% capfall, defined as the time when approximately 50% of the opercula had fallen from the flowers in a single cluster. Three flowers were randomly sampled from each of 20 clusters. Leaves were collected at different developmental stages from at least 10 different vines. The stages of leaf development were defined by measurement

of average leaf diameter, that is, 3 cm (young, 20 leaves sampled), 9 cm (half expanded, 15 leaves sampled), and 18 cm (fully expanded, 12 leaves sampled). Flower clusters were tagged at 50% capfall and three berries were sampled randomly from each of 20 bunches at fortnightly intervals until harvest. The onset of grape ripening, veraison, was defined as the stage at which there was a rapid increase in Total Soluble Solids (°Brix) as measured using a hand-held refractometer (refractometer model 10430, Reichert). Berries were physically separated into skin and pulp samples using sterile scalpels and tweezers. Plant material was immediately frozen in liquid nitrogen then stored at -80°C.

2.2.6.2 Pathogen-induced expression

E. necator infected leaves and berries were sampled from at least 10 separate vineyard-grown and potted glasshouse-grown vines that had not been treated with fungicides and had been naturally infected. Leaves and berries displaying infection were categorised as a mild infection (less than 20% of the surface covered in hyphae) or a severe infection (greater than 20% coverage). Leaves and berries showing no sign of infection to the naked eye were considered uninfected and used as controls. Berries were physically separated into skin and pulp samples using sterile scalpel and tweezers. *E. necator* conidia (Section 2.2.3.1) were inoculated on to attached leaves of *V. vinifera* and *P. tricuspidata* potted glasshouse-grown vines (grown as per Section 2.2.2) using a fine paintbrush. Figure 2.1 demonstrates regions of visible powdery mildew infection excised and sampled (designated infected) separately from areas on the same leaf, which were remote from the obvious infection and showed no sign of infection to the naked eye (designated remote).

For each detached leaf time-course inoculation, two young leaves of similar developmental stage (approximately 6 cm in diameter) were excised from separate glasshouse-grown Chardonnay vines (Section 2.2.2). Leaves were surface-sterilised (Section 2.2.3.1) and placed on plates containing 1% (w/v) bacto-agar (pH 7.0) and 0.04% (v/v) Pimaricin (Sigma) and inoculated with *E. necator* conidia (Section 2.2.3.1) using a settling tower (900 cm height, 23 cm diameter). Plates were incubated at 22°C under a 16 h light / 8 h dark cycle.

P. viticola infections were generated on glasshouse-grown attached Chardonnay leaves, grown as per Section 2.2.2, by spraying the abaxial leaf surface with a spore solution (Section 2.2.3.2). Each leaf was enclosed in a plastic bag overnight to maintain humidity. Triplicate samples of control leaves (water inoculation only), leaves with mild infection (12 d post inoculation) and severe infection (20 d post inoculation) were snap frozen in liquid nitrogen and stored at -80°C.

B. cinerea infections were generated by inoculating glasshouse-grown (as per Section 2.2.2) attached Chardonnay leaves (approximately 6 cm diameter) with active mycelium (Section 2.2.3.3). Fungus was placed in direct contact with leaves, which were then enclosed in plastic bags containing a small amount of water. Fungus was allowed to infect and develop for 3 d and leaves successfully infected were sampled. Regions immediately surrounding the infection site were excised and sampled separately from tissue, remote to, but on the same leaf as the lesion (Fig. 2.2). Tissue was snap frozen in liquid nitrogen and stored at -80°C.

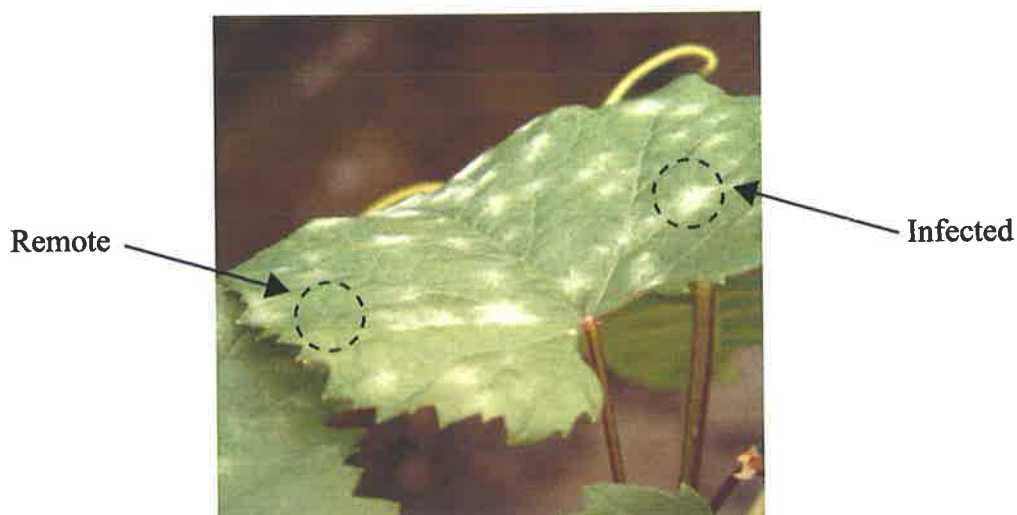


Figure 2.1. Regions of *E. necator* infection excised for expression analysis. Glasshouse-grown Chardonnay leaves (6 cm diameter) were inoculated with *E. necator* conidia. Regions of visible powdery mildew infection (infected) were excised and sampled separately from areas on the same leaf, which were remote from the obvious infection and showed no sign of infection to the naked eye (remote).

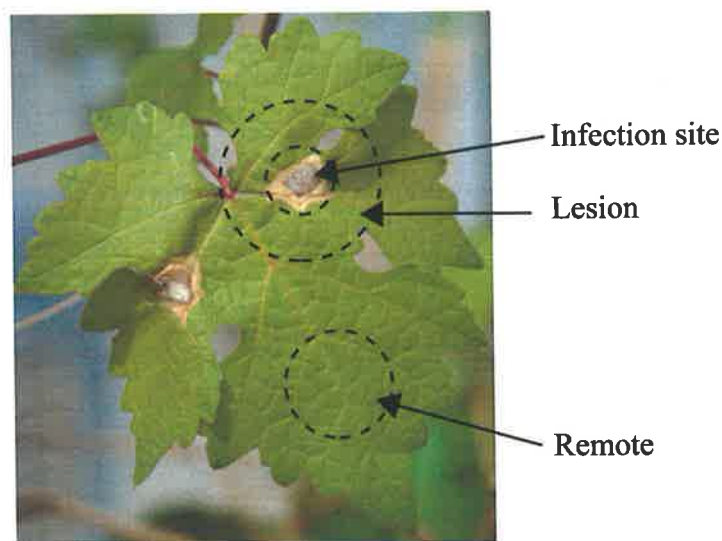


Figure 2.2. Regions of *B. cinerea* infection excised for expression analysis. Glasshouse-grown Chardonnay leaves (6 cm diameter) were inoculated with *B. cinerea* mycelium. Regions immediately surrounding the infection site containing fungal lesions (lesion) were sampled separately from tissue, remote to, but on the same leaf as the lesion (remote).

2.2.6.3 Wounding-induced expression

The adaxial surface of glasshouse-grown (as per Section 2.2.2) Chardonnay leaves were wounded by gently rubbing with fine sandpaper. Four replicate control and wounded leaves were sampled at 0, 4, 8 and 24 h post wounding, immediately frozen in liquid nitrogen and stored at -80°C.

2.2.6.4 Plant hormone and growth regulator-induced expression

Ethylene (released from ethephon) and methyl jasmonate treatments were carried out on glasshouse grown Chardonnay vines in sealed 20 L clear containers. Vines were either sprayed to run off with 10 mM ethephon [(2-chloroethyl) phosphonic acid, Ethrel, Bayer CropScience, Australia] or exposed to a cotton ball onto which 400 µL of 0.5% (v/v) methyl jasmonate had been pipetted. Control vines were treated in the same way as the individual chemical treatments but without the addition of methyl jasmonate or ethephon. Four replicate leaves were sampled 2 d post treatment, snap frozen in liquid nitrogen and stored at -80°C.

For detached leaf treatments, duplicate glasshouse-grown (as per Section 2.2.2) Chardonnay leaves were excised and the petioles submerged in distilled water containing 1 mM salicylic acid (SA), 50 µM abscisic acid (ABA) and 50 µM of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) respectively. Leaves were incubated at 22°C under a 16 h light / 8 h dark cycle. Duplicate control leaves were treated in the same way as the individual chemical treatments, but without the addition of chemicals. Leaves were sampled after 6 h and 24 h post-treatment, snap frozen in liquid nitrogen and stored at -80°C.

2.2.7 Reverse Transcriptase (RT)-PCR analysis

Total RNA (Section 2.2.5.1) extracted from tissue samples was DNase-treated using the RNase-free DNase kit (Qiagen) according to manufacturer's instructions. The concentration of DNase-treated RNA samples was determined spectrophotometrically and confirmed on ethidium bromide-stained agarose gels as per Section 2.2.5.1.

Semi-quantitative RT-PCR reactions were carried out using the Superscript™ One Step RT-PCR with Platinum® Taq System (Invitrogen) according to manufacturer's instructions. All primer pairs were used under identical conditions, 30 min at 50°C, followed by 30 cycles of 15 s at 94°C, 30 s at 55°C, 1 min at 68°C, and a final extension of 10min at 72°C. RT-PCR products were visualised on 1% (w/v) agarose gels stained with ethidium bromide as per Section 2.2.5.3.1. RT-PCR reactions were performed using a *GLP* gene-specific forward primer in combination with an oligo(dT)-adapter primer (B26). that binds to the poly(A) tail of mRNA. The concentration of total RNA used in each RT-PCR reaction was verified by carrying out RT-PCR reactions on each of the samples using 18S rRNA primers Vv18SF and Vv18SR (Appendix 1). The levels of 18S rRNA have been found to be very stable in plant cells (Kim *et al.* 2003) and are likely to be less influenced by treatments involving pathogen infection than other housekeeping genes such as glyceraldehyde-3-phosphate, tubulin or actin. RT-PCR figures were digitally inverted to show the negative image for greater resolution.

2.2.8 Northern blot analysis

Northern analysis was performed using total RNA (extracted as per Section 2.2.5.1). RNA samples were quantified on ethidium bromide-stained agarose gels (as per Section

2.2.5.1) to confirm concentrations. RNA samples (4 µg/lane) were heat denatured and fractionated on a 1.2% (w/v) agarose gel containing 7.4% (v/v) formaldehyde prepared in 1× MOPS/EDTA buffer (Table 2.1). Samples were adjusted to 6× agarose gel loading buffer (RNA) (Table 2.1) and incubated at 65°C for 15 min before loading. Gels were electrophoresed at approximately 100 V in 1× MOPS/EDTA buffer. RNA was transferred to Zeta-probe GT Genomic nylon membrane (Bio-Rad, CA, USA) via capillary action with 10X SSC (Table 2.1) for at least 15 h. Membranes were then rinsed in 2X SSC followed by UV-crosslinking (UV Stratalinker 1800, Stratagene, San Diego, USA). Radiolabeled (³²P) probes were generated using a Gigaprime labeling kit (GeneWorks, Adelaide, Australia). Membranes were pre-hybridised in hybridisation buffer (Table 2.1) for 10 min at 65°C and then hybridised for at least 15 h at 65°C in 7 mL of hybridisation buffer with the addition of denatured radiolabelled probe. Following hybridisation, membranes were washed with 2X SSC, 0.1% SDS at 65°C for 5 min, followed by high stringency washing with 0.1X SSC, 0.1% SDS at 65°C for 15 min. Membranes were exposed to Kodak Biomax MS film with intensifying screens at -80°C for approximately 24 to 72 h. Equality of RNA loadings was confirmed by re-hybridising membranes with an 18S ribosomal RNA cDNA probe.

Gene-specific probes used for Northern blot expression studies were generated by PCR amplification using gene-specific primers. PCR reactions (50 µL) contained 2.5 µL template quality DNA mini-preparation (1 in 50 dilution), 60 mM Tris-SO₄ (pH 8.9), 18 mM (NH₄)₂SO₄, 200 µM dNTPs, 2 mM MgSO₄, 0.2 µM gene-specific primer, 0.2 µM B26 primer, 1.25 U PLATINUM *Taq* High Fidelity DNA polymerase (Invitrogen). PCR conditions were as follows: 95°C for 3 min, 30 cycles of 94°C for 30 sec, 54°C for 30

sec, 72°C for 1 min, and a single cycle of 72 °C for 7 min. The PCR product was gel purified as per Section 2.2.5.4.1.

2.3 Results

2.3.1 Development of *E. necator* on members of the Vitaceae family

A scheme of six developmental stages, which are characterised by the differentiation of specific structures following germination, adapted from Leinhos *et al.* (1997), was defined for the development of *E. necator* on *V. vinifera* leaves (Fig. 2.3A). Stage 0 represents ungerminated conidia. Upon germination, a germ tube emerged from one end of the conidium (stage 1) and after a brief period of growth the germ tube terminated in a multi-lobed appressorium (stage 2). A primary hypha emerged from the end of the conidium opposite to the germ tube (stage 3) and subsequently a secondary hypha extended from the primary appressorium (stage 4). The initial primary hypha appressorium, was produced along the primary hypha (stage 5), shortly followed by the emergence of a tertiary hypha from the conidium adjacent to the germ tube (stage 6). Conidiophores developed 4 d post inoculation.

Detached leaves of *V. vinifera*, *M. rotundifolia*, *P. tricuspidata* and *C. antarctica* were inoculated with *E. necator* and the developmental stages of 50 conidia at 24 h post inoculation were recorded (Fig. 2.3B). Conidia germinated successfully and produced primary appressoria on all four Vitaceae species. The most startling difference in the growth of *E. necator* on *M. rotundifolia*, *P. tricuspidata* and *C. antarctica* compared to *V. vinifera*, was the proportion of conidia that did not progress past the primary appressoria stage (stage 2). On *M. rotundifolia*, *P. tricuspidata* and *C. antarctica*, 42%,

62% and 62% of germinated conidia respectively, exhibited the primary appressoria as the most developed structure, compared to only 16% on *V. vinifera*. This indicates that penetration was less successful on these relatives of *V. vinifera*. Furthermore, a higher proportion of the conidia on *V. vinifera* (44%) had developed primary hyphae appressoria (stage 5) 24 hours after inoculation (hai), while only 6% of conidia on *M. rotundifolia* developed to stage 5. Primary hyphae appressoria were not observed on either *P. tricuspidata* or *C. antarctica*. Also, a considerably smaller proportion of conidia on *M. rotundifolia*, *P. tricuspidata* and *C. antarctica* had produced secondary hypha (stage 4), 12%, 2% and 0%, respectively, compared to *V. vinifera* (22%).

At 24 hai there was evidence of papilla deposition beneath some primary appressoria. These papillae were observed as small brown refractive concentric bodies (data not shown). On *M. rotundifolia*, *P. tricuspidata* and *C. antarctica*, 8%, 28% and 20% of germinated conidia, respectively, were observed to have primary appressoria associated with visible papillae. Visible papillae were not found to be associated with primary appressoria on *V. vinifera* at 24 hai. In all instances that papillae were observed, the primary appressoria (stage 2) was the most developed conidia structure, indicating that penetration had not been successful when a papilla was known to be present.

A

Developmental stages of <i>E. necator</i>			
0	Ungerminated conidium (UnG)	0 h	
1	Germ tube (GT)	4 h	
2	Primary appressorium (PA)	6-8 h	
3	Primary hypha (PH)	12-20 h	
4	Secondary hypha (SH)	20-24h	
5	Primary hypha appressoria (PHA)	24 h	
6	Tertiary hypha (TH)	1-2 d	

B

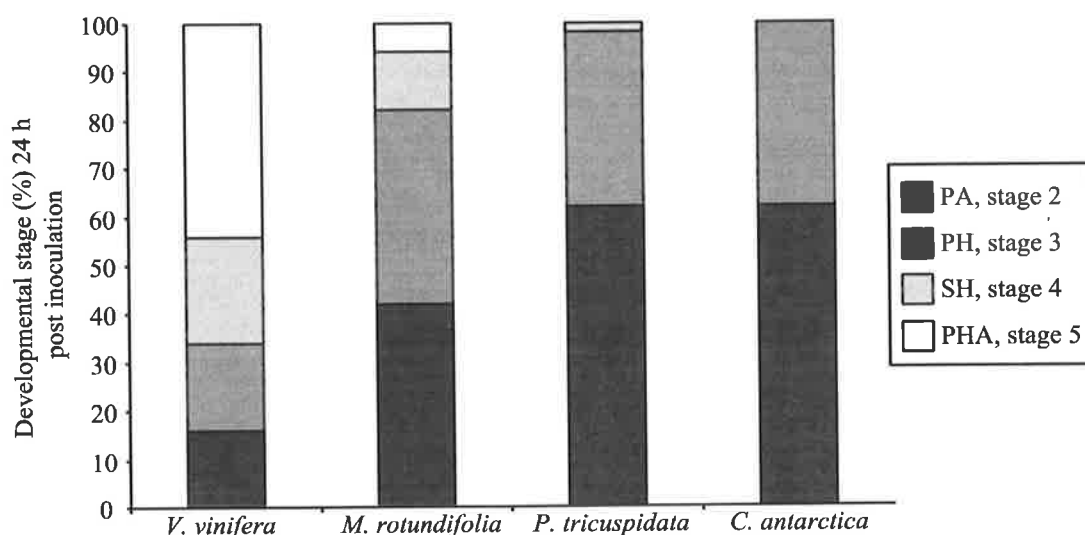


Figure 2.3. Development of *E. necator*. (A) Development of *E. necator* on detached *V. vinifera* cv. Chardonnay leaves at 22°C. The characteristic infection structure and approximate timing of each developmental stage is indicated. (B) Frequency of developmental stages (%) of *E. necator* conidia at 24 h post inoculation on detached *V. vinifera* cv. Chardonnay, *M. rotundifolia*, *P. tricuspidata* and *C. antarctica* leaves. The developmental stages of 50 conidia for each species were recorded.

2.3.2 Isolation of GLP genes

2.3.2.1 *V. vinifera* GLP genes

2.3.2.1.1 Cloning strategy

Two partial length clones, *VvL2* and *VvL3* (Appendix 3), with sequence homology to GLPs were kindly provided by Dr Ian Dry (CSIRO, Plant Industry, South Australia). These clones were isolated from *V. vinifera* cv. Chardonnay genomic DNA using degenerate PCR. Degenerate primers, OXOLP1 and OXOLP2 (Appendix 1) were based on known GLP sequences from other species. Using PCR amplification techniques and searches of the *V. vinifera* EST database (funded by the Cooperative Research Centre for Viticulture) a total of seven GLP full-length cDNA from *V. vinifera* were identified as outlined below.

VvGLP2 and *VvGLP3*

RACE-PCR was used to clone the full-length cDNA sequences, *VvGLP2* and *VvGLP3* (Appendix 3), from the partial length *V. vinifera* clones *VvL2* and *VvL3* (Appendix 3), respectively. Using sequence obtained from *VvL2* and *VvL3* clones, gene-specific primers OXL2Fb and OXL3F, respectively (Appendix 1; 2), were used to amplify 3' ends from powdery mildew-infected Chardonnay grape berry skins. Gene-specific primers, OXL2R and OXL2Rb, and OXL3R and OXL3Rb, respectively (Appendix 1; 2), were designed to the 3' end sequence and used to amplify 5' ends by 5' RACE.

VvGLP1

In addition to cloning the 5' end of *VvGLP3*, the 5' end of another clone, later designated *VvGLP1* (Appendix 3), was also amplified from powdery mildew-infected Chardonnay

grape berry skins using the *VvGLP3* 5' RACE primers OXL3R and OXL3Rb (Appendix 1; 2). Using sequence obtained from *VvGLP1*, the gene-specific primer OXL1F (Appendix 1; 2) was used to amplify 3' ends from powdery mildew-infected Chardonnay grape berry skins.

VvGLP4

The primer used to amplify the 3' end of *VvGLP1*, OXL1F (Appendix 1; 2), also generated the 3' end of an additional clone, designated *VvGLP4* (Appendix 3), from powdery mildew-infected Chardonnay grape berry skins. The 5' end of *VvGLP4* was obtained by 5' RACE using gene-specific primers, OXL4Rb, OXL4R and OXL4Rc (Appendix 1; 2), designed to the 3' end of *VvGLP4*.

VvGLP5 and *VvGLP6*

Two *V. vinifera* cv. Chardonnay GLP partial length sequences were identified from searches of the *V. vinifera* EST database, and designated *VvGLP5* and *VvGLP6* (Appendix 3). Sequencing identified that *VvGLP5* and *VvGLP6* were full-length clones.

VvGLP7

A gene-specific primer designed to *VvGLP5*, OXL5F (Appendix 1), was designed to amplify a *VvGLP5* fragment in combination with the oligo(dT) adapter primer B26 (Appendix 1). In addition to amplifying *VvGLP5*, the 3' end of a further *V. vinifera* GLP, designated *VvGLP7* (Appendix 3), was amplified from total RNA isolated from *B. cinerea* infected Chardonnay leaf tissue. The 5' end of *VvGLP7* was obtained by 5'

RACE using gene-specific primers, GLP7Rb and GLP7Ra (Appendix 1; 2), designed to the 3' end of *VvGLP7*.

2.3.2.1.2 Full-length *VvGLP* clones

PCR amplified DNA fragments were cloned and sequenced in both directions and separate clones were sequenced to verify the accuracy of sequencing. Regions of 100% nucleotide sequence overlap between 3' and 5' RACE clones indicated that corresponding 3' and 5' ends had been obtained. The *V. vinifera GLP* full-length cDNA clones, *VvGLP1* to *VvGLP7* (Fig. 2.4), were assembled *in silico* and ranged from 833 to 947 nt in length. The full-length cDNAs all consist of a 5'- untranslated region, coding region, 3'-untranslated region, ranging from 14 to 133, 624 to 678, and 138 to 258 nt in length, respectively (Appendix 3). Figure 2.5 shows that the deduced open reading frames for *VvGLP1* to *VvGLP7* range in length from 207 to 225 amino acid residues.

The seven *V. vinifera* GLPs contain germin boxes A, B and C characteristic of GLPs (Bernier and Berna, 2001). Highly conserved histidine and glutamate residues within boxes B and C, and two cysteine residues, one in box A and the other between boxes A and B, are also present in the grapevine sequences (Woo *et al.* 2000; Fig. 2.5). Putative N-terminal signal peptides (Fig. 2.5) for *VvGLP2*, *VvGLP3*, *VvGLP4*, *VvGLP6* and *VvGLP7* (21, 23, 23, 17 and 22 amino acids in length, respectively), were predicted using the PSORT online analysis tool (Nakai and Kanehisa 1992). N-terminal signal peptides were not predicted for either *VvGLP1* or *VvGLP5*. Possible N-glycosylation sites were predicted for *VvGLP1* (N41 and N58), *VvGLP2* (N50 and N68), *VvGLP3* (N71), *VvGLP4* (N71 and N79), *VvGLP5* (N41 and N66), *VvGLP6* (N59) and *VvGLP7*

(N54 and N71) between boxes A and B, are depicted in Figure 2.5. RGD (Arg-Gly-Asp)-like tripeptides, either KGD (Lys-Gly-Asp) or KGE (Lys-Gly-Glu), are present in all of the grapevine GLP sequences (Fig. 2.5).

A phylogenetic tree (Fig. 2.6A), constructed from the deduced amino acid sequences using the GCG WebANGIS program PileUp, represents the sequence similarities between the grapevine GLPs. VvGLP1, VvGLP3, VvGLP4, VvGLP5 and VvGLP7 group together based on amino acid sequence similarity, whereas the other two grapevine GLPs, VvGLP2 and VvGLP6, are more dissimilar. The sequence identity between all the *V. vinifera* GLP deduced amino acid sequences varies from 34.8 to 87.5% (Fig. 2.6B). VvGLP1, VvGLP3, VvGLP4, VvGLP5 and VvGLP7 share greater identity (66.5 to 87.5%) whereas VvGLP2 and VvGLP6 share less identity to the other GLPs (VvGLP2, 37.8 to 52.6%; VvGLP6, 34.8 to 38.3%).

To examine the phylogenetic relationships between derived amino acid sequences of the *V. vinifera* GLPs, with GLPs and germins from other plant species, protein sequences were aligned using ClustalW (Thompson *et al.* 1994) and sequence relationships visualised using a Neighbor Joining unrooted tree (Fig. 2.7). The germins and GLPs used in this study included 22 sequences from dicotyledonous species (*A. thaliana*, *Atriplex lentiformis*, *G. hirsutum*, *M. crystallinum*, *N. plumbaginifolia*, *P. nil*, *P. sativum*, *Prunus persica*, *S. alba*, *V. vinifera*), six from monocotyledonous species (*H. vulgare*, *Triticum aestivum*), two from gymnosperm species (*P. caribaea*, *Pinus radiata*) and two from bryophyte species (*B. unguiculata*, *P. patens*).

The VvGLPs grouped within the GLP subfamilies previously proposed by Carter and Thornburg (2000). VvGLP1, VvGLP3, VvGLP4, VvGLP5 and VvGLP7 grouped within GLP subfamily 1 and all shared highest amino acid identity (76, 73, 69, 72 and 59%, respectively) to an *A. thaliana* GLP (GenBank protein ID BAB10836) cloned from a genomic library (Asamizu *et al.* 1998). No functional data is available for this *A. thaliana* gene and TAIR Microarray Expression Searches (<http://www.arabidopsis.org/>) did not identify any expression data for this gene. VvGLP1, VvGLP3, VvGLP4, VvGLP5 and VvGLP7 also shared amino acid identity to the pathogen-induced, epidermal-specific *H. vulgare* protein, HvGLP4 (Wei *et al.* 1998), (66, 63, 56, 63 and 53%, respectively) and *A. lentiformis* AlGLP (Genbank protein ID BAA78563), (57, 57, 55, 55 and 53%, respectively), both with demonstrated SOD activities (Tabuchi *et al.* 2003, Christensen *et al.* 2004).

VvGLP2 shared highest amino acid identity, 74%, to an *O. sativa* GLP (GenBank protein ID AAT47457) within GLP subfamily 2. The gene encoding this protein was cloned from a genomic library. No information is available relating to the transcription of this gene. VvGLP2 also shared 70% amino acid identity to a Rhicadhesin receptor, PsGER1, from *P. sativa* (Swart *et al.* 1994) and a *N. plumbaginifolia* GLP, Nectarin I, with demonstrated Mn-SOD activity (Carter and Thornburg 2000).

Subfamily 3, includes VvGLP6, and this GLP shared highest amino acid identity (76%) to the *P. persica* auxin-binding GLP PpABP19 (Ohmiya *et al.* 1998). VvGLP6 also shared 70% amino acid identity with SaGLP from *S. alba* (Heintzen *et al.* 1994) and 69% to GhGLP from *G. hirsutum* (Kim and Triplett 2004, Kim *et al.* 2004). A *H.*

vulgare GLP, HvGLP2 with AGPPase activity is also grouped within subfamily 3 (Rodriguez-Lopez *et al.* 2001, Vallelian-Bindschedler *et al.* 1998).

Accession	Sequence	Position
VvGLP1	-----	-
VvGLP5	-----	-
VvGLP7	-----	-
VvGLP3	-----A	1
VvGLP4	-----A	1
VvGLP2	AACTCATCATTATAAATACACAACCCCACTTTTCTCTCTCCCCATTCTAATTTGAGATATCCAT	65
VvGLP6	-----	-
VvGLP1	-----	-
VvGLP5	-----	9
VvGLP7	-----ATCCCCGCCTAAAGATAAAGATTGTGATTGTTGGAGGCAGTACTCTAACAATGAAG	56
VvGLP3	CCTCTCACCCAAGG-----ACACAGATATC---GATTGTTGGAGA---GGGTCTAACAATGATG	54
VvGLP4	CCCTCACCTCAAGTTCTCAACACAAAGATCTGTGATTGTTGGAGATACTACTCTAACAATGAAG	66
VvGLP2	CTACTTTTCCCCTTCTCTCTTTTTCATATCATTATTATCTTTGTATTTTTTTTGTCTCCG	130
VvGLP6	-----A	1
VvGLP1	-----TAACTGCGCCCTCATGGCAATGGCTTCATCTTTGGCTCTGGCTC	46
VvGLP5	GACTAACAATGAAGTG TAGTTTCCG G C C C T C A T G G A A T T G C T T C C T C T C T G C C C T C G C C T T	65
VvGLP7	AGAGGCGTTAGTTTCC GGTAAACCG G C C T T C A T G G C A T T G C T T C C T T C T G C C C T G G C C T A	121
VvGLP3	AAAGGTGTTTGTTC TGTAACTG T C C C T C T G G C T T G C T T C C T C T C T G C C C T G G C C T C	119
VvGLP4	AAGGGTGTTAGTTTCC GGTAACTG T T C C C T G T G G C A T T G T T C C T C T C T G C C C T G G C C T C	131
VvGLP2	ACCATGGCGGCTGTTG GGCCTTGT T T T A T C A C A C T C G C T T C T C T C G G C A C C G T T C C T G C	195
VvGLP6	AACATTCTTCTTTATA TTGGTGAAGATGTTTCTCCCAATCTCGCATCTTTCCCTATCTTA	66
VvGLP1	-----TAACTGCGCCCTCATGGCAATGGCTTCATCTTTGGCTCTGGCTC	46
VvGLP5	GACTAACAATGAAGTG TAGTTTCCG G C C C T C A T G G A A T T G C T T C C T C T C T G C C C T C G C C T T	65
VvGLP7	AGAGGCGTTAGTTTCC GGTAAACCG G C C T T C A T G G C A T T G C T T C C T T C T G C C C T G G C C T A	121
VvGLP3	AAAGGTGTTTGTTC TGTAACTG T C C C T C T G G C T T G C T T C C T C T C T G C C C T G G C C T C	119
VvGLP4	AAGGGTGTTAGTTTCC GGTAACTG T T C C C T G T G G C A T T G T T C C T C T C T G C C C T G G C C T C	131
VvGLP2	ACCATGGCGGCTGTTG GGCCTTGT T T T A T C A C A C T C G C T T C T C T C G G C A C C G T T C C T G C	195
VvGLP6	AACATTCTTCTTTATA TTGGTGAAGATGTTTCTCCCAATCTCGCATCTTTCCCTATCTTA	66
VvGLP1	-----TAACTGCGCCCTCATGGCAATGGCTTCATCTTTGGCTCTGGCTC	46
VvGLP5	GACTAACAATGAAGTG TAGTTTCCG G C C C T C A T G G A A T T G C T T C C T C T C T G C C C T C G C C T T	65
VvGLP7	AGAGGCGTTAGTTTCC GGTAAACCG G C C T T C A T G G C A T T G C T T C C T T C T G C C C T G G C C T A	121
VvGLP3	AAAGGTGTTTGTTC TGTAACTG T C C C T C T G G C T T G C T T C C T C T C T G C C C T G G C C T C	119
VvGLP4	AAGGGTGTTAGTTTCC GGTAACTG T T C C C T G T G G C A T T G T T C C T C T C T G C C C T G G C C T C	131
VvGLP2	ACCATGGCGGCTGTTG GGCCTTGT T T T A T C A C A C T C G C T T C T C T C G G C A C C G T T C C T G C	195
VvGLP6	AACATTCTTCTTTATA TTGGTGAAGATGTTTCTCCCAATCTCGCATCTTTCCCTATCTTA	66
VvGLP1	-----TAACTGCGCCCTCATGGCAATGGCTTCATCTTTGGCTCTGGCTC	46
VvGLP5	GACTAACAATGAAGTG TAGTTTCCG G C C C T C A T G G A A T T G C T T C C T C T C T G C C C T C G C C T T	65
VvGLP7	AGAGGCGTTAGTTTCC GGTAAACCG G C C T T C A T G G C A T T G C T T C C T T C T G C C C T G G C C T A	121
VvGLP3	AAAGGTGTTTGTTC TGTAACTG T C C C T C T G G C T T G C T T C C T C T C T G C C C T G G C C T C	119
VvGLP4	AAGGGTGTTAGTTTCC GGTAACTG T T C C C T G T G G C A T T G T T C C T C T C T G C C C T G G C C T C	131
VvGLP2	ACCATGGCGGCTGTTG GGCCTTGT T T T A T C A C A C T C G C T T C T C T C G G C A C C G T T C C T G C	195
VvGLP6	AACATTCTTCTTTATA TTGGTGAAGATGTTTCTCCCAATCTCGCATCTTTCCCTATCTTA	66
VvGLP1	-----TAACTGCGCCCTCATGGCAATGGCTTCATCTTTGGCTCTGGCTC	46
VvGLP5	GACTAACAATGAAGTG TAGTTTCCG G C C C T C A T G G A A T T G C T T C C T C T C T G C C C T C G C C T T	65
VvGLP7	AGAGGCGTTAGTTTCC GGTAAACCG G C C T T C A T G G C A T T G C T T C C T T C T G C C C T G G C C T A	121
VvGLP3	AAAGGTGTTTGTTC TGTAACTG T C C C T C T G G C T T G C T T C C T C T C T G C C C T G G C C T C	119
VvGLP4	AAGGGTGTTAGTTTCC GGTAACTG T T C C C T G T G G C A T T G T T C C T C T C T G C C C T G G C C T C	131
VvGLP2	ACCATGGCGGCTGTTG GGCCTTGT T T T A T C A C A C T C G C T T C T C T C G G C A C C G T T C C T G C	195
VvGLP6	AACATTCTTCTTTATA TTGGTGAAGATGTTTCTCCCAATCTCGCATCTTTCCCTATCTTA	66
VvGLP1	-----TAACTGCGCCCTCATGGCAATGGCTTCATCTTTGGCTCTGGCTC	46
VvGLP5	GACTAACAATGAAGTG TAGTTTCCG G C C C T C A T G G A A T T G C T T C C T C T C T G C C C T C G C C T T	65
VvGLP7	AGAGGCGTTAGTTTCC GGTAAACCG G C C T T C A T G G C A T T G C T T C C T T C T G C C C T G G C C T A	121
VvGLP3	AAAGGTGTTTGTTC TGTAACTG T C C C T C T G G C T T G C T T C C T C T C T G C C C T G G C C T C	119
VvGLP4	AAGGGTGTTAGTTTCC GGTAACTG T T C C C T G T G G C A T T G T T C C T C T C T G C C C T G G C C T C	131
VvGLP2	ACCATGGCGGCTGTTG GGCCTTGT T T T A T C A C A C T C G C T T C T C T C G G C A C C G T T C C T G C	195
VvGLP6	AACATTCTTCTTTATA TTGGTGAAGATGTTTCTCCCAATCTCGCATCTTTCCCTATCTTA	66
VvGLP1	-----TAACTGCGCCCTCATGGCAATGGCTTCATCTTTGGCTCTGGCTC	46
VvGLP5	GACTAACAATGAAGTG TAGTTTCCG G C C C T C A T G G A A T T G C T T C C T C T C T G C C C T C G C C T T	65
VvGLP7	AGAGGCGTTAGTTTCC GGTAAACCG G C C T T C A T G G C A T T G C T T C C T T C T G C C C T G G C C T A	121
VvGLP3	AAAGGTGTTTGTTC TGTAACTG T C C C T C T G G C T T G C T T C C T C T C T G C C C T G G C C T C	119
VvGLP4	AAGGGTGTTAGTTTCC GGTAACTG T T C C C T G T G G C A T T G T T C C T C T C T G C C C T G G C C T C	13

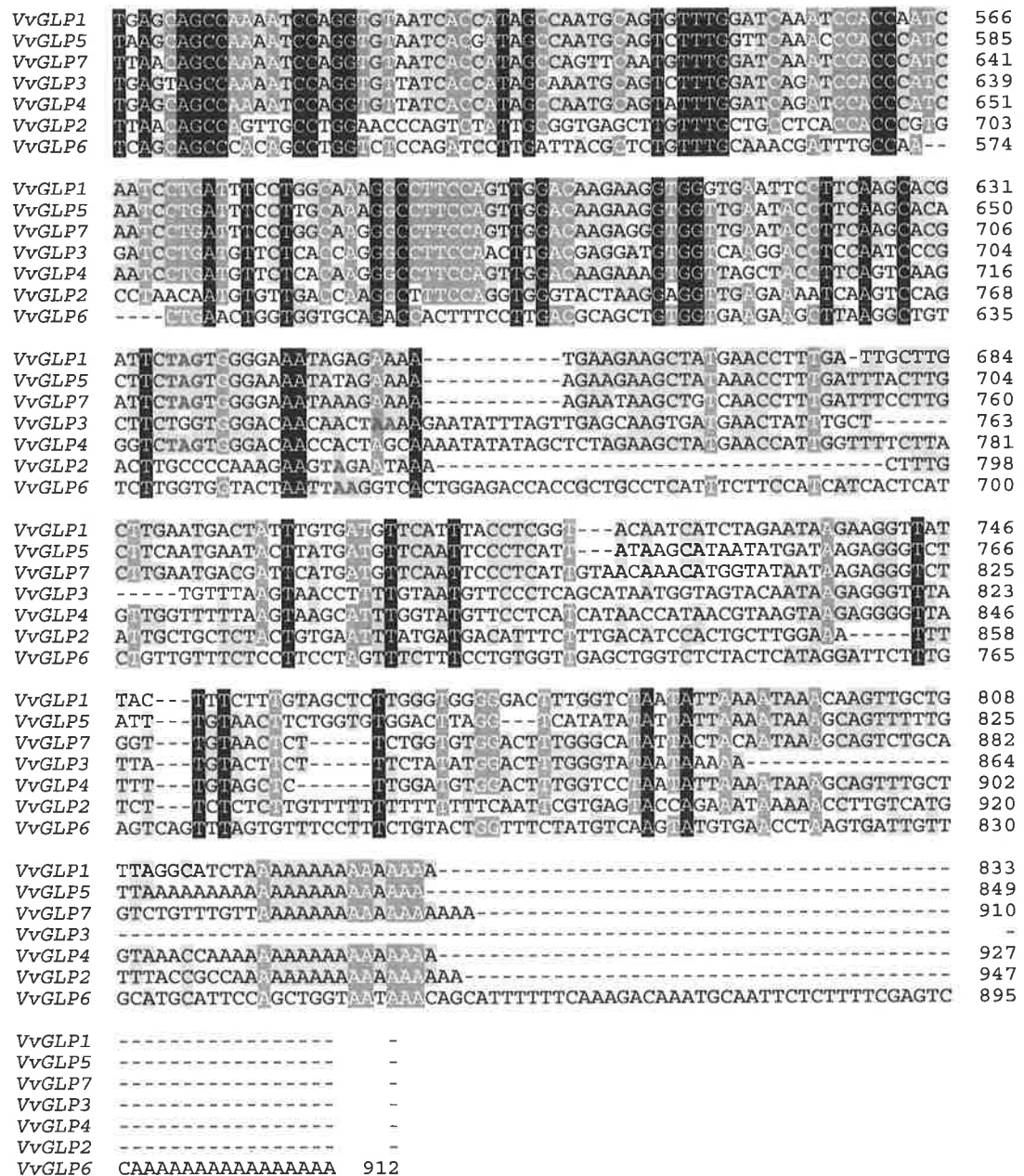


Figure 2.4. Nucleotide alignment of the *VvGLP* cDNA clones. Full-length sequences were joined together from 3' and 5' RACE clones. Sequences were aligned using the GCG WebANGIS Wisconsin software package program PileUp (GCG) and displayed by the GenDoc program. Dashes indicate introduced gaps. Shading represents the degree of sequence identity at each nucleotide position: nucleotides conserved between sequences are shaded in black, dark grey shading indicates conserved nucleotides in six out of seven of the sequences and light grey shading indicates conserved nucleotides in four or five of the sequences. Putative start and stop codons are shown in red.

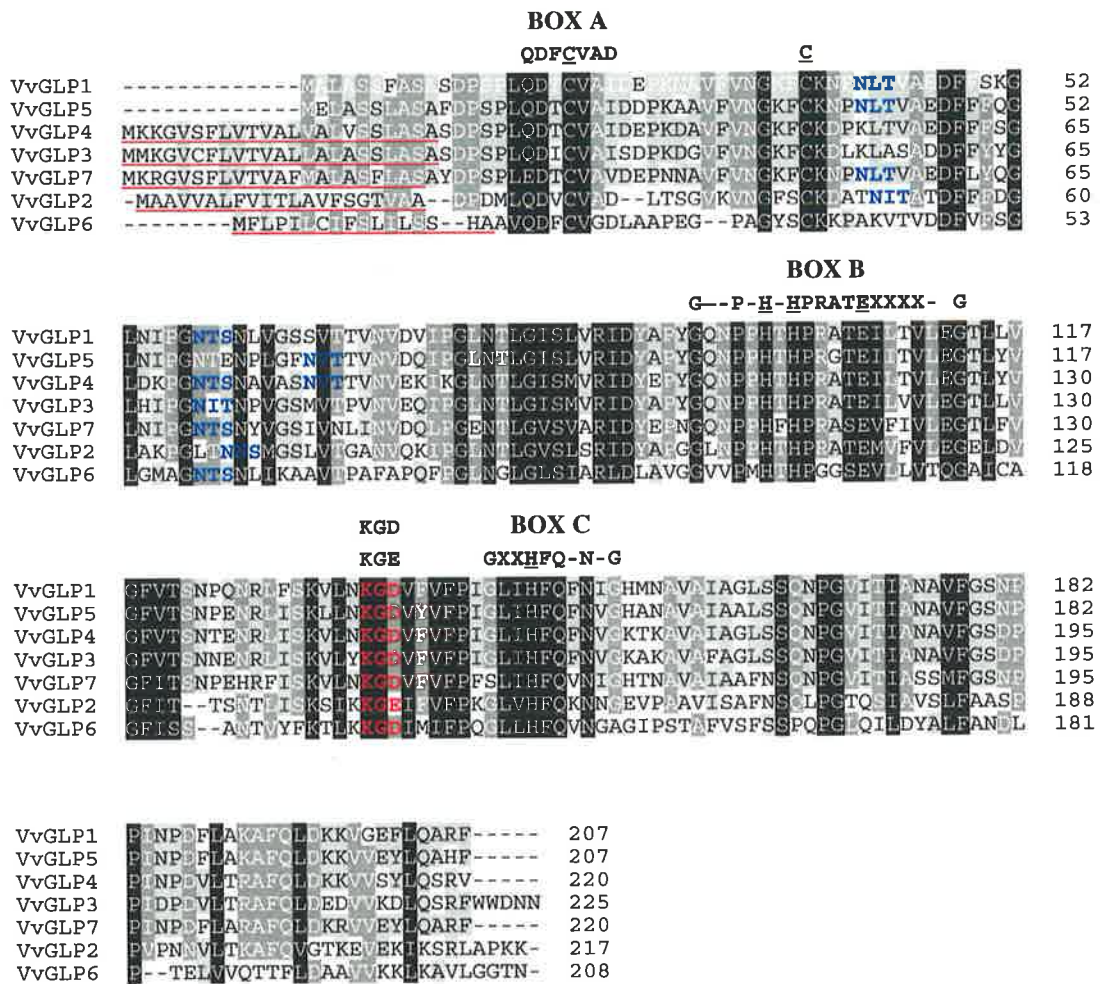
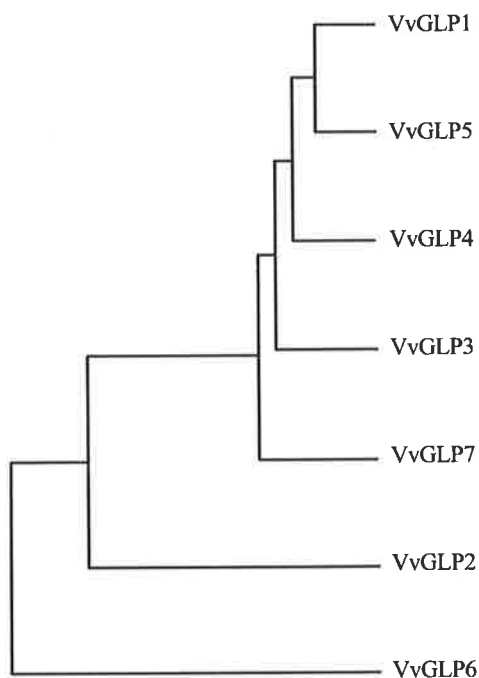


Figure 2.5. Deduced amino acid alignment of the VvGLPs. Alignment based on pairwise similarity using the GCG WebANGIS Wisconsin software package program PileUp (GCG) and displayed by the GenDoc program. Shading represents conservation of amino acid similarity at each position: residues conserved between sequences are shaded in black, dark grey shading indicates similar residues in six out of seven of the sequences and light grey shading indicates similar residues in four or five out of the seven sequences. Dashes indicate introduced gaps. Putative N-terminal signal sequences are underlined in red. The conserved germin motifs, boxes A, B and C, are indicated with the glutamate and the three histidines involved in metal binding and the conserved cysteine residues underlined. Putative glycosylation sites are shown in blue. RGD-like tripeptides (KGD/E) are shown in red.

A



B

VvGLP1							
VvGLP2	52.2						
VvGLP3	77.8	50.5					
VvGLP4	83.2	52.6	82.8				
VvGLP5	87.5	52.2	76.9	81.7			
VvGLP6	36.3	37.8	38.3	37.3	37.5		
VvGLP7	77.4	47.9	66.5	72.4	76.0	34.8	
	VvGLP1	VvGLP2	VvGLP3	VvGLP4	VvGLP5	VvGLP6	VvGLP7

Figure 2.6. Sequence similarity between sequences of VvGLPs. (A) Phylogenetic tree constructed using the GCG WebANGIS Wisconsin software package program PileUp. (B) Percent identity calculated using the GCG WebANGIS Wisconsin software package program Gap (GCG).

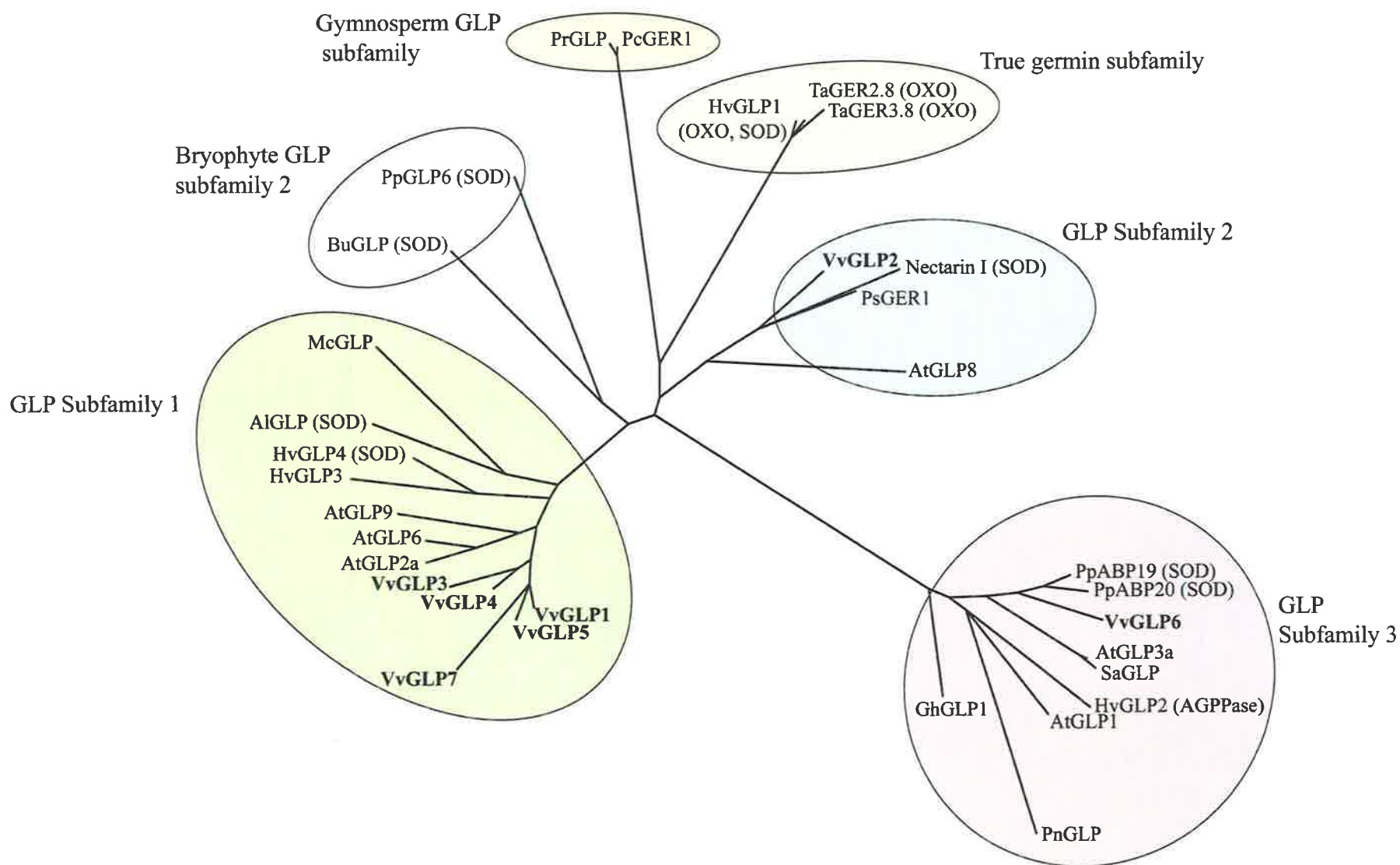


Figure 2.7. Unrooted phylogenetic tree of GLPs. Alignment of deduced amino acid sequences was based on pairwise similarity using ClustalW and ProtDist was used to calculate distance matrix to build a Neighbor Joining unrooted tree with Phylip. Branches representing the germin and GLP subfamilies are indicated. Where functional activity has been demonstrated by functional assay it is indicated: OXO (oxalate oxidase activity), SOD (superoxide dismutase), AGPPase (adenosine diphosphate glucose pyrophosphatase activity). The VvGLPs are shown in bold. The NCBI RefSeq/GenBank protein ID of the sequences used for the analysis in this study were AlGLP, BAA78563; AtGLP1, AAB51584; AtGLP2a, AAB51570; AtGLP3a, AAB51566; AtGLP6, AAB51572; AtGLP8, AAB51585; AtGLP9, NP_193199; BuGLP, BAC53790; GhGLP1, AAF21988; HvGLP1, CAA74595; HvGLP2, CAA75907; HvGLP3, AAG00426; HvGLP4, CAA63659; McGLP, AAA33030; Nectarin I, AAF03355; PcGER1, AAC99473; PnGLP, BAA08266; PpABP19, AAD00295; PpABP20, AAB51240; PpGLP6, BAD86502; PrGLP, AAC05146; PsGER1, CAB65369; SaGLP, CAA59257; TaGER2.8, AAA34270; TaGER3.8, AAA34271.

2.3.2.2 *P. tricuspidata* GLP genes

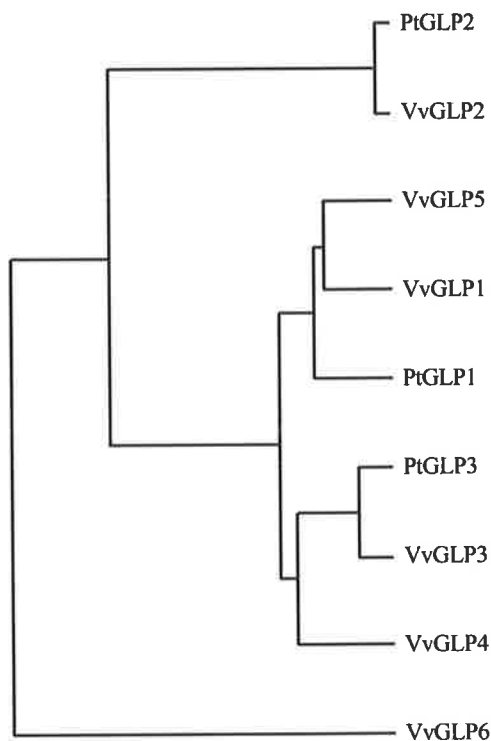
The altered development of *E. necator* on *M. rotundifolia*, *P. tricuspidata* and *C. antarctica*, compared to *V. vinifera*, indicated penetration was less successful on these Vitaceae family members (Fig. 2.3B). Furthermore, papillae were associated with failed penetration, suggesting these species exhibit enhanced penetration resistance. Consequently, it was of interest to determine the identity of GLPs within members of the Vitaceae family, which showed enhanced penetration resistance, and whether the expression of these genes could account for the variation in penetration success.

As visible papillae were most prevalent in *P. tricuspidata*, 24 hai with *E. necator*, attempts were made to isolate GLP genes from this species. A degenerate approach, using the degenerate grapevine primers, OXOLP1 and OXOLP2 (Appendix 1), originally used to clone the partial length fragments, VvL2 and VvL3, from grapevine proved unsuccessful (data not shown). However efforts to clone *P. tricuspidata* GLPs using gene-specific primers designed to *V. vinifera* GLP sequences proved more successful. Three partial length sequences were PCR amplified from *P. tricuspidata* leaf cDNA, 24 hai with *E. necator*, using *V. vinifera* primers as follows; *PtGLP1* (OXL5F and OxL3R), *PtGLP2* (OXL2Fb and B26) and *PtGLP3* (VvGLP3Fb and GLP3Ra) (Appendix 1; 2).

The partial length sequences of *PtGLP1*, *PtGLP2* and *PtGLP3* were 120, 644 and 488 bp, respectively, and encoded translation products of 40, 163 and 162 amino acids, respectively (Appendix 3). Comparison of the deduced amino acid sequences of *P. tricuspidata* and *V. vinifera* GLPs (Fig. 2.8 A & B) shows that *PtGLP1* shares the

highest amino acid sequence identity to VvGLP1 (90%), PtGLP2 to VvGLP2 (97%), and PtGLP3 to VvGLP3 (92%) (Fig. 2.8B). BLASTp searches indicate that PtGLP1 and PtGLP3 are most closely related (82% and 73% identity of amino acids, respectively) to *A. thaliana* GLPs (GenBank protein ID BAB10834 and BAB10832, respectively). These *Arabidopsis* genes were cloned from a genomic library (Asamizu *et al.* 1998), and there is no expression data available for these genes. PtGLP2 is 83% identical to *N. attenuata* (GenBank protein ID AAR97545).

A



B

PtGLP1	90.0	62.5	77.5	77.5	85.0	50.0	75.0
PtGLP2	55.6	96.9	54.6	58.1	55.6	39.1	51.9
PtGLP3	79.6	53.8	92.0	82.1	77.8	41.8	69.8
	VvGLP1	VvGLP2	VvGLP3	VvGLP4	VvGLP5	VvGLP6	VvGLP7

Figure 2.8. Deduced amino acid sequence similarity between sequences of PtGLPs and VvGLPs. (A) Phylogenetic tree constructed using the GCG WebANGIS Wisconsin software package program PileUp (B) Percent amino acid identity calculated using the GCG WebANGIS Wisconsin software package program Gap (GCG).

2.3.3 Expression analysis of grape *GLP* genes

For semi-quantitative RT-PCR analysis, amplification reactions were performed with DNase-treated RNA and using a *GLP* gene-specific forward primer in combination with an oligo(dT)-adapter primer (B26). As the oligo(dT)-adapter primer binds to the poly(A) tail of mRNA, the possibility of amplifying any contaminating genomic DNA was prevented. A 403 bp *VvGLP1* fragment was amplified using OXL1Fb (Appendix 1); a 692 bp *VvGLP2* fragment using OXL2Fb (Appendix 1); a 701 bp *VvGLP3* fragment using *VvGLP3Fb* (Appendix 1); a 269 bp *VvGLP4* fragment using OXL4F (Appendix 1); a 661 bp *VvGLP5* fragment using GLP5Fc (Appendix 1); a 461 bp *VvGLP6* fragment using *VvGLP6F* (Appendix 1) and a 697 bp *VvGLP7* fragment using GLP7Fa (Appendix 1). The concentrations of total RNA used in each RT-PCR reaction were verified by carrying out RT-PCR reactions on each of the samples using 18S rRNA primers. RT-PCR results were confirmed by performing repeat reactions with identical conditions.

In some cases, Northern Blot analysis was also used to detect *VvGLP3* transcript levels using a 215 bp PCR fragment probe amplified from *VvGLP3* cDNA sequence using OXL3Fb and B26 (Appendix 1). As the nucleotide identity between the *VvGLP3* probe sequence and the *VvGLP1*, *VvGLP2*, *VvGLP4*, *VvGLP5*, *VvGLP6* and *VvGLP7* sequences is 57, 44, 68, 60, 41 and 58% respectively, there is unlikely to be any significant cross-hybridisation between the *VvGLP3* probe and the other *VvGLP* gene transcripts under high stringency washing conditions. Equality of RNA loadings was confirmed by re-hybridising membranes with an 18S ribosomal RNA cDNA probe.

2.3.3.1 Differential expression of *VvGLP* genes in response to *E. necator* infection

Neither *VvGLP1* nor *VvGLP7* appeared to be expressed in either healthy uninfected leaves or berries (skin and pulp) or induced in these tissues by *E. necator* (Fig. 2.9). In contrast, both *VvGLP2* and *VvGLP6* were expressed in both leaves and berries (skin and pulp). *VvGLP2* expression was maintained during infection, whereas the *VvGLP6* transcript appeared to decrease in both leaves and berries with increasing level of infection. *VvGLP5* was not expressed in healthy uninfected leaves and appeared to show only low level expression in berry skin. However, *VvGLP5* transcript accumulated strongly in the berry pulp. As with *VvGLP6* expression, *VvGLP5* expression in the berry pulp appeared to be significantly down-regulated in response to *E. necator* infection.

Neither *VvGLP3* nor *VvGLP4* were expressed in either healthy uninfected leaves or berries. However, leaves and berries infected with *E. necator* exhibited a strong accumulation of these transcripts (Fig. 2.9). *VvGLP3* and *VvGLP4* transcripts were detected at low levels in leaves with mild infection (less than 20% hyphal coverage) and increased in leaves with severe infection (greater than 20% hyphal coverage). *VvGLP3* and *VvGLP4* transcripts also accumulated in powdery mildew-infected berries, but were only observed within the skin tissue, indicating that these genes may be specifically expressed in infected epidermal cells.

To confirm the results obtained with RT-PCR demonstrating the inducibility of *VvGLPs* in *E. necator*-infected samples, Northern analysis was performed using a probe specific to *VvGLP3* (Fig. 2.10). The pattern of transcript accumulation mirrored that observed using RT-PCR analysis with a *VvGLP3* gene-specific primer (Fig. 2.9). *VvGLP3* was

not expressed in either healthy uninfected leaves (approximately 6 cm diameter) or berries (8 weeks post capfall). Furthermore, *VvGLP3* transcript was present at low levels in leaves with mild *E. necator* infection and exhibited strong accumulation in leaves with severe infection, and in berries with both mild and severe *E. necator* infection. Northern analysis also confirmed that *VvGLP3* induction was observed specifically within the berry skin and not the pulp tissue (Fig. 2.10).

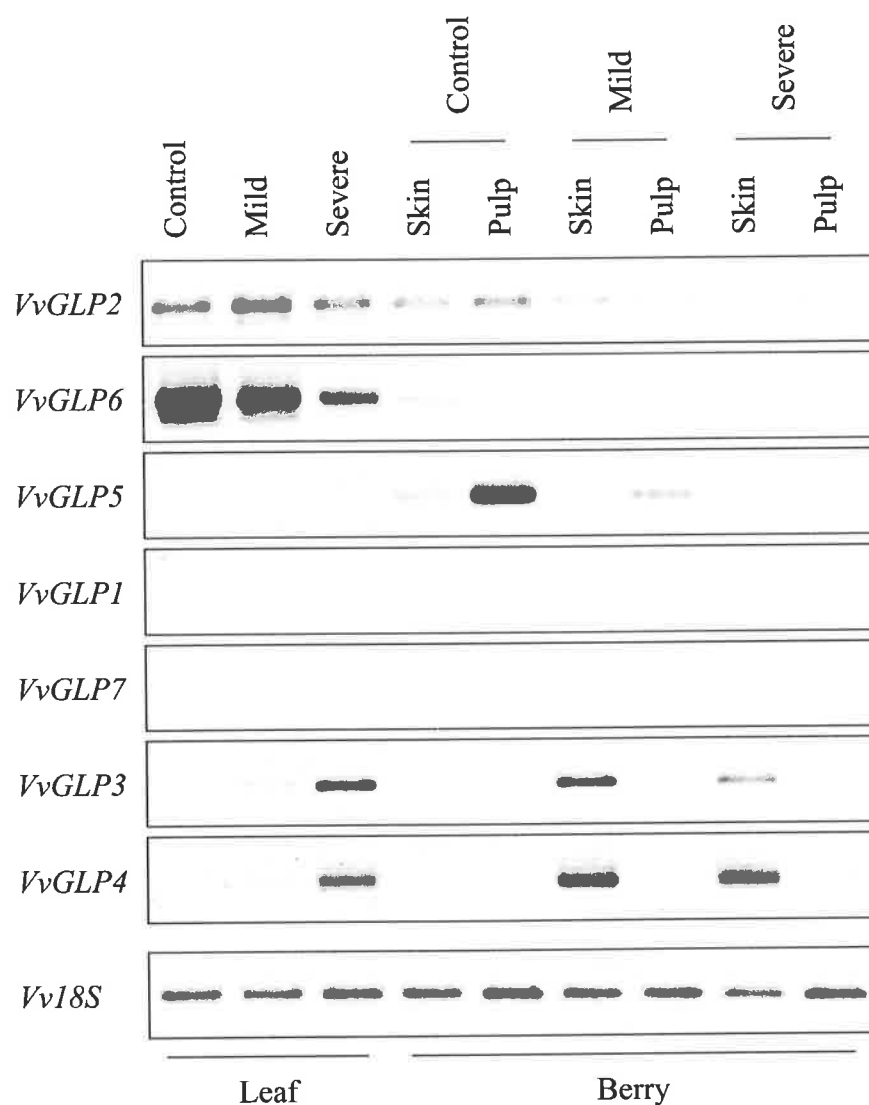


Figure 2.9. Accumulation of *VvGLP* transcripts in response to *E. necator* infection. Total RNA was extracted from Chardonnay leaves (approximately 6 cm in diameter) and berries (8 weeks post capfall) at different stages of infection: control (healthy), mild

infection (less than 20% of the surface was covered in hyphae) and severe infection (greater than 20% coverage). Berries were separated into skin and pulp components prior to RNA extraction. The transcripts were detected by RT-PCR analysis a gene-specific primer in combination with the oligo(dT) adapter primer (B26). The concentrations of total RNA used in each RT-PCR reaction were verified by carrying out RT-PCR reactions on each of the samples using 18S rRNA primers. The RT-PCR products were separated on agarose gels and stained with ethidium bromide. The negative image is shown for greater resolution.

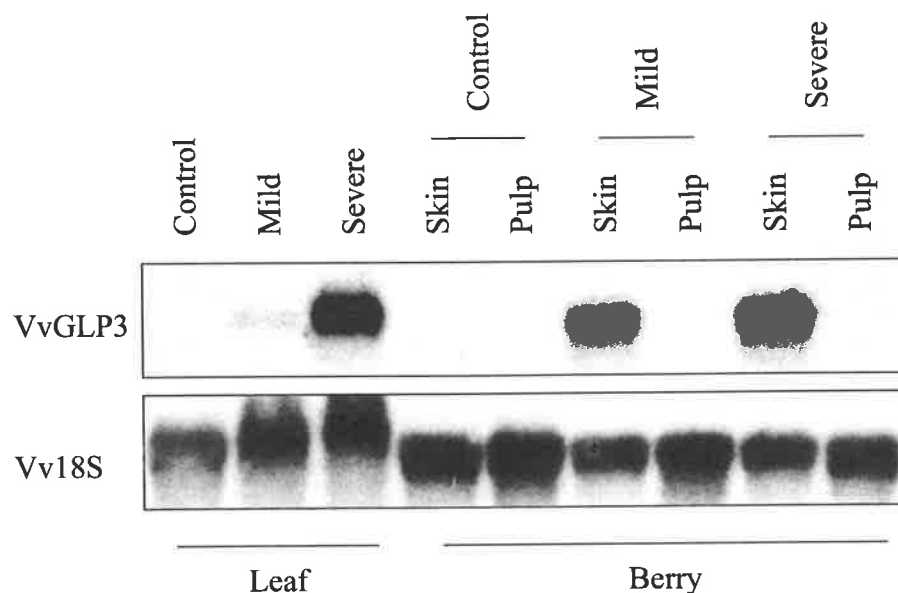


Figure 2.10. Accumulation of *VvGLP3* transcript in response to *E. necator* infection. Total RNA was extracted from healthy Chardonnay leaves (approximately 6 cm in diameter) and berries (8 weeks post capfall) at different stages of infection: control (healthy), mild infection (less than 20% of the surface was covered in hyphae) and severe infection (greater than 20% coverage). Berries were separated into skin and pulp components prior to RNA extraction. The transcripts were detected by Northern analysis with a *VvGLP3* gene-specific probe. Equality of RNA loadings was confirmed by re-hybridising the membrane with an 18S rRNA cDNA probe.

2.3.3.2 Localised induction of *VvGLP3* in *E. necator* -infected tissues

In order to determine if powdery mildew induced *VvGLP* gene expression was localised to the sites of powdery mildew infection or systemic within the infected leaf, regions of visible powdery mildew infection (infected) were excised and sampled separately from

areas on the same leaf, which were remote from the obvious infection (remote) as shown in Fig. 2.1. Accumulation of the powdery mildew-inducible *VvGLP3* transcript was found to be restricted to regions of visible infection and was not systemically induced within the infected leaf in regions physically remote from the site of powdery mildew infection (Fig. 2.11).

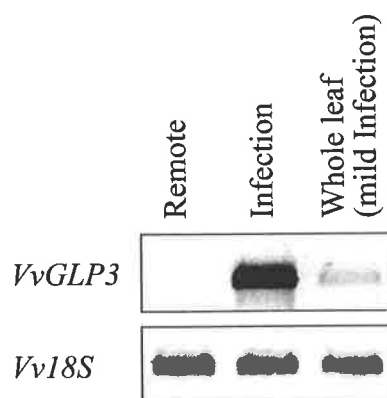


Figure 2.11. Localisation of *VvGLP3* transcript relative to *E. necator* infection. Glasshouse-grown Chardonnay leaves (6 cm diameter) were inoculated with *E. necator* conidia. Regions of visible powdery mildew infection (infected) were excised and sampled separately from areas on the same leaf which were remote from the obvious infection (remote). Total RNA was extracted from infected and remote regions on the same leaf, and from whole leaves displaying mild infection (less than 20% hyphal coverage). *VvGLP3* transcripts were detected by RT-PCR analysis with a gene-specific primer in combination with an oligo(dT) adapter primer (B26). The concentrations of total RNA used in each RT-PCR reaction were verified by carrying out RT-PCR reactions on each of the samples using 18S rRNA primers. The RT-PCR products were separated on agarose gels and stained with ethidium bromide. The negative image is shown for greater resolution.

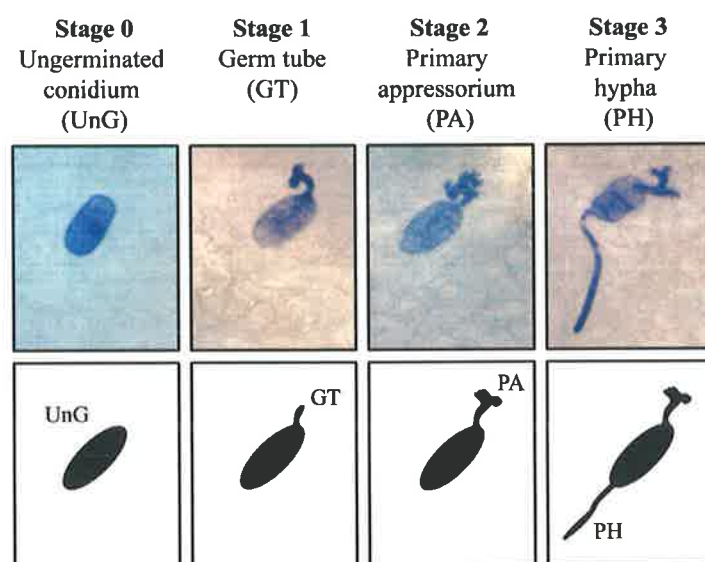
2.3.3.3 Time course of *VvGLP3* induction in response to *E. necator* infection

Having established that *VvGLP3* is induced in response to powdery mildew infection, the aim of this study was to attempt to establish at what stage of *E. necator* infection *VvGLP3* transcription commenced. The stages of early *E. necator* development (stage 0,

ungerminated; stage 1, germ tube, stage 2, primary appressorium; stage 3, primary hyphae; Fig 2.12A) of 100 conidia were determined for each sampling time post-inoculation as shown in Figure 2.12B. Within 4 hai approximately 30% of conidia had germinated. Within 12 hai most of the germinated conidia had a primary appressorium, indicating the timing of attempted penetration. The formation of primary hyphae, an indicator of successful haustorium formation within epidermal cells, was observed 24 hai. By 48 hai most of the germinated conidia had formed primary hyphae.

Figure 2.13 shows that *VvGLP3* transcript began accumulating between 12 and 24 hai and increased over the 72 h time course. Thus, the timing of induction of *VvGLP3* transcript coincides with the timing of penetration of the epidermal cells. No induction of *VvGLP3* transcript was observed in un-inoculated detached leaves.

A



B

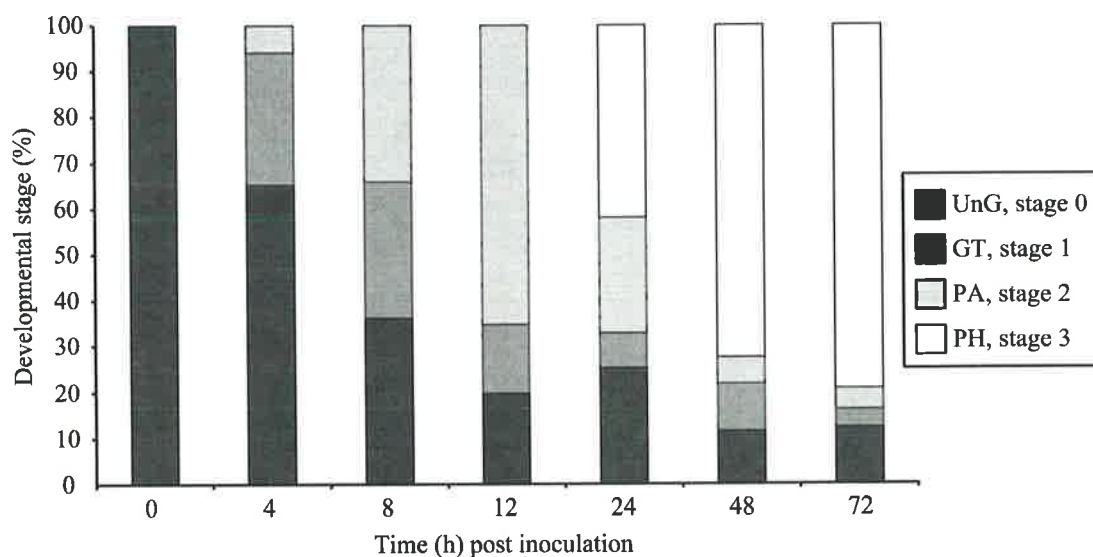


Figure 2.12. Development of *E. necator* on detached Chardonnay leaves. (A) Sequence of development. The characteristic infection structure of each stage is indicated. (B) Frequency of developmental stages (%) of *E. necator*. Detached glasshouse-grown leaves (6 cm diameter) were inoculated with *E. necator* conidia. Samples were collected at 0, 4, 8, 12, 24, 48 and 72 h post inoculation and examined at each time point for development stage of fungal infection. The developmental stages of 100 conidia at each time point were recorded.

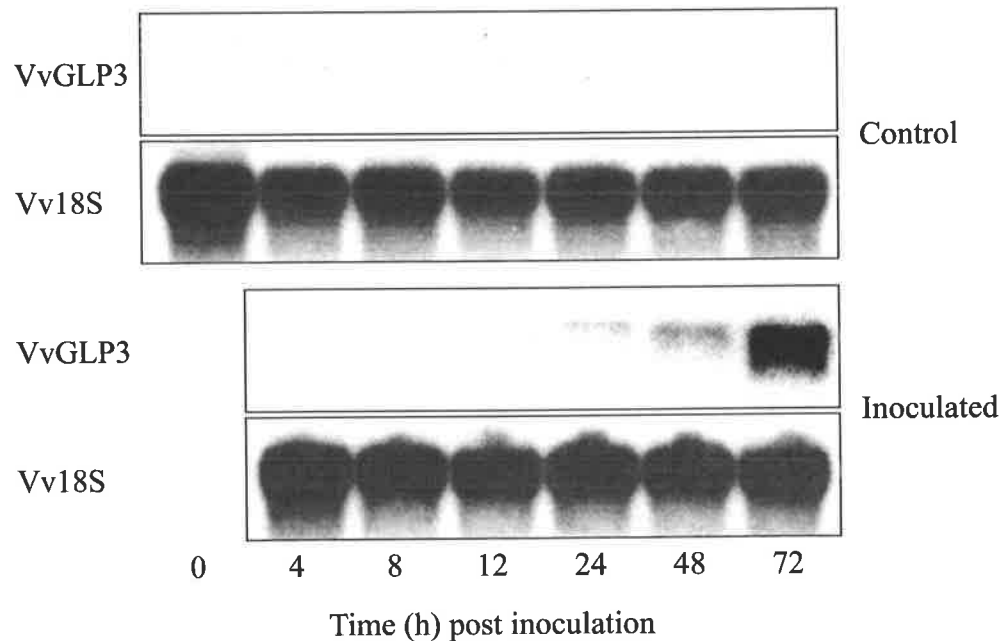


Figure 2.13. Time course of *VvGLP3* induction by *E. necator*. Detached glasshouse-grown Chardonnay leaves (6 cm diameter) were inoculated with *E. necator* conidia. Samples were collected at 0, 4, 8, 12, 24, 48 and 72 h post inoculation. Total RNA was extracted and transcripts were detected by Northern analysis with a *VvGLP3* gene-specific probe. Equality of RNA loadings was confirmed by re-hybridising the membrane with an 18S rRNA cDNA probe.

2.3.3.4 Response of *P. tricuspidata* GLP genes to *E. necator* infection

As papillae formation, in response to *E. necator* infection, is greatly enhanced in *P. tricuspidata* compared with *V. vinifera* 24 hai (Fig. 2.3B) it was of interest to examine GLP gene induction in *P. tricuspidata*. Young glossy leaves of potted glasshouse vines were tagged and inoculated with *E. necator* conidia. As *E. necator* does not complete its life cycle on *P. tricuspidata* it was not possible to sample tissue with established infection. Consequently, control and inoculated leaves were sampled at 24 hai when *E. necator* is attempting to infect.

Using RT-PCR analysis with a *GLP* gene-specific primer in combination with an oligo(dT) adapter primer (B26) *PtGLP1*, *PtGLP2* and *PtGLP3* transcripts were not detected in healthy uninoculated leaves nor did they accumulate in leaves inoculated with *E. necator* (data not shown). These results suggest that these *PtGLPs* are either expressed at very low levels or that the original clones may have been amplified from genomic contamination. Consequently, the remainder of this thesis focussed on the characterisation of *V. vinifera GLPs* alone.

2.3.3.5 Differential expression of *VvGLP* genes in response to *P. viticola* and *B. cinerea*

The aim of this experiment was to determine if induction of *VvGLP3* and *VvGLP4* is specific for *E. necator* infection, or whether they are also induced by other grapevine pathogens such as *P. viticola* or *B. cinerea*. RT-PCR analysis using a *GLP* gene-specific primer in combination with an oligo(dT) adapter primer (B26) was used to determine the expression of *VvGLPs* in response to *P. viticola* and *B. cinerea* infection (Fig. 2.14). When the expression of a particular *VvGLP* gene was not detected in any of the tissues examined, a positive control RNA was included to verify reaction conditions. Induction of a thaumatin-like protein *VvTI2* (Davies and Robinson 2000; accession number AJ237998) was used as a positive control for *P. viticola* and *B. cinerea* infection (Fig. 2.14). A *VvTI2* fragment was amplified using the gene-specific primer VvTL2F and B26 (Appendix 1). *VvTI2* was expressed at low levels in the control leaves and was markedly induced in response to mild and severe *P. viticola* infection and in tissue surrounding the *B. cinerea* lesion.

Significantly, the *E. necator* inducible GLPs, *VvGLP3* and *VvGLP4*, did not appear to be induced in leaves in response to *B. cinerea* infection. Nor was *VvGLP3* expressed in leaves infected with *P. viticola*. However, *VvGLP4* did show a low level of induction in leaves infected with *P. viticola*.

As shown in Fig. 2.9, *VvGLP2* and *VvGLP6* are expressed in healthy non-infected grape leaf tissue. *VvGLP2* appeared to be upregulated by *P. viticola* infection, but not by *B. cinerea* infection. In contrast, expression of *VvGLP6* appeared to decrease in response to both *P. viticola* infection and *B. cinerea* infection.

VvGLP1 and *VvGLP7* were not expressed in healthy non-infected grape leaf tissue and showed quite different responses to infection by *B. cinerea* and *P. viticola* (Fig. 2.14). *VvGLP1* was not detected in leaves infected with *B. cinerea* or in leaves mildly infected with *P. viticola*. However, *VvGLP1* transcript was detected in leaves which were severely infected with *P. viticola*. Interestingly, *VvGLP7* expression was induced in response to infection of leaves by both grape pathogens. *VvGLP5* was not expressed in healthy uninfected grape leaf tissue, nor was it induced in response to infection with *B. cinerea* or *P. viticola*.

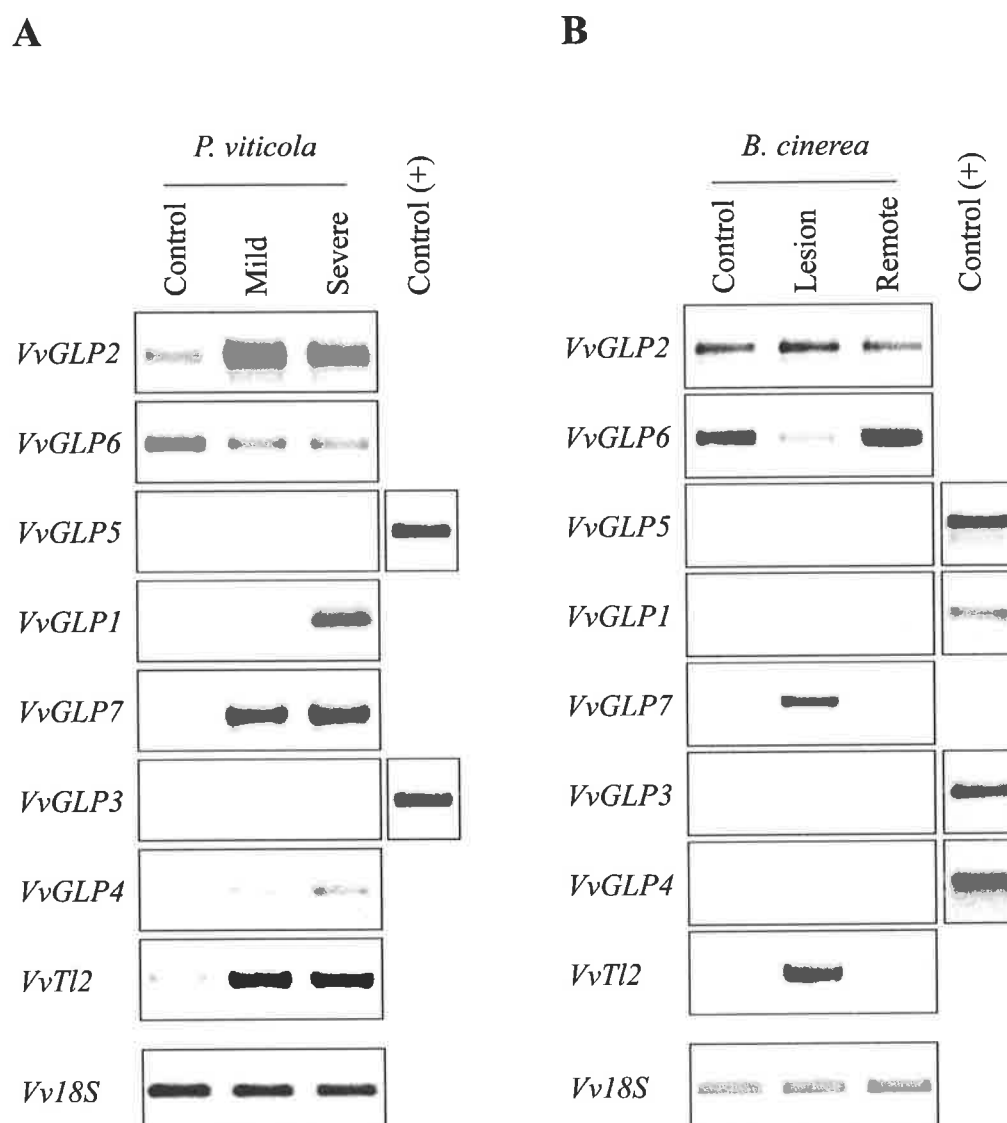


Figure 2.14. Expression of VvGLPs in response to infection by *P. viticola* and *B. cinerea*. Total RNA was extracted from control non-infected leaves, (A) from leaves with mild *P. viticola* infection (12 d post inoculation) and severe *P. viticola* infection (20 d post inoculation), (B) from regions of grapevine leaves containing *B. cinerea* lesions (lesion) and from tissue remote (remote) to but on the same leaf as the *B. cinerea* lesion. Transcripts were detected by RT-PCR analysis with a gene-specific primer in combination with an oligo(dT) adapter primer (B26). When the expression of a particular VvGLP gene was not detected in any of the tissues examined, a positive control RNA (shown in separate boxes) was included to verify reaction conditions as follows: VvGLP1, severe downy mildew leaf infection (20 d post inoculation); VvGLP3 and VvGLP4, severe powdery mildew leaf infection (greater than 20% of the surface was covered in hyphae); VvGLP5, healthy berry pulp (8 weeks post capfall). The concentrations of total RNA used in each RT-PCR reaction was verified by carrying out RT-PCR reactions on each of the samples using 18S rRNA primers. The RT-PCR products were separated on agarose gels and stained with ethidium bromide. The negative image is shown for greater resolution.

2.3.3.6 Expression of *VvGLP* genes in response to wounding

As several of the *VvGLP* genes were induced in response to fungal infection, experiments were carried out to determine if an abiotic stress, such as wounding, would also result in induction of these genes. Glasshouse-grown Chardonnay leaves (approximately 6 cm diameter) were treated with sandpaper to simulate wounding of the epidermal tissue.

The expression of *VvChi4a* (Accession number U97521), a pathogenesis related class IV acid chitinase, was used as a positive control for wounding treatment in grape tissues. A 1020 bp *VvChi4a* fragment was amplified using WHP5F and B26 (Appendix 1). *VvChi4a* was not expressed in the control leaves at any of the time points sampled or in the wounded leaves at 0 h post-wounding. Accumulation of *VvChi4a* transcript was detected at 4 h post-wounding and appeared to increase at 8 and 24 h post-wounding.

None of the pathogen-inducible forms, that is, *VvGLP1*, *VvGLP3*, *VvGLP4* or *VvGLP7* were found to be induced in response to the wounding treatment (Fig. 2.15), nor was there any clear effect of wounding on *VvGLP6* expression. In contrast, expression of *VvGLP2* appeared to be upregulated by the wounding treatment.

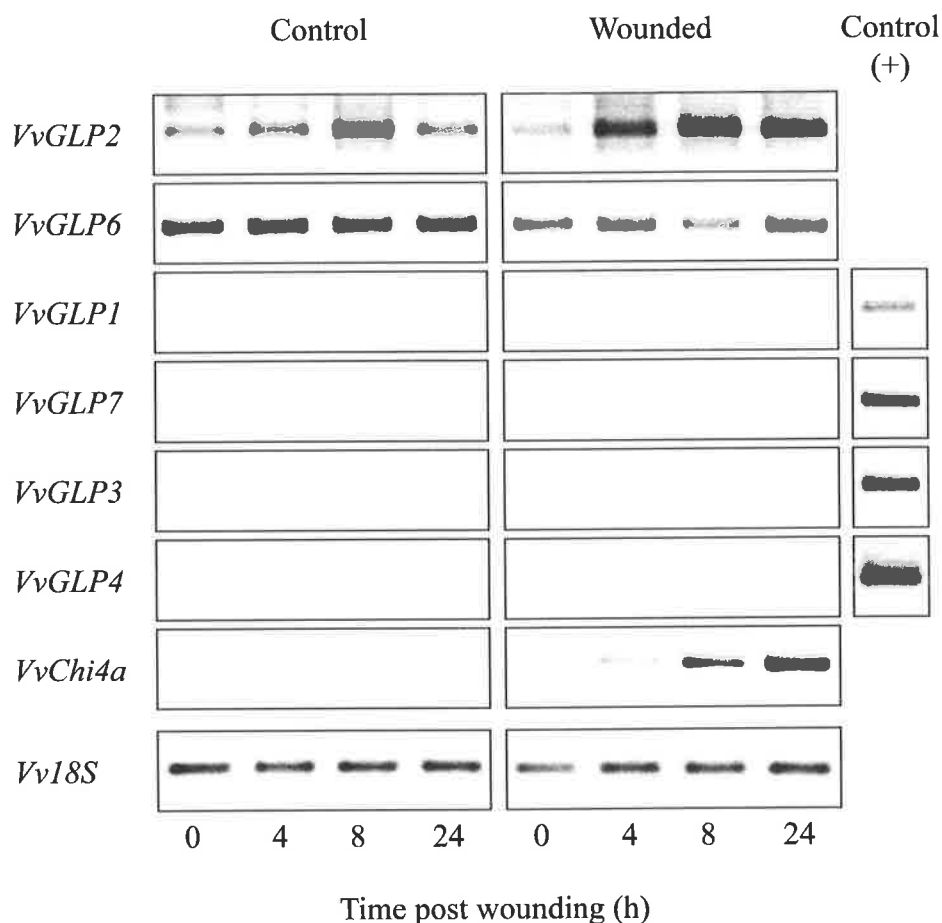


Figure 2.15. Expression of *VvGLPs* in response to wounding. Total RNA was extracted from Chardonnay leaves (approximately 6 cm diameter) 0, 4, 8 and 24 h post wounding with fine sandpaper. Transcripts were detected by RT-PCR analysis with a gene-specific primer in combination with an oligo(dT) adapter primer (B26). When the expression of a particular *VvGLP* gene was not detected in any of the tissues examined, a positive control RNA (shown in separate boxes) was included to verify reaction conditions as follows: *VvGPL1* and *VvGLP7*, severe downy mildew leaf infection (20 d post inoculation); *VvGLP3* and *VvGLP4*, severe powdery mildew leaf infection (greater than 20% of the surface was covered in hyphae). The concentrations of total RNA used in each RT-PCR reaction were verified by carrying out RT-PCR reactions on each of the samples using 18S rRNA primers. The RT-PCR products were separated on agarose gels and stained with ethidium bromide. The negative image is shown for greater resolution.

2.3.3.7 Expression of *VvGLP* genes in response to plant hormones and growth regulators

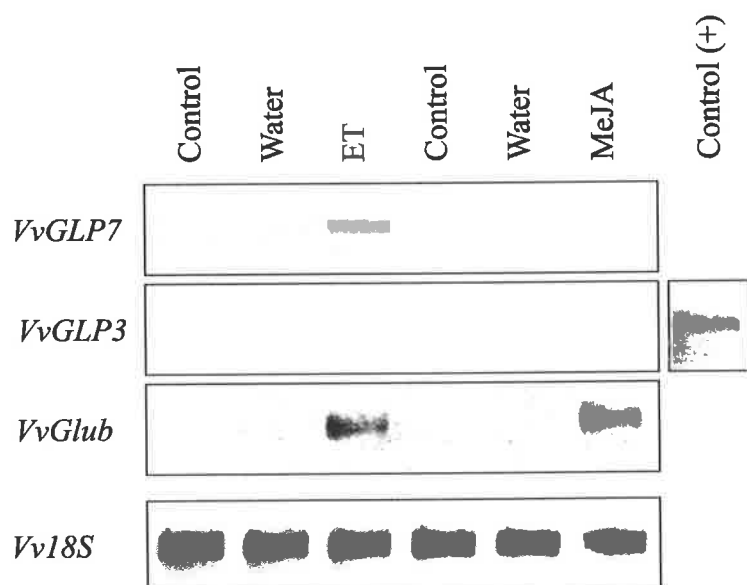
The expression of the pathogen-inducible grapevine *GLP* genes, *VvGLP3* and *VvGLP7* (Fig. 2.9; 2.14), in response to treatment with various plant hormones and growth regulators was investigated in an attempt to determine possible roles of signalling molecules involved in the induction of these genes. Chardonnay leaves were treated with ethylene (ET), methyl jasmonate (MeJA), salicylic acid (SA), abscisic acid (ABA) and the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D).

Induction of *VvGlub* (accession number AF053750), a pathogenesis-related basic β -1,3-glucanase, was used as a positive control for the ethephon and methyl jasmonate treatments (Fig. 2.16A). A 262 bp *VvGlub* fragment was amplified using the gene-specific primer *VvGlub1* and B26 (Appendix 1). *VvGlub* was not expressed in the control leaves or leaves bagged and treated with water alone. However, it was shown to accumulate in the ethephon- and methyl jasmonate-treated leaves confirming the vapour-based treatments had been successful.

Induction of the glutathione-S-transferase *VvGSTI* (Poinssot *et al.* 2003) was used as a positive control for SA, ABA and 2,4-D treatments in detached leaves (Fig. 2.16B). *VvGSTI* was amplified using the gene-specific primer GSTB and B26 (Appendix 1) and was not expressed in the control detached leaves at either 0, 6 or 24 h. *VvGSTI* transcript accumulated 6 h post treatment with SA, and 6 and 24 h post treatment with ABA, and 2,4-D, confirming that they had successfully infiltrated the detached leaves.

VvGLP3 was not induced in response to bagging, nor was it induced in response to any of the plant hormones or growth regulators tested (Fig. 2.16). Like *VvGLP3*, *VvGLP7* was not expressed in control leaves, or in response to bagging, JA, SA, ABA or 2,4-D treatments (Fig. 2.16). However, unlike *VvGLP3*, *VvGLP7* was induced in response to ethephon treatment (Fig. 2.16A), suggesting the signalling pathway involved in the induction of this gene involves ethylene.

A



B

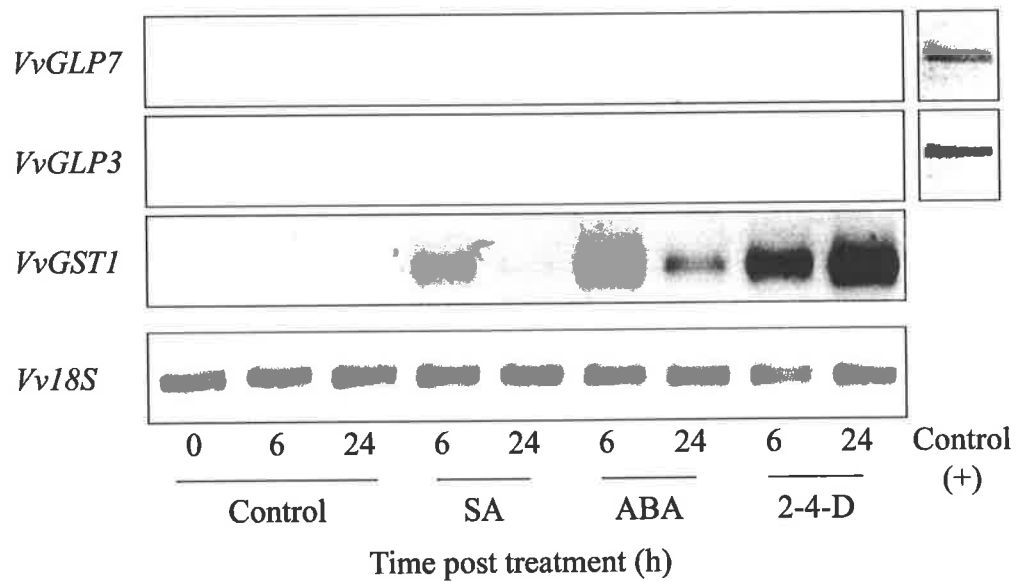


Figure 2.16. Expression of pathogen-inducible *VvGLPs* in response to various plant hormones and growth regulators. Total RNA was extracted from (A) Grapevine leaves excised from whole vines, 2 d post treatment, sprayed to run off with 10 mM ethephon (ET) or exposed to a cotton ball containing 400 μ L 0.5% (v/v) methyl jasmonate (MeJA) and placed in sealed 20 L clear containers. (B) Detached grapevine leaves, 6 and 24 h post treatment, where the petioles were submerged in distilled water containing 1 mM SA, 50 μ M ABA and 50 μ M 2,4-D. Transcripts were detected by RT-PCR analysis with a gene-specific primer in combination with an oligo(dT) adapter primer (B26). When the expression of a particular *VvGLP* gene was not detected in any of the tissues examined, a positive control RNA (shown in separate boxes) was included to verify reaction conditions as follows: *VvGLP7*, severe downy mildew leaf infection (20 d post inoculation); *VvGLP3*, severe powdery mildew leaf infection (greater than 20% of the surface was covered in hyphae). The concentrations of total RNA used in each RT-PCR reaction were verified by carrying out RT-PCR reactions on each of the samples using 18S rRNA primers. The RT-PCR products were separated on agarose gels and stained with ethidium bromide. The negative image is shown for greater resolution.

2.3.3.8 Expression patterns of *VvGLP* genes in various grape tissues

The pathogen-inducible grape *GLP* genes, i.e. *VvGLP3*, *VvGLP4* and *VvGLP7*, were not expressed in the healthy non-infected young grape leaf and pre-veraison stage berry tissues used for the biotic and abiotic stress treatments. It was of interest to determine therefore whether these genes are truly pathogen-inducible, or whether they are expressed in other grape tissues or at other stages of leaf and berry development.

No *VvGLP3*, *VvGLP4* or *VvGLP7* transcript could be detected in any of the different grape tissues and developmental stages examined (Fig. 2.17) supporting the notion that these *GLP* family members are truly pathogen-inducible. *VvGLP2* was found to be expressed in all tissues examined, including roots, flowers, leaves and the skin and pulp tissue of berries, at all developmental stages. *VvGLP6* expression was detected in all aerial parts of grapevine, but was absent from root tissue. *VvGLP6* expression appeared

to decrease in fully mature or senescing tissue as indicated by the considerably lower transcript levels in fully expanded leaves and in mature post-veraison berries. Of particular interest, were the tissue-specific expression patterns observed patterns for both *VvGLP1* and *VvGLP5* (Fig. 2.17). *VvGLP1* was only expressed in root tissue, whereas *VvGLP5* transcript was only detected in the pre-veraison berry where the expression appeared to be predominately within cells in the berry pulp.

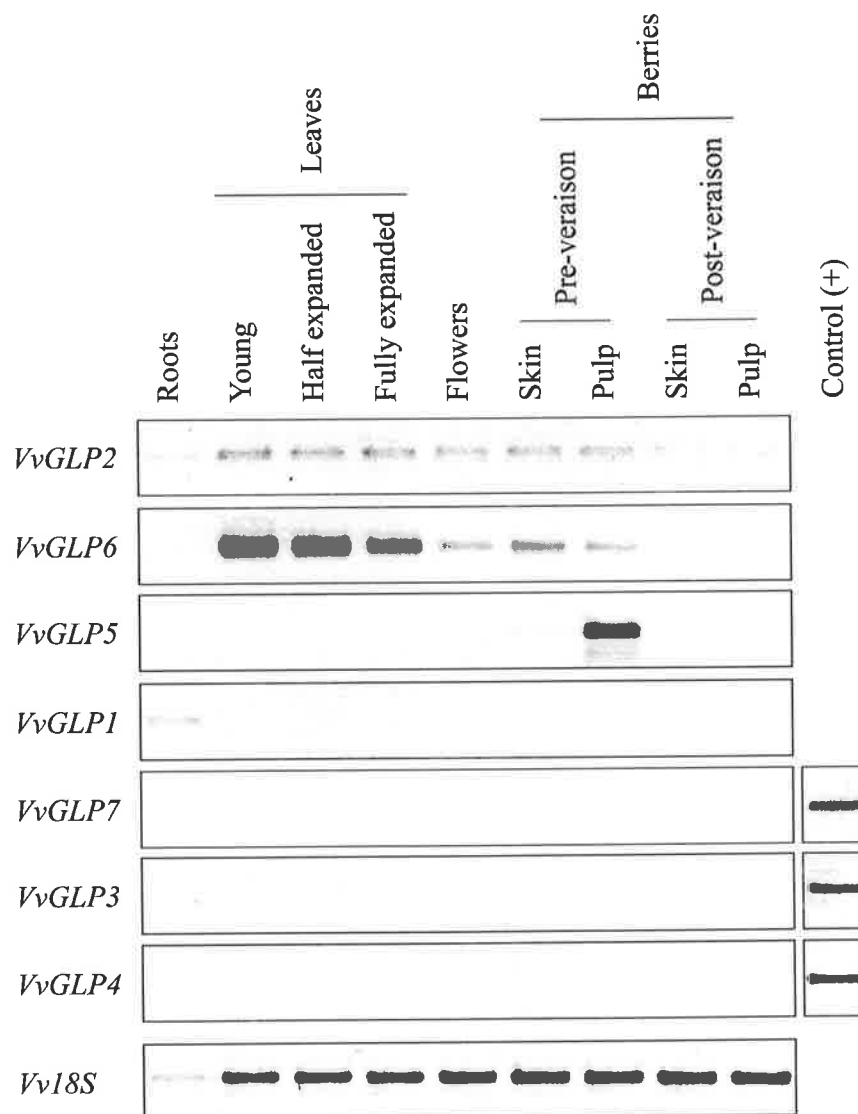


Figure 2.17. Expression of *VvGLPs* in various grape tissues. Accumulation of *GLP* transcripts (*VvGLP1* to *VvGLP7*) in Chardonnay roots, leaves, flowers and berries. Total RNA was extracted from flowers at 50% cap fall and from leaves and berries at various developmental stages. Leaves of approximate diameter 3 cm (young), 9 cm (half expanded), and 18 cm (fully expanded) were sampled. Berries were sampled at 4 weeks (pre-veraison) and 12 weeks (post-veraison) post flowering (50% cap fall) and berry skin and pulp were separated. Transcripts were detected by RT-PCR analysis with a gene-specific primer in combination with an oligo(dT) adapter primer (B26). When the expression of a particular *VvGLP* gene was not detected in any of the tissues examined, a positive control RNA (shown in separate boxes) was included to verify reaction conditions as follows: *VvGLP7*, severe downy mildew leaf infection (20 d post inoculation); *VvGLP3* and *VvGLP4*, severe powdery mildew leaf infection (greater than 20% of the surface was covered in hyphae). The concentrations of total RNA used in each RT-PCR reaction were verified by carrying out RT-PCR reactions on each of the samples using 18S rRNA primers. The RT-PCR products were separated on agarose gels and stained with ethidium bromide. The negative image is shown for greater resolution.

2.3.3.9 Expression of *VvGLP* genes in developing grape berries

As some of the *VvGLPs* (*VvGLP2*, *VvGLP5* and *VvGLP6*) are expressed in grape berries and the expression of *VvGLP5* and *VvGLP6* appeared to vary with berry age (Fig. 2.18), it was of interest to investigate the developmental expression patterns of these particular *VvGLP* genes in more detail. RNA was collected from Shiraz berries harvested at 1, 2, 4, 6, 8.5, 10 and 14 weeks post 50% capfall. Veraison, based on changes in sugar content and colour change, was determined to have occurred at 8.5 weeks post 50% capfall.

Figure 2.18 shows that the level of *VvGLP2* transcript did not appear to alter significantly during berry development. This is consistent with previous findings that *VvGLP2* is expressed in both pre- and post-veraison berries (Fig 2.17). In contrast, *VvGLP6* and *VvGLP5*, previously demonstrated (Fig 2.17) to be expressed at 4 weeks post 50% capfall (pre-veraison), but not at 12 weeks post 50% capfall (post-veraison), exhibited developmental transcript accumulation in the berry (Fig. 2.18). *VvGLP6* was only expressed in young berries up to 4 weeks post 50% capfall, after which point expression is markedly down-regulated. *VvGLP5* transcript, on the other hand, accumulated strongly at 4 weeks post 50% capfall and continued to be expressed until veraison. Low levels of expression were also observed post-veraison.

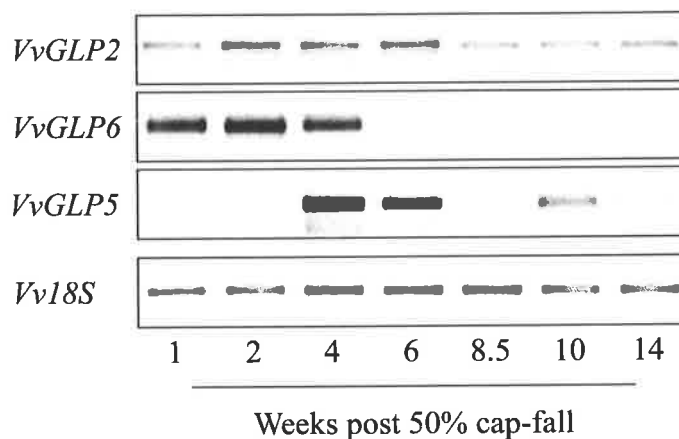


Figure 2.18. Developmental expressions of *VvGLPs* in developing grape berries. Total RNA was extracted from whole Shiraz berries 1, 2, 4, 6, 8.5, 10 and 14 weeks post 50% capfall. Transcripts were detected by RT-PCR analysis with a gene-specific primer in combination with an oligo(dT) adapter primer (B26). The concentrations of total RNA used in each RT-PCR reaction were verified by carrying out RT-PCR reactions on each of the samples using 18S rRNA primers. The RT-PCR products were separated on agarose gels and stained with ethidium bromide. The negative image is shown for greater resolution.

2.4 Discussion

2.4.1 Grapevine *GLP* gene structure

Prior to the work described in this thesis, *GLPs* from cereals (Dumas *et al.* 1995, Zhang *et al.* 1995, Hurkman and Tanaka 1996a, Hurkman and Tanaka 1996b Caliskan and Cuming 1998, Vallelan-Bindschedler *et al.* 1998, Woo *et al.* 2000) and *A. thaliana* (Membre *et al.* 1997, Carter *et al.* 1998, Staiger *et al.* 1999, Membre *et al.* 2000) were the primary focus. The impetus for identifying and characterising grapevine *GLP* genes arose from observations implicating these genes in the defence response against powdery mildew (Wei 1994, Dumas *et al.* 1995, Zhang *et al.* 1995, Wei *et al.* 1998).

This study identified seven novel *V. vinifera* cv. Chardonnay cDNA clones, designated *VvGLP1* to *VvGLP7*. The deduced amino acid sequences of the *VvGLP* genes (Fig. 2.5)

were found to contain the three highly conserved germin boxes (A, B & C) that define GLPs (Bernier and Berna 2001). Boxes B and C of the grapevine GLPs contain the highly conserved histidine and glutamate residues that are involved in binding manganese at the active site of the protein (Requena and Bornemann 1999). Grapevine GLPs also contain two conserved cysteine residues (Fig. 2.5) involved in stabilising the barley germin structure via a disulfide bond (Woo *et al.* 2000).

Previous investigations of the subcellular localisation of various GLPs, have all demonstrated that a proportion of each of the GLPs investigated has been localised to the extracellular space (Lane *et al.* 1992, Heintzen *et al.* 1994, Vallelían-Bindschedler *et al.* 1998, Ohmiya *et al.* 1998, Nakata *et al.* 2002, Kim *et al.* 2004). With two exceptions, VvGLP1 and VvGLP5, the grapevine GLP deduced amino acid sequences contain putative N-terminal signal peptides (Fig. 2.5), targeting the encoded proteins to the secretory pathway. The grapevine GLP N-terminal signal peptides vary in length from 17 to 23 amino acids and all exhibit classic N-terminal signal peptide characteristics including a positively charged N-terminal segment (*n*-region), a hydrophobic middle segment (*h*-region) and a polar C-terminal (*c*-region) (Emmanuelsson and von Heijne 2001).

Consistent with GLPs from other species (Carter *et al.* 1998), the grapevine GLPs either contain a single N-glycosylation site (VvGLP1, VvGLP3 and VvGLP6) or two putative N-glycosylation sites (VvGLP2, VvGLP4, VvGLP5 and VvGLP7) (Fig. 2.5). Although N-glycosylation sites, at a fixed location between boxes A and B (Bernier and Berna, 2001), have been identified in most GLP coding sequences, only a few studies have

actually demonstrated glycosylation of the purified protein (Yamahara *et al.* 1999, Carter and Thornburg 2000, Membre *et al.* 2000, Kim *et al.* 2004). Lerouge *et al.* (1998) determined that for the same glycoprotein, the quantity of side-chains may vary, as can the degree of side-chain modification. It is possible therefore that grapevine GLPs, with two putative glycosylation sites, may exist as either, or both, monoglycosylated or diglycosylated forms. Although putative N-glycosylation sites were identified for all grapevine GLPs, as neither VvGLP1 nor VvGLP5 have signal peptides, it is unlikely they will be exposed to the N-glycosylation machinery within the endoplasmic reticulum (ER). As such, it is unlikely these proteins are glycosylated even though they contain putative glycosylation sites.

Some GLPs have been proposed to act as plant cell wall receptors via RGD-like tripeptides, RGD, KGD, or KGE, thereby facilitating protein-protein interactions in a similar manner to that of the RGD motif in animal adhesion proteins (Swart *et al.* 1994, Dunwell 1998, Bernier and Berna 2001). RGD-mediated recognition in mammalian systems is mediated via interactions between plasma membrane bound receptors known as integrins, and extracellular protein ligands containing RGD motifs (Giancotti and Ruoslahti 1999). Although the RGD-like tripeptides, have only been identified in some 50% of GLP sequences (Bernier and Berna 2001), it is interesting to note the KGD/E tripeptide is present in all grapevine GLPs identified in this study. However, it is also important to note that not all proteins containing RGD-like tripeptides mediate cell attachment in the mammalian recognition system (Ruoslahti 1996). Therefore, although the KGD/E tripeptides, within the grapevine GLPs, are candidates for facilitating

protein-protein interactions, until this hypothesis has been functionally tested their role is only speculative.

2.4.2 Phylogenetic analysis of the grapevine GLPs

The seven grapevine GLP sequences analysed in this study grouped into three GLP subfamilies (Carter and Thornburg 2000; Nakata *et al.* 2004; Fig. 2.7), demonstrating a higher level of conserved amino acid identity exists between subfamily members than between grapevine GLPs belonging to different subfamilies. Although there is only limited functional data currently available, based on the high level of conservation of VvGLP1, VvGLP3, VvGLP4, VvGLP5 and VvGLP7 with two GLP subfamily 1 proteins with demonstrated SOD activity (Tabuchi *et al.* 2003; Christensen *et al.* 2004), it is possible to speculate these proteins may also exhibit a similar activity.

While VvGLP2 grouped in GLP subfamily 2 with a *N. plumbaginifolia* GLP with demonstrated SOD activity (Carter and Thornburg 2000), the extremely limited functional data for proteins in this subfamily renders it difficult to speculate on proposed enzyme activity for this grapevine GLP. In the case of GLP subfamily 3, the disparate enzyme activity of proteins within the subfamily prevents speculation on the putative enzyme activity of VvGLP6. This grapevine GLP shares a high level of conserved amino acid identity to *P. persica* auxin-binding GLPs with SOD activity (Ohmiya *et al.* 1998), but also groups with a *H. vulgare* GLP, HvGLP2, with AGPPase activity (Rodriguez-Lopez *et al.* 2001, Vallelian-Bindschedler *et al.* 1998).

2.4.3 Inducible expression of grapevine GLPs

The results presented in this chapter, illustrate that the grapevine GLPs are widely diverse in their expression patterns, signifying these proteins have diverse roles both in normal cellular development and in response to a range of biotic and abiotic factors. Table 2.2 summarises the expression of VvGLPs in healthy non-infected tissues, and in response to a range of biotic and abiotic conditions, as determined in this study

2.4.3.1 VvGLP3 and VvGLP4

Reverse Transcriptase (RT)-PCR and Northern expression analysis identified two grapevine GLPs, VvGLP3 and VvGLP4, which are not expressed in healthy non-infected roots, flowers, or various developmental stages of either leaves or berries, but are strongly induced in leaves and berries in response to powdery mildew (Fig. 2.9; 2.17). VvGLP3 was not induced in response to either *P. viticola* (Fig. 2.14A) or *B. cinerea* (Fig. 2.14B) infection, nor did VvGLP3 transcript accumulate in response to wounding (Fig. 2.15). These observations suggest that VvGLP3 is induced specifically in response to *E. necator*. Like VvGLP3, VvGLP4 was not induced in leaves in response to *B. cinerea* infection (Fig. 2.14B) or wounding (Fig. 2.15). However, VvGLP4 transcript does accumulate at low levels in leaves severely infected with *P. viticola* (Fig. 2.14A).

VvGLP3 was demonstrated to accumulate specifically in infected regions, rather than systemically throughout the whole leaf (Fig. 2.11), suggesting that VvGLP3 expression may be limited to either epidermal cells which have been penetrated by the powdery mildew fungus and/or cells surrounding the infected cell. In order to be able to determine the tissue specificity of powdery mildew-inducible VvGLP3 accumulation it

is necessary to be able separate the epidermal layer from the other tissue layers. The techniques used in barley (Wei *et al.* 1998) for the physical separation of epidermal tissue from the remainder of the leaf are inappropriate for grapevine leaves. However, it was possible to physically separate the skin and pulp tissue from *E. necator* infected grape berries, and by this means, powdery mildew-inducible *VvGLP3* expression was localised specifically to the grape berry skin (Fig. 2.9). The skin-specific expression of *VvGLP3* in the grape berry, and the confinement of *E. necator* infection to epidermal cells, makes it tempting to speculate that this gene is induced specifically within the epidermal cells. However, it is important to note that grape berry skin tissue is composed of several cell layers, comprising the epidermis and hypodermis (Ollat *et al.* 2002), and consequently, it is not possible to identify the exact tissue-specific location of powdery mildew-inducible *VvGLP3* transcript accumulation using this technique.

The temporal expression of *VvGLP3* induction, in response to *E. necator* infection, is consistent with a role in papillae formation. Accumulation of *VvGLP3* transcript initially accumulates 12 to 24 h post inoculation (Fig. 2.13). The timing is simultaneous with primary appressoria formation and the predicted penetration of the epidermal cell (Fig. 2.12B).

The expression pattern of *VvGLP3* is similar to that observed for barley *GLP*, *HvGLP4* (Wei *et al.* 1998). Like *VvGLP3*, *HvGLP4* is not expressed in healthy non-infected leaves, but is induced by powdery mildew and transcript accumulation appears epidermal-specific. The spatial and temporal expression of *HvGLP4* suggested a role for this protein in papillae formation in response to the formation of the fungal primary and

appressorial germ tubes in barley (Wei *et al.* 1998). One significant difference in the observed expression patterns for *HvGLP4* and *VvGLP3* is that *HvGLP4* appears induced much earlier in barley in response to powdery mildew spore inoculation than *VvGLP3* in grapevine. *HvGLP4* transcript accumulated in leaves within 3 h after inoculation with *B. graminis* (Wei *et al.* 1998), with maxima at 6 and 24 h, whereas *VvGLP3* transcript did not accumulate until 12 to 24 h following inoculation with *E. necator* (Fig. 2.13). The observed delay in *VvGLP3* transcript accumulation compared to *HvGLP4* may be related to the different levels of spore application between the two experiments. If regions of the leaves were not sufficiently covered with conidia, the level of transcript would have been diluted during the preparation of RNA. It is also important to note that following *B. graminis* inoculation of barley, papillae are formed in response to the generation of a primary germ tube, 3 to 4 h after inoculation, and in response to the appressorial germ tube, 10 to 18 h after inoculation. In comparison to the *B. graminis* infection process, *E. necator* conidia produce only a single germ tube and appressorium formation (and therefore any resultant papillae formation) occurs significantly later at around 6 to 8 h after inoculation. Thus, the later accumulation of *VvGLP3*, compared to *HvGLP4*, may simply reflect the different infection processes of *B. graminis* and *E. necator*.

Mutant studies with *A. thaliana* have identified a salicylic acid (SA)-dependent or a jasmonic acid/ethylene-dependent signalling pathway leading to the activation of defence genes in response to pathogen attack (Glazebrook 2001, Hammond-Kosack and Parker 2003). Significantly, *VvGLP3* was not induced by treatment of grape leaves with various plant hormones or elicitor molecules, including ethylene, methyl jasmonate,

salicylic acid, abscisic acid or synthetic auxin (Fig. 2.16). This suggests that other types of elicitor molecules must be involved in the induction of this gene.

It is also interesting to note that neither *VvGLP3* nor *VvGLP4* were induced by wounding (Fig. 2.15). In the past *B. graminis* penetration has been compared to wounding using fine microneedles (Aist 1976). Although the response to mechanical wounding using microneedle puncture seemingly mimics papillae response to attempted *B. graminis* penetration, Russo and Bushnell (1989) observed differences in the repair mechanisms. Following microneedle wounding, the puncture site was repaired within 2 min. Cytoplasmic aggregation was observed 5 min after wounding and in the subsequent 40 to 60 min a wound plug containing callose, cellulose and pectin was deposited. Unlike papillae formation, phenolics were not involved in the mechanical wounding response. Considering the wound response does not appear to involve the same processes as papillae formation in response to attempted fungal penetration, it is not surprising powdery-mildew inducible grapevine *VvGLP3* and *VvGLP4* are not induced by this abiotic stress.

2.4.3.2 *VvGLP7*

VvGLP7 appears to be another pathogen-inducible form of grapevine *GLP*. As with *VvGLP3* and *VvGLP4* this gene was not expressed in healthy non-infected roots, leaves, flowers or berries during any of the developmental stages examined (Fig. 2.17). In contrast to *VvGLP3* however, *VvGLP7* transcript accumulation was not induced in response to powdery mildew (Fig. 2.9), but was induced in response to infection by *P. viticola* (Fig. 14A) and *B. cinerea* (Fig. 14.B). Like *VvGLP3* and *VvGLP4* the inducible

expression was specifically in response to pathogen attack and not induced by wounding.

Studies with *A. thaliana* indicate defence against necrotrophic pathogens such as *B. cinerea* is often associated with induction of parallel jasmonic acid and ethylene signalling cascades whereas defence responses to biotrophic pathogens, such as *E. necator* and *P. viticola*, are more often associated with the SA signalling pathway (Hammond-Kosack and Parker 2003). Although *VvGLP7* was not induced in response to salicylic acid (Fig. 2.16B), it was the only grapevine *GLP* gene induced in response to ethylene treatment (Fig. 2.16A). Interestingly, *VvGLP7* transcript did not accumulate in response to treatment with MeJA whereas the marker gene encoding the PR protein β -1,3-glucanase (*VvGlub*) was induced by both elicitors (Fig. 2.16A). Clearly in grapevine the signalling pathways are more complicated than those described for *A. thaliana*.

Furthermore, *VvGLP7* is not induced by the biotrophic pathogen *E. necator*, but is by the biotrophic pathogen *P. viticola* and the necrotrophic pathogen *B. cinerea*. While it is tempting to speculate that *VvGLP3* and *VvGLP7* respond differentially to specific molecules derived from these two biotrophic pathogens, it is also possible that this observation may result from differences in the tissue-specificity of *VvGLP3* and *VvGLP7* expression. Unlike *E. necator*, *P. viticola* infects via the stomates and penetrates mesophyll cells. If *VvGLP7* expression is restricted to mesophyll cells, it would not be expected to respond to *E. necator* infection but may be induced by *P. viticola* infection.

Roles for GLPs against powdery mildew infection have been studied for some time, but expression data for *VvGLP7* presented in this study suggests a novel role for GLPs in defence against other pathogens including *P. viticola* and *B. cinerea*. However, until further investigations localise the tissue-specific expression of *VvGLP7* and the enzyme activity of the protein encoded by this gene, it is premature to speculate on the role *VvGLP7* may play in defence against these fungal pathogens.

2.4.3.3 *VvGLP5*

VvGLP5 was found to be expressed in healthy non-infected tissue, but in a highly tissue-specific manner. Of all the tissues examined, *VvGLP5* was only expressed in the berry (Fig. 2.17). *VvGLP5* does not appear to be either pathogen or stress-inducible as *VvGLP5* transcript did not accumulate in response to *E. necator* (Fig. 2.9), *P. viticola* (Fig. 2.14A) or *B. cinerea* (Fig. 2.14B) infection, nor did it accumulate in response to an abiotic stress such as wounding (Fig. 2.15). In fact, expression of *VvGLP5* appeared to be repressed by *E. necator* infection. Zimmerli *et al.* (2004) previously reported gene repression in *A. thaliana* in response to both host (*E. cichoracearum*) and non-host (*B. graminis*) powdery mildew pathogens. These repressed genes mainly encoded proteins associated with basic metabolism and photosynthesis. During pathogen attack coordination of defence responses and plant growth is thought to facilitate diversion of metabolic resources from general metabolism to defence related metabolism (Logemann *et al.* 1995, Zimmerli *et al.* 2004).

Within berries, the *VvGLP5* transcript strongly accumulated in the berry pulp (Fig. 2.17), suggesting the expression of this gene may be pulp-specific. The low level of

VvGLP5 expression detected in berry skin tissue may be an artefact resulting from contamination of the skin tissue with pulp tissue.

Grape berry development during the first 2 weeks after flowering, results predominantly from cell division. Cell division ceases 30 to 40 d after flowering and subsequent berry growth occurs primarily by cell enlargement (Ollat *et al.* 2002). *VvGLP5* transcript accumulated strongly at 4 weeks post 50% capfall and continued to be expressed until veraison, after which low levels accumulated (Fig. 2.18). Thus, *VvGLP5* expression is co-incident with a period of cell enlargement within the grape berry. Grape berry cell enlargement is thought to involve a complex process of cell wall loosening and cell expansion for which various models have been proposed (Huang and Huang 2001). It is thought that the orientation of the cell wall microfibrillar components may constrain the directions in which cell expansion can occur and the degree of crosslinking between the microfibrillar components determines the extent to which expansion can occur. Hydrogen peroxide has been implicated in the regulation of cell wall extensibility. Fry (1989) proposed a novel mechanism whereby H_2O_2 , in the presence of ascorbic acid and Cu^{2+} , can be used at the cell wall to produce hydroxyl radicals. Hydroxyl radicals could be involved in non-enzymatic scission of polysaccharides and, consequently, in wall loosening and cell expansion (Fry 1998, Schopfer *et al.* 2002, Schweikert *et al.* 2002). *VvGLP5* may therefore play a role in cell expansion in the pulp cells of berries during development by facilitating the apoplastic generation of H_2O_2 , via SOD activity.

2.4.3.4 VvGLP6

Accumulation of VvGLP6 transcript was found to be tissue-specific and developmentally regulated. VvGLP6 was expressed in leaves, flowers and berries, but not in roots, and expression in leaves and berries was down regulated as the tissue aged (Fig. 2.17). VvGLP6 does not appear to be either pathogen or stress-inducible as VvGLP6 transcript did not accumulate in response to *E. necator* (Fig. 2.9), *P. viticola* (Fig. 2.14A) or *B. cinerea* (Fig. 2.14B) infection, nor did it accumulate in response to an abiotic stress such as wounding (Fig. 2.15). Like VvGLP5, the expression of VvGLP6 was repressed by pathogen infection. Accumulation of VvGLP6 transcript was down regulated in response to *E. necator*, *P. viticola* and *B. cinerea* infection.

The deduced amino acid sequence of VvGLP6 is most similar to GLP subfamily 3 members (Fig. 2.7). Like VvGLP6, other GLP subfamily 3 members also exhibit tissue-specific and developmental expression. For example, *A. thaliana* AtGER3 transcripts accumulate in young leaves and reproductive tissues, but not in root tissue (Membre *et al.* 1997, Staiger *et al.* 1999). GLPs from *S. alba* and *P. nil* are primarily expressed in young leaves and reproductive tissue (Ono *et al.* 1993, Heintzen *et al.* 1994, Ono *et al.* 1996). Cotton GhGLP1 transcripts accumulate specifically within elongating fibre cells (Kim and Triplett 2003). Finally, barley HvGLP2 expression was shown to be strongest in young leaves and lacking in roots (Vallelian-Bindschedler *et al.* 1998).

During berry development, VvGLP6 transcript accumulates in young berries for the first 4 weeks after flowering (Fig. 2.18). The developmental expression of VvGLP6 coincides with a period of grape berry cell growth primarily by cell division (Ollat *et al.* 2002).

Following this period, cell division ceases and cell growth results entirely from cell enlargement. For protoplast division to occur, cell wall reconstitution is required (Katsirdakis and Roubelakis-Angelakis 1992). Treatment of *N. tabacum* protoplasts with peroxidase inhibitors, cyanide or DTT, or the H_2O_2 scavenger catalase, prevented cell division, and prompted a proposed role for apoplastic H_2O_2 in cell wall cross-linking reactions during cell wall reconstitution required for subsequent cell division (de Marco and Roubelakis-Angelakis 1996a, de Marco and Roubelakis-Angelakis 1996b). As VvGLP6 is most closely related to a peach GLP with SOD activity (Ohmiya *et al.* 1998), this may suggest a potential role for VvGLP6 in cell wall reconstitution during the early stages of cell division in developing grape berries through its involvement in H_2O_2 generation, via SOD activity.

It is also interesting to note that the transcript abundance of several GLP subfamily 3 members, including *HvGLP2* (Vallelian-Bindschedler *et al.* 1998), *AtGER3* (Membre *et al.* 1997, Staiger *et al.* 1999), *SaGLP* (Heintzen *et al.* 1994) and *PnGLP* (Ono *et al.* 1993, Heintzen *et al.* 1994, Ono *et al.* 1996), varies with the circadian cycle, whereas that of others, including *GhGLP1* (Kim and Triplett 2003), does not. The accumulation of VvGLP6 transcript during the photoperiod was not tested in this study. However, samples were consistently collected at the same point in the photoperiod to prevent distorted transcript accumulation patterns should VvGLP6 expression vary with the circadian cycle.

2.4.3.5 VvGLP2

VvGLP2 was expressed in all tissues examined (Fig. 2.17). Expression of VvGLP2 was not observed to vary considerably in leaves and berries of different developmental stages (Fig. 2.17; Fig. 2.18), nor was expression significantly altered in response to response to *E. necator* (Fig. 2.9) or *B. cinerea* (Fig. 2.14B) infection. However, VvGLP2 transcript appeared to be induced in response to *P. viticola* infection (Fig. 2.14A) and wounding (Fig. 2.15).

VvGLP2 is grouped within GLP subfamily 2 (Fig. 2.7) along with the *N. plumbaginifolia* GLP, Nectarin I (Carter and Thornburg 2000). Nectarin I was found to be the most abundant protein in tobacco floral nectar, representing approximately 50% of the total protein. Nectarin I protein has been shown to have SOD activity and has been proposed to have role in protecting the gynoecium and ovules from pathogen attack via the SOD catalysed dismutation of superoxide and its protonated form (HO_2/O_2^-) to H_2O_2 , resulting in H_2O_2 concentrations that are toxic to microorganisms (Carter and Thornburg 2000). The expression patterns of VvGLP2 and Nectarin I are disparate, suggesting that these GLPs have unrelated functions. For example, VvGLP2 is expressed in roots, leaves, flowers and berries (Fig. 2.17) whereas, Nectarin I transcript only accumulates in the nectary and at low levels in the ovary, and does not accumulated in leaves, stems, roots or other floral organs (Carter *et al.* 1999). Furthermore, VvGLP2 was not developmentally expressed in leaves and berries (Fig. 2.17; Fig. 2.18) whereas Nectarin I was expressed developmentally with transcripts only accumulating while the nectary was actively secreting nectar.

Also grouped with VvGLP2 in GLP subfamily 2 is the *P. sativum* rhicadhesin receptor, PsGER1 which is proposed to mediate the attachment of *Rhizobium* spp. to the plant cell wall during the nodulation process (Swart *et al.* 1994). The interaction between rhicadhesin and the *P. sativum* GLP occurs via an RGD tripeptide. The RGD-like tripeptide KGE is present in the VvGLP2 sequence and in a similar manner to PsGER1, VvGLP2 may also act as a cell wall receptor.

2.4.3.6 VvGLP1

Like VvGLP5, the expression of VvGLP1 also appears to be highly tissue-specific. Accumulation of VvGLP1 transcript in healthy non-infected tissue was only observed in grapevine root (Fig. 2.17). The encoded VvGLP1 sequence is most closely related to proteins with SOD activity (Fig. 2.7). Assuming VvGLP1 protein also exhibits SOD activity, this protein may be involved in the generation of H₂O₂ in root tissue. Reactive oxygen species, such as H₂O₂, are generally associated with abiotic and biotic stress responses and cell death in plants (Wojtaszek 1997, Hückelhoven and Kogel 2003, Overmyer *et al.* 2003). However, Liskay *et al.* (2004) has recently proposed a new role for reactive oxygen species in cell elongation of unstressed root cells. Although the biochemical mechanisms responsible for cell elongation are mostly unknown, it is generally thought that increase in the extensibility of cell walls, resulting in a reduction in cell turgor, is an initiator for elongation growth (Cosgrove 2001). Using histochemical assays, Liskay *et al.* (2004) observed HO₂/O₂⁻, H₂O₂, and [•]OH specifically within the growing zone of maize (*Zea mays*) seedling roots. Thus, a role for extracellular [•]OH, generated via peroxidase-mediated Fenton reactions from O₂⁻ and H₂O₂, in cell wall loosening via non-enzymatic cleavage of cell wall polysaccharides

(Fry 1998) and subsequent cell expansion was proposed. It is possible that VvGLP1 plays a similar role in elongating cells of grapevine roots.

2.4.3.7 *P. tricuspidata* GLPs

Resistance to powdery mildew varies significantly within species of the various genera comprising the Vitaceae family (Boubals 1961). The results of this study show that papillae formation, in response to *E. necator* infection, was much more enhanced in *P. tricuspidata* and *M. rotundifolia* than in *V. vinifera* (Fig. 2.3B). This finding inspired investigation into the possible role of GLPs in papillae-based resistance of *P. tricuspidata* to powdery mildew. Three novel *P. tricuspidata* GLPs, *PtGLP1*, *PtGLP2* and *PtGLP3*, were identified with a high degree of sequence homology to GLPs from *V. vinifera* (Fig. 2.8B). However, expression analysis of these genes indicated that these genes were neither expressed in healthy non-inoculated leaves, nor induced in response to *E. necator* inoculation, suggesting that these *PtGLPs* are either expressed at very low levels not detectable in the RT-PCR conditions used or that the original clones had been amplified from genomic contamination.

Although attempts at cloning a powdery mildew-inducible *P. tricuspidata* GLP were not successful, an additional GLP from *P. tricuspidata* has since been isolated (Dr Ian Dry, CSIRO Plant Industry, personal communication). Significantly, this GLP is not expressed in healthy non-infected leaves, but is induced 24 h after inoculation with *E. necator*. Of great interest is preliminary expression data suggesting that induction of this GLP either occurs earlier, or more strongly, than VvGLP3 (Dr Ian Dry, CSIRO Plant Industry, personal communication). Further work is now underway to determine if the

increased penetration resistance of *P. tricuspidata* to *E. necator*, compared to *V. vinifera*, may be a product of elevated *GLP* expression in this species.

Table 2.2. Summary of expression of *VvGLPs* in healthy non-infected tissues and in response to a range of biotic and abiotic conditions as determined in this study. The symbols used are as follows: (-), no expression; (+), expression; (↑), up-regulation; (~), no change; (↓), down-regulation. ¹ Expression is predominantly within the berry pulp and is developmentally regulated. ² Expression is developmentally regulated. Abbreviations used: ET, ethylene; MeJA, methyl jasmonate; SA, salicylic acid; ABA, abscisic acid, synthetic auxin 2,4-D, 2,4-dichlorophenoxyacetic acid; ND, not determined.

	<i>VvGLP1</i>	<i>VvGLP2</i>	<i>VvGLP3</i>	<i>VvGLP4</i>	<i>VvGLP5</i>	<i>VvGLP6</i>	<i>VvGLP7</i>
Healthy non-infected tissues:							
Roots	+	+	-	-	-	-	-
Leaves	-	+	-	-	-	+ ²	-
Flowers	-	+	-	-	-	+	-
Berries	-	+	-	-	+ ¹	+ ²	-
Response to various biotic and abiotic conditions:							
<i>E. necator</i>	-	~	↑	↑	↓	↓	-
<i>P. viticola</i>	↑	↑	-	-	-	↓	↑
<i>B. cinerea</i>	-	~	-	-	-	↓	↑
Wounding	-	↑	-	-	ND	~	-
ET	ND	ND	-	ND	ND	ND	+
MeJA	ND	ND	-	ND	ND	ND	-
SA	ND	ND	-	ND	ND	ND	-
ABA	ND	ND	-	ND	ND	ND	-
2-4-D	ND	ND	-	ND	ND	ND	-

2.4.4 Summary

This study has identified that the *V. vinifera* genome contains a multi-gene family of at least seven *GLP* genes. This finding is consistent with that of *GLP* families in other species including *A. thaliana* (Carter *et al.* 1998, Carter and Thornburg 1999), barley (Wu *et al.* 2000, Druka *et al.* 2002) and rice (Membre and Bernier 1998). Expression patterns for the grapevine *GLPs*, in various tissues, and in response to fungal pathogens,

abiotic stress and treatment with various plant hormone and growth regulators, have been characterised. This has demonstrated that each of the grapevine *GLPs* has specific spatial and temporal expression patterns induced under different conditions indicating, that the grapevine *GLP* gene family is composed of numerous members with apparently dissimilar functions. The expression of some grapevine *GLP* genes is consistent with closely related *GLPs* from other species. Most significant to this research, was the identification of a grapevine *GLP*, *VvGLP3*, which was strongly induced in leaves and berries in response to *E. necator* infection, but not in response to infection by *P. viticola* or *B. cinerea*, or wounding. Based on expression data obtained in this study, possible roles for the grapevine *GLPs* have been proposed. In addition, three novel *P. tricuspidata* *GLPs*, were identified. However, as these genes were neither expressed in healthy non-inoculated leaves, nor induced in response to *E. necator* inoculation, it is not possible to speculate on putative roles for the proteins encoded by these *GLP* genes.

Chapter 3. Tissue-specific localisation and targeting of grapevine GLPs

3.1 Introduction

Cloning and expression analysis of grapevine *GLPs* (Chapter 2) led to the identification of a *V. vinifera GLP*, *VvGLP3*, that was strongly induced in leaves and berries in response to *E. necator* infection (Fig. 2.9; 2.10), but not in response to *P. viticola* (Fig. 2.14A) or *B. cinerea* (Fig. 2.14B) infection. The timing of *VvGLP3* transcript accumulation, in response to *E. necator* inoculation, is consistent with attempted fungal penetration of the cell wall and host papillae formation (Fig. 2.12; 2.13). However, in order for this protein to have a role in papillae-based defence to *E. necator* it would need to be expressed within epidermal cells. Owing to the vascular complexity of grapevine leaves, the techniques used successfully in barley (Gregersen *et al.* 1997) to separate epidermal and mesophyll tissue, are inappropriate for the leaves of this species. Consequently, the tissue-specific localisation of powdery mildew-inducible *VvGLP3* expression in leaves, could not be determined by this method. In grape berries, *VvGLP3* transcript accumulates specifically within the grape berry skin tissue (Fig. 2.9; 2.10). However, as the grape berry skin is composed of epidermal and hypodermal cell layers (Ollat *et al.* 2002), the expression of *VvGLP3* could not be localised specifically to the layer of attacked epidermal cells.

Consequently, this chapter describes an alternative approach to localising powdery mildew-inducible expression using promoter: reporter gene studies. As reliable and efficient transformation protocols for grapevine are currently not available, a VvGLP3 promoter: *GUS* construct was transformed into the model plant *A. thaliana*. *A. thaliana* was chosen due to the relative ease of transformation and availability of a powdery mildew isolate, *E. cichoracearum*, which is capable of infecting this host.

This chapter also describes the results of studies examining the targeting of grapevine GLPs within cells. Most GLPs have a signal peptide and GLPs studied at the protein level are associated with the extracellular matrix (Lane *et al.* 1992, Heintzen *et al.* 1994, Vallelian-Bindschedler *et al.* 1998, Ohmiya *et al.* 1998). However, collectively the subcellular localisation of GLPs has received little attention. Isolation of extracellular proteins is impeded by the technical problems associated with extraction of proteins from the cell wall. Harvesting the extracellular compartment is hindered by potential contamination with intracellular proteins resulting from plasma membrane disruption, and soluble extracellular proteins are often lost from the cell wall extract during tissue homogenisation (Lee *et al.* 2004). Extracellular proteins are often glycosylated, plasma membrane-anchored or bonded to the cell wall, further complicating extraction (Lee *et al.* 2004).

Alternatively, immunolocalisation and GFP-fusion protein analysis are powerful techniques for localisation of putative extracellular proteins. Since the demonstration that proteins can be fused to GFP without inhibiting fluorescence (Wang and Hazelrigg 1994), GFP has been exploited to investigate the targeting of proteins of interest. A

major advantage of GFP over other reporter proteins is that GFP fusions can be localised non-invasively by external illumination, without the need for external substrates, in living cells. Particle bombardment of DNA-coated gold particles on onion epidermal peels is a model system for localisation of proteins fused to reporter proteins such as GFP (Quaedvlieg *et al.* 1998). Onion epidermal peels are ideal for GFP localisation as the cells are relatively large and transparent, comprising a single cell layer with insignificant background autofluorescence (Scott *et al.* 1999). Thus, the cellular targeting of grapevine GLP gene products was determined using GFP fusion constructs transiently expressed in onion (*Allium cepa* L.) cells.

The localisation studies described in this chapter were designed to enhance our understanding of the proposed role of powdery-mildew-inducible grapevine GLPs in defence against powdery mildew. In particular, the aims were to observe the tissue-specific localisation of powdery mildew-inducible grapevine *VvGLP3* expression and to determine the targeting of the encoded protein within the cell.

3.2 Materials and methods

3.2.1 Materials

Buffers and solutions, as summarised in Table 3.1, were prepared with nanopure water and autoclaved where appropriate. All chemicals were analytical or molecular biology grade.

Table 3.1. Solutions (and their composition) used in this research.

Solution	Composition
<i>Arabidopsis</i> DNA extraction buffer	200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS
DNA extraction buffer	0.25 M NaCl, 0.2 M Tris-HCl (pH 8), 2.5% (w/v) PVP, 0.05 M Na ₂ EDTA, 0.1% (v/v) β -mercaptoethanol
DNA extraction buffer (modified)	0.5 M NaCl, 1 M Tris-HCl (pH 8), 2.5% (w/v) PVP, 0.05 M EDTA, pH 8, 3% (v/v) sarkosyl, 20% (v/v) ethanol, 1% (v/v) β -mercaptoethanol
Germination plates	1 $\times$ Murashige and Skoog (MS) Salt Mixture (Invitrogen), 1% (w/v) agar, 1% (w/v) sucrose, pH 5.7
GUS staining solution	100 mM NaH ₂ PO ₄ , 10 mM Na ₂ EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% (v/v) Triton X-100, 0.5 mg/mL (w/v) 5-bromo-4-chloro-3-indolyl- β -D-glucuronide cyclohexylammonium salt (X-Gluc), pH 7.0
1 $\times$ PBS	137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, pH 7.4
Sterilising solution	2:1 100% (v/v) ethanol:bleach (active ingredient 3.5% (w/v) sodium hypochlorite), 0.1% (w/v) SDS

3.2.2 Plant materials and growth conditions

V. vinifera leaves were sampled from vineyard and potted glasshouse vines grown at the Waite Campus (Adelaide, South Australia) as described previously (Section 2.2.2). *A. thaliana* ecotype Columbia 4 (Col-4) seed was kindly provided by Elizabeth Lee (CSIRO, Plant Industry, South Australia). Individual *A. thaliana* plants were cultivated in sterile moistened soil containing sand:Perlite:peat moss:Vermiculite (1:1:1:1, v/v) with 1 g L⁻¹ iron sulphate, 3 g L⁻¹ Osmocote® slow release fertiliser (Yates, Australia), 2 g L⁻¹ dolomite, 0.5 g L⁻¹ gypsum, 1 g L⁻¹ agricultural lime and 0.4 g L⁻¹ hydrated lime. Plants were grown in 5 cm diameter pots at 22°C under constant light in controlled

temperature growth rooms. *A. thaliana* plants intended for floral dip transformation (Section 3.2.7.2) were planted five to ten per 12 cm diameter pot. Onions were purchased from Independent Grocers Australia Food Store, Highgate, South Australia.

3.2.3 Pathogen culture and maintenance

E. cichoracearum was obtained from naturally infected *A. thaliana* plants, grown at the Waite Campus (Adelaide, South Australia) as per Section 3.2.2. The isolate was kindly identified by Dr Ian Pascoe (Department of Natural Resources and Environment, Victorian Institute for Horticultural Development, Australia). *E. cichoracearum* was maintained on apple cucumber seedlings grown in Perlite at 22°C under a 12 h light / 12 h dark cycle. Petioles were inoculated by gently tapping conidia from infected leaves (14 d post inoculation) above one-week-old seedlings.

3.2.4 Cloning *V. vinifera* GLP promoters

3.2.4.1 Cloning *VvGLP* promoter fragments from a *V. vinifera* cv. Shiraz promoter-walking library

Promoter-walking libraries were obtained from Dr. Mark Thomas (CSIRO, Plant Industry, Adelaide). Libraries were constructed from *Pml*I, *Hpa*I, *Pvu*II, *Sca*I, *Sna*BI, *Ssp*I, *Stu*I and *Swa*I restriction digests of *V. vinifera* cv. Shiraz genomic DNA ligated to an adapter sequence following the method of Siebert *et al.* (1995). DNA regions upstream of the grapevine *GLP* coding regions were amplified using a nested PCR strategy. The first amplification reaction was performed using the forward O1 adaptor primer (Appendix 1; 4), corresponding to the adapter sequence, and a reverse gene-

specific primer. For the second round of amplification, a second reverse nested adaptor primer, O2 (Appendix 1; 4), was used with a nested gene-specific primer.

Primary PCR reactions were conducted in 25 μL volumes containing 0.5 μL of the promoter-walking library DNA, 60 mM Tris- SO_4 (pH 8.9), 18 mM $(\text{NH}_4)_2\text{SO}_4$, 1.5 mM MgSO_4 , 200 μM dNTPs, 0.5 μM nested adaptor-specific primer O2, 0.5 μM nested gene-specific primer and 1.25 μL of PLATINUM *Taq* High Fidelity DNA polymerase (Invitrogen, Australia). The cycle parameters were as follows: initial denaturation step at 94°C for 1 min, 35 cycles of denaturation at 94°C for 30 s and annealing/extension at 68°C for 6 min, and a final annealing/extension time at 68°C for 15 min. Second round PCR reactions were conducted in 20 μL volumes containing 1 μL of a 1/100 dilution of the first round reaction, 60 mM Tris- SO_4 (pH 8.9), 18 mM $(\text{NH}_4)_2\text{SO}_4$, 1.5 mM MgSO_4 , 200 μM dNTPs, 0.5 μM adaptor primer O1, 0.5 μM and gene-specific primer and 2.5 μL of PLATINUM *Taq* High Fidelity DNA polymerase (Invitrogen). The cycle parameters were as follows: initial denaturation step at 94°C for 1 min, 20 cycles of denaturation at 94°C for 30 s and annealing/extension at 68°C for 6 min, and a final annealing/extension time at 68°C for 15 min. PCR products were examined on an ethidium-bromide stained 0.75% (w/v) agarose gel (as per Section 2.2.5.3.1) and PCR products were gel purified as per Section 2.2.5.4.1. Purified DNA fragments were cloned into pGEM[®]-T Easy (Promega, Madison, USA) (as per Section 2.2.5.4) and clones containing the promoter fragments were verified by sequencing using the SP6 and T7 sequencing primers (Section 2.2.5.5; Appendix 1). Sequencing accuracy was verified by sequencing separate clones.

When the cloned promoter fragments were less than 1 kb, and therefore considered unlikely to contain the entire promoter, an additional promoter fragment was isolated from the Shiraz genomic DNA promoter-walking library. Using a second pair of nested reverse gene-specific primers (Appendix 1; 4), designed to the partial-length promoter sequence, DNA upstream of the initial *GLP* promoter fragment was amplified and cloned. Regions of 100% nucleotide overlap between grapevine *GLP* genes and promoter fragments indicated that the corresponding promoter fragment had been obtained. The merged promoter fragments were assembled *in silico* and the sequences, inclusive of the start codon, are shown in Appendix 5.

3.2.4.2 Isolation of full-length promoter clones from Chardonnay genomic DNA

Genomic DNA was prepared from 2 g of leaf tissue from *V. vinifera* cv. Chardonnay by grinding to a fine powder in liquid nitrogen using a pre-cooled mortar and pestle. The ground tissue was re-suspended in 25 mL of DNA extraction buffer (Table 3.1) by vortexing, followed by centrifugation at 4,000 *g* for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 5 mL modified DNA extraction buffer (Table 3.1) by brief vortex mixing and shaking at 100 rpm at room temperature for 30 min. The suspension was extracted by adding an equal volume of chloroform:isoamyl alcohol (24:1 v/v), mixing gently and centrifuging at 4,000 *g* for 20 min at 20°C. The upper aqueous phase was retained, 0.54 vol of 100% isopropanol added and left at room temperature for 10 min before pelleting via centrifugation at 23,000 *g* for 15 min at 20°C. The supernatant was discarded and the pellet was washed with 80% (v/v) ethanol and dried under vacuum for 10 min at room temperature. The pellet was re-suspended in 600 µL TE buffer (Table 2.1) containing 0.7% (v/v) RNase cocktail [500 U mL⁻¹ RNase

A, 20, 000 U mL⁻¹ RNase TI (Ambion, Geneworks, South Australia)] and incubated for 30 min at 37°C before centrifuging at 16,000 g for 10 min at room temperature. The supernatant was retained and 300 µL of 7.5 M sodium acetate and 0.54 volumes of 100% isopropanol were added. DNA was pelleted by centrifugation at 16,000 g for 10 min at room temperature. The pellet was then washed with 80% (v/v) ethanol and dried under vacuum for 10 min at room temperature. The DNA was re-suspended in 200 µL TE buffer and stored at -20°C.

Full-length promoter fragments were PCR amplified from Chardonnay genomic DNA using gene-specific forward (VvGLP3Fa) and reverse (OXL3Rd) gene-specific primers (Appendix 1) based on the *V. vinifera* cv. Shiraz sequence using PLATINUM *Taq* High Fidelity DNA polymerase (Invitrogen) as per Section 2.2.5.3.2 with the modification of a longer (2.5 min) extension time. PCR products were examined on ethidium-bromide stained 0.75% (w/v) agarose gel (as per Section 2.2.5.3.1). PCR products of the appropriate size were purified as per Section 2.2.5.4.1 and cloned into pGEM[®]-T Easy as per Section 2.2.5.4. Clones containing the promoter fragment were verified by sequencing using the SP6 and T7 sequencing primers (Section 2.2.5.5; Appendix 1).

3.2.5 *In silico* promoter analysis

Promoter sequences were analysed using the GCG WebANGIS Wisconsin software package operated by the Australian National Genomic Information Service (<http://www.angis.su.oz.au/>). The grapevine *GLP* gene promoter sequences and 5' UTR were scanned for the presence of *cis*-elements registered with the PLACE database (<http://www.dna.affrc.go.jp/htdocs/PLACE>; Higo *et al.* 1999) and the PlantCARE Web

site (<http://intra.psb.ugent.be:8080/PlantCARE>; Lescot *et al.* 2002). Pairwise sequence comparisons between the promoter sequences were done using the PromoterWise program from the European Bioinformatics Institute (EBI; <http://www.ebi.ac.uk/Wise2/promoterwise.html>), using the default settings. As selected by Duan and Schuler (2005) for the *A. thaliana* cytochrome P450 monooxygenase promoters, direct and inverted matches in a continuous region of seven or greater nucleotides containing at least two guanosines or cytosines were designated as conserved elements.

3.2.6 Vector construction

3.2.6.1 *VvGLP3* promoter: *GUS* construct

A 1803 nt fragment of the *VvGLP3* promoter was PCR amplified from *V. vinifera* cv. Chardonnay genomic DNA (as per Section 3.2.4.2) using PLATINUM *Taq* High Fidelity DNA polymerase (Invitrogen) as per Section 3.2.4.2 and appropriate primers (Appendix 1). The forward primer GLP3Fc (located 1810 nt upstream of the start codon; Appendix 1) contained a *Hind*III site and the reverse primer GLP3Rd (located 7 nt upstream of the start codon; Appendix 1) contained a *Bam*HI site. PCR products were examined on an ethidium-bromide stained 0.75% (w/v) agarose gel and PCR products were purified, and cloned into the pGEM[®]T-Easy vector (as per Section 2.2.5.4). Promoter fragments were digested with *Hind*III / *Bam*HI to release the desired promoter fragment for ligation into the multiple cloning site of pCAMBIA 1391Z binary vector (CAMBIA, Canberra, Australia; Fig. 3.1A) after cutting with *Hind*III / *Bam*HI.

3.2.6.2 VvGLP: GFP constructs

VvGLP ORFs (VvGLP2, VvGLP3 and VvGLP6) were PCR amplified (as per Section 2.2.5.3.2) from cDNA synthesised (Section 2.2.5.2) using the oligo(dT) adapter B26 primer (Appendix 1) from total RNA extracted (as per Section 2.2.5.1) from *V. vinifera* cv. Chardonnay powdery mildew-infected leaves. Primers were designed for GLP ORFs with the forward primer containing an *Xho*I site and the native start codon. The reverse primer contained an *Xba*I site with no stop codon. The VvGLP2 fusion contains 670 nt of VvGLP2 nucleotide sequence, and was amplified using GLP2Fa and GLP2Rf primers (Appendix 1). The VvGLP3 fusion contains 696 nt of VvGLP3 nucleotide sequence, and was amplified using GLP3Fa and GLP3Re primers (Appendix 1). The VvGLP6 fusion contains 645 nt of VvGLP6 nucleotide sequence, and was amplified using GLP6Fb and GLP6Rc primers (Appendix 1).

PCR products were examined on an ethidium bromide-stained 1.2% (w/v) agarose gel, purified, and cloned into pGEM[®]T-Easy vector as per Sections 2.2.5.4. Clones encoding the VvGLP ORFs were verified by sequencing using the SP6 and T7 sequencing primers (Section 2.2.5.5, Appendix 1). GLP ORF clones were digested with *Xho*I / *Xba*I (Roche Diagnostics) to release the ORF fragments for ligation into the multiple cloning site of *Xho*I / *Xba*I digested modified pART7 vector (Gleave, 1992). The modified vector contained the full-length GFP ORF, without a start codon but containing a stop codon (T. Franks, unpublished data), permitting in-frame C-terminal fusions of the GLP ORFs with the GFP ORF (Fig. 3.1B). Modified pART7 vector containing the full-length GFP ORF, with a start and stop codon (T. Franks, unpublished data), was used as a control in this experiment.

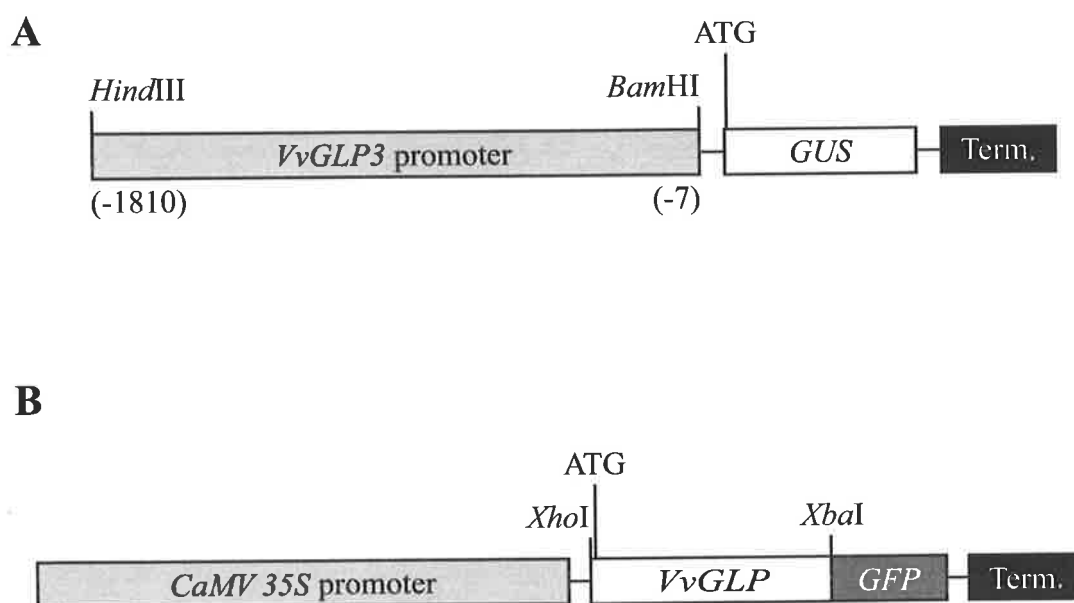


Figure 3.1. Constructs used for tissue-specific localisation and targeting of VvGLPs. **(A)** VvGLP3 promoter: *GUS* construct. A 1803 bp fragment of the VvGLP3 promoter was PCR amplified from *V. vinifera* cv. Chardonnay genomic DNA. The forward primer (located 1810 nt upstream of the start codon) contained a *Hind*III site and the reverse primer (located 7 nt upstream of the start codon) contained a *Bam*HI site. **(B)** VvGLP: *GFP* constructs. VvGLP ORFs (VvGLP2, VvGLP3 and VvGLP6) were PCR amplified from total RNA extracted from *V. vinifera* cv. Chardonnay powdery mildew-infected leaves. Primers were designed for VvGLP ORFs with the forward primer containing an *Xho*I site and the native start codon. The reverse primer contained an *Xba*I site with no stop codon. The constructs are not drawn to scale. Abbreviations used: CaMV 35S; Cauliflower Mosaic Virus 35S promoter; *GFP*, green fluorescent protein gene from *Aequorea victoria*; *GUS*, *gusA* gene; Term., nopaline synthase terminator sequence.

3.2.7 Transformation of *A. thaliana*

3.2.7.1 *Agrobacterium tumefaciens* transformation

Constructs were electroporated into *A. tumefaciens* strain EHA105 as per Section 2.2.5.4.3, except that cells were incubated at 28°C for 2 h to allow expression of antibiotic-resistance genes. Aliquots (100, 300 and 600 µL) of the transformed cells were spread on 1.2% LB agar (Table 2.1) containing 50 µg mL⁻¹ rifampicillin and 50 µg mL⁻¹ kanamycin, and incubated for 2 d at 28°C for selection of transformants. Single *A.*

tumefaciens colonies were used to inoculate LB (Table 2.1) liquid cultures with 50 µg mL⁻¹ rifampicillin and 50 µg mL⁻¹ kanamycin. Cultures were incubated for 2 d at 28°C with shaking at 200 rpm.

3.2.7.2 *A. thaliana* floral dip transformation

Transformation of *A. thaliana* Col-4 was performed using a modified floral dipping method (Clough and Bent, 1998). *Agrobacterium* was grown in 250 mL liquid LB culture (Table 2.1) containing rifampicillin (50 µg mL⁻¹) and kanamycin (50 µg mL⁻¹) at 28°C and shaking at 200 rpm. Cultures were started from a 1:25 dilution of a 10 mL overnight culture and grown for 24 to 32 h. Cells were harvested by centrifugation at 5,000 g for 5 min at 4°C and re-suspended in 5% sucrose to a final OD₆₀₀ of 0.6 to 1.0 (typically 400 to 600 mL). For floral dip the inoculum was transferred to a beaker and supplemented with 0.05% (v/v) Silwet L-77.

A. thaliana Col-4 plants were grown to flowering as per Section 3.2.2. After most plants had formed primary bolts, inflorescences were cut to encourage synchronised emergence of multiple secondary bolts. Plants were dipped when most secondary inflorescences were 2 to 10 cm long. Plants were inverted and the inflorescences were submerged in the liquid and subjected to gentle agitation for 30 s. Dipped plants were transferred to plastic trays and covered in clear plastic and left in a low light overnight. Plants were grown for a further week before re-dipping and subsequently grown for a further 3 to 5 weeks until siliques were brown and dry. Seeds were harvested over the following 2 weeks and stored at 4°C under desiccation.

3.2.7.3 Selection of *A. thaliana* transgenic plants

Seeds were vernalised for 3 to 5 d at 4°C and surface sterilised with 50 mL sterilising solution (Table 3.1) for 5 min followed by three washes with 100% (v/v) ethanol. Seeds were dried in a laminar flow for 1 to 2 h. Transgenic plants were selected on germination plates (Table 3.1) containing 35 µg mL⁻¹ hygromycin. Plates were sealed with surgical tape, cold treated for 2 d and grown for 2 weeks in a controlled environment at 24°C under constant light. Transformants were selected as seedlings that generated green leaves and extensive roots within the selective media. After the development of three to five adult leaves transformants were transferred to moistened potting soil and covered in clear plastic. Plastic covers were gradually opened over the subsequent 2 weeks.

3.2.7.4 PCR screening of *A. thaliana* transformants

Putative transformants were PCR screened using *GUS* primers, GUSFWD and GUSREV (Appendix 1) designed to amplify an 805 bp product of the *GUS* gene. DNA was extracted using a method adapted from Edwards *et al.* (1991). Leaf tissue (3 to 4 mg) was collected in an Eppendorf tube and macerated using a disposable pestle for 10 s at room temperature. Six hundred µL of *Arabidopsis* extraction buffer (Table 3.1) was added and the sample was vortexed for 5 s. The mixture was left at room temperature until all the samples had been extracted (up to 1 h). The extracts were centrifuged at 16,000 g for 90 s and 300 µL of the supernatant was transferred to a clean Eppendorf tube. The supernatant was mixed with 300 µL of 100% isopropanol and left at room temperature for 2 min. Following centrifugation at 16,000 g for 5 min, the pellet was washed twice with 80% (v/v) ethanol and dried under vacuum for 10 min. The resulting

pellet was resuspended in 100 μ L of TE Buffer (Table 2.1) containing 0.7% (v/v) RNase cocktail [500 U mL^{-1} RNase A; 20,000 U mL^{-1} RNase TI (Ambion)] and stored at -20°C . PCR reactions (20 μ L) contained 1 μ L genomic DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 μ M dNTPs, 1.5 mM MgCl_2 , 0.5 μ M forward primer, 0.5 μ M reverse primer and 0.5 U PLATINUM *Taq* DNA polymerase (Invitrogen). PCR conditions were as follows: 94°C for 3 min, 30 cycles of 94°C for 45 sec, 55°C for 30 sec, and 72°C for 2.5 min, with a final extension for 10 min at 72°C .

3.2.8 Transient transformation of onion epidermal cells

Gold particles, 1.0 μm in diameter (12 mg), were vortexed in 200 μ L of 100% (v/v) ethanol for 1 min, centrifuged for 10 s, washed twice with 100 μ L sterile water and resuspended in 200 μ L glycerol to produce a final concentration of 60 $\mu\text{g } \mu\text{L}^{-1}$. This suspension (6.5 μ L) was removed and added to 2 μ L of 0.75 $\mu\text{g } \mu\text{L}^{-1}$ construct. While gently vortexing, 10 μ L of cold 0.1 M spermidine and 25 μ L of 2.5 M CaCl_2 were added slowly. After vortexing for 3 min the resulting mixture was incubated on ice for 10 min. The particles were spun down for 10 s in a microfuge, washed once with 100 μ L cold 70% (v/v) ethanol, once with 100 μ L cold absolute ethanol and resuspended in 22 μ L cold absolute ethanol. The final mixture (6 μ L) was placed on a sterile filter holder and dried in a desiccator for 15 min. Epidermal strips were removed from onion bulbs using sterile tweezers and were placed on 1.2% (w/v) agar and bombarded at 620 kPa of helium after evacuating the chamber to -90 kPa.

3.2.9 Visualisation of GUS in *A. thaliana*

Histochemical GUS staining was carried out according to the procedure of Jefferson (1987). Tissues were immersed in GUS staining solution (Table 3.1) and placed in a vacuum chamber and evacuated until the solution began to bubble. The plates were sealed and incubated at 37°C for 2 h. GUS staining was observed using a Zeiss Axioskop2 microscope and recorded using a Spot digital camera (Diagnostic Instruments, SciTech, Australia).

3.2.10 Visualisation of GFP in onion epidermal cells

Tissue was stored in the dark for 48 h, and observed using a Leica stereomicroscope coupled to a fluorescence module. Epidermal peels were mounted on microscope slides in 1× PBS (Table 3.1). To visualise GFP in the nucleus, epidermal peels were incubated in 1.25 µg ml⁻¹ 4', 6-diamino-2-phenylindole dihydrochloride (DAPI) for 1 h before being mounted on microscope slides in 1× PBS. GFP in the cell wall was visualised by incubating epidermal peels in 0.8 M mannitol solution for 1 h before being mounted on microscope slides in 1× PBS. Detailed images were collected using a Bio-Rad Radiance 2100 Confocal Laser Scanning Microscope System (The Hanson Institute Detmold Family Trust Cell Imaging Centre, Institute for Medical and Veterinary Science, Adelaide, Australia). The excitation wavelength used for GFP detection was 488nm.

3.3 Results

3.3.1 Cloning of *VvGLP* promoter regions

Promoter fragments were isolated from a Shiraz genomic DNA promoter-walking library for six of the grapevine *GLP* genes (*VvGLP1* to 6). Several attempts to isolate the *VvGLP7* promoter from the Shiraz library were unsuccessful, suggesting that this promoter fragment was not present in the library. As the *VvGLP3* promoter fragment was originally isolated from Shiraz tissue, and the *VvGLP3* cDNA was cloned from Chardonnay tissue (Chapter 2), a *VvGLP3* promoter fragment was also isolated from Chardonnay genomic DNA for sequence comparison and functional analysis. The *VvGLP3* promoters isolated from Shiraz and Chardonnay share 99% nucleotide identity (data not shown). For the remaining grapevine *GLPs*, for which functional analyses were not undertaken, the Shiraz promoter sequence alone was isolated and subjected to *in silico* analysis. For *VvGLP1*, *VvGLP2*, *VvGLP3*, *VvGLP4*, *VvGLP5* and *VvGLP6*, 1145, 1879, 1849, 1149, 2155 and 2109 bp promoter sequence was obtained, respectively (Appendix 5).

3.3.2 Tissue-specific localisation of *VvGLP3* expression

Genomic DNA from hygromycin resistant *A. thaliana* progeny were PCR screened for the presence of the *VvGLP3* promoter: *GUS* fusion (Fig. 3.2). All eighteen T₁ hygromycin resistant progeny examined were positive for the 805 bp PCR product (1-18) and were transferred to soil.

Histochemical analysis of GUS activity in leaves of two representative T₁ transgenic plants (lines 13 and 14) was undertaken. To determine whether the *VvGLP3* promoter:

GUS construct was functional in *A. thaliana*, leaves naturally infected with *E. cichoracearum*, were chosen for GUS staining. Significantly, GUS staining was only observed in epidermal cells (Fig. 3.3). By varying the focal plane, it was also possible to observe that GUS staining was only present in cells in contact with fungal hyphae, but was not present in adjacent epidermal cells, nor was it present in mesophyll cells beneath the attacked epidermal cell. Furthermore, GUS staining was not present in all epidermal cells in contact with fungal hyphae, but rather appeared to be associated with fungal hyphae that produced appressoria. Visualisation of haustoria was not possible using this technique. Consequently, it could not be determined whether GUS staining was differentially present in cells infected with *E. cichoracearum* compared to cells that were successful in preventing penetration.

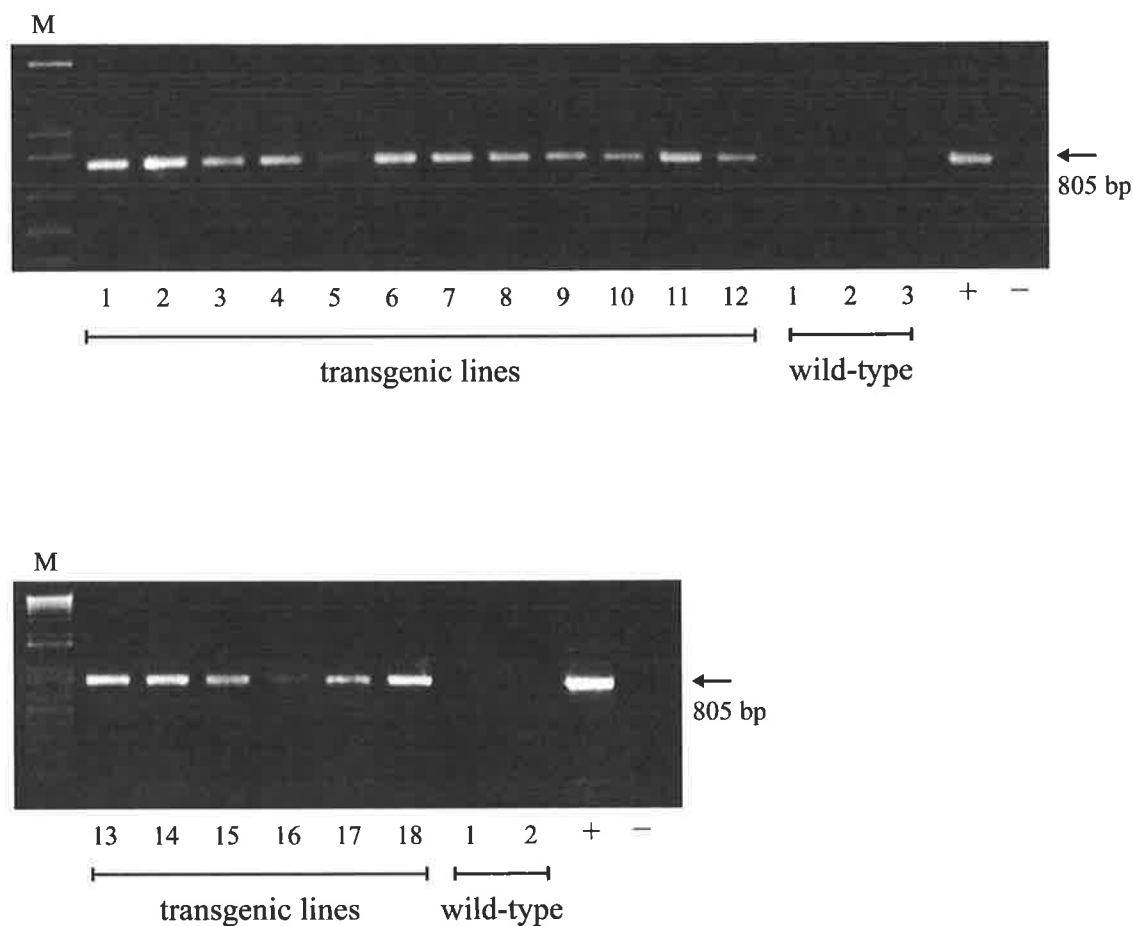


Figure 3.2. PCR analysis of T₁ *A. thaliana* transgenic lines. Gene-specific primers PCR amplified a 805 bp fragment of the *GUS* gene. Genomic DNA was extracted from hygromycin resistant T₁ plants (lines 1 to 18) transformed with the *VvGLP3* promoter: *GUS* fusion and untransformed wild-type *A. thaliana* Col-4 plants (1 to 3). Positive control (+), DNA extracted from *Agrobacterium* transformed with the *GUS* gene. Negative control (-), minus DNA. M, 1kb+ DNA marker (Invitrogen). PCR products were separated on ethidium bromide-stained agarose gels.

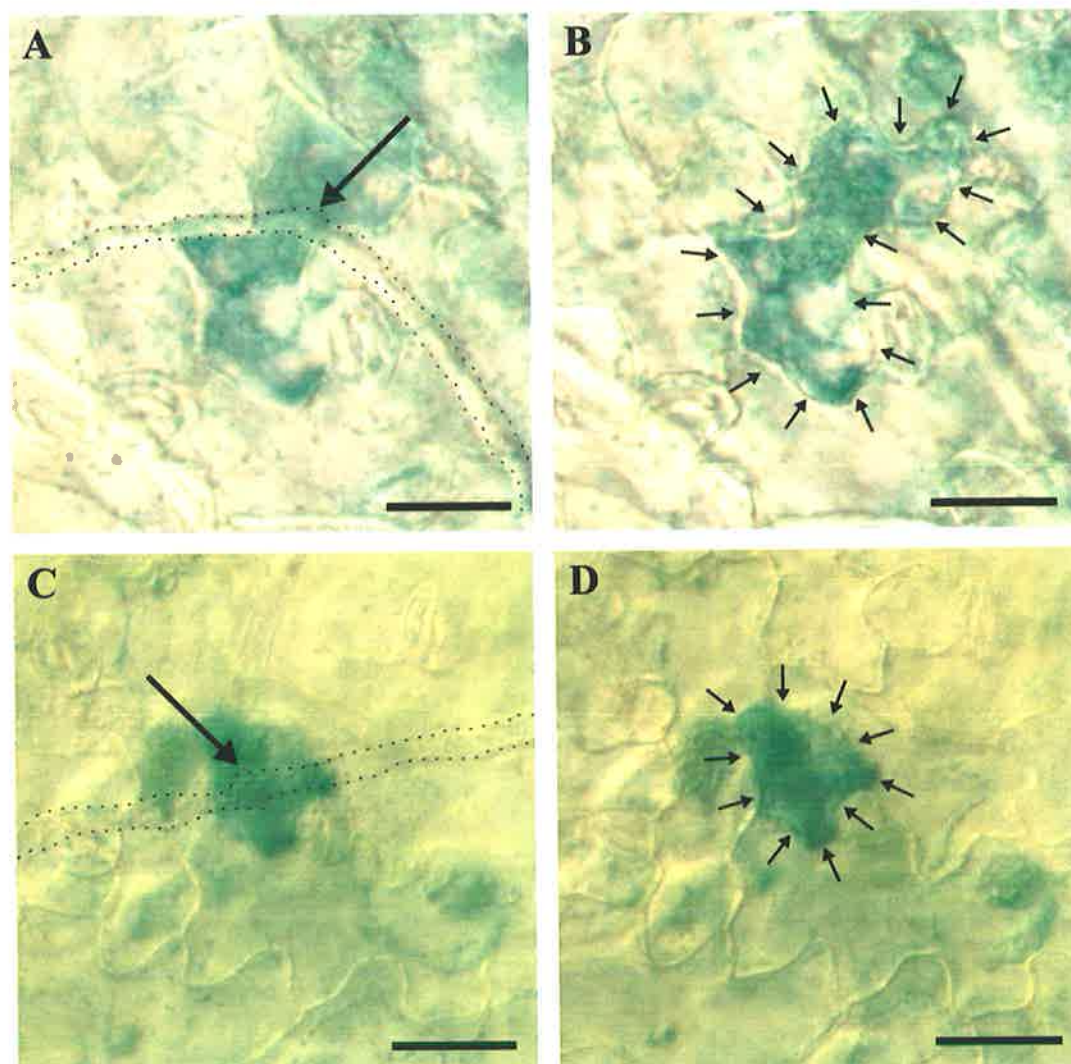


Figure 3.3. Tissue-specific expression of *VvGLP3*. Histochemical staining for GUS in leaves of transgenic *A. thaliana* plants transformed with the *VvGLP3* promoter: *GUS* fusion. Leaves were infected with *E. cichoracearum*. Two representative T_1 lines are presented: line 3 (A and B) and line 4 (C and D). In panels (A) and (C) the focal plane is at the level of the hyphae on the leaf surface whereas panels (B) and (D) are focussed at the level of the epidermal cell. Dots, fungal hyphae; large arrows, fungal appressorium; small arrows, GUS stained epidermal cell. Scale bar denotes 20 μ M.

3.3.3 *In silico* analysis of VvGLP promoters

Global analysis of the grapevine *GLP* promoters was undertaken in an attempt to identify putative *cis*-elements responsible for the specific expression patterns observed (Chapter 2, Fig. 3.3). Analysis was restricted to 1000 nt immediately upstream from the translational start codon as used Seki *et al.* (2001) for analysis of *A. thaliana* promoter sequences. For *VvGLP1*, *VvGLP2*, *VvGLP3*, *VvGLP4*, *VvGLP5* and *VvGLP6*, potential TATA boxes (Shirsat *et al.* 1989, Tjaden *et al.* 1995, Grace *et al.* 2004) were located at 125, 164, 81, 94, 77 and 76 nt immediately upstream of the translational start codon, respectively (Appendix 5). Potential CAAT boxes (Shirsat *et al.* 1989) were identified for *VvGLP1*, *VvGLP4*, *VvGLP5* and *VvGLP6* at 63, 164, 121 and 119 nt immediately upstream of the translational start codon, respectively (Appendix 5). For *VvGLP2* and *VvGLP3* CAAT motifs were located much further upstream at 465 and 287 nt respectively.

Numerous regulatory elements from tissue-specific and stress response genes were identified in the grapevine *GLP* promoters using *in silico* analysis (Table 3.2), including known elements responsive to salicylic acid, wounding, ethylene, methyl jasmonate, auxin, gibberellic acid, abscisic acid, heat stress, low temperature and drought. Although many regulatory elements were identified within the promoter sequences, there does not appear to be consistent agreement between the known *cis*-elements and the expression of the individual grapevine *GLP* genes as determined using RT-PCR analysis (Chapter 2). For example, *VvGLP3* is not induced in grape leaves in response to treatment with methyl jasmonate, salicylic acid, synthetic auxin or abscisic acid (Fig. 2.16) and, in agreement with this, was not found to contain putative promoter elements for these

inducers (Table 3.2). However, *VvGLP3* was also not induced by treatment with ethylene (Fig. 2.16) or following wounding (Fig. 2.15) but analysis of the cloned *VvGLP3* promoter fragment indicated it did contain putative elements responsive to these two abiotic treatments (Table 3.2). In fact, the wounding responsive WUN motif (Pastuglia *et al.* 1997) is present in all of the grapevine promoter fragments analysed (Table 3.2) whereas, *VvGLP2* was the only grapevine *GLP* identified by RT-PCR expression analysis (Fig. 2.15) to be wound-responsive. Of the disease-responsive elements identified (Table 3.2), the W box element WBOXATNPR1 (Yu *et al.* 2001, Chen *et al.* 2002) was present in all *GLP* promoter fragments analysed whereas, the W box element WBOXPCWRKY1 (Eulgem *et al.* 1999, Eulgem *et al.* 2000; Table 3.2) was only present in the promoter fragments of powdery mildew-inducible *HvGLP4* gene from barley (Wei *et al.* 1998) and *VvGLP2* which is not responsive to *E. necator* infection (Fig. 2.9).

In order to try and identify elements responsible for the powdery mildew-inducible nature of *VvGLP3*, its promoter sequence was compared directly with the promoter sequences of other powdery mildew-inducible genes *VvGLP4* (Fig. 2.9) and barley *HvGLP4* for any novel conserved *cis*-elements. A similar approach was used to identify novel elements in the promoters of P450 monooxygenase genes (Duan and Schuler 2005). Direct and inverted matches in a continuous region of seven or greater nucleotides containing at least two guanosines or cytosines were designated as conserved elements. The results shown in Table 3.3 indicate that the *VvGLP3/VvGLP4* promoter regions share ten conserved sequence elements, whereas fourteen are shared by *VvGLP3/HvGLP4*. The longest conserved sequence was 16 nt between the

VvGLP3/VvGLP4 promoter pair. However, using the specified search parameters no conserved sequences were identified which were common in all three powdery mildew-inducible *GLP* promoter sequences.

Table 3.2. *In silico* promoter analysis. *Cis*-elements identified within the *HvGLP4* and *VvGLP* promoter sequences from searches of the PLACE and PlantCARE databases. *HvGLP4* promoter GenBank accession number, AJ310534. Analysis was restricted to 1000 nt immediately upstream from the translational start codon. Promoter elements are described in: WBOXATNPR1 (Yu *et al.* 2001, Chen *et al.* 2002), WBOXPCWRKY1 (Eulgem *et al.* 1999, Eulgem *et al.* 2000), WUN motif (Pastuglia *et al.* 1997), TCA element (Pastuglia *et al.* 1997), CGTCA motif (Rouster *et al.* 1997), ERE (Itzhaki *et al.* 1994), AuxRR-core (Sakai *et al.* 1996), AuxRE (Liu *et al.* 1997), TGA box (Liu *et al.* 1997), ABRE (Baker *et al.* 1994), P box (Washida *et al.* 1999), TATC box (Washida *et al.* 1999), HSE (Pastuglia *et al.* 1997), LTR (Dunn *et al.* 1998), MBS binding site (Yamaguchi-Shinozaki and Shinozaki 1994), As1 (Yamaguchi-Shinozaki and Shinozaki 1994). I, same element in inverted direction on the promoter.

<i>Element</i>	<i>Inducer</i>	<i>HvGLP4</i>	<i>VvGLP3</i>	<i>VvGLP4</i>	<i>VvGLP1</i>	<i>VvGLP2</i>	<i>VvGLP5</i>	<i>VvGLP6</i>
WBOXAT NPR1	Disease	383, 483, 790, 956	265 (I), 419, 836	88, 836 (I), 921	118	309 (I), 642, 900	814 (I)	319, 365
WBOXP CWRKY1	Disease	484				310		
WUN motif	Wounding	14 (I), 74, 163, 304, 340, 845 (I), 876	310, 345 (I), 453, 656 (I), 794 (I), 902, 950, 998	69, 498, 711 (I), 730 (I), 910 (I)	303 (I), 801 (I)	154, 481, 506 (I), 748 (I), 154 (I)	51, 177 (I), 200 (I), 236 (I), 384 (I), 424 (I), 444 (I), 480 (I), 664, 700 (I), 932	310 (I), 354 (I), 383, 613 (I), 640 (I), 731 (I), 938 (I), 780 (I), 908
TCA element	SA	392 (I), 533			625	105 (I)		895, 940, 959
CGTCA motif	MeJA					200, 641 (I)		272, 318 (I)

Element	Inducer	<i>HvGLP4</i>	<i>VvGLP3</i>	<i>VvGLP4</i>	<i>VvGLP1</i>	<i>VvGLP2</i>	<i>VvGLP5</i>	<i>VvGLP6</i>
ERE	Ethylene	161	346 (I), 584 (I)		304 (I), 765 (I)	975 (I), 976	930	355 (I), 381, 732 (I), 733
AuxRR-core	Auxin	168 (I)			297 (I), 569			500 (I)
AuxRE	Auxin						847 (I)	
TGA box	Auxin					203 (I)		318
ABRE	ABA			54 (I)				317
P box	GA	215 (I), 888	232 (I), 296 (I), 844	28 (I)	67 (I), 417 (I), 439 (I), 810 (I)	276, 521 (I), 723, 966 (I)	307 (I), 565 (I)	
TATC box	GA	62	673	139 (I)	652, 674 (I)	79 (I)	842 (I)	459 (I)
HSE	Heat stress	800 (I), 202, 315, 366 (I), 392 (I), 416 (I), 692 (I), 932, 979	180 (I), 318, 342 (I), 344, 402 (I), 415 (I), 891 (I)	70 (I), 276 (I), 943 (I)	99 (I), 101, 153, 619, 661 (I), 755	22 (I), 774 (I), 856	52 (I), 519, 745, 999 (I)	102, 147 (I), 228, 251 (I), 343 (I), 384 (I), 384, 878, 892 (I), 897 (I), 933, 953, 997
LTR	Low temp	337 (I)				406 (I)		
MBS binding site	Drought	139 (I), 494		286, 570 (I)			652, 819 (I)	
As1	Root		836					275 (I), 318

Table 3.3. Elements conserved between the *VvGLP3*, *VvGLP4* and *HvGLP4* promoters. Numbers indicate the position of the elements identified relative to the ATG start codon. Pairwise sequence comparisons were performed using the European Bioinformatics Institute program PromoterWise. With the exception of *VvGLP4* (1146 nt), analysis was restricted to 1800 nt immediately upstream from the translational start codon. Direct and inverted matches in a continuous region of seven or greater nucleotides containing at least two guanines or cytosines were designated as conserved elements. I, same element in inverted direction on the promoter. *HvGLP4* promoter GenBank accession number, AJ310534.

Sequence	Length	<i>VvGLP3</i>	<i>VvGLP4</i>	<i>HvGLP4</i>
GAATGGTTCT	10	1743	808 (I)	
GTTTCATT	7	1196	1120 (I)	
AAAATGAAG	9	918	347 (I)	
AAATCCTTGT	10	791	37 (I)	
TGTAGTGCTAG	11	599	1145 (I)	
TAACCATGGA	10	368	599 (I)	
GAAGACT	7	190	161	
GACTAATTCAAGGACT	16	103	116	
CATGTTTCC	9	71	84	
ATTTCTCACC	10	55	67	
GCAAGCA	7	1538		100
TCAAGAAAATGA	12	1377		392 (I)
GAGAAACA	8	1318		316
ACAGAATGC	9	1287		1791 (I)
TGATGATGGA	10	1230		917 (I)
ATTGCAA	7	1072		1414 (I)
CATTGCTT	8	1533		244
ATCAAATG	8	935		1159 (I)
TCATGAAATCC	11	696		724 (I)
TGATGTGT	8	488		377
CCATGTCA	8	270		509 (I)
AAAAGGAGA	9	231		891 (I)
TAGTTTGAAAA	11	214		683
CAAGAAAAA	9	116		1312 (I)

3.3.4 Cellular targeting of VvGLPs

To further investigate the potential roles of grapevine GLPs *in vivo*, the reporter gene *GFP* was fused C-terminal to the *VvGLP3*, *VvGLP2* and *VvGLP6* ORFs downstream of the CaMV 35S promoter. The fusion proteins were transiently expressed in onion epidermal cells following biolistic transformation and detected 48 h post-bombardment using a confocal laser-scanning microscope. The targeting of VvGLP: GFP fusion proteins were observed in multiple independent bombardment experiments. Untargeted free GFP (35S-GFP) was distributed throughout the cytoplasm and nucleus (Fig. 3.4) whereas distinct patterns of localisation were observed for each of the VvGLP: GFP fusion proteins (Fig 3.4).

VvGLP3:GFP was found to be localised to both the cell periphery and the nuclear envelope (Fig 3.4). The nuclear localisation of VvGLP3:GFP was confirmed by staining transformed cells with the nucleic acid stain (DAPI) and merging the DAPI and GFP images (Fig. 3.5). In order to determine whether the VvGLP3:GFP fusion protein at the cell periphery is located internal or external to the plasma membrane, onion epidermal cells were plasmolysed, resulting in shrinkage of the cell protoplasts and retraction of the plasma membranes from the cell walls. Although VvGLP3:GFP was observed to retract with the plasmolysed plasma membrane, a significant proportion of VvGLP3 remained within the cell wall, suggesting that the VvGLP3 protein is targeted to the extracellular space (Fig 3.6).

The cellular localisation of powdery mildew-inducible VvGLP3, grouped in GLP subfamily 1 (Fig. 2.7) was compared to the localisation of VvGLP2 (GLP subfamily 2)

and VvGLP6 (GLP subfamily 3). VvGLP2:GFP was found to be associated with punctate bodies at the cell periphery (Fig. 3.6) consistent with either a Golgi-localised (Baldwin *et al.* 2001, Nebenfuhr *et al.* 1999) or ER-localised protein (Kim *et al.* 2004). Unlike VvGLP3:GFP however, VvGLP2:GFP did not localise to the cell wall in plasmolysed onion cells (Fig. 3.6), suggesting that this protein is not extracellular.

The targeting of VvGLP6:GFP was distinctly different from that observed for VvGLP3:GFP and VvGLP2:GFP (Fig. 3.4). Although VvGLP6:GFP was observed at the cell periphery, it was also present throughout the cell in filamentous bundles reminiscent of that observed for proteins labelling the actin in the cytoskeleton (Sheahan *et al.* 2004).

Figure 3.4. Targeting of VvGLP: GFP fusion proteins within onion cells. *VvGLP3:GFP*, *VvGLP2:GFP*, *VvGLP6:GFP* and free *GFP* were expressed in onion epidermal cells using a CaMV 35S promoter following biolistic delivery of vector DNA. Tissue was stored in the dark for 48 h. Epidermal peels were mounted on microscope slides in 1× PBS. Cells were analysed for GFP fluorescence using a confocal laser-scanning microscope. Merged z-series images and individual z-series images are shown in the left and right column, respectively. Nucleus localised protein is indicated by arrows. Bar = 100µm.

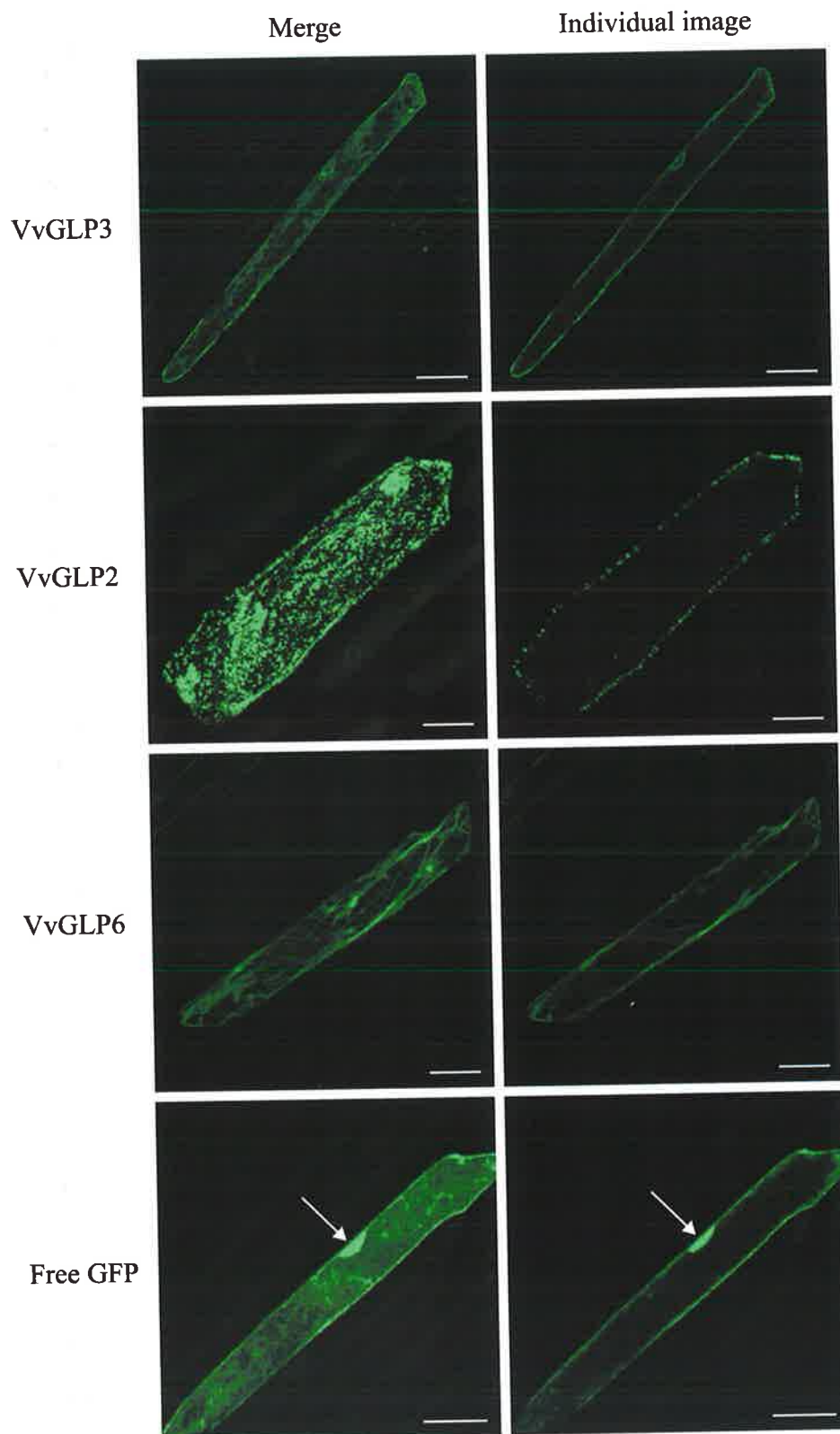
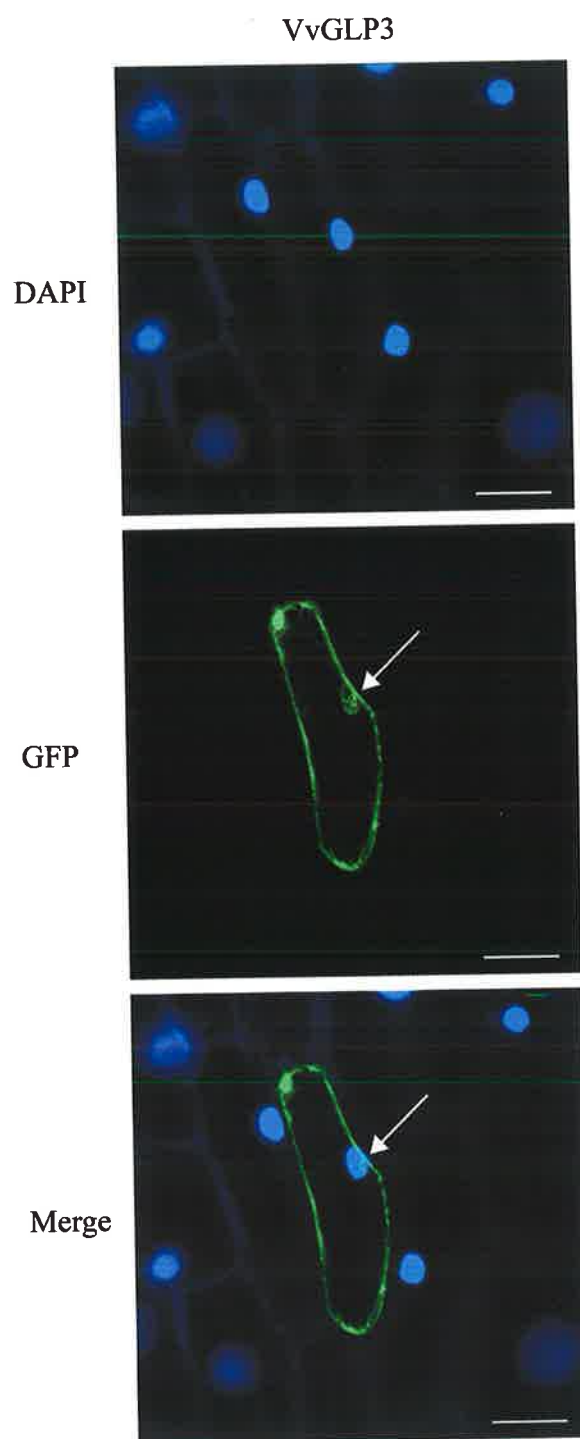


Figure 3.5. Targeting of VvGLP3:GFP within onion cells treated with DAPI. *VvGLP3:GFP* was expressed in onion epidermal cells using CaMV 35S promoter following biolistic delivery of vector DNA. Tissue was stored in the dark for 48 h. To visualise GFP in the nucleus, epidermal peels were incubated in $1.25 \mu\text{g ml}^{-1}$ DAPI for 1 h before being mounted on microscope slides in $1\times$ PBS. Cells were analysed for GFP (middle column) and DAPI (left column) fluorescence using a confocal laser-scanning microscope. GFP/DAPI merge image is shown in the right column. Nucleus localised protein are indicated by arrows. Bar = $100\mu\text{m}$.



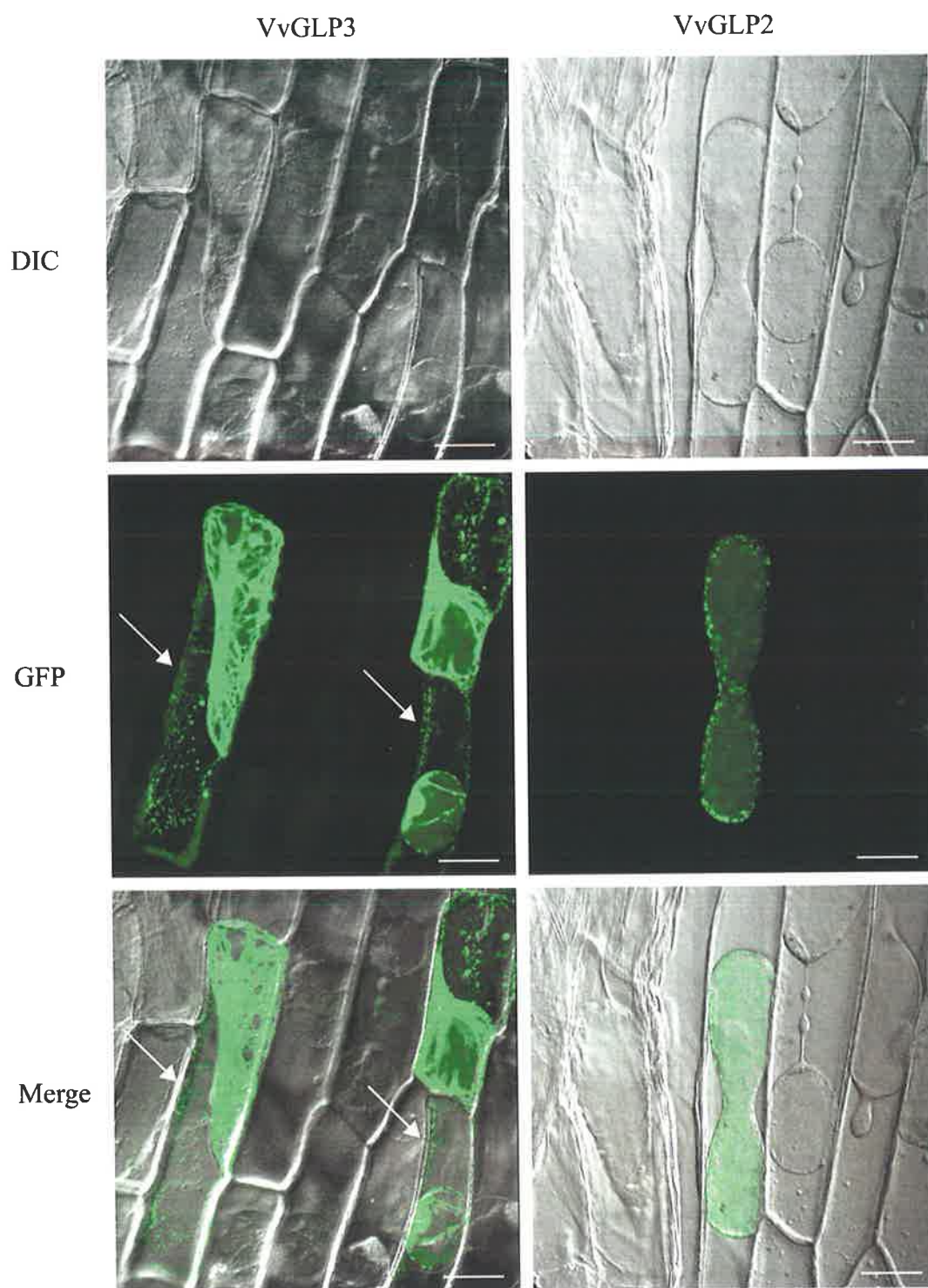


Figure 3.6. Targeting of VvGLP3:GFP and VvGLP2:GFP within onion cells treated with mannitol. VvGLP3:GFP localises to the cell wall of onion cells (top row) and VvGLP2:GFP localises to the plasma membrane (bottom row). *VvGLP3:GFP* and *VvGLP2:GFP* were expressed in onion epidermal cells using CaMV 35S promoter following biolistic delivery of vector DNA. Tissue was stored in the dark for 48 h. To visualise GFP in the cell wall, epidermal peels were incubated in 0.8 M mannitol solution for 1 h before being mounted on microscope slides in 1× PBS. Cells were analysed for GFP fluorescence (middle column) using a confocal laser-scanning microscope. Differential interference contrast (DIC) images and GFP/DIC merge images are shown in the left and right columns, respectively. Arrows indicate cell wall localised protein. Bar = 100µm.

3.4 Discussion

3.4.1 Tissue-specific expression of VvGLP3

The localisation of *VvGLP3* promoter driven powdery mildew-induced expression specifically in epidermal tissue (Fig. 3.3), where *E. cichoracearum* infection takes place (Adam and Somerville 1996), supports a role for this *GLP* in defence against powdery mildew. Significantly, the *VvGLP3* promoter is induced not only in *V. vinifera* in response to *E. necator*, but also functions in *A. thaliana* in response to *E. cichoracearum*. This finding demonstrates that the *VvGLP3* promoter functions not only in heterologous species, but also responds to infection by other powdery mildew species.

A number of plant promoters have been previously identified that are responsive to infection by biotrophic fungal pathogens (Ayliffe *et al.* 2002, Fotopoulos *et al.* 2003). The *fis1* gene of flax (*Linum usitatissimum*) was originally identified as a host gene up-regulated during a successful flax rust (*Melampsora lini*) infection (Roberts and Pryor 1995). This gene was induced during a compatible flax rust infection, but its induction was not detectable during an incompatible infection when rust growth was halted by the

action of the *L6* resistance gene, suggesting that this gene some role in supporting metabolism associated with the biotrophic interaction. Using promoter: *GUS* gene studies, Ayliffe *et al.* (2002) demonstrated the *fis1* promoter fragment directed *GUS* expression to leaf mesophyll cells within and immediately surrounding *M. lini* infection sites. Like *VvGLP3*, the *fis1* promoter is induced specifically by the rust pathogen and not by other fungal pathogens or by wounding (Mitchell *et al.* 2006). However, the *fis1* promoter has also been found to direct weaker but significant endogenous expression in reproductive structures such as the developing seed capsule, connective tissue between anther lobes and vascular tissues (Ayliffe *et al.* 2002, Mitchell *et al.* 2006).

Promoters controlling the expression of powdery mildew-inducible genes have also been characterised using promoter: reporter gene studies. Fotopoulos *et al.* (2003) demonstrated that the *A. thaliana* monosaccharide transporter *AtSTP4* promoter was induced in mature leaves following *E. cichoracearum* infection. The *AtSTP4* promoter was also responsible expression in root tissue. Furthermore, *E. cichoracearum*-inducible expression controlled by the *AtSTP4* promoter was not confined to infected epidermal cells, but also present in many other leaf cells.

A number of studies have also previously demonstrated the activity of *GLP* promoters, isolated from various plant species, in transgenic plants (Berna and Bernier 1997, Berna and Bernier 1999, Staiger *et al.* 1999, Carter and Thornburg 2003). Staiger *et al.* (1999) used promoter: reporter gene studies to demonstrate that *A. thaliana* *GLP Atger3* (GLP subfamily 3; Fig 2.7) promoter mediates organ-specific gene expression in transgenic *A. thaliana*. The *N. plumbaginifolia* *Nectarin I* (GLP subfamily 2; Fig. 2.7) promoter also

regulates highly specific expression in the mature floral nectaries of transgenic *N. tabacum* (Carter and Thornburg 2003). Expression mediated by the wheat germin, *gf-2.8* (true germin GLP subfamily; Fig. 2.7) promoter was also studied in transgenic *N. tabacum* plants (Berna and Bernier 1997, Berna and Bernier 1999). These studies demonstrated that *gf-2.8* promoter driven expression was developmentally regulated, stimulated by treatment with auxin (Berna and Bernier 1997) and induced in response to biotic and abiotic stresses, including TMV, wounding and treatment with heavy metals cadmium, copper and cobalt (Berna and Bernier 1999). Collectively, these reports demonstrate that the promoters of *GLP* genes grouped in different GLP subfamilies and isolated from various plant species are responsible for highly specific gene expression patterns. This study is the first reported characterisation of a promoter for a *GLP* gene grouped in GLP subfamily 1.

3.4.2 *In silico* promoter analysis

In an attempt to identify putative *cis*-elements responsible for powdery mildew-inducible expression of *VvGLP3*, promoter fragments for another five of the grapevine *GLP* genes (*VvGLP1*, *VvGLP2*, *VvGLP4*, *VvGLP5* and *VvGLP6*), in addition to powdery mildew-inducible *VvGLP3* promoter, were isolated. *In silico* analysis of these *VvGLP* promoter regions, together with the promoter sequence for the powdery mildew inducible *HvGLP4* gene from barley (Wei *et al.* 1998), identified a number of putative *cis*-elements responsive to disease, wounding, temperature stress, drought and various hormones and related molecules (including ethylene, methyl jasmonate, salicylic acid, synthetic auxin, abscisic acid and gibberellic acid) (Table 3.2).

Of the known disease responsive elements, GCC-like elements (Ohme-Tagaki *et al.* 2000) and W boxes (Eulgem *et al.* 2000) are well characterised. Although the GCC box is often found in the promoter regions of defence genes (Rushton *et al.* 2002), it was not present in the promoter fragments of powdery mildew-inducible *VvGLP3*, *VvGLP4* or *HvGLP4*. W Boxes, elicitor-responsive elements, are the binding sites for WRKY transcription factors (Rushton *et al.* 2002). Of the disease responsive W boxes identified (Table 3.2), WBOXATNPR1 (Yu *et al.* 2001, Chen *et al.* 2002) was identified in all *GLP* promoter fragments analysed whereas, WBBOXPCWRKY1 (Eulgem *et al.* 1999, Eulgem *et al.* 2000) was only present in the promoter fragments of powdery mildew-inducible *HvGLP4* (Wei *et al.* 1998) and *VvGLP2*. These results suggest that novel elements within the grapevine *GLP* gene promoters, that are not currently present in the databases searched, are likely to be responsible for the powdery mildew-inducible expression of *VvGLP3*.

Interestingly, the wounding responsive WUN motif (Pastuglia *et al.* 1997) was found to be present in all of the grapevine promoter fragments analysed (Table 3.2) but *VvGLP2* was the only grapevine *GLP* gene which responded to wounding treatment (Fig. 2.15).

Elements responsive to various plant hormones and related molecules were also identified in the grapevine *GLP* promoter sequences. However, there was no apparent relationship between the presence or absence of these putative promoter elements and the observed expression of the *VvGLP* genes in response to the various inducer treatments. Furthermore, the only tissue-specific element identified in the *VvGLP* promoter sequences was an As1 root-specific element (Yamaguchi-Shinozaki and

Shinozaki 1994) identified in the *VvGLP3* and *VvGLP6* sequences (Table 3.2). The presence of this tissue-specific element does not correlate with the observed expression of the *VvGLP* genes as neither *VvGLP3* nor *VvGLP6* are expressed in roots (Fig. 2.17). Similarly, the *As1* element was not present in grapevine *GLPs*, *VvGLP2* or *VvGLP1*, which are expressed in roots (Fig. 2.17).

Collectively, the specific expression patterns of the *VvGLP* genes observed using RT-PCR analysis (Chapter 2), could not be consistently correlated with the presence/absence of any published *cis*-acting regulatory elements (Table 3.2), emphasising that caution must be used in interpreting the output of *in silico* promoter analyses without empirical data to confirm the prediction.

An alternative *in silico* analysis approach was also undertaken to identify novel sequence elements conserved specifically in the promoter regions of the powdery mildew-inducible genes *VvGLP3*, *VvGLP4* and *HvGLP4*. This approach was used by Duan and Schuler (2005) to identify novel elements in the promoters of P450 monooxygenase genes. However, it should be noted that as functional analysis of these novel elements is yet to be undertaken, the usefulness of this approach is currently unknown. Using this approach, novel sequence elements of 7-16 nt in size were identified that were conserved in the promoter sequences of *VvGLP3* and either *VvGLP4* or *HvGLP4* (Table 3.3). Significantly, no sequence elements were identified that were conserved within all three promoter regions; however this may have been a function of the stringency of the analysis used. Alternatively, the powdery mildew inducible expression of these three genes may not be mediated by the same transcription factor

binding at the same promoter sequence element. For example, although there is good evidence to indicate that the *VvGLP3* (Figs. 2.9 and 3.3) and *HvGLP4* (Wei *et al.* 1998) genes are expressed specifically in epidermal cells, the actual tissue location of *VvGLP4* expression is unknown. The epidermal-specific expression of *VvGLP4* has been extrapolated from the observation that *VvGLP4* transcripts accumulate specifically within in the grape berry skin (Fig. 2.9). However, as the grape berry skin is actually composed of several cell layers including the epidermis and hypodermis (Ollat *et al.* 2002) it is possible that *VvGLP4* expression may not in fact be epidermal-specific. Consequently, different host or pathogen factors may be involved in the induction of *VvGLP4* expression, in response to powdery mildew infection, than with *VvGLP3* and *HvGLP4*.

3.4.3 Subcellular localisation of *VvGLP*

As many cellular processes are spatially constrained to distinct cellular compartments, protein subcellular localisation often provides clues as to the functional role of a protein (Nair and Rost 2002). In this study the localisation of *VvGLP* gene products was determined using GFP fusion constructs transiently expressed in onion cells. The results obtained with the *VvGLP3*:GFP expression construct suggest that *VvGLP3* is localised to the nuclear envelope and the cell periphery (Fig 3.4) and that at least a proportion of the *VvGLP3* protein is present within the extracellular compartment (Fig 3.6). The association of a GLP with the nuclear envelope was not predicted based on protein sequence analysis. One possible explanation for the observed nuclear association is that it may have arisen from over-expression, resulting in spill-over of protein to other cellular compartments (Brandizzi *et al.* 2003). As the nuclear envelope and the ER are

continuous, saturation of secretion to the extracellular space may have led to build-up of VvGLP3 in the nuclear envelope.

Extracellular proteins are thought to play a central role in plant defence responses against pathogens (Lee *et al.* 2004) and changes in abundance of plant-secreted proteins have been identified in response to fungal pathogens (Rep *et al.* 2002, Zareie *et al.* 2002). Significantly, Schweizer *et al.* (1999a) used a transient expression system (Schweizer *et al.* 1999b) to show that the reduced penetration efficiency of *B. graminis* resulting from over-expression of wheat GLP *TaGLP2a* relies on the presence of the TaGLP2 N terminal signal peptide. This finding demonstrates that secretion of TaGLP2a is essential for reduced susceptibility to *B. graminis* resulting from there transient over-expression of *TaGLP2a*.

The extracellular localisation of VvGLP3 is also consistent with the reported localisation of other GLPs. For many of the GLPs, at least a proportion of the protein is localised within the extracellular space (Lane *et al.* 1992, Heintzen *et al.* 1994, Ohmiya *et al.* 1998, Vallelian-Bindschedler *et al.* 1998, Membre *et al.* 2000). Lane *et al.* (1992) used colloidal gold-conjugated secondary antibody to identify that 40% of germin in germinating wheat embryos is localised to the cell wall. This extracellular localisation of germin was later confirmed by Membre *et al.* (2000) using transgenic tobacco plants over-expressing wheat germin. Heintzen *et al.* (1994) used immunogold labelling of *S. alba* leaves to demonstrate SaGLP protein is also predominantly associated with cell walls. Using cell fractionation studies, Vallelian-Bindschedler *et al.* (1998) established that HvGLP2 is loosely associated with the cell wall.

The localisation of powdery mildew-inducible VvGLP3, grouped in GLP subfamily 1 (Fig. 2.7) was also compared to the localisation of grapevine GLPs from the other two GLP subfamilies. Subfamily 2 GLP, VvGLP2, was associated with punctate bodies at the cell periphery (Fig. 3.4), comparable to that observed for the ER (Kim *et al.* 2004) and Golgi (Baldwin *et al.* 2001, Nebenfuhr *et al.* 1999) localised proteins. The punctate cell peripheral localisation observed for VvGLP2 is similar to that observed for cotton GLP, GhGLP1, using immunofluorescent labelling (Kim *et al.* 2004). By double-labelling fibre cells, 2 to 4 days post anthesis, with an antibody that recognises Golgi bodies, Kim *et al.* (2004) were able to demonstrate that there was no overlap in the signals. This result prompted the authors to suggest that GhGLP is targeted to the ER during early fibre development. Unlike VvGLP3, VvGLP2 did not localise to the cell wall in plasmolysed onion cells (Fig. 3.6), suggesting that this protein is not extracellular.

Like VvGLP2, the targeting of subfamily 3 grapevine GLP, VvGLP6 (Fig. 2.7), was also distinctly different to that observed for VvGLP3. Although VvGLP6 was observed at the cell periphery, it was also present throughout the cell in filamentous bundles (Fig. 3.4) similar to that observed for proteins labelling the actin in the cytoskeleton (Sheahan *et al.* 2004). The distinct cellular localisation observed for VvGLP6 has not been previously reported for GLPs from other plant species.

One interesting conclusion to be drawn from the results of the GFP targeting studies presented in this chapter is that they do not always agree with the predictions based on software prediction programs. VvGLP3, VvGLP2 and VvGLP6 all have putative N-

terminal signal peptides (Fig. 2.5), suggesting these proteins are targeted to the secretory pathway. Signal peptides target proteins to the ER, and it is known that proteins containing a C-terminal KDEL (Lys-Asp-Glu-Leu) or HDEL (His-Asp-Glu-Leu) sequence may be retained in the ER, whereas proteins without these sequences may continue to the Golgi complex (Denecke *et al.* 1992). As neither KDEL nor HDEL are present in the VvGLP3, VvGLP2 or VvGLP6 sequences (Fig. 2.5), these proteins would be predicted to proceed to the Golgi complex for further processing, rather than be retained in the ER. Furthermore, as these GLPs do not appear to contain predicted signal sequences targeting them to particular organelles, they may follow the default pathway and be transported to the plasma membrane. Proteins containing extensive hydrophobic regions may be embedded in the plasma membrane (Slabas *et al.* 2004) and the absence of extensive hydrophobic regions in the VvGLP3, VvGLP2 and VvGLP6 sequences suggest these proteins should be secreted into the extracellular space.

This apparent disagreement between predicted protein localisations based on localisation prediction programs and the observed localisation using fusion proteins has become apparent with the recent development of high-throughput screening techniques to functionally determine targeting of proteins within cells (Escobar *et al.* 2003). Curiously, these large-scale screens, involving the fusion of DNA encoding GFP to cDNA libraries, have frequently observed targeting of proteins which was not predicted by web-based localisation programs (Chivasa *et al.* 2002). Although in some instances these contradictory findings may have been the result of screening artefacts, others are thought to represent the correct localisation. More recently, Koroleva *et al.* (2005) used a high-throughput screen to determine the localization of 155 *A. thaliana* proteins in *A.*

thaliana. They found that in only 53% of the cases did the proteins show localisation patterns consistent with those predicted using web-based software localisation prediction programs. Furthermore, many proteins appear to be localised to multiple sub-cellular compartments (Silva-Filho 2003). These findings, together with the results of this study, demonstrate that localisation prediction programs may inaccurately predict protein targeting, and therefore should only be used as a prediction to be tested, rather than taken as indisputable evidence.

Chapter 4. *In planta* demonstration of grapevine GLP enzyme activity

4.1 Introduction

The *V. vinifera* GLP, *VvGLP3*, is strongly induced in leaves and berries in response to powdery mildew (Fig. 2.9; 2.10). The timing of *VvGLP3* induction, in response to *E. necator* inoculation, correlates with attempted penetration of the cell wall and host papillae formation (Fig. 2.12; 2.13). Significantly, using promoter: reporter gene studies, the *VvGLP3* promoter was demonstrated to drive *GUS* expression specifically in epidermal cells of *A. thaliana* leaves infected with *E. cichoracearum* (Fig. 3.3). Furthermore, using transient expression of *GFP* fusion constructs in onion cells, the *VvGLP3* gene product was localised to the cell wall (Fig. 3.6). The spatial and temporal expression of *VvGLP3*, together with the targeting of *VvGLP3* to the cell wall, suggests a possible role for this gene in papillae-based defence to powdery mildew. However, to further elucidate the role of *VvGLP3*, it is necessary to determine the enzyme activity of the protein encoded by this gene.

Although the enzyme activity of most of the GLPs has yet to be determined, cereal germin has been demonstrated to have both OXO activity and SOD activity (Woo *et al.* 2000), barley HvGLP2 has AGPPase activity (Rodriguez-lopez *et al.* 2001) and a number of GLPs have been shown to have SOD activity (Yamahara *et al.* 1999, Carter and Thornburg 2000, Christensen *et al.* 2004, Kim *et al.* 2004). Of particular relevance

is the finding that the protein encoded by powdery mildew-inducible barley *HvGLP4*, has SOD activity (Christensen *et al.* 2004). Following *B. graminis* inoculation, *HvGLP4* was shown to be induced to a much higher level in cells with papillae effective in preventing penetration, compared to haustorium-containing infected cells (Gjetting *et al.* 2004). In barley H_2O_2 accumulates at sites of papillae formation in response to *B. graminis* inoculation and may be involved in cross-linking cell wall components during papillae formation (Thordal-Christensen *et al.* 1997).

As GLPs are highly glycosylated proteins, it is thought that a plant system rather than bacterial or yeast expression system would be required for the successful expression of recombinant enzyme activity (Dr Hans Thordal-Christensen, The Royal Veterinary and Agricultural University, Denmark, personal communication). As rapid and efficient transformation protocols for grapevine are currently not available, it was decided to determine the enzyme activity of recombinant grapevine VvGLP3 protein expressed in the model plant *A. thaliana*. For comparison, the enzyme activity of recombinant grapevine GLP from a different subfamily, VvGLP2 (GLP subfamily 2; Fig. 2.7), was also determined.

4.2 Materials and Methods

4.2.1 Materials

Buffers and solutions, as summarised in Table 4.1, were prepared with nanopure water and autoclaved where appropriate. All chemicals were analytical or molecular biology grade.

Table 4.1. Solutions (and their composition) used in this research.

Solution	Composition
2× loading dye	4% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 125 mM Tris-HCl (pH 6.8), 2% (v/v) β-mercaptoethanol
<i>Arabidopsis</i> liquid culture	1× Murashige and Skoog (MS) Salt Mixture (Invitrogen), 2% dextrose (w/v), pH 5.7
SDS running buffer	0.29% (w/v) Trizma base, 1.44% (w/v) glycine, 0.1% (w/v) SDS, pH 8.3

4.2.2 Plant and pathogen materials and maintenance

V. vinifera leaves and berries were sampled from vineyard and potted glasshouse vines grown at the Waite Campus (Adelaide, South Australia) as described previously (Section 2.2.2). *A. thaliana* was cultivated in growth rooms as per Section 3.2.2. *Arabidopsis* liquid cultures (Table 4.1) were grown in 250 mL Erlenmeyer flasks based on the method used by Ramonell *et al.* (2002). Seeds for liquid cultures were surface sterilised in 15 mL tubes by adding 5 mL of 50% (v/v) ethanol to approximately 200 seeds. The ethanol was aspirated and the seeds were sterilised in 5 mL of 50% bleach (active ingredient 3.5% (w/v) sodium hypochlorite) containing 0.1% (v/v) Tween for 10 min. The seeds were washed three times in sterile water before being transferred into 100 mL *Arabidopsis* liquid culture. Cultures were grown under constant light at 22°C with constant agitation at 90 rpm for 2 weeks before harvesting. *E. cichoracearum* was obtained from naturally infected *A. thaliana* plants, grown at the Waite Campus (Adelaide, South Australia) as per Section 3.2.2. The identity of the isolate was kindly verified by Dr Ian Pascoe (Department of Natural Resources and Environment, Institute for Horticultural Development, Australia).

4.2.3 Vector construction

Constructs for 35S promoter-driven constitutive expression of *VvGLP3* and *VvGLP2* were generated. These constructs included an in-frame C-terminal histidine (His)-tag to permit His-purification of the recombinant protein, if necessary. As Christensen *et al.* (2004) found that C-terminal His-tagged barley *GLP*, *HvGLP4*, encoded a protein with SOD activity, but N-terminal His-tagged *HvGLP4* did not, His-tags were fused in-frame with the C-terminal of the grapevine *GLPs*.

VvGLP ORFs (*VvGLP2* and *VvGLP3*) were PCR amplified (as per Section 2.2.5.3.2) from cDNA synthesised (Section 2.2.5.2) using the oligo(dT) adapter B26 primer (Appendix 1) from berry skins of *V. vinifera* cv. Chardonnay infected with *E. necator*. Forward primers were designed to the ORF sequences to include the native start codon and contained a 5'-*XhoI* site. The reverse primers, also designed to the ORF sequences, contained six consecutive histidine residues (generating an in-frame C-terminal 6×His tag), an introduced stop codon and a terminal *BamHI* site. The *VvGLP3* construct contains 677 bp of *VvGLP3* nucleotide sequence and was amplified using GLP3Fa and GLP3Rb (Appendix 1). The *VvGLP2* construct contains 654 bp of *VvGLP2* nucleotide sequence and was amplified using GLP2Fa and GLP2Rd primers (Appendix 1).

PCR products were examined on a 1.2% (w/v) agarose gel. Purified DNA fragments were cloned into pGEM[®] T-easy vector (Promega) as per Section 2.2.5.4 and verified by sequencing with the SP6 and T7 sequencing primers (Section 2.2.5.5; Appendix 1). Clones were digested with *XhoI* / *BamHI* (Roche Diagnostics) to release the ORF fragment for ligation to the multiple cloning site of the pART7 (Gleave 1992) vector

previously cut with *Xho*I / *Bam*HI. Fragments containing the CaMV 35S promoter, the GLP ORF, and the nopaline synthase (NOS) transcription terminator sequence were *Not*I-digested from pART7 and ligated into the binary vector pART27 (Gleave 1992) previously cut with *Not*I to generate *VvGLP* ORF constructs (Figure 4.1).

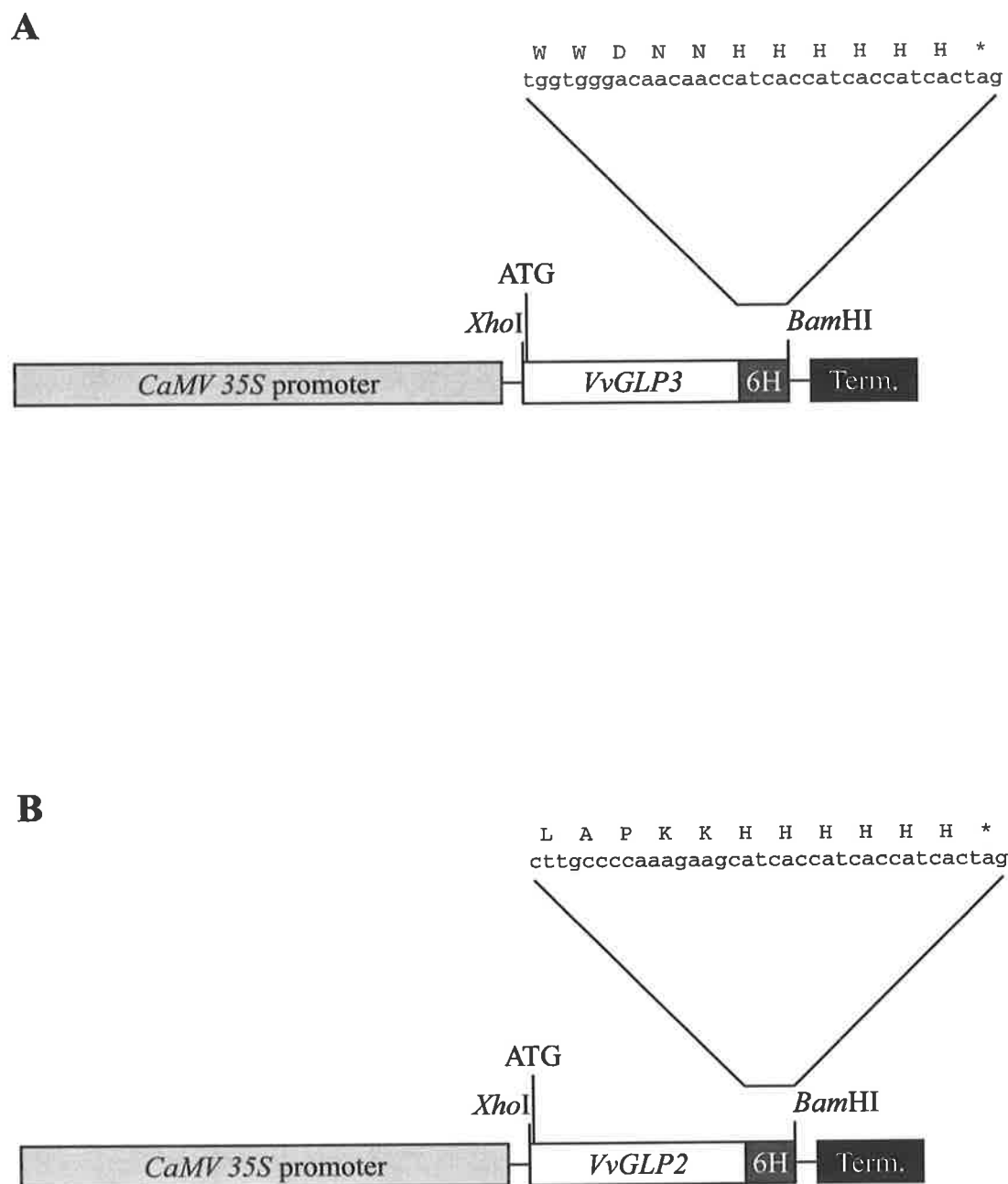


Figure 4.1. Constructs used for expression of recombinant grapevine GLPs in *A. thaliana*. In-frame C-terminal His-tagged (A) VvGLP3 and (B) VvGLP2 were expressed in *A. thaliana*. The VvGLP ORF fragments were PCR amplified from *V. vinifera* cv. Chardonnay cDNA. The constructs are not drawn to scale. Abbreviations used: 6H, six histidine residues; Term., nopaline synthase terminator sequence.

4.2.4 *A. thaliana* floral dip transformation

The *GLP* ORF fusions were electroporated into *A. tumefaciens* strain EHA105, as per Section 3.2.7.1. Constructs were transformed into *A. thaliana* Col-4 plants using a modified floral dipping method (Clough and Bent 1998) as per Section 3.2.7.2. As the T-DNA of the pART27 vector carries a kanamycin resistance gene, transgenic plants were selected on germination plates containing 35 $\mu\text{g mL}^{-1}$ kanamycin, as per Section 3.2.7.4.

The presence of the *GLP* coding sequence in kanamycin resistant lines was verified by PCR analysis (as per Section 3.2.7.4) using gene-specific primers. The forward primers, GLP3Fa and GLP2Fa (Appendix 1), corresponded to the *VvGLP3* and *VvGLP2* sequences, respectively. The reverse primer, OCS300 (Appendix 1), was designed to octopine synthase terminator sequence derived from the pART7 vector.

4.2.5 Expression of grapevine recombinant protein

Total RNA was extracted from 100 mg leaf tissue from wild-type and transgenic *A. thaliana* using the RNeasy Plant Mini Kit (Qiagen). To eliminate genomic contamination, RNA was DNase treated, using the RNase-free DNase kit (Qiagen). The concentration of DNase-treated RNA samples was determined spectrophotometrically and confirmed on ethidium bromide-stained agarose gels (as per Section 2.2.5.1).

Total RNA was analysed for the expression of recombinant grapevine *GLP* using RT-PCR. RT-PCR amplification reactions were performed using forward and reverse *GLP* gene-specific primers. A 677 bp *VvGLP3* fragment was amplified using GLP3Fa and

GLP3Rb primers, and a 654 bp *VvGLP2* fragment using GLP2Fa and GLP2Rd. For each of the samples, the *A. thaliana* β -tubulin (At5g23860; NCBI RefSeq, NP_568437) housekeeping gene was amplified using β -tubF and β -tubR primers (Appendix 1) to verify that the concentrations of total RNA used in each of the RT-PCR reactions were consistent.

RT-PCR reactions were carried out using the SuperscriptTM One Step RT-PCR with Platinum[®] Taq System (Invitrogen) according to manufacturer's instructions. Cycling conditions were: 30 min at 50°C, 30 cycles (except β -tubulin, 10 cycles) of 15 s at 94°C, 30 s at annealing temperature, 1 min at 68°C, and a final extension of 10min at 72°C. Annealing temperatures were 55°C except for β -tubulin (52°C). RT-PCR products were visualised on 1% (w/v) agarose gels stained with ethidium bromide as per Section 2.2.7.

4.2.6 Partial purification of recombinant VvGLPs

A. thaliana was grown in liquid culture to facilitate the rapid production of a large amount of tissue (250 mg) in a space efficient manner for assaying the enzyme activity of the recombinant proteins. Liquid culture grown *A. thaliana* seedlings were frozen with liquid nitrogen and ground into a fine powder using chilled mortar and pestle. Tissue (250 mg) was resuspended in 600 μ L of 150 mM Tris-HCl (pH 7.2). Heat unstable proteins were removed by denaturation at 65°C for 5 min, a temperature conservatively less than denaturation temperatures reported for GLPs (90°C; Carter and Thornburg 2000). Following heat denaturation the sample was centrifuged at 10,000 g for 10 min. The supernatant was retained and applied to a YM-30 Microcon column (Millipore, Adelab, South Australia) and centrifuged at 10,000 g for 10 min to

concentrate the crude extract. The concentrated sample, typically 150 to 200 μL , was transferred to a clean YM-30 Microcon column and centrifuged at 10,000 g for 10 min to concentrate the sample to less than 10 μL .

4.2.7 In-gel SOD enzyme assay

Crude extractions to be assayed for SOD activity were corrected to 10 μL with 150 mM Tris-HCl (pH 7.2). Samples were adjusted to 2x loading dye (Table 4.1) and loaded into a 4 to 20% Tris-glycine-SDS polyacrylamide gel (Life-Gels, Clarkston, GA). Gels were electrophoresed at approximately 150 volts for 1 h in SDS running buffer (Table 4.1). A modification of the photochemical method of Beauchamp and Fridovich (1971) was used to detect SOD activities on gels. The gel was washed in 50 mM Tris-HCl (pH 7.8) for 60 min to remove SDS, soaked in 0.3 mM nitroblue tetrazolium (NBT) for 20 min in the dark and washed briefly with 36 mM potassium phosphate buffer (pH 7.8). The gel was subsequently incubated in 36 mM potassium phosphate buffer (pH 7.8) containing 28 mM Tetramethylethylenediamine (TEMED) and 2.8 μM riboflavin for 15 min in the dark, and rinsed in 36 mM potassium phosphate buffer (pH 7.8). The gel was illuminated on a light box for 15 min to initiate the photochemical reaction. All procedures were carried out at room temperature.

4.3 Results

4.3.1 Expression of grapevine GLPs in transgenic *A. thaliana*

Grapevine GLPs, *VvGLP3* and *VvGLP2*, were expressed under the control of the CaMV 35S promoter, in transgenic *A. thaliana* plants. Three kanamycin resistant T_1 lines were

obtained for *VvGLP3*, and two for *VvGLP2*. The presence of the *VvGLP* coding sequence in kanamycin resistant lines was verified by PCR analysis using gene-specific primers (Fig. 4.2). In addition to amplifying a fragment of recombinant *VvGLP2*, a spurious band of approximately 1300 bp was also amplified using the *VvGLP2* gene-specific primers (Fig. 4.2B). As this band was also amplified from the wild-type *A. thaliana* Col 4 genomic DNA, it does not indicate the presence of the *VvGLP2* coding sequence.

Significantly, expression of recombinant grapevine *GLP* genes was detected using RT-PCR in all positive transgenic lines (Fig. 4.3). The T₁ transgenic plants were transferred to soil to set seed (T₂). T₂ lines were selected on kanamycin and verified for the presence of the *VvGLP* expression construct by PCR (data not shown) and one line of each construct was chosen for protein analysis.

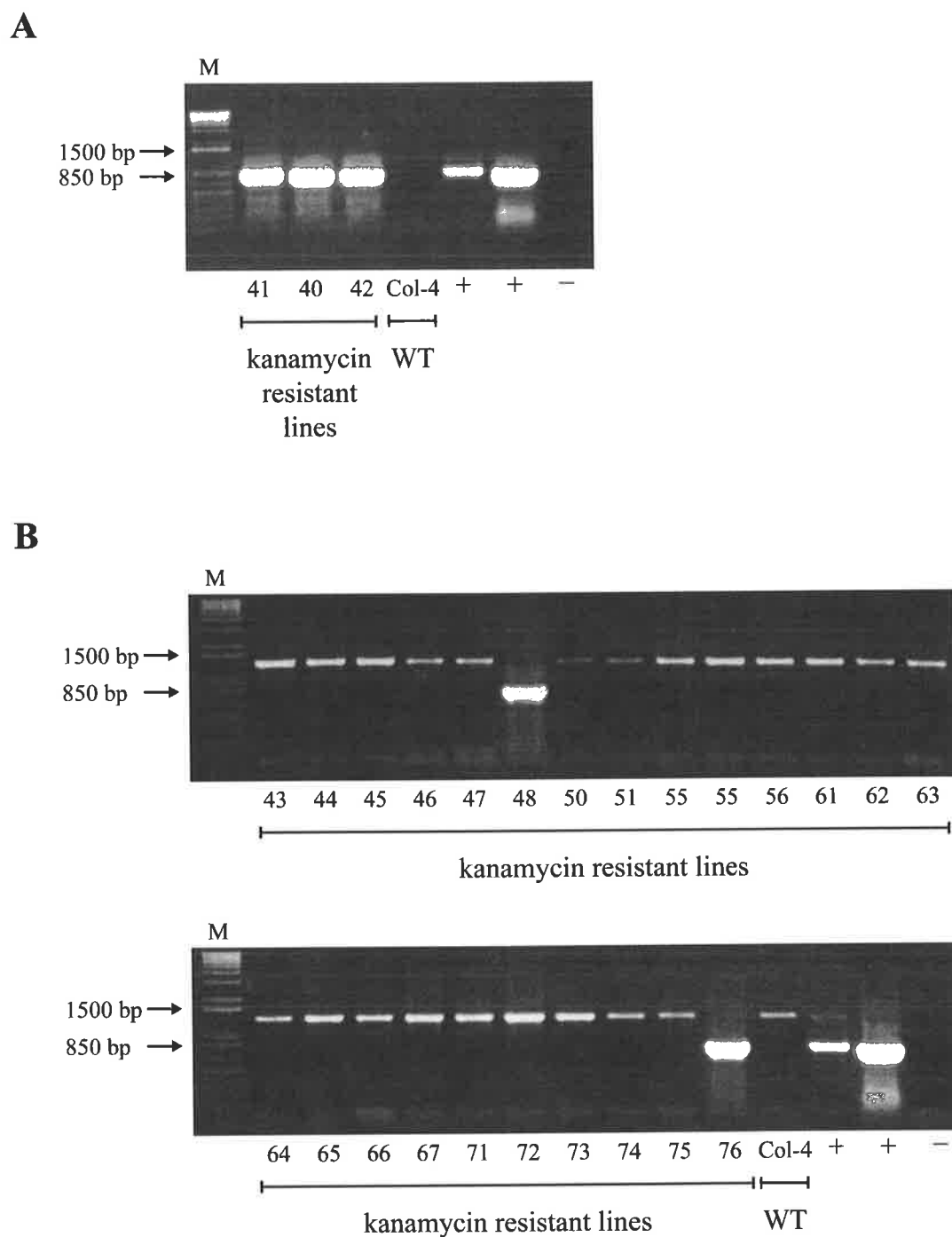


Figure 4.2. PCR analysis of T₁ *A. thaliana* kanamycin resistant lines. Gene-specific primers amplified a fragment of the recombinant (A) *VvGLP3* and (B) *VvGLP2* constructs in lines 40, 41 and 42, and lines 48 and 76, respectively. Genomic DNA was extracted from kanamycin resistant T₁ *A. thaliana* plants and untransformed wild-type (WT) *A. thaliana* Col 4. Positive controls (+), DNA extracted from *E. coli* (left) and *Agrobacterium* (right) transformed with the *VvGLP* constructs. Negative control (-), minus DNA. M, 1kb+ DNA marker (Invitrogen). PCR products were separated on ethidium bromide-stained agarose gels.

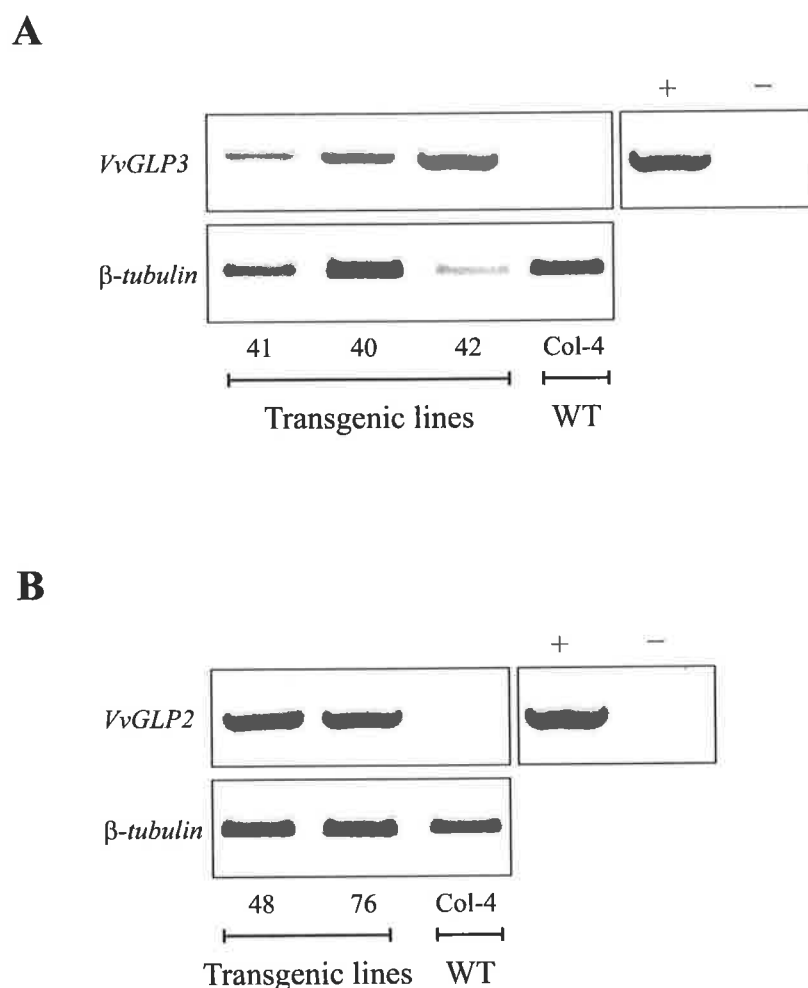


Figure 4.3. Expression of recombinant *VvGLP* in T₁ transgenic *A. thaliana* lines. Total RNA was extracted from leaves wild-type *A. thaliana* and T₁ transgenic *A. thaliana* expressing (A) *VvGLP3* (lines 41, 40 and 42) or (B) *VvGLP2* (lines 48 and 76) under the control of the CaMV 35S promoter. Transcripts were detected by RT-PCR analysis with gene-specific primers. Controls (shown in separate boxes) were included in the same RT-PCR experiment to verify reaction conditions as follows: positive control, DNA extracted from *Agrobacterium* transformed with the recombinant protein construct; negative control, minus DNA. The concentrations of total RNA used in each RT-PCR reaction was verified by carrying out RT-PCR reactions on each of the samples using primers designed to amplify β -Tubulin from *A. thaliana*. The RT-PCR products were separated on agarose gels and stained with ethidium bromide. The negative image is shown for greater resolution.

4.3.2 Identification of VvGLPs with SOD activity

Germinated seed collected from *A. thaliana* transgenic T₂ lines (T₃ seed) originating from line 41 (*VvGLP3*) and line 76 (*VvGLP2*) was used for assay of SOD activity. Achromatic bands, indicative of SOD activity (Beauchamp and Fridovich 1971), were present in extracts from both wild-type and transgenic *A. thaliana* lines expressing either *VvGLP3* or *VvGLP2* (Fig. 4.4). The presence of native *A. thaliana* proteins with SOD activity is consistent with previous reports (Pan *et al.* 2001). Significantly, a strong band of SOD activity, representing a protein with a molecular mass of approximately 100 kDa, was observed in extracts from transgenic *A. thaliana* lines expressing either *VvGLP3* or *VvGLP2* (Fig. 4.4). In contrast, this band of SOD activity was not present in extracts from wild-type *A. thaliana* (Fig. 4.4), signifying that C-terminal His-tagged *VvGLP3* and *VvGLP2* are catalytically active proteins with SOD activity. As comparisons between crude enzyme extracts were able to demonstrate *VvGLP3* and *VvGLP2* encode proteins with SOD activity, it was not deemed necessary to further purify the recombinant protein using the His tag.

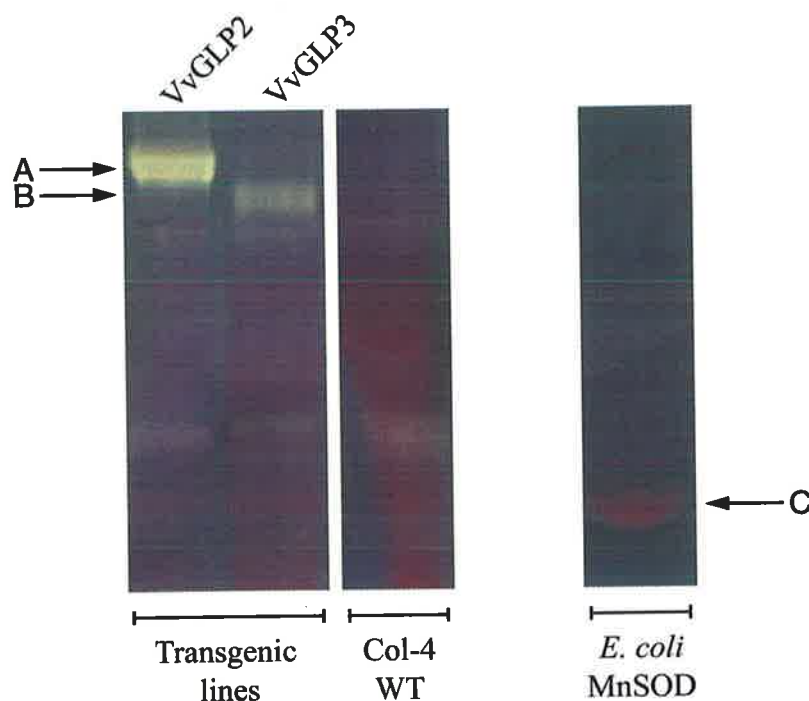


Figure 4.4. In-gel superoxide dismutase (SOD) assay. Achromatic bands are indicative of SOD activity. Seed collected from wild-type (WT) *A. thaliana* Col-4 and transgenic *A. thaliana* T₂ lines (T₃ seed), originating from T₁ lines 41 (VvGLP3) and 76 (VvGLP2), were grown in liquid culture. Tissue (250 mg) was extracted in 150 mM Tris-HCl (pH 7.2), heat denatured at 65°C for 5 min and concentrated on micron columns. Crude extracts were corrected to 10 µL with 150 mM Tris-HCl (pH 7.2) and subjected to 4-20% SDS polyacrylamide gel electrophoresis (PAGE) followed by in-gel SOD activity staining (Beauchamp and Fridovich 1971). The gel was stained with nitroblue tetrazolium (NBT) and then soaked in riboflavin to generate superoxide radicals. NBT reacts with superoxide to form a blue precipitate. The stained gel was illuminated on a light box for 15 min to initiate the photochemical reaction. Protein that exhibits SOD activity produces a white band against a blue background. Labels A and B indicate bands of SOD activity observed in extracts from transgenic *A. thaliana* lines expressing either VvGLP2 or VvGLP3, respectively, that were not present in extracts from wild-type *A. thaliana*. Label C indicates the position of SOD activity representing *E. coli* MnSOD (250 U; Keele *et al.* 1970) used as a positive control for SOD activity. The size of the proteins was estimated using Precision Plus Protein Standards (BioRad, Australia) during the early stages of the photochemical initiation when both the ladder and the bands of SOD were apparent.

4.3.3 Phenotype of transgenic plants

The transgenic *A. thaliana* lines expressing *VvGLP3* or *VvGLP2* under the control of the 35S constitutive promoter did not appear to exhibit any obvious altered morphological phenotype. Transgenic *A. thaliana* plants, over-expressing *VvGLP3* or *VvGLP2*, were naturally infected with *E. cichoracearum*. Although thorough inoculation studies, including measurement of relative penetration efficiencies were not undertaken, the transgenic lines did not appear to have altered susceptibility to powdery mildew infection compared to untransformed lines (data not shown).

4.4 Discussion

The biochemical functions of GLPs are currently receiving a great deal of consideration and numerous roles for these proteins have been proposed (reviewed in Chapter 1). It is becoming apparent that SOD activity is common throughout the GLP clades. SOD activity has been demonstrated for proteins within the true germin subfamily (Woo *et al.* 2000), the bryophyte subfamily 2 (Yamahara *et al.* 1999, Nakata *et al.* 2004), subfamily 1 (Christensen *et al.* 2004), subfamily 2 (Carter and Thornburg 2000) and subfamily 3 (Ohmiya *et al.* 1998). In comparison, OXO activity has not been reported for proteins beyond the true germin subfamily (Dumas *et al.* 1993, Lane *et al.* 1993). Subfamily 3 also includes proteins with diverse biochemical activities including AGPPase activity (Rodriguez-lopez *et al.* 2001) and the auxin-binding proteins (Ohmiya *et al.* 1998).

The temporal and spatial expression of *VvGLP3* (Chapters 2 and 3) and the extracellular localisation of *VvGLP3* protein (Chapter 3), strongly support a role for *VvGLP3* in production of H₂O₂ in defence against powdery mildew attack similar to that proposed

for powdery mildew-inducible barley GLP, HvGLP4 (Wei *et al.* 1998, Christensen *et al.* 2004) as described in Chapter 1. To better understand the proposed role of VvGLP3 (GLP subfamily 1; Fig. 2.7), the protein encoded by this gene was biochemically characterised. Grapevine GLP was expressed in the model plant *A. thaliana* and recombinant protein was assayed for SOD activity (Beauchamp and Fridovich 1971). For comparison, grapevine GLP from a different subfamily, VvGLP2 (GLP subfamily 2; Fig. 2.7), was also expressed in *A. thaliana* and recombinant protein assayed for SOD activity.

It was necessary to extract large quantities of transgenic *A. thaliana* tissue for enzyme activity to be detected using an in-gel SOD assay following SDS-PAGE (Beauchamp and Fridovich 1971). Growth of selected lines in liquid culture was found to be an extremely useful technique for rapidly generating large quantities of tissue. GLPs are extremely heat tolerant proteins as demonstrated by the fact that tobacco GLP Nectarin I retains SOD activity at temperatures up to 90°C (Carter and Thornburg 2000). Thus, heat was used to denature unstable proteins permitting the extracts to be concentrated by filtration prior to SDS-PAGE.

Multiple minor proteins with SOD activity are present in wild-type non-transgenic plants (Pan *et al.* 2001; Fig. 4.4) and were also observed in transgenic *A. thaliana* expressing VvGLP3 and VvGLP2. Additional proteins of approximately 100 kDa, with strong SOD activity, were also present in the transgenic *A. thaliana* plants (Fig. 4.4). Crystallography of cereal germin has demonstrated it to have a homohexameric structure, existing as a trimer or dimers, with a molecular mass of approximately 100

kDa (Woo *et al.* 2000). Germin monomers are stabilised by a disulphide bond between two conserved cysteine residues, which are also present in the grapevine GLPs (Fig. 2.5). Hydrophobic interactions are thought to stabilise the dimers and trimers (Woo *et al.* 2000). Providing the samples are not boiled, heat-treated GLP migrates on SDS-polyacrylamide gels in an oligomeric form (Carter and Thornburg 2000, Christensen *et al.* 2004). Based on the size of the proteins (approximately 100 KDa) found exclusively in either extract from the different transgenic *Arabidopsis* plants, we may conclude that the proteins demonstrating strong SOD activity are recombinant VvGLP2 and VvGLP3.

Curiously, recombinant VvGLP3 is more electrophoretically mobile than recombinant VvGLP2 under the SDS-PAGE conditions applied. SDS treatment reduces the charge and conformational differences between proteins. Consequently, molecular weight is theoretically the main determinant of electrophoretic mobility during SDS-PAGE. The difference in the grapevine GLP mobilities can not be attributed to the difference in size based on putative peptide sequences; the products encoded by recombinant VvGLP3 and VvGLP2 are 225 and 217 amino acids, respectively. The organisation of the hexameric protein structure of GLPs can impact their ability to be reduced by SDS (Woo *et al.* 2000). Hence, the disparity between the electrophoretic mobility of VvGLP2 and VvGLP3 may be explained by their resistance to SDS reduction. The apparent relative sizes of these recombinant proteins may therefore be due to charge or conformational differences influencing electrophoretic mobility.

Plants possess three types of “true” SOD proteins: CuZn-SOD, Mn-SOD and Fe-SOD (Bowler *et al.* 1994). SOD plays an important role in protecting living cells against

superoxide anion toxicity. Superoxide and its protonated form ($\text{HO}_2^-/\text{O}_2^-$) are generated as a by-product of numerous physiological reactions including electron flow in the chloroplasts and mitochondria and various redox reactions (Bowler *et al.* 1994). Superoxide can react with H_2O_2 to produce the highly reactive hydroxyl radical ($\cdot\text{OH}$). The hydroxyl radical can lead to the peroxidation of membrane lipids, breakage of DNA strands, and enzyme inactivation (Bowler *et al.* 1992, Mehdy 1994). SOD has evolved as a mechanism to scavenge $\text{HO}_2^-/\text{O}_2^-$. The remaining H_2O_2 may then be detoxified by catalase or peroxidase. As VvGLP2 is expressed in roots, leaves and berries (Fig. 2.17), various protective roles for VvGLP2 SOD activity throughout the plant can be envisaged and will not be discussed further.

The transgenic *A. thaliana* lines expressing either VvGLP3 or VvGLP2 under the control of the 35S constitutive promoter did not appear to exhibit any altered developmental phenotype. This was also true of transgenic *Arabidopsis* lines expressing active HvGLP4 under the control of the 35S constitutive promoter (Christensen *et al.* 2004).

Although transient over-expression of HvGLP4 in barley leaves significantly reduced the penetration efficiency of *B. graminis* in barley, constitutive expression of HvGLP4 in *A. thaliana* did not affect susceptibility to *E. cichoracearum* (Christensen *et al.* 2004). Furthermore, transient over-expression of HvGLP4 only significantly reduced the penetration efficiency of *B. graminis* in only one out of four tested barley cultivars (Christensen *et al.* 2004). The authors speculate that for HvGLP4 action, one or more genotype-dependent host components may also be required. Therefore, it is not surprising the transgenic *A. thaliana* lines constitutively expressing VvGLP3, or

VvGLP2, did not appear to have altered susceptibility to *E. cichoracearum*. This may be the product of another limiting factor in the process of papillae formation such as availability of the SOD substrate (superoxide and its protonated form; $\text{HO}_2^-/\text{O}_2^-$). Thus, despite this negative result in *Arabidopsis*, *VvGLP3* has the potential to reduce susceptibility to *E. necator*, in grapevine, in the presence of these genotype-dependent host factors.

Chapter 5. Identification of a powdery mildew-inducible *GLP* in *Arabidopsis thaliana*

5.1 Introduction

The aim of this research project was to characterise the grapevine *GLP* gene family and to investigate its potential role in defence against powdery mildew. Cloning and expression analysis identified a grapevine *GLP*, *VvGLP3*, which was strongly induced in leaves and berries in response to *E. necator* infection (Fig. 2.9; 2.10). *VvGLP3* was not expressed in the various grapevine tissues analysed (Fig. 2.17), nor was it induced in response to other pathogens (Fig. 2.14) or wounding (Fig. 2.15), suggesting that this gene is induced specifically in response to powdery mildew. In response to *E. necator* inoculation, the timing of *VvGLP3* induction correlates with attempted penetration of the cell wall and host papillae formation (Fig. 2.12; 2.13). Using promoter: reporter gene studies in *A. thaliana*, a *VvGLP3* promoter fragment was shown to express *GUS* specifically in epidermal cells infected with *E. cichoracearum* (Fig. 3.3). Furthermore, the protein encoded by *VvGLP3* was shown to be targeted to the cell wall (Fig. 3.6) and to catalyse SOD activity (Fig. 4.4).

All of this data strongly suggests a role for this grapevine *GLP* in papillae-based defence to powdery mildew. However, none of these findings provides direct evidence for a role of *VvGLP3* in defence against powdery mildew. A more direct approach to determining gene function would be to determine the phenotype of *VvGLP3* mutants in grapevine.

As the creation of gene knockouts in grapevine is still a very long and labour intensive process it was decided to try and investigate the role of powdery mildew-inducible *GLP* in papillae-based defence in the model plant *A. thaliana* using T-DNA insertion lines. *A. thaliana* was chosen due to the completion of the *A. thaliana* genome sequencing (*Arabidopsis* Genome Initiative 2000), the existence of publicly available T-DNA insertion lines (Sessions *et al.* 2002, Alonso *et al.* 2003), and the availability of a powdery mildew isolate, *E. cichoracearum*, which is capable of infecting this host. This chapter describes the identification of powdery mildew-inducible *A. thaliana* *GLP* and the characterisation of T-DNA insertion mutants for this *GLP* gene. The tissue-specific expression of powdery mildew-inducible *AtGLP* was also examined using promoter: reporter gene studies.

5.2 Materials and methods

5.2.1 Materials, plant materials and pathogen culture

Buffers and solutions were prepared with nanopure water and autoclaved where appropriate. All chemicals were analytical or molecular biology grade. *A. thaliana* ecotype Columbia 4 (Col-4) seed was kindly provided by Elizabeth Lee (CSIRO, Plant Industry, South Australia) and cultivated as per Section 3.2.2. *E. cichoracearum* was maintained as per Section 3.2.3.

5.2.2 *A. thaliana* GLPs

A. thaliana *GLPs* were identified from gene name searches of the TIGR *A. thaliana* Database (<http://www.tigr.org/tdb/e2k1/ath1/>) and analysed using the GCG WebANGIS

Wisconsin software package operated by the Australian National Genomic Information Service (<http://www.angis.su.oz.au/>). To examine the relationship between the *A. thaliana* GLPs and GLPs from other plant species, deduced amino acid sequences were aligned using ClustalW with default settings (Thompson *et al.* 1994) at the ANGIS BioManager server (<http://biomanager.angis.org.au/>). The aligned sequences were used to build a Neighbor Joining unrooted tree with Phylip. The distance matrix for the multiple sequence alignment was calculated using ProtDist (Felsenstein 1989).

The NCBI RefSeq/Genbank protein ID of the peptide sequences used for the analysis in this study were: At1g09560, NP_172427; At1g10460, NP_563870; At1g72610, NP_177405; At1g74820, NP_177620; At1g18970, NP_564067; At1g18980, NP_173332; At3g04150, NP_187065; At3g04170, NP_187067; At3g04180, NP_187068; At3g04190, NP_187069; At3g04200, NP_187070; At3g05930, NP_187244; At3g05950, NP_187246; At3g10080, NP_187619; At3g62020, NP_974477; At4g14630, NP_193199; At5g20630, NP_197563; At5g26700, NP_850875; At5g38910, NP_198707; At5g38930, NP_198709; At5g38940, NP_198710; At5g38950, NP_198711; At5g38960, NP_198712; At5g39100, NP_568562; At5g39110, NP_198727; At5g39120, NP_198728; At5g39130, NP_198729; At5g39150, NP_198731; At5g39160, NP_198732; At5g39180, NP_198734; At5g39190, NP_198735; At5g61750, NP_200983; BuGLP, BAC53790; HvGLP1, CAA74595; HvGLP2, CAA75907; HvGLP4, CAA63659; Nectarin I, AAF03355; PnGLP, BAA08266; PpABP19, AAD00295; SaGLP, CAA59257; TaGER3.8, AAA34271.

5.2.3 Powdery mildew-inducible *A. thaliana* GLPs

Total RNA was isolated from healthy *A. thaliana* leaves and leaves infected with *E. cichoracearum* as per section 4.2.5. Total RNA was analysed for the expression of GLPs using RT-PCR. *Arabidopsis*-specific primers (Appendix 1) were designed to conserved regions within all members of a homologous group (based on amino acid sequence) clustered by PileUp. (Fig 5.3). AtGLP6Fa and AtGLPR primers amplified members of group 1 (*At5g39100*, *At5g39110*, *At5g39120*, *At5g39180* and *At5g39150*); AtGLP2Fa and AtGLPR primers amplified group 2 members (*At5g39130*, *At5g39190* and *At5g39160*); AtGLP9Fa and AtGLP9Ra primers amplified group 3 members (*At4g14630*, *At5g38960*, *At5g38910*, *At5g38940* and *At5g38930*); AtGLPFb and AtGLPRb primers amplified group 4 members (*At3g05950* and *At3g04200*); AtGLPFa and AtGLPRa primers amplified group 5 members (*At3g04170*, *At3g04150*, *At3g04190* and *At3g04180*). Functionality of primer pairs was tested by PCR (as per Section 3.2.7.4) using genomic DNA as a template.

For each of the RNA samples used, the *A. thaliana* β -tubulin gene (*At5g23860*; NCBI RefSeq, NP_568437) was amplified, using β -tubF and β -tubR primers (Appendix 1), to verify that the concentrations of total RNA used in each of the RT-PCR reactions were consistent. RT-PCR reactions were carried out using the SuperscriptTM One Step RT-PCR with Platinum[®] Taq System (Invitrogen) according to manufacturer's instructions. Cycling conditions were: 30 min at 50°C, 30 cycles (except β -tubulin, 10 cycles) of 15 s at 94°C, 30 s at annealing temperature, 1 min at 68°C, and a final extension of 10min at 72°C. Annealing temperatures were 55°C except for β -tubulin (52°C). RT-PCR products were visualised on 1% (w/v) agarose gels stained with ethidium bromide as per

Section 2.2.7. RT-PCR products were purified and cloned into pGEM[®]-T Easy as per Section 2.2.5.4. The identity of the RT-PCR products was determined by sequencing using the SP6 and T7 sequencing primers (as per Section 2.2.5.5; Appendix 1).

5.2.4 Identification of *AtGLP9* T-DNA insertion mutants

For functional analysis of *AtGLP9* (*At4g14630*), the T-DNA insertion pools generated by the Salk Institute Genome Analysis Laboratory (SIGnAL) and the Syngenta *Arabidopsis* Insertion Library (SAIL; formerly Gilroy *Arabidopsis* Reverse Lethal Insertion Collection), were searched for *AtGLP9* T-DNA insertion mutants (Sessions *et al.* 2002, Alonso *et al.* 2003).

A. thaliana T-DNA insertion lines were grown as per Section 5.2.1 and insertion mutants were identified using a PCR-based screen to confirm the presence of T-DNA flanking regions for T-DNA lines. The sequences for gene-specific PCR primers that flank the T-DNA were provided by Syngenta. To determine whether an individual plant was homozygous for the absence of the T-DNA insertion, heterozygous for the T-DNA insertion, or homozygous for the T-DNA insertion, two sets of PCR were performed. The first, using a gene-specific primer pair and the second using a gene-specific primer and the T-DNA border primer, LB (Appendix 1; Fig. 5.1B). The following primer pairs were used to screen the insertion mutants: SAIL_270_D05, 270FWD and 270REV; SAIL_534_D01, 534FWD and 534REV; SAIL_593_E06, 534FWD and 534REV (Appendix 1).

PCR reactions (20 μ L) contained 1 μ L genomic DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 μ M dNTPs, 1.5 mM MgCl₂, 0.5 μ M forward primer, 0.5 μ M reverse primer and 0.5 U PLATINUM *Taq* DNA polymerase (Invitrogen). PCR conditions were as follows: 94°C for 3 min, 30 cycles of 94°C for 45 sec, 55°C for 30 sec, and 72°C for 1 min, with a final extension for 10 min at 72°C.

To determine whether the T-DNA insertion modified the induction of *AtGLP9*, the expression of this gene was analysed in *E. cichoracearum*-infected plants. RNA was extracted from healthy *A. thaliana* leaves and *A. thaliana* leaves infected with *E. cichoracearum* and expression of *AtGLP9* was determined using RT-PCR as per Section 5.2.3.

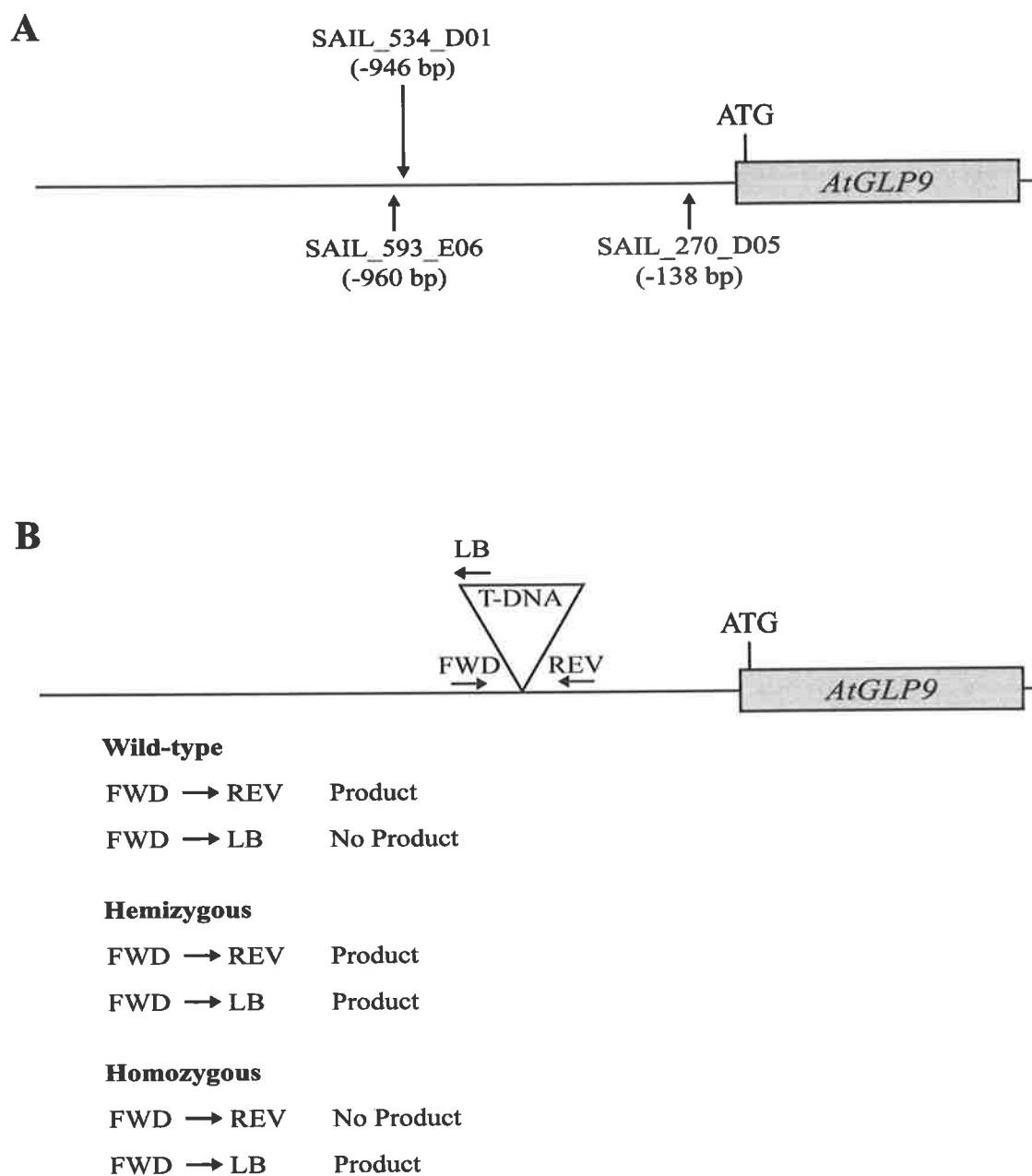


Figure 5.1. *AtGLP9* (*At4g14630*) T-DNA insertion lines. (A) Scheme of *AtGLP9* and the predicted insertions in lines SAIL_270_D05, SAIL_534_D01 and SAIL_593_E06, which lie at positions 138, 946 and 960 nt upstream of the start codon, respectively. (B) Insert confirmation using PCR with T-DNA left border (LB) and gene-specific forward (FWD) and reverse (REV) primers.

5.2.5 *In silico* promoter analysis

The *AtGLP9* (*At4g14630*) promoter sequence (NCBI RefSeq, NM_117545) and 5' UTR were scanned for the presence of *cis*-elements registered with the PLACE database (<http://www.dna.affrc.go.jp/htdocs/PLACE>; Higo *et al.* 1999) and the PlantCARE Web site (<http://intra.psb.ugent.be:8080/PlantCARE>; Lescot *et al.* 2002). Pairwise sequence comparisons between the *AtGLP9* promoter and promoter sequences for powdery mildew-inducible GLPs from grapevine (*VvGLP3* and *VvGLP4*; Chapter 3) and barley (*HvGLP4*; GenBank accession number, AJ310534) were carried out using the PromoterWise program from the European Bioinformatics Institute (EBI; <http://www.ebi.ac.uk/Wise2/promoterwise.html>), using the default settings. As selected for the *in silico* analysis of the grapevine promoters (Section 3.2.5), direct and inverted matches in a continuous region of seven or greater nucleotides containing at least two guanosines or cytosines were designated as conserved elements.

5.2.6 *AtGLP9* promoter: *GUS* construct

A 1334 bp fragment of the *AtGLP9* promoter was PCR amplified from *A. thaliana* genomic DNA (as per Section 3.2.7.3) using PLATINUM *Taq* High Fidelity DNA polymerase (Invitrogen) as per Section 3.2.4.2 with appropriate primers. The forward primer *AtGLP9Fc* (located 1336 nt upstream of the start codon; Appendix 1) contained a *HindIII* site and the reverse primer *AtGLP9Rc* (located 2 nt upstream of the start codon; Appendix 1) contained a *BamHI* site. PCR products were examined on an ethidium-bromide stained 0.75% (w/v) agarose gel and PCR products were purified, and cloned into the pGEM[®]T-Easy vector (as per Section 2.2.5.4). Promoter fragments were digested with *HindIII* / *BamHI* to release the desired promoter fragment for ligation into

the multiple cloning site of pCAMBIA 1391Z binary vector (CAMBIA, Canberra, Australia; Fig. 5.2) after cutting with *Hind*III / *Bam*HI.

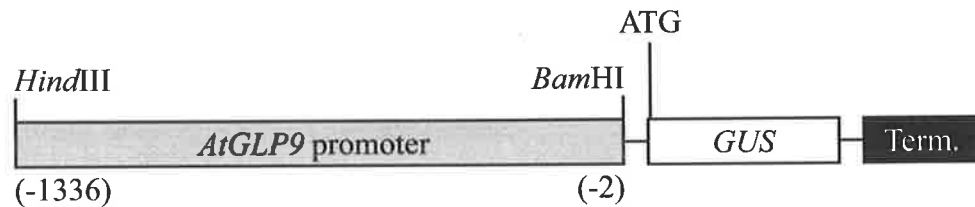


Figure 5.2. Construct used for tissue-specific localisation of AtGLP9. A 1334 bp fragment of the *AtGLP9* promoter was PCR amplified from *A. thaliana* genomic DNA. The forward primer (located 1336 nt upstream of the start codon) contained a *Hind*III site and the reverse primer (located 2 nt upstream of the start codon) contained a *Bam*HI site. The construct is not drawn to scale. Abbreviations used: *GUS*, *gusA* gene; Term., nopaline synthase terminator sequence.

5.2.7 Transformation of *A. thaliana*

The *AtGLP9* promoter: *GUS* construct was electroporated into *A. tumefaciens* strain EHA105 and putative transformants were PCR screened as per Section 3.2.7.1. *A. thaliana* Col-4 plants were transformed with the *AtGLP9* promoter: *GUS* construct using a modified floral dipping method (Clough and Bent, 1998) as per Section 3.2.7.2. Transgenic plants were selected on germination plates containing 35 µg mL⁻¹ hygromycin as per Section 3.2.7.4. Putative transformants were PCR screened using PLATINUM *Taq* DNA polymerase as per Section 3.2.7.4. Histochemical GUS staining was carried out as per Section 3.2.9.

5.3 Results

5.3.1 Powdery mildew-inducible *A. thaliana* GLP

5.3.1.1 Identification of *A. thaliana* GLP sub-groups

Thirty-two *A. thaliana* GLPs were identified by gene name searches of the TIGR *A. thaliana* Database (<http://www.tigr.org/tdb/e2k1/ath1/>). Sequence relationships between derived GLP protein sequences from *A. thaliana* and other translated GLP sequences from other plant species are shown in Fig. 5.3.

All but six of the *A. thaliana* GLP sequences (At1g74820, At1g18980, At1g18970, At5g61750, At1g10460 and At3g10080) could be subdivided within the GLP clades proposed by Carter and Thornburg (2000). Four GLPs were grouped in subfamily 2 (At5g26700, At3g05930, At1g09560 and At3g62020) and two in GLP subfamily 3 (At1g72610 and At5g20630). Significantly, twenty AtGLPs (At5g38930, At4g14630, At5g38960, At5g39190, At5g38940, At5g38910, At3g04190, At3g04180, At3g04200, At3g05950, At5g38950, At3g04150, At3g04170, At5g39160, At5g39130, At5g39180, At5g39120, At5g39110, At5g39150, At5g39100) were grouped in GLP subfamily 1. Figure 5.3 illustrates that this subfamily also includes the powdery mildew-inducible GLPs from barley (HvGLP4) and grapevine (VvGLP3 and VvGLP4; Chapter 2). On the basis of sequence homology, this group was considered as the most likely to contain a powdery mildew-inducible GLP from *A. thaliana*.

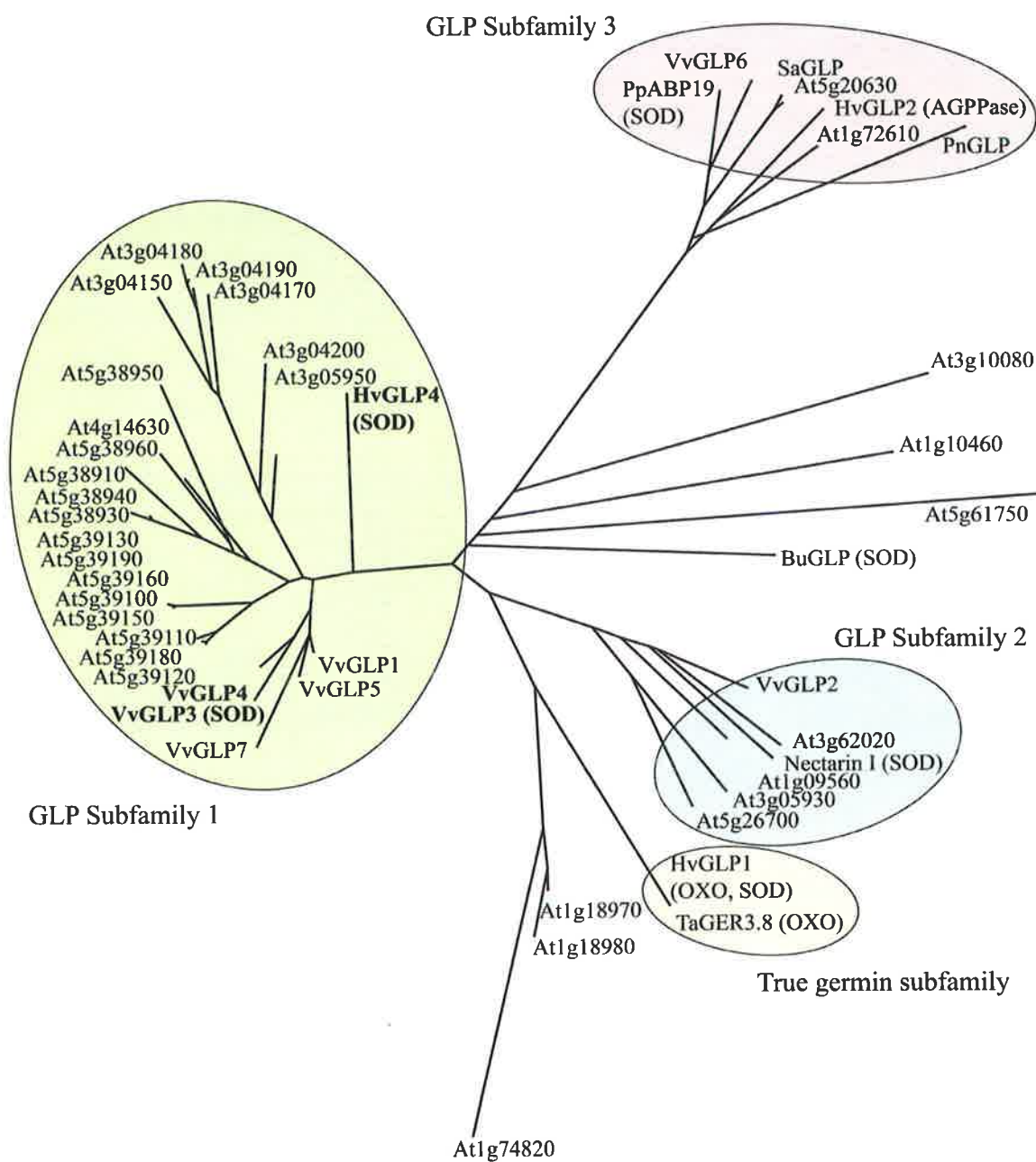


Figure 5.3. Unrooted phylogenetic tree of GLPs. Alignment of deduced amino acid sequences was based on pairwise similarity using ClustalW and Protdist was used to calculate distance matrix to build a Neighbor Joining unrooted tree with Phylip. Branches representing the germin and GLP subfamilies are indicated. Where functional activity has been demonstrated by functional assay it is indicated: OXO (oxalate oxidase activity), SOD (superoxide dismutase), AGPPase (adenosine diphosphate glucose pyrophosphatase activity). Powdery mildew-inducible GLPs as determined by this project (VvGLP3 and VvGLP4; Chapter 2) or previously published (HvGLP4; Wei *et al.* 1998) are highlighted in bold. The NCBI RefSeq/GenBank protein ID of the peptide sequences used for the analysis in this study were At1g09560, NP_172427; At1g10460, NP_563870; At1g72610, NP_177405; At1g74820, NP_177620; At1g18970, NP_564067; At1g18980, NP_173332; At3g04150, NP_187065; At3g04170, NP_187067; At3g04180, NP_187068; At3g04190, NP_187069; At3g04200, NP_187070; At3g05930, NP_187244; At3g05950, NP_187246; At3g10080, NP_187619; At3g62020, NP_974477; At4g14630, NP_193199; At5g20630, NP_197563; At5g26700, NP_850875; At5g38910, NP_198707; At5g38930, NP_198709; At5g38940, NP_198710; At5g38950, NP_198711; At5g38960, NP_198712; At5g39100, NP_568562; At5g39110, NP_198727; At5g39120, NP_198728; At5g39130, NP_198729; At5g39150, NP_198731; At5g39160, NP_198732; At5g39180, NP_198734; At5g39190, NP_198735; At5g61750, NP_200983; BuGLP, BAC53790; HvGLP1, CAA74595; HvGLP2, CAA75907; HvGLP4, CAA63659; Nectarin I, AAF03355; PnGLP, BAA08266; PpABP19, AAD00295; SaGLP, CAA59257; TaGER3.8, AAA34271.

5.3.1.2 Identification of a powdery mildew-inducible *AtGLP*

In order to determine which T-DNA mutants should be analysed for phenotype, it was first necessary to determine which of the members of the *AtGLP* gene family are induced in response to powdery mildew. Based on sequence alignments between the derived protein sequences (Fig. 5.3), the twenty candidate GLPs clustered into five groups for which group-specific primers could be designed which should amplify all members of that group using RT-PCR (Fig. 5.4). Group 1, 2 and 4 primers did not amplify a product in either the healthy uninfected *A. thaliana* leaves or leaves infected with *E. cichoracearum* (Fig. 5.5). Group 5 primers amplified a product in both healthy and infected leaves. However, group 3 primers amplified a product in the *E.*

cichoracearum-infected leaves that was absent in the healthy uninfected leaves. Thirty individual clones of the RT-PCR amplified group 3 fragment were subjected to diagnostic restriction digest analysis. The restriction digest patterns were exclusively consistent with that predicted for *AtGLP9* (*At4g14630*). Moreover, four individual clones were sequenced and found to be 100% homologous to the Genbank sequence for *AtGLP9* (*At4g14630*).

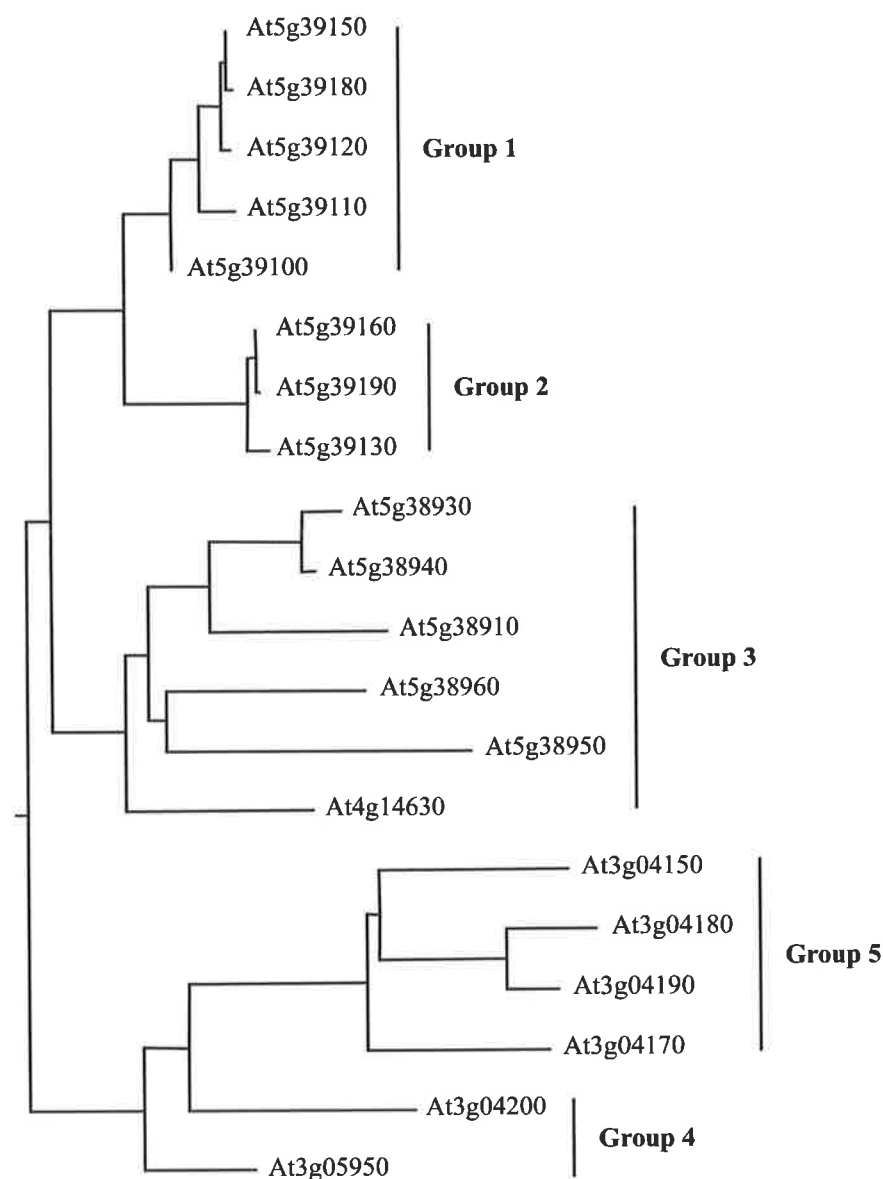


Figure 5.4. Sequence homology of *A. thaliana* GLPs from subfamily 1. Phylogenetic tree constructed using the GCG WebANGIS Wisconsin software package program PileUp. The NCBI RefSeq of the peptide sequences used for the analysis in this study were At3g04150, NP_187065; At3g04170, NP_187067; At3g04180, NP_187068; At3g04190, NP_187069; At3g04200, NP_187070; At3g05950, NP_187246; At4g14630, NP_193199; At5g38910, NP_198707; At5g38930, NP_198709; At5g38940, NP_198710; At5g38950, NP_198711; At5g38960, NP_198712; At5g39100, NP_568562; At5g39110, NP_198727; At5g39120, NP_198728; At5g39130, NP_198729; At5g39150, NP_198731; At5g39160, NP_198732; At5g39180, NP_198734; At5g39190, NP_198735.

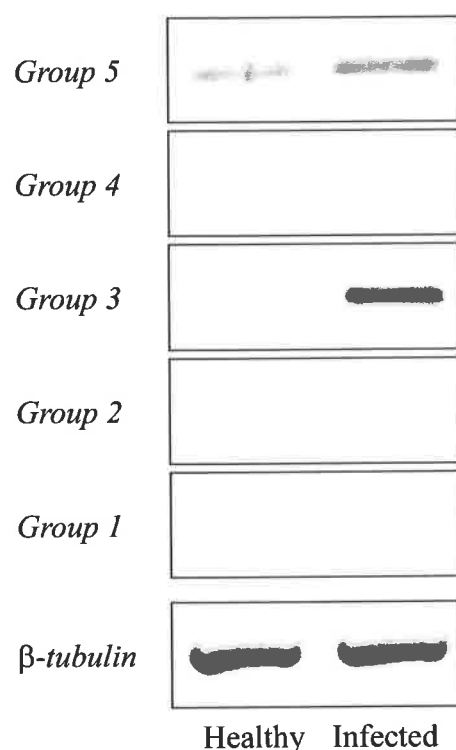


Figure 5.5. Accumulation of *A. thaliana* GLP transcripts in response to *E. cichoracearum* infection. Total RNA was extracted from leaves of healthy and *E. cichoracearum*-infected wild-type *A. thaliana* plants. Transcripts were detected by RT-PCR analysis with group-specific primers. The concentrations of total RNA used in each RT-PCR reaction was verified by carrying out RT-PCR reactions on each of the samples using primers designed to amplify β -Tubulin from *A. thaliana*. The RT-PCR products were separated on agarose gels and stained with ethidium bromide. The negative image is shown for greater resolution.

5.3.2 T-DNA insertion mutants

Analysis of the Salk Institute Genome Analysis Laboratory (SIGnAL) and the Syngenta *Arabidopsis* Insertion Library (SAIL) databases did not identify any T-DNA insertion lines with insertions within the *AtGLP9* coding region. However, three lines with insertions upstream of the coding region were identified. Seed was obtained for the following three T-DNA lines with insertions positioned upstream of the translation

initiation start codon as indicated: SAIL_270_D05 (-138 nt), SAIL_534_D01 (-946 nt) and SAIL_593_E06 (-960 nt).

PCR amplification was used to confirm the presence of T-DNA flanking regions for T-DNA lines using a left-border primer and a gene-specific primer. For line SAIL_270_D05 wild-type, heterozygous and homozygous plants were identified (Fig. 5.6), whereas for lines SAIL_534_D01 and SAIL_593_E06 all plants were identified as wild-type (data not shown).

To determine whether the T-DNA insertion in the promoter of *AtGLP9* in line SAIL_270_D05 resulted in disruption of gene expression, plants homozygous for the T-DNA insertion were analysed for the expression of *AtGLP9* using RT-PCR. *AtGLP9* transcript was found to accumulate in leaves of homozygous plants infected with powdery mildew (Fig. 5.7), indicating that the T-DNA insertion 138 nt upstream of the start codon had not resulted in disruption of *AtGLP9* expression. It was therefore concluded that these T-DNA mutants could not be used to analyse the phenotype of plants following the removal of *AtGLP9* protein.

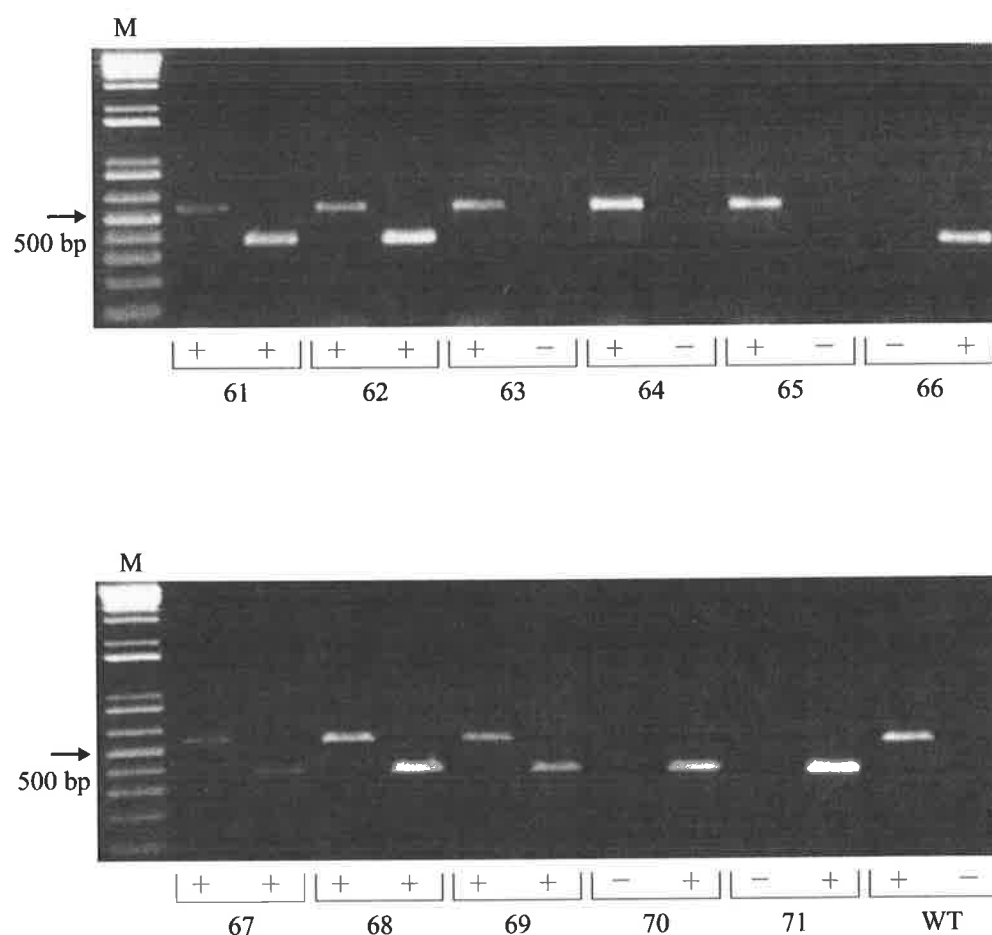


Figure 5.6. Insert confirmation using PCR with T-DNA left border (LB) and gene-specific forward (FWD) and reverse (REV) primers. PCR analysis of the predicted insertion in wild-type (WT) *A. thaliana* and eleven progeny plants of line SAIL_270_D05. Products of PCR using the primer sets FWD/REV and FWD/LB (Appendix 1) were run in adjacent lanes for each sample. Plants 61, 62, 67, 68, and 69 are hemizygous for the insertion, whereas plants 66, 70, and 71 are homozygous for the insertion. Plants 63, 64 and 65 are wild-type. The presence and absence of PCR amplified products in each lane is depicted by + and -, respectively.

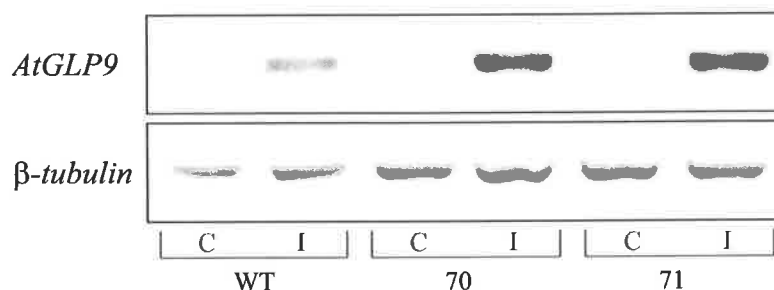


Figure 5.7. Accumulation of *AtGLP9* transcripts in T-DNA insertion mutants in response to *E. cichoracearum* infection. Total RNA was extracted from healthy control leaves (C) and *E. cichoracearum*-infected leaves (I) of wild-type (WT) *A. thaliana* and homozygous plants 70 and 71 of line SAIL_270_D05. The transcripts were detected by RT-PCR analysis using *AtGLP9Fa* and *AtGLP9Ra* primers (Appendix 1). The concentrations of total RNA used in each RT-PCR reaction was verified by carrying out RT-PCR reactions on each of the samples using primers designed to amplify β -*Tubulin* from *A. thaliana*. The RT-PCR products were separated on agarose gels and stained with ethidium bromide. The negative image is shown for greater resolution.

5.3.3 Localisation of *AtGLP9* expression

Despite the fact that T-DNA insertion lines did not provide any information on the functional role of powdery mildew-inducible *AtGLP9*, the tissue-specific expression of *AtGLP9* was still investigated. An *AtGLP9* promoter: *GUS* fusion construct was transformed into *A. thaliana* and hygromycin resistant progeny were PCR screened for the presence of the *AtGLP9* promoter: *GUS* fusion (Fig. 5.8). Fourteen T₁ hygromycin resistant and PCR positive transgenic plants (1-14) were obtained and transferred to soil.

Histochemical analysis of *GUS* activity in leaves of representative T₁ transgenic plants (lines 1 and 2) was undertaken. No *GUS* expression was observed in healthy leaves. Leaves infected with *E. cichoracearum* showed identical patterns of *GUS* expression associated with infection. Interestingly, *GUS* staining was not observed in the epidermal

cell directly beneath the fungal appressorium at *E. cichoracearum* infection sites but, instead, was localised in the mesophyll cell directly beneath the attacked epidermal cell (Fig. 5.9). Significantly, GUS staining was specifically observed in the mesophyll cell directly beneath the attacked epidermal cell, and not in the surrounding mesophyll cells.

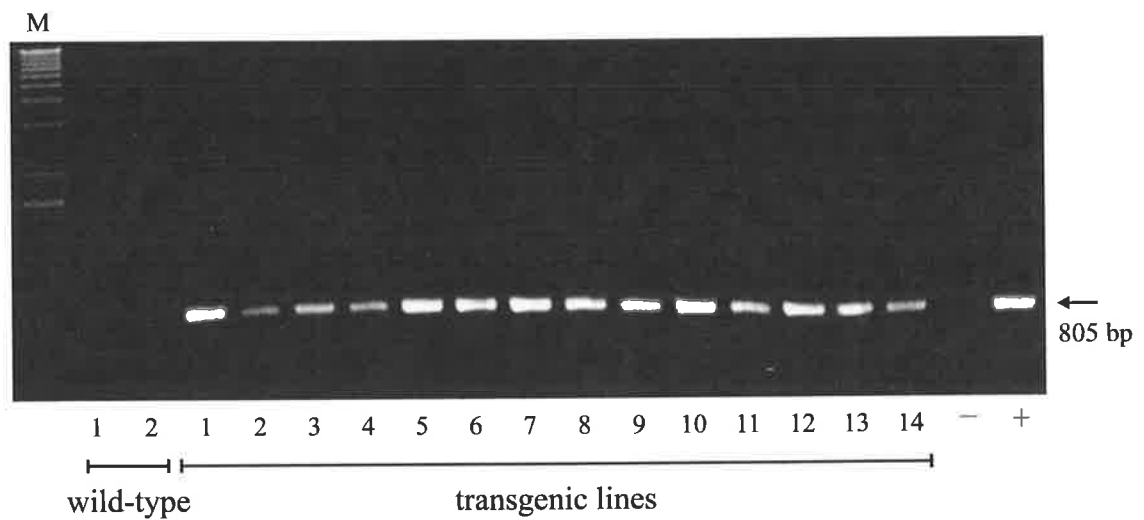


Figure 5.8. PCR analysis of *AtGLP9* promoter: *GUS* fusion *A. thaliana* transgenic (T₁) lines. Gene-specific primers PCR amplified a 805 bp fragment of the *GUS* gene. Genomic DNA was extracted from hygromycin resistant T₁ *A. thaliana* plants (lines 1 to 14) transformed with the *AtGLP9* promoter: *GUS* fusion and untransformed wild-type *A. thaliana* Col-4 plants (1 and 2). Positive control (+), DNA extracted from *Agrobacterium* transformed with the *GUS* gene. Negative control (-), minus DNA. M, 1kb+ DNA marker (Invitrogen). PCR products were separated on ethidium bromide-stained agarose gels.

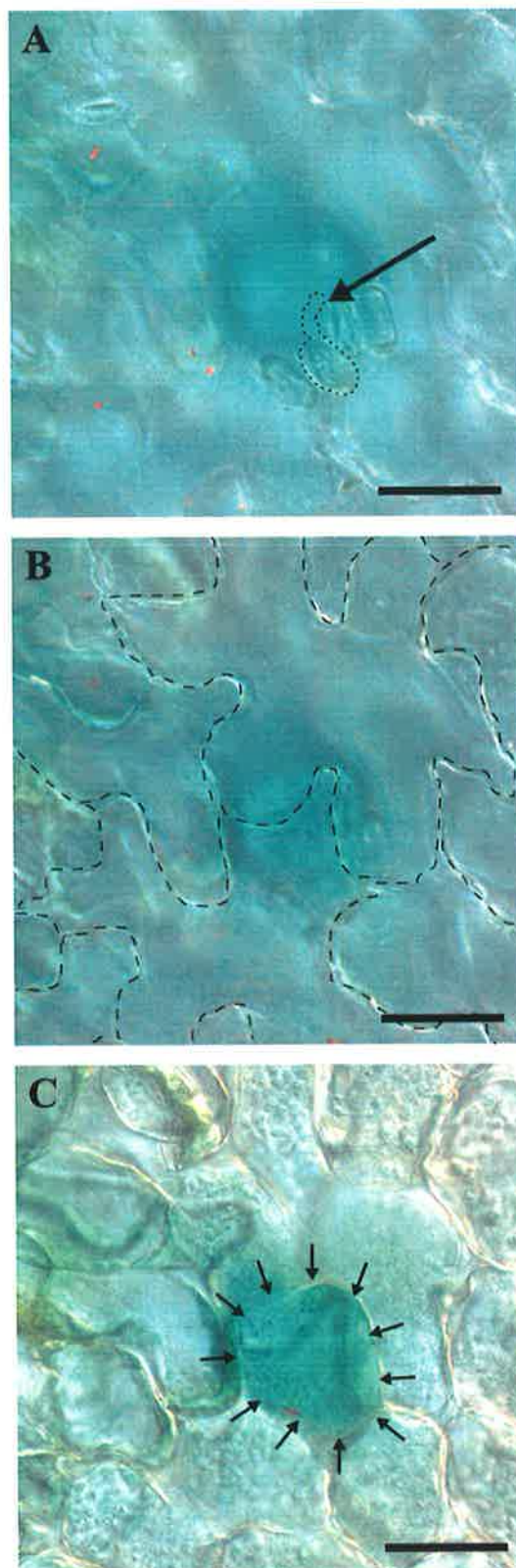


Figure 5.9. Cell-specific localisation of *AtGLP9* expression. Histochemical staining for GUS in leaves of transgenic *A. thaliana* plants transformed with the *AtGLP9* promoter: *GUS* fusion (line 1). Leaves were infected with *E. cichoracearum*. In panel (A) the focal plane is at the level of the fungus on the leaf surface whereas panel (B) is focussed at the level of the epidermal cell, and panel (C) is focussed at the mesophyll cell. Dotted line outlines fungal spore; large arrow, fungal appressorium; dashes, epidermal cells; small arrows, GUS stained mesophyll cell. Scale bar denotes 20 μm .

5.3.4 *In silico* analysis of the *AtGLP9* promoter

In silico analysis of the *AtGLP9* promoter was undertaken to identify *cis*-elements responsible for the specific expression of *AtGLP9* in mesophyll cells directly beneath epidermal cells challenged with *E. cichoracearum* (Fig. 5.9). Searches using the PLACE and PlantCARE databases identified numerous putative *cis*-elements within the *AtGLP9* promoter sequence. Analysis was restricted to 1000 nt immediately upstream from the translational start codon as undertaken for the analysis of the grapevine promoter sequences (Chapter 3). Putative *cis*-elements responsive to disease, wounding, ethylene, heat stress, gibberellic acid, low temperature and drought were identified (Table 5.1A).

The disease-responsive W box element WBOXATNPR1 (Yu *et al.* 2001, Chen *et al.* 2002) found to be present in all *GLP* promoter fragments (Table 3.2), was also present in the *AtGLP9* promoter region. The disease-responsive W box element WBOXPCWRKY1 (Eulgem *et al.* 1999, Eulgem *et al.* 2000) present in the promoter fragment of powdery mildew-inducible barley *HvGLP4* (Wei *et al.* 1998), but not within the promoter of powdery mildew-inducible grapevine *VvGLP3* or *VvGLP4* (Table 3.2), was also absent from the *AtGLP9* promoter.

In an attempt to identify novel elements within the promoter sequence that are responsible for the powdery mildew-inducible nature of *AtGLP9*, the promoter sequence of this gene were compared with the promoter sequences powdery mildew inducible grapevine, *VvGLP3* and *VvGLP4* (Fig. 2.9), and barley *HvGLP4* (Wei *et al.* 1998) genes. The conserved sequences, shown in Table 5.1B, indicate that the *AtGLP9/VvGLP3* promoter pair share six conserved sequences, one between *AtGLP9/VvGLP4* and two between *AtGLP9/HvGLP4*. *AtGLP9/VvGLP3/VvGLP4* share one conserved sequence and *AtGLP9/VvGLP3/HvGLP4* share two. Using the specified parameters no conserved sequences were identified in all four powdery mildew-inducible *GLP* promoter sequences. The longest conserved sequence was 12 nt between the *AtGLP9/VvGLP3* promoter pair.

Table 5.1. *In silico* analysis of the *AtGLP9* promoter. (A) *Cis*-elements identified within the *AtGLP9* promoter sequence from searches of the PLACE and PlantCARE databases. Analysis was restricted to 1000 nt immediately upstream from the translational start codon. WBOXATNPR1 (Yu *et al.* 2001, Chen *et al.* 2002), WUN motif (Pastuglia *et al.* 1997), ERE (Itzhaki *et al.* 1994), P box (Washida *et al.* 1999), TATC box (Washida *et al.* 1999), HSE (Pastuglia *et al.* 1997), LTR (Dunn *et al.* 1998), MBS binding site (Yamaguchi-Shinozaki and Shinozaki 1994). (B) Elements conserved between the *AtGLP9*, *VvGLP3*, *VvGLP4* and *HvGLP4* promoters. Numbers indicate the position of the elements identified relative to the ATG start codon. Pairwise sequence comparisons were performed using the European Bioinformatics Institute program PromoterWise. Direct and inverted matches in a continuous region of seven or greater nucleotides containing at least two guanines or cytosines were designated as conserved elements. With the exception of *VvGLP4* (1146 nt), analysis was restricted to 1800 nt immediately upstream from the translational start codon. *AtGLP9* promoter, NCBI RefSeq, NM_117545. *HvGLP4* promoter GenBank accession number, AJ310534. I, same element in an inverted direction on the promoter.

A

Element	Inducer	<i>AtGLP9</i>
WBOXATNPR1	disease	66, 599 (I), 615 (I), 624, 949 (I)
WUN motif	wounding	161, 189, 444, 811, 924 (I)
ERE	ethylene	297, 357 (I), 442, 448 (I), 449, 809
P box	GA	37, 429
TATC box	GA	80, 762 (I)
HSE	heat stress	42, 52, 164, 891
LTR	low	121
MBS	drought	422 (I)

B

Sequence	Length	<i>AtGLP9</i>	<i>VvGLP3</i>	<i>VvGLP4</i>	<i>HvGLP4</i>
AAATCTTTGT	10	1283	791	37 (I)	
GAGAAACA	8	527	1318		316
ATTGCAA	7	1583 1582 (I)	1072		1414 (I)
GAAAGAGAGAG	11	726	1397		
GATGATG	7	1763 (I)	1229 490		
TGAAATCTTTGT	12	1285	793		

5.4 Discussion

The specific aim of the research described in this chapter was to find direct evidence supporting a role of *E. necator*-induced *VvGLP3* (Fig. 2.9; 2.10) in papillae-based defence to powdery mildew. Due to experimental practicalities, as described in Section 5.1, it was not feasible to characterise the phenotype of *VvGLP3* mutants in grapevine within the timeframe of this project. The alternative approach selected was to investigate the role of powdery mildew-inducible *GLP* in the model plant, *A. thaliana*, using T-DNA insertion lines. In order to perform these studies a powdery mildew-inducible *A. thaliana GLP* needed to be identified.

Of the thirty-two putative *GLP* genes identified in the *A. thaliana* genome, twenty were found to cluster (based on amino acid sequence homology) within the same *GLP* subfamily 1 as the *E. necator*-inducible grapevine *GLPs*, *VvGLP3* and *VvGLP4* (Fig. 2.9) and *B. graminis*-inducible barley *HvGLP4* (Wei *et al.* 1998). However, expression and sequence analysis indicated that only one (*AtGLP9*) of this group of twenty *AtGLP* candidates was induced in response to *E. cichoracearum* infection (Fig. 5.5).

In order to verify that *AtGLP9* is an ortholog of *VvGLP3*, tissue localisation studies were undertaken with an *AtGLP9* promoter: *GUS* fusion in transgenic *A. thaliana*. Surprisingly, in tissue sections of *E. cichoracearum*-infected *A. thaliana* leaves, *AtGLP9* promoter driven *GUS* expression was not found to be localised to infected epidermal cells, as observed for *E. cichoracearum*-induced *VvGLP3* promoter driven expression (Fig. 3.3), but rather, was localised to the mesophyll cell directly beneath the attacked epidermal cell (Fig. 5.9). Interestingly, in addition to powdery mildew-inducible barley

HvGLP4 (Wei *et al.* 1998) another barley GLP, *HvGLP1*, is induced during *B. graminis* infection (Zhou *et al.* 1998) but in contrast epidermal-specific *HvGLP4*, *HvGLP1* transcript is found to accumulate specifically in the leaf mesophyll. This mesophyll-specific barley *HvGLP1* has demonstrated OXO activity (Zhou *et al.* 1998). Therefore, while it is tempting to speculate that *AtGLP9* and *HvGLP1* are orthologues, this is not supported by the clustering based on amino acid sequence homology (Fig. 5.3).

Alternatively, *AtGLP9* may be orthologous to *VvGLP4*. Powdery mildew-inducible *VvGLP4* transcript was found to accumulate specifically within the skin tissues of grape berries, suggesting an epidermal location, like *VvGLP3* (Fig. 2.9). However, the grape berry skin is actually composed of several cell layers including the epidermis and hypodermis (Ollat *et al.* 2002). Thus, it is possible that *VvGLP4*, like *AtGLP9*, may be expressed within the mesophyll layer immediately beneath the penetrated epidermal cell. This hypothesis is supported by the observation that unlike *VvGLP3*, *VvGLP4* does show some low level induction in response to *P. viticola* (Fig. 2.14) which penetrates via stomates and infects mesophyll cells (Kiefer *et al.* 2002), as opposed to epidermal cells.

Attempts to determine *AtGLP9* function using T-DNA insertion mutants were not successful. T-DNA lines with insertions within the *AtGLP9* coding region were not available. Furthermore, a T-DNA line, SAIL_270_D05, with an insertion positioned 138 nt upstream of the *AtGLP9* translation initiation start codon (Fig. 5.1A) did not show any significant reduction in *E. cichoracearum*-induced *AtGLP9* expression (Fig. 5.7),

demonstrating, at least, that the *AtGLP9* promoter region 138 nt upstream of the start codon is not responsible for the induction of *AtGLP9* in response to *E. cichoracearum*.

In silico promoter analysis of the *AtGLP9* promoter region was undertaken to identify elements responsible for the expression of *AtGLP9* (Table 5.1A and B). Putative promoter elements within the *AtGLP9* promoter region were compared to those identified in the grapevine and barley *GLP* promoter regions (Table 3.2). There is good evidence to indicate that *AtGLP9* is mesophyll-specific (Fig. 5.9) and that *VvGLP3* (Figs. 2.9 and 3.3) and *HvGLP4* (Wei *et al.* 1998) genes are expressed specifically in epidermal cells. Also, the tissue location of *VvGLP4* expression is unknown and therefore, the comparison of the promoter regions from these four promoter sequences is only useful for looking at *cis*-elements that might be involved in the powdery mildew response, and not related to tissue-specific expression.

In silico analysis of the *AtGLP9* promoter identified a number of known *cis*-elements responsive to disease, wounding, temperature stress, drought and various plant hormones and related molecules (including ethylene and gibberellic acid) (Table 5.1A). These elements were also identified within the promoter regions of the grapevine (*VvGLP1*, *VvGLP2*, *VvGLP3*, *VvGLP4*, *VvGLP5* and *VvGLP6*) and barley (*HvGLP4*) *GLP* genes analysed (Table 3.2). Only one disease responsive element, W box element WBOXATNPR1 (Yu *et al.* 2001, Chen *et al.* 2002), was identified within the *AtGLP9* promoter region (Table 5.1A). This element was also present in the promoter regions of all the grapevine *GLP* genes analysed (Table 3.2), including those which are not powdery mildew-inducible (Fig. 2.9). As the presence/absence of regulatory elements

within the grapevine *GLP* promoters (Table 3.2) could not be consistently correlated with specific expression patterns (Chapter 2), empirical data is needed to verify predictions based on the output of the *AtGLP9 in silico* promoter analyses.

Cis-elements responsible for powdery mildew-induced expression have not been identified, but with the identification of another powdery mildew-inducible *GLP*, *AtGLP9*, from a different species, it is possible to speculate that common motifs exist within the promoter regions of the powdery mildew-inducible *GLPs*. Using the same approach as in Chapter 3 for *in silico* promoter analysis, novel sequences present in the *AtGLP9* promoter fragment and other powdery mildew-inducible genes, *VvGLP3*, *VvGLP4*, and *HvGLP4* (Table 5.1B), were identified.

Significantly, no sequence elements were identified that were conserved within all four promoter regions. This may indicate that the powdery mildew-inducible expression of these genes is not all mediated by the same promoter element. Alternatively, the stringency of the analysis undertaken may have excluded elements conserved within the four promoter regions.

Chapter 6. Discussion

6.1 Grapevine GLPs

GLPs are members of large multi-gene families exhibiting diverse patterns of expression (Bernier and Berna 2001). Consistent with the *GLP* families of other species, including *A. thaliana* (Carter *et al.* 1998, Carter and Thornburg 1999), barley (Wu *et al.* 2000, Druka *et al.* 2002) and rice (Membre and Bernier, 1998), this project has confirmed that the grapevine genome contains a multi-gene family of at least seven *GLP* genes, designated *VvGLP1* to *VvGLP7* (Chapter 2).

While the original aim of this research was to investigate the role of *GLPs* and, in particular, *VvGLP3* in the defence response of grapevines against *E. necator* infection (Fig. 2.9; 2.10), additional members of the grapevine *GLP* gene family were found to be expressed in a range of grapevine tissues and induced in response to other fungal pathogens, wounding and plant growth regulators. This suggests that the grapevine *GLPs* are likely to play diverse roles in both normal cellular development and in response to biotic and abiotic stresses (Chapter 2).

It is important to note that the current classification of *GLPs* into subfamilies (Carter and Thornburg 2000) is based entirely on sequence homology and not on demonstrated biochemical activity (Fig. 2.7). Subfamily 3, for example, currently includes proteins with diverse biochemical activities including peach auxin-binding proteins with SOD

activity (Ohmiya *et al.* 1998) and barley GLP with AGPPase activity (Rodriguez-lopez *et al.* 2001). Furthermore, while the patterns of expression of some of the grapevine GLPs are consistent with closely related GLPs from other species, for many GLPs this is not the case. For example, the powdery mildew-inducible grapevine GLPs, *VvGLP3* and *VvGLP4* (Fig. 2.10), are grouped in subfamily 1 (Fig. 2.7) with barley *HvGLP4* which is also induced in response to powdery mildew (Wei *et al.* 1998). However, this subfamily also contains the grapevine GLPs, *VvGLP1*, *VvGLP5* and *VvGLP7* (Fig. 2.10) which are not induced by this fungal pathogen. Thus, GLP sequence similarity alone is insufficient to confidently predict GLP expression or function.

It also raises the question as to whether these diverse proteins should in fact be referred to as germin-like proteins or whether a more suitable nomenclature could be proposed. While the cupin domain (the region spanning germin boxes B to C) is conserved amongst proteins in the cupin superfamily (Dunwell 1998), the GLPs exclusively contain germin box A. This characteristic may be used to differentiate these proteins from other members of the cupin superfamily by referring to them as 'box A cupins'. It may be appropriate to investigate the role of the conserved box A beyond the function of the cysteine residue it contains in protein stabilisation through disulphide bonding (see Woo *et al.* 2000). Further subdivision of the group could then be proposed on the basis of characteristics other than overall sequence homology, for example, function, expression patterns or regional sequence conservation.

A number of pieces of evidence suggest that *VvGLP3*, *VvGLP4* and *VvGLP7* may be paralogs (i.e. arising from gene duplication) that have evolved different pathogen and/or

tissue-specific expression patterns. These genes may represent three highly conserved open reading frames which have evolved differences in two primary inducible characteristics of their promoters, being their tissue and pathogen specificities.

VvGLP3 expression appears to be restricted to the epidermis and is specifically induced in response to the biotrophic pathogen *E. necator* (Fig. 2.9; 3.3). *VvGLP3* is not induced in response to infection by either the biotrophic pathogen *P. viticola* (Fig. 2.14A), or the necrotrophic pathogen *B. cinerea* (Fig. 2.14B).

Like *VvGLP3*, *VvGLP4* transcript accumulates following infection by *E. necator* (Fig. 2.9) and is not induced in response to colonisation by the necrotrophic pathogen *B. cinerea* (Fig. 2.14B). However, in contrast to *VvGLP3*, *VvGLP4* is weakly induced in response to infection by *P. viticola* (Fig. 2.14A) which does not infect epidermal cells (Kiefer *et al.* 2002). This leads to the hypothesis, as outlined in Chapter 5, that *VvGLP4*, like *AtGLP9*, may be expressed within the mesophyll layer immediately beneath the penetrated epidermal cell which may only be penetrated by *P. viticola* at a low frequency.

Finally, like *VvGLP3* and *VvGLP4*, *VvGLP7* is also induced in response to biotrophic pathogen attack, but unlike *VvGLP3* and *VvGLP4*, it is not induced in response to infection by *E. necator* (Fig. 2.9) but is strongly induced in response to *P. viticola* (Fig. 2.14A), suggesting that *VvGLP7* expression may be restricted to the mesophyll tissue. However, *VvGLP7* also shows further diversification from *VvGLP3* and *VvGLP4* in that it is induced during infection by the necrotrophic pathogen *B. cinerea* (Fig. 2.14B).

Significantly, it was also the only grapevine *GLP* gene induced in response to ethylene (Fig. 2.16A). This result is in agreement with studies in *A. thaliana* indicating that defence against necrotrophic pathogens is often associated with induction of ethylene signalling cascades (Hammond-Kosack and Parker 2003).

We can therefore construct a scenario where a number of gene duplication events, followed by evolution of their promoter regions, has produced three closely related genes with different tissue (*VvGLP3*, epidermal; *VvGLP4* and *VvGLP7*, mesophyll) and pathogen (*VvGLP3* and *VvGLP4*, biotrophic; *VvGLP7*, biotrophic and necrotrophic) specificities. However, from the information currently available, it is difficult to ascertain whether the differential induction patterns of *VvGLP3*, *VvGLP4* and *VvGLP7* are a function of the specific pathogen involved, the tissues that the pathogen infects or a combination of both. Functional characterisation of the *VvGLP4* and *VvGLP7* promoter regions using a promoter: reporter strategy as employed for *VvGLP3* (Chapter 3) and *AtGLP9* (Chapter 5) will be required to confirm the tissue- and pathogen-specific nature of these genes.

6.2 Functional dissection of epidermal-specific powdery mildew-inducible grapevine *GLP* expression

The results presented in Chapter 2 demonstrate that epidermal-specific powdery mildew-inducible *VvGLP3* is not expressed in healthy non-infected roots, flowers, or various developmental stages of either leaves or berries. It was not induced by other fungal pathogens, wounding or in response to treatment with various plant hormone and growth regulators including ethylene, methyl jasmonate, salicylic acid, abscisic acid or

synthetic auxin. This is of particular significance as both salicylic acid-dependent and jasmonic acid/ethylene-dependent signalling pathways are known to lead to the activation of defence genes in response to pathogen attack (Glazebrook 2001, Hammond-Kosack and Parker 2003).

This raises the question as to the source (i.e. fungal or plant) and type of elicitor molecules responsible for *VvGLP3* induction. One possibility is that *VvGLP3* is induced in response to the release of oligogalacturonides from the epidermal cell wall during powdery mildew penetration. These biologically active carbohydrates have previously been shown to induce defence responses in a range of plants (Ridley *et al.* 2001) including grapevine (Aziz *et al.* 2004), but their effect on *GLP* expression has not yet been examined.

Clearly one area for future investigation relates to dissection of the epidermal-specific powdery mildew-inducible *VvGLP3* promoter to identify key regulatory regions. Promoter deletion studies, similar to those undertaken by Mitchell *et al.* (2006) on the rust fungus-induced *fis1* promoter from flax, would enable the identification of promoter regions controlling both the epidermal-specific and powdery mildew-specific nature of *VvGLP3* expression. Such techniques have also been used successfully to characterise other *GLP* promoters. For example, Carter and Thornburg (2003) demonstrated that nectary-specific expression controlled by the *N. plumbaginifolia* *GLP Nectarin I* promoter is the product of multiple elements. Also, elements contributing to circadian expression as regulated by the *Atger3* promoter were found to reside between 299 and 967 nt upstream of the transcriptional start (Staiger *et al.* 1999).

Given the identification of novel domains within the *VvGLP3* promoter, yeast one hybrid studies could then be used to identify transcription factors which control epidermal and powdery mildew specific induction.

6.3 Potential application of *VvGLP* genes for reduced susceptibility to powdery mildew

In light of the results presented in this study, and evidence from the published literature, there is potential for the application of *VvGLP3* for reduced susceptibility of grapevines to powdery mildew, through novel biotechnology applications. Consistent with a potential role in papillae formation, the temporal expression of *VvGLP3*, in response to *E. necator* infection, was found to coincide with appressoria formation and predicted fungal penetration of the epidermal cell wall (Fig. 2.13). Significantly, promoter-driven *GUS* expression was not present in all epidermal cells, but rather appeared to be localised to epidermal cells in contact with fungal appressoria (Fig. 3.3). Gjetting *et al.* (2004) recently showed that barley epidermal-specific powdery mildew-inducible *HvGLP4* transcript accumulates to a significantly greater level in cells with papillae effective in preventing penetration, compared to haustorium-containing infected cells. These findings provide strong evidence for a role for *HvGLP4* in papillae-based defence to powdery mildew. However, it should be noted that *HvGLP4* may be induced as a consequence of the defence rather than being directly involved. It would lend support to the potential use of a gene technology application if *VvGLP3* is also expressed differentially in cells containing papillae effective in preventing penetration, compared to infected cells, as observed for *HvGLP4*.

Recombinant *VvGLP3* was successfully expressed in transgenic *A. thaliana* and the partially purified recombinant protein demonstrated to exhibit SOD activity (Chapter 4). This finding is consistent with that observed for barley epidermal-specific powdery mildew-inducible *HvGLP4* (Christensen *et al.* 2004). Furthermore, using *VvGLP3*:GFP fusion constructs, transiently expressed in onion cells, at least a proportion of recombinant *VvGLP3* protein was shown to be targeted to the extracellular compartment (Fig. 3.6) which is consistent with the proposed role for this protein in papillae-based defence to *E. necator*.

Given the evidence to suggest that *GLPs* are involved in penetration defence to powdery mildew in cereals (Schweizer *et al.* 1999a, Christensen *et al.* 2004), and the shared characteristics between these *HvGLP4* and *VvGLP3*, there is a good possibility that *VvGLP3* would reduce susceptibility in grapevine when expressed under the control of a constitutive promoter. The failure of constitutively expressing *VvGLP3* in transgenic *A. thaliana* lines to alter the susceptibility of *A. thaliana* to *E. cichoracearum* (Chapter 4) may be due to the absence of genotype-dependent host factors. Alternatively, *VvGLP3* induction may be a consequence of the defence response rather than being directly involved.

Recent studies suggest that *GLPs* may reduce susceptibility by a mechanism other than H_2O_2 mediated cell wall cross-linking at sites of papillae formation. Schweizer *et al.* (1999a) demonstrated that transient over-expression of non-enzymatic *GLPs* reduced the penetration efficiency of *B. graminis*. Furthermore, Christensen *et al.* (2004) demonstrated that there was no obvious link between H_2O_2 levels and loss of *HvGLP4*

protein in RNAi lines. This led Christensen *et al.* (2004) to propose an alternative role for HvGLP4 as a structural protein, reinforcing the cell wall during attempted fungal penetration. This proposed structural role is also supported by the observation that the transgene product becomes insolubilised at sites of attempted fungal ingress in cells transiently over-expressing GLP (Schweizer *et al.* 1999a).

Although there have been numerous attempts to engineer disease resistance using transgenic approaches, development of resistance to plant pathogens has generally been unsuccessful (Stuiver and Custers 2001, Gurr and Rushton 2005). Significantly, constitutive expression of transgene products can be detrimental to the plant, leading to metabolic disorders or undesirable side effects (Kasuga *et al.* 1999). In addition, constitutive expression of transgenes often results in inadequate levels of expression. As such, tissue-specific promoters are highly desirable for genetic engineering. Prior to this study, epidermal-specific and/or powdery mildew-specific grapevine promoters were not known.

Genetic modification techniques for engineering plant disease resistance are becoming increasingly sophisticated (Stuiver and Custers 2001). The use of pathogen-inducible promoters to minimise the cost of resistance to plant yield has been proposed (Hammock-Kosack and Parker 2003). The apparently highly localised powdery mildew-induced expression of *VvGLP3* suggests its promoter is an ideal candidate for expressing synthetic powdery mildew resistance genes. Significantly, the fact that the *VvGLP3* promoter is functional in heterologous pathosystems (Chapter 3) suggests that

this strategy may also be applicable to other powdery mildew susceptible crops and powdery mildew isolates.

One approach to generate synthetic powdery mildew resistance would be to use the *VvGLP3* promoter to express antifungal genes. Another approach would be to use the *VvGLP3* promoter to promote programmed cell death. Kawai-Yamada *et al.* (2005) recently described such a gene from *A. thaliana*, *Cdf1*, which when over-expressed resulted in cell death in *Saccharomyces cerevisiae*. The grapevine homolog to *Cdf1* would be such a candidate for generating synthetic powdery mildew resistance.

For these approaches to be successful in generating synthetic powdery mildew resistance, the regulation of the *VvGLP3* promoter would need to be highly specific. Ayliffe *et al.* (2002) identified the putative rust-specific promoter from flax, *fis1*, as a promising candidate for generating synthetic resistance, but later, the *fis1* promoter was found to confer weaker, yet significant, endogenous expression (Mitchell *et al.* 2006). Attempts to modify the *fis1* promoter using deletion analysis successfully reduced the functional rust-inducible promoter to 282 nt fragment, but as yet have not been successful in uncoupling the constitutive expression (Mitchell *et al.* 2006).

Promoter deletion experiments, which are designed to characterise tissue- and powdery mildew-specific regulatory elements within the *VvGLP3* promoter, would provide valuable data in evaluating the feasibility of this promoter in mediating powdery mildew-specific expression. In addition, it may permit the uncoupling of pathogen-inducible expression from any endogenous expression, should it be identified.

Uncoupling of pathogen-induced expression from constitutive expression has been successful for the *TobRB7* promoter from tobacco (Opperman *et al.* 1994), where elements responsible for nematode-inducible expression were separated from root-specific, promoter components.

In another study, Altpeter *et al.* (2005) attempted to use the wheat *GstA1* promoter to drive epidermal-specific powdery mildew-inducible expression of the defence-related gene, *TaPERO* peroxidase, in wheat. *GstA1* was thought to be exclusively powdery mildew-inducible, but was unexpectedly shown to be constitutively expressed in the epidermis. Nevertheless, stable and transient over-expression of the *TaPERO* peroxidase gene reduced susceptibility to *B. graminis* f. sp. *tritici*, demonstrating that an epidermal-specific promoter can be effective in reducing susceptibility to powdery mildew in cereals.

In summary, two potential strategies available to reduce susceptibility to powdery mildew, incorporating the novel *VvGLP3* identified in this study, have been described. First and most obvious, is the constitutive over-expression of *VvGLP3* in transgenic grapevine. This strategy has shown success in reducing susceptibility in cereals (Schweizer *et al.* 1999a, Christensen *et al.* 2004). The second strategy described uses the *VvGLP3* promoter to induce an artificial HR to powdery mildew attack. This type of construct could not only be used in grapevine, to produce a non-race specific *E. necator* resistance, but based on the results with *Arabidopsis*, could be extended into other powdery mildew susceptible crops, and against other powdery mildew species.

6.4 Summary

The strategic focus of this project was to investigate possible strategies for control of powdery mildew disease of grapevine. By gaining a greater understanding of the biological processes involved in the defence response of grapevine against powdery mildew, novel strategies for the engineering of disease resistance have emerged.

Genetic modification approaches to disease resistance are an important alternative when traditional approaches to breeding or control are insufficient. Powdery mildew of grapevine is an example. Attempts at producing powdery mildew-resistant cultivars through breeding have realised limited success due to the difficulty of selecting for quantitative traits controlling both resistance and fruit quality (Boubals 1961, Eibach *et al.* 1989). Furthermore, isolates of *E. necator* showing reduced sensitivity to commonly applied fungicides are now emerging, in turn, limiting the effectiveness of their use in controlling the disease (Gubler *et al.* 1996, Erickson and Wilcox 1997, Savocchia *et al.* 2004).

A major public concern relating to the use of genetically modified organisms, as compared to conventional plant breeding, is the genetic distance between the source of the transgene and the target organism (Nielsen 2003). Consequently, molecular strategies are changing, and the choice of genetic material for transgenes has become significant (Rommens *et al.* 2004). The *VvGLP3* epidermal-specific powdery-mildew inducible grapevine promoter, identified in this study, is an excellent candidate to drive the targeted expression of anti-fungal or programmed cell death-inducing genes in cells specifically attacked by powdery mildew. The outcomes from this research may then be

used to reduce susceptibility of winegrape cultivars to powdery mildew using genetic manipulation technologies.

Appendix 1

The oligodeoxyribonucleotides used in this study were obtained from GeneWorks (Adelaide, South Australia). All were designed as per Chapters 2, 3, 4 and 5 except B26, AAP and AUAP (universal primers), SP6 and T7 (sequencing primers), O1 and O2 (Siebert *et al.* 1995), OXOLP1, OXOLP2, Vv18SF, Vv18SR, GSTB (kindly provided by Dr. Ian Dry, CSIRO, Plant Industry, South Australia), 270 FWD, 270 REV, 534 FWD, 534REV and LB (Syngenta), and β -tubF and β -tubR (kindly provided by Dr. Marc Goetz, CSIRO, Plant Industry, South Australia). I = inosine.

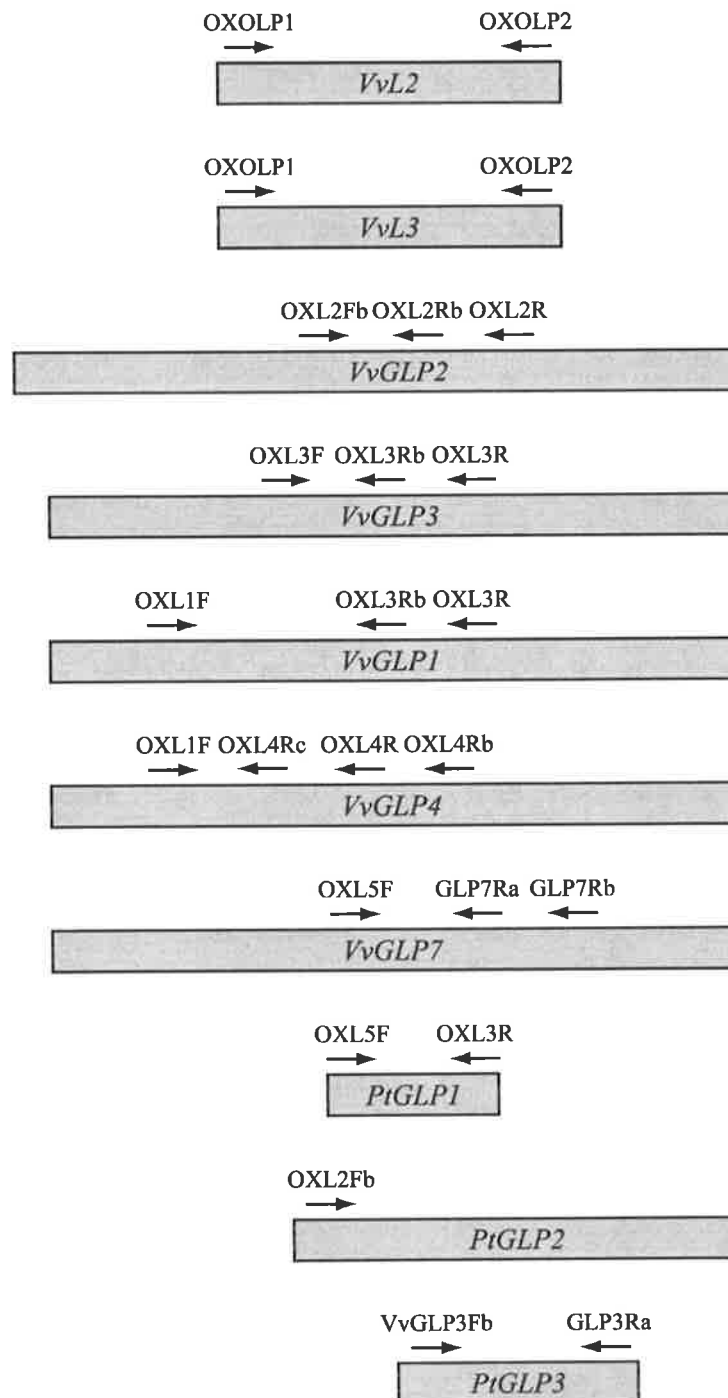
Primer	Size (nt)	Primer sequence (5' to 3'-end)
O1	27	GGATCCTAATACGACTCACTATAGGGC
O2	17	AATAGGGCTCGAGCGGC
270FWD	22	AAGTGCCCTCCTTTTTCAGTTGA
270REV	19	CCGCCATCTGCTGGTGTGT
534FWD	24	CAATCGGTCAATGAAATAATTAAC
534REV	21	GGGTTGCTTGATTCGTTAAGC
AAP	36	GGCCACGCGTCGACTAGTACGGGIIIGGGIIIGGGIIIG
AtGLP2Fa	22	TCCCTTTTGCCATAATTGCTTT
AtGLP6Fa	23	AAAAATGGTGTTTTTGTGAATGG
AtGLP9Fa	22	AAGCCAACCTCAAGACTTTTGT
AtGLP9Ra	22	GTCCCTCTGGAAACACAAATAC
AtGLPFa	20	CCTGGTCTCAACACTCTTGG
AtGLPFb	24	AACTTCATTTGTATCTTGTTACGA
AtGLP9Fc	27	ACGAAGCTTCCAGTATTACGGATTG
AtGLPR	23	TGAAAATGGATCATTCCTATGGG
AtGLPRa	20	GTCCAACGCAAAAGCTTTAG
AtGLPRb	21	TCTATTGTTGTCTTGTTTCA
AtGLP9Rc	29	TAGGGATCCGTATGTATGGAGACGATTG
AUAP	20	GGCCACGCGTCGACTAGTAC

Primer	Size (nt)	Primer sequence (5' to 3'-end)
B26	36	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTT
β -tubF	24	GGGTGCTGGAAACAATTGGGCTAA
β -tubR	21	ACTGCTCACTCACGCGCCTAA
GLP1Ra	31	TTGATGTGTTTCCTGGAATATTCAGCCCTTT
GLP1Rb	30	TCAGGTTTGGGTTCTTGCAGAACTTTCCAT
GLP2Fa	25	ATACTCGAGACCATGGCGGCTGTTG
GLP2Ra	28	GGCCTTGGTAGGCTGTTTGCAAAGTCAA
GLP2Rb	29	AAGCCTACGGTTTCTAAGTGGGCCCCATA
GLP2Rd	46	TATGGATCCTAGTGATGGTGATGGTGATGCTTCTTTGGGGCAAGTC
GLP2Rf	27	CCTCTAGACTTCTTTGGGGCAAGTCTG
GLP3Fa	28	GCGCTCGAGACAATGATGAAAGGTGTTT
GLP3Fc	26	GCGAAGCTTGGTTCTAGAGCACTTCA
GLP3Ra	27	CGTGGATCCTTAGTTGTTGTCCCACCA
GLP3Rb	44	CGTGGATCCTAGTGATGGTGATGGTGATGGTTGTTGTCCCACCA
GLP3Rd	25	CGCGGATCCCTCTCTAACAATCGAT
GLP3Re	26	CGCTCTAGAGTTGTTGTCCCACCAGA
GLP4Ra	26	AGAAGAAATCCTCGGCGACGGTCAGC
GLP4Rb	29	GCCACGAGGGCAACAGTTACCAGGAAACT
GLP4Rc	30	TTTCTGCATTGCCATAGACTGTCAAGGCAT
GLP5Fc	22	CACAGAAAATCCACTGGGGTTT
GLP5Ra	28	CCCTTGAAAGAAGAAATCCTCGGCCACA
GLP5Rb	27	AGCCAATTCCATGAGGGCCAGGAAACT
GLP5Rc	29	CAAAACGGCATCTTCCATTGCTGAATTG
GLP5Rd	28	AGTGGCCAATTCTGCCAGCAGTATGCTG
GLP6Fb	28	GCGCTCGAGAAGATGTTTCTCCCAATTC
GLP6Rc	29	GCGTCTAGAATTAGTACCACCAAGAACAG
GLP7Fa	24	ATTCCAGGAAACACATCGAATTAT
GLP7Ra	28	CCTGGTAATTGATCAACATTTATAAGAT
GLP7Rb	21	TGACACCCCAAGAGTGTTC
GSTB	19	CATTGGTTTTCTCCCATCG
GUSFWD	16	CATGGTAGATCTGAGG
GUSREV	16	CGTGGTTACAGTCTTG
LB	22	TTCATAACCAATCTCGATACAC
OCS300	20	TGCACAACAGAATTGAAAGC
OXL1F	20	AGAACCCAAACCTGACCGTC
OXL1Fb	25	CTTCCAATTCAATATTGGGCACATG
OXL2Fb	22	CGCGACAAACATAACTGCAACG

Primer	Size (nt)	Primer sequence (5' to 3'-end)
OXL2R	20	GTGCGTAGTCGATGCGGGAG
OXL2Rb	20	GACGCCAAGGGTGTGAGAC
OXL2Rf	30	CCGCCATGGTCGGAGAAACAAAAAATAC
OXL2Rg	29	GCGAGTGTGATGACAAACAAGGCCACAAC
OXL3F	22	TGACCCCAAGGATGGTGTGTTC
OXL3Fb	18	TTCTCACCAGGGCCTTCC
OXL3R	23	GTGCATAATCAATACGAACCATG
OXL3Rb	22	TGCCAAGGGTGTTAAGTCCTGG
OXL3Rc	29	CCAAAGCCAAGAGGGCAACAGTTACAAGG
OXL3Rd	29	AACACAAATGTCCTGCAGTGGGCTAGGGT
OXL4F	26	TCCAGTTGGACAAGAAAGTGGTTAGC
OXL4R	30	TTTCTGCATT GCCATAGACT GTCAAGGCAT
OXL4Rb	19	GTTTTGGCCATATGGTTCA
OXL4Rc	28	TCAACATTTACAGTAGTGACATTTGAGG
OXL5F	24	TGCAAGAACCCAAATCTGACTGTG
OXL6Ra	27	ACGCAGAAGTCCTGCACTGCAGCATGA
OXL6Rc	30	GGGAAAAGATGCAGAGAATTGGGAGAAACA
OXOLP1	19	(C/T)TICA(A/G)GA(C/T)ITITG(T/C)GTIG
OXOLP2	22	C(T/G)IGG(G/A)TGI(G/A)I(G/A)TGIGGIGG(G/A)T
SP6	19	GATTTAGGTGACACTATAG
T7	20	TAATACGACTCACTATAGGG
Vv18SF	19	CAACAAACCCCGACTTCTG
Vv18SR	20	TGTCACTACCTCCCCGTGTC
VvGLP3Fa	28	AACTCCATGATTTTCAGCTTTTGCTCGCC
VvGLP3Fb	25	CTGCAAGGATCTAAAGCTTGCTTCA
VvGLP6F	20	ATGGGGCTGGGATACCATCC
VvGlub1	25	GTTTGCCATGTTTAATGAGAACAGG
VvTL2F	20	CGCACTTAACCAATTCAGCA
WHP5F	20	ACAGTCCTTCTTGTGGGGC

Appendix 2

Stylised diagrams indicating the location and orientation of primers used to amplify *V. vinifera* and *P. tricuspidata* GLP genomic and cDNA clones (Chapter 2).



Appendix 3

GLP sequences (Chapter 2) are shown below. Genomic or cDNA sequences are shown first, followed by the predicted translation products. Putative start and stop codons in cDNAs are shown in red. C-terminal ends of protein sequences are indicated by an asterisk (*).

VvL2

Genomic DNA

TTGCAGGATGTGTGTGTGGCCGATCTCACTTCTGGTGTAAGGTGAATGGGTTCTAGCTGTAAGGACGCGACAAAC
ATAACTGCAACGGATTTCTTCTTCGACGGCCTGGCGAAACCGGGTCTGACCAACAACCTCCATGGGGTCTCTGGTA
ACAGGTGCTAATGTGCAGAAGATTCTGGTCTCAACACCCTTGGCGTCTCTCTCTCCCGCATCGACTACGCACCA
GGTGGTCTCAATCCCCCCCCACCCCCACCCCCG

Protein

LQDVCVADLTSGVKVNGFSCKDATNITATDFFFDGLAKPGLTNNSMGS�VTGANVQKIPGLNTLGVSLSRIDYAP
GGLNPPHPHP

VvL3

Genomic DNA

CTGCAGGACGTGTGTGTGGCAATTAGTGACCCCAAGGATGGTGTGTTCTGTAATGGAAAGTTCTGCAAGGATCCA
AAGCTTGCTTCAGCTGATGATTTTTTCTACTATGGGCTCCACATTCCAGGAAACATAACGAACCCAGTTGGCTCA
ATGGTCACTCCTGTAAATGTTGAACAAATACCAGGACTTAACACCCTTGGCATATCCATGGTTCGTATTGATTAT
GCACCATACGGTCAAAACCCCCCCCCACCTCCATCCCCG

Protein

LQDVCVAISDPKDGVFVNGKFCKDPKLASADDFYYGLHIPGNITNPVGSMTVPVNVEQIPGLNTLGISMVRIDY
APYQONPPHLHP

VvGLP1

cDNA

TAACTGTGGCCCTCATGGCATTGGCTTCATCTTTTGCCCTCTGCCTCTGATCCAAGCCCACTGCAGGACACTTGTG
TTGCCATTGATGAACCAAGAATGCTGTGTTTGTAATGGAAAGTTCTGCAAGAACCCAAACCTGACCGTCGCCG
AGGATTTCTTCTCTAAAGGGCTGAATATTCCAGGAAACACATCAAATCTAGTGGGCTCAAGTGTCACTACAGTAA
ATGTTGATGTGATACCAGGACTGAACACTCTTGGGATATCACTGGTTCGTATCGATTATGCACCCTATGGTCAAA
ACCCTCCTCACACTCACCTCGTGCCACTGAGATCCTCACCGTCTTGGAGGGAACCTCTTAGTTGGCTTTGTCA
CATCCAACCCCTCAAAACCGCCTCTTTAGCAAAGTCTCAACAAGGGTGATGTTTTGTGTTCCCTATTGGTCTCA
TTCACTTCCAATTCAATATTGGGCACATGAATGCAGTAGCCATTGCTGGTCTGAGCAGCCAAAATCCAGGTGTAA
TCACCATAGCCAATGCAGTGTGTTGGATCAAATCCACCAATCAATCCTGATTTCTGGCAAAGGCCTTCCAGTTGG
ACAAGAAGGTGGGTGAATTCCTTCAAGCACGATTCTAGTGGGGAAATAGAGAAAATGAAGAAGCTATGAACCTTT
GATTGCTTGCTTGAATGACTATTTGTGATGTTTACCTCGGTACAATCATCTAGAATAAGAAGGTTATTACT
TTCTTTGTAGCTCTTGGGTGGGGGACTTTGGTCTAATATTAATAAACAAGTTGCTGTTAGGCATCTAAAAAA
AAAAAAA

Protein

MALASSFASASDPSPLQDTCVAIDEPKNAVVFVNGKFCKNPNTLVAEDFFSKGLNIPGNTSNLVGSSVTTVNVDVI
PGLNTLGLISLVRIDYAPYQONPPHTHPRATEILTVLEGTLLVGFVTSNPQNRLFSKVLNKGDFVFPIGLIHFQF
NIGHMNAVAIAGLSSQNPGVITIANAVFGSNPPINPDFLAKAFQLDKKVGEFLQARF*

VvGLP2**cDNA**

AACTCATCATTATAAATACACAACCCCACTTTTTCTCTCTCCCATTTCTAATTTGAGATATCCATCTACTTTTTC
CCCTTTCTTCTCTTTTTTCATATCATTATTATCTTTGTATTTTTTTTGTCTCCGACCATGGCGGCTGTTGTGGC
CTTGTTTGTTCATCACACTCGCTGTCTTCTCCGGCACCGTTGCTGCAGATCCGGACATGCTTCAGGACGTCTGTGT
TGCCGATCTCACTTCTGGTGTAAAGGTGAATGGGTTTCTGCTGTAAGGACGCGACAAACATAAAGTGAACGGATTT
CTTCTTCGACGGCTGGCGAAACCGGGTCTGACCAACAACCTCCATGGGGTCTCTGGTAACAGGTGCTAATGTGCA
GAAGATTCTGGTCTCAACACCCCTTGGCGTCTCTCTCTCCCGCATCGACTACGCACCAGGTGGTCTCAACCCACC
CCACACTCACCCCTCGTGCTACAGAGATGGTGTTCGTGCTTGAAGGCGAGTTGGATGTGGGGTTTCATCACCACCTC
CAACACTCTCATTTCCAAGTCCATTAAAGAAAGGGGAGATCTTTGTGTTCCCAAAGGGGTTGGTCCACTTCCAGAA
GAACAACGGCGAAGTCCCTGCTGCCGTCTATCTGCTTTTAAACAGCCAGTTGCTGGAACCCAGTCTATTGCGGT
GAGCTTGTGTTGCTGCCTCACCACCCGTGCCTAACAATGTGTTGACCAAGGCTTTCAGGTGGGTACTAAGGAGGT
TGAGAAAATCAAGTCCAGACTTGCCCAAGAAAGTAGAATAAACTTTGATTGCTGCTCTACTGTGAATTTATGAT
GACATTTCTTTGACATCCACTGCTTGGAAATTTTCTTCTCTCTGTTTTTTTTTTTTTCAATTCGTGAGTACCA
GAAATAAAAAACCTTGTCTGTTTACCGCCAAAAA

Protein

MAAVVALFVITLAVFSGTVAADPDMLQDVCVADLTSGVKVNGFSCKDNTITATDFFFDGLAKPGLTNNMGSGLV
TGANVQKIPGLNTLGVSLSRIDYAPGGLNPPHPTHPRATEMVFVLEGLDVGFIITTSNTLISKSIKKGEIFVFPKG
LVHFQKNNGEVPAAVISAFNSQLPGTQSIIVSLFAASPPVPNNVLTAKFQVGTKEVEKIKSRLAPKK*

VvGLP3**cDNA**

ACCTCTCACCCAAGGACACAGATATCGATTGTTGGAGAGGGTCTAACAATGATGAAAGGTGTTGTTTCTTGTGTA
ACTGTTGCCCTCTTGGCTTTGGCTTCCCTCTCTTGCCTCTGCCTCTGACCCTAGCCCACTGCAGGACATTTGTGTT
GCAATTAGTGACCCCAAGGATGGTGTGTTCTGTAATGGAAAGTTCTGCAAGGATCTAAAGCTTGCTTCAGCTGAT
GATTTTTTCTACTATGGGCTCCACATTCCAGGAAACATAACGAACCCAGTTGGCTCAATGGTCACTCCTGTAAAT
GTTGAACAAATACCAGGACTTAACACCCCTTGGCATATCCATGGTTCGTATTGATTATGCACCATACGGTCAAAAC
CCTCCTCACACACACCCTCGTGCTACTGAGATCCTAGTTGTCTTGGAGGGAACCCCTCTTAGTTGGCTTTGTGACA
TCCAACAACGAAACCGCCTCATCAGCAAAGTCTTTTACAAGGGTGTGTTTTTGTGTTTCCAATCGGTCTCATT
CACTTCCAATTCATGTTGGGAAGGCTAAAGCGGTTGCCTTTGCTGGTCTGAGTAGCCAAAATCCAGGTGTTATC
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GAGGATGTGGTCAAGGACCTCCAATCCCGCTTCTGGTGGGACAACAACATAAAGAAATATTTAGTTGAGCAAGTGA
TGAATATTTGCTTGTGTTAAGTAACCTTTGTAATGTTCCCTCAGCATAATGGTAGTACAATAAGAGGGTTTATT
ATGTACTTCTTTCTATATGGACTTTGGGTATAATAAAAA

Protein

MMKGVCFVLTVALLLALASSLASADPSPLQDVCVAISDPKDGVFVNGKFCKDLKLASADDDFFYYGLHIPGNITNP
VGSMVTPVNVEQIPGLNTLGISMVRIDYAPYQNPPTHPRATEILVLEGLTLVGFTSNNENRLISKVLYKGD
VFVFPGLIHFQFNVGKAKAVAFAGLSSQNPVITIANAVFGSDPPIDPDVLTRAFQLLEDVVKDLQSRFWWDNN
*

VvGLP4**cDNA**

ACCCCTCACCTCAAGTTCTCAACACAAAGATCTGTGATTGTTGGAGATACTACTCTAACAAATGAAGAAGGGTGT
AGTTTCTCTGGTAACGTGTTGCCCTCGTGGCATTGGTTTCTCTCTTGCCTCTGCCTCTGATCCAAGCCCACTGCAG
GACACCTGTGTTGCCATTGATGAGCCCAAGGATGCTGTATTTGTGAATGGAAAGTTCTGCAAGGACCCAAAGCTG
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TATGGCCAAAACCCCTCCTCACACTCACCCCTCGCGCCACCGAGATCCTAACTGTCTTGGAGGGAACCCCTACGTT
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TTCCAGTTGGACAAGAAAGTGGTTAGCTACCTTCAGTCAAGGGTCTAGTGGGACAACCCTAGCAAAATATATAG
CTCTAGAAGCTATGAACCATGGTTTTCTTAGTTGGTTTTTAAAGTAAGCATTGGTATGTTCTCATCATAACCA
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CTGTAAACCAAAAAA

Protein

MKKGVSFVLTVALVALVSSLASASDPSPLQDTCVAIDEPKDAVFVNGKFKCKDPKLTVAEDFFFSGLDKPGNTSNA
VASNVTTVNVEKIKGLNTLGISMVRIDYEPYQONPPHPTHPRATEILTVLEGLTYVGFVTSNTENRLISKVLNKG
VFVFPGLIHFQFNVGKTKAVAIAGLSSQNPVITIANAVFGSDPPINPDVLTRAFQLDKKVVSYLQSRV*

VvGFP5**cDNA**

GACTAACAAATGAAGTGTTAGTTTCTGGCCCTCATGGAATTGGCTTCTCTCTTGCCTCTGCCTTTGATCCAAGC
CCACTGCAGGACACTTGTGTTGCCATTGATGACCCCAAGGCTGCTGTATTGTAAATGGAAAGTTCTGCAAGAAC
CCAAATCTGACTGTGGCCGAGGATTTCTTCTTCAAGGGCTGAATATTCAGGAAACACAGAAAATCCACTGGGG
TTTAATGTCACTACTGTAAATGTTGATCAAATACCAGGACTTAACACTCTTGGGATATCACTGGTTCGTATCGAT
TATGCACCCTATGGTCAAAACCCTCCTCACACTCACCTCGTGGCACTGAGATCATCACTGTCTTGGAGGGAACA
CTCTACGTAGGCTTTGTACATCCAACCCTGAAAACCGCCTCATTAGCAAACCTCTCAACAAGGGGGATGTTTAT
GTGTTCCCTATTGGTCTTATTCACTTCCAATTCAATGTGGGCACGCTAACGCAGTTGCCATTGCTGCTCTAAGC
AGCCAAAATCCAGGTGTAATCACGATAGCCAATGCAGTCTTTGGTTCAAACCCACCCATCAATCCTGATTTCTT
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GAAGAAGCTATAAACCTTTGATTTACTTGTCTCAATGAATACTTATGATGTTCAATTCCTCATTATAAGCATAA
TATGATAAGAGGCTCTATTTGTAACCTCTGGTGTGGACTTAGGTTCATATATATTATTAATAAAGCAGTTTTTG
TAAAAAAAAAAAAAAAAAAAAA

Protein

MELASSLASAFDPSPLQDTCVAIDDPKAAVFVNGKFKCNPNLTVAEDFFQGLNIPGNTENPLGFNVTTTVNVDQI
PGLNTLGLISLVRIDYAPYQONPPHPTHPRGTEIITVLEGLTYVGFVTSNPNENRLISKLLNKGDVYVFPGLIHFQF
NVGHANAVAIAALSSQNPVITIANAVFGSNPPINPDFLAKAFQLDKKVVEYLQAHF*

VvGFP6**cDNA**

AAACATTCTCTTTATATTTGGTGAAGATGTTTCTCCCAATTCTCTGCATCTTTTCCCTTATCTTATCATCTCAT
GCTGCAGTGCAGGACTTCTGCGTCGGGGACCTTGACGCCCCAGAGGGGCTGCAGGCTACTCTTGCAAGAAGCCT
GCAAAGGTTACTGTTGATGATTTTGTCTTCTCCGGCTTAGGCATGGCAGGGAACACCTCAAATCTTATCAAAGCT
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GCTGTAGGAGGGGTTGTGCCAATGCACACACACCTGGAGGTTGAGAAGTCTGCTTGTACGCAGGGGGCAATC
TGTGCTGGGTTCAATTCCTCAGCCAATACCGTCTACTTCAAACTCTTAAGAAGGGTGACATTATGATTTTCTCT
CAAGGACTACTGCATTTTCAAGTGAATGGGGCTGGGATACCATCCACCGCTTTGTAGCTTTCAGCAGCCACAG
CCTGGTCTCCAGATCCTTGATTACGCTCTGTTTGCAAACGATTTGCCAACTGAAGTGGTGGTGACAGACCACTTTC
CTTGACGCAGCTGTGGTGAAGAAGCTTAAGGCTGTTCTTGGTGGTACTAATTAAGGTCAGTGGAGACCACCGCTG
CCTCATTTTCTTCCATCATCACTCATCTGTTGTTTCTCTTCCCTAGTTTCTTCTCTGTGGTTGAGCTGGTCTCTAC
TCATAGGATTCTTTGAGTCAGTTTAGTGTTTCTTTCTGTACTGGTTTCTATGTCAAGTATGTGAACCTAAGTGA
TTGTTGCATGCATTCCAGCTGGTAATAAACAGCATTTTTTCAAAGACAAATGCAATTCCTTTTCGAGTCCAAAA
AAAAAAAAAAAA

Protein

MFLPILCIFSLILSSHAHVQDFCVGDLAAPEGPAGYSCKKPAKVTVDDFVFSGLGMAGNTSNLIKAAVTPAFAPQ
FPGLNGLGLSIARLDLAVGGVPMHPTHGGSEVLLVTQGAICAGFISSANTVYFKTLKKGDIMIFPQGLLHFQVN
GAGIPSTAFVSFSSPQGLQILDYALFANDLPTELVVQTFDLDAVVKKLKAVLGGTN*

VvGFP7**cDNA**

ATCCCCGCTAAAGATAAAGATTGTGATTGTTGGAGGCAGTACTCTAACAATGAAGAGAGGCGTTAGTTTCTCTGG
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CATCCAACCTGAACACCGCTTCATTAGCAAAGTCTGAACAAGGGGATGTTTTTGTGTTCCCTTTCACTCTCA
TTCACTTCCAAGTCAACATTGGGCACACTAATGCAGTAGCCATTGCGCTTTTAAACAGCCAAAATCCAGGTGTAA
TCACCATAGCCAGTTCAATGTTTGGATCAAATCCACCCATCAATCCTGATTTCTTGGCAAGGGCCTTCCAGTTGG
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GATTTCTTGCTGAATGACGATTATGATGTTCAATTCCCTCATTTGTAACAAACATGGTATAATAAGAGGGTCT
GGTTGTAACCTCTTCTGGTGTGGACTTTGGGCATATTACTACAATAAAGCAGTCTGCAGTCTGTTTGTAAAAAA
AAAAAAAAAA

PtGLP1

cDNA

Protein

PtGLP2

cDNA

Protein

PtGLP3

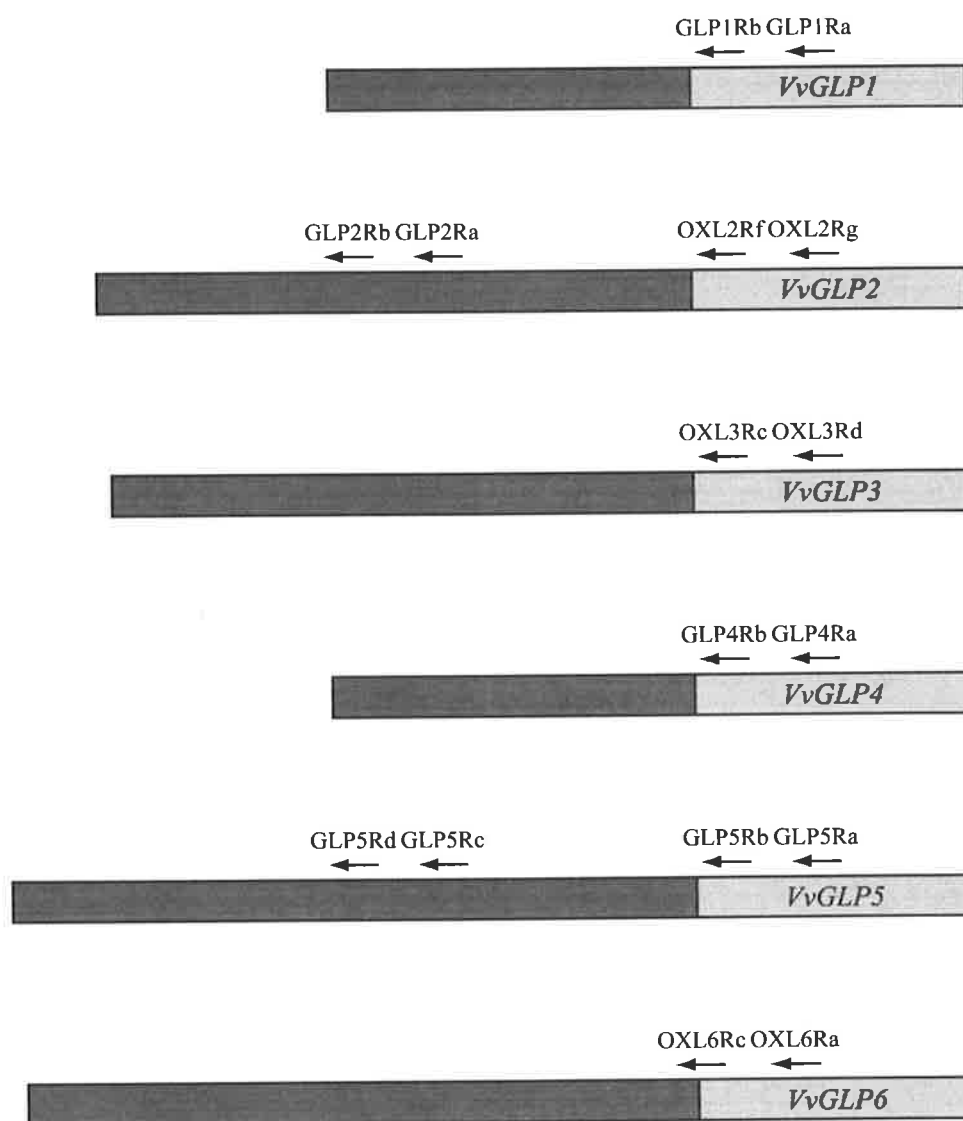
cDNA

Protein

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Appendix 4

Stylised diagrams indicating the location and orientation of primers used to amplify *Vitis vinifera* cv. Shiraz GLP promoter clones (Chapter 3).



Appendix 5

Vitis vinifera GLP promoter sequences (Chapter 3) are shown below. Promoter fragments were isolated from Shiraz (*VvGLP1*, *VvGLP2*, *VvGLP4*, *VvGLP5* and *VvGLP6*) and Chardonnay (*VvGLP3*) genomic DNA. Putative start codons are shown in bold. Potential TATA and CAAT boxes (Shirsat *et al.* 1989, Tjaden *et al.* 1995, Grace *et al.* 2004) located in the sequence immediately upstream from the translational start codon are boxed. TATA and CAAT boxes are on the negative strand are indicated in boxed italics. Unresolved nucleotide residues are indicated by an N.

VvGLP1 Promoter

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1 CCTTTGTTCTCTCTCTGGATTTTTTACCTGCAGAATCTTCTAAGACAAGTTGCTTGACTG
61 AACACAGGATATGCATAAAGCCAAAATCATTTTCAGTTTCATTTTCACATTAATTCAAATAC
121 CCAAGTCCTATATGACCTAGAAATGGTCTTGGAGGGAACCCCTACCCTGATTTGCAAAC
181 AAAACACTAATGGGTCATGACATTAATCACAACTGATCATAGATGGCTTGTAGTCCACA
241 GGTTTACACCCCTTAGCTTTACCCCTGGACTAAGTCCTGAAACCCAAAGATTATGCAGAATC
301 AAACCTCTAAAGTCTACAGCATGAGTTGAGGAAAAAAGGATGAGTAATTAATGTAGTAGT
361 TGAGGAATGATTCAATGTTTGAAATAGAAAAAATGCAATCCATTGAACACTTAGCTGGCC
421 TATCTGCTTACTGTATACATAAAACATAGACTAATCCGAAGACTTCTATGTGATAACTAG
481 AGAATTTTCATATCCAAGGTTTTTCACTTCTTTCTCATCAGAAGACAAAATTGTAAGTGT
541 GGAGAAAACCTAACAACCTAGTTTATTTGTTAAGGTCCCTTTCTCTCATAAAACATAAAT
601 ATATTCACTATCAATGACTATAGTGATAATAATAATGGTCTTCTTTTTGCTGTATATACA
661 CTACTTTAGAGTTAATTTGTTCAATGTTTGGTTACCAAGAAAACAGAAGGACTGATCTGC
721 TGCATTAAAAGGGTAAGTAATCCTGAATACTTTCACTTCATTTTGCCATATGTAGTTCTT
781 CCATTAGCTAGCCATTTGGAACCTAAATCTGGGGGAGAACTTTTCATATGGGAGATGGATA
841 TGAAATTGGACCTGCCTCCGCCTGATTGAACCTTCACTTTCAACATATGCATTGAGGAATA
901 TGTTAAGATCAGTATATACTAATAACCTACATAAAATTAACATGTATTGGTGAAAACACTT
961 ATCCTTGTAAGTGAGAAAGCCAAAAAATACCTACCCATTGAAGACCATCTAT
1021 AAATTTGACATGTTTCTCATGCAAAATTTTACCCCTCACCTAAACACAAAGATTTGTGA
1081 TTGTTGGAGACAGTACTCTAACAATGGAGAAAGGTGTTAGTTTCCTAGTAACTGTGGCCC
1141 TCAATG
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VvGLP2 Promoter

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1   ATTATATTTAAGCACACATGTCTAGATATCCACCGTCCGCTCCACGACAAAACCTTAATTT
61  TGGTTCAAAATGACCATTTAGAACAAACAAAAGATCTATTTAAAGTATATTTTGAAATTT
121 TGTATAAAAAGAACTCTCTCTCATATTTTATCTTTTATTTATTTATTTAAAAAAAACCT
181 TTCAAAATATGATTTTCTAATAATTTAAAAAGAGAAAAAAGGAGGACTACTTTGGAC
241 AGTAATATGAAAACATGATTAAAATGAAAATTTTGTGGGTAAAACTTCGAAGTGCCCAT
301 TCGACCCAAAATTCAGAAATCTTCCCGTAGTTTGTCTTTGGAGTCGGTGCACCAAAAGC
361 CCAATTTAAGGAGGGTGCATGGCTCATACCAAATGGGTGACTGCTGACTGCTGACTGCTC
421 TTGTAAAGTCATCTAATTAGAACCTTTGTATGACACCTTGGAATTTGGAAATCCACAAA
481 TAATACAAATCAACCGATCCAAGAATTGACTAGCTGCAAGCATTACAGCATTGCAAGAAA
541 GCACAACCGTTAACAATAAGAGATATTATGGAGAAGAAAGCAACTGGCATTACAGAAAAG
601 GAGAAATAGGCAAAGCAATGAACCTTGAAGGAAAAATAAGCAGGAAGGGGTATGAGGCC
661 ACTTAGAAACCGTAGGCTTTGGTTGAATTTTCTTCTTGAGACTTGACTTTGCAAACAGC
721 CTACCAAGGCCCAAACCTCCACGAAACAGCGACCTCATCCCTTGCAATTCAAGGCGATTTCA
781 TAAGTATTCTCCATATTCAACTCCGCCATTATCTTCCAATAGAAAGACAAAATGCAGA
841 GCTCATCCACAAGCCATTTAGTCTAATTCAGGTTTGCAATTGAAGGCAAATTTATAGACA
901 TTTGAAATACTAAAGGATAATCACGACAACTCCCATCAAAATGCTAACGGTTAAAATTCA
961 CCTAAAGCTCATAATTTGACATTGAAACAACTAGATAAATCTTCTCTTCCCCCTTCCAA
1021 GAAAATGGAAGACAAAATGCAGAGTTCTCTCTACAAATATAAAGCAATTTAATAACAAA
1081 CATAAGAAGATTTGATACTCAAAATTTTCAGTGAAAGGATATTAGAAAGGAAATTCAAAA
1141 TATCATGCACCACCTTATGAATGAGGATGACAACTCCTACAGAAGTAGCAGCATCCTAAA
1201 AGAATCCGCTAAAATGCTAATACAACTCACAATTTGACGCCCAAAGAGCTACCCACTTCA
1261 ATTGAAAACAGCATAATTGAGAGCCCACTTACAGCTTAGATAACTTAGAGCATGACATAT
1321 TTCAGGAAACAAAGCAAGGCCATGGGCCCCAAGGCAGAAGGCACCCAACATGAAATTTAA
1381 CTTGGCACACCTGTAAATTACATTAGTAAAACAATGAATGAACCTTGATTAATCGATAAA
1441 ATAATAATATTATGAGCAAAGGTTAAAGCTTTCGGTGGTTGAAAGGGCAGTGCAACGCTG
1501 CTTCTTGAGGACTACGCACAGCCAAATGGGTGAGATCCCGTGGACGGCACGATGAGAAA
1561 TGGGGAGTCAAATGGGCATAACAAAGGCAGAGTAAAGGGCCTTCTGATGCCTTTACTTTG
1621 GGTGGAAGAAGGCATGTTTCGTGCTTTTTTAGTTGCGCAGTCTCCACCAAATCAACGTCA
1681 CCACCTCTCGTCTTTCCCAAGTCCCAACACACAAATAATATCATTCCCTCAAGCTCATA
1741 CTTAACTCATCATTATAAATACACAACCCCACTTTTTCTCTCTCCCCATTCTAATTTGAG
1801 ATATCCATCTACTTTTTCCCCCTTCTCTCTTTTTTCATATCATTATTATCTTTGTATTTT
1861 TTTTGTTCCTCCGACCATG

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VvGLP3 Promoter

1 AACTCCATGATTTTCAGCTTTTGCTCGCCATGGGAATGGTTCCTAGAGCACTTCAGTTGTAT
 61 GAAGAGATGAGATTAGAAGGTGTTTGGCCAACAGATGTTACATTTCTCTCTGCTTCAT
 121 GCTTGTGCCCATGTGGGTTTAGTTGAAAAGGGCATGGGGTTCCTTGAATCCATGGCTAAA
 181 GATTATGGGATTGGTCCAAGGATGGAACACTATGCTTGTGTGTTGTGACATGATGGGCCGA
 241 GCAGGGCTTCTTAATGAAGCCAAGAAGTTTATTGAGAGATTGCCTGAAAAACCTGGTACT
 301 CTTGTTTGGCAAGCATTTGCTTGGAGCTTGTAGCATTTCATGGTAATTCGAAATGGGAAAA
 361 TATGCAGCTAATCAATTGTTTGTGCAAGCACCTGAGAGCCCAGCCCCCTTATATTTTACTG
 421 GCTAATATATATTCTTCTGAAGGGAATGGAAAGAGAGAGCAAGGACCATCAAGAAAATG
 481 AAAGATATGGGTGTGACAAAAGAAACAGGCATAAGTTGGATTGAGATTGAGAAACAAATC
 541 CACAGTTTGTGTTGAGGACAGAATGCATCCACATGCCGAGATTATTTATGGCGTTCTA
 601 GGGGAGTTGTTTAAACTGATGATGGATGAAGGGTATGTGCCTGATAAGAGGTTTCATTCTC
 661 TATTACTTGGATCAACATGGAAAAGAATAGTCAATAGTTATTCTAAGAGAAATTTGAAAA
 721 ACGAAATCAGCTTTTAGGAATGCATCATATGGTGCATTTGGTATGACCTTGAAAAATTGCA
 781 ATTGCTCGCTCAGACAGATGGGGGCCAACATTTCTTCATTTACTGAAGAAGCATGAATAA
 841 GAAACTCAACATTTTCATTGAAGATGCAAACTATTTTCATGAGAGTGACTTTACACCTTCAT
 901 TGCTTTATTTTATCAAAATGCTGAAGCCTAAAATGAAGAGTAAGTTAATTCCTTTCTTATT
 961 TTTTTTTTTATTTTGGAGACTTTTACTTCAATCCAATCCAATTCCTTCTGTTGACTTCAAT
 1021 CAGGTTATTGGTATATTTTTGGACTCAATAACATGAAATCTTTGTTGTTTGGCATATGTA
 1081 CTTTGTGAACATTTATGCCATCACCATGCATGAAACCGAAGGTGAGCTACTCCTCTTAAC
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 1381 GAATTGCTGATATTTATTACCTATTCTGGATGCTTCCATCTCCTCTTGACATAGTTTTT
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 1561 AATCTTCTAGATAAGTCCATGTCAAGATATATCAGTTAAAGCAAAGGGAAAAACGAAAAAG
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 1681 TGGTGTAGACTGGCAAAACAAAGACTACTTCATCTGATAGAAAAAAAACCAAGAAAAAC
 1741 AAAGACTAATTCAAGGACTATTATCTATAAAATAGCATGTTTCCATGCAAGATTTCTCAC
 1801 CTCTCACCCAAAGGACACAGATATCGATTGTTAGAGAGGGTCTAACAATG

VvGLP4 Promoter

1 CCTAGCACTACATATATATAAAAGAAAATGAACTGCAATTAATTTTCAGATTAGAGAAGCC
 61 GGCTGGAAATCTTCTAGATCAGTTCCAACATAATTACAAAATTGAAAAATAAAAATGAGA
 121 GCACCTAGAATTTGACTTTGGAAATTGAAGAAAGAAGGCCACTCCAAGAATGGGAAAACC
 181 TCTAGAGGTTTTCTATTGGTGCATAATTTTCTATGCATTTATGCCTTGACAGTCTATGGC
 241 AATGCAGAAAGGGAGAGAGATGATAGTGTCTATGCTTTGAAAACCTGACCAGGGCAATGCA
 301 TACATTCTAAGTCAAGTGCATATATATATACGAGAGAGAGAGAGAGAAGTGATCAGTTCC
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 421 AATGACCCAAAGCTTTTCGTAATGTGATTTTAAGGACTAATTTAGGCATCACCCAAAGATT
 481 AAGAATGGATGTTAGAAGTCTCAGGTGGATGGTTTTATTAATAACATTCTCTCAAAGTGC
 541 AAGGGACTCCATGGTTAGTTTACTGAATTCATTTGGCAGTTGCAGATGAATTCAGGATC
 601 TTCACTTTAACAATAAGATTGCAAAAGAAAGATAAAGAAGAACCGGAGTTATGCCTGGT
 661 CTAAGCCTGCACCTACTCTACAATCTTCTATTTGCTTGTCCATAAACCCTTTTAAGGGT
 721 TTTCAATGTTTGGTACTGGGAAAACAGAAAGCTCTACTGTGTTATATGCTACTAGTTAA
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 841 TAAGTTGAAGATGTCTAACACAACCTGGAGTTTAGTTTTGACACGATCTTCTAGATAAG
 901 TAACCGAGAAAGAAAAACACTTAACGAGTAGTTGTTGAAGAACCATTTCATCATTGAAAGT
 961 TCTTTTAAAAAAGAACTATTCTATTGAAGACTTGGTTAGTTTATGCATGTGATATACTAG
 1021 TAAAACAAAAGACTAATTCAAGGACTCTCGTCTATAAAATGACATGTTTCCCATGCAAAA
 1081 TTTCTACCCCTCACGTCTAGCTCTCAACACAAAGATTTGTGATTGTTGGAGATACTACT
 1141 CTAACAATG

VvGFP5 Promoter

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1   CTGCCATTGGTGCCCAAGTAAAAACACACTCATTAGCAAATTAACTTGAAACCGTTTAA
61  GATATGCACCAAGTCTCAAGAAAAGAAAAGTACTTTCTAACAAAAACAAAGGAAAAGAAT
121 TGAATACATACAATATAATCAATCAAGGGCATTTCATAAGAAAATCTACCAATGGAAAAA
181 GGTTTGGCCATAGAGAAACCTAGTATCAAATAAATGTGCATAAGAACGTTTTAGTAAAAA
241 CGCAAGGCTGCACTCATGAATAGTTTTTCTAAAAATGGACTAATTAAATTATTTGGAACCCA
301 TAAGAGTTAATTAATTAGTTAGGACCCAAGTGGGCTGGATTAGGTGATCCAAACTCAATT
361 TAGGCTCAAGTCACTTAACCCCATAGGGAAGCCTCAGTAAACCCCTTAAGGTTTAAAGT
421 TTTAGCCTTATTGTTAAGTTTTTTTAAAAATTCAGAAAAGGAACCCCTAGCTACCTTCTAGA
481 GAGAGAAAGAGAAGTTTACCCTTCTTTTATACCAAGACCCATGGAGAGAGAGATCGGGTG
541 AAAGATTCTTGGATAGACGATTCTACTATGACCACAGTGGTTTTCAAAGTATTCATCGGAT
601 GAAGATATTATTATTAAAGTAAAGAAAAGAAAATGAAAGTCAATCATTTTTTTTATTATTT
661 GTTTTATTACCTTCTTAATTTTAATTTTGTAATAAATTTTAAAAATAAATAATTATAAAAT
721 AGTACCAATTAATAAATGTAAGATTTGTTTATTATTTTATATTAAAAATAACATTTTAT
781 TATGTAATTTTAATATATAAATCATATTATAATATTAAATTATTTTTTTATTGGTTAAATGA
841 CAATCCAAAAATAATTAAATATGATTTTTTATATTGATATTAAATATTGATTTTTTTTCAAT
901 AAATAAAAAAATTAATAAAAAGAATAATCTTCAAAACCTATTTTCTATAAATATATTTTCAT
961 GATTAAATATATAAGTATTTAGTCTTGAGTAAATATGAATAAATTTGATAATCAAAGGTA
1021 ACTTTTCNCCATGGATATAAACTCTGTGGCTAGACTTGATATTTTCCACAAAGGCATCA
1081 CTCATCNCAAAAAAAAAAAAAAAAAAGGAAAAGTTTTAAATTTGTTGGGAAAAGTGGGCTT
1141 TTGGCAAACAGGAAAATTTGTTGTAGTGTATCCATCAATATATGAAAGGAAAGTTATTCA
1201 CTAACCTTCCAAGTTTAACTCAATTTGAAAAAGTGCACCTCTTTTTCATATCTAGTAAGA
1261 GAAGCCATGCACTCTTAAGCTAATGTACAGTCCATTCTGAAGTACTTATTGAGATATCTA
1321 TGCTAATGGATGCAGTTGTCAAGTAGCTATAAGTTACCTAACCATGCATAATATTGATTA
1381 AGAAGCAATACTTAAAAATAGTTGTAAAAAAATTTCTCTTACTTCCAGCATACTGTGGCAG
1441 AATTGGCCACTAAGAAATGAGCATTATGGCAAGTCCTAACACCTAATTCATTACTCTAAC
1501 AACTGCTTACAATTCAGCAATGGAAAGATGCCGTTTTTGCTAATTCTCTAAATTAATCCTC
1561 CCATTTTTAATTTTAACTTGGTGTGGTAAAAGGGTTATATAACCCATCAAATGAGGTTAA
1621 AGTAGCTACTTCATAAAATGTCCCAAGTGAGCCATTTATATTGTTAGATCAAAGAAATTG
1681 GAGAGGGTGGGAGAAGTATAAGTGTATGGAATAGTGAAGAAAGAATGGAAATGAAAAC
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1921 ATTGAGATGGTGGGAGAAGTGTAAGTGTATGGAATAGTGAAGGAAAGAATGGAGAAA
1981 TGAAAATCCTTTGAAGACTTGTCTTGTAAAGTGAATAAAAAGAACTGCCCATTTGAAGATT
2041 TGGTGAGTGCGAAGGCTAATTCAGTACTCTTGTCTATAAAATTAACATCTTTCCCATGCA
2101 AAATTTCTCACCCCTCACTGACTAACATGAAGTGTTAGTTTCTGGCCCTCATG

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VvGLP6 Promoter

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1   CTGCTAAAGGTTATATTCACCATCCTGTAAATTCTCTACTCACTGTAGAGTGCGTGGTAG
61  ATACNGTAAACCCTAACACCACAAACGCCAACCTTTGATACATGGGGTGTAAGTTGCTC
121 CTTAAACCCTAACACCACAAACATCGAGTCTTGATACGCGAGGCGTATAGTTGCTCCATA
181 AACCTAACACCGCAAACACTTATTCTTAATAGGCAGAGCCAATTGAACAATCATCTTTT
241 CTAAAAGCTTAAGTTGTCGGGATTTGAATACTTTCTTCCAGCTCATGTAGAGATGGAAA
301 CAAGTGGGTAGACGATCATAAATTTGTGATAATAGATAAATAGATGGATGGAGGTCTCAG
361 AGTTCCGATAATGAGAGCTCTACTCTAAAGTCGTTTTAGGAACGCTGGAATTTTCATTCT
421 CATTAATTAATGTGTTTGGGTTTTATTTTATCTCACTCCTGATAAAATATATATATAAAA
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541 TTTTTTAATGAATTATATAATTATTTCTTGAAATACCATTTTCACTGATTATGTCATCAC
601 TAATAATTGAAAATTAGATTTCGTCCATATAAATGTTTTCTTCTTTGAGATATGTTCTT
661 TTATTATTATTATTATTTATGATTTTTTTTTCTCCAACAAATAATTACATAGTAGAAAAAG
721 TGGAGAATTATTTTGTCTTTTATAAACAAACATCTCATAGCAAATATGAGGTAGTTTCGAA
781 GAGCGTGATCATTAGAAATAGTGAGTCTGATAGATTATAGATGAGTTTGAGTTTTATTAT
841 GAATAAAATTTATGTTTTTTTTTCATGGTTTCTTTATGAATTTTTTAATTAGAGCAAATTGA
901 GGTTAATTTTAAGGATTCTGGATTATTCAAGGCATTTACTTTATTTTTTACCTTCACGTTT
961 AATTCAAGAAATAAAATACAAATAAAAGGTAATATATTGTTATTTTTTAAAGACAAATG
1021 AGTTTGAGTTCCTCATGGAATAGCTATTGATGCAGAGAAGGGAAACAAAAATAAAATAA
1081 AATCCTTATGAGGTGTTTGTTTTTTGAAAAAAAATCTTAGATAATAATAATAAACAT
1141 ATCTAAAAGAAGAAAAACATTTATGCAGAAAAAGTGTAGTCGTGAATTGGTACAGATGACA
1201 TTACAAGTAAAACTATTTTTTGTCTAAAAAAATTCATCATCGTGGAACCCAAATCTCAC
1261 TCTCATTTCGTATCATGATTGATTCCTCTCTTTTCCATTCCCTATTTCCCGGTACTAAAGCC
1321 TAAAAGAGTAATTTGAATAAGCTAGGCTAATACTGGGGAAGGATAAGTTGAAATTTGAAA
1381 TGCAGAAGCAAGTGAGATGGTTGAACAATCCGGACAATTCTATGAGAATCTTGCAAGTGGG
1441 GTATCCACAAGATGCCCTTATCCTCTGGCAATCAACAAAAGCCCTTCAAGATGGGCAATG
1501 TCTAGGAACCACACATTGGGCAATGGCTACCGTTTGAAGGGGTGGGAGTCTGGGACGAC
1561 TTGCAGGAGTGCCCTTCTAGCTGTAGAAAAATGTTTTGTTATACTAATGGACCACCTTCTC
1621 ACATCTCCCTATCTTGTTTGTATGTTGGGATAAGACCAGATCAGATATCAGAAGATGTG
1681 ATTTGGGTGGTGGTGAAAGATCGTAAATGAGGGGTTTGCTGAAAATTTCAAGCTTACGGA
1741 TTGACTGTGTGTTGAAATATTGAAGTTTTCTTACTGCCTCACTGGCTTGACGTGATGGAA
1801 ATTGAAAAGGCCAAAGATGTTTTCACTCCTTGCCGTCATCATGAGCCAAAAAGAGGTT
1861 TCTTGGTTTTTTCTGAAAGAAAAGATTGCAGAATCACATGCATCACAGCTCAGAATCTGT
1921 CATTTTTCAAGCTTTCATTTTAGGGTACACTGCATGTGAACTTTTTGGCCGTATATCTC
1981 TAGCAATCAATGGAAGATGCTGAAAAAATATTCCGCATTCTGATTCTATATATAAGGG
2041 TGCCAAGCCTGATCAGGACGGCAAAGAAGGGCTAAGGCAAACATTCTTCTTTATATTTG
2101 GTGAAGATG

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