Identification and Characterisation of *Vitis vinifera* Pathogenesis-Related Proteins That Accumulate During Berry Ripening

by

David Bruce Tattersall (B. Sc. Hons)

A thesis submitted for the degree

of Doctor of Philosophy

Department of Horticulture, Viticulture and Oenology

Faculty of Agricultural and Natural Resource Sciences

The University of Adelaide

May, 1999

TABLE OF CONTENTS

ABSTRACT

The profile of soluble proteins found in the juice of ripe *V. vinifera* berries often appears surprisingly simple, with the predominance of a few low molecular mass proteins that are known to be associated with white wine haze formation. To understand the biology and function of these proteins in the berry, and with the long term view to reduce the problem of haze formation in wine, the following study was performed.

Four prominent juice proteins were purified and through amino acid sequence analysis identified as pathogenesis-related (PR) like proteins. A detailed study was conducted on two of these proteins, namely VVPR-4a, a 13 kDa PR-4 like protein, and VVTL1, a 21 kDa PR-5 like protein. cDNA sequence analysis and electrospray mass spectrometry suggested both proteins undergo post-translational modifications, including the removal of an N-terminal signal sequence. Analysis of grapevine genomic DNA suggested that VVPR-4a and VVTL1 are encoded by genes that belong to multigene families. Both genes appeared to be expressed mainly in the berry, coordinated with the onset of sugar accumulation and softening. The genes of VVPR-4a and VVTL1 were sequenced, and putative promoter elements possibly involved in the developmental control of gene expression were detected in both cases. It is believed that PR proteins play an important role in plant defence, particularly in the protection of plants against fungal attack. In this study, *in vitro* fungal growth inhibition assays also suggested berry PR-like proteins may perform this role. We propose therefore that a function of the developmentally regulated accumulation of PR-like proteins in the grape berry is to provide a pre-emptive measure against pathogen attack.

STATEMENT OF AUTHORSHIP

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 is 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.

David B. Tattersall

ACKNOWLEDGEMENTS

Foremost, thanks to my PhD supervisors, Prof Peter Høj and Dr Robyn van Heeswijck for their excellent guidance, support and patience throughout my studies. Your advice and encouragement has been invaluable.

I thank Dr Elizabeth Waters, Dr Simon Robinson, Dr Yoji Hayasaka and Ken Pocock for the highly informative discussions about protein-associated wine haze. Additionally, I thank Yoji for mass spectrometric analysis, and also Dr Neil Shirley and Jelle Lahnstein for amino acid sequence analysis.

Thanks to Dr Eileen Scott, Dr Belinda Stummer, Robyn McMahon, Michael Priest and Dr Trevor Wicks for providing fungal isolates and associated advice. Also, thank you to Andrew Jacobs and Dr Ian Dry for the RNA samples prepared from powdery mildew infected grapevines, and Richard Stewart for the gift of the GII β-1,3-glucanase expression vector.

A big thank you to past and present members of the Høj and van Heeswijck labs, in particular to my long term colleagues, Anna Stines, Dean Naylor, Patrik Jones, Alison Kellow, Anne Morgan, and Kathy Adams. It has been a great pleasure working with you all and I am grateful for the friendships, help, advice and comic relief. A special thanks to my good friend Chris Ford for his guidance since my arrival in Adelaide, and informative discussions about science, life matters, and most importantly fishing. Also thanks to all the staff and students of the 'Waite' I have known over the years, for the support, friendships, and cake!

A sincere tribute to my family, in particular my parents, Kevin and Sylvia, for their understanding and encouragement. Thank you to my best friend and partner Kylie, for the ongoing emotional support and love.

Financial support from the Grape and Wine Research Development Corporation and the receipt of an Australian Postgraduate Award is gratefully acknowledged.

ABBREVIATIONS

A	adenine
ABA	abscisic acid (5-[1-Hydroxy-2,6,6-trimethyl-4-oxycyclohex-2-en-yl]-3-methyl-pentadienoic acid)
bp	base pair
°Brix	degrees Brix (a refractive index measure of g solute/100 g solution)
BSA	bovine serum albumin
BTOA	benzothiazole-2-oxyacetic acid
C	cytosine
°C	degrees Celsius
cDNA	complementary DNA to an RNA
conc.	concentration
C-terminal	carboxy-terminal
cv	cultivar
Da	dalton
dCTP	2'-deoxycytidine 5'-triphosphate
DNA	deoxyribonucleic acid
DTT	1,4-dithithreitol
EDTA	ethylenediamine tetra-acetic acid
g	gravity
G	guanine
h	hours
HPLC	high performance liquid chromatography
HR	hypersensitive response
I	inosine
IAA	indole-3-acetic acid
IPTG	isopropyl-β-D-thiogalactaside
kb	kilobase
kDa	kilodalton
L	litre
L.	Linnaeus
LB	Luria-Bertani
MCS	multi-cloning site
µg	microgram
mg	milligram
min	minute
mm	millimetre
µL	microlitre
mL	millilitre
M	molar
mM	millimolar
MOPS	3-(N-morpholino)propane sulfonic acid
M_r	relative molecular mass
mRNA	messenger RNA
MW	molecular weight
nm	nanometre
NMR	nuclear magnetic resonance

nt	nucleotide
N-terminal	amino-terminal
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pers. commun.	personal communication
pI	isoelectric point
Ponceau S	3-hydroxy-4-[2-sulfo-4-(sulfophenylazo)phenylazo]-2,7-naphthalenedisulfonic acid
PR	pathogenesis-related
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
SA	salicylic acid (2-hydroxybenzoic acid)
SAR	systemic acquired resistance
SDS	sodium dodecyl sulfate
SSC (20x)	3 M NaCl, 0.3 M Na_3citrate buffer
syn	synonym
T	thymine
TAE (50x)	2 M tris-HCl buffer, 1 M acetic acid, 50 mM EDTA, pH 8
TCA	trichloroacetic acid
TE	10 mM tris-HCl buffer, pH 8, 1 mM EDTA
TFA	trifluoroacetic acid
T_m	melting temperature
TMV	tobacco mosaic virus
Tricine	N-tris-(hydroxymethyl)-methylglycine
Tris	tris(hydroxymethyl)aminomethane
Tween-20	polyoxyethylene-sorbitan monolaurate
UTR	untranslated region
UV	ultraviolet
vol	volume
v/v	volume/volume
w/v	weight/volume
WPF	weeks post flowering
WWW	world wide web

One and three letter codes for amino acids

A	Ala	alanine	L	Leu	leucine
R	Arg	arginine	K	Lys	lysine
N	Asn	asparagine	M	Met	methionine
D	Asp	aspartic acid	F	Phe	phenylalanine
C	Cys	cysteine	P	Pro	proline
E	Glu	glutamic acid	S	Ser	serine
Q	Gln	glutamine	T	Thr	threonine
G	Gly	glycine	W	Trp	tryptophan
H	His	histidine	Y	Tyr	tyrosine
I	Ile	isoleucine	V	Val	valine

PUBLICATIONS

Parts of the work described in this thesis have been published or submitted for publication in the following:

Tattersall DB, van Heeswijck R, Høj PB (1997) Identification and characterization of a fruit-specific, thaumatin-like protein that accumulates at very high levels in conjunction with the onset of sugar accumulation and berry softening in grapes. Plant Physiol 114: 759-769

Tattersall DB, van Heeswijck R, Jacobs AK, Høj PB (1999) Ripening induced expression of a PR-4 like gene protein in fruit: Identification and characterization of the grapevine berry protein and its gene. Planta submitted

Waters EJ, Hayasaka Y, **Tattersall DB**, Adams KS, Williams PJ (1998) Sequence analysis of grape (*Vitis vinifera*) berry chitinases that cause haze formation in wines. J Agric Food Chem 46: 4950-4957

CHAPTER 1

General introduction

1.1 Berry proteins and haze formation in wine

Whilst a great deal is known about some of the simple chemical constituents of the grapevine (*Vitis vinifera* L.) berry, and the qualitative and quantitative changes that occur during berry development (Kanellis and Roubelakis-Angelakis, 1993), little is known about berry protein metabolism and the associated ripening physiology. The profile of soluble proteins found in the juice of ripe berries often appears surprisingly simple with a predominance of a few low molecular mass proteins (Hsu and Heatherbell, 1987a). Unfortunately for the wine industry, these soluble proteins survive the vinification process and are known to be associated with wine haze formation (Waters *et al.*, 1991). To understand the biology and function of these proteins in the berry, and with the long term view to reducing the problem of haze formation in wine, the following study was performed.

1.1.1 Protein-associated wine haze

Wine, in particular white wine, can develop a cloudiness or sediment due to the precipitation of protein from solution (Bayly and Berg, 1967; Hsu and Heatherbell, 1987a; Waters *et al.*, 1992). The presence of precipitated protein, usually caused by elevated storage temperature, is often termed 'wine haze' or 'heat haze', and a wine with the potential to form a haze is classified as 'unstable'. To many consumers of white wine, protein associated haze is unattractive and therefore constitutes an undesirable characteristic.

The soluble proteins in wine which have the potential to aggregate to form hazes and precipitates are derived from the grapevine berry. The early study by Koch and Sajak (1959) was the first to demonstrate that several distinct soluble proteins exist in wine. Further research has revealed that wines made from a single variety contain at

least 25 different berry proteins, with molecular masses of between 11 to 65 kDa (Hsu and Heatherbell, 1987a; Murphey *et al.*, 1989; Waters *et al.*, 1991; Yokotsuka *et al.*, 1991; Pueyo *et al.*, 1993; Dorrestein *et al.*, 1995; Weiss *et al.*, 1998). The most prominent juice and wine proteins are acidic and have relatively low molecular masses (approximately 20 to 30 kDa).

The concentration of soluble protein in 'unfined' wine is commonly in the 50 to 100 mg/L range but has been reported to be as high as several hundred mg/L (Koch and Sajak, 1959; Bayly and Berg, 1967; Somers and Ziemelis, 1973a; Hsu and Heatherbell, 1987a; Murphey *et al.*, 1989; Yokotsuka *et al.*, 1991; Dorrestein *et al.*, 1995; Santoro, 1995). Some variations in protein concentrations are most likely dependent on the method of protein determination, and the variety of grape, vintage and location. Most white grape varieties contain high amounts of soluble protein in their juice and wine derived therefrom. Particularly high protein concentrations are commonly found in the varieties Muscat of Alexandria (syn Muscat Gordo Blanco), Sauvignon Blanc, White Riesling, Sylvaner, and Gewürztraminer (Bayly and Berg, 1967; Somers and Ziemelis, 1973a; Hsu and Heatherbell, 1987a; Rankine, 1989; Ough, 1992). Additionally, despite many common phenological characteristics, grapevine varieties do exhibit individuality in their juice and wine soluble protein profiles (Luis, 1983; Hsu and Heatherbell, 1987a, b; Murphey *et al.*, 1989; Pueyo *et al.*, 1993; Dorrestein *et al.*, 1995; Santoro, 1995; Weiss *et al.*, 1998). However, notwithstanding the varietal differences, the biggest factor determining the amount of soluble protein in juice, and the wine made therefrom, is probably berry maturity (Luis, 1983; Murphey *et al.*, 1989). Murphey *et al.* (1989) reported that the concentration of soluble juice protein of White Riesling and Gewürztraminer juices almost doubled in the last six weeks of ripening, suggesting synthesis of soluble protein is at its greatest in the late stages of berry development.

Several studies have shown that only up to a 50% reduction in the protein concentration occurs upon transformation of a juice into wine (Hsu and Heatherbell, 1987a; Murphey *et al.*, 1989; Yokotsuka *et al.*, 1991; Pueyo *et al.*, 1993; Santoro, 1995). Furthermore, electrophoretic polypeptide banding patterns of juice and wine differ only slightly due to size polymorphisms, and appearance/disappearance of bands is negligible (Hsu and Heatherbell, 1987a, b; Murphey *et al.*, 1989; Yokotsuka *et al.*, 1991; Pueyo *et al.*, 1993; Santoro, 1995). This suggests the soluble proteins are resistant to proteolytic attack by fermenting yeast (Lagace and Bisson, 1990; Waters *et al.*, 1992).

1.1.2 Removal of haze-associated protein during winemaking

Potential protein 'instability' in wine is usually estimated by incubation at an elevated temperature, followed by visual or photometric inspection for clarity and precipitate formation (Berg and Akiyoshi, 1961; Pocock and Rankine, 1973). A wine is considered to be 'heat stable' if no haze is observed, typically following an incubation at 80°C for 6 hours. Although all major wine proteins appear to contribute to wine haze (Hsu and Heatherbell, 1987b; Waters *et al.*, 1991), a 24 kDa protein is believed, based upon heating in a model wine, to be the main contributor to wine haze on a per unit weight basis (Waters *et al.*, 1992).

In addition to elevated temperature, early research also suggested that the presence of phenolics may enhance the formation of protein-associated wine haze (Koch and Sajak, 1959; Somers and Ziemelis, 1973a). Although phenolics such as procyanidins represent only a minor proportion of a haze (less than 5% w/w), and are only weakly associated with protein (Waters *et al.*, 1995b), specific interactions between proteins and wine phenolics have been reported (Yokotsuka *et al.*, 1991). Moreover, soluble wine proteins require phenolics in order to precipitate in a heated model wine (Waters *et al.*, 1995b).

The preferred method for removing 'unstable' proteins from juice or wine is by the addition of bentonite (Rankine, 1989; Ough, 1992). Bentonite, an aluminium-silicate based clay, possesses a net negative charge at wine pH (typically pH 3 to pH 4) and acts as a cation exchanger. Through this property, exchangeable cations such as Na^{+}, Ca^{2+} and Mg^{2+} are released and proteins, with isoelectric points greater than the pH of the juice or wine, are bound to the clay. Bentonite has the ability to quickly remove the majority of proteins from juice or wine over a range of different ethanol concentrations and temperatures (Somers and Ziemelis, 1973b, Hsu and Heatherbell, 1987b; Blade *et al.*, 1988; Dawes *et al.*, 1994). Unfortunately, several disadvantages are associated with bentonite use. After bentonite has been added to juice or wine, it must be removed, which can result in ~3% loss of wine volume (D. McWilliam and T. Jones, pers. commun.). In addition, some favourable characteristics, such as various aroma components, may be reduced in a wine following a bentonite treatment (Miller *et al.*, 1985; Voilley *et al.*, 1990). Environmental problems concerning disposal of used bentonite are also an issue (Rankine, 1989).

Bentonite fining is the most prevalent method for protein fining of wine but other methods previously tested include ultrafiltration (Hsu *et al.*, 1987; Flores *et al.*, 1988; Peri *et al.*, 1988; Flores *et al.*, 1990), addition of proteolytic enzymes (Lagace and Bisson, 1990), and flash pasteurisation (Koch and Sajak, 1959; Hsu and Heatherbell, 1987b). Whilst ultrafiltration removes most proteins, in its current format, unattractive for most large scale winemaking due to the presence of residual proteins in the filtrate, high set up costs, and possible loses of flavour, aroma and colour (Miller *et al.*, 1985; Voilley *et al.*, 1990). Proteolytic enzymes, although most attractive in principle, have proven to be ineffective under standard winemaking conditions (Ngaba-Mbiakop, 1981; Feuillat and Ferrari, 1982; Heatherbell *et al.*, 1984; Waters *et al.*, 1992), whilst flash pasteurisation has been judged to have too detrimental an effect on wine quality (Ferenczy, 1966).

A possible alternative solution to the stabilisation of wine against protein haze formation is the addition of specific yeast derived glycoproteins, known as haze protective factors. These glycoproteins appear to reduce the particle size of denatured wine protein to a level which cannot be detected with the naked eye (Waters *et al.*, 1993). This in turn is believed to reduce the amount of protein needed to be removed to make a wine appear 'stable'. Both yeast derived (Ledoux *et al.*,1992; Waters *et al.*, 1993; Waters *et al.*, 1994) and grape derived (Waters *et al.*, 1994) polysaccharide-linked proteins have been deemed to act as haze protective factors. Further research and development is needed to determine the effectiveness of haze protective factors in winemaking.

1.1.3 Identification of haze-associated proteins as pathogenesis-related proteins

During the early stages of this study, which was commenced in April 1995, there were two reports detailing the purification and characterisation through amino acid sequencing of wine haze-associated proteins (Santoro, 1995; Waters *et al.*, 1996). Santoro (1995) purified three proteins from a Chardonnay wine, one protein with a molecular mass of 28 kDa and two other proteins with molecular masses of 35 kDa. Amino terminal sequence of only 9, 8 and 7 amino acid residues, respectively, were obtained from the proteins, although no sequence homology to other known proteins was reported. A more defining study by Waters *et al.* (1996) resulted in the purification and characterisation of proteins present in three dominant and separable protein fractions of a Muscat of Alexandria wine, with molecular masses of 24, 28 and 32 kDa. Extensive sequencing revealed that two 24 kDa proteins (major and minor isoforms) shared extensive homology with thaumatin-like proteins, while the 28 kDa and 32 kDa proteins were both homologous to a number of plant chitinases. Thaumatin-like proteins and plant chitinases can be classified as pathogenesis-related (PR) proteins (Stintzi *et al.*, 1993). PR proteins generally resist proteolytic cleavage and are soluble at acidic pH

(Stintzi *et al.*, 1993), which are properties exhibited by the majority of juice and wine proteins. Recognition that the major haze-forming proteins in wine are plant PR-like proteins helps explain the selective survival of these proteins through the winemaking process.

1.2 Plant pathogenesis-related proteins

Early descriptions of pathogenesis-related (PR) proteins appeared in studies involving the infection of resistant tobacco plants with the tobacco mosaic virus (TMV) (Gianinazzi *et al.*, 1970; van Loon and Van Kammen, 1970). Infection resulted in a hypersensitive response (HR) and the appearance of new soluble leaf proteins. These soluble proteins were later termed 'pathogenesis-related proteins' and defined as 'proteins coded for by the host plant but induced only in pathological or related situations' (Antoniw *et al.*, 1980). PR proteins have since been characterised from many dicotyledonous and monocotyledonous plant species (for reviews see Stintzi *et al.* (1993) and van de Rhee *et al.* (1994)). However, as tobacco was originally used as a model plant for the study of PR proteins, the majority of PR proteins first purified and characterised were from tobacco.

The induction of PR proteins can also be caused by non-viral pathogens, including viroids, bacteria and fungi. During infection, especially in incompatible reactions leading to necrosis, PR proteins rapidly accumulate to high concentrations, with individual proteins accounting for up to 10 % of the soluble protein content of tobacco leaves (van Loon, 1997). van Loon and Van Kammen (1970) proposed that PR proteins might play an antipathogenic role against invading pathogens considering they are associated with the hypersensitive response. Moreover, it was found that PR proteins were found to accumulate in the uninfected parts of the plant and not just locally around the sites of infection (Kassanis *et al.*, 1974; van Loon, 1975). This association of PR protein accumulation with the phenomenon of systemic acquired resistance (SAR), a condition in which an infected plant has systemic resistance against subsequent pathogen attack (Ross, 1961, Ryals *et al.*, 1994), also suggests a role of PR proteins in plant defence.

Although conclusive evidence for involvement of PR proteins in plant disease resistance has not yet been recorded (van Loon, 1997), PR proteins have been shown to possess inhibitory activities toward the growth of fungal pathogens in both *in vitro* and *in vivo* studies. When a plant is attacked by a fungal pathogen, PR proteins are presumed to challenge the invading organism, possibly by interfering with the pathogen's cell wall or membrane integrity, and through this activity perhaps releasing elicitors which could serve to induce further PR protein production (Stintzi, 1993). This proposed scenario is illustrated in Figure 1.1.

The production of PR proteins can also be modulated by a number of factors other than pathogen attack, such as the application of plant hormones and signalling molecules like salicylic acid, ethylene, abscisic acid (ABA), as well as changes in the balance of auxins and cytokinins (Shinshi *et al.*, 1987; Yalpani *et al.*, 1991; Zhu *et al.*, 1993; Coupe *et al.*, 1997). Additionally, other forms of stress such as bacterial toxins (Beffa *et al.*, 1995), air pollutants (Sandermann *et al.*, 1998), wounding (Ponstein *et al.*, 1994), ultraviolet light (Yalpani *et al.*, 1994; Green and Fluhr, 1995), heavy metals (Didierjean *et al.*, 1996) and osmotic or salt stress (Singh *et al.*, 1987) can turn on PR protein production possibly via the modulation of hormone levels (Jung *et al.*, 1995). Interestingly, subsets of PR proteins also exist in the organs of healthy plants apparently in the absence of any stress. The presence of PR proteins in 'healthy plants' often appears in an organ specific fashion and has been observed in fruit (Clendennen and May, 1997), flowers (Neale *et al.*, 1990; Harikrishna *et al.*, 1996), and senescent leaves (Hanfrey *et al.*, 1996; Tornero *et al.*, 1997). Such an expression of PR proteins may represent a pre-emptive response of plants to potential pathogen attack, or indicate some PR proteins may have role in plant development processes (eg. De Jong *et al.* (1992)).

PR proteins have routinely been classified into groups, commonly referred to as families (Antoniw *et al.*, 1980). Initial studies involving tobacco resulted in the

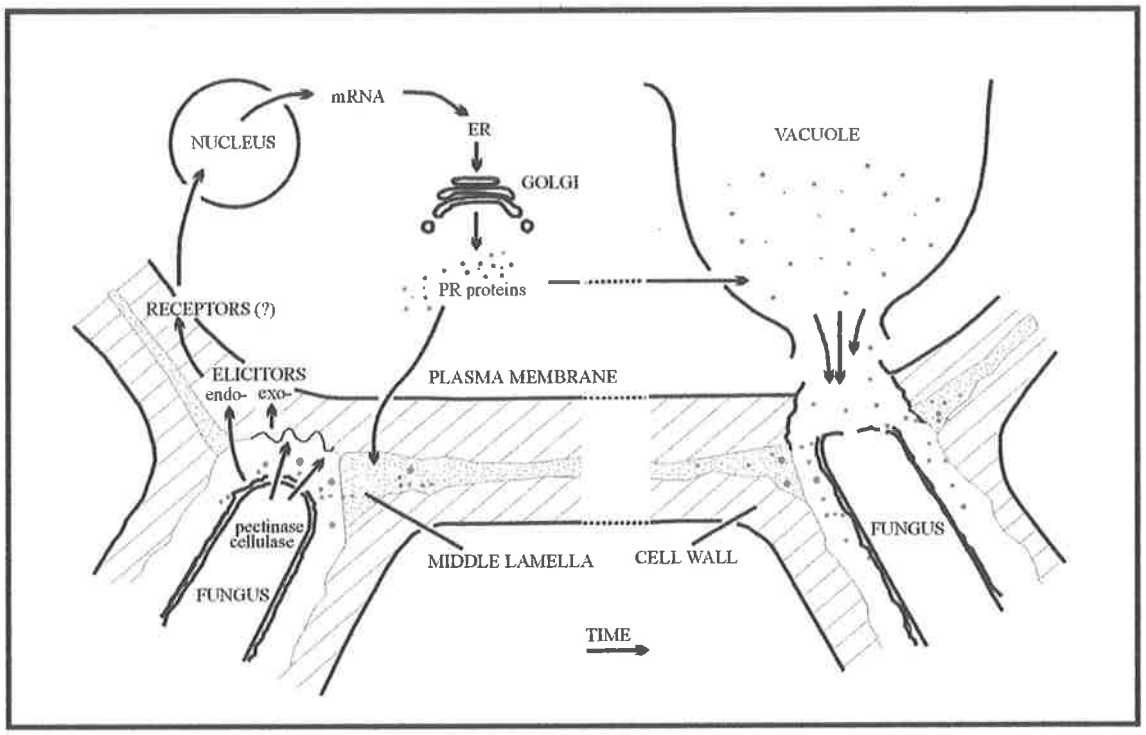


Figure 1.1 Proposed interaction between invading fungal hyphae and plant PR proteins. Initially, the approaching fungus makes contact with extracellularly located PR proteins (shown in green). This inhibits fungal growth and/or results in the release oligosaccharide elicitors from the hydrolysed hyphal cell wall. With time, the fungus may breach the plant cell membrane which would result in contact with intracellular PR proteins located in the vacuole (shown in red). Redrawn from Mauch and Staehelin (1989).

establishment of five distinct families, named PR-1 to PR-5. Newly identified PR proteins were ascribed to these families in order of their decreasing mobility when subjected to native gel electrophoresis. PR proteins were also being identified in other plants. Unfortunately this resulted in an array of different nomenclature for related PR proteins present in distinct individual plant species. Eventually, a system which classified PR proteins from any source into a family according to amino acid sequence, serological relationship and/or enzymatic activity was devised (van Loon *et al.*, 1994). Despite this advance it has been proposed that an additional six PR families (PR-6 to PR-11) be adopted along with the five well established PR protein families (PR-1 to PR-5) (van Loon *et al.*, 1994). These newly defined PR protein families consist of proteinase inhibitors (PR-6) (Heitz *et al.*, 1993), endoproteinases (PR-7) (Vera and Conejero, 1988), peroxidases (PR-9) (Lagramini and Rothstein, 1987), 'ribonuclease-like' proteins (PR-10) (Swoboda *et al.*, 1994) and novel classes of chitinase/lysozymes (PR-8 and PR-11) (Melchers *et al.*, 1994; Yeboah *et al.*, 1998). Indeed, even more families may be adopted in the future as additional classes of pathogen induced proteins are characterised (eg. Broekaert *et al.* (1997).

In the sections below, the characteristics of the five well established PR protein families (PR-1 to PR-5) are briefly reviewed.

1.2.1 PR-1 Family

Due to their high mobility during native electrophoresis PR-1 proteins were the focus of most early studies involving PR proteins (Gianinazzi *et al.*, 1970; van Loon and Van Kammen, 1970), although the first characterisation of PR-1 proteins by amino acid sequencing was reported much later (Lucas *et al.*, 1985) (Table 1.1). Despite extensive sequence similarities between the PR-1 proteins from tomato and tobacco, little could be elucidated about their biological function. Initial experiments involving immunoblotting

with PR-1 antibodies also revealed the existence of structurally related PR-1 type proteins in a wide range of plant species (Nassuth and Sanger, 1986; White *et al.*, 1987) without any further clue being revealed about their possible biological function.

cDNAs and genomic clones encoding PR-1 proteins have been cloned from a wide range of plants including elder berry (Coupe *et al.*, 1997), barley (Mouradov *et al.*, 1993; Bryngelsson *et al.*, 1994; Mouradov *et al.*, 1994; Stevens *et al.*, 1996), tobacco (Hooft van Huijsduijnen *et al.*, 1986; Oshima *et al.*, 1987; Payne *et al.*, 1989; Oshima *et al.*, 1990), tomato (Tornero *et al.*, 1994; Tornero *et al.*, 1997), maize (Casacuberta *et al.*, 1991), *Arabidopsis thaliana* (Metzler *et al.*, 1991) and *Medicago truncatula* (Szybiak Strozycka *et al.*, 1995). Sequence analysis of PR-1 genes, which encode for mature proteins of 14-17 kDa, has revealed they do not contain introns within their open reading frames. Many plants contain several PR-1 related sequences within their genomes, with at least four functional genes in tobacco and three in tomato (Niderman *et al.*, 1995). PR-1 pseudogenes are also known to exist (Pfitzner *et al.*, 1991).

Table 1.1 - PR-1 like proteins isolated and characterised by amino acid sequencing.

Protein	Plant species and tissue	Antifungal activity *	M_r (kDa)	Reference
PR-1	*Oryza sativa* (rice) leaves treated with jasmonic acid.	nd	16.5	(Moons *et al.*, 1997)
P14a P14b P14c	*Lycopersicon esculentum* (tomato) leaves infected with *Phytophthora infestans*; also leaves infected with viroid (PSTV).	yes	14-15	(Lucas *et al.*, 1985; Niderman *et al.*, 1995)
PR-1a PR-1b PR-1c PR-1g	*Nicotiana tabacum* (tobacco) leaves infected with virus (TMV).	yes	14-15	(Lucas *et al.*, 1985; Niderman *et al.*, 1995)
HvPR-1a HvPR-1b	*Hordeum vulgare* (barley) leaves infected with *Erysiphe graminis*	nd	15	(Bryngelsson *et al.*, 1994)

* yes = growth inhibition of one or more fungal species *in vitro*; nd = growth inhibition potential not determined or reported.

By a combination of cDNA and protein sequencing data, it is known PR-1 proteins are typically synthesised as preproteins. An N-terminal signal peptide directs passage of PR-1 proteins through the endoplasmic reticulum, after which most of the proteins are released by the cell into the apoplastic space due to the lack of additional topogenic signals (Linthorst, 1991). However, some PR-1 proteins are known to have short C-terminal extensions thought to act as signal peptides for vacuolar targeting (Tornero *et al.*, 1994). Both basic and acidic PR-1 proteins of tobacco appear to be located extracellularly (Niderman *et al.*, 1995).

Alexander *et al.* (1993) was the first to report the antifungal properties of PR-1 proteins. It was found transgenic tobacco constitutively expressing high amounts of an acidic PR-1 protein had an increased tolerance to *Perenospora tabacina* and *Phytophthora parasitica* when challenged under controlled conditions. Also, Niderman *et al.* (1995) found that the three basic PR-1 isoforms from tomato and a basic PR-1 protein from tobacco had inhibitory activity against *Phytophthora infestans* both in *in vitro* and *in vivo* assays. Interestingly, the acidic PR-1 proteins from tobacco were reported to have little or no antifungal activity. Additionally, the constitutive transgenic expression of a tobacco PR-1 protein in tobacco plants resulted in no decrease in susceptibility to infection by two different viruses (Linthorst *et al.*, 1989).

Recently, it was discovered that a human pathogenesis-related protein (GliPR), believed to play an important role in tumour growth, and PR-1 proteins share significant similarities (Szyperski *et al.*, 1998). In particular, the tomato PR-1 protein (P14a) (Lucas *et al.*, 1985) and GliPR share a 35% amino acid sequence identity. By comparing the known three-dimensional structure of P14a obtained by NMR (Fernandez *et al.*, 1997) to the proposed GliPR structure, it was suggested that PR-1 proteins and GliPR may operate according to the same molecular mechanisms, although these still remain unknown.

1.2.2 PR-2 and PR-3 Families: the polysaccharide endohydrolases

PR-2 (endo-β-1,3-glucanases; classes I, II and III; van Loon *et al.*, 1994) and PR-3 (endo-chitinases; classes I, II, IV, V and VI; Neuhaus *et al.*, 1996) protein families both contain members which have been assigned enzymatic functions. Moore and Stone (1972) were the first to discover the increase in β-1,3-glucanase activity of tobacco plants when infected with TMV, however, the identification of PR-2 and PR-3 proteins as endohydrolases was only realised later (Legrand *et al.*, 1987; Kauffmann *et al.*, 1988). The β-1,3-glucanases hydrolyse β-1,3-glucan (poly(1,3-β-D-glucose)), whilst the chitinases can hydrolyse chitin (poly(1,4-(*N*-acetyl-β-D-glucosamine))) into small oligomeric subunits. Additionally, some chitinases are capable of hydrolysing peptidoglycan present in bacterial cell walls (lysozyme activity). A large number of β-1,3-glucanases and chitinases have been purified and characterised from a wide range of plant species, and the encoding cDNAs and genes obtained (for reviews see Meins Jr. *et al.* (1992), Collinge *et al.* (1993), Raikhel *et al.* (1993), Stone and Clarke (1993), Beintema (1994), Graham and Sticklen (1994), Simmons (1994), Høj and Fincher (1995), Beffa and Meins Jr. (1996)). The PR-2 and PR-3 families consist of proteins which have similar molecular masses of approximately 25 to 35 kDa. These proteins are located either within vacuoles or extracellularly, depending on the topogenic signal present in the primary translation product (some have C-terminal extensions) (Linthorst *et al.*, 1990; Melchers *et al.*, 1993).

Interestingly, the substrate for PR-2 proteins, β-1,3-glucan, is only present in small quantities *in planta* (Stone and Clarke, 1993), whilst the substrate for the PR-3 proteins, chitin, is yet to be found in higher plants. Although PR-2 and PR-3 proteins may have a role in plant development (Bucciaglia and Smith, 1994; Vögeli-Lange *et al.*, 1994; Dong and Dunstan, 1997), it has been widely proposed that PR-2 and PR-3 proteins play a role in plant defence, especially as β-1,3-glucan and chitin are major

components of many fungal cell walls (Bartnicki-Garcia, 1968; Wessels, 1993). The first strong indication for such a role was provided by Schlumbaum *et al.* (1986) when a purified pathogen-inducible bean chitinase was demonstrated to inhibit the growth of *Trichoderma viride* on agar plates. Subsequent *in vitro* studies found β-1,3-glucanases purified from pea pods to be antifungal and furthermore, plant chitinases and β-1,3-glucanases were demonstrated to act synergistically to inhibit hyphal extension (Mauch *et al.*, 1988). Many *in vitro* studies have now demonstrated the effectiveness of PR-2 and PR-3 proteins from a number of different plants, to inhibit the growth of fungi (Roberts and Selitrennikoff, 1988; Leah *et al.*, 1991; Verburg and Huynh, 1991; Huynh *et al.*, 1992b; Gomes *et al.*, 1996; Lawrence *et al.*, 1996; Anderson *et al.*, 1998; Salzman *et al.*, 1998). Additionally, it is known specific isoforms vary in their effectiveness to inhibit fungal growth *in vitro* (Sela-Buurlage *et al.*, 1993) suggesting some PR-2 and PR-3 proteins may have roles other than the direct lysis of hyphae. A possibility is that some isoforms are specifically involved in the hydrolysis of fungal cell wall polysaccharides whilst others primarily serve to specifically induce other plant defence pathways (eg. phytoalexin synthesis) via the release of specialised glucan or chitin oligosaccharides which have an elicitor function (Dixon and Harrison, 1990). If this were the case it is perhaps not surprising that certain fungal pathogens secrete proteins that act as inhibitors to a specific subset of plant hydrolases of the PR-2 family (Ham *et al.*, 1997).

In an attempt to extend the observations on the *in vitro* antifungal activities of PR proteins to an *in planta* scenario researchers have produced transgenic plants that synthesise PR-2 and/or PR-3 proteins to a high level constitutively. Transgenic tobacco, alfalfa, carrot and oilseed rape plants expressing either plant β-1,3-glucanases (Yoshikawa *et al.*, 1993; Zhu *et al.*, 1994; Jach *et al.*, 1995; Masoud *et al.*, 1996), or chitinases (Broglie *et al.*, 1991; Vierheilig *et al.*, 1993; Zhu *et al.*, 1994; Jach *et al.*, 1995; Grison *et al.*, 1996; Punja and Raharjo, 1996) have indeed demonstrated decreased

susceptibility to a number of fungal pathogens under controlled glasshouse conditions. Furthermore, in an extension of these studies, enhanced coexpression of chitinases and β-1,3-glucanase in transgenic tobacco (Zhu *et al.*, 1994; Jach *et al.*, 1995) and tomato (Jongedijk *et al.*, 1995) plants demonstrated a synergistic effect between the transgenes and thus enhanced protection against specific pathogens. The functions of PR-2 and PR-3 proteins have also been examined through suppression of gene expression using antisense technology. Thus Beffa *et al.* (1996) unexpectedly found that tobacco plants with a reduced β-1,3-glucanase activity showed a decrease in disease symptoms upon TMV infection, along with a delayed spread of symptoms and a lower virus yield as compared to control plants. The transgenic plants were proposed to have an inhibited virus movement *in planta* because physical barriers such as callose (β-1,3-glucans) were less prone to degradation due to a decrease in β-1,3-glucanase activity. This led to the tentative conclusion that PR-2 proteins may also have a role in viral pathogenesis. Using antisense technology, Samac and Shah (1994) also attempted to down-regulate chitinase synthesis in *Arabidopsis* plants. However, suppression of chitinase expression during fungal attack was not evident and the disease resistance of the transgenic plants was not significantly different to that of control plants. Conclusive proof of the direct involvement of PR-2 and PR-3 proteins in the defence response is therefore still lacking although on balance, such a role seems plausible.

1.2.3 PR-4 Family

Two distinct classes of PR-4 type proteins have so far been characterised from a number of different plant species (see Table 1.2). Class I proteins have molecular masses of approximately 20 kDa and, through sequence similarities, have been proposed to consist of two domains (Ponstein *et al.*, 1994). The proposed N-terminal domain (approx. 6 kDa) is homologous to hevein, a lectin present in the sap of the rubber tree, thus some PR-4 type proteins are described as hevein-like. Indeed these proteins do

exhibit lectin-like characters with the ability to bind chitin (Ponstein *et al.*, 1994). It has furthermore been proposed that class I PR-4 proteins exhibit chitinase activity (Neuhaus *et al.*, 1996), however, the enzyme activity detected was extremely low (Ponstein *et al.*, 1994) and the presence of a contaminating chitinase cannot be ruled out. The 14 kDa C-terminus of the class I proteins is homologous to the smaller class II PR-4 proteins. The function of the 14 kDa class II proteins is unknown although a weak affinity for chitin has been observed for barley PR-4 proteins (Hejgaard *et al.*, 1992; Ludvigsen and Poulsen, 1992b). Furthermore, class I and II PR-4 proteins are highly similar to the putative products of two wound inducible, tandemly arranged potato genes, *win1* and *win2* (Stanford *et al.*, 1989). Hence, some researchers also refer to PR-4 proteins as win-like.

Table 1.2 - PR-4 like proteins isolated and characterised by amino acid sequencing

Protein	Plant species and tissue	Antifungal activity *	M_r (kDa)	Reference
PR-4	*Nicotiana tabacum* (tobacco) leaves infected with virus (TMV)	nd	13.5[b]	(Friedrich *et al.*, 1991)
PR-P2	*Lycopersicon esculentum* (tomato) leaves infected with *Cladosporium fulvum*	nd	13.5[b]	(Joosten *et al.*, 1990; Linthorst *et al.*, 1991)
CBP20	*Nicotiana tabacum* (tobacco) leaves infected with virus (TMV)	yes	20[a]	(Ponstein *et al.*, 1994; Hensel *et al.*, 1997)
Prohevein	*Hevea brasiliensis* (rubber tree) latex	nd	20[a]	(Broekaert *et al.*, 1990; Van Parijs *et al.*, 1991)
wheatwin1 wheatwin2	*Triticum aestivum* (wheat) grain	yes	13[b]	(Caruso *et al.*, 1993; Caruso *et al.*, 1996)
barwin	*Hordeum vulgare* (barley) grain	nd	13.7[b]	(Svensson *et al.*, 1992)
CBPN CBP4 CBP5	*Hordeum vulgare* (barley) grain (CBPN) and *Erysiphe graminis* infected leaf (CBP4 and CBP5)	yes	13.7[b] 13.6[b] 13.7[b]	(Hejgaard *et al.*, 1992)

* yes = growth inhibition of one or more fungal species *in vitro*; nd = growth inhibition potential not determined or reported; no = no growth inhibition observed *in vitro*.
[a] Class I
[b] ClassII

cDNAs encoding PR-4 like proteins have been cloned from a wide range of plants including tobacco (Friedrich *et al.*, 1991; Linthorst *et al.*, 1991; Ponstein *et al.*, 1994), tomato (Linthorst *et al.*, 1991; Harris *et al.*, 1997), maize (Chevalier *et al.*, 1995), elderberry (Coupe *et al.*, 1997), *Arabidopsis* (Potter *et al.*, 1993), potato (Shirras and Northcote, 1984) and barley (Gregersen *et al.*, 1997). Analysis of the deduced amino acid sequences has revealed the presence of both N- and C-terminal topogenic signals. An N-terminal signal peptide, alone, results in PR-4 proteins being exported to the extracellular matrix (Friedrich *et al.*, 1991). Alternatively, PR-4 proteins are retained intracellularly, most likely in a vacuolar location, when a C-terminal signal peptide is also present in the translated protein (Ponstein *et al.*, 1994; Hensel *et al.*, 1997).

The genomes of tobacco and tomato contain two to four genes encoding PR-4 like proteins (Linthorst *et al.*, 1991; Ponstein *et al.*, 1994) while potato plants possibly contain a minimum of five functional genes (Stanford *et al.*, 1989). Two wound-induced genes that encode putative class I PR-4 like proteins in potato have been isolated and shown to contain one intron (Stanford *et al.*, 1989). No class II PR-4 encoding genes have been cloned from any source.

The chitin-binding activity of some PR-4 proteins suggests they may play an antifungal role (see Table 1.2). Both class I and II PR-4 like proteins from tobacco, barley and wheat do indeed display antifungal activity in *in vitro* studies (Hejgaard *et al.*, 1992; Ponstein *et al.*, 1994; Caruso *et al.*, 1996). Barley PR-4 proteins exhibited antifungal activity against *Trichoderma harzianum*, an activity which was enhanced in a synergistic fashion when mixed with PR-3 or PR-5 proteins (Hejgaard *et al.*, 1992). Similarly, Ponstein *et al.* (1994) demonstrated that the tobacco PR-4 protein CBP20 alone inhibited the growth of the fungal pathogen *Trichoderma viride* whilst its combination with tobacco chitinase or β-1,3-glucanase led to synergistic inhibition of *Fusarium solani* and *Alternaria radicina* growth *in vitro*.

The three-dimensional structure of barwin, a class II PR-4 type protein from barley, has been solved using NMR (Ludvigsen and Poulsen, 1992b). A globular fold of the peptide backbone was observed and identified as a potential binding site for chitin but binding studies revealed affinities to be weak. The exact role of PR-4 proteins thus remains unknown.

1.2.4 PR-5 Family: the thaumatin-like, osmotin and permatin proteins

PR-5 proteins exhibit molecular masses typically in the 20 to 30 kDa range. Members of this protein family have been assigned roles in: defence against plant pathogens, osmotic stress (Singh *et al.*, 1987), development (Neale *et al.*, 1990; Kuboyama *et al.*, 1997; Sassa and Hirano, 1998) and even freezing tolerance (Hon *et al.*, 1995). PR-5 proteins are often classified as thaumatin-like proteins as they exhibit extensive amino acid sequence identity to thaumatin, a sweet tasting protein present in the fruit of the African shrub *Thaumatococcus daniellii* (van der Wel and Loeve, 1972; Cornelissen *et al.*, 1986a). An alternative designation of PR-5 family members is that of osmotin-like. Osmotin, a protein originally purified from salt stressed tobacco plants (Singh *et al.*, 1987; Singh *et al.*, 1989) exhibits >50% positional identity to several PR-5 proteins. Finally, due to a proposed fungal membrane permeabilising activity originally suggested by Roberts and Selitrennikoff (1990), PR-5 protein members have also been referred to as permatins (Vigers *et al.*, 1991).

PR-5 proteins have been purified from many plants (Table 1.3), and numerous cDNA and genomic sequences encoding these proteins have been cloned. Information obtained from mature protein and cDNA sequences reveals the possibility of two topogenic signals within the primary translation product. As seen for other PR protein family members, a transient N-terminal peptide exists for PR-5 proteins. This directs the

protein to the extracellular matrix, unless a second C-terminal peptide is present to target the protein intracellularly, usually to the vacuole (Melchers *et al.*, 1993).

Table 1.3 - PR-5 like proteins isolated and characterised by amino acid sequencing

Protein	Plant Species and tissue	Antifungal activity *	M_r (kDa)	Reference
BFTP	*Brassica campestris* flower buds	yes	27	(Cheong *et al.*, 1997b)
PR-5d (also known as OLP)	*Nicotiana tabacum* (tobacco) cultured cells	yes	25	(Koiwa *et al.*, 1994; Koiwa *et al.*, 1997)
PLTP	*Cucurbita* sp. (pumpkin) leaves	yes	28	(Cheong *et al.*, 1997a)
Osmotin (isoforms I and II). Osmotin II also known as AP24.	*Nicotiana tabacum* (tobacco) cultured cells (NaCl stressed), also leaves infected with *Phytophthora infestans* or virus (TMV)	yes	24	(Singh *et al.*, 1987; Singh *et al.*, 1989; Stintzi *et al.*, 1991; Woloshuk *et al.*, 1991; Vigers *et al.*, 1992; Abad *et al.*, 1996)
Zeamatin	*Zea mays* (maize) seeds	yes	22	(Roberts and Selitrennikoff, 1990; Vigers *et al.*, 1991; Batalia *et al.*, 1996)
Trimatin	*Triticum aestivum* (wheat) grain	yes	22	(Vigers *et al.*, 1991)
BP-R BP-S	*Hordeum vulgare* (barley) grain	yes	23	(Hejgaard *et al.*, 1991)
NP24 (isoforms I and II).	*Lycopersicon esculentum* (tomato) fruit, also leaves infected with *Phytophthora infestans* (NP24 I)	yes	24	(King *et al.*, 1988; Woloshuk *et al.*, 1991; Pressey, 1997)
Thaumatin (isoforms T0, TI and TII)	*Thaumatococcus daniellii* (ketemfe berry) fruit	yes	22	(van der Wel and Loeve, 1972; Iyengar *et al.*, 1979; Vigers *et al.*, 1991)
27 kDa protein	*Diospyros texana* (persimmon) fruit	yes	27	(Vu and Huynh, 1994)
Linusitin	*Linum usitatissimum* (flax) seed	yes	25	(Borgmeyer *et al.*, 1992; Anzlovar *et al.*, 1998)
ATLP1 ATLP3	*Arabidopsis thaliana* cDNAs expressed in *E. coli*	yes (ATLP3)	23	(Hu and Reddy, 1997)
GO	*Vitis labruscana* (American grapevine) fruit	yes	27	(Salzman *et al.*, 1998)

* yes = growth inhibition of one or more fungal species *in vitro*.

Table 1.3 continued

Protein	Plant Species and tissue	Antifungal activity*	M_r (kDa)	Reference
ZLP	*Zea mays* (maize) cDNA expressed in tomato, *Arabidopsis thaliana* and insect cells	yes	22	(Malehorn *et al.*, 1994)
PR-S	*Nicotiana tabacum* (tobacco) leaves infected with virus (TMV)	yes	24	(Kauffmann *et al.*, 1990; Vigers *et al.*, 1992)
MAI	*Zea mays* (maize) seeds	yes	22	(Richardson *et al.*, 1987; Huynh *et al.*, 1992a)
P23	*Lycopersicon esculentum* (tomato) leaves infected with viroid (CEV)	nd	23	(Rodrigo *et al.*, 1991a)
Protein C	*Solanum tuberosum* (potato) salicylate treated leaves	nd	21	(Pierpoint *et al.*, 1990)
IFW 19 IFW 16 IFW 15	*Hordeum vulgare* (barley) leaves treated with silver nitrate	nd	19 16 15	(Trudel *et al.*, 1998)
Hv-1	*Hordeum vulgare* (barley) leaves infected with *Erysiphe graminis*	nd	19	(Bryngelsson and Green, 1989)
gp22	*Nicotiana tabacum* (tobacco) leaves infected with virus (TMV)	nd	22	(Edelbaum *et al.*, 1991)
P21	*Glycine max* (soybean) leaves	nd	21	(Graham *et al.*, 1992)
Rye TLs	*Secale cereale* (Winter rye) leaves	nd	16 and 25	(Hon *et al.*, 1995)
Pru a 2, also known as CHTL	*Prunus avium* (cherry) fruit	no	23	(Fils-Lycaon *et al.*, 1996; Inschlag *et al.*, 1998)
SRgp24	*Mesembryanthemum crystallinum* (common ice plant) callus	nd	24¶	(Yen *et al.*, 1994)
SE39b	*Nicotiana tabacum* (tobacco) flower stigmas	nd	39¶	(Kuboyama *et al.*, 1997)
PsTL1	*Pyrus serotina* (Japanese pear) flower styles	nd	32¶	(Sassa and Hirano, 1998)
AHCSP33	*Arachis hypogaea* (groundnut) leaves	nd	33¶	(Dave and Mitra, 1998)

* yes = growth inhibition of one or more fungal species *in vitro*; nd = growth inhibition potential not determined or reported; no = no growth inhibition observed *in vitro*.

¶ protein possibly modified by glycosylation.

There appears to be at least six genes encoding PR-5 isoforms in some plant genomes (Zhu *et al.*, 1995; Loulakakis, 1997a). Notably, no introns exist within any of the PR-5 genes so far characterised (van Kan *et al.*, 1989; Nelson *et al.*, 1992; Zhu *et al.*, 1995). Various putative promoter sequences controlling PR-5 gene expression in both pathological and other stress situations have been studied using transgenic constructs and through this critical elements have been identified (Albrecht *et al.*, 1992; Kononowicz *et al.*, 1992; Nelson *et al.*, 1992; Raghothama *et al.*, 1993; Zhu *et al.*, 1995; Sato *et al.*, 1996).

PR-5 proteins impede the growth of a wide range of fungal pathogens *in vitro* (see Table 1.3). Additionally, a synergistic effect can occur when PR-5 proteins are mixed with members of other PR protein families (Hejgaard *et al.*, 1991; Hejgaard *et al.*, 1992; Lorito *et al.*, 1996) or other components known to affect fungal cell walls/membranes (Roberts and Selitrennikoff, 1990; Vigers *et al.*, 1991; Lorito *et al.*, 1996). Liu *et al.* (1994) and Zhu *et al.* (1996) demonstrated that transgenic tobacco and potato plants constitutively expressing high levels of osmotin showed an increased tolerance to the fungal pathogen *Phytophthora infestans*. Conversely, Liu *et al.* (1994) found that transgenic tobacco plants with enhanced levels of osmotin displayed no change in the development of disease symptoms when challenged with *Phytophthora parasitica*, even though *in vitro* inhibition was observed with the purified osmotin. The basis for these apparently contradictory results is not yet understood. Furthermore, transgenic tobacco constitutively expressing a PR-5 protein showed no signs of increased resistance to virus infection (Linthorst *et al.*, 1989).

The mechanism by which PR-5 proteins cause lysis of fungal hyphae and spores, *in vitro*, is not firmly established. The PR-5 proteins from maize seed (Roberts and Selitrennikoff, 1990; Batalia *et al.*, 1996), tobacco (Abad *et al.*, 1996) and flax seed (Anzlovar *et al.*, 1998) are proposed to somehow permeabilise fungal membranes and

induce cytoplasmic leakage. Apart from permeabilising fungal membranes, there is evidence that several, but not all, thaumatin-like proteins bind β-1,3-glucans (Trudel *et al.*, 1998). Likewise, it has been suggested PR-5 proteins have the ability to bind actin (Takemoto *et al.*, 1997) and to act as a receptor for other unknown ligands (Wang *et al.*, 1996). It has also been reported that a PR-5 like protein from maize seeds possesses α-amylase/trypsin inhibition activity (Richardson *et al.*, 1987), however, no such activity has been demonstrated for the near identical maize seed protein, zeamatin (Vigers *et al.*, 1991; Batalia *et al.*, 1996). The three-dimensional crystal structures of three PR-5 like proteins, thaumatin (from *Thaumatococcus daniellii*) (Kim *et al.*, 1988; Ogata *et al.*, 1992; Ko *et al.*, 1994), zeamatin (from *Zea mays*) (Batalia *et al.*, 1996), and PR-5d (from *Nicotiana tabacum*) (Koiwa *et al.*, 1999), have been solved by protein crystallography. Analysis of these structures does reveal the presence of a pronounced cleft, the function of which still remains to be elucidated although it clearly is compatible with the binding of large molecules.

1.2.5 PR proteins of the *Vitis* species

Initial studies on PR-like proteins in grapevine focussed on factors influencing β-1,3-glucanase activity. Clarke and Stone (1962) were the first to report β-1,3-glucanase activity in the xylem and phloem of *V. vinifera* canes. They hypothesised the β-1,3-glucanase activity to have a role in the removal of dormancy callose although their results revealed there were no significant differences in total β-1,3-glucanase activity between dormant and activated canes. In a subsequent study, Hawker *et al.* (1974) showed that β-1,3-glucanase activity gradually increased in leaves during the growing season but also that β-1,3-glucanase activity was not induced in grapevine leaves infected with a spectrum of viruses causing leafroll, yellow speckle, fleck or fanleaf symptoms.

Since the commencement of this study in April 1995, there have been many papers dealing with grapevine proteins homologous to members of the PR-2, PR-3, PR-5 and PR-8 families. Firstly, as many as thirteen chitinase isoforms may exist in healthy, wounded, and salicylic acid treated grapevine tissues (Derckel *et al.*, 1996). Chitinases have also been detected in cell cultures (Deloire *et al.*, 1996; Busam *et al.*, 1997), leaves infected by various fungal pathogens, and leaves treated with chemical and fungal elicitors (Busam *et al.*, 1997). A chitinase has also been purified from mature berries and identified by amino acid sequencing as a class IV chitinase (Derckel *et al.*, 1998). This chitinase has extensive homology to a 32 kDa wine haze-associated protein (Waters *et al.*, 1996; Waters *et al.*, 1998) and to two ripening induced berry chitinases (Robinson *et al.*, 1997). Moreover, chitinases are now known to be abundant constituents of mature *Vitis labruscana* L. berries (Salzman *et al.*, 1998). Secondly, β-1,3-glucanase like proteins have been detected in cell cultures (Deloire *et al.*, 1996), *Botrytis cinerea* infected and salicylic acid treated leaves (Renault *et al.*, 1996), and two partial cDNAs encoding different isoforms have been cloned (Kraeva *et al.*, 1998). Finally, a cDNA encoding a thaumatin-like protein has also been isolated from cell cultures (Loulakakis, 1997a; Loulakakis, 1997b) which is homologous to a previously characterised wine haze-associated protein (Waters *et al.*, 1996). Also, in a very recent study, a thaumatin-like protein was purified from mature *V. labruscana* berries (Salzman *et al.*, 1998). As most of the above developments occurred subsequent to experimentation described in this thesis, and indeed subsequent to publication of some of the results reported herein (Tattersall *et al.*, 1997), relevant discussion and reference to these papers will be given in the following chapters.

1.3 Aims of this study

Despite the ubiquitous presence of PR proteins in plants, and notwithstanding the hundreds of reports describing aspects of their properties, the exact role of PR proteins in plant development or defence, and their mode(s) of action remain largely unknown. Initial results from this study, and other research (Waters *et al.*, 1996), indicated that PR-like proteins are highly abundant in the grapevine berry. This observation not only presented an opportunity to further define the role of PR proteins in plant function but also had clear technical implications for viticulture and winemaking. Understanding whether different management practices have an impact on the accumulation of these proteins, and whether this in turn can modulate pathogen resistance of the vine is of great practical importance, as is the definition of individual PR protein functions. Further insights in these areas may in turn facilitate the development of superior ways of stabilising wine therefore avoiding the formation of wine haze with a minimum of intervention.

The general aim of this study was to develop molecular tools to define the complement of prominent PR-like proteins in *V. vinifera* berries and to carefully examine their accumulation during development of the fruit from flowering to berry maturity. Specific aims of the project were to:

- purify and identify abundant PR-like soluble juice proteins (Chapter 2)
- obtain and characterise the cDNAs encoding the above proteins (Chapter 3)
- use Northern and Western blot analyses to define the tissue-specific and temporal expression pattern of the cloned genes encoding these proteins (Chapter 4)
- examine the potential antifungal properties of these proteins *in vitro* (Chapter 5).

CHAPTER 2

Purification and identification of four prominent berry PR-like proteins

2.1 Introduction

For over a decade, the small number of berry-derived proteins that are associated with white wine haze have been known to have relatively low molecular masses (~12 to 30 kDa) and isoelectric points (~4.1 to 5.8) (Hsu and Heatherbell, 1987a). This study was undertaken to gain a better understanding of the properties, synthesis and functions of these proteins within the berry. As found in wine, soluble berry proteins in juice are relatively abundant, and few in number. Indeed, the protein composition of a juice and a wine made therefrom are highly similar. Accordingly, berry juice provided the ideal source from which soluble grape proteins that persist through the winemaking process could be purified readily.

The initial step for this examination involved the purification of four prominent proteins from Muscat of Alexandria berry juice that were to be the subject of further detailed characterisation. The purified proteins, as judged by SDS-PAGE, included a major and a minor 24 kDa protein, a 13 kDa protein, and a 28 kDa protein. As described in the following chapter, protein sequencing revealed the major 24 kDa protein (named VVTL1), and minor 24 kDa protein (named VVTL2), to be thaumatin-like (PR-5 like) proteins. Additionally, the partial amino acid sequence of the 13 kDa protein (named VVPR-4a) exhibited high homology to known PR-4 like proteins, and the partial amino acid sequence of the 28 kDa protein (named VVCHIT) suggests it may be a chitinase (PR-3 like). Since the commencement of this study, four prominent proteins have been purified from a Muscat of Alexandria wine (Waters *et al.*, 1996; Waters *et al.*, 1998). Examination of the partial amino acid sequences of the soluble wine proteins reported by Waters *et al.* (1996) reveals that they are highly similar, if not identical, to that of three berry PR-like proteins characterised here (VVTL1, VVTL2 and VVCHIT).

2.2 Materials and methods

2.2.1 Protein purification

Ammonium sulfate precipitation and anion exchange fractionation of soluble juice proteins

Approximately 2 L of juice from Muscat of Alexandria berries (BRL Hardy Berri Estate, Australia or Alverstoke vineyard, The University of Adelaide) was subjected to $(NH_4)_2SO_4$ fractionation. $(NH_4)_2SO_4$ was added to 80% saturation and stirred overnight at 4°C. The pellet obtained after centrifugation (10,000*g* for 30 min, 4°C) was extensively dialysed at 4°C against 20 mM Tris-HCl, pH 8.0 before loading onto a 1.5 x 16 cm Q-Sepharose column (Pharmacia, Sweden) equilibrated with the above buffer. Proteins were eluted with a linear 0 to 0.35 M NaCl gradient (in equilibration buffer) over 33.3 h, at a flow rate of 30 mL per h. Fractions of 8 mL were collected and those containing prominent 24 kDa proteins (VVTL1: fractions 9-13; VVTL2: fractions 19-22), a 13 kDa protein (VVPR-4a: fractions 35-39), and a 28 kDa protein (VVCHIT: fractions 43-47), as judged by SDS-PAGE were individually pooled, concentrated to approximately 4 mL, and exchanged into respective buffers (as described below), by ultrafiltration using a Diaflo YM 10 (VVTL1, VVTL2, VVCHIT) or YM3 (VVPR-4a) membrane (Amicon, USA).

VVTL1, VVPR-4a and VVCHIT purification by gel filtration chromatography

VVTL1, VVPR-4a and VVCHIT (in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl) were then individually loaded onto a Superdex 200 Hiload™ 26/60 column (Pharmacia, Sweden), equilibrated in the same buffer, and chromatographed using a flow rate of 100 mL per h. Fractions (4 mL) containing either VVTL1, VVPR-4a or VVCHIT were

identified, pooled and concentrated as above. VVPR-4a was rechromatographed on the Superdex 200 Hiload™ 26/60 column until other contaminating proteins could not be detected by SDS-PAGE.

VVTL2 purification by cation exchange chromatography

VVTL2 (in 20 mM formic acid, pH 4.0) was loaded onto a SP Sepharose Hiload™ 26/10 cation exchange column (Pharmacia, Sweden) equilibrated with the same buffer, and eluted with a linear 0 to 100 mM NaCl gradient (in equilibration buffer) over 6.67 h, at a flow rate of 150 mL per h. Fractions of 8 mL were collected and those containing a 24 kDa protein, which was judged to be pure by reverse phase HPLC (as described below), were pooled.

Protein yield and purity

The yield of essentially pure protein was approximately 30 mg/L, 2 mg/L, 2 mg/L and 10mg/L of juice (Alverstoke vineyard) for VVTL1, VVTL2, VVPR-4a and VVCHIT, respectively.

The purity of VVTL1, VVTL2, VVPR-4a and VVCHIT was finally assessed by reverse phase HPLC using a Beckman System Gold apparatus (128 diode array detector module, 126 pumps, 507E autosampler) and Gold Noveau software. An equal volume of denaturing buffer (6.0 M guanidine hydrochloride, 0.1 M Tris-HCl, pH 7.6, 1 mM EDTA) was added before proteins were applied to a C_8 column (4.6 x 250 mm; Vydac, USA) equilibrated in buffer A (0.1% TFA) and operated with a flow rate of 0.6 mL/min. The column was washed for 5 min with buffer A followed by a linear gradient of 0 to 100% buffer B (80% (v/v) CH_3CN, 0.085% (v/v) TFA) developed over 60 min at the same flow rate.

2.2.2 SDS-PAGE

Loading buffer (final concentration of 60 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 8% (v/v) glycerol, 100 mM DTT, 0.001% bromophenol blue) was added and proteins then incubated at 80°C for 2 min before resolution by SDS-PAGE in 12.5% (w/v) (50:1.35; acrylamide:bisacrylamide) Tris-glycine gels (Fling and Gregerson, 1986) or 16% (w/v) (32:1; acrylamide:bisacrylamide) Tris-tricine gels (Schägger and von Jagow, 1987). Post electrophoresis, gels were stained (0.1% (w/v) Coomassie Brilliant Blue R-250 (Sigma, USA), 40% (v/v) ethanol, 7% (v/v) acetic acid) at approximately 60°C for 30 min and then destained (20% (v/v) ethanol, 7% (v/v) acetic acid). Gels were preserved by drying between cellophane sheets (Biorad, USA) which had been previously soaked in 50% (v/v) ethanol.

2.2.3 Protein digestion, acid hydrolysis and sequence analysis

Prior to proteolytic digestion, VVTL1 (~400 μg) was denatured by TCA precipitation as follows. Essentially, ice cold TCA (60% (w/v)) was added to a final concentration of 10% (w/v) before a 15 min incubation on ice followed by centrifugation (12,000*g*, 15 min). The pellet was washed twice with ice cold ethanol:ethyl acetate (2:1 v/v) before resuspension in 50 μL of 6 M Urea, 50 mM Tris-HCl, pH 8.0 and 5 mM DTT, incubated at 60°C for 30 min, and then diluted with 3 vol of 25 mM Tris-HCl, pH 7.7 and 1 mM EDTA. VVTL1 was either digested with endoproteinase Glu-C (Promega) or endoproteinase Lys-C (Promega, USA), which was added at a final ratio of substrate to protease of approximately 20:1 (w/w). Digestions were performed at 37°C for 42 h. Before proteolytic digestion of VVPR-4a (~100 μg) by endoproteinase Lys-C, essentially as described above, and acid hydrolysis of VVTL1 (~1 mg), both proteins were reductively alkylated with iodoacetamide as described previously (Høj *et al.*, 1987). Acid hydrolysis of VVTL1 was performed in 70% (v/v) TFA (HPLC grade, Pierce, USA) at 40°C for 20 h and terminated by addition of 10 vol of H_2O before

vacuum concentration. Peptides generated by endoproteinase digestion and acid hydrolysis were purified by reverse phase HPLC essentially as described in Section 2.2.1 except peptides were eluted with a gradient of 0 to 75% buffer B developed over 125 min. Collected peptides were then subjected to sequencing as described below. VVCHIT (~100 μg) was resupended in 150 μL of reaction buffer (100 mM NaH_2PO_4, pH 8, containing 10 mM EDTA, 5 mM DTT, 5% (v/v) glycerol, 1 M urea) before incubation with 5 μg pyroglutamate aminopeptidase (Boehringer Mannheim, Germany) at 4°C for 18 h, then 25°C for 4 h. The VVCHIT reaction mixture was then concentrated and buffer exchanged with an Ultrafree MC 10 filter unit (Millipore, USA) before sequencing as described below.

Proteins and peptides were sequenced using an automated Edman G1000A protein sequencer (Hewlett-Packard, USA), by Neil Shirley and Jelle Lahnstein (Department of Plant Science, The University of Adelaide).

2.2.4 Protein database analyses

Amino acid sequences were compared to known sequences within the GenBank database via the BLAST suite of programs (Altschul *et al.*, 1990). Access to the GenBank database and related analysis programmes was provided by the Australian National Genomic Information Service (ANGIS), located at The University of Sydney.

2.3 Results

2.3.1 Polypeptide pattern of berries, juice and wine

When berry proteins are extracted using protective buffering agents and analysed by SDS-PAGE, a very complex pattern of proteins ranging from less than 10 kDa to greater than 100 kDa is evident (Figure 2.1, lane 1). By contrast, when juice is expressed in the absence of protective agents, a very simple pattern of polypeptides is visible (Figure 2.1, lane 2) indicating a selective extraction of soluble, relatively low molecular mass components has taken place. From previous studies it is known that these proteins are associated with wine haze (Hsu and Heatherbell, 1987a; Waters *et al.*, 1995a).

The polypeptide patterns of both Muscat of Alexandria juice and wine consist mainly of relatively low molecular mass components in the range of approximately 20 to 30 kDa (Figure 2.1). Additionally, less abundant polypeptides ranging in molecular masses from 13 kDa to approximately 60 kDa are observed. Most, if not all, polypeptides observed in the Muscat of Alexandria juice and wine are consistently reported to be present in the juices and wines from many different varieties and locations (Hsu and Heatherbell, 1987a; Murphey *et al.*, 1989; Waters *et al.*, 1991; Yokotsuka *et al.*, 1991; Pueyo *et al.*, 1993; Dorrestein *et al.*, 1995; Santoro, 1995). Reported compositional differences may be accounted for by variations in analytical methods such as SDS-PAGE techniques. This is exemplified when the same juice proteins are analysed by Tris-tricine (Figure 2.1A) and Tris-glycine (Figure 2.1B) SDS-PAGE.

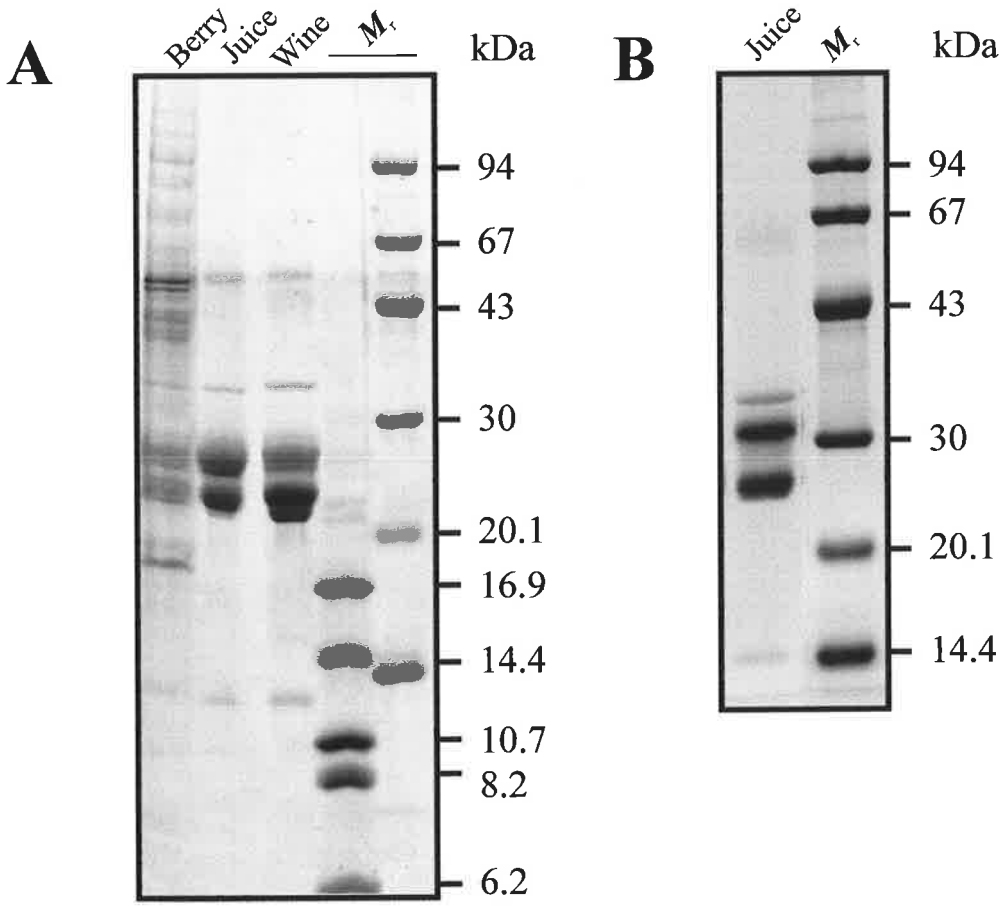


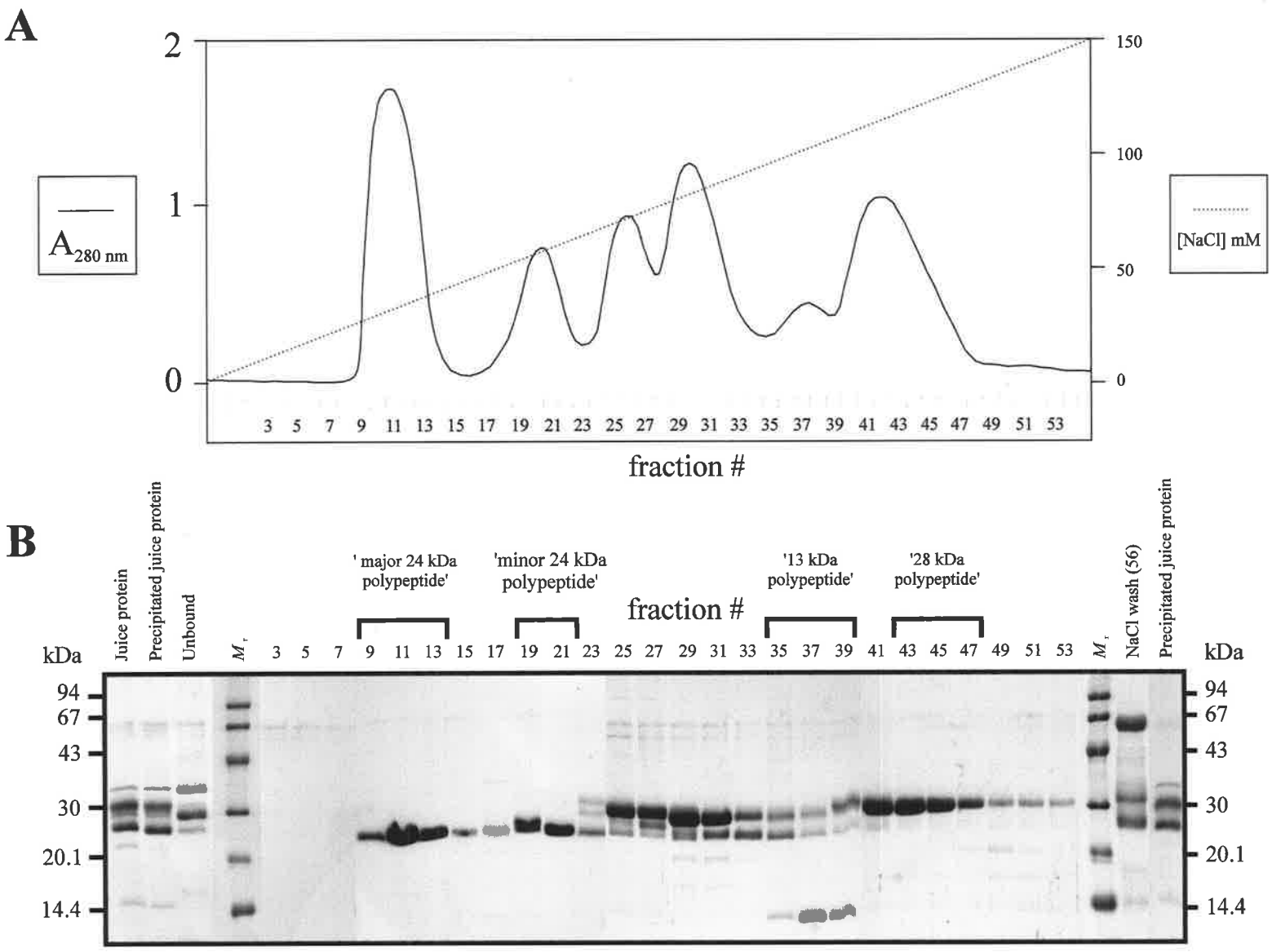
Figure 2.1 The complement of soluble proteins in grape juice and wine are highly similar and represent only a small subset of total proteins present in the berry. (A) Tris-tricine SDS-PAGE was used to analyse protein extracted from Muscat of Alexandria berries by boiling in SDS-PAGE loading buffer (berry), $(NH_4)_2SO_4$ precipitated juice (juice) and wine protein (wine). Berries were obtained from the Alverstoke vineyard (The University of Adelaide), juice from BRL Hardy Berri Estate, and the wine protein was a gift from Dr Elizabeth Waters (The Australian Wine and Research Institute). M_r, Molecular mass standards. (B) Tris-glycine SDS-PAGE analysis of the same juice proteins analysed in (A) illustrates the variations in apparent molecular mass estimates of polypeptides obtained when using different SDS-PAGE techniques.

2.3.2 Purification of four soluble juice proteins

As a first step in the understanding the biochemistry of some of the principle protein components of a Muscat of Alexandria juice, and hence wine, four prominent polypeptides were purified to apparent homogeneity. The molecular masses of these polypeptides, as judged by Tris-tricine SDS-PAGE, were 24 (2 polypeptides - a major and a minor form), 13 and 28 kDa. The purification of the major 24 kDa polypeptide, and 13 and 28 kDa polypeptides was readily achieved from clarified juice, using ammonium sulfate precipitation followed by anion exchange (Figure 2.2) and gel filtration chromatography (Figure 2.3). All three polypeptides appeared to have been purified to homogeneity following the final rounds of gel filtration chromatography. The minor 24 kDa polypeptide was purified as described above except, following anion exchange chromatography (Figure 2.2), the polypeptide was subjected to cation exchange chromatography (Figure 2.4) before apparent homogeneity was achieved. The purification of all polypeptides was monitored by SDS-PAGE (Figure 2.5) and reverse phase HPLC (Figure 2.6).

The majority of soluble juice proteins bound to an anion exchange column (pH 8.0) and were eluted with less than 150 mM NaCl (Figure 2.2). Several major peaks of protein were resolved, many containing two or more polypeptides. This result was consistent with the two-dimensional PAGE study of Hsu and Heatherbell (1987a) where up to 25 different polypeptides were found in Gewürztraminer and White Riesling juice, the majority with a low pI (4.1 to 5.8), and the outcome similar to that obtained by Waters *et al.* (1995) when Muscat of Alexandria wine was subjected to anion exchange chromatography. The anion exchange fractions (Figure 2.2) contained a major 24 kDa polypeptide (fractions 9-13), a minor 24 kDa polypeptide (fractions 19-22) eluting at lower salt concentration, and a later eluting 13 kDa polypeptide (fractions 35-39) and 28 kDa polypeptide (fractions 43-47).

Figure 2.2 Separation of soluble grape juice proteins by anion exchange chromatography. (A) Proteins bound to the Q-sepharose column were eluted by a linear NaCl gradient and monitored by absorbance at 280 nm. (B) Aliquots (20 μL) of every second eluted fraction (8 mL) were analysed by Tris-glycine SDS-PAGE and protein visualised by Coomassie brilliant blue. Fractions containing major and minor 24 kDa polypeptides, and 13 kDa and 28 kDa polypeptides, which were the subject of further investigation, are highlighted. Clarified Muscat of Alexandria juice (juice protein), $(NH_4)_2SO_4$ precipitated juice protein (precipitated juice protein), protein which failed to bind to the column (unbound), and protein which eluted from the column after a 350 mM NaCl wash (NaCl wash) are shown. M_r, Molecular mass standards.


A
2
1
0
150
100
50
0
A280 nm
[NaCl] mM
3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53
fraction #
B
Juice protein
Precipitated juice protein
Unbound
Mr
' major 24 kDa polypeptide'
'minor 24 kDa polypeptide'
fraction #
'13 kDa polypeptide'
'28 kDa polypeptide'
NaCl wash (56)
Precipitated juice protein
kDa
94
67
43
30
20.1
14.4

The major 24 kDa polypeptide, and 13 and 28 kDa polypeptides were subjected to further purification by gel filtration chromatography. All three proteins behaved as monomers (Figure 2.3). Interestingly, the 24 kDa polypeptide was estimated to have a considerably smaller molecular mass of 16 kDa when its elution volume was compared to that of protein standards. This suggests one of two possibilities. Firstly, the polypeptide may have a very compact structure, or secondly, even in the presence 150 mM NaCl, the polypeptide may have interacted with the column matrix, which consisted of cross-linked agarose (a complex range of polysaccharide chains having alternating 1,3-α- and 1,4-β-linkages) and dextran (1,6-α-D-glucan).

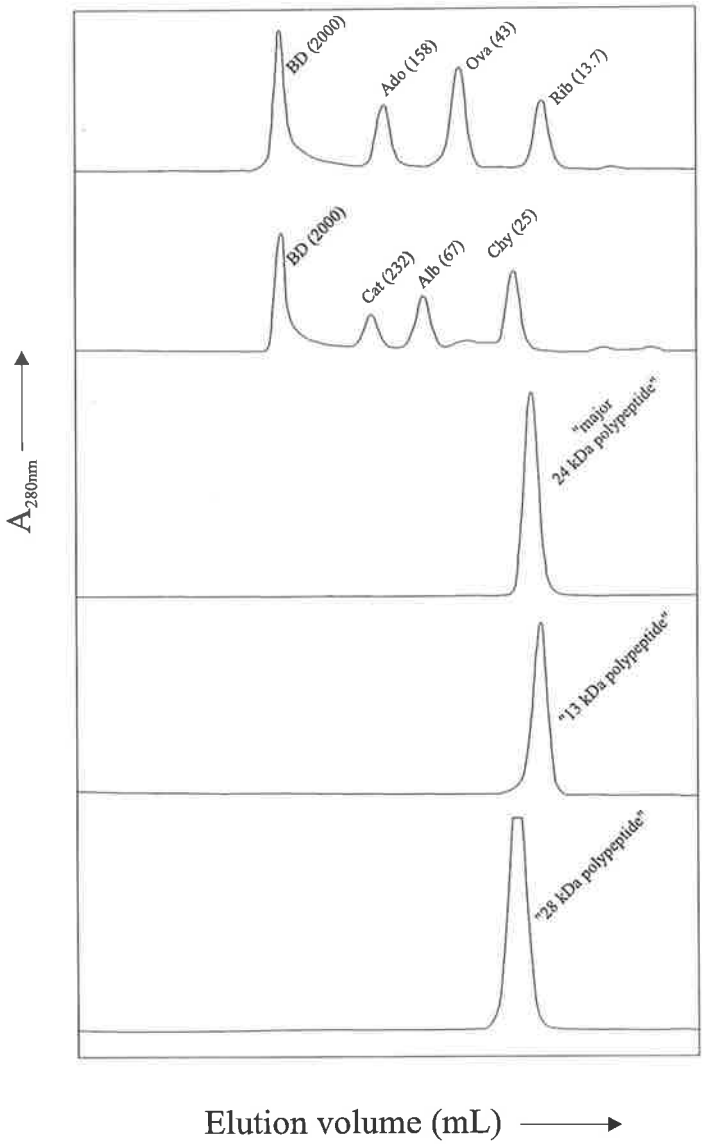
SDS-PAGE (Figure 2.5B, lane 3) and reverse phase HPLC analysis (results not shown) revealed the pooled 'minor 24 kDa polypeptide' fractions from anion exchange chromatography (Figure 2.2) contained two 24 kDa polypeptide species. The N-terminal amino acid sequence (NH_2-Ala-Thr-Phe-Asp-Ile-Leu-Asn-Lys-X-Thr-) of the less abundant species (Figure 2.4) was identical to that of the 'major 24 kDa polypeptide' (as described below). The 'minor 24 kDa polypeptide' of interest was purified by cation exchange chromatography which enabled its separation from the less abundant 'major 24 kDa polypeptide' species (Figure 2.4).

2.3.3 Identification of juice proteins as PR-like proteins

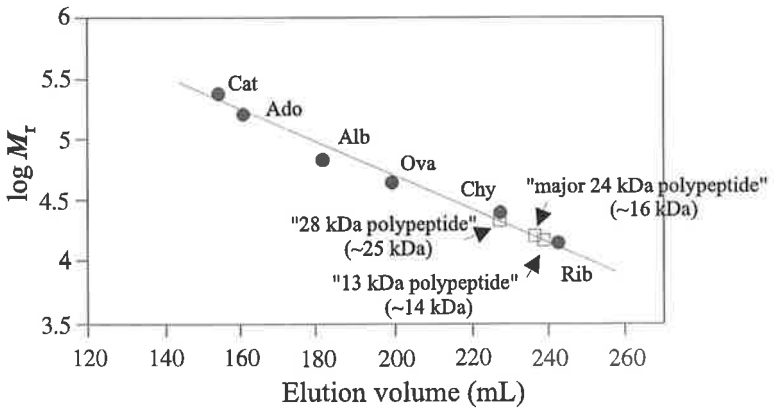
The N-terminal sequences of the purified major 24 kDa polypeptide (NH_2-Ala-Thr-Phe-Asp-Ile-Leu-Asn-Lys-X-Thr-), and minor 24 kDa polypeptide (NH_2-Ala-Thr-Phe-Asn-Ile-Gln-Asn-His-X-Ser-), are identical to that of major wine haze-associated proteins isolated by Waters *et al.* (1996). These proteins are homologous to thaumatin, an immensely sweet protein (10^5 times sweeter than sucrose on a molar basis) found in the arils of the ripe fruits of *Thaumatococcus daniellii* (van der Wel and Loeve, 1972), a West African shrub. Therefore, the major 24 kDa polypeptide was named VVTL1 (for *Vitis vinifera* thaumatin-like protein 1; Tattersall *et al.*, 1997), and similarly the minor 24

Figure 2.3 The major 24 kDa polypeptide, and 13 and 28 kDa soluble juice polypeptides behave as monomers when subjected to gel filtration. The 24, 13 and 28 kDa polypeptides, judged to be essentially pure by SDS-PAGE, were applied to a Superdex 200 Hiload column and elution monitored by absorbance at 280 nm. (A) Elution volumes were compared to that of protein standards (Pharmacia, Sweden) and (B) the log M_r values vs elution volumes were graphed. The M_r standards (M_r shown in parentheses) were as follows: BD, blue dextran (≥2,000 kDa); Cat, catalase (232 kDa); Ado, adolase (158 kDa); Alb, bovine serum albumin (67 kDa); Ova, ovalbumin (43 kDa); Chy, chymotrypsin A (25 kDa); Rib, ribonuclease A (13.7 kDa).

A


BD (2000)
Ado (158)
Ova (43)
Rib (13.7)
BD (2000)
Cat (232)
Alb (67)
Chy (25)
"major 24 kDa polypeptide"
"13 kDa polypeptide"
"28 kDa polypeptide"
A_{280nm}
Elution volume (mL)

B


log M_r
6
5.5
5
4.5
4
3.5
120
140
160
180
200
220
240
260
Elution volume (mL)
Cat
Ado
Alb
Ova
Chy
Rib
"major 24 kDa polypeptide"
(~16 kDa)
"28 kDa polypeptide"
(~25 kDa)
"13 kDa polypeptide"
(~14 kDa)

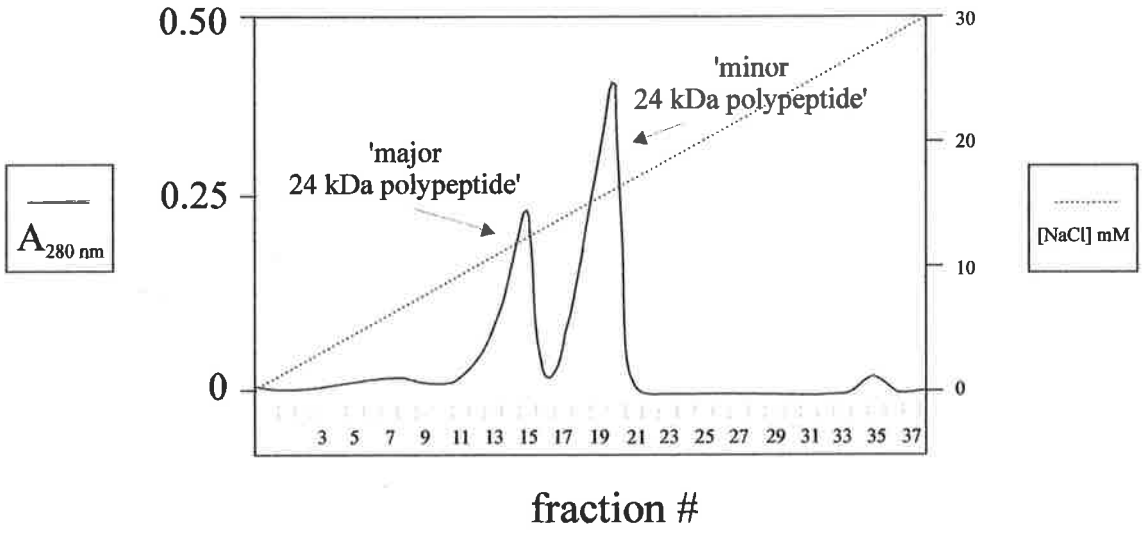


Figure 2.4 Purification of a 'minor 24 kDa juice polypeptide' by cation exchange chromatography. Proteins bound to a cation exchange column were eluted by a linear NaCl gradient and monitored by absorbance at 280 nm. Aliquots (20 μL) of eluted fractions (8 mL) were analysed by SDS-PAGE and reverse phase HPLC (results not shown). Both peaks consisted of 24 kDa components as judged by SDS-PAGE, however, reverse phase HPLC analysis (see Figure 2.6) confirmed peak 2 contained the 'minor 24 kDa polypeptide' of interest.

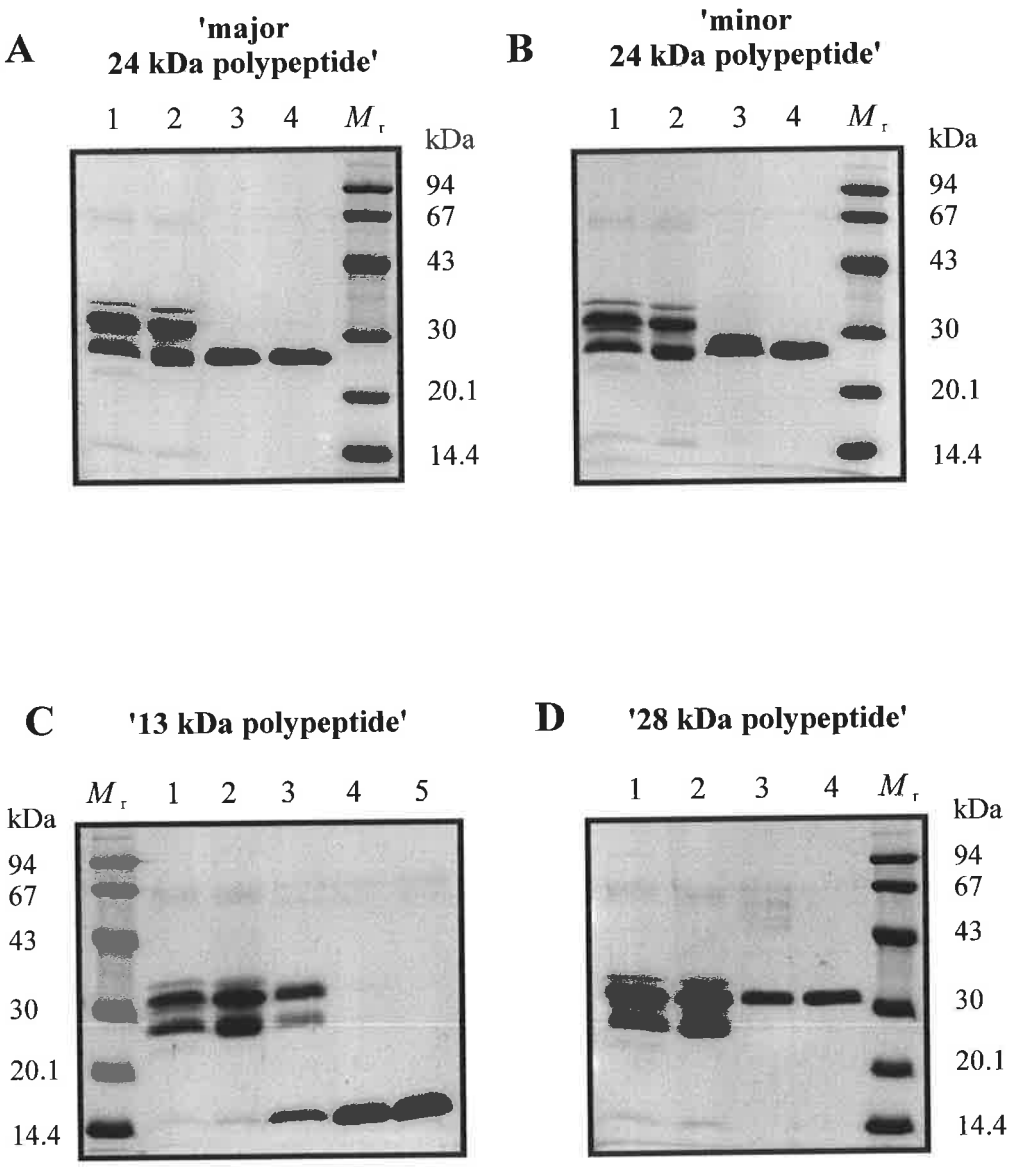
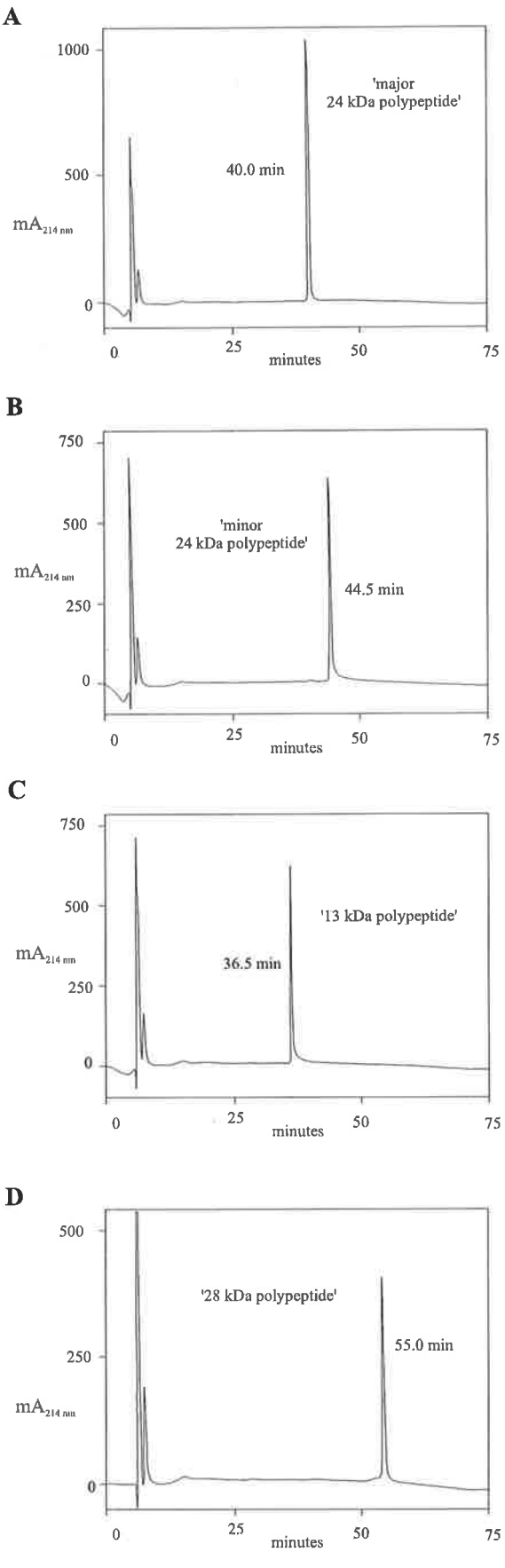


Figure 2.5 SDS-PAGE analysis of the purification steps of four polypeptides from juice. (A) SDS-PAGE analysis of: juice (lane 1), precipitated juice protein (lane 2), pooled anion exchange fractions containing the 'major 24 kDa polypeptide' (lane 3), and pooled gel filtration fractions containing the 'major 24 kDa polypeptide' (lane 4). (B) SDS-PAGE analysis of: juice (lane 1), precipitated juice protein (lane 2), pooled anion exchange fractions containing the 'minor 24 kDa polypeptide' (lane 3), and pooled cation exchange fractions containing the 'minor 24 kDa polypeptide' (lane 4). (C) SDS-PAGE analysis of: juice (lane 1), precipitated juice protein (lane 2), pooled anion exchange fractions containing the '13 kDa polypeptide' (lane 3), pooled first round gel filtration fractions containing the '13 kDa polypeptide' (lane 4), and second round gel filtration fractions containing the '13 kDa polypeptide' (lane 5). (D) SDS-PAGE analysis of: juice (lane 1), precipitated juice protein (lane 2), pooled anion exchange fractions containing the '28 kDa polypeptide' (lane 3), and pooled gel filtration fractions containing the '28 kDa polypeptide' (lane 4). M_r, Molecular mass standards.

Figure 2.6 Major and minor 24 kDa polypeptides, and 13 and 28 kDa polypeptides elute as single peaks when subjected to reverse phase HPLC. Approximately 25 μg of the (A) major 24 kDa, (B) minor 24 kDa, (C) 13 kDa, and (D) 28 kDa polypeptides were applied to a C_8 column and then eluted with a 0 to 80% (v/v) acetonitrile gradient developed over 60 min as described in the Materials and Methods. Approximate retention times are shown.


A
1000
500
0
mA214 nm
'major
24 kDa polypeptide'
40.0 min
0
25
50
75
minutes
B
750
500
250
0
mA214 nm
'minor
24 kDa polypeptide'
44.5 min
0
25
50
75
minutes
C
750
500
250
0
mA214 nm
'13 kDa polypeptide'
36.5 min
0
25
50
75
minutes
D
500
250
0
mA214 nm
'28 kDa polypeptide'
55.0 min
0
25
50
75
minutes

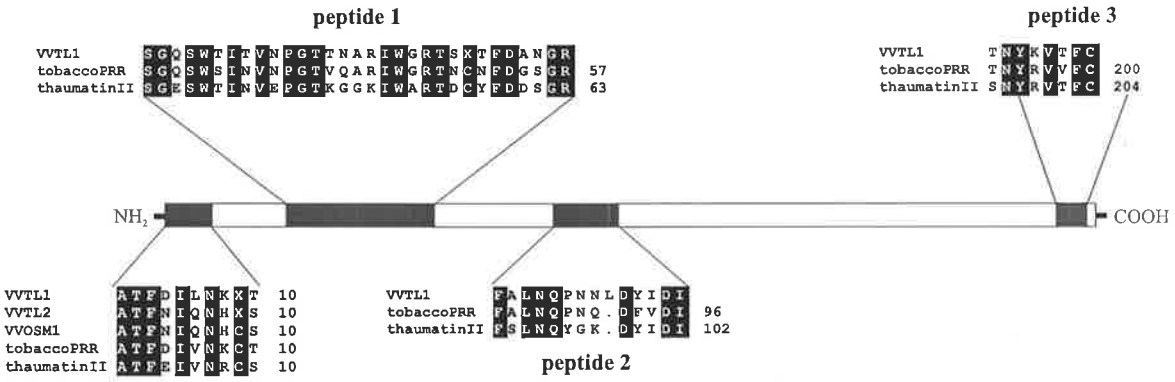
kDa polypeptide was named VVTL2. The N-terminal sequence of VVTL2 (Figure 2.7A) also reveals that it is highly similar, if not identical, to a *V. vinifera* (cv Sultana) protein (VVOSM1) encoded by a cDNA isolated from cell culture (Loulakakis, 1997b). Interestingly, it is known that VVTL1 (Peng *et al.*, 1997), and other thaumatin-like proteins tested (Singh *et al.*, 1987; Roberts and Selitrennikoff, 1990; Fils-Lycaon *et al.*, 1996) lack any sweet characteristics detectable by humans.

To further characterise VVTL1, proteolytic digestion was undertaken. Initial attempts to digest this protein confirmed its inherent resistance to proteolytic digestion as inferred from its survival during winemaking procedures. Indeed, despite the use of strong denaturing agents and carboxymethylation, VVTL1 resisted complete digestion by both endoproteinase Lys-C and endoproteinase Glu-C (results not shown). Nevertheless, following reverse phase HPLC separation of the peptides present in the digestion mixtures, two pure peptides were obtained and their sequences determined (Peptides 1 and 2, Figure 2.7A). Major similarities of these internally located VVTL1 peptides to other PR-5 like proteins were found when a database search was performed. When the VVTL1 peptide sequences were aligned with highly homologous PR-5 like proteins (Figure 2.7A), it was apparent that sequence towards the C-terminus of VVTL1 had yet to be obtained. To overcome this lack of sequence, reductively alkylated VVTL1 was initially subjected to limited acid hydrolysis in TFA. Subsequently, a short peptide was purified by reverse phase HPLC and sequenced (Peptide 3, Figure 2.7A). The protein data presented in Figure 2.7A proved sufficient to enable the design of degenerate oligonucleotide primers and the eventual cloning of a VVTL1 encoding cDNA (see Chapter 3).

To determine the identity of the purified 13 kDa polypeptide it was firstly subjected to Edman degradation. No N-terminal sequence was obtained, indicating that the 13 kDa polypeptide had a modified N-terminal amino acid. In an attempt to obtain peptide sequence, and to overcome difficulties of proteolytic digestion, the protein was

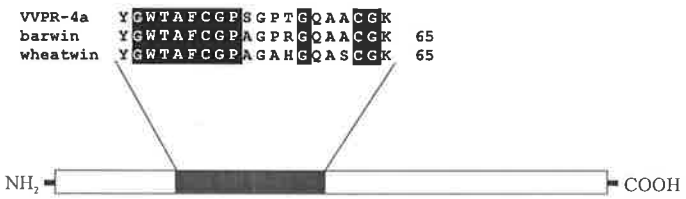
A

VVTL1 and VVTL2 ('24 kDa polypeptides')



B

VVPR-4a ('13 kDa polypeptide')



C

VVCHIT ('28 kDa polypeptide')

firstly reductively alkylated. When the endoproteinase Lys-C digest was subjected to reverse phase HPLC, a peptide was purified and sequence obtained (Tyr-Gly-Trp-Thr-Ala-Phe-Cys-Gly-Pro-Ser-Gly-Pro-Thr-Gly-Gln-Ala-Ala-Cys-Gly-Lys). Database searches revealed a very high degree of sequence similarity to members of the PR-4 family of pathogenesis-related proteins (Figure 2.7B). The purified protein was named VVPR-4a (for *Vitis vinifera* PR-4 protein a) and represents the first reported PR-4 like protein purified from grapevine or any fruit. As with VVTL1, the peptide sequence information of VVPR-4a was utilised for the eventual cloning of a VVPR-4a encoding cDNA (see Chapter 3).

Amino acid sequence of the 28 kDa polypeptide was obtained subsequent to the identification of wine and juice proteins by Waters *et al.* (1996, 1998). Both these studies revealed the potential for N-terminal modification of abundant proteins of approximate molecular masses 28 to 32 kDa. These proteins, which have been classified as chitinases, commonly possess an N-terminal pyroglutamate residue which blocks Edman degradation (Waters *et al.*, 1998). To avoid potential sequence problems, the 28 kDa polypeptide was first incubated with pyroglutamate aminopeptidase, an enzyme which removes N-terminal pyroglutamate residues. When the treated 28 kDa polypeptide was subjected to Edman degradation the following sequence was obtained (Gly-Tyr-X-Gly-Thr-Gly-Ser-Glu-Tyr-X-Gly-Asp-). This amino acid sequence is identical to that of ChitD, a chitinase purified from Muscat of Alexandria juice, and the Muscat of Alexandria wine protein Ia (Waters *et al.*, 1998). It is also highly similar to the amino acid sequence of a chitinase (CHV 5) purified from *V. vinifera* (cv Pinot noir) berries (Derckel *et al.*, 1998), and to other plant chitinases (Figure 2.7C). Furthermore, a cDNA encoding a highly similar grapevine berry class IV chitinase (PR-3 like), Vv*Chi*4A, has been cloned (Robinson *et al.*, 1997). As further characterisation of the 28 kDa polypeptide is needed to prove its possible identity to ChitD (Waters *et al.*, 1998),

for the purpose of this study, it will be referred to as VVCHIT (for *Vitis vinifera* chitinase).

2.4 Discussion

The preparation of berry juice constitutes a selective extraction procedure of a small number of relatively low molecular weight proteins (Figure 2.1) which, once extracted, can persist throughout the winemaking process (Hsu and Heatherbell, 1987a). This selective extraction of a small subset of proteins from the berry appears to be result of two major factors. Firstly, a large proportion of berry proteins most likely form insoluble precipitates when complexed with phenolics from cell vacuoles soon after cell lysis (Anderson, 1968), and/or secondly, many proteins may simply denature when exposed to the acidic conditions (pH ~3 to 4) of the berry juice. To negate these problems often encountered when studying active soluble enzymes from recalcitrant grapevine tissue such as berries, 'complex' detergent based buffers were developed (Hawker, 1969a, 1969b; Downton and Hawker, 1973; Ford and Høj, 1998). These 'complex' buffers are designed to inhibit polyphenolic:protein interactions and therefore contain many additives such as copper chelating agents (Cu^{2+} is a cofactor for polyphenol oxidase), antioxidants and polyphenol binding compounds. The use of such buffers results in efficient extraction of a complex array of berry polypeptides (see a comparison by Ford and Høj (1998)). Fortunately, from the point of view of purifying wine haze proteins, inclusion of the protective agents is not required. Instead the expression of berry juice alone represented an ideal first step in making the purification process a relatively simple task. With the purification and sequencing of four prominent soluble juice proteins it is now apparent that the haze associated proteins of wine are in fact PR-like proteins derived from the grapevine berry, a phenomena also reported by Waters *et al.* (1996).

Comparison of the soluble proteins present in a Muscat of Alexandria juice and commercial wine reveals a highly similar polypeptide pattern (Figure 2.1, lanes 2 and 3). The very minor differences observed between the wine and juice polypeptide pattern may

be explained by the fact that the wine analysed in lane 3 was not made from the juice analysed in lane 2. The conservation of polypeptide pattern between juice and wines has been reported previously by Pueyo *et al.* (1993). Overall, this suggests the soluble berry proteins extractable into juice and wine tolerate low pH conditions (pH ~3 to 4) and ethanol concentrations of at least 10% (v/v). Resistance to proteolytic cleavage by peptidases secreted by fermenting yeasts is also evident (Waters *et al.*, 1992) and appears to be intrinsic rather than a function of protease inhibiting substances (Waters *et al.*, 1995a). As yet, attempts to isolate wine yeasts that produce effective proteases against wine haze proteins during fermentation have been unsuccessful (Lagace and Bisson, 1990; Charoenchai *et al.*, 1997), and the use of current commercial protease preparations at winemaking temperatures of 20°C or below appears ineffectual (Feuillat and Ferrari, 1982; Waters *et al.*, 1992). Now that it is evident wine haze associated proteins are berry PR-like proteins, an alternative and more appropriate approach to finding effective proteases for the degradation of wine haze proteins may be to first understand the mechanisms of PR protein turnover in plants. In fact, it has been previously reported that extracellular proteases isolated from tomato (Rodrigo *et al.*, 1989) and tobacco (Rodrigo *et al.*, 1991b) can degrade some PR proteins *in vitro*. Such specialised proteases possess activity at low pH, making them potentially suitable candidates for application in juice and wine processing.

The soluble proteins present in grape juice and wine from around the world appear to share several properties including amino acid sequence, mass and charge (Hsu and Heatherbell, 1987a; Murphey *et al.*, 1989; Waters *et al.*, 1991; Yokotsuka *et al.*, 1991; Pueyo *et al.*, 1993; Dorrestein *et al.*, 1995; Santoro, 1995). Interestingly, possible genuine small variations in varietal protein compositions (both protein molecular mass and presence) are currently being considered as a method of distinguishing ('fingerprinting') wines (Weiss *et al.*, 1998; Y. Hayasaka, pers. commun.). Such 'fingerprinting', as opposed to DNA analyses, may be used in the future to guarantee

wine varietal authenticity of bottled products to governing organisations and consumers. Notwithstanding these minor differences, the ubiquitous nature of juice and wine protein suggests common physiological signals are responsible for their production by grapevine berries. Indeed, it has been proposed these proteins accumulate primarily as a result of developmental processes that occur during berry ripening (Murphey *et al.*, 1989).

Chitinases and thaumatin-like proteins have now been purified from a number of fleshy fruits including tomato (Pressey, 1997), cherry (Fils-Lycaon *et al.*, 1996) and persimmon (Vu and Huynh, 1994). This study confirms the presence of these proteins in grapevine berries, a phenomenon which now has been widely reported (Derckel *et al.*, 1996; Robinson *et al.*, 1997; Tattersall *et al.*, 1997; Derckel *et al.*, 1998; Salzman *et al.*, 1998; Waters *et al.*, 1998). The reasons for the location of PR-like proteins within fleshy fruit have not been fully established and this will be more extensively discussed in following chapters. These PR-3 and PR-5 like proteins may play a role in defence against pathogens, especially those of fungal origin (see Section 1.2.4), as suggested by *in vitro* assays involving a thaumatin-like protein purified from persimmon (Vu and Huynh, 1994) and *Vitis labruscana* (cv Concord) grapes (Salzman *et al.*, 1998). Chitinases are thought to degrade the chitin present in the cell walls of fungal pathogens, however, the mechanism by which thaumatin-like proteins exert antifungal effects is unknown. It has been observed that some thaumatin-like proteins have an affinity for β-1,3-glucan (Trudel *et al.*, 1998), also a component of fungal cell walls. However, although VVTL1 appeared to interact with the gel filtration column matrix during purification (see Section 2.3.2), it was reported that grapevine berry thaumatin-like proteins do not possess β-1,3-glucan binding activity (Trudel *et al.*, 1998). It is of course still possible that VVTL1 and related fruit thaumatin-like proteins such as VVTL2 have an affinity for polysaccharides which are present in fungal cell walls.

This study also represents the first report of the identification a PR-4 like protein from fleshy fruit. PR-4 proteins have also been implicated as being important for plant

defence via unknown mechanisms (Hejgaard *et al.*, 1992; Caruso *et al.*, 1996). Such a possible role for PR-4 like proteins in berries will be discussed in the following chapters.

CHAPTER 3

Molecular cloning of cDNAs encoding the berry PR-like proteins - VVTL1 and VVPR-4a

3.1 Introduction

Of the four predominant grape berry PR-like proteins identified in Chapter 2, two were further characterised by a combination of molecular biology and protein biochemistry techniques. Further definition of the properties of these proteins may help facilitate the discovery of superior ways of removing unwanted protein from 'unstable' wine. For example, it may be possible to design specific affinity based fining agents, or to find more suitable and efficient proteases to replace the widespread use of bentonite clay in winemaking. Such a replacement would be advantageous for several reasons. Bentonite is believed to have detrimental effects on the sensory properties of treated wines (Miller *et al.*, 1985; Voilley *et al.*, 1990), it leads to typical losses of ~3% of wine volume during removal from fined wine (D. McWilliam and T. Jones, pers. commun.), and there are environmental problems concerning its disposal (Rankine, 1989).

A detailed characterisation of berry PR-like proteins may also help reveal their role within fruits in general and the grapevine in particular. Both the abundance and stability of these proteins suggests they may be integral to important processes within the berry, for example, development or defence against pathogens. These issues need to be addressed if genetic manipulation is to be considered as an option for reducing PR-like protein levels in berries, and wine made therefrom. Conversely, if these proteins have roles in plant defence, enhanced expression of respective genes within the grapevine may be beneficial.

Described in this chapter is the examination of several characteristics of the berry PR-like proteins, VVTL1 and VVPR-4a. Sequence analysis of cloned cDNAs has allowed for the deduction of the complete primary structures for both proteins. Also, combined cDNA sequence analysis and electrospray mass spectrometry data has been used to predict post-translational modifications including the identification of likely

topogenic signals responsible for protein subcellular localisation, and the formation of intramolecular disulfide bonds. Finally, based on X-ray crystallographic data for PR-5 proteins (Ko *et al.*, 1994; Koiwa *et al.*, 1999), and NMR data for a PR-4 protein (Ludvigsen and Poulsen, 1992b), predictions of tertiary structure have been made for VVTL1 and VVPR-4a.

3.2 Materials and Methods

All molecular biology procedures were performed essentially as described by Sambrook *et al.* (1989) unless otherwise noted.

3.2.1 Berry RNA Isolation

Total RNA was extracted from ripening Muscat of Alexandria grapevine berries essentially as described by Levi *et al.* (1992), except that the extraction buffer contained 500 mM Tris-HCl, pH 8.0 while thiourea and aurintricarboxylic acid were excluded. With the aid of a small coffee grinder (Black and Decker, Australia), berries (stored at -70°C) were ground to a powder in liquid N_2 before addition of buffer (2 mL of buffer to 1 g tissue). RNA concentrations and purity were determined by scanning UV spectroscopy (assuming 1 OD_{260} = 40 μg/mL). Typical total RNA yields were ~20 μg/g fresh berry weight.

3.2.2 cDNA Synthesis

All oligonucleotides were synthesised by Gibco BRL Lifescience (Australia) and PCRs performed in a Perkin Elmer GeneAmp PCR System 2400 machine (USA).

VVTL1 cDNA

The cDNA sequence of VVTL1 was obtained by combination of the information contained in an 'internal' cDNA, a 5'-RACE product and a 3'-RACE product. First strand cDNAs were generated with SUPERSCRIPT II RNase H^- Reverse Transcriptase (GIBCO BRL, USA) according to the manufacturer's instructions in a volume of 20 μL, using 2 μg of post-veraison berry RNA as template and 150 pmoles of primer. A $(dT)_{15}$ primer was used in the preparation of first strand cDNA for cloning of the 'internal'

cDNA (nucleotides 154 to 738, Figure 3.2) and 3' RACE-PCR product (nucleotides 610 to 954, Figure 3.2), while the primer TL6 (described below) was employed to prepare the first strand cDNA used to generate the 5' RACE-PCR product (nucleotides 1 to 254, Figure 3.2).

'Internal' cDNA: Based on amino acid sequences obtained from VVTL1, degenerate oligonucleotides representing all possible codon usages were synthesised. The oligonucleotides and corresponding amino acid sequences are as follows: TL1, 5'-TT(C/T)GA(C/T)AT(A/T/C)(C/T)TIAA(C/T)AA(A/G)TG(T/C)ACITA-3' (FDILNKCT); TLRT2, 5'-(A/G)CA(A/G)AAIGTIAC(C/T)TT(A/G)TA(A/G)TT-3' (NYKVTFC). Reaction mixtures typically contained one unit of *Taq* polymerase (GIBCO BRL, USA), 50 pmoles of each primer and 2 μL of the first strand cDNA product as template in a final volume of 25 μL, buffered according to the manufacturer's instructions. Following incubation at 94°C for 2 min, a total of 37 cycles were performed (cycle 1 to 2: 94°C for 1 min, 37°C for 2 min, and 72°C for 1 min 30 s; cycles 3 to 37: 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min 30 s) then terminated by a final extension reaction at 72°C for 7 min.

RACE-Products: RACE-PCR, performed essentially as described by Frohman *et al.* (1988), was employed to obtain the 5' and 3' ends of the final cDNA (Figure 3.2). Oligonucleotides described by Frohman *et al.* (1988) were used in conjunction with TL5, 5'-TTGACGGTGATTGTCCAGGACTG-3' (nucleotides 232 to 254, Figure 3.2) and TL6, 5'-CATTGGCGTCGAAGGTGCATGAG-3' (nucleotides 291 to 313, Figure 3.2) to generate the 5' RACE-PCR product. The Frohman *et al.* (1988) oligonucleotides and primers TL3, 5' - AA(C/T)AA(C/T)(C/T)TIGA(C/T)TA(C/T)ATIGA(C/T)AT - 3' (nucleotides 415 to 437, Figure 3.2) and TL7, 5'-AGCTGTGGTCCGACCACATACTC-3' (nucleotides 610 to 632, Figure 3.2) were used to generate the 3'-RACE-PCR product.

VVPR-4a cDNA

The cDNA sequence of VVPR-4a was obtained by combination of the information contained in an 'internal' cDNA (nucleotides 205 to 437, Figure 3.6), a 5'-RACE product (nucleotides 1 to 320, Figure 3.6) and a 3'-RACE product (nucleotides 321 to 569, Figure 3.6). A $(dT)_{15}$ primer was used in the preparation of first strand cDNA for cloning of the 'internal' cDNA and 3' RACE-PCR product, whilst the primer WIN4 (described below) was employed to prepare the first strand cDNA for generation of the 5' RACE-PCR product.

'Internal' cDNA: Based on amino acid sequence obtained from VVPR-4a and related proteins from other plant species (see Figure 3.7), degenerate oligonucleotides representing all possible codon usages were synthesised and employed in PCR reactions as described for cloning of VVTL1 encoding cDNAs (see above). The oligonucleotides and corresponding amino acid sequences are as follows: WIN1, 5'-TA(C/T)GGITG GACIGCITT(C/T)TG(C/T)GG-3' (YGWTAFCG); WINRT, 5'-CA(A/G)T(C/T)IAC (A/G)AAI(C/T)C(A/G)TA(A/G)T(C/T)IAC-3' (V(N/D)Y(Q/E)FV(N/D)C).

RACE-Products: RACE-PCR, performed essentially as described for VVTL1 cDNA synthesis, was employed to obtain the 5' and 3' ends of the final cDNA (Figure 3.6). Oligonucleotides described by Frohman *et al.* (1988) were used in conjunction with WIN4, 5'-ATCCAATCCTCCATTGCTGCATTG-3' (nucleotides 323 to 346, Figure 3.6) and WIN3, 5'-CCACTATTCTTCACCGTTGCCTG-3' (nucleotides 299 to 320, Figure 3.6) to generate the 5' RACE-PCR product. The oligonucleotides of Frohman *et al.* (1988) combined with the WIN1-primer (described above) and WIN5-primer, 5'-ACCAATGCAGCAATGGAGGATTG-3' (nucleotides 321 to 343, Figure 3.6) were used to generate the 3'-RACE-PCR product.

3.2.3 Cloning and sequencing of RT- and RACE-PCR products

PCR products were purified from TAE buffered agarose gels using the Wizard DNA purification system (Promega, USA), ligated into the pGEM-T vector (Promega, USA) and transformed into either competent *E. coli* XL1 blue (Stratagene, USA) or JM109 (Promega, USA) cells. Competent cells were prepared using a $CaCl_2$ based method as described by Miller (1987). Transformed cells were grown (37°C overnight) on LB agar (1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 2% (w/v) agar, pH 7) containing 100 μg/mL ampicillin. Double stranded plasmid templates were prepared from *E. coli* cultures, grown (37°C overnight) in 2YT broth (0.5% (w/v) NaCl, 1% (w/v) yeast extract, 1.6% (w/v) tryptone) containing 50 μg/mL ampicillin, using the alkaline lysis based method described elsewhere (Birnboim and Doly, 1979).

Sequencing was performed by the dideoxynucleotide chain termination method using a Sequenase™ Version 2.0 sequencing kit (Amersham Lifescience, UK), as described in the manufacturer's instructions, and [α-^{35}S]dATP labelled products were separated on a 6% (w/v) denaturing polyacrylamide gel and detected by autoradiography. Automated DNA sequencing (Dyedeoxy Terminator™ Chemistry; Perkin Elmer, USA) was occasionally performed by one of the following facilities: Newcastle DNA, The University of Newcastle (Australia); Flinders Medical Centre DNA sequencing core facility, Flinders University (Australia); Nucleic Acid and Protein Chemistry Unit, The University of Adelaide. All facilities used a Perkin Elmer Applied Biosystems Model 373A DNA sequencer (USA) for the detection of products.

3.2.4 Mass spectrometry

The masses of VVTL1 and VVPR-4a, respectively, were determined on a API-300 triple quadrupole mass spectrometer coupled with an ionspray interface (Perkin Elmer Sciex, Thornhill, Ontario, Canada) by Yoji Hayasaka at the Mass Spectrometry

Facility, Waite Campus, The University of Adelaide. Briefly, the ion spray voltage was 5.5 kV and the orifice voltage was 30 V. The curtain (N_2) and nebuliser (air) gases were set at 8 and 10 units, respectively. Solutions of proteins (~1 pmol/μL) were introduced into the mass spectrometer by a flow injector (8125, Rheodyne, Cotati, California, USA) with a 5 or 20 μL sample loop connected to the ion sprayer. The injected solution was delivered by 50% acetonitrile containing 2.5% acetic acid at a rate of 5 μL/min, using a syringe pump (Cole-Parmer, Niles, Illinios, USA). Mass spectra were processed to determine the molecular weight of the protein by deconvolution of the multiply charged ions of the protein using Bio-Multiview software (Pekin Elmer Sciex, Canada).

3.2.5 Protein database searching, sequence alignments, pI and mass predictions

The GenBank and sister databases were searched for sequences related to VVTL1 and VVPR-4a using the BLASTP Version 1.4.9 program (Altschul *et al.*, 1990). Protein alignments and dendrograms were made using the PILEUP program. The dendrogram was displayed using the FIGURE program. Protein pI and mass predictions were made using the PEPSORT program. PILEUP, PEPSORT and FIGURE were provided within the Wisconsin package, Version 8.1.0 (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711). All programs were provided by the Australian Genomic Information Service (ANGIS), located at The University of Sydney.

3.2.6 Three-dimensional protein structure prediction

VVTL1 and VVPR-4a tertiary structure predictions were made using the SWISS-MODEL tools and software packages available through the world wide web (WWW) server of the Swiss Institute of Bioinformatics (Internet address = http//:www.expasy.ch/swissmod/ SWISS-MODEL.html). The First Approach Mode was used to obtain predictions. Briefly, VVTL1 and VVPR-4a protein sequence was

compared, via the BLASTP2 program (Altschul *et al.*, 1990), to sequences of known tertiary structure held within the Brookhaven Protein Data Bank. Areas of sequence identities with model templates were selected with the SIM program (Huang and Miller, 1991), and model predictions generated with the ProModII program (Peitsch, 1996). Energy minimisation of predicted models was performed by the Gromos96 program (BIOMOS, Zürich, Switzerland).

Model coordinate files obtained from Brookhaven Protein Data Bank and SWISS-MODEL database were displayed using the MOLMOL molecular graphics program, version 2.6 (Koradi *et al.*, 1996), which is available at the following internet address: http://odin.ethz.ch/wuthrich/software/molmol/index.html.

3.3 Results

3.3.1 Isolation of cDNAs encoding VVTL1 and VVPR-4a

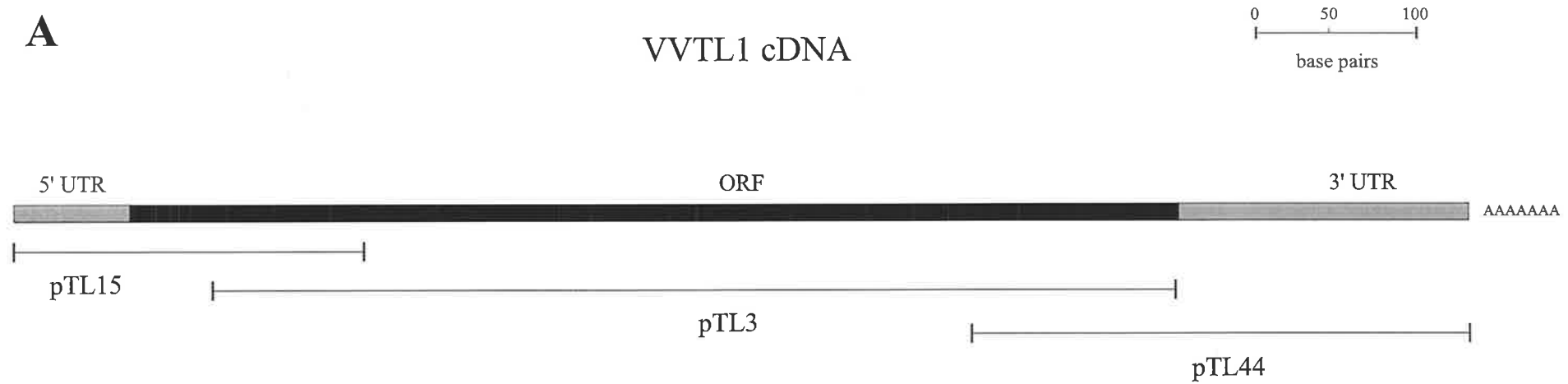
In the absence of a suitable grapevine berry cDNA library, partial cDNAs encoding VVTL1 and VVPR-4a were obtained by amplifying target cDNAs using the technique of RT-PCR. Based on the peptide sequence of the purified proteins, and also in the case of VVPR-4a, conserved sequences of other PR-4 like proteins (see Figure 3.7), several degenerate primers (as described in the materials and methods Section) were synthesised and employed in PCR reactions to amplify respective cDNAs from reverse transcribed grapevine berry mRNA. The source of mRNA template for cDNA generation was post-veraison Muscat of Alexandria berries (~17° Brix). The reason for this choice was two-fold. Firstly, both VVTL1 and VVPR-4a had been purified from a Muscat of Alexandria juice, and secondly, it was assumed that both VVTL1 and VVPR-4a were synthesised towards the latter stages of berry development (see Section 1.1.1).

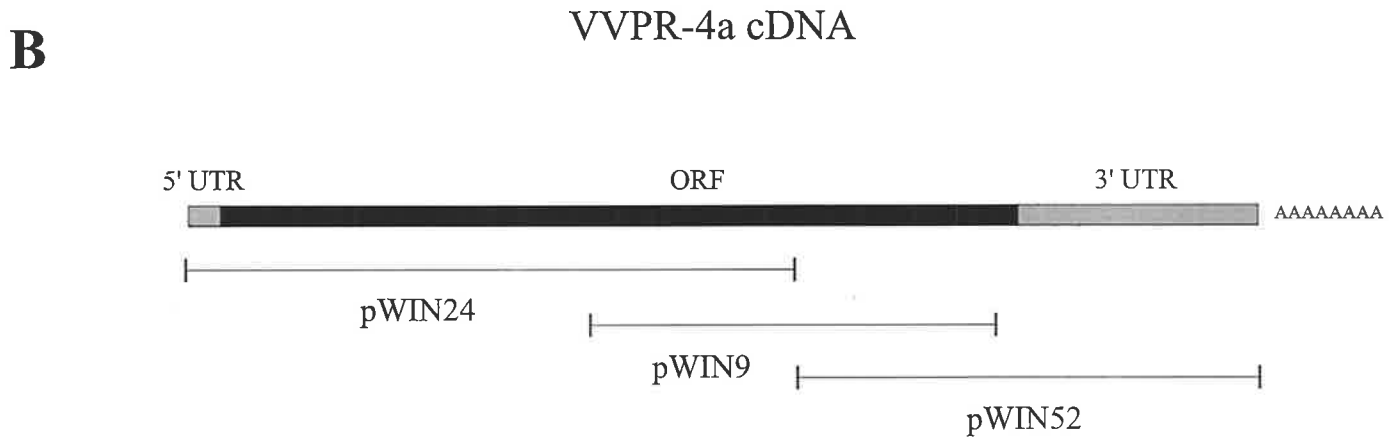
The cloning and sequencing of partial VVTL1 and VVPR-4a cDNAs allowed the further design of cDNA specific primers (see Section 3.2.2) for use in RACE-PCR reactions (Frohman *et al.*, 1988), allowing the remaining 5' and 3' regions of the encoding cDNAs to be amplified. Subsequently, the cloning of the RACE-PCR products facilitated the sequence characterisation of the full length cDNAs encoding VVTL1 and VVPR-4a (Figure 3.1).

3.3.2 Characterisation of the cDNA encoding VVTL1

The sequence of the VVTL1 encoding cDNA and the deduced amino acid sequence is shown in Figure 3.2. Comparison of the deduced amino acid sequence and the N-terminal sequence data for the purified protein suggests that VVTL1 is synthesised

Figure 3.1 Subclone map of (A) VVTL1 and (B) VVPR-4a cDNAs. Each cDNA sequence is a combination of information contained in an 'internal' cDNA, 5' RACE-PCR product, and a 3' RACE-PCR product (as described in the Materials and Methods section). The inserts and corresponding names of the individual plasmid subclones are shown. The respective open reading frames (ORF) are shown in black, and the 5' and 3' untranslated regions (UTR) are shown in grey.


A
VVTL1 cDNA
0
50
100
base pairs
5' UTR
ORF
3' UTR
AAAAAAA
pTL15
pTL3
pTL44


B
VVPR-4a cDNA
5' UTR
ORF
3' UTR
AAAAAAAA
pWIN24
pWIN9
pWIN52

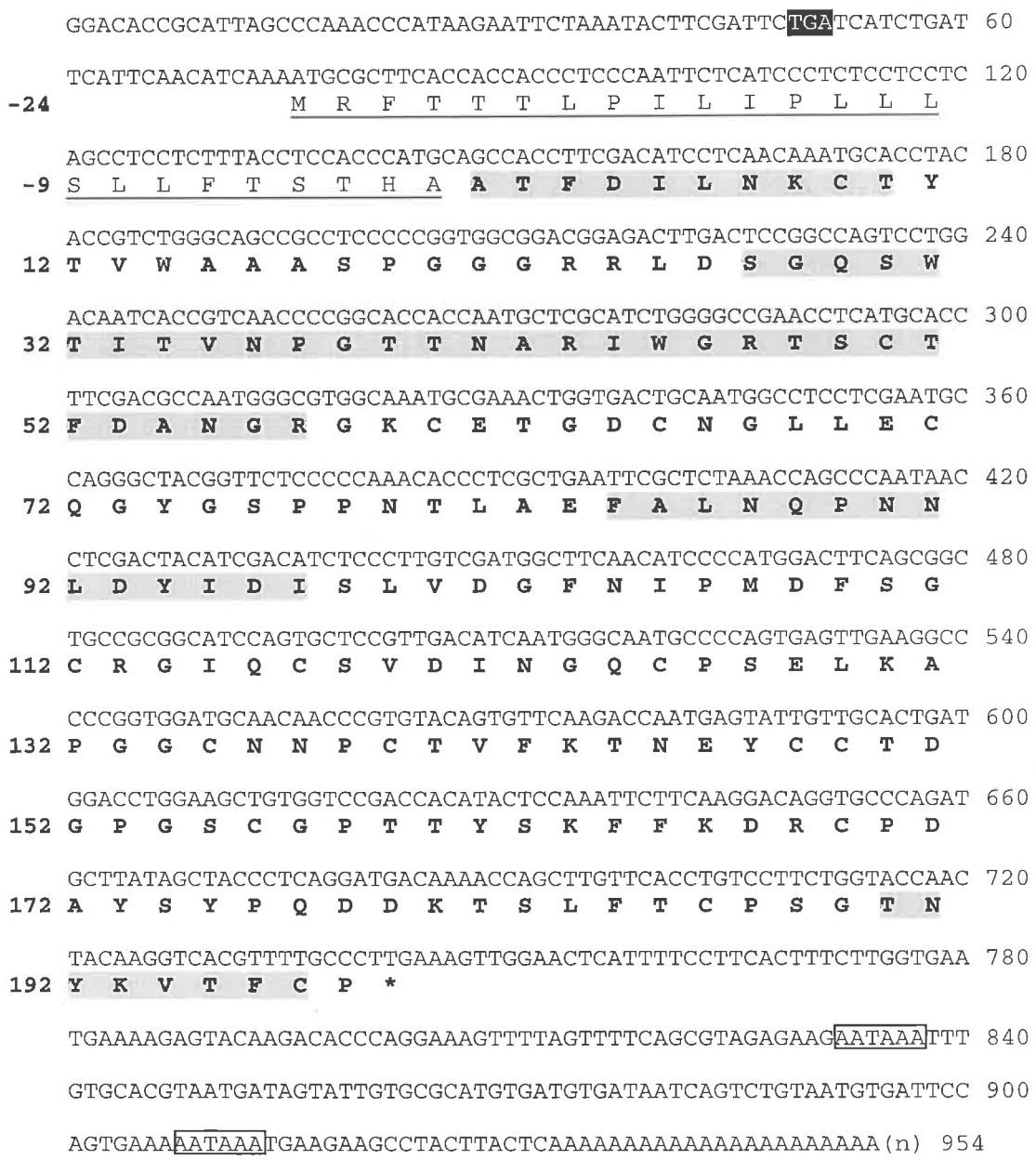


Figure 3.2 Nucleotide and deduced amino acid sequence of a VVTL1 encoding cDNA. Peptide sequences obtained from purified VVTL1 are highlighted, a cleavable presequence is underlined and putative polyadenylation signals are boxed. Nucleotides are numbered on the right hand side while amino acids are numbered on the left hand side with the first residue of the mature protein designated +1. The translational stop codon is denoted by an asterisk (*) and an in-frame stop codon upstream of the initiation codon is shaded black. This sequence was compiled from an 'internal' cDNA (154 to 738), a 5'-RACE product (1 to 254) and a 3'-RACE product (610 to 954). No sequence discrepancies were noted in the overlaps.

as a precursor protein with a typical transient presequence (Bar-Peled *et al.*, 1996) of 24 residues preceding Ala^{+1} of the mature protein. The presequence, which is assumed to be necessary for transport in the secretory system, suggests that VVTL1 is an apoplastic protein as it is not synthesised with a C-terminal propeptide which may act as a vacuolar targeting signal (Melchers *et al.*, 1993). The predicted mass of the mature protein is 21 286.45 Da whilst that obtained by mass spectrometry is 21 272.90 +/- 1.88 Da. The discrepancy of 16 Da can readily be accounted for by the formation of eight disulfide bonds as seen in the crystallised PR-5 like proteins, thaumatin (Ogata *et al.*, 1992; Ko *et al.*, 1994), zeamatin (Batalia *et al.*, 1996) and PR-5d (Koiwa *et al.*, 1999). Thus, it was concluded not only that the cDNA sequence (Figure 3.2) exactly specifies the purified VVTL1 but also that the protein undergoes no post-translational modification other than the removal of the presequence and formation of intramolecular disulfide bonds. The predicted pI (4.60) is consistent with the protein's behaviour during anion exchange chromatography (see Section 2.3.2). Within the 3' untranslated region of the VVTL1 cDNA there are two consensus polyadenylation signals (Li and Hunt (1997); Hunt and Messing, 1998), one within 19 bp of the poly (A) tail.

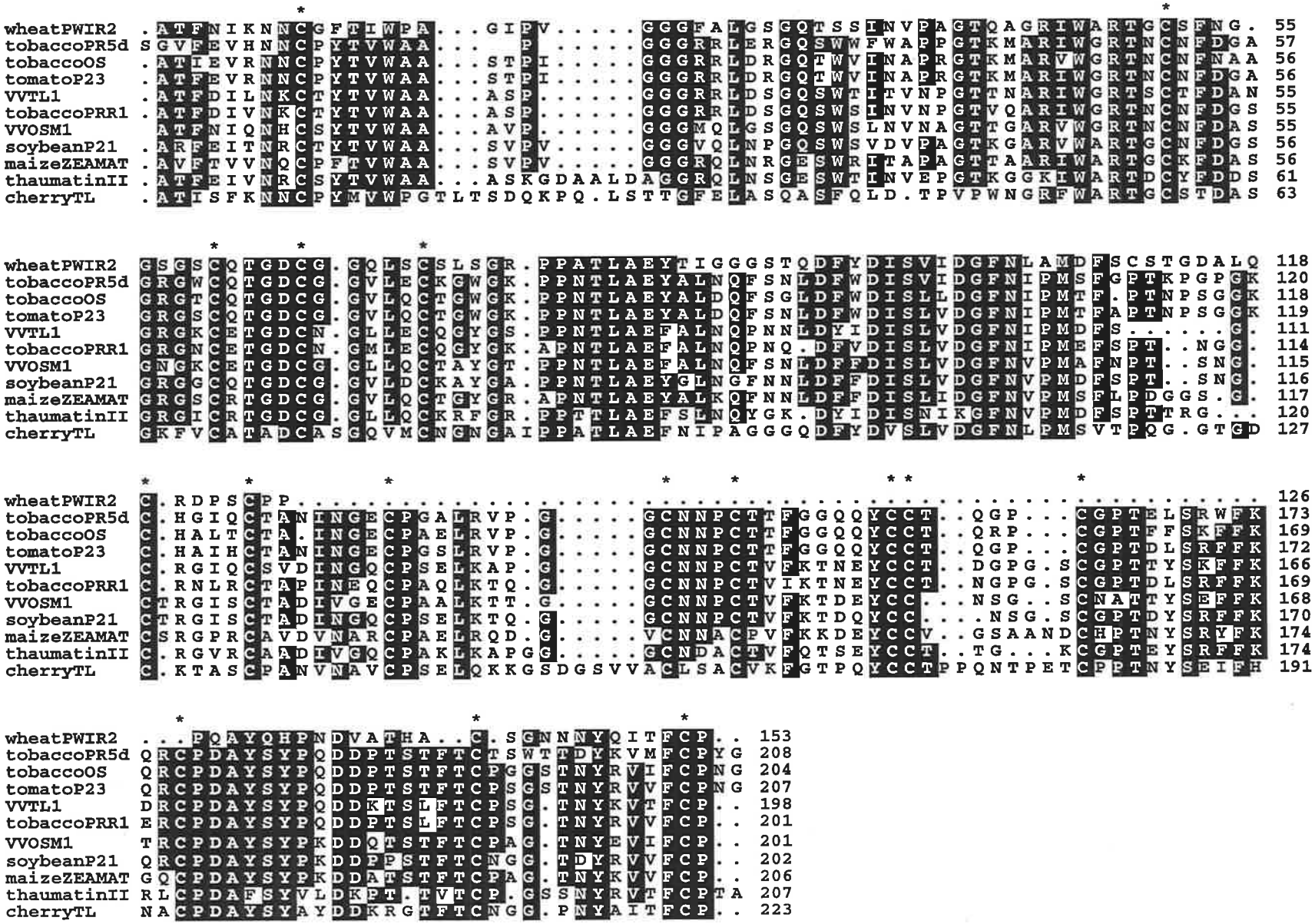
A cDNA encoding a grapevine protein, VVOSM1, has been cloned from cultured cells (Loulakakis, 1997b). The predicted N-terminal amino acid sequence of VVOSM1 is identical to that of VVTL2 (see Figure 2.7A). Also, the predicted mass of VVOSM1, accounting for the formation of a proposed eight disulfide bonds and the removal of a putative signal peptide, is 21 248 Da, which is in good agreement with the predicted mass of purified VVTL2 (21 246 Da). Therefore, it is highly likely that VVTL2 and VVOSM1 are identical proteins.

3.3.3 Comparison of VVTL1 to other PR-5 protein sequences

The primary structure of VVTL1 is highly similar to those of a large number of PR-5 like proteins present within the GenBank and sister databases (Figure 3.3). To date, the known proteins most similar to VVTL1 are two isoforms of the tobacco PR-R protein (PRR1 and PRR2) (Cornelissen *et al.*, 1986b; Payne *et al.*, 1988) which exhibit positional amino acid sequence identity of 82% and 79% to that of VVTL1, respectively. The PR-R genes are induced by TMV infection (Cornelissen *et al.*, 1986b). Other highly related proteins include PR-5B (76% positional identity; encoded by a cDNA isolated from chickpea (*Cicer arietinum*); GenBank accession no. O81926), and ATOSM from *Arabidopsis* (74% positional identity) (Capelli *et al.*, 1997), the mRNA of which is most abundant in older leaves. Also, the VVTL1 amino acid sequence is 72% identical to that of VVOSM1 (Loulakakis, 1997b), and therefore presumably VVTL2. Apart from the berry, the VVOSM1 gene is also expressed in various other grapevine tissues including roots, stems and leaves (Loulakakis, 1997a).

A common feature to all the PR-5 like proteins analysed was the existence of 16 positionally conserved cysteine residues (see Figure 3.3). As previously mentioned (see Section 3.3.2), these residues are believed to be involved in the formation of disulfide bonds. Because of the large number of PR-5 like protein sequences available within various protein databases, the relationship of VVTL1 to other PR-5 like proteins could be examined by the use of a dendrogram (Figure 3.4). Apart from the group of highly related proteins within VVTL1 is placed, it is apparent that there is high degree of relatedness amongst a group of proteins from several cereal plants including wheat, barley, oats, and rye, and amongst a separate group of proteins from several Solanaceous species (*Nicotiana tabacum, Solanum commersonii* and *Lycopersicon esculentum*). The grouping of the cereal PR-5 proteins is mostly likely accounted for by the fact these smaller proteins lack an approximately 50 amino acid internal sequence present in other

Figure 3.3 Multiple amino acid sequence alignment of some PR-5 protein family members using the PILEUP program. Residues that are identical in at least seven of the eleven sequences are highlighted. Actual or deduced mature amino acid sequences were obtained from the following sources: wheatPWIR2 (cDNA isolated from powdery mildew challenged wheat leaves (Rebmann *et al.*, 1991)), PR-5d (gene isolated from tobacco (Sato *et al.*, 1996) encoding a neutral protein expressed in cultured cells (Koiwa *et al.*, 1997)), tobaccoOS (cDNA isolated from NaCl stressed tobacco culture cells encoding osmotin (Singh *et al.*, 1989)), tomatoP23 (Citrus exocortis viroid-induced cDNA from tomato leaves encoding the vacuolar PR protein P23 (Rodrigo *et al.*, 1993)), VVTL1 (cDNA isolated from grapevine berries), tobaccoPRR (cDNA encoding PR protein R (major form) isolated from tobacco leaves infected with TMV (Payne *et al.*, 1988)), VVOSM1 (cDNA isolated from grapevine cell culture (Loulakakis, 1997b); VVOSM1 is possibly identical to VVTL2), soybeanP21 (protein purified from healthy soybean leaves (Graham *et al.*, 1992)), maizeZEAMAT (protein purified from maize seed (Batalia *et al.*, 1996)), thaumatinII (cDNA encoding for thaumatin, an intensely sweet protein from the seed arils of the West African shrub, *Thaumatococcus daniellii* (Edens *et al.*, 1982)) and cherryTL (cDNA encoding a protein purified from ripe cherry fruit (Fils-Lycaon *et al.*, 1996)). Amino-terminal presequences and transient carboxy-terminal propeptides are not included. Conserved cysteine residues are denoted by asterisks (*).


wheatPWIR2 . ATFNIKNNCGFTIWPA...GIPV.....GGGFALGSGQTSSINVPAGTQAGRIWARTGCSFNG. 55
tobaccoPR5d SGVFEVHNNCPYTVWAA... P.....GGGRRLERGQSWWFWAPPGTKMARIWGRTNCNFDGA 57
tobaccoOS . ATIEVRNNCPYTVWAA...STPI.....GGGRRLDRGQTWVINAPRGTKMARVWGRTNCNFNAA 56
tomatoP23 . ATFEVRNNCPYTVWAA...STPI.....GGGRRLDRGQTWVINAPRGTKMARIWGRTNCNFDGA 56
VVTL1 . ATFDILNKCTYTVWAA...ASP.....GGGRRLDSGQSWTITVNPGTTNARIWGRTSCTFDAN 55
tobaccoPRR1 . ATFDIVNKCTYTVWAA...ASP.....GGGRRLDSGQSWSINVNPGTVQARIWGRTNCNFDGS 55
VVOSM1 ATFNIQNHCSYTVWAA...AVP.....GGGMQLGSGQSWSLNVNAGTTGARVWGRTNCNFDAS 55
soybeanP21 . ARFEITNRCTYTVWAA...SVPV.....GGGVQLNPGQSWSVDVPAGTKGARVWARTGCNFDGS 56
maizeZEAMAT . AVFTVVNQCPFTVWAA...SVPV.....GGGRQLNRGESWRITAPAGTTAARIWARTGCKFDAS 56
thaumatinII . ATFEIVNRCSYTVWAA...ASKGDAALDAGGRQLNSGESWTINVEPGTKGGKIWARTDCYFDDS 61
cherryTL . ATISFKNNCPYMVWPGTLTSDQKPQ.LSTTGFELASQASFQLD.TPVPWNGRFWARTGCSTDAS 63
wheatPWIR2 GSGSCQTGDCG.GQLSCSLSGR.PPATLAEYTIGGGSTQDFYDISVIDGFNLAMDFSCSTGDALQ 118
tobaccoPR5d GRGWCQTGDCG.GVLECKGWGK.PPNTLAEYALNQFSNLDFWDISVIDGFNIPMSFGPTKPGPGGK 120
tobaccoOS GRGTCQTGDCG.GVLLQCTGWGK.PPNTLAEYALDQFSGLDFWDISLLDGFNIPMTF.PTNPSGGGK 118
tomatoP23 GRGSCQTGDCG.GVLLQCTGWGK.PPNTLAEYALDQFSNLDFWDISLVDGFNIPMTFAPTNPSGGGK 119
VVTL1 GRGKCETGDCN.GLLEECQGYGS.PPNTLAEFALNQPNNLDYIDISLVDGFNIPMDFS.....G. 111
tobaccoPRR1 GRGNCETGDCN.GMLEECQGYGK.APNTLAEFALNQPNQ.DFVDISLVDGFNIPMEFSPT..NGG. 114
VVOSM1 GNGKCETGDCG.GLLQCTAYGT.PPNTLAEFALNQFSNLDFFDISLVDGFNVPMAFNPTT..SNG. 115
soybeanP21 GRGGCQTGDCG.GVLDCKAYGA.PPNTLAEYGLNGFNNLDFFDISLVDGFNVPMDFSPTT..SNG. 116
maizeZEAMAT GRGSCRTGDCG.GVLQCTGYGR.APNTLAEYALKQFNNLDFFDISLIDGFNVPMSFLPDGGS.G. 117
thaumatinII GRGICRTGDCG.GLLQCKRFGR.PPTTLAEFSLNQYGK.DYIDISNIKGFNVPMDFSPTTRG... 120
cherryTL GKFVCATADCASGQVMCNGNGAIPPATLAEFNIPAGGGQDFYDVSLVDGFNLPMSVTPQG.GTGD 127
wheatPWIR2 C.RDPSCPP..... 126
tobaccoPR5d C.HGIQCTANINGECPGALRVP.G.....GCNNPCTTFGGQQYCCT..QGP...CGPTELSRWFK 173
tobaccoOS C.HALTCTA.INGECPGAELRVP.G.....GCNNPCTTFGGQQYCCT..QRP...CGPTTFFSKFFK 169
tomatoP23 C.HAIHCTANINGECPGSLRVP.G.....GCNNPCTTFGGQQYCCTT..QGP...CGPTTDLSRFFK 172
VVTL1 C.RGIQCSVDINGQCPSELKAP.G.....GCNNPCTVFKTNEYCCTT..DGPG.SCGPTTYSKFFK 166
tobaccoPRR1 C.RNLRCTAPINEQCPAQLKTQ.G.....GCNNPCTVIKTNEYCCTT..NGPG.SCGPTDLSRFFK 169
VVOSM1 CTRGISCTADIVGECPAALKTTT.G.....GCNNPCTVFKTDEYCC....NSG..SCNATTYSEFFK 168
soybeanP21 CTRGISCTADINGQCPSELKTQ.G.....GCNNPCTVFKTDQYCC....NSG.SCGPTDYSRFFK 170
maizeZEAMAT CSRGPRCAVDVNARCPAELRQD.G.....VCNNACPVFKKDEYCCV..GSAANDCHPTNYSRYFK 174
thaumatinII C.RGVRCAADIVGQCPAKLKAPGG.....GCNDACTVFQTSEYCCTT..TG...KCGPTEYSRFFK 174
cherryTL C.KTASCPANVNAVCPSELQKKGSDGSVVACLSACVKFGTPQYCCTPPQNTPETCPPTNYSEIFH 191
wheatPWIR2 ...PQAYQHPNDVATHA..C.SGNNNYQITFCP.. 153
tobaccoPR5d QRCPDAYSYPQDDPTSTFTCTSWTTDYKVMFCPYG 208
tobaccoOS QRCPDAYSYPQDDPTSTFTCPGGSTNYRVIFCPNG 204
tomatoP23 QRCPDAYSYPQDDPTSTFTCPSGSTNYRVVFCPNG 207
VVTL1 DRCPDAYSYPQDDKTSLFTCPSG.TNYKVTFCP.. 198
tobaccoPRR1 ERCPDAYSYPQDDPTSLFTCPSG.TNYRVVFCP.. 201
VVOSM1 TRCPDAYSYPKDDQTSTFTCPAG.TNYEVIFCP.. 201
soybeanP21 QRCPDAYSYPKDDPPSTFTCNGG.TDYRVVFCP.. 202
maizeZEAMAT GQCPDAYSYPKDDATSTFTCPAG.TNYKVVFCP.. 206
thaumatinII RLCPDAFSYVLDKPT.TVTCP.GSSNYRVTFCPTA 207
cherryTL NACPDAYSYAYDDKRGTFTCNGG.PNYAITFCP.. 223

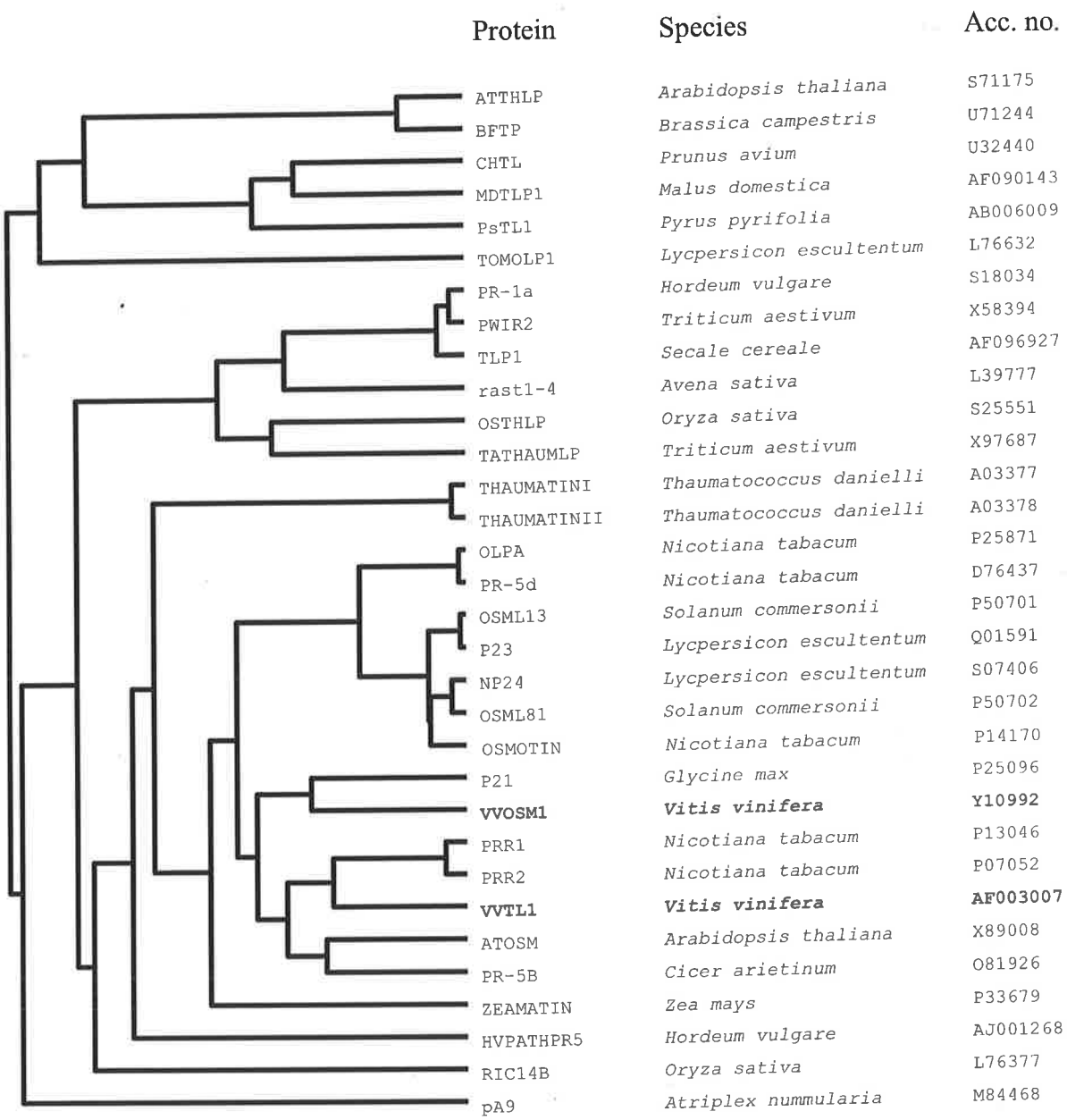


Figure 3.4 Dendrogram illustrating phylogenic relationships among a number PR-5 like proteins. Actual or deduced amino acid sequences were obtained from the SWISSPROT, GenBank, TREMBL and PIR databases. Sequences encoding known or presumed signal peptides were deleted and following alignment of mature protein sequences the dendrogram was constructed using the PILEUP program. Relevant database accession numbers and the species of protein origin are shown. VVTL1 and VVOSM1 (VVTL2), are shown in bold.

PR-5 proteins (see Figure 3.3). The significance of the absence of this stretch of amino acids remains unknown as these proteins still possess antifungal activity (Vigers *et al.*, 1991).

3.3.4 Modelling of the putative three-dimensional structure of VVTL1

Comparative computer modelling is a relatively new phenomenon which has been proven to be highly useful for many structural predictions, particularly when the degree of sequence identity between target and template sequences are >70% (Mosimamm *et al.*, 1995). Such protein models have allowed for the design of specific ligands (eg. new drugs), and although not all models are useful (Peitsch and Guex, 1997), large-scale comparative protein modelling is now believed to be sufficiently accurate for the use in genomic projects (Peitsch and Guex, 1997; Adzhubei *et al.*, 1998; Bork *et al.*, 1998; Sánchez and Šali, 1998). Comparative modelling involves three basic steps. Firstly, a search is conducted with the sequence of interest (target sequence) to determine if related proteins exist with known three-dimensional structures obtained by X-ray crystallography or NMR (template sequences). Secondly, target sequences are aligned with template sequences, and then, if more than one target-template combination exists, areas of best 'fit' are chosen between different template and target sequences. Finally, a model is generated based on the previous alignments and refined.

Through the availability of the highly resolved crystal structures of the PR-5 like proteins, PR-5d (Koiwa *et al.* (1999); Brookhaven accession code: 1IAUN.pdb), and thaumatin (Ko *et al.* (1994); Brookhaven accession codes: 1ITHV.pdb, 1ITHU.pdb), it was possible to predict the tertiary structure for the highly similar VVTL1 sequence (see Figure 3.3 for a comparison) using the SWISS-MODEL suite of programs. Essentially, these PR-5 like proteins, and the predicted VVTL1, are tightly folded globular proteins (Figure 3.5). They consist of a hydrophobic core composed primarily of a β-sandwich,

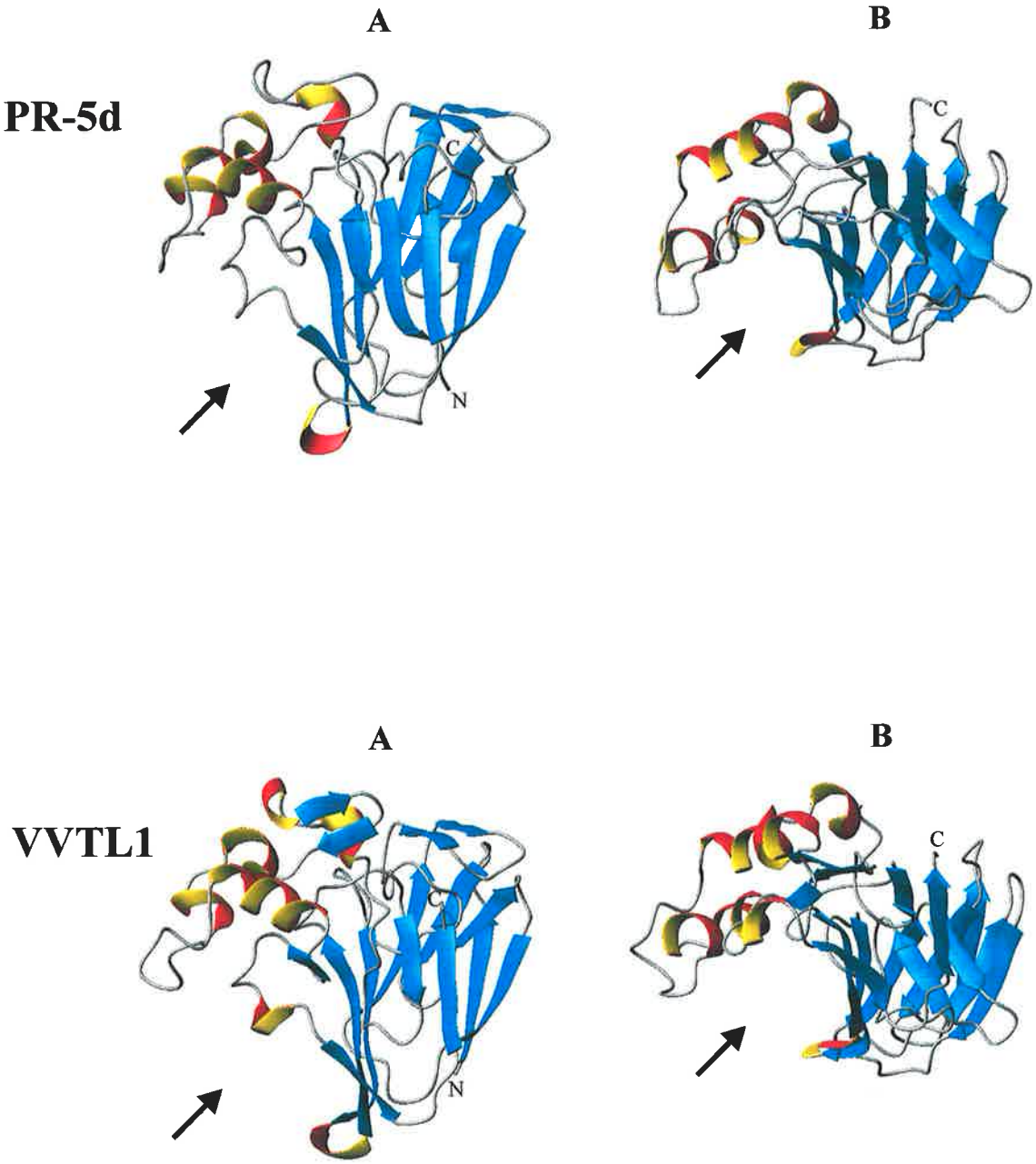


Figure 3.5 Comparison of the known tertiary structure of PR-5d, with the SWISS-MODEL predicted structure of VVTL1. Shown is a 'ribbon' representation of the two protein structures (A) from a side perspective as in a previous study of the PR-5 protein zeamatin, and (B) with the molecules rotated 60° around the horizontal X axis (ie. a top perspective). β-strands are shown in blue and α-helical regions in red and yellow. The arrows indicate the position of a pronounced cleft. The positions of the amino (N) and carboxy (C) termini are shown. Actual and predicted structural coordinates were obtained from the Brookhaven Protein Data Bank and the SWISS-MODEL database, respectively, and graphical models displayed using the MOLMOL program.

consisting mostly of eight β-strands, and several loops which extend from this core. This structure is held together by eight disulfide bonds and well over one hundred hydrogen bonds. Examination of the X-ray crystal structures of thaumatin, zeamatin (Batalia *et al.*, 1996) and PR-5d (Koiwa *et al.*, 1999) reveals the presence of a cleft, or groove - a feature that VVTL1 is also predicted to possess (Figure 3.5B). The significance of minor structural differences between PR-5d and the putative VVTL1 model will remain unknown until specific amino acids or regions are implicated, for example, in possible binding activities. Moreover, surface charges, which have not been examined here, have been suggested to be major determinants in the activities of the PR-5 like proteins, zeamatin, PR-5d and thaumatin (Batalia *et al.*, 1996; Koiwa *et al.*, 1999).

3.3.5 Characterisation of the cDNA encoding VVPR-4a

Comparison of the amino acid sequence deduced from the VVPR-4a cDNA (Figure 3.6) with electrospray mass spectrometry data obtained for the purified protein suggests that VVPR-4a undergoes a number of post-translational modifications. Firstly, it appears VVPR-4a is synthesised with an N-terminal presequence of 21 residues, typical of a signal peptide needed for translocation to the endoplasmic reticulum (Bar-Peled *et al.*, 1996). The VVPR-4a cDNA does not reveal the presence of a C-terminal signal peptide (Melchers *et al.*, 1993), indicating the mature VVPR-4a may not be transported to the vacuole but rather to the apoplastic space, as suggested for most small PR-4 like proteins which are known to exist in the intercellular fluid of leaves (Joosten *et al.*, 1990; Friedrich *et al.*, 1991; Hejgaard *et al.*, 1992). Secondly, based on the NMR structure determination of barwin (a PR-4 like protein from barley) (Svensson *et al.*, 1992), all PR-4 like proteins so far characterised, including VVPR-4a, most likely contain three highly conserved disulfide bridges. Thirdly, the N-terminal blockage of the protein can be assumed to be due to the formation of a pyroglutamyl residue at Gln^{+1} following proteolytic removal of residues -21 to -1 inclusive. Accounting for the removal

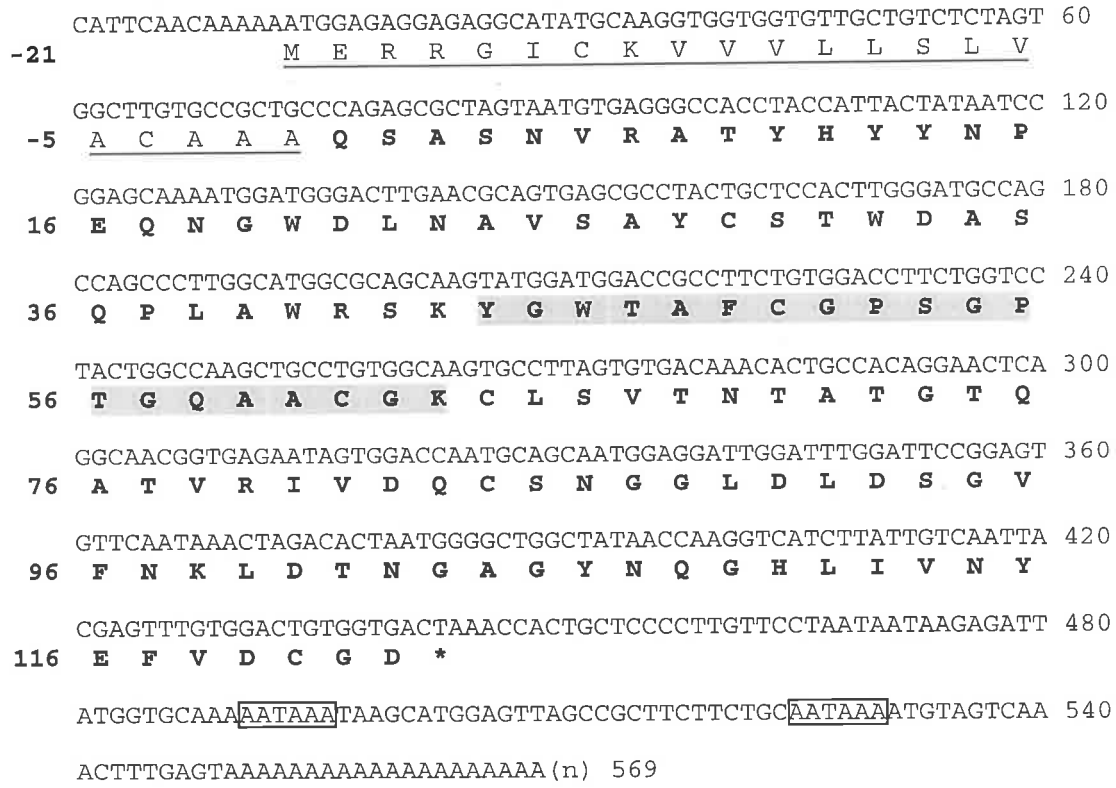


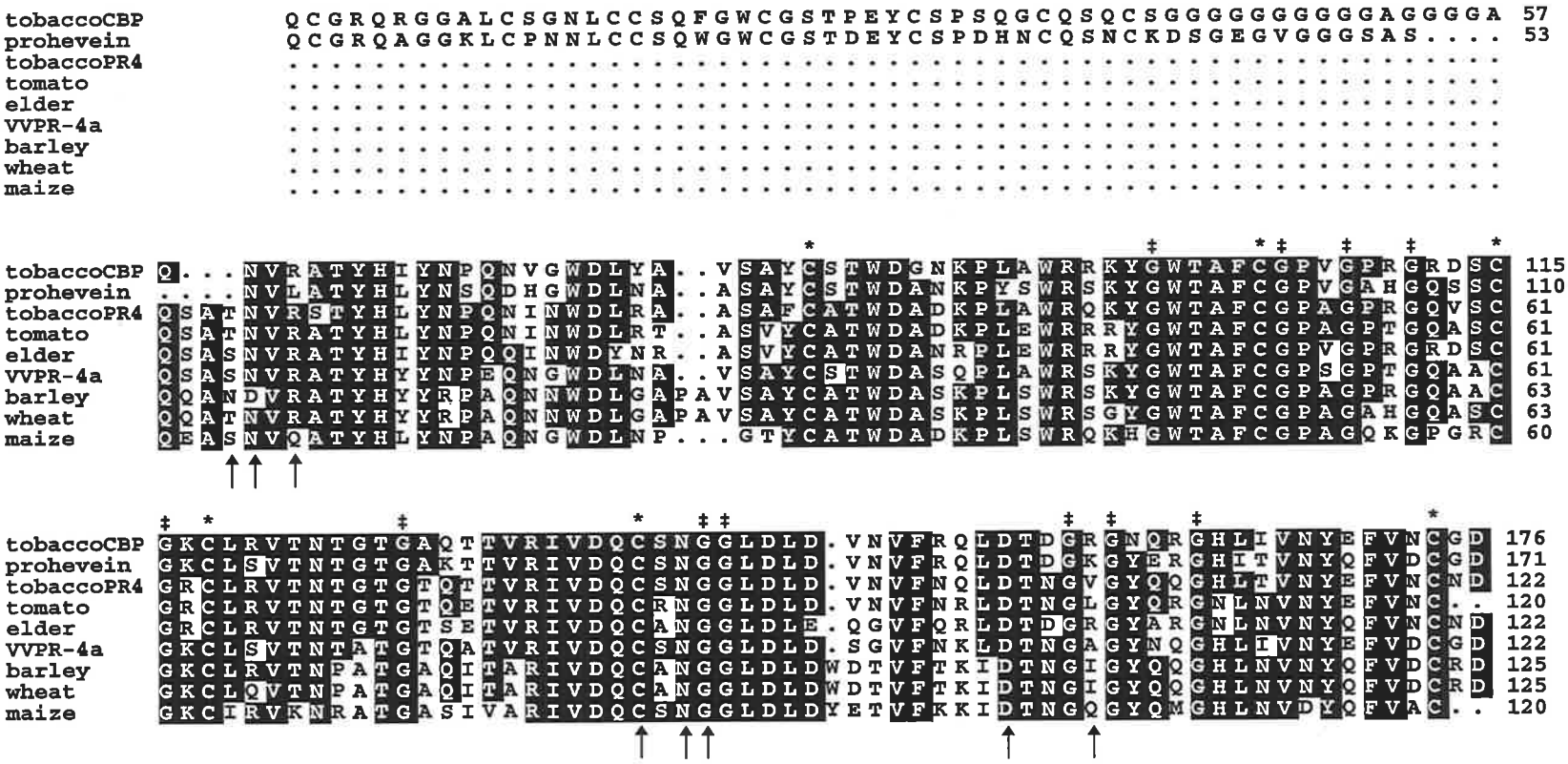
Figure 3.6 The VVPR-4a encoding cDNA and deduced amino acid sequence. Nucleotides are numbered on the right side and amino acids are numbered on the left, with the first residue of the mature protein designated as +1. Peptide sequence obtained from purified VVPR-4a is high-lighted, a cleavable presequence is underlined and the translational stop codon is denoted by an asterisk (*). Putative polyadenylation signals are boxed. This sequence was compiled from an 'internal' cDNA (205 to 437), a 5'-RACE product (1 to 320) and a 3'-RACE product (321 to 569). No sequence discrepancies were noted in the overlaps.

of the signal peptide, formation of three disulfide bonds and formation of the pyroglutamyl residue at Gln^{+1}, the predicted mass of mature VVPR-4a is 13019.11 Da whilst that obtained by mass spectrometry is 13021 +/- 0.6 Da. Thus, it was concluded that the cDNA sequence specifies the purified VVPR-4a protein and, based on strong experimental evidence, that the suggested post-translational modifications take place. The predicted pI (4.61) of VVPR-4a is consistent with the protein's behaviour during anion-exchange chromatography (see Section 2.3.2). Analogous to the VVTL1 cDNA, two consensus polyadenylation signals are present in the 3' untranslated region of the VVPR-4a cDNA - one within 19 bp of the poly (A) site.

3.3.6 VVPR-4a is highly homologous to other PR-4 (class II) proteins

When the deduced amino acid sequence of mature VVPR-4a was aligned with other known protein sequences a number of similarities were revealed (Figure 3.7). Foremost, VVPR-4a is highly homologous to a number of small PR-4 (class II)-like proteins from a range of pathogen challenged and healthy plants. Positional identity of VVPR-4a to these proteins is as high as 78% for barwin, a basic protein from barley seed (Svensson *et al.*, 1992), suggesting a highly similar tertiary structure (Ludvigsen and Poulsen, 1992b). A near identical protein to barwin, also purified from barley was reported as possessing antifungal properties (Hejgaard *et al.*, 1992) as were two PR-4 like proteins purified from wheat (Caruso *et al.*, 1993; Caruso *et al.*, 1996). VVPR-4a is also homologous to PR-4 like proteins encoded by cDNAs isolated from tobacco mosaic virus infected tobacco (Friedrich *et al.*, 1991; Linthorst *et al.*, 1991), *Cladosporium fulvum* infected tomato (Linthorst *et al.*, 1991), *Sambucus nigra* leaf abscission zones (Coupe *et al.*, 1997), maize glucose starved root tips (Chevalier *et al.*, 1995) and barley leaves infected with the fungus *Blumeria graminis* (Gregersen *et al.*, 1997).

Figure 3.7 Multiple amino acid sequence alignment of PR-4 like proteins using the PILEUP program. Putative signal peptides have not been included. The aligned sequences are as follows: tobaccoCBP (CBP20 cDNA isolated from TMV infected tobacco (Ponstein *et al.*, 1994)), prohevein (cDNA isolated from laticifers of *Hevea brasiliensis* (Broekaert *et al.*, 1990)), tobaccoPR4 (PR-4a cDNA isolated from TMV infected tobacco (Linthorst *et al.*, 1991)), tomato (PR-P2 cDNA isolated from *Cladosporium fulvum* infected tomato (Linthorst *et al.*, 1991)), elder (cDNA isolated from *Sambucus nigra* leaflet abscission zone (Coupe *et al.*, 1997)), VVPR-4a (cDNA isolated from grapevine berries), barley (barwin isolated from grain (Svensson *et al.*, 1992)), wheat (wheatwin1 isolated from grain (Caruso *et al.*, 1993)) and maize (cDNA isolated from glucose starved maize root tips (Chevalier *et al.*, 1995)). Highlighted residues are identical in at least five of the nine aligned proteins. Conserved cysteine and glycine residues are denoted by asterisks (*) and hatches (‡), respectively. Eight residues believed to be involved in the binding of chitin (Ludvigsen and Poulsen, 1992b) are indicated by arrows.


tobaccoCBP QCGRQRGGALCSGNLCCSQFGWCGSTPEYCSPSQGCQSQCSGGGGGGGGGGGGAGGGGA 57
prohevein QCGRQAGGKLCPNNLCCSQWGWCGSTDEYCSPDHNCQSNCKDSGEGVGGGSAS.... 53
tobaccoPR4 ....
tomato ....
elder ....
VVPR-4a ....
barley ....
wheat ....
maize ....
tobaccoCBP Q...NVRATYHIYNPQNVGWDLYA..VSAYCSTWDGNKPLAWRRKYGWTAFCGPVGPRGRDSC 115
prohevein ....NVLATYHLYNSQDHGWDLNA..ASAYCSTWDANKPYSWRSKYGWTAFCGPVGAHGQSSC 110
tobaccoPR4 QSATNVRSTYHLYNPQNINWDLRA..ASAFCATWDADKPLAWRQKYGWTAFCGPAGPRGQVSC 61
tomato QSATNVRATYHLYNPQNINWDLRT..ASVYCATWDADKPLEWRRRYGWTAFCGPAGPTGQASC 61
elder QSASNVRATYHIYNPQQINWDYNR..ASVYCATWDANRPLEWRRRYGWTAFCGPVGPRGRDSC 61
VVPR-4a QSASNVRATYHYYNPEQNGWDLNA..VSAYCSTWDASQPLAWRSKYGWTAFCGPSGPTGQAAC 61
barley QQANDVRATYHYYRPAQNNWDLGAPAVSAYCATWDASKPLSWRSKYGWTAFCGPAGPRGQAAC 63
wheat QQATNVRATYHYYRPAQNNWDLGAPAVSAYCATWDASKPLSWRSGYGWTAFCGPAGAHGQASC 63
maize QEASNVQATYHLYNPAQNGWDLNP...GTYCATWDADKPLSWRQKHGWTAFCGPAGQKGPGRC 60
tobaccoCBP GKCLRVTNTGTGAQTTVRIVDQCSNGGLDLD.VNVFRQLDTDGRGNQRGHLIVNYEFVNCGDD 176
prohevein GKCLSVTNTGTGAKTTVRIVDQCSNGGLDLD.VNVFRQLDTDGKGYERGHITVNYQFVDCGDD 171
tobaccoPR4 GRCLRVTNTGTGTQTTVRIVDQCSNGGLDLD.VNVFNQLDTNGVGYQQGHLTVNYEFVNCND 122
tomato GRCLRVTNTGTGTQETVRIVDQCRNGGLDLD.VNVFNRLDTNGLGYQRGNLNVNYEFVNC.. 120
elder GRCLRVTNTGTGTSETVRIVDQCANGGLDLE.QGVFQRLDTDGRGYARGNLNVNYQFVNCND 122
VVPR-4a GKCLSVTNTATGTQATVRIVDQCSNGGLDLD.SGVFNKLDTNGAGYNQGHLLVNYEFVDCGDD 122
barley GKCLRVTNPATGAQITARIVDQCANGGLDLDWDTVFTKIDTNGIGYQQGHLNVNYQFVDCRDD 125
wheat GKCLQVTNPATGAQITARIVDQCANGGLDLDWDTVFTKIDTNGIGYQQGHLNVNYQFVDCRD 125
maize GKCIRVKNRATGASIVARIVDQCSNGGLDLDYETVFKKIDTNGQGYQMGHLNVDYQFVAC.. 120

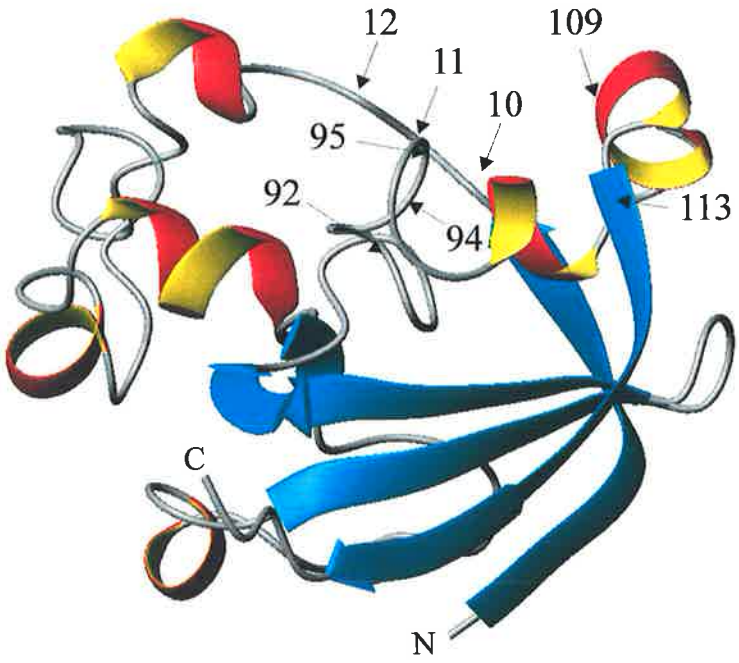
The larger class I PR-4 like proteins, which contain an N-terminal chitin binding domain, possess C-terminal domains which are also highly homologous to VVPR-4a. These are CBP20 from tobacco (Ponstein *et al.*, 1994), prohevein from the latex of the rubber tree (Broekaert *et al.*, 1990), the gene products of *win1* and *win2* from potato (Stanford *et al.*, 1989) and the putative proteins encoded by the HEL and TAB7 cDNAs from *Arabidopsis* (Potter *et al.*, 1993) and tomato (Harris *et al.*, 1997), respectively.

3.3.7 Modelling of the putative three-dimensional structure of VVPR-4a

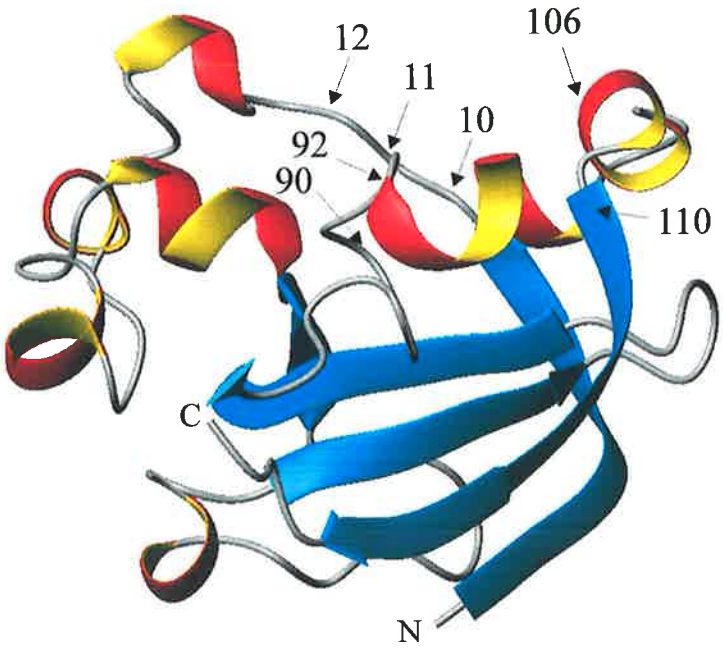
The three-dimensional structure of barwin, a barley class II PR-4 like protein, has been determined by NMR (Ludvigsen and Poulsen (1992); Brookhaven accession code: 1IBW3.pdb) and, given the high degree of sequence conservation, VVPR-4a is likely to have an almost identical structure (Varghese *et al.*, 1994). Indeed, computer modelling predicts that VVPR-4a would have a similar globular fold (Figure 3.8). Barwin has a core which is composed primarily of β-sheet structures between which two major hydrophobic regions exist. The β-sheet region is flanked by several relatively short α-helical and loop regions. This tightly packed structure is held together by three disulfide bonds and over sixty hydrogen bonds. By examination of the primary structure of known PR-4 like proteins (Figure 3.7) it is apparent that most glycine residues are highly conserved. It is believed that these residues, which are normally susceptible to high mutation rates in other proteins, are important in the correct folding of the peptide chain (Ludvigsen and Poulsen, 1992b). Eight residues believed to be involved in the binding of chitin tetramers (Ludvigsen and Poulsen, 1992b) are indicated in Figure 3.7 and Figure 3.8. Seven of these residues are positionally conserved in VVPR-4a.

Figure 3.8 Comparison of the known tertiary structure of barwin with the SWISS-MODEL predicted structure of VVPR-4a. Shown is a 'ribbon' representation of the two protein structures from a side perspective chosen in a previous NMR study of the barwin structure (Ludvigsen and Poulsen, 1992b). β-strands are shown in blue and α-helical regions in red and yellow. Residues proposed to form a binding site for chitin are indicated. The positions of the amino (N) and carboxy (C) termini are shown. Actual and predicted structural coordinates were obtained from the Brookhaven Protein Data Bank and the SWISS-MODEL database, respectively, and graphical models are displayed using the MOLMOL program.

Barwin



VVPR4-a



3.4 Discussion

Characterisation of VVTL1 and VVPR-4a by sequence and mass spectrometric analyses, combined with sequence analyses of the corresponding cDNAs, has allowed the determination of the post-translational modifications to which the primary VVTL1 and VVPR-4a translation products are subjected. Thus, both VVTL1 and VVPR-4a appear to be synthesised as preproteins with 24 and 21 amino acid N-terminal leader sequences. These presequences presumably ensure passage through the secretory pathway to an extracellular location (Stintzi *et al.*, 1993; van de Rhee *et al.*, 1994). Additionally for VVPR-4a, as is commonly speculated for a number of PR-4 proteins (Svensson *et al.*, 1992; Caruso *et al.*, 1993; Caruso *et al.*, 1996), the N-terminal glutamine residue is converted to a pyroglutamate residue, after the removal of the signal peptide. The functional significance of this modification is unknown but it may serve to reduce the susceptibility to amino peptidase degradation. The mass spectrometric data also strongly imply that VVTL1 and VVPR-4a contain multiple disulfide bridges. The sixteen conserved cysteine residues of VVTL1 most likely form eight disulfide bonds as observed in the tertiary structures of thaumatin (Ogata *et al.*, 1992; Ko *et al.*, 1994), zeamatin (Batalia *et al.*, 1996) and PR-5d (Koiwa *et al.*, 1999). The six conserved cysteine residues of VVPR-4a are likely to form three intermolecular sulfide bridges, as observed for the barley PR-4 like protein, barwin (Ludvigsen and Poulsen, 1992a).

Comparison of the putative VVTL1 tertiary structure, predicted by computational simulation (Figure 3.5), to that of known PR-5 like protein structures reveals several common features. Foremost, the most prominent surface feature of the known PR-5 like tertiary structures is the presence of a pronounced cleft. The function of this cleft remains unknown, however, it may play a role in the binding of molecules such as β-1,3-glucans (Trudel *et al.*, 1998) or actin (Takemoto *et al.*, 1997). Further

circumstantial evidence for an unknown ligand binding role of PR-5 proteins has been provided by the isolation of a cDNA from *Arabidopsis* encoding the protein PR5K (Wang *et al.*, 1996). PR5K, which is presumed to play a role in cell signal transduction, has been proposed to consist of three domains - an extracellular domain which is highly homologous to PR-5 proteins, a transmembrane domain, and an intracellular serine/threonine kinase domain, which is active when expressed as a recombinant protein. Moreover, osmotin (a PR-5 protein) appears to have a specific interaction with plasma membrane components of yeast and this interaction in turn produces a cytotoxic effect (Yun *et al.*, 1997, 1998), an activity which may be extended to other antifungal PR-5 proteins. Lastly, authentic thaumatin has a specific ability to bind to the membrane located taste receptors of old world primates and humans. This binding elicits an extremely sweet sensation and is on a molar basis 10^5 times more potent than sucrose (van der Wel and Loeve, 1972).

Considering the range of known and proposed binding functions of PR-5 like proteins it is highly likely that specific affinities for different ligands exist. For example, only a subset of PR-5 proteins bind β-1,3-glucans (Trudel *et al.*, 1998). Similarly, not all PR-5 proteins bind yeast membrane components (Yun *et al.*, 1997), and only thaumatin has been found to be sweet tasting. This differential affinity for specific ligands is perhaps also reflected in the observation that PR-5 proteins differ markedly in their ability to inhibit the growth of a number of different fungal genera (Stintzi *et al.*, 1993). The structural basis for such differences is unknown although it is believed that only minor tertiary structure differences are responsible for the difference in sweetness of authentic thaumatin and its close homologues (Ko *et al.*, 1994; Slootstra *et al.*, 1995; Suami *et al.*, 1997), whilst surface charges may be integral to the antifungal activity of zeamatin (Batalia *et al.*, 1996). Nonetheless the exact mechanism(s) by which PR-5 like proteins function remains largely unknown.

As discussed previously, it is becoming more evident that PR-4 proteins can be grouped into two major classes (Ponstein *et al.*, 1994). The class I PR-4 proteins, of which CBP20 (Ponstein *et al.*, 1994), prohevein (Broekaert *et al.*, 1990), the putative products of *win1*, *win2* (Stanford *et al.*, 1989), HEL (Potter *et al.*, 1993) and TAB7 (Harris *et al.*, 1997) can be included, are either known to be plant lectins (Van Parijs *et al.*, 1991; Ponstein *et al.*, 1994), or homologues thereof. Many of these proteins are therefore implicated in plant defence (Peumans and Van Damme, 1995). As illustrated in Figure 3.9, these class I proteins are proposed to consist of two domains, which may undergo post-translational processing into an N-terminal and C-terminal domain as observed for prohevein (Lee *et al.*, 1991). It is the N-terminal lectin domain, often referred to as the hevein (homologous in size and sequence to mature hevein) domain, that is known to give the class I PR-4 proteins their lectin properties. Interestingly, other proteins such as a class I chitinase from tobacco contain this conserved domain indicating that a possible transposition of DNA between genes may give rise to new proteins containing lectin domains (Shinshi *et al.*, 1990). In contrast, the smaller class II PR-4 type proteins, of which VVPR-4a is a representative, lack the lectin domain, however are highly homologous in both size and sequence to the C-terminal domain of the class I PR-4 proteins.

The class II PR-4 like protein, barwin, has been proposed to bind chitin (Hejgaard *et al.*, 1992), albeit weakly, and a potential binding site for a tetramer of N-acetylglucosamine has been determined (Ludvigsen and Poulsen, 1992b). The potential site (see Figure 3.8) is proposed to involve eight residues - Tyr10, His11, Tyr12, Asp92, Asp94, Trp95, Tyr109 and His113. Seven of these residues are present in VVPR-4a suggesting it may have a similar affinity for chitin. VVPR-4a does not contain a Trp95 residue at the expected position 93 (refer to Figure 3.7). As chitin is present in the cell wall of pathogenic fungi, the above data is not at odds with the suggestion that VVPR-

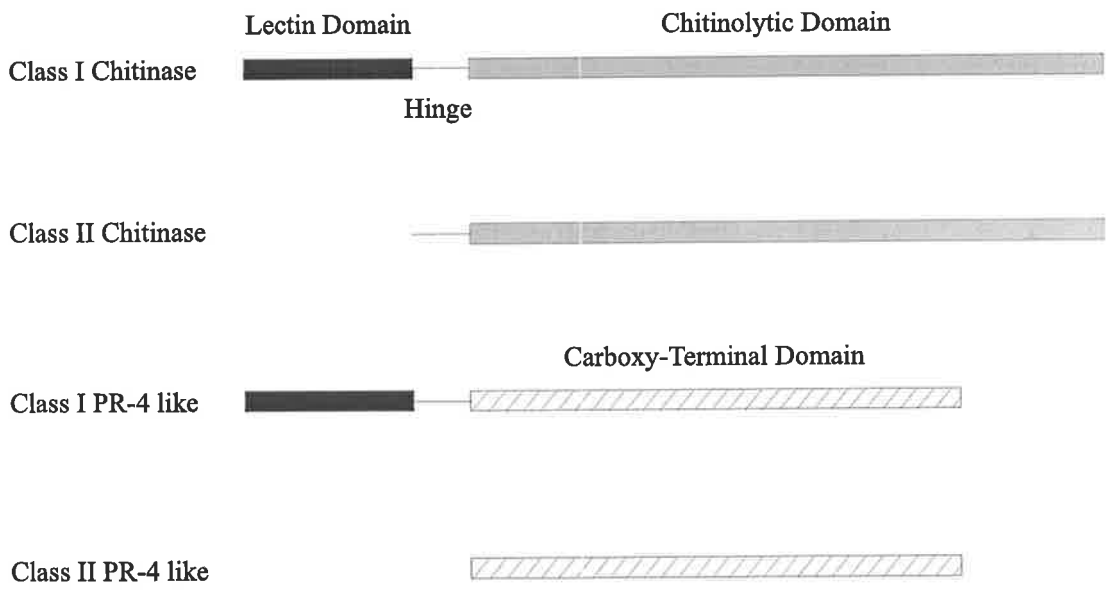


Figure 3.9 Diagrammatical representation of the proposed domains common to PR-4 like and other related proteins. These domains include a lectin (hevein) domain present in class I chitinases and class I PR-4 like proteins. The class I PR-4 like proteins possess a carboxy-terminal domain which is highly similar to the class II PR-4 like proteins, to which VVPR-4a can be classified. The diagram is modified from Friedrich *et al.* (1991). Both class I and class II chitinases are classified as PR-3 proteins (van Loon *et al.*, 1994).

4a may play a role in plant defence. Such implications will be discussed in the following chapters.

In conclusion, due to their highly conserved primary structures, it is likely that VVTL1 and VVPR-4a have highly similar secondary and tertiary structures to previously characterised proteins, a suggestion which is further confirmed by the simulated prediction of the tertiary structures of the two grape proteins. Given that such tightly packed globular structures have the potential to provide stability over a broad pH and temperature range, and resistance to proteolytic attack (Stintzi *et al.*, 1993), it is now understandable why these berry PR-like proteins persist throughout the winemaking process. Further comparison of protein structures, mutational studies, and more extensive binding studies will be required to determine how possible ligands might interact with PR-5 and PR-4 like proteins. These studies, in turn, may provide further insights to the *in planta* function of these proteins, including VVTL1 and VVPR-4a. As a complement to such studies, a full understanding of the temporal and spatial synthesis, and concentration, of these proteins in the plant is required. In the following chapter, tools generated through protein purification (Chapter 2) and cDNA cloning (Chapter 3) will be used to address this issue.

CHAPTER 4

Characterisation and expression patterns of the ripening induced VVTL1 and VVPR-4a encoding genes

4.1 Introduction

The grapevine berry is a fleshy fruit (Coombe, 1976), consisting primarily of three basic structures: the skin (exocarp), flesh (mesocarp) and seeds (see Figure 4.1A). Like a small number of other fruits, the ripening of grapes is non-climacteric and its growth pattern follows a double sigmoid curve encompassing three stages (Coombe (1976); Figure 4.1B). Stage I, immediately following flowering, is characterised by a short period of rapid cell division followed by marked cell enlargement (Nakagawa and Nanjo, 1965; Considine and Knox, 1979) concomitant with high rates of metabolism and rapid accumulation of acid (Kliewer, 1965). Stage II is a lag-phase, some 7 to 10 weeks post flowering (WPF), which constitutes a short period of slow, or no, growth (Coombe and Bishop, 1980). Towards the end of this period, the berries lose chlorophyll and acidity reaches a maximum. Berry softening together with the rapid accumulation of sugars and amino acids (for review see Kanellis and Roubelakis-Angelakis (1993)) signals entry into stage III, in which cell expansion rather than cell division is chiefly responsible for the continued growth in berry size (Nakagawa and Nanjo, 1965; Considine and Knox, 1979). Viticulturists use the term veraison (from the French véraison) to describe the entry into stage III and hence the inception of ripening.

Although a great deal is known about some of the chemical constituents of grapevine berries and the changes that occur through the three stages of berry growth, little is known about their protein composition and respective gene expression. The profile of soluble proteins found in the juice of ripe berries often appears surprisingly simple with a predominance of a few low molecular weight proteins (see Chapter 2). The simplicity of this profile is to a large extent a result of incomplete extraction of total cellular protein. To better understand the accumulation of these soluble berry PR-like proteins during the development of the berry, a systematic sampling, throughout stages I,

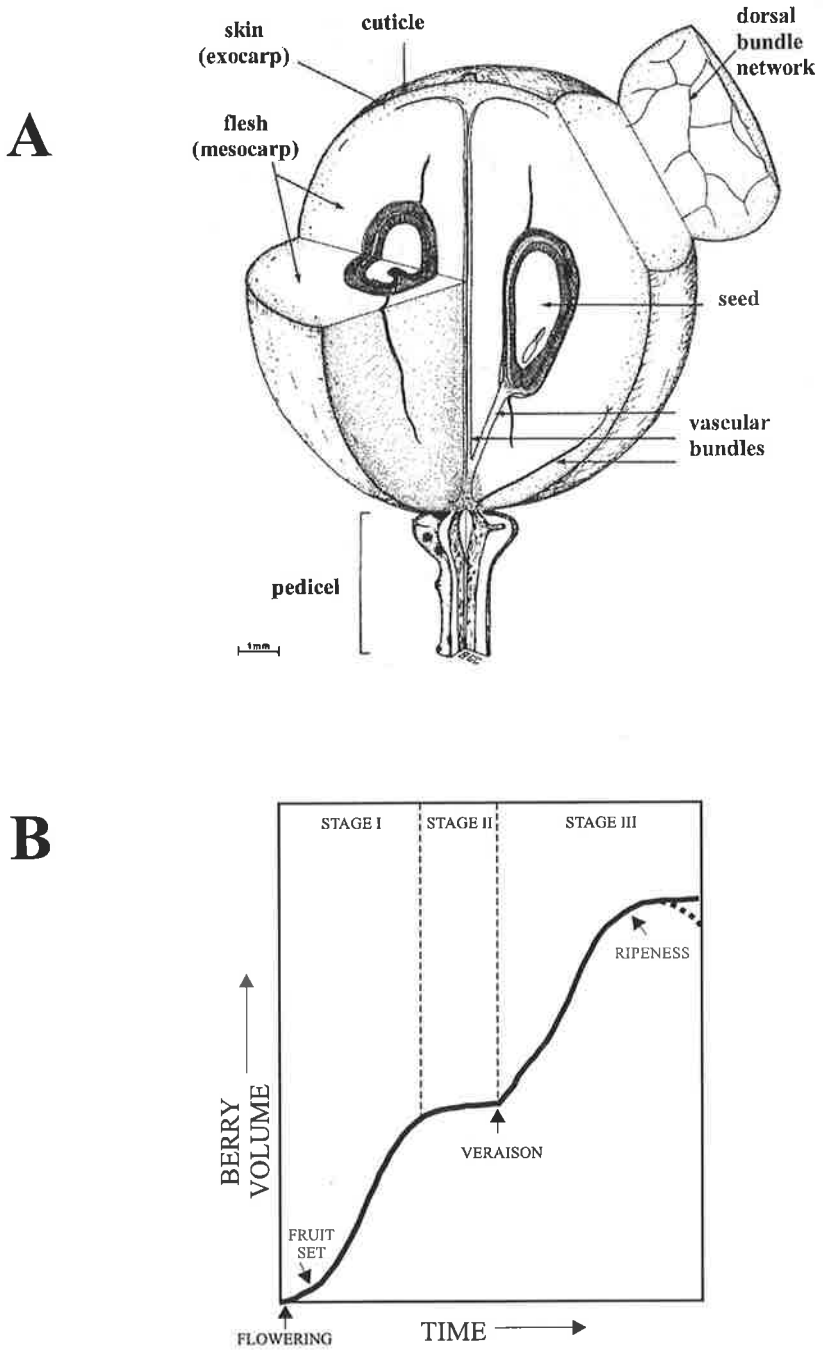


Figure 4.1 The morphology and growth of the grapevine berry. (A) Illustration of the berry showing the three major structures- the skin, flesh and seed (reproduced from Coombe (1987)). (B) Graph demonstrating the three major phases of berry growth (volume) - stages I, II and III (redrawn from Coombe and Hale (1973)).

II and III, was performed. Through Northern and Western blot analyses it was possible to demonstrate that the genes encoding VVTL1 and VVPR-4a are predominantly expressed in the berry at the onset of ripening. Furthermore, the respective genes, which appear to be coordinately regulated, have been cloned. Sequence analysis has revealed that both genes contain similar putative sequence elements which could be the targets of transcription factors which ensure tissue and developmentally specific expression.

4.2 Materials and Methods

All common molecular biology procedures were performed, and solutions prepared, as described by Sambrook *et al.* (1989) unless otherwise noted.

4.2.1 Berry sampling

Berries were harvested in the 1995/96 season from 26 *V. vinifera* cv Muscat of Alexandria vines (planted 1981) at the Alverstoke vineyard (Waite Campus, The University of Adelaide; Latitude: 35° South). Synchronously flowering bunches were tagged and their development followed to maturity. Ten bunches were taken from the vines every 1 to 2 weeks. No more than 10% of the a particular vine's bunches were harvested. A 50 berry sample, consisting of five berries taken from the top, middle and bottom of each bunch, was subjected to weight and deformability measurements. Deformability measurements were performed with a calibrated Harpenden Skinfold Caliper Gauge (British Indicators, West Sussex, UK) as previously described (Coombe and Bishop, 1980) and illustrated (Coombe, 1992). Remaining berries were frozen in liquid N_2 and powdered in a Waring Blendor™ before storage at -70°C. Sub-samples of the frozen berries (2 to 5 g) from different developmental stages were used for protein, RNA and 'free run' juice extraction. 'Free run' juice was obtained by thawing powdered berries and centrifuging at 12,000*g* for 5 min. Both °Brix (Erma digital refractometer, Japan) and pH of the juice were measured.

4.2.2 Tissue sampling and protein extraction

All grapevine tissues were obtained from the Muscat of Alexandria vines used for berry sampling except roots which were obtained from a potted vine of the same cultivar grown under shadehouse conditions. Skin, pulp and seed tissue was obtained from

mature berries harvested 17 WPF. Skin was removed from frozen berries and washed extensively in distilled water prior to protein extraction. Tissue (2 to 5 g) was frozen in liquid N_2 and ground to a fine powder using a mortar and pestle. Protein was extracted by addition of 2 mL of buffer (500 mM Tris-HCl, pH 8.0, 5% (w/v) SDS, 10 mM DTT, 10 mM sodium diethyldithiocarbamate) to 1 g of powdered tissue followed by incubation at 95°C for 5 min and then centrifugation at 12,000*g* for 5 min. Protein was concentrated by precipitation with ice-cold TCA at a final concentration of 10% (w/v). After a 15 min incubation on ice the precipitated protein was collected by centrifugation for 15 min at 12,000*g* and the resulting pellet washed twice with ice-cold ethanol:ethyl acetate (2:1, v/v). Following brief drying, proteins were resuspended in 20 mM Tris-HCl, pH 8.0 before analysis by SDS-PAGE and immunoblotting. Juice from various fruits, purchased from a local fruit shop, was obtained by squeezing the pulp gently, followed by centrifugation at 12,000*g* for 5 min. For the measurement of (1,3)-β-D-glucan endohydrolase activity, berry proteins were extracted in 50 mM sodium acetate, pH 5.0, 2 mM DTT and assayed as described previously (Chen *et al.*, 1993).

4.2.3 Southern blot analyses

Grapevine genomic DNA was extracted from Muscat of Alexandria leaves as described previously Steenkamp *et al.* (1994).

VVTL1 gene

DNA (4 µg) was digested to completion with respective restriction enzymes (Promega, USA), resolved in a TAE buffered 0.8% (w/v) agarose gel, capillary blotted with 20 x SSC onto Hybond N^+ membrane (Amersham, UK) according to the manufacturer's instructions, and then fixed with 0.4 M NaOH for 15 min. The blot was incubated at 65°C for 1 h in prehybridisation solution (5 x SSC, 0.5% (v/v) SDS, 5 x Denhardt's reagent, 100 µg/mL denatured and sheared salmon testes DNA) before

addition of a denatured DNA probe (nucleotides 154-738, Figure 3.2), which was [α-^{32}P]dCTP labelled using the Megaprime™ DNA labelling system (Amersham, UK) according to the manufacturer's instructions. For high-stringency screening, hybridisation for 16 h at 65°C was followed by washes of the membrane at 65°C in 2 x SSC, 0.1% (w/v) SDS for 2 x 10 min, 1 x SSC, 0.1% (w/v) SDS for 15 min and then 0.1 x SSC, 0.1% (w/v) SDS for 15 min. Labelled DNA was detected with a PhosphorImaging™ screen (Kodak, USA) and analysed using a Storm 860 PhosphorImager™ (Molecular Dynamics, USA) and ImageQuaNT software (Molecular Dynamics, USA). Screenings at lower stringency were performed exactly as for the high stringency screens except that filters were washed only in 2 x SSC at 65°C for 3 x 10 min.

VVPR-4a gene

Southern blot analyses were performed essentially as described above with the following modifications. Digested DNA (8 µg) was resolved then blotted onto Hybond N membrane (Amersham, UK). DNA was fixed with UV light (as described by the membrane's manufacturer) with a UV crosslinker (Amersham, UK). Prehybridisation was performed at 60°C for 1 h, followed by hybridisation with [α-^{32}P]dCTP labelled probes. VVPR-4a cDNA probes were hybridised at 60°C for 16 h before the membrane was washed at 60°C in 2 x SSC, 0.1% (w/v) SDS for 2 x 10 min, 1 x SSC, 0.1% (w/v) SDS for 15 min and then 0.5 x SSC, 0.1% (w/v) SDS for 15 min. Labelled DNA was detected as described above.

4.2.4 Northern blot analyses

Total RNA was extracted from berries and other grapevine tissues, and quantified by UV spectroscopy, as described in Section 3.2.1. Total RNA (5 µg) was denatured by incubation (65°C for 15 min) in denaturing buffer (final conc. of 6.5% (v/v)

formaldehyde, 50% (v/v) formamide, 1 x MOPS buffer (20 mM MOPS, pH 7.0, 10 mM sodium acetate, 1 mM EDTA)) and then chilled on ice (5 min). RNA loading buffer was then added (final conc. 10% (v/v) glycerol, 0.05% bromophenol blue) before the RNA was resolved by electrophoresis through a 1.25% (w/v) agarose gel, containing 6.5% (v/v) formaldehyde, in 1 x MOPS buffer. Following blotting onto a Hybond N membrane (Amersham, UK) according to the manufacturer's instructions, the RNA was fixed with a UV crosslinker (Amersham, UK), and the blot hybridised and washed before the labelled DNA was detected as described for VVTL1 or VVPR-4a Southern blot analyses (Section 4.2.3) under stringent conditions.

Total RNA from *Uncinula necator* infected leaves and pre-veraison berries (6 WPF) was a kind gift from A. Jacobs, I. Dry, and S. Robinson (CSIRO Plant Industry, Adelaide) . Infections were assessed by the naked eye and judged to be mild if hyphae covered up to 20% of leaf or berry surface areas, and to be severe if hyphae covered greater than 50% of the leaf or berry surface area.

4.2.5 SDS-PAGE and Western transfer

Proteins were resolved by SDS-PAGE as described previously (Section 2.2.2). Gels were stained with Coomassie Brilliant Blue R-250 or blotted onto nitrocellulose membrane (MSI laboratories, USA) using a semi-dry transfer unit (LKB broma, Sweden) as described elsewhere (Harlow and Lane, 1988). Protein transfer was confirmed by staining the nitrocellulose membranes for 2 min at room temperature with 0.2% (w/v) Ponceau S (Sigma, USA) in 0.2% (w/v) TCA and 3% (w/v) sulfosalicylic acid, before destaining with deionised H_2O.

4.2.6 Western blot analyses

Anti-VVTL1 antibodies were produced by the Institute of Medical and Veterinary Science (Adelaide, Australia) in New Zealand White rabbits. Reverse phase HPLC purified VVTL1 (~100 μg in phosphate buffered saline) was mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly. The rabbit was given a further two booster injections (~100 μg of VVTL1 in Freund's incomplete adjuvant) at 3 weekly intervals, before bleeding one week after the final boost. The IgG fraction was stored in 30% (v/v) glycerol containing 0.02% (v/v) sodium azide at 4°C. A dilution of 1:10,000 (v/v) in 3% (w/v) BSA was used for immunoblotting experiments. Prior to probing, the immunoblots were blocked overnight at 4°C in a 5% (w/v) low fat milk powder dissolved in TBS-T (137 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl, pH 8.0, 0.1% (v/v) Tween 20 (Sigma, USA)). Immunoblots were probed (1 h at room temperature) with anti-VVTL1 antibodies, followed by horseradish peroxidase-coupled anti-rabbit antibodies (Promega, USA; dilution of 1:10,000 (v/v) in 3% (w/v) BSA) before detection using ECL™ reagents (Amersham, UK) and subsequent exposure to Hyperfilm-MP™ (Amersham, UK). Before and after antibody additions immunoblots were washed for 4 x 10 min with TBS-T at room temperature.

4.2.7 Genomic library construction and gene isolation

A genomic library was constructed by ligating *V. vinifera* cv. Muscat of Alexandria DNA, which had been partially digested with *Mbo*I then partially filled using Klenow DNA polymerase (Promega, USA) leaving 5'-AG-3' overhangs, into the λFIXII vector (Stratagene, USA), which had been digested with *Xho*I and partially filled in leaving 3'-CT-5' overhangs. The DNA was then packaged into Gigapack III Gold packaging extract (Stratagene, USA) according to the manufacturer's instructions. *E. coli* XL1-Blue MRA (P2) cells (Stratagene, USA) were used as the library's host.

Approximately 4.5 x 10^5 plaques were screened in duplicate firstly with a [α-^{32}P]dCTP labelled VVTL1 probe under high stringency conditions as described in Section 4.2.3. After the detection of VVTL1 related DNA, filters were stripped of probe, with boiling 0.5% (w/v) SDS. The stripped filters were reprobed at 65°C with a combination of two [α-^{32}P]dCTP labelled probes (pWIN24 and pWIN52 insert DNA; Figure 3.1), representing the entire VVPR-4a cDNA, and then washed under high stringency conditions (0.1 x SSC, 0.1% (w/v) SDS at 65°C). Hybridising phage were purified through two additional rounds of screening. The DNA of hybridising phage was purified (Section 4.2.8) and the presence of VVTL1 or VVPR-4a encoding DNA confirmed by Southern blot hybridisation, and by PCR employing gene specific primers. Respective genes were subcloned from λ phage inserts into the pBluescript II SK (-) vector (Stratagene, USA), and transformed into competent *E. coli* XL1-Blue cells (Stratagene, USA) before sequencing as previously described (Section 3.2.3).

4.2.8 λ phage DNA extraction

For the preparation of λ phage DNA, 100 µL of host *E. coli* cells (prepared as described in the λFIXII manual (Stratagene, USA)) were transfected by incubation (37°C, 15 min) with 100 µL of transfection buffer (10 mM $MgCl_2$, 10 mM $CaCl_2$) and 100 µL of a suitable phage stock. Phage stock typically consisted of an agar plug containing a monoclonal plaque of interest stored at 4°C in 1 mL of SM buffer (0.1M NaCl, 10 mM Mg_2SO_4, 20 mM Tris-HCl, pH 7.5, 0.01% (w/v) gelatin), with 25 µL of chloroform added as a preservative. After addition of 10 mL of NZY broth (0.5% (w/v) NaCl, 0.2% (w/v) Mg_2SO_4, 0.5% yeast extract, 1% (w/v) casein hydrolysate), the culture was grown overnight at 37°C, or until cell lysis was evident. Chloroform (200 µL) was then added and the lysate was clarified by centrifugation (4,000*g*, 10 min). Soluble phage particles were precipitated by adding an equal volume of PEG solution (20% w/v

PEG 6000, 2.5 M NaCl). The mixture was incubated on ice for 60 min, before centrifugation (2,000*g*, 10 min), and resuspension of the phage pellet in SM buffer. Remaining cellular contents were removed by incubation (with mixing for 10 min) with an equal volume of a Q-Sepharose (Pharmacia, Sweden) suspension (20% (v/v) equilibrated in SM buffer). The Q-Sepharose was removed by centrifugation (12,000*g*, 5 min) before RNAse A (20 µg) was added to the clarified supernatant. Following a 15 min incubation at 37°C, the phage particles were lysed as previously described (Helms *et al.*, 1985), then extracted with phenol:chloroform:isoamylalcohol (25:24:1). The phage DNA was precipitated with isopropanol, the pellet washed with 70% (v/v) ethanol, dried and resuspended in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). Typical DNA yields were ~20 µg/mL of phage lysate.

4.2.9 Searches for putative transcription factor binding sites

DNA sequences of the respective genes were scanned for putative transcription factor binding sites by the TFSEARCH program (Yutaka Akiyama: "TFSEARCH: Searching Transcription Factor Binding Sites"; internet address = http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html). The TFSEARCH program employed the TRANSFAC database (Heinemeyer *et al.*, 1998), which contains the known binding sites of previously characterised plant transcription factors.

4.3 Results

4.3.1 Sampling of developing Muscat of Alexandria berries

As a first step in understanding the processes that affect grapevine berry PR-like gene expression, berries from Muscat of Alexandria vines were sampled routinely from flowering to berry maturity (17 WPF). Values obtained for berry weight, deformability, °Brix and pH are shown in Figure 4.2. A double sigmoid growth curve typical of grape berries (Coombe, 1976) was observed. The onset of veraison, signalled by the sudden increase in berry deformability and the onset of rapid accumulation of soluble solids, was clearly observed nine WPF, shortly before the resumption of rapid berry growth.

4.3.2 VVTL1 and VVPR-4a genes belong to small multigene families

Before defining when and in which tissues the VVTL1 and VVPR-4a encoding genes are expressed, it was first determined how many closely related genes exist within the diploid genome of *V. vinifera*.

VVTL1 gene

Through availability of the VVTL1 encoding cDNA it was possible to ascertain the likely copy number of VVTL1 encoding genes. Southern blot hybridisation analysis of restriction endonuclease digested genomic DNA under highly stringent conditions (0.1 x SSC, 65°C), in which only targets of greater than about 90% identity were detected (the T_m of the probe in 0.1 x SSC is 75.2°C; Meinkoth and Wahl (1984)), revealed that VVTL1 is most likely encoded by a single gene (Figure 4.3A). Hybridisation under less stringent conditions (2 x SSC, 65°C), in which only targets of greater than about 70% identity will be detected (the T_m of the probe in 2 x SSC is 96.8°C; Meinkoth and Wahl (1984)), reveals additional hybridising DNA fragments (Figure 4.3B). Indeed, through

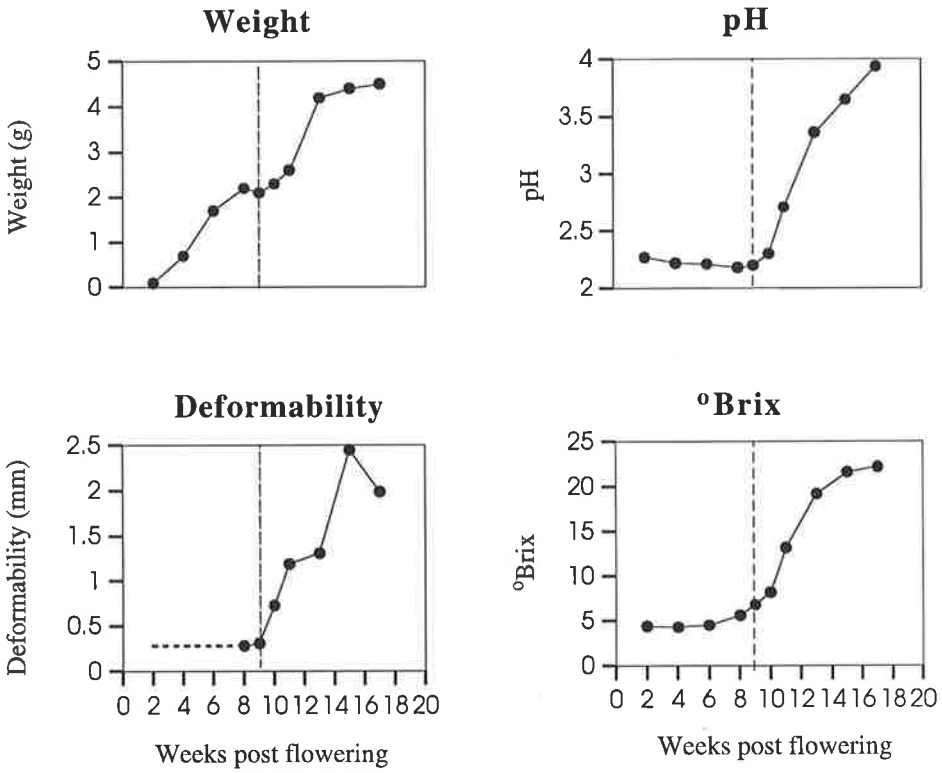


Figure 4.2 Physical and chemical changes in grape berries from flowering to ripeness. Muscat of Alexandria berries were sampled from 2 to 17 weeks post flowering. Weight and deformability measurements were performed on whole berries whilst pH and °Brix measurements were performed on 'free run' juice. The approximate time of veraison is indicated by a dashed line (~9 weeks post flowering).

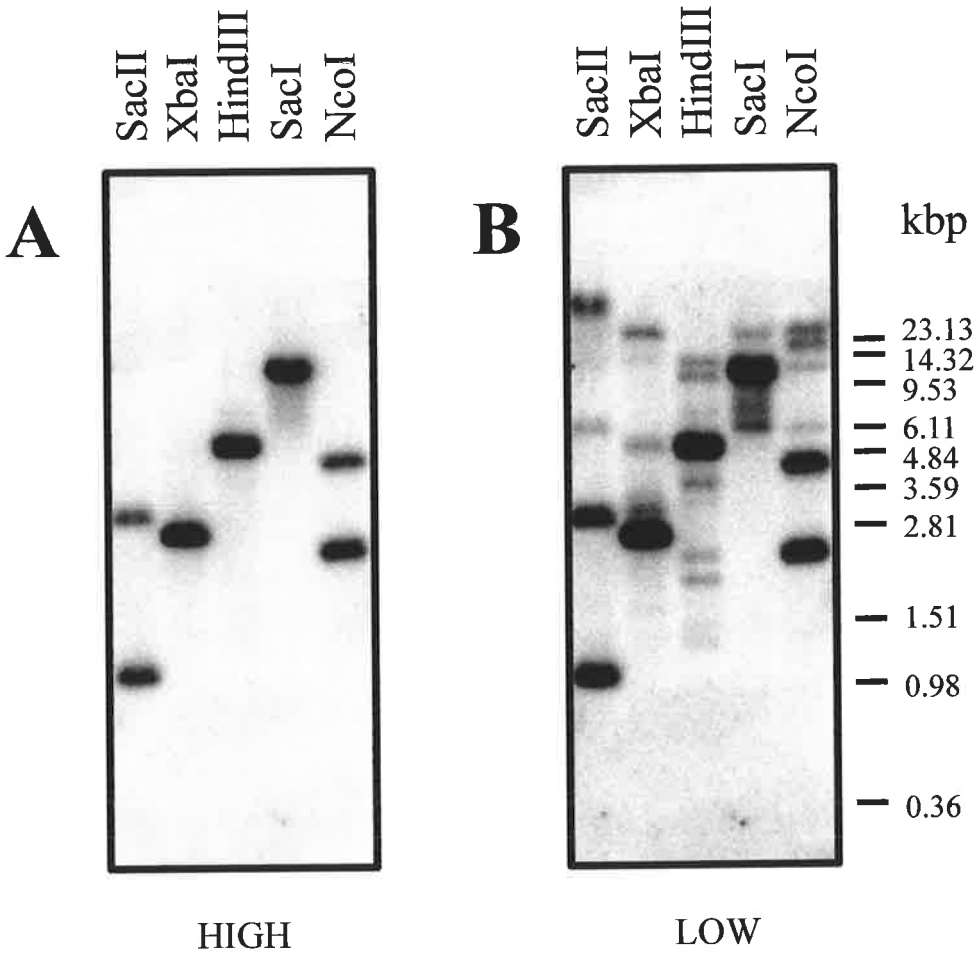


Figure 4.3 Genomic Southern blot analysis of *V. vinifera* DNA indicates VVTL1 is encoded by a single gene and that related genes exist. Muscat of Alexandria DNA was digested with the restriction enzymes *Sac*II, *Xba*I, *Hind*III, *Sac*I and *Nco*I, blotted onto a nylon membrane and probed with VVTL1 cDNA. Membranes were then screened under (A) high and (B) low stringency conditions as detailed in Materials and Methods. Note: *Sac*II and *Nco*I cut the VVTL1 encoding cDNA.

the purification of VVTL2 (see Chapter 2) it is known that other genes encoding distinct proteins with some degree of sequence similarity to VVTL1 do exist within the grape genome. VVTL2 is likely to be identical to the grapevine protein VVOSM1 (see Sections 2.3.3 and 3.3.2), a protein of 72% positional identity to VVTL1 (Loulakakis, 1997b).

VVPR-4a gene

To estimate the copy number of VVPR-4a encoding genes, genomic DNA from *V. vinifera* cv Muscat of Alexandria was analysed by Southern blot hybridisation, using a 114 bp probe primarily representing the 3' untranslated region (nucleotides 436 to 550 in Figure 3.6) of the VVPR-4a cDNA. Employing the highest stringency practically possible for this relatively short probe, the results (Figure 4.4B) indicate that two highly similar sequences encoding VVPR-4a may exist within the diploid *V. vinifera* genome. Whether these sequences are allelic or represent distinct genes is not clear. When the same membrane was stripped and reprobed with a probe (nucleotides 77 to 321 in Figure 3.6) representing the coding region of VVPR-4a (Figure 4.4A) it is evident the VVPR-4a gene belongs to small, highly related multigene family, consisting of at least two, possibly five genes.

4.3.3 The VVTL1 and VVPR-4a genes are highly expressed in ripening berries

To determine when during fruit development the VVTL1 and VVPR-4a encoding genes are expressed, equal amounts of RNA, extracted from berries at different stages of development, were subjected to Northern analysis (Figure 4.5), using firstly a 114 bp VVPR-4a specific probe, and secondly a VVTL1 specific probe. There was a clear correlation between the onset of veraison (the initiation of sugar accumulation and softening), at nine WPF, and substantial accumulation of both the VVTL1 and VVPR-4a messages. A maximum level of message was observed at 11 WPF, after which the

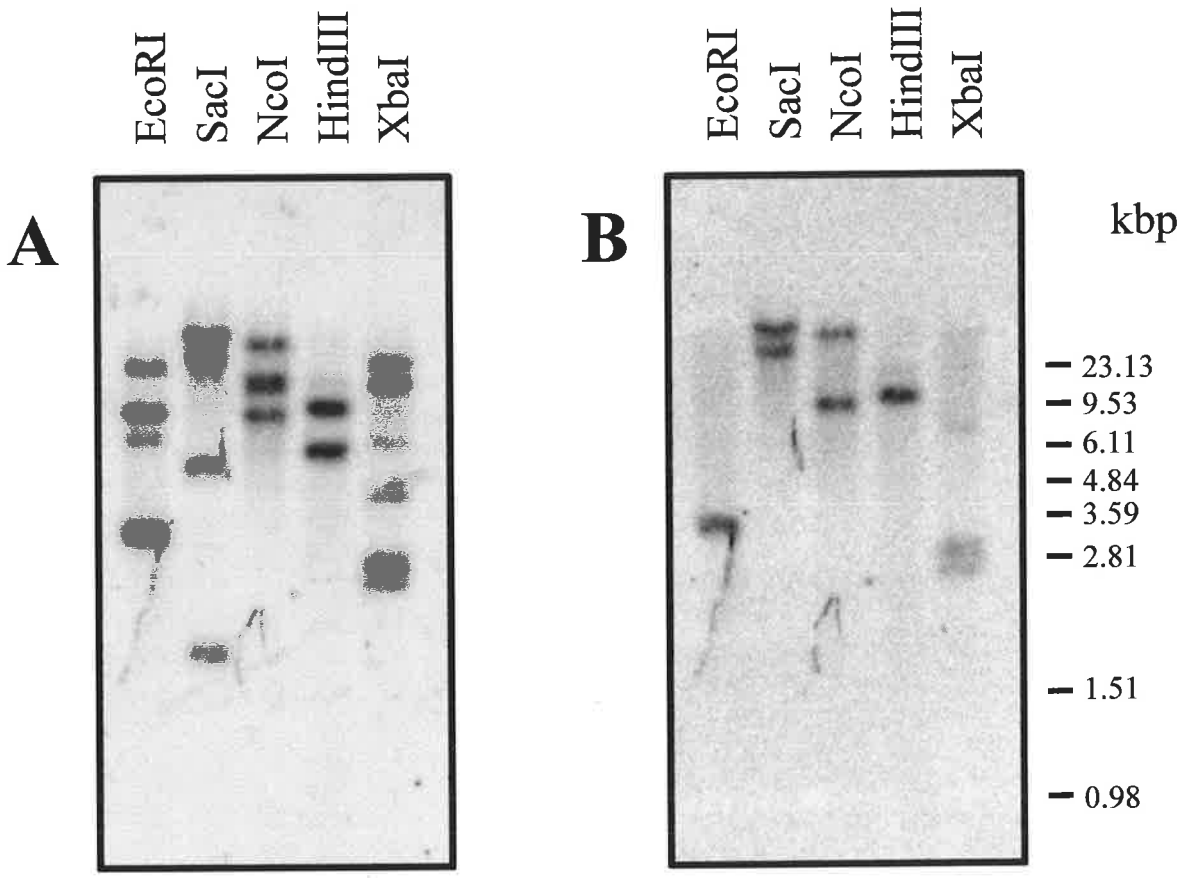


Figure 4.4 Genomic Southern analysis reveals that the gene encoding VVPR-4a belongs to a multigene family. Grapevine DNA was digested with the restriction enzymes *Eco*RI, *Sac*I, *Nco*I, *Hind*III, *Xba*I, blotted onto a nylon membrane and probed with partial VVPR-4a cDNA probes representing (A) the protein coding region (nucleotides 77-321, Figure 3.6) and (B) the 3' untranslated region (nucleotides 436-550, Figure 3.6). None of the restriction enzymes employed cut the VVPR-4a encoding cDNA.

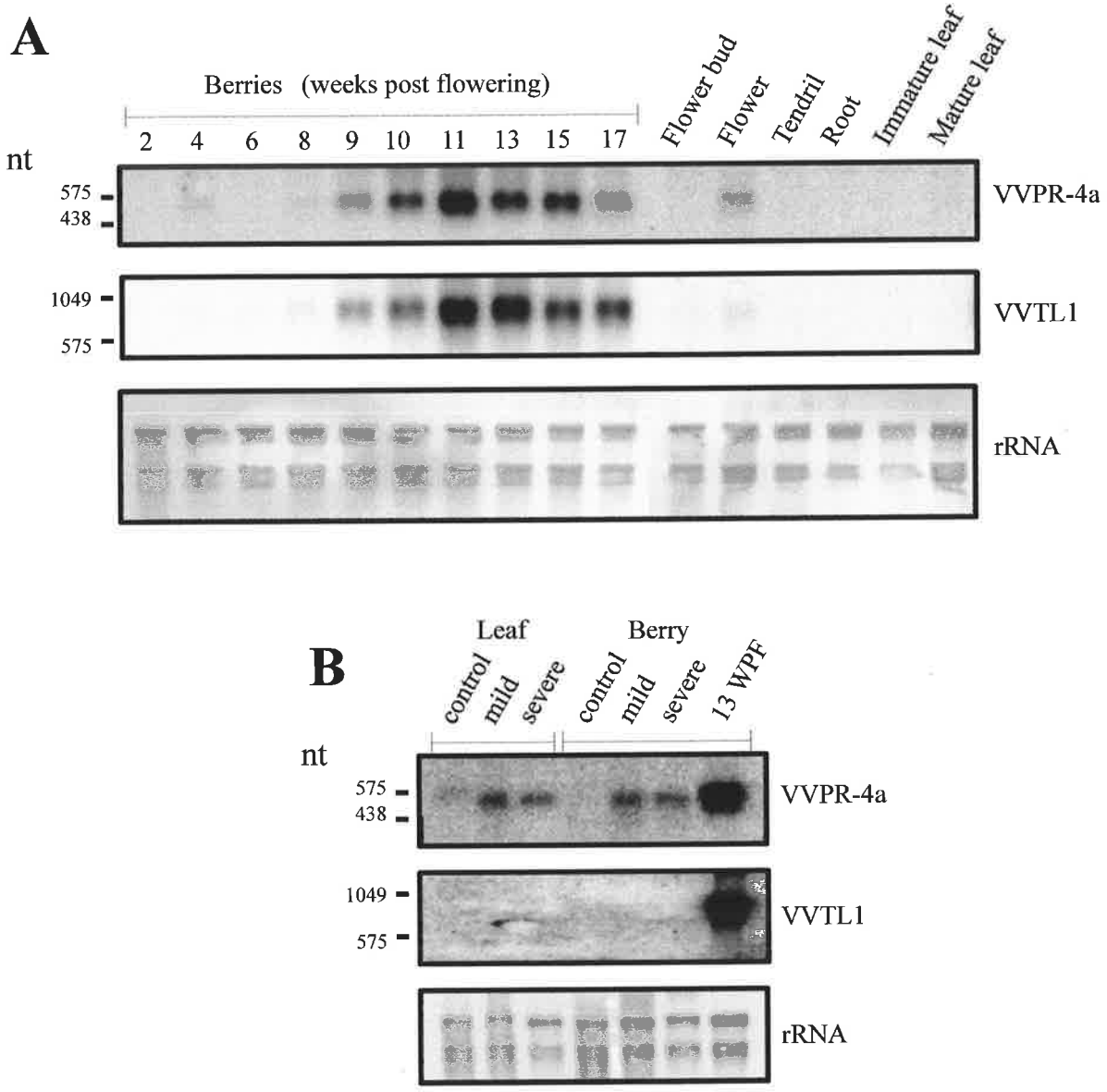


Figure 4.5 Northern analysis of RNA extracted from (A) developing grapevine berries and various grapevine tissues, and (B) *Uncinula necator* infected leaves and pre-veraison berries. Total RNA (5 μg) was loaded in each lane, electrophoresed, blotted onto a nylon membrane and probed in succession with the 114 bp probe representing the 3' untranslated region of the VVPR-4a cDNA, and the VVTL1 cDNA as described in the Materials and Methods. Equal loading was assessed by staining blotted RNA with methylene blue (Wilkinson *et al.*, 1991). The positions, and sizes (nt = nucleotides), of single stranded RNA markers are shown.

VVTL1 and VVPR-4a mRNA appeared to decrease in abundance. This suggests that near berry maturation either a higher degradation rate of VVTL1 and VVPR-4a mRNA occurs, or that gene activation may diminish. Flowers were the only other tissue that contained detectable levels of message hybridising to both probes, whilst very low levels or no VVTL1 or VVPR-4a message is present in roots, leaves, tendrils or flower buds, suggesting a highly tissue specific pattern of the expression of the VVTL1 and VVPR-4a genes (Figure 4.5). The induction of expression at veraison, followed by sustained high levels of message throughout ripening is a pattern also demonstrated for a chitinase (Vv*Chi*4) (Robinson *et al.*, 1997). Together, these results suggest that a coordinate regulation of PR protein genes occurs in ripening grapevine berries in response to developmental signals.

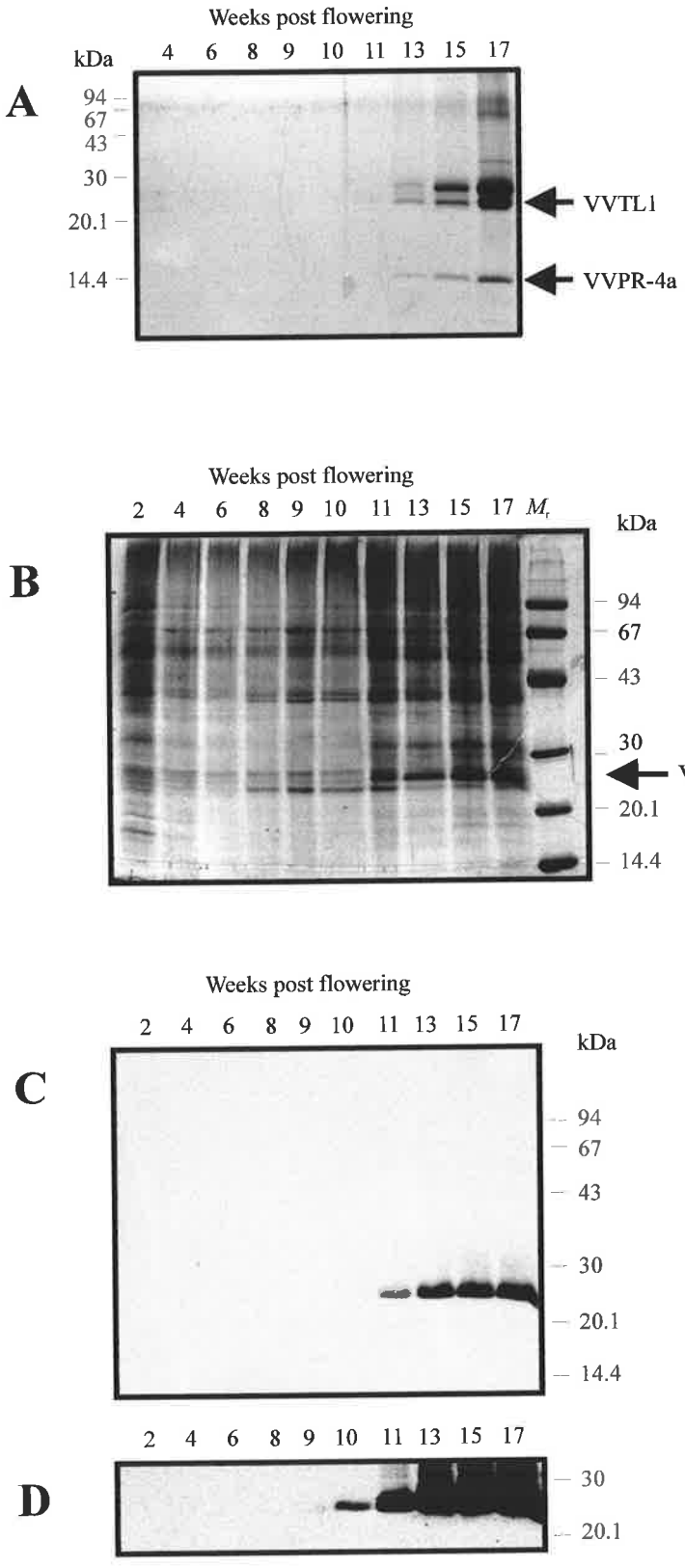
4.3.4 The VVPR-4a gene is induced by powdery mildew infection

An important feature of PR proteins is the inducibility of their genes in tissues challenged with a pathogen (van Loon *et al.*, 1994). Powdery mildew, a major pathogen of both leaves and pre-veraison berries of *V. vinifera* was used to challenge these tissues under controlled conditions. Northern analysis (Figure 4.5B) showed that the VVPR-4a mRNA levels were higher in infected tissue whilst no VVTL1 mRNA could be detected. The highly similar pattern of expression of the genes encoding VVPR-4a and VVTL1 during fruit development can therefore not be extended to situations of pathogen challenge.

4.3.5 Protein profiles of developing grape berries

Extraction of berry proteins in a 5% (w/v) SDS based buffer followed by electrophoresis reveals a relatively complex polypeptide pattern with few obvious changes during development other than a general increase in the amount of extractable protein (Figure 4.6B). A prominent feature is the continuous accumulation of two

Figure 4.6 Protein profiles of berries at different stages of development. (A) Protein extracted from juice of Muscat of Alexandria berries (4 to 17 weeks post flowering) was resolved by SDS-PAGE and stained with Coomassie brilliant blue. Each lane contains protein TCA precipitated from 400 µl of juice. The estimated positions of VVTL1 and VVPR-4a are indicated by arrows. (B) Protein extracted from whole Muscat of Alexandria berries (2 to 17 weeks post flowering) into an SDS containing buffer was resolved by SDS-PAGE and stained with Coomassie brilliant blue. Each lane contains protein extracted from approximately 50 mg fresh weight of tissue. The arrow indicates the approximate position in the gel of VVTL1. (C) VVTL1 accumulates as the grape matures. Proteins in a replica gel to that shown in B were transferred to a nitrocellulose membrane and subjected to Western blot analysis with anti-VVTL1 antibodies. (D) A longer exposure of the immunoblot shown in C. The positions of molecular mass standards are indicated on the sides of respective gels and immunoblots.


A
Weeks post flowering
kDa
4 6 8 9 10 11 13 15 17
94
67
43
30
20.1
14.4
VVTL1
VVPR-4a
B
Weeks post flowering
2 4 6 8 9 10 11 13 15 17 M_r
kDa
94
67
43
30
VVTL1
20.1
14.4
C
Weeks post flowering
2 4 6 8 9 10 11 13 15 17
kDa
94
67
43
30
20.1
14.4
D
2 4 6 8 9 10 11 13 15 17
30
20.1

proteins with apparent masses of 24 kDa and 32 kDa, clearly distinguishable at and after 13 WPF. These are likely to represent VVTL1/VVTL2 and VVCHIT, based on the protein purification and characterisation data obtained using Muscat of Alexandria juice described previously (Section 2.3.2 and 2.3.3). When clarified 'free-run' juice, was extracted from the same berry samples without any protective buffering agents, a very simple polypeptide pattern consisting almost exclusively of 13, 24 and 32 kDa components was observed (Figure 4.6A). Again, based on previous data, these are likely to represent VVPR-4a, VVTL1/VVTL2, and VVCHIT. All of these protein species accumulated to significant levels during the latter stages of ripening (Figure 4.6A).

4.3.6 VVTL1 is specific to ripening fruit of *V. vinifera*

Northern blot analysis suggested a tight temporal and site-specific control of the expression of VVTL1 and VVPR-4a encoding genes, indicating that the berry may the sole site of VVTL1 and VVPR-4a accumulation (Section 4.3.3). Additionally, analysis of total and soluble protein by SDS-PAGE strongly indicated both proteins accumulate during ripening (Section 4.3.5). To investigate these aspects further, polyclonal antibodies were raised to VVTL1* purified from juice (Chapter 2) and Western blots were performed. The antibodies produced were shown to be highly specific for VVTL1, and had a cross-reactivity of approximately 20 times less against VVTL2 (results not shown), which is proposed to have a positional identity of 72% to VVTL1 (see Section 4.3.2). The Western blot analysis therefore of total berry protein extracts (Figure 4.6C) and of free run juice samples (data not shown) confirmed that a 24 kDa protein accumulating in ripening fruit was most likely VVTL1. High levels of the VVTL1 mRNA appeared 10 WPF and remained high throughout ripening (stage III) (see Figure

* Despite several independent attempts in three different rabbits, efforts to raise antibodies against VVPR-4a were unsuccessful as no immunological response appeared to be mounted.

4.5). VVTL1, or a close homologue thereof, was readily detectable 11 WPF and although its level appeared to increase throughout ripening, the relatively slow rate of accumulation from weeks 13 to 17 post flowering could, given the high mRNA levels, indicate that some protein turnover or post-transcriptional control is taking place, as previously observed for the tobacco PR-5 protein, osmotin (LaRosa *et al.*, 1992).

The availability of the highly specific antibodies allowed a further search for VVTL1 and highly similar proteins in other tissues of the grapevine. Equal amounts of protein extracted from berry skins, berry pulp, seeds, roots, flowers, tendrils, young leaves and mature leaves were electrophoresed and subjected to Western blotting. It was found that only the berry pulp and skin extracts contained detectable amounts of VVTL1 or a close homologue thereof (Figure 4.7). The level of VVTL1 or like protein in the pulp extract prepared as described here was many-fold higher than the levels found in the skin, a scenario similar to that found for berry chitinases (Derckel *et al.*, 1998). In fact, it cannot be ruled out that the low amount of VVTL1 or like protein detected in the skin extract is derived from small amounts of pulp adhering to the skin preparation. Conversely, it cannot ruled out that VVTL1 or like protein was lost from the skin during preparation which included washing in distilled water to remove the majority of adhering pulp. Indeed, Pocock *et al.* (1998) found that thaumatin-like proteins and berry chitinases were readily extractable, with model grape juice, from skin samples prepared from Pinot noir and Sauvignon blanc berries.

4.3.7 PR-5 like proteins are major protein species in a range of fruits

The Muscat family of grapes is renowned in the wine industry for the high levels of protein that may be found in the wines prepared from them, with concomitant problems of precipitation and so-called 'haze formation' during product storage (see Chapter 1). However, extraction of 'free-run' juice from the ripe berries of six different

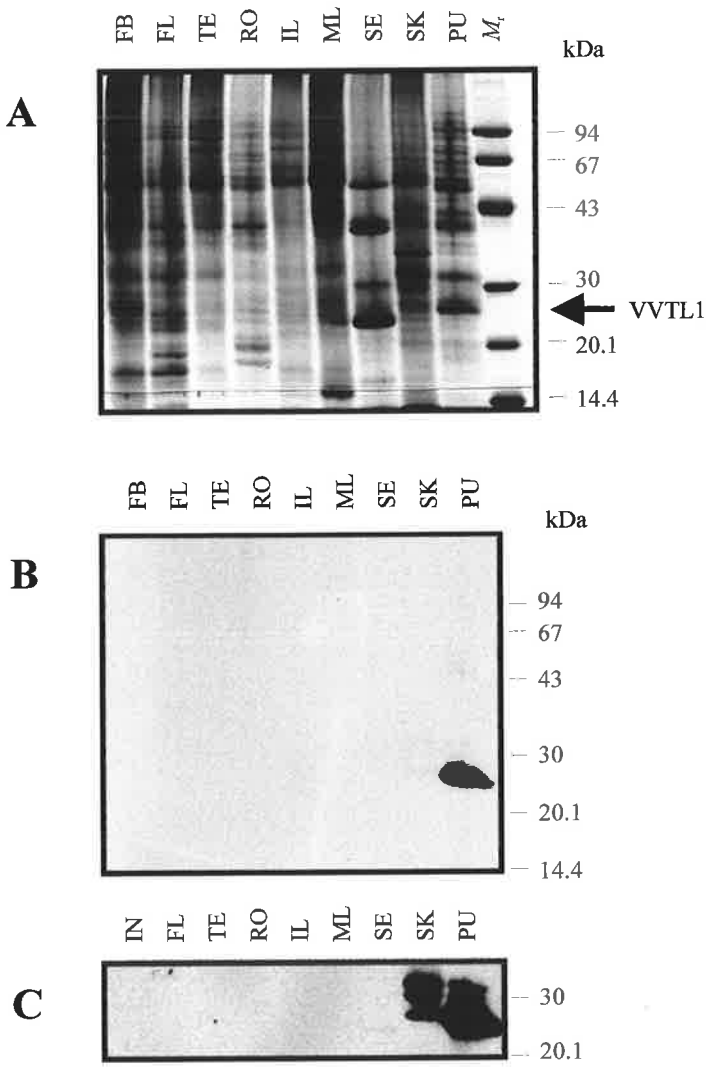


Figure 4.7 VVTL1 is specific to the grape berry and present in high levels in the pulp. (A) Proteins extracted from flower buds (FB), flowers (FL), tendrils (TE), roots (RO), immature leaves (IL), mature leaves (ML), seeds (SE), berry pulp (PU) and skins (SK) were resolved by SDS-PAGE and stained with Coomassie brilliant blue. The arrow indicates the position in the gel of VVTL1. (B) As for A except proteins were transferred to a nitrocellulose membrane and VVTL1 detected by Western blotting. (C) A longer exposure of the immunoblot shown in B.

grape cultivars (Figure 4.8A) shows that the accumulation of VVTL1, or its close homologues, is widespread (Figure 4.8B). The highly conserved accumulation of VVTL1, or closely related protein, in the fruits of *V. vinifera* raises the question of how ubiquitous this phenomenon is. To address this question a variety of commercially available fruits with no apparent disease symptoms were obtained and the presence of VVTL1 homologues in their juice analysed by Western blot analysis (Figure 4.8C). In some fruits (banana and kiwifruit) large amounts of VVTL1-like soluble proteins had accumulated whilst others appeared to contain little or no cross-reactive protein. Thus the accumulation of PR-5 like proteins in fruits may be widespread but possibly not universal. It should be borne in mind that the antibodies raised against VVTL1 may not recognise PR-5 like proteins from all species since significant sequence diversity may exist. For example, a thaumatin-like protein purified from cherry (CHTL) (Fils-Lycaon *et al.*, 1996) exhibits only 45% positional identity with VVTL1 whilst a tobacco PR-5 protein (PRR-1) (Payne *et al.*, 1988) exhibits 82% positional identity with VVTL1 (see Figure 3.3). Notably, since the commencement of this study, it is has been reported that PR-5 like proteins do exist in tomato fruit (Pressey, 1997), and PR-5 like cDNAs have been cloned from apple (unpublished; GenBank accession no. AF090143) and banana (Clendennen and May, 1997; Medina-Suárez *et al.*, 1997) fruit. Whether the onset of PR-like protein synthesis in banana and kiwi fruit is tightly coupled to ripening has yet to be established, as has the nature of the regulatory signals controlling the promoters of these highly inducible genes.

4.3.8 Isolation of VVTL1 and VVPR-4a encoding genes

To further characterise the genetic material responsible for the production of VVTL1 and VVPR-4a, their respective genes were isolated. A genomic library was constructed from *V. vinifera* cv. Muscat of Alexandria DNA and approximately 4.5 x 10^5 plaques were screened at high stringency using partial cDNAs encoding VVTL1 or

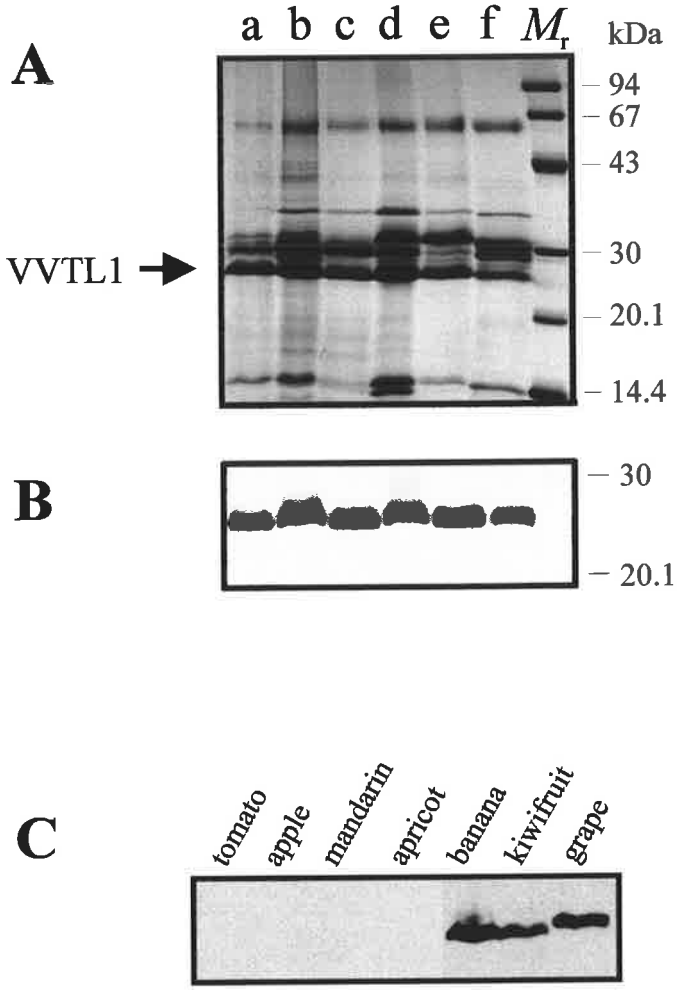


Figure 4.8 VVTL1 homologues are present in the 'free run' juice of several *V. vinifera* cultivars and other fleshy fruits. (A) Proteins from the juices (°Brix in parentheses) of the following cultivars (a) Chardonnay (23.8), (b) Pinot noir (27.8), (c) Palomino (18.5), (d) Shiraz (23.4), (e) Semillon (26.3) and (f) Sultana (syn Thompson seedless) (21.7) were subjected to SDS-PAGE and the gel stained with Coomassie brilliant blue. The arrow represents the position of VVTL1 homologues. (B) Proteins in a replica gel to that shown in A were subjected to Western analysis using anti-VVTL1 antibodies. (C) Juice (20 μL) from the ripe fruits of the designated plants was resolved by SDS-PAGE and the separated proteins transferred to a nitrocellulose membrane. The blot was subjected to Western analysis using anti-VVTL1 antibodies. The fruits (°Brix of juices in parentheses) are as follows: tomato (4.1), apple (12.9), mandarin (13.4), apricot (18.3), banana (20.9), kiwi fruit (14.1), and grape (Muscat of Alexandria) (22.2).

VVPR-4a. Six phage clones were isolated which hybridised to the VVTL1 cDNA probe, whilst only three phage clones hybridised to the VVPR-4a cDNA probes. To confirm the presence of the targeted genes Southern blot hybridisation was performed on several candidate phage (data not shown). It was revealed that the phage isolate λ7A1 contained DNA restriction fragments indicative of the presence of the VVTL1 gene, and hybridisation using a probe primarily representing the 3' untranslated region of the VVPR-4a cDNA, revealed that the phage isolate λ4C2 most likely contained the VVPR-4a gene. The above results were confirmed by PCR employing gene specific primers (data not shown).

4.3.9 Sequence characterisation of the VVTL1 and VVPR-4a genes

To characterise the VVTL1 and VVPR-4 encoding genes by DNA sequencing, several different plasmid subclones were constructed, from λ7A1 DNA (Figure 4.9) and λ4C2 DNA (Figure 4.10), respectively. Through the employment of both gene specific primers and vector sequencing primers, the VVTL1 encoding gene (Figure 4.11) and VVPR-4a encoding gene (Figure 4.12) were sequenced on both DNA strands. Notably, this study represents the first published report of the cloning and sequence characterisation of a PR-4 like class II gene.

By comparison of the cDNA sequences (Chapter 3) and gene sequences it was possible to predict putative transcription sites for both VVTL1 and VVPR-4a encoding genes (Figure 4.11 and Figure 4.12). Transcription start sites appear to be located 26 and 25 nucleotides, respectively, downstream of putative 'TATA boxes' - distances within the suggested limits of 31 +/- 7 nucleotides (Joshi, 1987). Like other previously characterised PR-5 genes (van Kan *et al.*, 1989; Nelson *et al.*, 1992), the VVTL1 gene contains no introns. Conversely, the VVPR-4a encoding gene contains a relatively small 107 bp intron (nucleotides 1059-1166, Figure 4.12). This intron has splice sites that

conform with consensus sequences suggested for higher plants (5' GT, 3' AG), and contains a pyrimidine tract (CT_8-G-CT_7) which is proposed to be recognised by intron splicing proteins (Simpson and Filipowicz, 1996). Furthermore, the positioning of the intron within the VVPR-4a open reading frame is identical to that of an intron within the PR-4 like class I potato gene, *win1* (Stanford *et al.*, 1989).

Utilising the TFMATRIX transcription factor binding site profile database (Heinemeyer *et al.*, 1998) and associated software, the VVTL1 and VVPR-4a encoding genes were scanned for regions homologous to the binding sites of previously characterised transcription factors. Each gene contained two putative binding sites for the same two types of transcription factors. The first site (VVTL1 gene: nucleotides 706-711, Figure 4.11; VVPR-4a gene: nucleotides 646-651, Figure 4.12) is highly similar to a consensus sequence to which a *myb*-like P protein from maize has been shown to bind (Grotewold *et al.*, 1994), whilst the other site constitutes a sequence to which the protein factor SBF-1 from bean binds (Lawton *et al.*, 1991) (VVTL1 gene: nucleotides 155-160, Figure 4.11; VVPR-4a gene: nucleotides 486-490, Figure 4.12). Comparison of the 5' flanking regions of the VVTL1 and VVPR-4a genes, upstream from the respective start codons, failed to reveal any extensive DNA sequence similarities (data not shown).

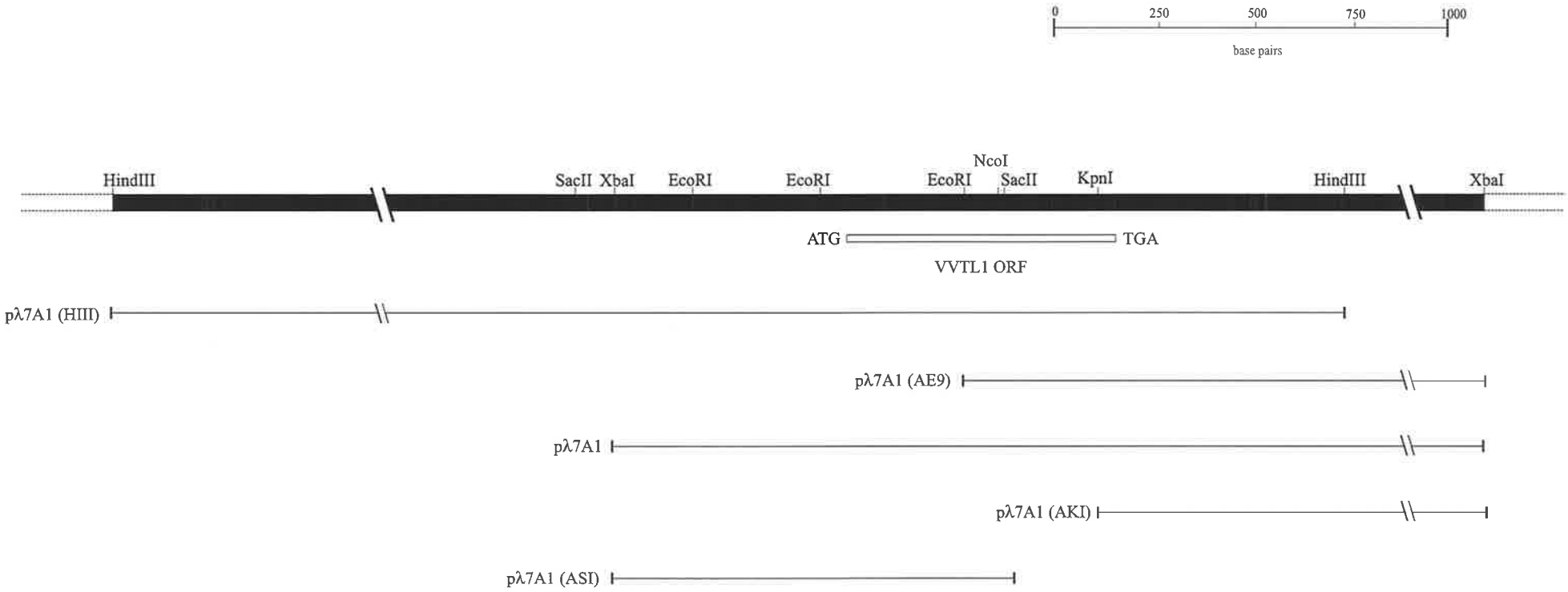


Figure 4.9 Partial subclone map of the λ7A1 isolate which contains the VVTL1 encoding gene. Shown is the VVTL1 open reading frame (ORF) and selected restriction enzyme sites. For simplicity, the diagram has been truncated. The pλ7A1 (HIII) subclone, consists of a ~5.5 kbp *Hind*III fragment, and the pλ7A1 subclone contains a ~2.4 kbp *XbaI* fragment.

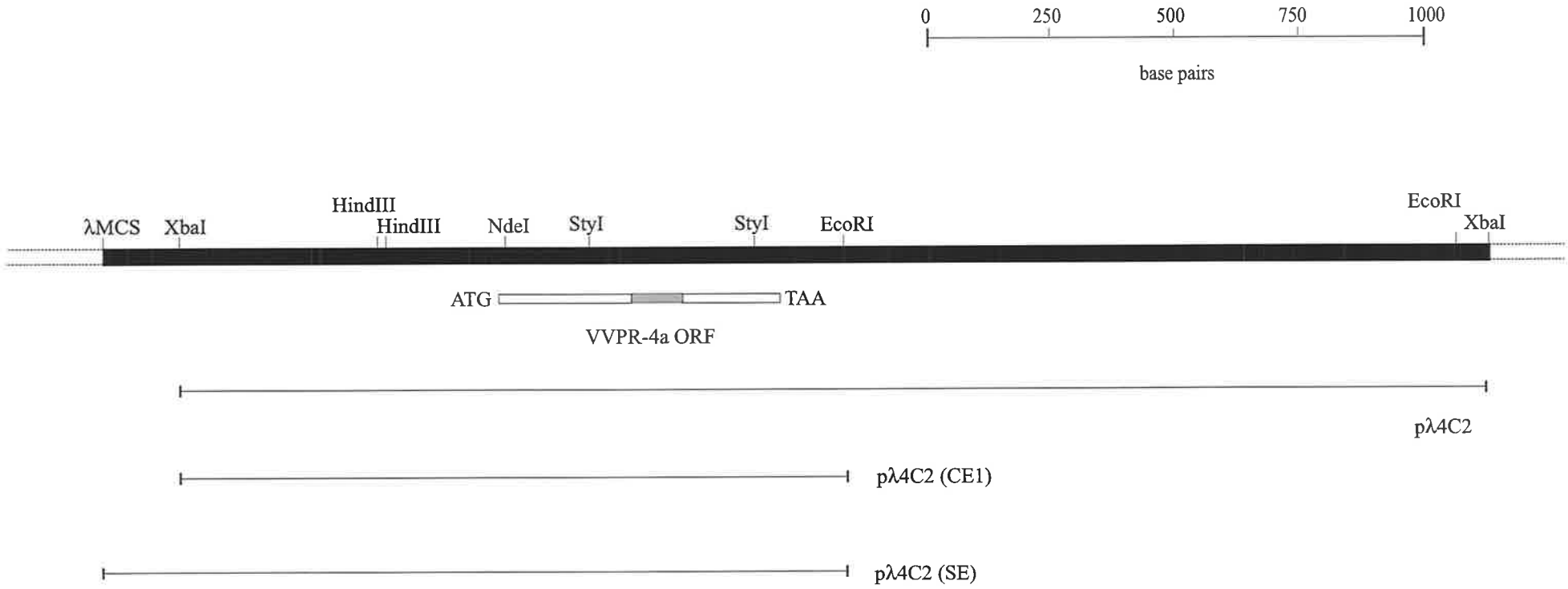
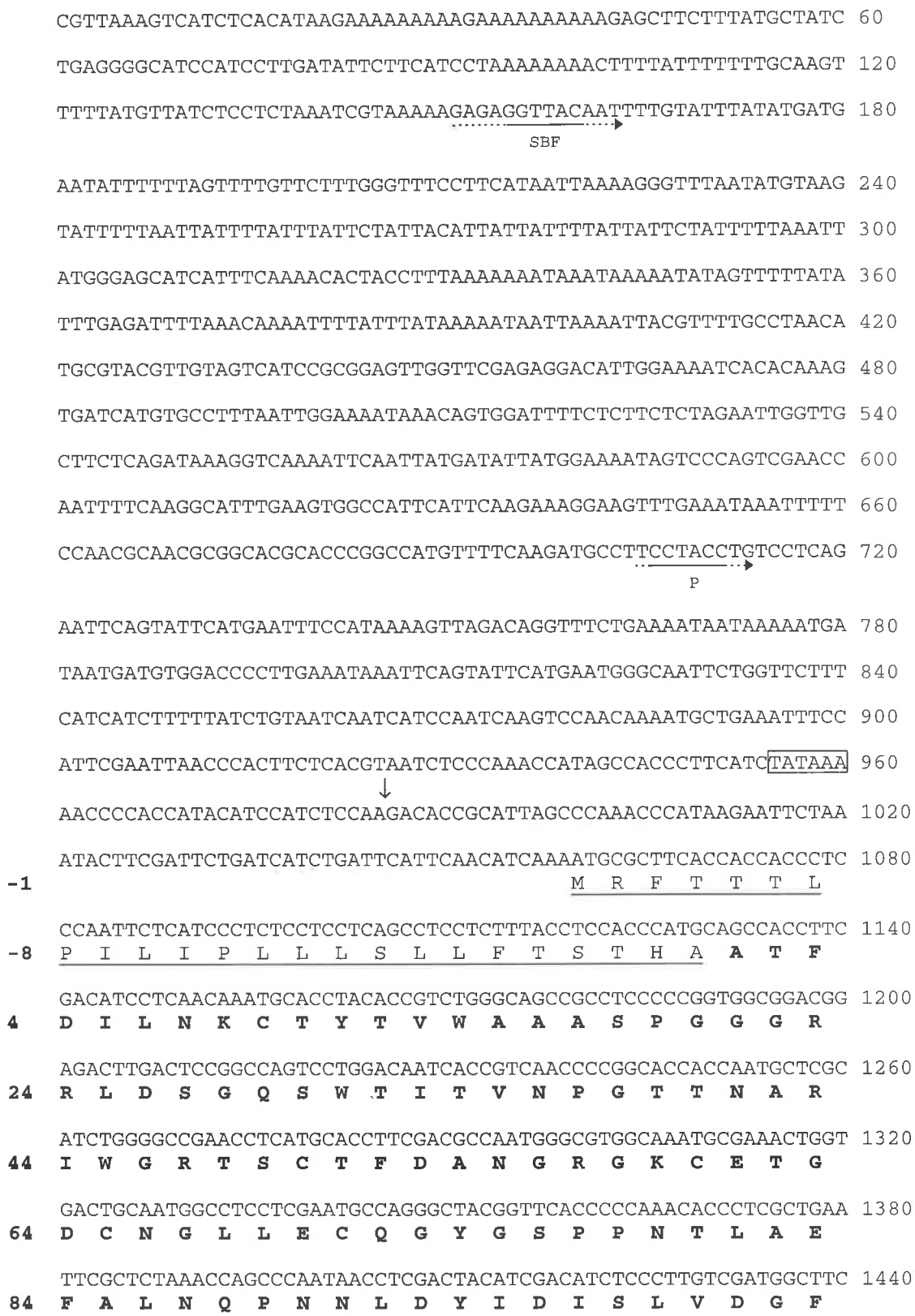


Figure 4.10 Partial subclone map of the λ4C2 isolate which contains the VVPR-4a encoding gene. Illustrated is the VVPR-4a open reading frame (ORF), which is interrupted by a small 107 bp intron (shown in grey), and selected restriction enzyme sites. The pλ4C2 (SE) subclone contains the entire VVPR-4a ORF and 798 bp of sequence preceding the start ATG codon. The λFIXII multiple cloning site (λMCS) containing the T3 promoter sequence is located adjacent to the 5' end of the pλ4C2 sequence.

Figure 4.11 The VVTL1 encoding gene and deduced amino acid sequence. The start and finish of the corresponding cDNA sequence (see chapter 3) is denoted by downward arrows. Nucleotides are numbered on the right side and amino acids are numbered on the left, with the first residue designated as +1. A cleavable presequence is underlined, the translational stop codon is denoted by an asterisk (*), and a putative 'TATA box' is boxed. Two putative transcription factor (SBF and P) binding sites are underscored by horizontal arrows.


CGTTAAAGTCATCTCACATAAGAAAAAAAAAGAAAAAAAAAAGAGCTTCTTTATGCTATC 60
TGAGGGGCATCCATCCTTGATATTCTTCATCCTAAAAAAAACTTTTATTTTTTTGCAAGT 120
TTTTATGTTATCTCCTCTAAATCGTAAAAAGAGAGGTTACAATTTTGTATTTATATGATG 180
SBF
AATATTTTTTAGTTTTGTTCTTTGGGTTTCCTTCATAATTAAAAGGGTTTAATATGTAAG 240
TATTTTTAATTATTTTATTTATTCTATTACATTATTATTTTATTATTCTATTTTTAAATT 300
ATGGGAGCATCATTTCAAAACACTACCTTTAAAAAAATAAATAAAAATATAGTTTTTATA 360
TTTGAGATTTTAAACAAAATTTTATTTATAAAAATAATTAAAATTACGTTTTGCCTAACA 420
TGCGTACGTTGTAGTCATCCGCGGAGTTGGTTCGAGAGGACATTGGAAAATCACACAAAG 480
TGATCATGTGCCTTTAATTGGAAAATAAACAGTGGATTTTCTCTTCTCTAGAATTGGTTG 540
CTTCTCAGATAAAGGTCAAAATTCAATTATGATATTATGGAAAATAGTCCCAGTCGAACC 600
AATTTTCAAGGCATTTGAAGTGGCCATTCATTCAAGAAAGGAAGTTTGAAATAAATTTTT 660
CCAACGCAACGCGGCACGCACCCGGCCATGTTTTCAAGATGCCTTCCTACCTGTCCTCAG 720
P
AATTCAGTATTCATGAATTTCCATAAAAGTTAGACAGGTTTCTGAAAATAATAAAAATGA 780
TAATGATGTGGACCCCTTGAAATAAATTCAGTATTCATGAATGGGCAATTCTGGTTCTTT 840
CATCATCTTTTTATCTGTAATCAATCATCCAATCAAGTCCAACAAAATGCTGAAATTTCC 900
ATTCGAATTAACCCACTTCTCACGTAATCTCCCAAACCATAGCCACCCTTCATCTATAAA 960
AACCCCACCATACATCCATCTCCAAGACACCGCATTAGCCCAAACCCATAAGAATTCTAA 1020
ATACTTCGATTCTGATCATCTGATTCATTCAACATCAAAATGCGCTTCACCACCACCCTC 1080
-1 M R F T T T L
CCAATTCTCATCCCTCTCCTCCTCAGCCTCCTCTTTACCTCCACCCATGCAGCCACCTTC 1140
-8 P I L I P L L L S L L F T S T H A A T F
GACATCCTCAACAAATGCACCTACACCGTCTGGGCAGCCGCCTCCCCCGGTGGCGGACGG 1200
4 D I L N K C T Y T V W A A A S P G G G R
AGACTTGACTCCGGCCAGTCCTGGACAATCACCGTCAACCCCGGCACCACCAATGCTCGC 1260
24 R L D S G Q S W T I T V N P G T T N A R
ATCTGGGGCCGAACCTCATGCACCTTCGACGCCAATGGGCGTGGCAAATGCGAAACTGGT 1320
44 I W G R T S C T F D A N G R G K C E T G
GACTGCAATGGCCTCCTCGAATGCCAGGGCTACGGTTCACCCCCAAACACCCTCGCTGAA 1380
64 D C N G L L E C Q G Y G S P P N T L A E
TTCGCTCTAAACCAGCCCAATAACCTCGACTACATCGACATCTCCCTTGTCGATGGCTTC 1440
84 F A L N Q P N N L D Y I D I S L V D G F

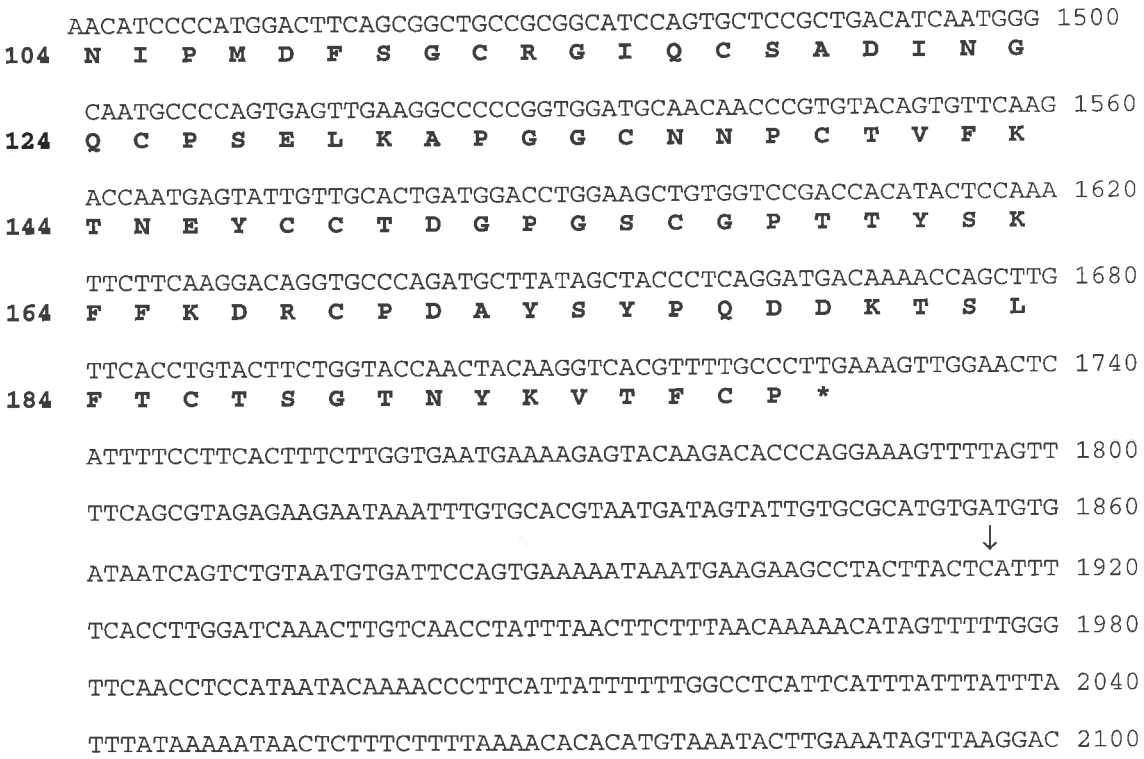
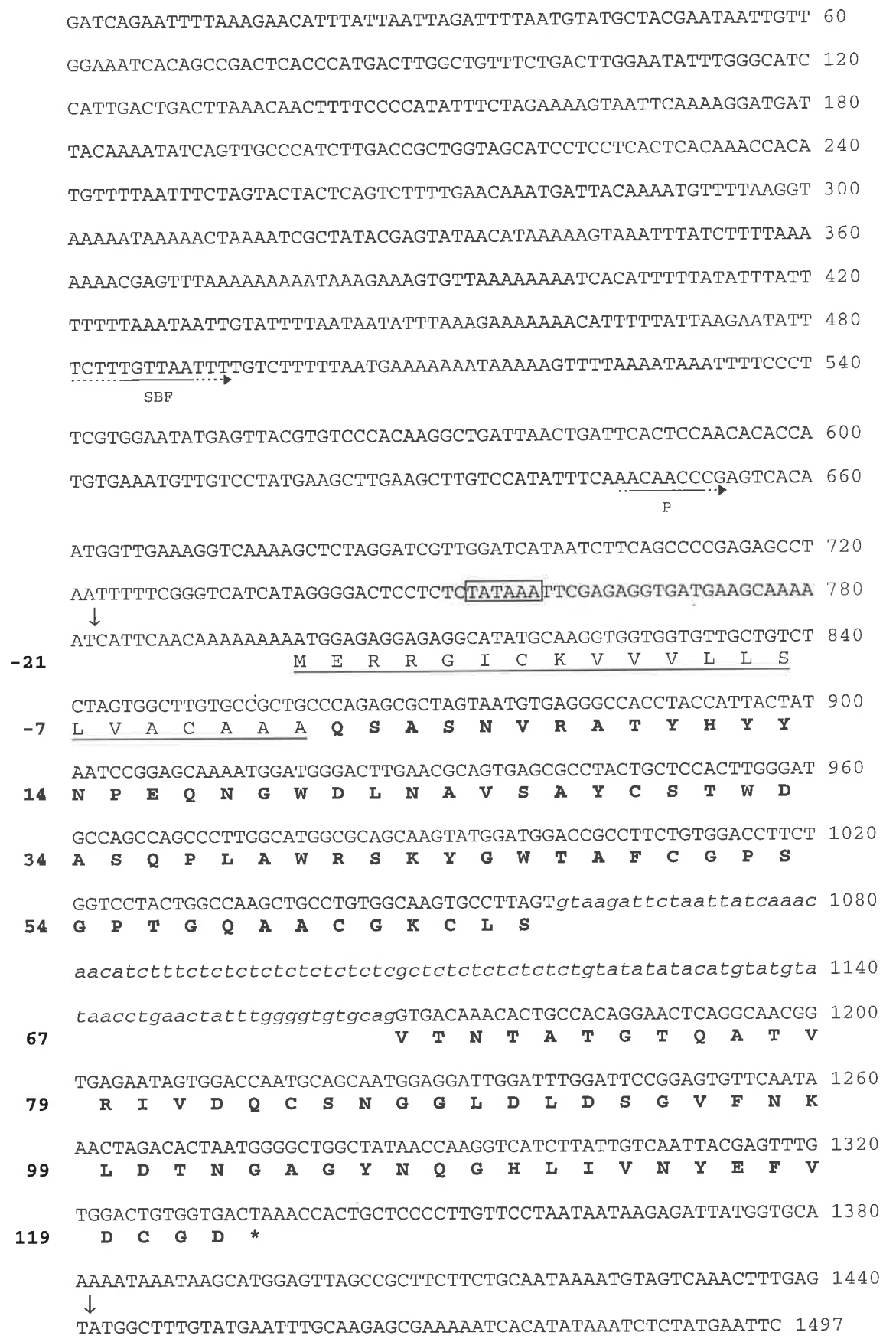


Figure 4.11 continued


GATCAGAATTTTAAAGAACATTTATTAATTAGATTTTAATGTATGCTACGAATAATTGTT 60
GGAAATCACAGCCGACTCACCCATGACTTGGCTGTTTCTGACTTGGAATATTTGGGCATC 120
CATTGACTGACTTAAACAACTTTTCCCCATATTTCTAGAAAAGTAATTCAAAAGGATGAT 180
TACAAAATATCAGTTGCCCATCTTGACCGCTGGTAGCATCCTCCTCACTCACAAACCACA 240
TGTTTTAATTTCTAGTACTACTCAGTCTTTTGAACAAATGATTACAAAATGTTTTAAGGT 300
AAAAATAAAAACTAAAATCGCTATACGAGTATAACATAAAAAGTAAATTTATCTTTTAAA 360
AAAACGAGTTTAAAAAAAAATAAAGAAAGTGTTAAAAAAAATCACATTTTTATATTTATT 420
TTTTTAAATAATTGTATTTTAATAATATTTAAAGAAAAAAACATTTTTATTAAGAATATT 480
TCTTTGTTAATTTTGTCTTTTTAATGAAAAAAATAAAAAGTTTTAAAATAAATTTTCCCT 540
SBF
TCGTGGAATATGAGTTACGTGTCCCACAAGGCTGATTAACTGATTCACTCCAACACACCA 600
TGTGAAATGTTGTCCTATGAAGCTTGAAGCTTGTCCATATTTCAAACAACCCGAGTCACA 660
P
ATGGTTGAAAGGTCAAAAGCTCTAGGATCGTTGGATCATAATCTTCAGCCCCGAGAGCCT 720
AATTTTTCGGGTCATCATAGGGGACTCCTCTCTATAAATTCGAGAGGTGATGAAGCAAAA 780
ATCATTCAACAAAAAAAAATGGAGAGGAGAGGCATATGCAAGGTGGTGGTGTTGCTGTCT 840
-21 M E R R G I C K V V V L L S
CTAGTGGCTTGTGCCGCTGCCCAGAGCGCTAGTAATGTGAGGGCCACCTACCATTACTAT 900
-7 L V A C A A A Q S A S N V R A T Y H Y Y
AATCCGGAGCAAAATGGATGGGACTTGAACGCAGTGAGCGCCTACTGCTCCACTTGGGAT 960
14 N P E Q N G W D L N A V S A Y C S T W D
GCCAGCCAGCCCTTGGCATGGCGCAGCAAGTATGGATGGACCGCCTTCTGTGGACCTTCT 1020
34 A S Q P L A W R S K Y G W T A F C G P S
GGTCCTACTGGCCAAGCTGCCTGTGGCAAGTGCCTTAGTgtaagattctaattatcaaac 1080
54 G P T G Q A A C G K C L S
aacatctttctctctctctctctcgctctctctctctgtatatatacatgtatgta 1140
taacctgaactatttggggtgtgcagGTGACAAACACTGCCACAGGAACTCAGGCAACGG 1200
67 V T N T A T G T Q A T V
TGAGAATAGTGGACCAATGCAGCAATGGAGGATTGGATTTGGATTCCGGAGTGTTCAATA 1260
79 R I V D Q C S N G G L D L D S G V F N K
AACTAGACACTAATGGGGCTGGCTATAACCAAGGTCATCTTATTGTCAATTACGAGTTTG 1320
99 L D T N G A G Y N Q G H L I V N Y E F V
TGGACTGTGGTGACTAAACCACTGCTCCCCTTGTTCCTAATAATAAGAGATTATGGTGCA 1380
119 D C G D *
AAAATAAATAAGCATGGAGTTAGCCGCTTCTTCTGCAATAAAATGTAGTCAAACTTTGAG 1440
TATGGCTTTGTATGAATTTGCAAGAGCGAAAAATCACATATAAATCTCTATGAATTC 1497

4.4 Discussion

Despite the many reports in the literature concerned with wine haze-associated proteins little is known about the temporal patterns of accumulation within the grapevine berry. To address this gap in knowledge, the accumulation of two representative wine haze proteins, the berry PR-like proteins VVTL1 and VVPR-4a, was studied in grapevine berry samples collected from fruit set to maturity. Northern and Western blot analyses revealed that the presence of the VVTL1 and VVPR-4a transcripts and proteins, and potentially therefore, transcriptional activation of their corresponding genes, coincides with the transition of berries from the stage II lag phase to stage III, and the onset of sugar accumulation and berry softening (veraison). Also, it appears that the proteins accumulate exclusively in the ripening berry, and not in other grapevine tissues.

The trigger at veraison for the onset of VVTL1 and VVPR-4a synthesis and accumulation throughout the remainder of berry ripening is unknown. In a variety of plants, a number PR proteins are induced in response to a suite of signals including pathogen attack, wounding, and signalling molecules such as salicylic acid, abscisic acid and ethylene (see Section 1.2). It would be tempting to speculate that the accumulation of berry PR-like proteins is a result of pathogen attack although this now appears unlikely. Berry PR-like proteins appear to be ubiquitous in berry juice from apparently healthy grape samples from a range of different varieties. Their homologues are also found in a number of other, apparently disease free, ripe fleshy fruits (Figure 4.7). Additionally, no β-1,3-glucanase activity, a potential indicator of pathogen attack (Stintzi *et al.*, 1993), could be detected in our berry extracts (results not shown), and has not been detected in other ripening berries in which PR-like proteins are known to accumulate (Robinson *et al.*, 1997; Derckel *et al.*, 1998; Kraeva *et al.*, 1998), despite the

recent cloning of a partial cDNA encoding a β-1,3-glucanase from cultured berry cells (Kraeva *et al.*, 1998).

A factor which might induce berry PR-like accumulation is osmotic stress. A change in the osmotic pressure of grape berry juice occurs very rapidly at the beginning of stage III, and over the entire ripening period there can be an increase in osmotic pressure of about 2,000 kilopascals (Downton and Loveys, 1978). The major solutes that contribute to this osmotic pressure change are fructose and glucose, both of which accumulate to near molar levels in grape juice (Kliewer, 1965). Interestingly, four tobacco PR-3 protein family members (Yun *et al.*, 1996), and the PR-5 protein family members, osmotin and NP24, which were originally purified from salt stressed tobacco and tomato cells, respectively (Singh *et al.*, 1987; King *et al.*, 1988), all accumulate at levels directly related to the degree of osmotic stress. Furthermore, the osmotin gene is known to be expressed during the later stages of tobacco fruit development (Kononowicz *et al.*, 1992), and an NP24 isoform accumulates during tomato fruit ripening (Pressey, 1997). Osmotin and NP24 are known to have a predominantly intracellular location, as opposed to the presumed extracellular location of VVTL1 and VVPR-4a. Therefore, whether VVTL1 and VVPR-4a have a role in osmotic stress adaptation or accumulate non-specifically in response to such stresses remains unknown. However, the gene encoding the potentially extracellular PR-5 like protein, VVOSM1 from grapevine, is known to be induced in NaCl stressed cultured grapevine cells (Loulakakis, 1997a).

High levels of free hexoses have now been shown to repress the synthesis of chlorophyll a/b-binding proteins (Sheen, 1990) and induce the synthesis of a number of defence-related genes such as proteinase inhibitor II (Johnson and Ryan, 1990), chalcone synthase (Tsukaya *et al.*, 1991) and four PR proteins (PAR-1, PR-1b, PR-Q and SAR 8.2) in the leaves of transgenic tobacco plants (Herbers *et al.*, 1995). Such

photoassimilate induced gene expression occurs only above a certain threshold hexose concentration and the situation is thus reminiscent of that seen in berries when the onset of stage III (veraison) occurs. Therefore, it is suspected that the temporally and spatially defined induction of not only VVTL1 and VVPR-4a, but perhaps of a majority of ripening related genes, directly or indirectly is caused by the onset of sugar accumulation, possibly due to the presence of regulatory 'sugar boxes' (Tsukaya *et al.*, 1991) within the promoters of these genes. The mechanism by which such hypothetical 'sugar sensing' and intermediate signals operate remains unclear. It has recently been demonstrated that the presence of an invertase in the vacuole in tobacco leaves leads to SAR (Herbers *et al.*, 1996a) and that hexose sensing therefore appears to take place in the secretory pathway. In this context it is important to note that vacuolar grape invertases are synthesised well in advance of veraison (Davies and Robinson, 1996) with no apparent VVTL1 and VVPR-4a gene expression as a result, possibly because the free hexoses generated do not reach critical threshold levels at early stages of berry development due to a multitude of competing metabolic demands. Preliminary experiments performed in this study (results not shown) to detect an effect of high sugar concentration (1 M glucose or 1 M fructose) on PR-like protein gene expression in pre-veraison berries were inconclusive. No induced expression of the VVTL1 or VVPR-4a genes was observed, either at the mRNA or protein level, when berries were floated in respective sugar solutions.

Another consideration on the matter of what triggers VVTL1, VVPR-4a and indeed class IV chitinase (Robinson *et al.*, 1997) gene expression at veraison is that of the role of signalling molecules. For example, salicylic acid (SA) could play a role in gene induction as it is a product of the phenylpropanoid pathway (Yalpani *et al.*, 1993), which is induced in grape berries at veraison (Boss *et al.*, 1996). The concentration of SA during berry ripening remains unknown, however, exogenous application of SA is known to induce chitinase activity in wounded grapevine leaves (Derckel *et al.*, 1996)

and berries (Derckel *et al.*, 1998). Conversely, it has now been demonstrated that sugar-dependent induction of PR proteins discussed previously can occur independent of SA (Herbers *et al.*, 1996b). Abscisic acid (ABA) is a plant hormone which is known to accumulate to high levels during grapevine berry sugar accumulation (Coombe and Hale, 1973), and to be capable of inducing PR protein encoding genes in several plants (Broekaert *et al.*, 1990; Zhu *et al.*, 1995; Raghothama *et al.*, 1997) including grapevine (Loulakakis, 1997a). ABA is known to be upregulated during osmotic adaptation of plant cells (Skriver and Mundy, 1990). Furthermore, the treatment of berries with a synthetic auxin, benzothiazole-2-oxyacetic acid (BTOA), delayed the onset of ripening, ABA production, and the induction of four different ripening related genes in parallel (Davies *et al.*, 1997). Alongside the potential role of ABA, that of indole acetic acid (IAA) must also be considered. IAA, which is present at high levels during early berry development (Cawthorn and Morris, 1982), may repress berry PR-like protein gene expression until after veraison, when IAA levels become very low and ABA levels peak. As grapevine berries are a nonclimacteric fruit (Kanellis and Roubelakis-Angelakis, 1993) it is unlikely that ethylene, which is an inducer of PR protein encoding genes in many plants (Cabello *et al.*, 1994; Coupe *et al.*, 1997; Tornero *et al.*, 1997), has a major role in berry PR-like protein accumulation.

The isolation and characterisation of the VVTL1 and VVPR-4a encoding genes provides the potential materials needed to elucidate which factors trigger the accumulation of VVTL1 and VVPR-4a at the onset of ripening, and indeed possibly which factors trigger ripening of this non-climacteric fruit - a process relatively little is known about. Sequence characterisation of the VVTL1 and VVPR-4a genes revealed the existence of two different putative plant promoter elements which are present in both genes. This suggests VVTL1 and VVPR-4a may accumulate in the berry as a result of a specific developmental program coordinated by the action of common transcription factors. One site (nucleotides 706-711, Figure 4.11; nucleotides 646-651, Figure 4.12)

is highly similar to silencer elements present in the promoter of the proposed bean defence gene encoding chalcone synthase (CHS15) (Lawton *et al.*, 1991). These silencer elements bind a protein factor, SBF-1 (Harrison *et al.*, 1991), the presence of which has been suggested to repress transcription of the chalcone synthase gene in hypocotyls, yet enhance activity in young cotyledons (Lawton *et al.*, 1991). The other putative promoter element (nucleotides 155-160, Figure 4.11; nucleotides 486-490, Figure 4.12) is near identical to a core sequence required to bind the *myb*-like transcription factor, P, from maize (Grotewold *et al.*, 1994). P binds to and activates transcription of the *A1* gene, which is required for the formation of pigment compounds in the pericarp of the cob (Grotewold *et al.*, 1991). A similar protein may control transcription of the VVTL1 and VVPR-4a genes, which are highly expressed in the ripening pericarp tissue of the grape. The involvement of promoter elements homologous to the fore-mentioned may indeed drive a berry specific transcription, unique to the ripening phase. Unfortunately the non-coding DNA sequences of other ripening induced berry PR-like protein genes of *V. vinifera* have yet to be defined. Further progress must await the elucidation of such sequences as well as manipulation of VVTL1 and VVPR-4a expression *in vivo*, a task which is well outside the scope of this PhD study considering the time required for such manipulations in the woody perennial *V. vinifera*. Attempts to characterise ripening related transcription factors through the one-hybrid selection in yeast are underway in other laboratories (S. Robinson, pers. commun.) but no definitive outcomes have yet been reported.

In conclusion, it has been clearly demonstrated that VVTL1 and VVPR-4a accumulate in a ripening induced manner in parallel with other berry PR-like proteins (Robinson *et al.*, 1997; Derckel *et al.*, 1998; Salzman *et al.*, 1998). The mechanism of induction and the exact roles of these developmentally regulated berry PR-like proteins remains unknown, however, their potential to act in plant defence has been assessed through *in vitro* antifungal assays as reported in the following chapter.

CHAPTER 5

Antifungal properties of berry PR-like proteins

5.1 Introduction

During ripening, the grapevine berry softens dramatically and accumulates both amino acids and sugars to very high levels (Kanellis and Roubelakis-Angelakis, 1993), a scenario which, everything else being equal, is expected to render the fruit susceptible to pathogen attack. In this context it is particularly interesting to note that this study and others (Robinson *et al.*, 1997; Tattersall *et al.*, 1997; Derckel *et al.*, 1998; Salzman *et al.*, 1998; Waters *et al.*, 1998) have recently identified PR-3, PR-4 and PR-5 like proteins as major protein components of the berry. Furthermore, research on the temporal and spatial accumulation of these fruit-specific PR-like proteins showed them to accumulate to very high levels in the ripening berries from veraison onwards (Robinson *et al.*, 1997; Tattersall *et al.*, 1997) - Chapter 4. Notably, accumulation of PR-like proteins in ripening fruit is not restricted to grapevine berries. PR-5 like (thaumatin-like) proteins have also been reported to be present in tomatoes (Pressey, 1997), cherries (Fils-Lycaon *et al.*, 1996), persimmons (Vu and Huynh, 1994), and the fruit of the West African shrub, *Thaumatococcus daniellii* (van der Wel and Loeve, 1972). Also, through the cloning of cDNAs (Dopico *et al.*, 1993; Clendennen and May, 1997; Medina-Suárez *et al.*, 1997; Nairn *et al.*, 1997), the purification of chitinase proteins (Clendennen *et al.*, 1998), and the detection of chitinase and β-1,3-glucanase activity (Esaka *et al.*, 1993; McLeod and Poole, 1994; McCollum *et al.*, 1997; Wurms *et al.*, 1997), it has become evident PR-like proteins are present in a wide range of fruit.

The presence of PR-3 and PR-5 like proteins in ripening grapevine berries has been suggested to be part of the natural defence system against fungal growth and invasion (Derckel *et al.*, 1998; Salzman *et al.*, 1998). It is well documented that many PR proteins from other plants inhibit fungal growth *in vitro* (see Section 1.2) with the greatest anti-fungal activity often observed using synergistic combinations of proteins

from different PR protein families (Leah *et al.*, 1991; Hejgaard *et al.*, 1992; Ponstein *et al.*, 1994). The enhanced efficacy of combinations of PR proteins appears to be reflected in their coordinate regulation *in vivo*, especially as a range of biotic and abiotic stresses have been shown to induce synthesis of different families of PR proteins concurrently in vegetative tissues (for reviews see Stintzi *et al.* (1993) and van de Rhee *et al.* (1994)). In view of this, PR-like proteins purified from ripening berries (Chapter 2) were examined for their potential antifungal properties. Additionally, other soluble proteins present in fractionated berry extracts were also examined for their potential antifungal properties. The results suggest that some of these proteins may indeed play a direct role in plant defence.

5.2 Materials and Methods

5.2.1 Assay for inhibition of fungal growth

Antifungal activity of protein solutions was determined essentially as previously described (Roberts and Selitrennikoff, 1986; Abad *et al.*, 1996). Briefly, all fungi were grown on potato dextrose agar (PDA; Sigma, USA) except *Phytophthora cinnamomi* and *Spaceloma ampelina* which were grown on corn meal agar (CMA; Difco, USA). An agar plug containing mycelia of an actively growing test fungus was placed in the centre of an agar plate, which was then incubated at 25°C (typically 1 to 2 days) allowing hyphae to grow outwards in a circle with a diameter of ~2 to 4 cm. Sterile blank paper (3 MM chromatography paper; Whatman, UK) discs of 6 mm diameter were then placed on the agar adjacent to the hyphal front before addition of a 50 µL protein or buffer solution. All solutions were filter sterilised by centrifugation through Costar Spin-X 0.22 µm columns (Corning, USA). The agar plates were then incubated at 25°C until the hyphal front had grown past the paper discs. Antifungal activity of a protein solution was visually assessed as a crescent of growth inhibition around the paper disc of interest compared to that of control discs, which contained either deionised H_2O, bovine serum albumin or boiled protein (100°C for 10 min). Inhibition experiments were performed at least twice to confirm reproducibility of results. Combinations of proteins were also tested for possible synergistic effects using the above assay. Amounts and combinations of proteins tested are shown in Table 5.1.

Table 5.1 Various protein combinations tested in hyphal growth inhibition assays.

Protein combinations and amounts
Total soluble juice protein (10 µg, 30 µg and 100 µg)
VVTL1 (30 µg and 100 µg)
VVTL2 (30 µg and 100 µg)
VVPR-4a (30 µg and 100 µg)
VVCHIT (30 µg and 100 µg)
GII β-1,3-glucanase (10 µg)
VVTL1 (15 µg) + VVTL2 (15 µg)
VVTL1 (15 µg) + VVPR-4a (15 µg)
VVTL1 (15 µg) + VVCHIT (15 µg)
VVTL2 (15 µg) + VVPR-4a (15 µg)
VVTL2 (15 µg) + VVCHIT (15 µg)
VVPR-4a (15 µg) + VVCHIT (15 µg)
VVTL1 (22.5 µg) + GII β-1,3-glucanase (7.5 µg)
VVTL2 (22.5 µg) + GII β-1,3-glucanase (7.5 µg)
VVPR-4a (22.5 µg) + GII β-1,3-glucanase (7.5 µg)
VVCHIT (22.5 µg) + GII β-1,3-glucanase (7.5 µg)
Total soluble juice protein anion exchange chromatography fractions: U, 11, 21, 25, 29, 43 and 56 (30 µg each)

5.2.2 Fungal strains

Fungi tested were obtained, or originated, from the New South Wales Department of Agriculture (NSW Ag.) Fungal Collection (Orange, NSW, Australia), South Australian Research and Development Institute (SARDI) Fungal Collection (Urrbrae, SA, Australia), or Lenswood Research Centre (LRC) Fungal Collection (Lenswood, SA, Australia).

The fungi tested, their culture collection number and relevant collection details are as follows: *Alternaria alternata* (DAR 45878, NSW Ag.), *Botrytis cinerea* (DAR 48939, NSW Ag.), *B. cinerea* (B27 (grape), SARDI), *B. cinerea* (B44 (rose), SARDI), *Colletotrichum gloeosporioides* (DAR 29821, NSW Ag.), *Phytophthora cinnamomi* (C2-77, LRC), *Rhizopus stolonifer* (DAR 27904, NSW Ag.), *Rhizoctonia solani* (AG4

1701, SARDI), *Sclerotinia sclerotiorum* (DAR 43140, NSW Ag.), *Spaceloma ampelina* (DAR 43140, NSW Ag.), *Trichoderma harzianum* (DAR 41916, NSW Ag.), *Verticillium dahliae* (DAR 44537, NSW Ag.).

5.2.3 Protein purification

VVTL1, VVTL2, VVPR-4a and VVCHIT were purified as described in Chapter 2. To reduce the potential negative effects of salts on antifungal activity (Roberts and Selitrennikoff, 1990), desalting of these proteins was accomplished by repeated concentration by ultrafiltration using a Diaflo YM10 (VVTL1, VVTL2, VVCHIT) or YM3 (VVPR-4a) membrane (Amicon, USA) followed by redilution with deionised H_2O. Protein fractions of soluble Muscat of Alexandria grape juice protein separated by anion exchange chromatography, were also prepared as described in Chapter 2 (see Figure 2.2). The protein fractions (1 mL) tested included: fractions 11, 21, 25, 29, 37, 43, 56 and the unbound protein fraction (U). Each protein fraction was prepared for fungal bioassays by overnight dialysis, using SPECTRA/POR membrane (MW cut-off 6-8,000; Spectrum Medical Industries, Los Angeles, California, USA), against deionised H_2O before concentration with Centricon 10 (Amicon, USA) ultrafiltration units.

A barley (*Hordeum vulgare*) β-1,3-glucanase (isoenzyme GII; Høj *et al.* (1989)) was recombinantly expressed and purified as described below. A modified pET3a plasmid expression vector (Novagen, USA) containing the barley GII β-1,3-glucanase ORF was constructed by Richard Stewart (Department of Plant Science, University of Adelaide). The recombinant GII β-1,3-glucanase contained an engineered polyhistidine 'tag' of six residues at the carboxy terminus which subsequently enabled the purification of the protein by nickel column chromatography (Ni^{2+}-NTA spin columns; Qiagen, USA) according to the manufacturer's instructions. The protein was expressed in *E. coli* BL21 (DE3) cells (Novagen, USA), a cell line which produces lysozyme in the presence of

IPTG. The *E. coli* culture was initially grown in LB media (1% (w/v) NaCl, 1% (w/v) bactotryptone (Difco, USA), 0.5% (w/v) yeast extract), containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol, at 37°C until an OD_{600} of ~0.4 was achieved. Synthesis of GII β-1,3-glucanase was then induced by incubating the culture at 25°C for 3.5 h in the presence of 0.5 mM IPTG. *E. coli* cell pellets containing GII β-1,3-glucanase were lysed by freezing in liquid N_2, then resuspended in wash buffer (50 mM sodium phosphate, pH 7.8, 300 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol) before the soluble protein fraction was applied to Ni^{2+}-NTA spin columns equilibrated the same buffer. Fractions containing GII β-1,3-glucanase were eluted by application of elution buffer (50 mM sodium phosphate, pH 7.8, 300 mM NaCl, 250 mM imidazole, 10 mM β-mercaptoethanol), combined, then dialysed against 20 mM Tris-HCl, pH 8.0 before passing through a 0.5 x 1.0 cm Q-Sepharose column (Pharmacia, Sweden), equilibrated with the same buffer, at a flow rate of ~1 mL per min. Unbound fractions containing essentially pure GII β-1,3-glucanase, as judged by SDS-PAGE, were concentrated and resuspended in deionised H_2O by ultrafiltration using a Diaflo YM10 membrane (Amicon, USA) as described above. The yield of GII β-1,3-glucanase was ~500 μg per 100 mL of induced culture. For the measurement of 1,3-β-D-glucan endohydrolase activity, purified protein was diluted in 50 mM sodium acetate, pH 5.0 and assayed at 37°C as previously described (Chen *et al.*, 1993).

Protein concentrations were estimated by a Coomassie blue binding assay (Sedmak and Grossberg, 1977) using bovine serum albumin (Hämosan, Austria) as a standard. Proteins were analysed by SDS-PAGE as previously described (Section 2.2.2)

5.3 Results

5.3.1 Berry PR-like proteins inhibit fungal growth *in vitro*

Ten different fungal strains were screened for suitability for use in hyphal growth inhibition studies through their ability to grow quickly (able to reach the agar plate perimeter within 3 to 5 days after inoculation) in a consistent radial manner. Eventually the following four species of fungi were chosen: *B. cinerea* (three isolates), *P. cinnamomi*, *T. harzianum*, and *R. solani*. Of these, only *B. cinerea* and *T. harzianum* were noticeably inhibited by as little as 10 μg of total soluble protein (resuspended in H_2O) from Muscat of Alexandria grape juice (Figure 5.1), a protein mixture consisting primarily of PR-like proteins (see Chapter 2 and Figure 2.1). Further examination revealed that three *B. cinerea* isolates - two isolates from grapevine and an isolate from rose - were equally susceptible (results not shown). The growth inhibition of *B. cinerea*, a pathogen which can inflict serious damage to grapevine berries near maturity, was unexpected but after several more days of growth the inhibitory effects of the protein were overcome (results not shown). A previous trial had suggested the growth of *S. sclerotiorum* was also inhibited by total soluble proteins from grape juice, however, strain instability and irregular growth patterns of this fungus prevented repetition of this finding.

Because of the observed susceptibility of *B. cinerea* and *T. harzianum* to the total complement of soluble Muscat of Alexandria juice proteins, these two fungi were chosen as candidates for further studies on the effect of the four purified juice proteins - VVTL1, VVTL2, VVCHIT and VVPR-4a (Figure 5.2). Considerable growth inhibition was only observed with discs containing VVCHIT, some inhibition was observed with discs containing VVTL1 or VVTL2, but little, if any, growth inhibition appeared to be

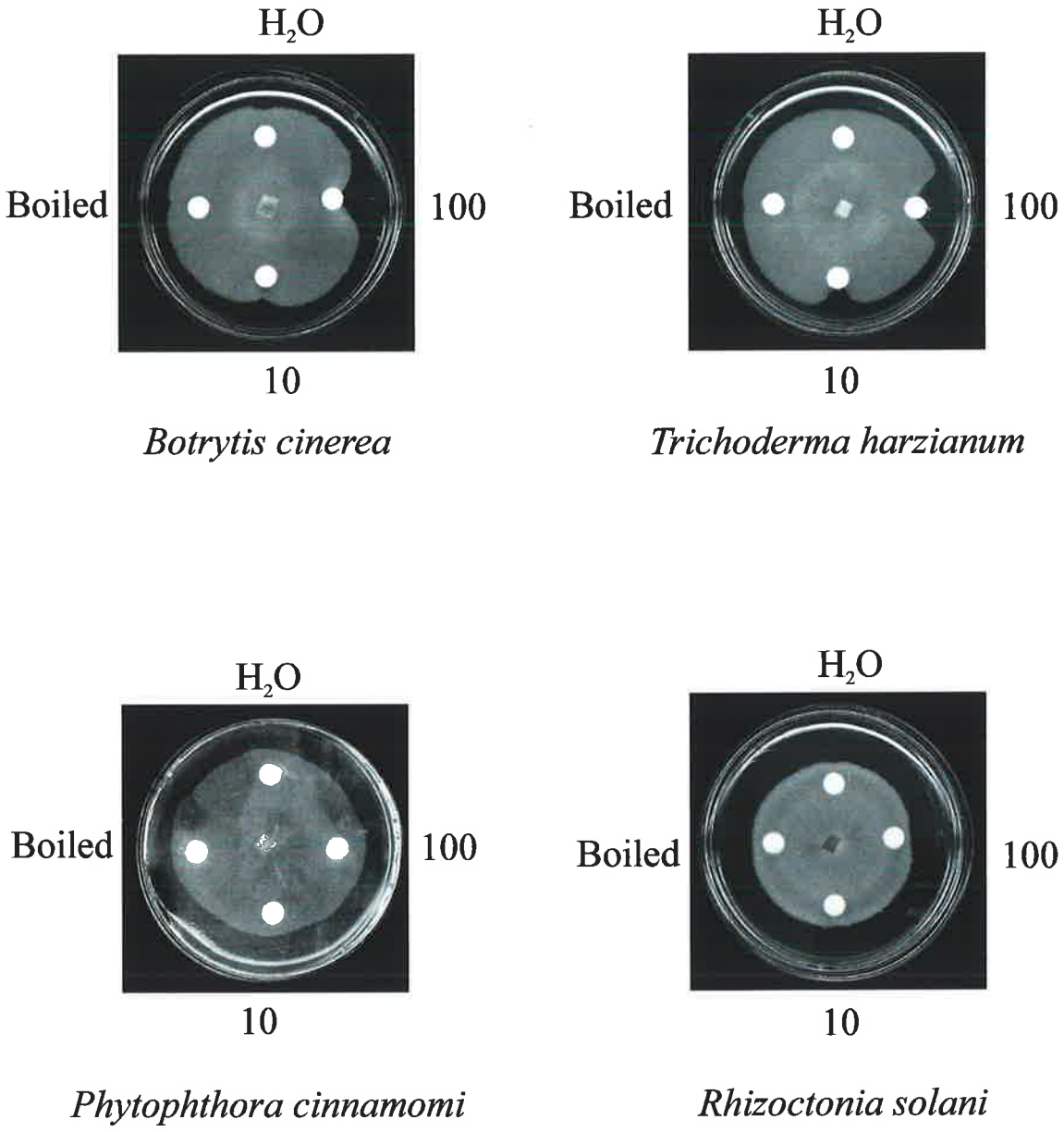


Figure 5.1 Antifungal activity of the total soluble protein present in grape juice. The hyphal growth inhibition assay was performed as described in the Materials and Methods with the following fungi: *B. cinerea* (B27), *T. harzianum* (DAR 41916), *P. cinnamomi* (C2-77), *R. solani* (AG4 1701). Discs contained 100 μg (100) or 10 μg (10) of total juice protein. Controls consisted of 100 μg total juice protein (boiled; 100°C for 10 min) or H_2O. All proteins were resuspended in 50 μL of H_2O.

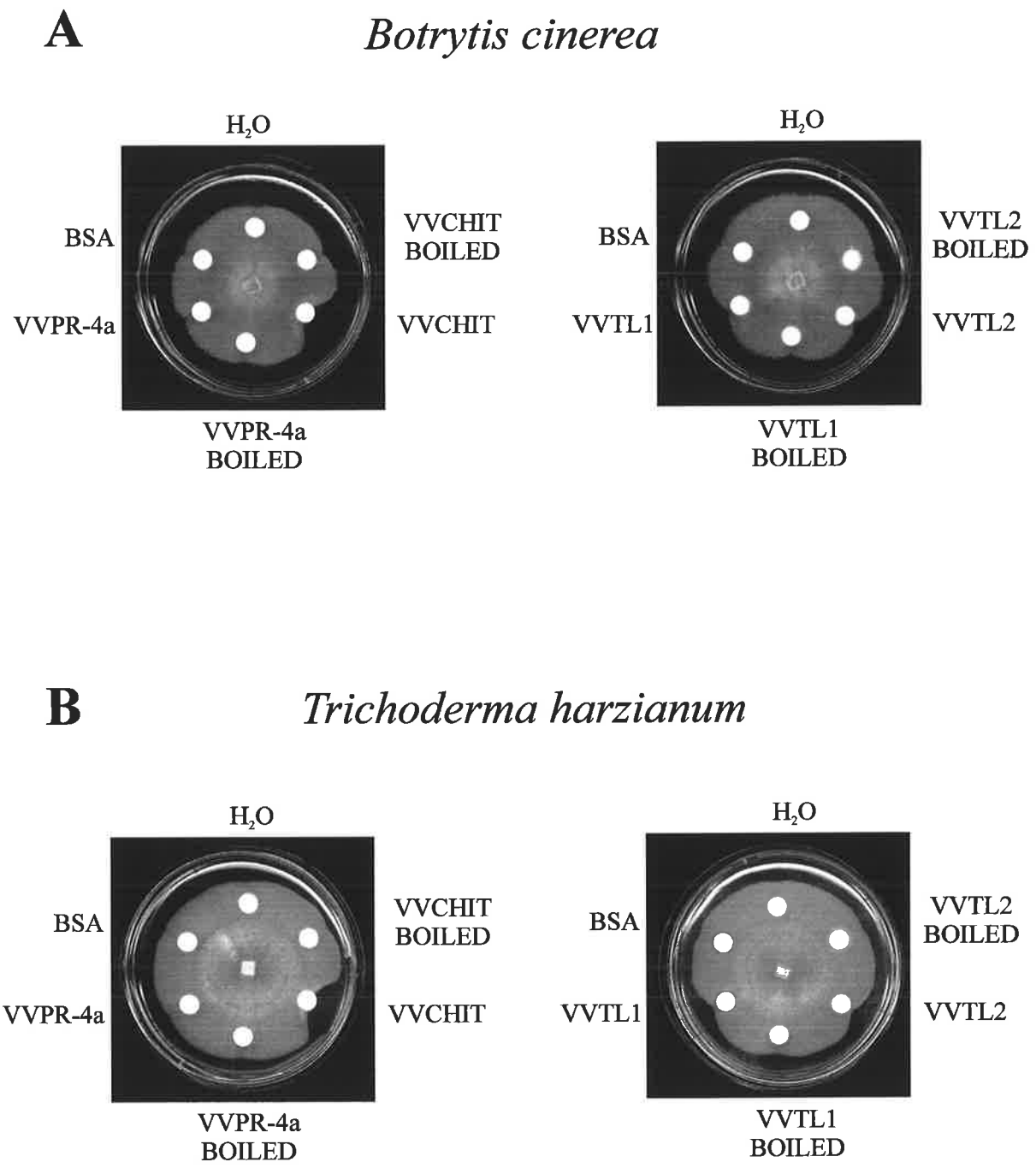


Figure 5.2 Antifungal activity of VVTL1, VVTL2, VVPR-4a, and VVCHIT proteins. The hyphal growth inhibition assay was performed using (A) *B. cinerea* (B27) and (B) *T. harzianum* (DAR 41916). Discs contained 100 μg of indicated proteins. Control discs contained 100 μg bovine serum albumin (BSA), 100 μg of respective proteins (boiled; 100°C for 10 min), or H_2O as indicated. All proteins were resuspended in 50 μL of H_2O.

associated with VVPR-4a. Boiling for 10 min appeared to destroy most, if not all, of the antifungal activity of the tested proteins.

5.3.2 Combinations of berry PR-like proteins, and a β-1,3-glucanase did not result in a synergistic antifungal effect.

It has previously been observed that combinations of PR proteins from PR-3, PR-4 and PR-5 families, can act in a synergistic manner to inhibit fungal growth *in vitro* (Roberts and Selitrennikoff, 1990; Hejgaard *et al.*, 1991). Additionally, members of the PR-2 family, the β-1,3-glucanases, have been reported to act synergistically with other PR proteins, especially when combined with chitinases (PR-3 proteins) in *in vitro* antifungal assays (Mauch *et al.*, 1988; Leah *et al.*, 1991; Sela-Buurlage *et al.*, 1993). As little or no β-1,3-glucanase activity appears to be present in ripening berries of *V. vinifera* (Robinson *et al.*, 1997; Derckel *et al.*, 1998), it was of interest to determine if enhanced antifungal activity could be detected using combinations of a barley β-1,3-glucanase (isoform GII), a protein previously shown to have *in vitro* antifungal activity (Leah *et al.*, 1991), and VVTL1, VVTL2, VVCHIT, or VVPR-4a. The availability of a plasmid expression vector encoding barley GII β-1,3-glucanase allowed for relatively quick production of this enzyme in large quantities in *E. coli*. Also, purification of this polyhistidine 'tagged' enzyme was readily performed through the application of nickel resin and anion exchange chromatography (Figure 5.3).

Unlike some previous studies, no synergism could be observed with combinations of berry PR-like proteins, or a combination of berry PR-like proteins and barley β-1,3-glucanase (isoform GII) protein when tested against *B. cinerea* and *T. harzianum*. All of the combinations tested are listed in Table 5.1, however, only some of the representative results are illustrated in Figure 5.4. Nevertheless, these findings are similar to those of

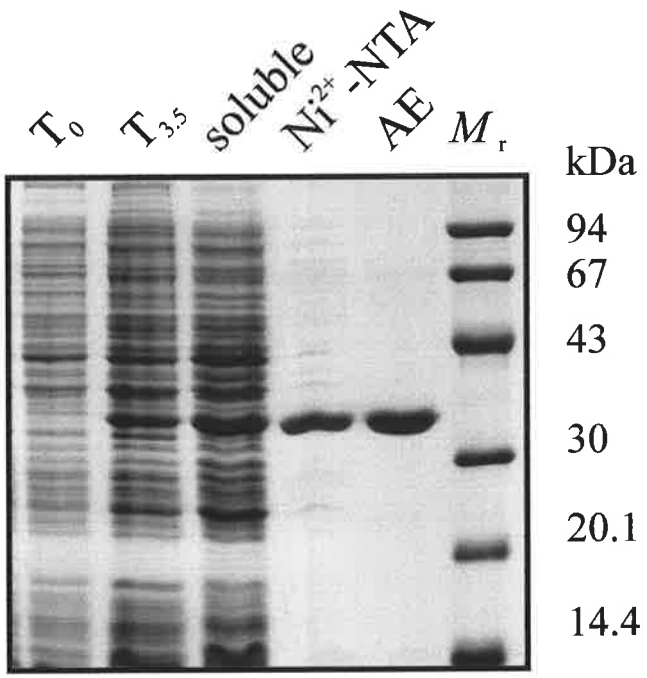


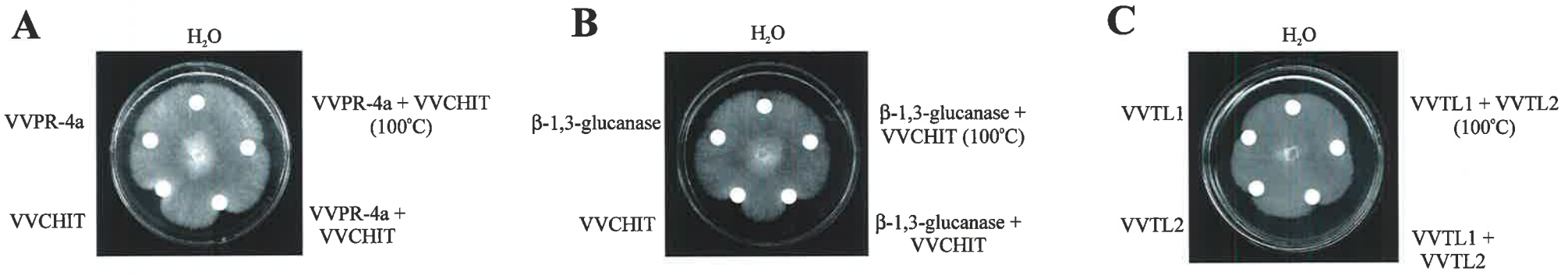
Figure 5.3 Synthesis of a barley β-1,3-glucanase (isoform GII) in *E. coli* and subsequent purification. The recombinant β-1,3-glucanase was expressed as described in the Materials and Methods. SDS-PAGE analysis of total *E. coli* cellular protein at induction (T_0), and 3.5 h after induction ($T_{3.5}$) with IPTG, total soluble *E. coli* protein at $T_{3.5}$, β-1,3-glucanase after nickel column purification (Ni^{2+}-NTA), and after anion exchange chromatography (AE).

Figure 5.4 Analysis of possible synergistic antifungal activities between selected berry PR-like proteins and a barley β-1,3-glucanase. Only selected tests are shown. The hyphal growth inhibition assays employed *B. cinerea* (B27) and *T. harzianum* (DAR 41916). Discs contained 30 μg of indicated berry PR-like proteins, 10 μg of β-1,3-glucanase, or combinations of proteins as indicated below. Control discs contained respective protein combinations (boiled; 100°C for 10 min), or H_2O. All proteins were resuspended in 50 μL of H_2O.

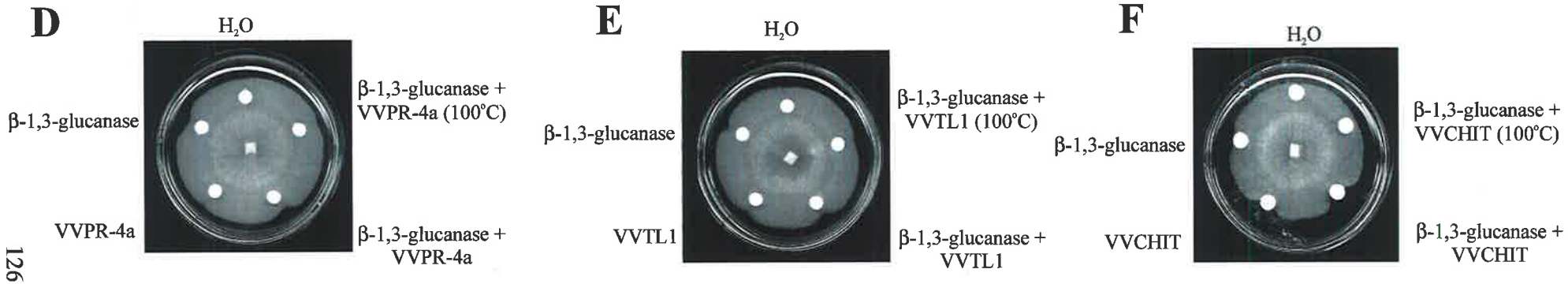
Various protein combinations tested in hyphal growth inhibition assays.

Panel	Protein combinations and amounts	Panel	Protein combinations and amounts
A	VVPR-4a (30 μg) VVCHIT (30 μg) VVPR-4a (15 μg) + VVCHIT (15 μg) VVPR-4a (30 μg) + VVCHIT (30 μg); 100°C	**D**	GII β-1,3-glucanase (10 μg) VVPR-4a (30 μg) GII β-1,3-glucanase (7.5 μg) + VVPR-4a (22.5 μg) GII β-1,3-glucanase (10 μg) + VVPR-4a (30 μg); 100°C
B	GII β-1,3-glucanase (10 μg) VVCHIT (30 μg) GII β-1,3-glucanase (7.5 μg) + VVCHIT (22.5 μg) GII β-1,3-glucanase (10 μg) + VVCHIT (30 μg); 100°C	**E**	GII β-1,3-glucanase (10 μg) VVTL1 (30 μg) GII β-1,3-glucanase (7.5 μg) + VVTL1 (22.5 μg) GII β-1,3-glucanase (10 μg) + VVTL1 (30 μg); 100°C
C	VVTL1 (30 μg) VVTL2 (30 μg) VVTL1 (15 μg) + VVTL2 (15 μg) VVTL1 (30 μg) + VVTL2 (30 μg); 100°C	**F**	GII β-1,3-glucanase (10 μg) VVCHIT (30 μg) GII β-1,3-glucanase (7.5 μg) + VVCHIT (22.5 μg) GII β-1,3-glucanase (10 μg) + VVCHIT (30 μg); 100°C

Botrytis cinerea



Trichoderma harzianum



Salzman *et al.* (1998) who challenged a *B. cinerea* isolate with a combination of PR-3 and PR-5 like proteins purified from *Vitis labruscana* berries. It should be noted that the purified recombinant barley GII β-1,3-glucanase had a specific activity of ~20 μmol of glucose equivalents/min/mg protein which was 5 to 10 fold lower than that of previously reported values for the native enzyme (Høj *et al.*, 1989; Leah *et al.*, 1991; Hrmova and Fincher, 1993). However, as some antifungal effect was observed when the barley β-1,3-glucanase was used alone, at least with *T. harzianum*, it was deemed satisfactory for the determination of potential synergistic effects when combined with other proteins.

5.3.3 Several uncharacterised soluble grape juice proteins possess antifungal activity

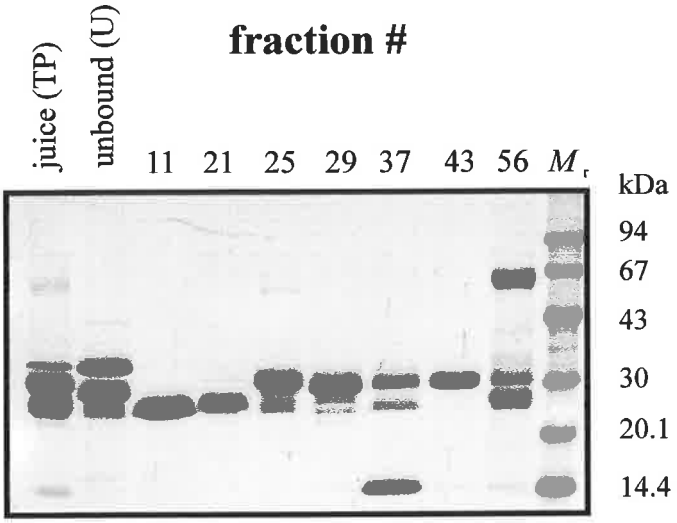
The relatively strong inhibitory activity of the total juice protein extract, suggested that proteins other than the purified PR-3, PR-4 and PR-5 components might be antifungal. In order to evaluate this proposition, fractions obtained by anion exchange chromatography of total juice protein (Figures 2.2 and 5.5A) were included in hyphal growth inhibition assays involving *B. cinerea* and *T. harzianum* (Figure 5.5B). Most fractions tested appeared to contain some antifungal activity, particularly against *T. harzianum*, and in addition, it appeared that different fractions varied in their relative potency, on an equal weight basis, to inhibit either *B. cinerea* or *T. harzianum*. For example, the unbound protein fraction (U) was the strongest inhibitor of *T. harzianum* growth, whilst fraction 43 was the strongest inhibitor of *B. cinerea* growth. This is not inconsistent with the previous suggestion that certain PR-like proteins may have specific activity against certain pathogens (Abad *et al.*, 1996).

Some of the protein fractions investigated for antifungal activity are known to possess substantial amounts of the previously characterised proteins (see figure legend for Figure 5.5). As expected, similar antifungal activity of these fractions to that of the

Figure 5.5 SDS-PAGE analysis of juice proteins in fractions obtained after anion exchange chromatography and examination of their respective antifungal activity *in vitro*. (A) Total soluble protein from a Muscat of Alexandria juice was fractionated by anion exchange chromatography (see Figure 2.2) and (B) hyphal growth inhibition assays were performed as described in the Materials and Methods with *B. cinerea* (B27) and *T. harzianum* (DAR 41916). The discs contained 30 μg of protein from the indicated fractions or 30 μg of total soluble juice protein (TP). Control discs contained H_2O. All proteins were resuspended in 50 μL of H_2O. Some fractions contained previously characterised proteins as shown below.

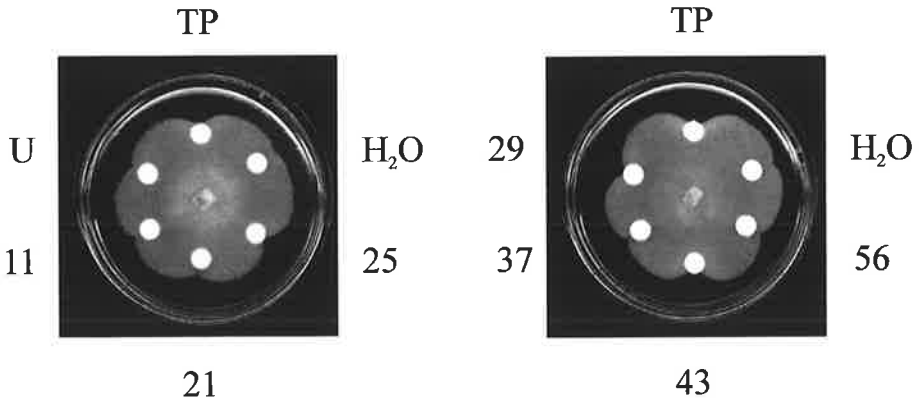
Fraction #	Includes protein
11	VVTL1
21	VVTL2
37	VVPR-4a
43	VVCHIT

A

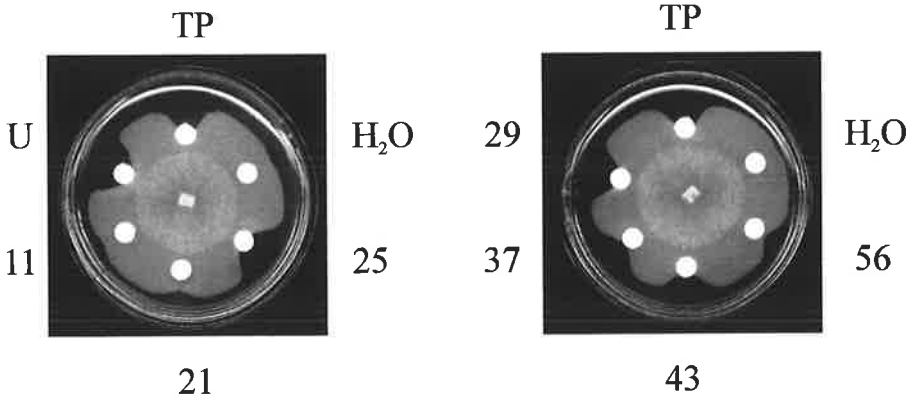


B

Botrytis cinerea



Trichoderma harzianum



purified proteins (Figure 5.2) was observed (Figure 5.5B), with fraction 43 being the most effective inhibitor of growth. Other fractions with relatively strong antifungal activity (U, 25 and 29) contained prominent polypeptides with approximate molecular masses of 28 to 32 kDa. These polypeptides may be other PR-3 like proteins (chitinases), four of which have been recently purified from Muscat of Alexandria juice and characterised by amino acid sequencing (Waters *et al.*, 1998).

5.4 Discussion

Many genes encoding PR proteins are known to be induced, often systemically, when plants are challenged with pathogens (Stintzi *et al.*, 1991; van de Rhee *et al.*, 1994). In line with this, the results of both *in vitro* and *in vivo* studies have strongly implied that these proteins have a direct role in plant defence, especially against fungal pathogens. In some cases however, PR-like proteins are reported to accumulate most likely as a result of developmental signals and not in response to pathogen attack. These proteins may exist in relatively high concentrations in specific tissues, such as dormant or germinating seeds (Roberts and Selitrennikoff, 1990; Hejgaard *et al.*, 1991; Vigers *et al.*, 1991; Caruso *et al.*, 1993; Vögeli-Lange *et al.*, 1994), and floral organs (Lotan *et al.*, 1989; Neale *et al.*, 1990; Ori *et al.*, 1990; Richard *et al.*, 1992; Harikrishna *et al.*, 1996). The role of developmentally regulated PR proteins in plant growth and development remains unknown, although again they are suggested to be involved in plant defence. For example, *in vitro* assays with PR-like proteins that accumulate in seed have demonstrated fungal growth inhibition (Roberts and Selitrennikoff, 1990; Hejgaard *et al.*, 1991; Vigers *et al.*, 1991) suggesting they are part of a pre-emptive strategy for protection against pathogen attack. However, a direct contribution to developmental processes cannot be excluded since a class II endochitinase is apparently necessary for carrot somatic embryo development (De Jong *et al.*, 1992). It is now clear that grapevine berries also accumulate PR-like proteins to high levels in a coordinated manner, probably as a result of a developmental signal. *In vitro* assays performed here suggest these proteins may play a role in defence of this presumably vulnerable organ against fungal attack. However, it cannot be ruled out that they may also be required for tissue or organ development, or that they provide a protective mechanism against physiological stresses such as that associated with increased osmotic pressure - a

function which has been previously proposed for some PR-like proteins (Singh *et al.*, 1987; King *et al.*, 1988; Yun *et al.*, 1996).

Although not all berry PR-like proteins exhibit *in vitro* antifungal activity, as detected in the plate assays described here, their activity could be enhanced *in vivo* in the presence of other antimicrobial proteins (Meyer *et al.*, 1996), for example, the as yet uncharacterised antifungal proteins detected in anion exchange chromatography fractions of grape juice (Figure 5.5). In addition, their *in vivo* activity could be enhanced by the presence of phytoalexins (Paxton and Groth, 1994) or other physiological factors such as high sugar concentrations (Salzman *et al.*, 1998). Considering the complexity of plant defence mechanisms (Guest and Brown, 1997), the interaction between grapevine PR-like proteins and potential pathogens is not expected to be simple and can only be approximated by *in vitro* assays.

The accumulation of PR-like proteins as described in this study, in developing berries, may suggest a new interpretation of previous research that studied the effect of fruit maturity on the ability of powdery mildew (*Uncinula necator)* to establish an infection. Delp (1954) noted that new powdery mildew infections had never been observed on ripe grape berries in the field and further established that they do not occur on berries from the varieties Muscat of Alexandria, Tokay, Sultana (syn Thompson seedless) and Carignane at a level of maturity greater than about 8°Brix. In contrast, berries at a more immature stage, at or below 6°Brix, were consistently susceptible. A more recent study (Chellemi and Marois, 1992) has confirmed these observations and demonstrates that grape berries become resistant to new powdery mildew infections above 7°Brix. The concentration of soluble solids at which berries are resistant or susceptible to new powdery mildew infections correlates well with the presence or absence, respectively, of berry PR-like proteins, suggesting that these proteins may play a role in this phenomenon. *U. necator* is an obligate parasite of the grapevine and

therefore difficult to study, however, *in vitro* assays have recently been developed (Giannakis *et al.*, 1998) which could be useful in providing some clues to the significance of PR-like protein accumulation in berry resistance to powdery mildew infection.

It is also interesting to note that the foliar disease susceptibility of plants has for decades been recognised to be correlated with the sugar content of leaves (reviewed by Vanderplank (1984)). From this, the concept of 'low' and 'high' sugar diseases was developed. For example, low sugar diseases are those where resistance is increased as leaf sugar content increases. Overall, results presented in this study suggest a link between high sugar concentration, PR protein expression and disease resistance in grape berries. In view of the demonstrated expression of PR-like proteins in other fruits at maturity, it would be interesting to examine the relative resistance or susceptibility of these fruits to new infections of known pathogens throughout their maturation in order to determine how universal this link may be.

CHAPTER 6

Summary and future directions

The work described in this thesis was undertaken to identify and investigate the properties, function, and patterns of accumulation of prominent berry proteins that are associated with white wine haze.

In Chapter 2, the purification of four soluble proteins from a Muscat of Alexandria juice was described. These proteins persist throughout the vinification process and three of them have subsequently been identified as major proteins of Muscat of Alexandria wine (Waters *et al.*, 1996). Amino acid sequence determination of the purified proteins revealed they were homologous to plant PR proteins belonging to the PR-3, PR-4 and PR-5 families. PR proteins have relatively low molecular weights, are soluble at low pH and resist proteolytic attack. Such properties are intrinsic to wine haze associated proteins, and it is now clear from the results of this study, and other recent research (Waters *et al.*, 1996; Waters *et al.*, 1998), that the vast majority of soluble wine proteins are PR-like proteins derived from the grapevine berry.

The research described in Chapter 3 resulted in the isolation of cDNAs encoding the soluble berry PR-5 like and PR-4 like proteins, VVTL1 and VVPR-4a respectively. Characterisation of the cDNAs enabled the complete primary structures of these proteins to be deduced, including topogenic signals which suggest they are extracellular proteins. They are highly similar in sequence to other previously characterised proteins for which tertiary structures have been solved through X-ray crystallography and NMR studies (Ludvigsen and Poulsen, 1992b; Ko *et al.*, 1994; Koiwa *et al.*, 1999). Therefore, through the use of computer modelling software, three-dimensional structural predictions could be made for VVTL1 and VVPR-4a. These may prove useful for future studies on the definition of potential binding substrates for both proteins, and hence PR-4 and PR-5 proteins in general. Additionally, such information may be used to aid the search for effective high affinity binding agents that could be used as alternatives to wine fining agents such as bentonite.

In Chapter 4 the expression patterns of the genes encoding VVTL1 and VVPR-4a were examined at both the mRNA and protein level. The careful sampling of grapevine berries from flowering to maturity, and other healthy grapevine tissues, provided material essential for these studies. The VVTL1 and VVPR-4a encoding genes appear to be expressed in a coordinated berry specific manner from veraison and throughout the remainder of the ripening period, the protein products accumulating predominantly towards berry maturity. Similar expression patterns of other grapevine berry PR-like proteins have also been reported (Robinson *et al.*, 1997; Derckel *et al.*, 1998) suggesting this may be a ubiquitous phenomenon, at least in *V. vinifera* berries. Additionally, the results of this study and others suggest soluble juice and wine proteins are highly similar irrespective of grapevine variety. Indeed, the molecular weights of several prominent soluble juice proteins from several white and red varieties have now been shown to be almost identical (Y. Hayasaka, pers. commun.).

Although it is now known that grapevine berry PR-like proteins accumulate during ripening, the signal(s) driving the expression of the respective genes have yet to be elucidated. As a first step in this process, a genomic library of Muscat of Alexandria was constructed and the genes encoding VVTL1 and VVPR-4a were isolated and characterised by DNA sequencing. The future study of the promoter regions of these genes, combined with the characterisation of other ripening related genes, may reveal common elements, which could lead to the isolation of regulatory transcription factors. Elucidation of the signal transduction pathways leading to the modulation of the activity of these transcription factors could then provide further insights into why berry PR-like protein encoding genes are expressed predominantly during ripening. Through studies of this kind, it is hoped that the factors controlling the ripening process eventually will be found. Such a discovery would be highly beneficial to the viticultural industry which currently has no control over the irregular ripening pattern of grapes, a process that can vary dramatically even within a single bunch. The isolation of gene promoter sequences

which can drive the expression of transgenes in a berry and ripening specific manner would also be useful considering current strategies for producing transgenic grapevines with improved properties.

As the berry ripens it accumulates high levels of sugar and softens, a scenario which is expected to render the berry susceptible to pathogen attack. In Chapter 5 experiments were detailed which revealed that some of the berry PR-like proteins (VVTL1, VVTL2 and VVCHIT) do have an antifungal effect *in vitro*. However, despite the sequence similarity of the protein VVPR-4a to other known antifungal proteins (eg wheatwin2; (Caruso *et al.*, 1996)) no antifungal activity was detected for this PR protein. Although the *in vitro* assays performed in this study may not be a true reflection of protein activity *in vivo*, it cannot be ruled out that berry PR-like proteins have a role in processes other than plant defence against pathogen attack, for example, in tissue development and/or osmotic stress adaptation. Through the recent development of transformation systems for *V. vinifera* (Mauro *et al.*, 1995; Perl *et al.*, 1996; Scorza *et al.*, 1996; Franks *et al.*, 1998) important clues to functions of the berry PR proteins may now be gained from attenuation of their expression in transgenic grapevines. Parallel studies in other ripening fruits where PR proteins have been shown to accumulate to high levels would also be of interest. Further information gained through these studies could be supported by berry PR-like protein localisation studies, both at the cellular and tissue level, which are now possible through the availability of the highly specific antibodies made against VVTL1, and also VVTL2 (this study, results not shown), and berry chitinases (E. Waters and S. Robinson, pers. commun.). The production of highly specific antibodies against VVPR-4a may also be achieved by the use of an alternative animal other than a rabbit, or the modification of this protein to make it more antigenic (Harlow and Lane, 1988).

Finally, whilst it may be beneficial for the winemaking industry to reduce the accumulation of berry PR-like proteins that are associated with white wine haze by, for example, genetic manipulation, this may not be a suitable option if the disease resistance or other physiological processes of the grapevine are compromised. Instead it might be highly attractive to find an efficient practice for depleting juice or wine of these proteins without the use of fining agents such as bentonite which are detrimental to wine quality, costly and difficult to dispose of. The possibility of identifying other more superior fining agents through studies on PR-protein structure and ligand binding have already been mentioned. Alternatively, the identification of a protease that degrades PR proteins under premium white winemaking conditions (pH 3 to 4, and <20°C) may provide an inexpensive and easy to use option. Continued study of the *in vivo* turnover of plant PR proteins in general (Rodrigo *et al.*, 1989; Rodrigo *et al.*, 1991b), and *V. vinifera* berry PR-like proteins, in particular, may not only lead to insights into PR protein degradation but reveal the existence of a useful protease for winemaking.

References

Abad LR, D'Urzo MP, Liu D, Narasimhan ML, Reuveni M, Zhu JK, Niu XM, Singh NK, Hasegawa PM, Bressan RA (1996) Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. Plant Sci 118: 11-23

Adzhubei IA, Adzhubei AA, Neidle S (1998) An integrated sequence-structure database incorporating matching mRNA sequence, amino acid sequence and protein three-dimensional structure data. Nucleic Acids Res 26: 327-331

Albrecht H, van de Rhee MD, Bol JF (1992) Analysis of *cis*-regulatory elements involved in induction of a tobacco PR-5 gene by virus infection. Plant Mol Biol 18: 155-158

Alexander D, Goodman RM, Gutrella M, Glascock C, Weymann K, Friedrich L, Maddox D, Ahlgoy P, Luntz T, Ward E, Ryals J (1993) Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein-1a. Proc Nat Acad Sci USA 90: 7327-7331

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic alignment search tool. J Mol Biol 215: 403-410

Anderson JW (1968) Extraction of enzymes and subcellular organelles from plant tissues. Phytochem 7: 1973-1988

Anderson MD, Chen ZX, Klessig DF (1998) Possible involvement of lipid peroxidation in salicylic acid-mediated induction of PR-1 gene expression. Phytochem 47: 555-566

Antoniw JF, Ritter CE, Pierpoint WS, Loon LCV (1980) Comparison of three pathogenesis-related proteins from plants of two cultivars of tobacco infected with TMV. J Gen Virol 47: 79-87

Anzlovar S, Dallaserra M, Dermastia M, Menestrina G (1998) Membrane permeabilizing activity of pathogenesis-related protein linusitin from flax seed. Mol Plant Microbe Interact 11: 610-617

Bar-Peled M, Bassham DC, Raikhel NV (1996) Transport of proteins in eukaryotic cells: more questions ahead. Plant Mol Biol 32: 223-249

Bartnicki-Garcia S (1968) Cell wall chemistry, morphogenesis, and taxonomy of fungi. Ann Rev Microbiol 22: 87-108

Batalia MA, Ernst S, Roberts W, Robertus JD (1996) The crystal structure of the antifungal protein zeamatin, a member of the thaumatin-like, PR-5 family. Nature Struct Biol 3: 19-23

Bayly FC, Berg HW (1967) Grape and wine proteins of white wine varietals. Am J Enol Vitic 18: 18-32

Beffa R, Meins Jr. F (1996) Pathogenesis-related functions of plant β-1,3-glucanases investigated by antisense transformation - a review. Gene 179: 97-103

Beffa R, Szell M, Meuwly P, Pay A, Vögeli-Lange R, Métraux JP, Neuhaus G, Meins Jr. F, Ferenc N (1995) Cholera toxin elevates pathogen resistance and induces pathogenesis-related gene expression in tobacco. EMBO J 14: 5753-5761

Beffa RS, Hofer RM, Thomas M, Meins Jr. F (1996) Decreased susceptibility to viral disease of β-1,3-glucanase-deficient plants generated by antisense transformation. Plant Cell 8: 1001-1011

Beintema JJ (1994) Structural features of plant chitinases and chitin-binding proteins. FEBS Lett 350: 159-163

Berg HW, Akiyoshi M (1961) Determination of protein stability in wine. Am J Enol Vitic 12: 107-110

Birnboim H, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7: 1513-1523

Blade WH, Boulton R (1988) Absorption of protein by bentonite in a model wine solution. Am J Enol Vitic 39: 193-199

Borgmeyer JR, Smith CE, Khai Huynh Q (1992) Isolation and characterization of a 25 kDa antifungal protein from flax seeds. Biochem Biophys Res Commun 187: 480-487

Bork P, Dandekar T, Diaz-Lazcoz Y, Eisenhaber F, Huynen M, Yuan Y (1998) Predicting function: From genes to genomes and back. J Mol Biol 283: 707-725

Boss PK, Davies C, Robinson SP (1996) Expression of anthocyanin biosynthesis pathway genes in red and white grapes. Plant Mol Biol 32: 565-569

Broekaert W, Lee HI, Kush A, Chua NH, Raikhel N (1990) Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). Proc Nat Acad Sci USA 87: 7633-7637

Broekaert WF, Cammue BPA, De Bolle MFC, Thevissen K, De Samblanx GW, Osborn RW (1997) Antimicrobial peptides from plants. Crit Rev Plant Sci 16: 297-323

Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254: 1194-1197

Bryngelsson T, Green B (1989) Characterization of a pathogenesis-related, thaumatin-like protein isolated from barley challenged with an incompatible race of mildew. Physiol Mol Plant Path 35: 45-52

Bryngelsson T, Sommer Knudsen J, Gregersen PL, Collinge DB, Ek B, Thordal Christensen H (1994) Purification, characterization, and molecular cloning of basic PR-1-type pathogenesis-related proteins from barley. Mol Plant Microbe Interact 7: 267-275

Bucciaglia PA, Smith AG (1994) Cloning and characterization of *Tag 1*, a tobacco anther β-1,3-glucanase expressed during tetrad dissolution. Plant Mol Biol 24: 903-914

Busam G, Kassemeyer HH, Matern U (1997) Differential expression of chitinases in *Vitis vinifera* L. responding to systemic acquired resistance activators of fungal challenge. Plant Physiol 115: 1029-1038

Cabello F, Jorrin JV, Tena M (1994) Chitinase and β-1,3-glucanase activities in chickpea (*Cicer arietinum*) - induction of different isoenzymes in response to wounding and ethephon. Physiol Plant 92: 654-660

Capelli N, Diogon T, Greppin H, Simon P (1997) Isolation and characterization of a cDNA clone encoding an osmotin-like protein from *Arabidopsis thaliana*. Gene 191: 51-56

Caruso C, Caporale C, Chilosi G, Vacca F, Bertini L, Magro P, Poerio E, Buonocore V (1996) Structural and antifungal properties of a pathogenesis-related protein from wheat kernel. J Protein Chem 15: 35-44

Caruso C, Caporale C, Poerio E, Facchiano A, Buonocore V (1993) The amino acid sequence of a protein from wheat kernel closely related to proteins involved in the mechanisms of plant defence. J Protein Chem 12: 379-386

Casacuberta JM, Puigdomenech P, San Segundo B (1991) A gene coding for a basic pathogenesis-related (PR-like) protein from *Zea mays*. Molecular cloning and induction by a fungus (*Fusarium moniliforme*) in germinating maize seeds. Plant Mol Biol 16: 527-536

Cawthorn DL, Morris JR (1982) Relationship of seed number and maturity to berry development, fruit maturation, hormonal changes, and uneven ripening of 'Concord' (*Vitis labrusca* L.) grapes. J Am Soc Hort Sci 107: 1097-1104

Charoenchai C, Fleet GH, Henschke PA, Todd BEN (1997) Screening of non-Saccharomyces wine yeasts for the presence of extracellular hydrolytic enzymes. Aust J Grape Wine Res 3: 2-8

Chellemi DO, Marois JJ (1992) Influence of leaf removal, fungicide applications, and fruit maturity on incidence and severity of grape powdery mildew. Am J Enol Vitic 43: 53-57

Chen L, Fincher GB, Høj PB (1993) Evolution of polysaccharide hydrolase substrate specificity: Catalytic amino acids are conserved in barley 1,3-1,4- and 1,3-β-glucanases. J Biol Chem 268: 13318-13326

Cheong NE, Choi YO, Kim WY, Bae IS, Cho MJ, Hwang I, Kim JW, Lee SY (1997a) Purification and characterization of an antifungal PR-5 protein from pumpkin leaves. Mol Cells 7: 214-219

Cheong NE, Choi YO, Kim WY, Kim SC, Cho MJ, Lee SY (1997b) Purification of an antifungal PR-5 protein from flower buds of *Brassica campestris* and cloning of its gene. Physiol Plant 101: 583-590

Chevalier C, Bourgeois E, Pradet A, Raymond P (1995) Molecular cloning and characterization of six cDNAs expressed during glucose starvation in excised maize (*Zea mays* L.) root tips. Plant Mol Biol 28: 473-485

Clarke AE, Stone BA (1962) β-1,3-glucan hydrolases from the grapevine (*Vitis vinifera*) and other plants. Phytochem 1: 175-188

Clendennen SK, Lopezgomez R, Gomezlim M, Arntzen CJ, May GD (1998) The abundant 31-kilodalton banana pulp protein is homologous to class-III acidic chitinases. Phytochem 47: 613-619

Clendennen SK, May GD (1997) Differential gene expression in ripening banana fruit. Plant Physiol 115: 463-469

Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993) Plant chitinases. Plant J 3: 31-40

Considine JA, Knox RB (1979) Development and histochemistry of the cells, cell walls, and cuticle of the dermal system of fruit of the grape, *Vitis vinifera* L. Protoplasma 99: 347-365

Coombe BG (1976) The development of fleshy fruits. Ann Rev Plant Physiol 27: 507-528

Coombe BG (1992) Research on the development and ripening of the grape berry. Am J Enol Vitic 43: 101-110

Coombe BG, Bishop GR (1980) Development of the grape berry. II. Changes in the diameter and deformability during veraison. Aust J Agric Res 31: 499-509

Coombe BG, Hale CR (1973) The hormone content of ripening grape berries and the effects of growth substance treatments. Plant Physiol 51: 629-634

Cornelissen BJC, Hooft van Huijsduijnen RAM, Bol JF (1986a) A tobacco mosaic virus-induced tobacco protein is homologous to the sweet-tasting protein thaumatin. Nature 321: 531-532

Cornelissen BJC, Hooft Van Huijsduijnen RAM, van Loon LC, Bol JF (1986b) Molecular characterization of messenger RNAs for 'pathogenesis-related' proteins 1a, 1b and 1c induced by TMV infection of tobacco. EMBO J 5: 37-40

Coupe SA, Taylor JE, Roberts JA (1997) Temporal and spatial expression of mRNAs encoding pathogenesis-related proteins during ethylene-promoted leaflet abscission in *Sambucus nigra*. Plant Cell Environ 20: 1517-1524

Dave RS, Mitra RK (1998) A low temperature induced apoplastic protein isolated from *Arachis hypogaea*. Phytochem 49: 2207-2213

Davies C, Boss PK, Robinson SP (1997) Treatment of grape berries, a nonclimacteric fruit with a synthetic auxin, retards ripening and alters the expression of developmentally regulated genes. Plant Physiol 115: 1155-1161

Dawes H, Boyes S, Keene J, Heatherbell D (1994) Protein instability of wines - influence of protein isoelectric point. Am J Enol Vitic 45: 319-326

De Jong AJ, Cordewener J, Schiavo FL, Terzi M, Vandekerckhove J, Van Kammen A, De Vries SC (1992) A carrot somatic embryo mutant is rescued by chitinase. Plant Cell 4: 425-433

Deloire A, Kraeva E, Mauro MC, Bonnet E, Renault AS (1996) Immunodetection of chitinase-like and 1,3-glucanase-like proteins secreted in vitro by embryogenic and non-embryogenic cells of grapevines. Vitis 36: 51-52

Delp CJ (1954) Effect of temperature and humidity on the grape powdery mildew fungus. Phytopathol 44: 615-626

Derckel J, Audran J, Haye B, Lambert B, Legrande L (1998) Characterization, induction by wounding and salicylic acid, and activity against *Botrytis cinerea* of chitinases and β-1,3-glucanases of ripening grape berries. Physiol Plant 104: 56-64

Derckel J, Legrande L, Audran J, Haye B, Lambert B (1996) Chitinases of the grapevine (*Vitis vinifera* L.): five isoforms induced by salicylic acid are constitutively expressed in other tissues. Plant Sci 119: 31-37

Didierjean L, Frendo P, Nasser W, Genot G, Marivet J, Burkard G (1996) Heavy-metal-responsive genes in maize: identification and comparison of their expression upon various forms of abiotic stress. Planta 199: 1-8

Dixon RA, Harrison MJ (1990) Activation, structure, and organization of genes involved in microbial defense in plants. Adv Genet 28: 165-234

Dong JZ, Dunstan DI (1997) Endochitinase and β-1,3-glucanase genes are developmentally regulated during somatic embryogenesis in *Picea glauca*. Planta 201: 189-194

Dopico B, Lowe AL, Wilson ID, Merodio C, Grierson D (1993) Cloning and characterization of avocado fruit mRNAs and their expression during ripening and low-temperature storage. Plant Mol Biol 21: 437-449

Dorrestein E, Ferreira RB, Laureano O, Teixeira AR (1995) Electrophoretic and FPLC analysis of soluble proteins in four Portuguese wines. Am J Enol Vitic 46: 235-242

Downton WJS, Hawker JS (1973) Enzymes of starch metabolism in leaves and berries of *Vitis vinifera*. Phytochem 12: 1557-1563

Dowton WJS, Loveys BR (1978) Compositional changes during grape berry development in relation to abscisic acid and salinity. Aust J Plant Physiol 5: 415-423

Edelbaum O, Sher N, Rubinstein M, Novick D, Tal N, Moyer M, Ward E, Ryals J, Sela I (1991) Two antiviral proteins, gp35 and gp22, correspond to β-1,3-glucanase and an isoform of PR-5. Plant Mol Biol 17: 171-173

Esaka M, Toyota A, Kitabayashi M (1993) Marked induction of basic chitinase in pumpkin in response to wounding. Phytochem 34: 631-635

Ferenczy S (1966) Étude proteines et substances azotées. Bull OIV 39: 1313-1336

Fernandez C, Szyperski T, Bruyere T, Ramage P, Mosinger E, Wuthrich K (1997) NMR solution structure of the pathogenesis-related protein P14a. J Mol Biol 266: 576-593

Feuillat M, Ferrari G (1982) Hydrolyse enzymatique des protéines du raison en vinification. C R Seances Acad Agric Fr 68: 1070-1075

Fils-Lycaon BR, Wiersma PA, Eastwell KC, Sautiere P (1996) A cherry protein and its gene, abundantly expressed in ripening fruit, have been identified as thaumatin-like. Plant Physiol 111: 269-273

Fling SP, Gregerson DS (1986) Peptide and protein molecular weight determination by electrophoresis using high-molarity Tris buffer system without urea. Anal Biochem 155: 83-88

Flores JH, Heatherbell DA, Hsu JC, Watson BT (1988) Ultrafiltration (UF) of White Riesling juice: Effect of oxidation and pre-UF juice treatment on flux, composition and stability. Am J Enol Vitic 39: 180-187

Flores JH, Heatherbell DA, McDaniel MR (1990) Ultrafiltration of wine: Effect of ultrafiltration on White Riesling and Gewurztraminer wine composition and stability. Am J Enol Vitic 41: 207-214

Ford CM, Høj PB (1998) Multiple glucosyltransferase activities in the grapevine *Vitis vinifera* L. Aust J Grape Wine Res 4: 48-58

Franks T, He DG, Thomas M (1998) Regeneration of transgenic *Vitis vinifera* L. Sultana plants - Genotypic and phenotypic analysis. Mol Breeding 4: 321-333

Friedrich L, Moyer M, Ward E, Ryals J (1991) Pathogenesis-related protein 4 is structurally homologous to the carboxy-terminal domains of hevein, Win-1 and Win-2. Mol Gen Genet 230: 113-119

Frohman MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc Nat Acad Sci USA 85: 8998-9002

Gianinazzi S, Martin C, Vallée JC (1970) Hypersensibilité aux virus, température et protéines solubles chez le *Nicotiana* Xanthi nc. Apparition de nouvelles macromolécules lorsde la repression de la synthése virale. C R Acad Sci Paris (Ser D) 270: 2383-2386

Giannakis C, Bucheli CS, Skene KGM, Robinson SP, Scott NS (1998) Chitinase and β-1,3-glucanase in grapevine leaves: a possible defence against powdery mildew infection. Aust J Grape Wine Res 4: 14-22

Gomes VM, Oliveira AEA, Xavier J (1996) A chitinase and a β-1,3-glucanase isolated from the seeds of cowpea (*Vigna unguiculata* l walp) inhibit the growth of fungi and insect pests of the seed. J Sci Food Agric 72: 86-90

Graham JS, Burkhart W, Xiong J, Gillikin JW (1992) Complete amino acid sequence of soybean leaf P21. Similarity to thaumatin-like polypeptides. Plant Physiol 98: 163-165

Graham LS, Sticklen MB (1994) Plant chitinases. Can J Bot 72: 1057-1083

Green R, Fluhr R (1995) UV-B-induced PR-1 accumulation is mediated by active oxygen species. Plant Cell 7: 203-212

Gregersen PL, Thordal-Christensen H, Förster H, Collinge DB (1997) Differential gene transcript accumulation in barley leaf epidermis and mesophyll in response to attack by *Blumeria graminis* f.sp. *hordei* (syn. *Erysiphe graminis* f.sp. *hordei*). Physiol Mol Plant Path 51: 85-97

Grison R, Grezes-Besset B, Schneider M, Lucante N, Olsen L, Leguay J, Toppan A (1996) Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a chimeric chitinase gene. Nature Biotech 14: 643-646

Grotewold E, Athma P, Peterson T (1991) Alternatively spliced products of the maize *P* gene encode proteins with homology to the DNA-binding domain of *myb*-like transcription factors. Proc Nat Acad Sci USA 88: 4587-4591

Grotewold E, Drummond BJ, Bowen B, Peterson T (1994) The *myb*-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavanoid biosynthetic gene subset. Cell 76: 543-553

Guest D, Brown J (1997) Plant defences against pathogens. In: Brown JE, Ogle HJ (eds), Plant pathogens and plant diseases. University of New England Printery, Armidale, Australia, pp 263-286

Ham KS, Wu SC, Darvill AG, Albersheim P (1997) Fungal pathogens secrete an inhibitor protein that distinguishes isoforms of plant pathogenesis-related endo-β-1,3-glucanases. Plant J 11: 169-179

Hanfrey C, Fife M, Buchanan Wollaston V (1996) Leaf senescence in *Brassica napus*: expression of genes encoding pathogenesis-related proteins. Plant Mol Biol 30: 597-609

Harikrishna K, Jampates-Beale R, Milligan SB, Gasser CS (1996) An endochitinase gene expressed at high levels in the stylar transmitting tissue of tomatoes. Plant Mol Biol 30: 899-911

Harlow E, Lane D (1988) Antibodies. A laboratory manual. Cold Spring Harbor Laboratory Press, New York, pp 417-510

Harris N, Taylor JE, Roberts JA (1997) Characterization and expression of an mRNA encoding a wound-induced (Win) protein from ethylene-treated tomato leaf abscission zone tissue. J Exp Bot 48: 1223-1227

Harrison MJ, Lawton MA, Lamb CJ, Dixon RA (1991) Characterization of a nuclear protein that binds to three elements within the silencer region of a bean chalcone synthase gene promoter. Proc Nat Acad Sci USA 88: 2515-2519

Hawker JS (1969a) Changes in the activities of enzymes concerned with sugar metabolism during the development of grape berries. Phytochem 8: 9-17

Hawker JS (1969b) Insoluble invertase from grapes: an artifact of extraction. Phytochem 8: 337-344

Hawker JS, Woodham RC, Downton WJS (1974) Activity of β-1,3-glucan hydrolase in virus infected grapevines and other plants. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 81: 100-107

Heatherbell DA, Ngaba P, Fombin J, Watson B, Garcia Z, Flores J, Hsu J (1984) Recent developments in the application of ultafiltration and protease enzymes to grape juice and wine processing. In: Proceedings of the International Symposium on Cool Climate Viticulture and Enology, Corvallis, USA (Oregon State University): pp 418-445

Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Anako EA, Podkolodnaya OA, Kolpakov FA, Podkolondny NL, Kolchanov NA (1998) Databases on transcriptional regulation: TRANSFAC, TRRD, and COMPEL. Nucleic Acids Res 26: 364-370

Heitz T, Geoffroy P, Stintzi A, Fritig B, Legrand M (1993) cDNA cloning and gene expression analysis of the microbial proteinase inhibitor of tobacco. J Biol Chem 268: 16987-16992

Hejgaard J, Jacobsen S, Bjorn SE, Kragh KM (1992) Antifungal activity of chitin-binding PR-4 type proteins from barley grain and stressed leaf. FEBS Lett 307: 389-392

Hejgaard J, Jacobsen S, Svendsen I (1991) Two antifungal thaumatin-like proteins from barley grain. FEBS Lett 291: 127-131

Helms C, Graham MY, Dutchik JE, Olson MV (1985) A new method for purifying lambda DNA from phage lysates. DNA 4: 39-49

Hensel C, Brosius M, Maeting I, Wartmann T, Horstmann C, Kunze G, Kunze I (1997) Cloning of the wound-inducible protein CBP20 and expression in suspension cultures of tobacco. Plant Sci 128: 199-206

Herbers K, Meuwly P, Frommer WB, Métraux JP, Sonnewald U (1996a) Systemic acquired resistance mediated by the ectopic expression of invertase: possible hexose sensing in the secretory pathway. Plant Cell 8: 793-803

Herbers K, Meuwly P, Métraux JP, Sonnewald U (1996b) Salicylic acid-independent induction of pathogenesis-related protein transcripts by sugars is dependent on leaf developmental stage. FEBS Lett 397: 239-244

Herbers K, Monke G, Badur R, Sonnewald U (1995) A simplified procedure for the subtractive cDNA cloning of photoassimilate-responding genes: isolation of cDNAs encoding a new class of pathogenesis-related proteins. Plant Mol Biol 29: 1027-1038

Høj PB, Fincher GB (1995) Molecular evolution of plant β-glucan endohydrolases. Plant J 7: 367-379

Høj PB, Hartman DJ, Morrice NA, Doan DNP, Fincher GB (1989) Purification of (1→3)-β-glucan endohydrolase isoenzyme II from germinated barley and determination of its primary structure from a cDNA clone. Plant Mol Biol 13: 31-42

Høj PB, Svendsen I, Scheller HV, Møller BL (1987) Identification of a chloroplast-encoded 9-kDa polypeptide as a 2[4Fe-4S] protein carrying centers A and B of Photosystem I. J Biol Chem 262: 12676-12684

Hon WC, Griffith M, Mlynarz A, Kwok YC, Yang DSC (1995) Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. Plant Physiol 109: 879-889

Hooft van Huijsduijnen RAM, van Loon LC, Bol JF (1986) Complementary DNA cloning of six messenger RNA species induced by tobacco mosaic virus infection of tobacco and a characterization of their translation products. EMBO J 5: 2057-2062

Hrmova M, Fincher GB (1993) Purification and properties of three (1,3)-β-D-glucanase isoenzymes from young leaves of barley (*Hordeum vulgare*). Biochem J 289: 453-461

Hsu JC, Heatherbell DA (1987a) Isolation and characterization of soluble proteins in grapes, grape juice, and wine. Am J Enol Vitic 38: 6-10

Hsu J, Heatherbell DA (1987b) Heat-unstable proteins in wine. I. Characterization and removal by bentonite fining and heat treatment. Am J Enol Vitic 38: 11-16

Hsu J, Heatherbell DA, Flores JH, Watson BT (1987) Heat-unstable proteins in wine. II. Characterization and removal by ultrafiltration. Am J Enol Vitic 38: 17-22

Hu X, Reddy ASN (1997) Cloning and expression of a PR5-like protein from *Arabidopsis*: inhibition of fungal growth by bacterially expressed protein. Plant Mol Biol 34: 949-959

Huang X, Miller M (1991) A time-efficient, linear-space local similarity algorithm. Adv Appl Math 12: 337-367

Hunt AG, Messing J (1998) mRNA polyadenylation in plants. In: Bailey-Serres J, Gallie DR (eds), A look beyond transcription: Mechanisms determining mRNA stability and translation in plants. American Society of Plant Physiologists, Rockville, pp 29-39

Huynh QK, Borgmeyer JR, Zobel JF (1992a) Isolation and characterization of a 22 kDa protein with antifungal properties from maize seeds. Biochem Biophys Res Commun 182: 1-5

Huynh QK, Hironaka CM, Levine EB, Smith CE, Borgmeyer JR, Shah DM (1992b) Antifungal proteins from plants. Purification, molecular cloning, and antifungal properties of chitinases from maize seed. J Biol Chem 267: 6635-6640

Inschlag C, Hoffmann-Sommergruber K, O'Riordain G, Ahorn H, Ebner C, Scheiner O, Breiteneder H (1998) Biochemical characterization of Pru a 2, a 23-kD thaumatin-like protein representing a potential major allergen in cherry (*Prunus avium*). Int Arch Allergy Appl Immunol 116: 22-28

Iyengar RB, Smits P, van der Ouderaa F, van der Wel H, van Brouwershaven J, Ravestein P, Richters G, van Wassenaar PD (1979) The complete amino-acid sequence of the sweet protein thaumatin I. Eur J Biochem 96: 193-204

Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf P, Leah R, Schell J, Maas C (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J 8: 97-109

Jongedijk E, Tigelaar H, van Roekel JSC, Bres-Vloemans SA, Dekker I, van den Elzen PJM, Cornelissen BJC, Melchers LS (1995) Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. Euphytica 85: 173-180

Joosten MHAJ, Bergmans CJB, Meulenhoff EJS, Cornelissen BJC, De Wit PJGM (1990) Purification and serological characterization of three basic 15-kilodalton pathogenesis-related proteins from tomato. Plant Physiol 94: 585-591

Joshi CP (1987) An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucleic Acids Res 15: 6643-6653

Jung JL, Maurel S, Fritig B, Hahne G (1995) Different pathogenesis-related-proteins are expressed in sunflower (*Helianthus annuus* L.) in response to physical, chemical and stress factors. J Plant Physiol 145: 153-160

Kanellis AK, Roubelakis-Angelakis KA (1993) Grape. In: Seymour GB, Taylor JE, Tucker GA (eds), Biochemistry of fruit ripening. Chapman and Hall, Cambridge, pp 189-234

Kassanis B, Gianinazzi S, White RF (1974) A possible explanation of the resistance of virus-infected tobacco plants to second infection. J Gen Virol 23: 11-16

Kauffmann S, Legrand M, Fritig B (1990) Isolation and characterization of six pathogenesis-related (PR) proteins of Samsun NN tobacco. Plant Mol Biol 14: 381-390

Kauffmann S, Legrand M, Geoffroy P, Fritig B (1988) Biological function of 'pathogenesis-related' proteins: four PR proteins of tobacco have 1,3-β-glucanase activity. EMBO J 6: 3209-3212

Kim SH, de Vos A, Ogata C (1988) Crystal structures of two intensely sweet proteins. TIBS 13: 13-15

King GJ, Turner VA, Hussey CE, Jr., Wurtele ES, Lee SM (1988) Isolation and characterization of a tomato cDNA clone which codes for a salt-induced protein. Plant Mol Biol 10: 401-412

Kliewer WM (1965) Changes in the concentration of malates, tartrates, and total free acids solids in flowers and berries of *Vitis vinifera*. Am J Enol Vitic 16: 92-100

Ko TP, Day J, Greenwood A, McPherson A (1994) Structures of three crystal forms of the sweet protein thaumatin. Acta Crystallogr Sect D Biol Cryst 50: 813-825

Koch J, Sajak E (1959) A review and some studies on grape protein. Am J Enol Vitic 18: 114-123

Koiwa H, Kato H, Nakatsu T, Oda J, Yamada Y, Sato F (1997) Purification and characterization of tobacco pathogenesis-related protein PR-5d, an antifungal thaumatin-like protein. Plant Cell Physiol 38: 783-791

Koiwa H, Kato H, Nakatsu T, Oda J, Yamada Y, Sato F (1999) Crystal structure of tobacco PR-5d protein at 1.8 Å resolution reveals a conserved acidic cleft structure in antifungal thaumatin-like proteins. J Mol Biol 286: 1137-1145

Koiwa H, Sato F, Yamada Y (1994) Characterization of accumulation of tobacco PR-5 proteins by IEF-immunoblot analysis. Plant Cell Physiol 35: 821-827

Kononowicz AK, Nelson DE, Singh NK, Hasegawa PM, Bressan RA (1992) Regulation of the osmotin gene promoter. Plant Cell 4: 513-524

Koradi R, Billeter M, Würthrich K (1996) MOLMOL: a program for display and analysis of macromolecular structures. J Mol Graphics 14: 51-55

Kraeva E, Tesniere C, Terrier N, Romieu C, Sauvage FX, Bierne J, Deloire A (1998) Transcription of a β-1,3-glucanase gene in grape berries in a late developmental period, or earlier after wounding treatments. Vitis 37: 107-111

Kuboyama T, Yoshida KT, Takeda G (1997) An acidic 39-kDa protein secreted from stigmas of tobacco has an amino-terminal motif that is conserved among thaumatin-like proteins. Plant Cell Physiol 38: 91-95

Lagace LS, Bisson LF (1990) Survey of yeast acid proteases for effectiveness of wine haze reduction. Am J Enol Vitic 41: 147-155

Lagramini LM, Rothstein S (1987) Tissue-specificity of tobacco peroxidase isoenzymes and their induction by wounding and tobacco mosaic infection. Plant Physiol 84: 438-442

LaRosa PC, Chen Z, Nelson DE, Singh NK, Hasegawa PM, Bressan RA (1992) Osmotin gene expression is posttranscriptionally regulated. Plant Physiol 100: 409-415

Lawrence CB, Joosten MHA, Tuzun S (1996) Differential induction of pathogenesis-related proteins in tomato by *Alternaria solani* and the association of a basic chitinase isozyme with resistance. Physiol Mol Plant Path 48: 361-377

Lawton MA, Dean SM, Dron M, Kooter JM, Kragh KM, Harrison MJ, Yu L, Tanguay L, Dixon RA, Lamb CJ (1991) Silencer region of a chalcone synthase promoter contains multiple binding sites for a factor, SBF-1, closely related to GT-1. Plant Mol Biol 16: 235-249

Leah R, Tommerup H, Svendsen I, Mundy J (1991) Biochemical and molecular characterization of three barley seed proteins with antifungal properties. J Biol Chem 266: 1564-1573

Ledoux V, Dulau L, Dubourdieu D (1992) Interprétation de l'amélioration de la stabilité protéique des vins au cours de l'élevage sur lies. J Int Sci Vigne Vin 26: 239-251

Lee HI, Broekaert WF, Raikhel NV (1991) Co- and post-translational processing of the hevein preproprotein of latex of the rubber tree (*Hevea brasiliensis*). J Biol Chem 266: 15944-15948

Legrand M, Kauffmann S, Geoffroy P, Fritig B (1987) Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinases. Proc Nat Acad Sci USA 84: 6750-6754

Levi A, Galau GA, Wetzstein HY (1992) A rapid procedure for the isolation of RNA from high-phenolic-containing tissues of pecan. Hortscience 27: 1316-1318

Linthorst HJM (1991) Pathogenesis-related proteins of plants. Crit Rev Plant Sci 10: 123-150

Linthorst HJM, Danhash N, Brederode FT, van Kan JAL, De Wit PJGM, Bol JF (1991) Tobacco and tomato PR proteins homologous to *win* and pro-hevein lack the "hevein" domain. Mol Plant Microbe Interact 4: 586-592

Linthorst HJM, Meuwissen RLJ, Kauffmann S, Bol JF (1989) Constitutive expression of pathogenesis-related proteins PR-1, GRP, and PR-S in tobacco has no effect on virus infection. Plant Cell 1: 285-291

Linthorst HJM, van Loon LC, van Rossum CMA, Mayer A, Bol JF, van Roekel JSC, Meulenhoff EJS, Cornelissen BJC (1990) Analysis of acidic and basic chitinases from tobacco and petunia and their constitutive expression in transgenic tobacco. Mol Plant Microbe Interact 3: 252-258

Liu D, Raghothama KG, Hasegawa PM, Bressan RA (1994) Osmotin overexpression in potato delays development of disease symptoms. Proc Nat Acad Sci USA 91: 1888-1892

Lorito M, Woo SL, D'Ambrosia M, Harman GE, Hayes CK, Kubicek CP, Scala F (1996) Synergistic interaction between cell wall degrading enzymes and membrane affecting compounds. Mol Plant Microbe Interact 9: 206-213

Lotan T, Ori N, Fluhr R (1989) Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell 1: 881-887

Loulakakis KA (1997a) Genomic organization and expression of an osmotin-like gene in *Vitis vinifera* L. Vitis 36: 157-158

Loulakakis KA (1997b) Nucleotide sequence of a *Vitis vinifera* L. cDNA (Ascession No. Y10992) encoding for osmotin-like protein. (PGR97-064). Plant Physiol 113: 1464

Lucas J, Henriuez AC, Lottspeich F, Henschen A, Sänger HL (1985) Amino acid sequence of the 'pathogenesis-related' leaf protein P14 from viroid-infected tomato reveals a new type of structurally unrelated protein. EMBO J 4: 2745-2749

Ludvigsen S, Poulsen FM (1992a) Secondary structure in solution of barwin from barley seed using ^{1}H nuclear magnetic resonance spectroscopy. Biochem 31: 8771-8782

Ludvigsen S, Poulsen FM (1992b) Three-dimensional structure in solution of barwin, a protein from barley seed. Biochem 31: 8783-8789

Luis ES (1983) A study of proteins during grape maturation, juice preparation and wine processing. Thesis, Doctor of Philosophy, The University of New South Wales, Sydney, Australia

Malehorn DE, Borgmeyer ER, Smith CE, Shah DM (1994) Characterization and expression of an antifungal zeamatin-like protein (*Zlp*) gene from *Zea mays*. Plant Physiol 106: 1471-1481

Masoud SA, Zhu Q, Lamb C, Dixon RA (1996) Constitutive expression of an inducible β-1,3-glucanase in alfalfa reduces disease severity caused by the oomycete pathogen *Phytophthora megasperma* f. sp *medicaginis*, but does not reduce disease severity of chitin-containing fungi. Transgenic Res 5: 313-323

Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol 88: 936-942

Mauch F, Staehelin LA (1989) Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell 1: 447-457

Mauro MC, Toutain S, Walter B, Pinck L, Otten L, Coutos-Thevenot P, Deloire A, Barbier P (1995) High efficiency regeneration of grapevine plants transformed with the GFLV coat protein gene. Plant Sci 112: 97-106

McCollum TG, Doostdar H, Mayer RT, McDonald RE (1997) Characterization of chitinases and β-1,3-glucanases in grapefruit flavedo during fruit development. Physiol Plant 99: 486-494

McLeod LC, Poole PR (1994) Changes in enzymic activities after harvest and in early stages of *Botrytis cinerea* infection of kiwifruit. J Sci Food Agric 64: 95-100

Medina-Suárez R, Manning K, Fletcher J, Aked J, Bird CR, Seymour GB (1997) Gene expression in the pulp of ripening bananas: Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of in vitro translation products and cDNA cloning of 25 different ripening-related mRNAs. Plant Physiol 115: 453-461

Meinkoth J, Wahl G (1984) Hybridization of nucleic acids immobilized on solid supports. Anal Biochem 138: 267-284

Meins Jr. F, Neuhaus JM, Speisen C, Ryals J (1992) The primary structure of plant pathogenesis-related glucanhydrolases and their genes. In: Meins Jr. F, Boller T (eds), Genes Involved in Plant Defense. Springer-Verlag, New York, pp 245-282

Melchers LS, Apotheker-de-Groot M, van der Knaap J, Ponstein AS, Sela-Buurlage MB, Bol JF, Cornelissen BJC, van den Elzen PJM, Linthorst HJM (1994) A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. Plant J 5: 469-480

Melchers LS, Sela-Buurlage MB, Vloemans SA, Woloshuk CP, Van Roekel JSC, Pen J, van den Elzen PJM, Cornelissen BJC (1993) Extracellular targeting of the vacuolar tobacco proteins AP24, chitinase and β-1,3-glucanase in transgenic plants. Plant Mol Biol 21: 583-593

Metzler MC, Cutt JR, Klessig DF (1991) Isolation and characterization of a gene encoding a PR-1-like protein from *Arabidopsis thaliana*. Plant Physiol 96: 346-348

Meyer B, Houlne G, Pozuetaromero J, Schantz ML, Schantz R (1996) Fruit-specific expression of a defensin-type gene family in bell pepper - upregulation during ripening and upon wounding. Plant Physiol 112: 615-622

Miller GC, Amon JM, Gibson RL, Simpson RF (1985) Loss of wine aroma attributable to protein stabilization with bentonite or ultrafiltration. Aust Grapegrower Winemaker 256: 46-50

Miller H (1987) Practical aspects of preparing phage and plasmid DNA: Growth, maintenance, and storage of bacteria and bacteriophage. In: Berger SL, Kimmel AR (eds), Methods in Enzymology: Guide to molecular cloning techniques. Academic Press, London, pp 145-170

Moons A, Prinsen E, Bauw G, Van Montagu M (1997) Antagonistic effects of abscisic acid and jasmonates on salt stress-inducible transcripts in rice roots. Plant Cell 9: 2243-2259

Moore AE, Stone BA (1972) Effect of infection with TMV and other viruses on the level of a β-1,3-glucan hydrolase in the leaves of *Nicotiana glutinosa*. Virology 50: 791-798

Mosimamm S, Meleshko R, James MNG (1995) A critical assessment of comparative molecular modeling of tertiary structures of proteins. Proteins: Stuct Funct Genet 23: 301-317

Mouradov A, Mouradova E, Scott KJ (1994) Gene family encoding basic pathogenesis-related 1 proteins in barley. Plant Mol Biol 26: 503-507

Mouradov A, Petrasovits L, Davidson A, Scott KJ (1993) A cDNA clone for a pathogenesis-related protein 1 from barley. Plant Mol Biol 23: 439-442

Murphey JM, Spayd SE, Powers JR (1989) Effect of grape maturation on soluble protein characteristics of Gewürztraminer and White Riesling juice and wine. Am J Enol Vitic 40: 199-207

Nairn CJ, Niedz RP, Hearn CJ, Osswald WF, Mayer RT (1997) cDNA cloning and expression of a class II chitinase from sweet orange. Biochem Biophys Acta 1351: 22-26

Nakagawa S, Nanjo Y (1965) A morphological study of Delaware grape berries. J Jpn Soc Hortic Sci 34: 85-95

Nassuth A, Sanger HL (1986) Immunological relationship between "pathogenesis-related" leaf proteins from tomato, tobacco and cowpea. Virus Res 154: 229-242

Neale AD, Wahleithner JA, Lund M, Bonnett HT, Kelly A, Meeks-Wagner DR, Peacock WJ, Dennis ES (1990) Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell 2: 673-684

Nelson DE, Raghothama KG, Singh NK, Hasegawa PM, Bressan RA (1992) Analysis of structure and transcriptional activation of an osmotin gene. Plant Mol Biol 19: 577-588

Neuhaus JM, Fritig B, Linthorst HJM, Meins Jr. F, Mikkelsen JD, Ryals J (1996) A revised nomenclature for chitinase genes. Plant Mol Biol Reptr 14: 102-104

Ngaba-Mbiakop PR (1981) Investigation of methods for determination and prevention of protein instability in wines. Thesis, Doctor of Philosophy, Oregon State University, Corvallis, USA

Niderman T, Genetet I, Bruyere T, Gees R, Stintzi A, Legrand M, Fritig B, Mosinger E (1995) Pathogenesis-related PR-1 proteins are antifungal. Isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against *Phytophthora infestans*. Plant Physiol 108: 17-27

Ogata CM, Gordon PF, de Vos AM, Kim S (1992) Crystal structure of a sweet tasting protein thaumatin I, at 1.65 Å resolution. J Mol Biol 228: 893-908

Ori N, Sessa G, Lotan T, Himmelhoch S, Fluhr R (1990) A major stylar matrix polypeptide (sp41) is a member of the pathogenesis-related proteins superclass. EMBO J 9: 3429-3436

Oshima M, Harada N, Matsuoka M, Ohashi Y (1990) The nucleotide sequence of the pathogenesis-related (PR) 1b protein gene of tobacco. Nucleic Acids Res 18: 181

Oshima M, Matsuoka M, Yamamota N, Tanaka Y, Kano-Murakami Y, Ozeki Y, Kato A, Harada N, Oshashi Y (1987) Nucleotide sequence of the PR-1 gene of *Nicotiana tabacum*. FEBS Lett 225: 243-246

Ough CS (1992) Winemaking basics. Food Products Press, Norwood (Australia)

Paxton JD, Groth J (1994) Constraints on pathogens attacking plants. Crit Rev Plant Sci 13: 77-95

Payne G, Middlesteadt W, Desai N, Williams S, Dincher S, Carnes M, Ryals J (1989) Isolation and sequence of a genomic clone encoding the basic form of pathogenesis-related protein 1 from *Nicotiana tabacum*. Plant Mol Biol 12: 595-596

Payne G, Middlesteadt W, Williams S, Desai N, Parks TD, Dincher S, Carnes M, Ryals J (1988) Isolation and nucleotide sequence of a novel cDNA clone encoding the major form of pathogenesis-related protein R. Plant Mol Biol 11: 223-224

Peitsch MC (1996) ProMod and Swiss-Model: Internet-based tools for automated comparative protein modelling. Biochem Soc Trans 24: 274-279

Peitsch MC, Guex N (1997) Large-scale comparative protein modelling. In: Wilkins MR, Williams KL, Appel RO, Hochstrasser DF (eds), Proteome research: new frontiers in functional genomics. Springer-Verlag, Berlin, pp 177-186

Peng ZK, Pocock KF, Waters EJ, Francis IL, Williams PJ (1997) Taste properties of grape (*Vitis vinifera*) pathogenesis-related proteins isolated from wine. J Agric Food Chem 45: 4639-4643

Peri C, Riva M, Decio P (1988) Crossflow membrane filtration of wines: Comparison of performance of ultrafiltration, microfiltration, and intermediate cut-off membranes. Am J Enol Vitic 39: 162-168

Perl A, Lotan O, Abuabied M, Holland D (1996) Establishment of an *Agrobacterium*-mediated transformation system for grape (*Vitis vinifera* L.) - the role of antioxidants during grape-*Agrobacterium* interactions. Nature Biotech 14: 624-628

Peumans WJ, Van Damme EJM (1995) Lectins as plant defense proteins. Plant Physiol 109: 347-352

Pfitzner AJP, Beilmann A, Goodman HM, Pfitzner UM (1991) Molecular analysis of two PR-1 pseudogenes from tobacco. Plant Mol Biol 16: 129-139

Pierpoint WS, Jackson PJ, Evans RM (1990) The presence of a thaumatin-like protein, a chitinase and a glucanase among the pathogenesis-related proteins of potato (*Solanum tuberosum*). Physiol Mol Plant Path 36: 325-338

Pocock KF, Hayasaka Y, Peng Z, Williams PJ, Waters EJ (1998) The effect of mechanical harvesting and long-distance transport on the concentration of haze-forming proteins in grape juice. Aust J Grape Wine Res 4: 23-29

Pocock KF, Rankine BC (1973) Heat test for detecting protein stability in wine. Aust Wine Brew Spirit Rev 91: 42-43

Ponstein AS, Bres-Vloemans SA, Sela-Buurlage MB, van den Elzen PJM, Melchers LS, Cornelissen BJC (1994) A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. Plant Physiol 104: 109-118

Potter S, Uknes S, Lawton K, Winter AM, Chandler D, DiMaio J, Novitzky R, Ward E, Ryals J (1993) Regulation of a hevein-like gene in *Arabidopsis*. Mol Plant Microbe Interact 6: 680-685

Pressey R (1997) Two isoforms of NP24: a thaumatin-like protein in tomato fruit. Phytochem 44: 1241-1245

Pueyo E, Dizy M, Polo MC (1993) Varietal differentiation of must and wines by means of protein fraction. Am J Enol Vitic 44: 255-260

Punja ZK, Raharjo SHT (1996) Response of transgenic cucumber and carrot plants expressing different chitinase enzymes to inoculation with fungal pathogens. Plant Disease 80: 999-1005

Raghothama KG, Liu D, Nelson DE, Hasegawa PM, Bressan RA (1993) Analysis of an osmotically regulated pathogenesis-related osmotin gene promoter. Plant Mol Biol 23: 1117-1128

Raghothama KG, Maggio A, Narasimhan ML, Kononowicz AK, Wang GL, Durzo MP, Hasegawa PM, Bressan RA (1997) Tissue-specific activation of the osmotin gene by ABA, C_2H_4 and NaCl involves the same promoter region. Plant Mol Biol 34: 393-402

Raikhel NV, Lee HI, Broekaert WF (1993) Structure and function of chitin-binding proteins. Ann Rev Plant Physiol Plant Mol Biol 44: 591-615

Rankine BC (1989) Making good wine : a manual of wine making practice for Australia and New Zealand. Sun books, Melbourne

Renault AS, Deloire A, Bierne J (1996) Pathogenesis-related proteins in grapevines induced by salicylic acid and *Botrytis cinerea*. Vitis 35: 49-52

Richard L, Arro M, Hoebeke J, Meeks-Wagner DR, Tran Thanh Van K (1992) Immunological evidence of thaumatin-like proteins during tobacco floral differentiation. Plant Physiol 98: 337-342

Richardson M, Valdes-Rodriguez S, Blanco-Labra A (1987) A possible function for thaumatin and a TMV-induced protein suggested by homology to a maize inhibitor. Nature 327: 432-434

Roberts WK, Selitrennikoff CP (1986) Isolation and partial characterization of two antifungal proteins from barley. Biochem Biophys Acta 880: 161-170

Roberts WK, Selitrennikoff CP (1988) Plant and bacterial chitinases differ in antifungal activity. J Gen Microbiol 134: 169-176

Roberts WK, Selitrennikoff CP (1990) Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity. J Gen Microbiol 136: 1771-1778

Robinson SP, Jacobs AK, Dry IB (1997) A class IV chitinase is highly expressed in grape berries during ripening. Plant Physiol 114: 771-778

Rodrigo I, Vera P, Conejero V (1989) Degradation of tomato pathogenesis-related proteins by an endogenous 37-kDa aspartyl endoproteinase. Eur J Biochem 184: 663-669

Rodrigo I, Vera P, Frank R, Conejero V (1991a) Identification of the viroid-induced tomato pathogenesis-related (PR) protein P23 as the thaumatin-like tomato protein NP24 associated with osmotic stress. Plant Mol Biol 16: 931-934

Rodrigo I, Vera P, van Loon LC, Conejero V (1991b) Degradation of tobacco pathogenesis-related proteins. Evidence for conserved mechanisms of degradation of pathogenesis-related proteins in plants. Plant Physiol 95: 616-622

Ross AF (1961) Systemic aquired resistance induced by localized infections in plants. Virology 14: 340-358

Ryals J, Uknes S, Ward E (1994) Systemic acquired resistance. Plant Physiol 104: 1109-1112

Salzman RA, Tikhonova I, Bordelon BP, Hasegawa PM, Bressan RA (1998) Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense response during fruit ripening in grape. Plant Physiol 117: 465-472

Samac DA, Shah DM (1994) Effect of chitinase antisense RNA expression on disease susceptibility of *Arabidopsis* plants. Plant Mol Biol 25: 587-596

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual (2nd ed.). Cold Spring Harbor Laboratory Press, New York

Sánchez R, Šali A (1998) Large-scale protein structure modeling of the *Saccharomyces cerevisiae* genome. Proc Nat Acad Sci USA 95: 13597-13602

Sandermann H, Ernst D, Heller W, Langebartels C (1998) Ozone - an abiotic elicitor of plant defence reactions. TIPS 3: 47-50

Santoro M (1995) Fractionation and characterization of must and wine proteins. Am J Enol Vitic 46: 250-254

Sassa H, Hirano H (1998) Style-specific and developmentally regulated accumulation of a glycosylated thaumatin/PR5-like protein in japanese pear (*Pyrus serotina* rehd.). Planta 205: 514-521

Sato F, Kitajima S, Koyama T, Yamada Y (1996) Ethylene-induced gene expression of osmotin-like protein, a neutral isoform of tobacco PR-5, is mediated by the AGCCGCC *cis*-sequence. Plant Cell Physiol 37: 249-255

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166: 368-379

Schlumbaum A, Mauch F, Vögeli U, Boller T (1986) Plant chitinases are potent inhibitors of fungal growth. Nature 324: 365-367

Scorza R, Cordts JM, Gray DJ, Gonsalves D, Emershad RL, Ramming DW (1996) Producing transgenic 'Thompson Seedless' grape (*Vitis vinifera* L.) plants. J Am Soc Hort Sci 121: 616-619

Sedmak JJ, Grossberg SE (1977) A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Anal Biochem 79: 544-552

Sela-Buurlage MB, Ponstein AS, Bres-Vloemans SA, Melchers LS, van den Elzen PJM, Cornelissen BJC (1993) Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol 101: 857-863

Shinshi H, Mohnen D, Meins Jr. F (1987) Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc Nat Acad Sci USA 84: 89-93

Shinshi H, Neuhaus J, Ryals J, Meins Jr. F (1990) Structure of a tobacco endochitinase gene: Evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine rich domain. Plant Mol Biol 14: 357-368

Shirras AD, Northcote DH (1984) Molecular cloning and characterisation of cDNAs complementary to mRNAs from wounded potato (*Solanum tuberosum*). Planta 162: 353-360

Simmons CR (1994) The physiology and molecular biology of plant 1,3-β-D-glucanases and 1,3;1,4-β-D-glucanases. Crit Rev Plant Sci 13: 325-387

Simpson GG, Filipowicz W (1996) Splicing of precursors to messenger RNA in higher plants: mechanism, regulation and sub-nuclear organisation of the spliceosomal machinery. Plant Mol Biol 32: 1-41

Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA, Pfankoch E, Regnier FE, Bressan RA (1987) Characterisation of osmotin. A thaumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiol 85: 529-536

Singh NK, Nelson DE, Kuhn D, Hasegawa PM, Bressan RA (1989) Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. Plant Physiol 90: 1096-1101

Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell 2: 503-512

Slootstra JW, De Geus P, Haas H, Verrips CT, Meloen RH (1995) Possible active site of the sweet-tasting protein thaumatin. Chem Senses 20: 535-543

Somers TC, Ziemelis G (1973a) Direct determination of wine proteins. Am J Enol Vitic 24: 47-50

Somers TC, Ziemelis G (1973b) The use of gel column analysis in evaluation of bentonite fining procedures. Am J Enol Vitic 24: 51-54

Stanford A, Bevan M, Northcote D (1989) Differential expression within a family of novel wound-induced genes in potato. Mol Gen Genet 215: 200-208

Steenkamp J, Wiid I, Lourens A, van Helden P (1994) Improved method for DNA extraction from *Vitis vinifera*. Am J Enol Vitic 45: 102-106

Stevens C, Titarenko E, Hargreaves JA, Gurr SJ (1996) Defence-related gene activation during an incompatible interaction between *Stagonospora* (*Septoria*) *nodorum* and barley (*Hordeum vulgare* L.) coleoptile cells. Plant Mol Biol 31: 741-749

Stintzi A, Heitz T, Kauffmann S, Legrand M, Fritig B (1991) Identification of a basic pathogenesis-related thaumatin-like protein of virus-infected tobacco as osmotin. Physiol Mol Plant Path 38: 137-146

Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B (1993) Plant 'pathogenesis-related' proteins and their role in defense against pathogens. Biochimie 75: 687-706

Stone BA, Clarke AE (1993) The chemistry and biology of (1→3)-β-glucans. La Trobe University Press, Bundora, Victoria, Australia

Suami T, Hough L, Machinami T, Watanabe N, Nakamura R (1997) Molecular mechanisms of sweet taste 7 - the sweet protein, thaumatin I. Food Chem 60: 277-285

Svensson B, Svendsen I, Højrup P, Roepstorff P, Ludvigsen S, Poulsen FM (1992) Primary structure of barwin: A barley seed protein closely related to the C-terminal domain of proteins encoded by wound-induced plant genes. Biochem 31: 8767-8770

Swoboda I, Scheiner O, Kraft D, Breitenbach M, Heberle Bors E, Vicente O (1994) A birch gene family encoding pollen allergens and pathogenesis-related proteins. Biochem Biophys Acta 1219: 457-464

Szybiak Strozycka U, Lescure N, Cullimore JV, Gamas P (1995) A cDNA encoding a PR-1-like protein in the model legume *Medicago truncatula*. Plant Physiol 107: 273-274

Szyperski T, Fernández C, Mumenthaler C, Wüthrich K (1998) Structure comparison of human glioma pathogenesis-related protein GliPR and the plant pathogenesis-related protein P14a indicates a functional link between the human immune system and a plant defense system. Proc Nat Acad Sci USA 95: 2262-2266

Takemoto D, Furuse K, Doke N, Kawakita K (1997) Identification of chitinase and osmotin-like protein as actin-binding proteins in suspension-cultured potato cells. Plant Cell Physiol 38: 441-448

Tattersall DB, van Heeswijck R, Høj PB (1997) Identification and characterization of a fruit-specific, thaumatin-like protein that accumulates at very high levels in conjunction with the onset of sugar accumulation and berry softening in grapes. Plant Physiol 114: 759-769

Tornero P, Conejero V, Vera P (1994) A gene encoding a novel isoform of the PR-1 protein family from tomato is induced upon viroid infection. Mol Gen Genet 243: 47-53

Tornero P, Gadea J, Conejero V, Vera P (1997) Two PR-1 genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development. Mol Plant Microbe Interact 10: 624-634

Trudel J, Greiner J, Potvin C, Asselin A (1998) Several thaumatin-like proteins bind to β-1,3-glucans. Plant Physiol 118: 1431-1438

Tsukaya H, Ohshima T, Naito S, Chino M, Komeda Y (1991) Sugar-dependent expression of the CHS-A gene for chalcone synthase from petunia in transgenic *Arabidopsis*. Plant Physiol 97: 1414-1421

van de Rhee MD, Linthorst HJM, Bol JF (1994) Pathogen-induced gene expression. In: Basra AS (ed) Stress-induced gene expression in plants. Harwood Academic Publishers, Chur, pp 249-284

van der Wel H, Loeve K (1972) Isolation and characterization of thaumatin I and II, the sweet-tasting proteins from *Thaumatococcus daniellii* Benth. Eur J Biochem 31: 221-225

van Kan JAL, van de Rhee MD, Zuidema D, Cornelissen BJC, Bol JF (1989) Structure of tobacco genes encoding thaumatin-like proteins. Plant Mol Biol 12: 153-155

van Loon LC (1975) Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. 'Samsun' and 'Samsun NN'. IV. Similarity of qualitive changes of specific proteins after infection with different viruses and their relationship to acquired resistance. Virology 67: 566-575

van Loon LC (1997) Induced resistance in plants and the role of pathogenesis-related proteins. Eur J Plant Pathol 103: 753-765

van Loon LC, Pierpoint WS, Boller T, Conjero V (1994) Recommendations for naming plant pathogenesis-related proteins. Plant Mol Biol Reptr 12: 245-264

van Loon LC, Van Kammen A (1970) Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. 'Samsun' and 'Samsun NN'. Changes in protein constitution after infection with TMV. Virology 40: 199-201

Van Parijs J, Broekaert WF, Goldstein IJ, Peumans WJ (1991) Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis*) latex. Planta 183: 258-264

Vanderplank JE (1984) Sink-induced loss of disease resistance and high-sugar disease processes and biotrophy. In: Vanderplank JE (ed) Disease resistance in plants. Academic Press, Orlando, pp 107-116

Varghese JN, Garrett TPJ, Colman PM, Chen L, Høj PB, Fincher GB (1994) 3-Dimensional structures of two plant β-glucan endohydrolases with distinct substrate specificities. Proc Nat Acad Sci USA 91: 2785-2789

Vera P, Conejero V (1988) Pathogenesis-related proteins of tomato. Plant Physiol 87: 58-63

Verburg JG, Huynh QK (1991) Purification and characterization of an antifungal chitinase from *Arabidopsis thaliana*. Plant Physiol 95: 450-455

Vierheilig H, Alt M, Neuhaus J, Boller T (1993) Colonization of transgenic *Nicotiana sylvestris* plants, expressing different forms of *Nicotiana tabacum* chitinase, by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae*. Mol Plant Microbe Interact 6: 261-264

Vigers AJ, Roberts WK, Selitrennikoff CP (1991) A new family of plant antifungal proteins. Mol Plant Microbe Interact 4: 315-323

Vigers AJ, Wiedemann S, Roberts WK, Legrand M, Selitrennikoff CP, Fritig B (1992) Thaumatin-like pathogenesis-related proteins are antifungal. Plant Sci 83: 155-161

Vögeli-Lange R, Frundt C, Hart CM, Beffa R, Nagy F, Meins Jr. F (1994) Evidence for a role of β-1,3-glucanase in dicot seed germination. Plant J 5: 273-278

Voilley A, Lamer C, Dubois P, Feuillat M (1990) Influence of macromolecules and treatments on the behaviour of aroma compounds in a model wine. J Agric Food Chem 38: 248-251

Vu L, Huynh QK (1994) Isolation and characterization of a 27-kDa antifungal protein from the fruits of *Diospyros texana*. Biochem Biophys Res Commun 202: 666-672

Wang X, Zafian P, Choudhary M, Lawton M, Wang XQ (1996) The PR5K receptor protein kinase from *Arabidopsis thaliana* is structurally related to a family of plant defense proteins. Proc Nat Acad Sci USA 93: 2598-2602

Waters EJ, Hayasaka Y, Tattersall DB, Adams KS, Williams PJ (1998) Sequence analysis of grape (*Vitis vinifera*) berry chitinases that cause haze formation in wines. J Agric Food Chem 46: 4950-4957

Waters EJ, Pellerin P, Brillouet JM (1994) A wine arabinogalactan-protein that reduces heat-induced wine protein haze. Biosci Biotech Biochem 58: 43-48

Waters EJ, Peng Z, Pocock K, Williams PJ (1995a) Proteins in white wine, II: Their resistance to proteolysis is not due to either phenolic association or glycosylation. Aust J Grape Wine Res 1: 94-99

Waters EJ, Peng Z, Pocock KF, Williams PJ (1995b) Proteins in white wine, I: Procyanidin occurence in soluble proteins and insoluble protein hazes and its relationship to protein instability. Aust J Grape Wine Res 1: 86-93

Waters EJ, Shirley NJ, Williams PJ (1996) Nuisance proteins of wine are grape pathogenesis-related proteins. J Agric Food Chem 44: 3-5

Waters EJ, Wallace W, Tate ME, Williams PJ (1993) Isolation and partial characterization of a natural haze protective factor from wine. J Agric Food Chem 41: 724-730

Waters EJ, Wallace W, Williams PJ (1991) Heat haze characteristics of fractionated wine proteins. Am J Enol Vitic 42: 123-127

Waters EJ, Wallace W, Williams PJ (1992) Identification of heat-unstable wine proteins and their resistance to peptidases. J Agric Food Chem 40: 1514-1519

Weiss KC, Yip T, Hutchens TW, Bisson LF (1998) Rapid and sensitive fingerprinting of wine proteins by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Am J Enol Vitic 49: 231-239

Wessels JGH (1993) Wall growth, protein excretion and morphogenesis in fungi. New Phytol 123: 397-413

White RF, Rybiki EP, Von Wechmar MB, Dekker JL, Antoniw JF (1987) Detection of PR-1 type proteins in *Amaranthaceae, Chenopodiaceae, Gramineae* and *Solanace* by immuno-electroblotting. J Gen Virol 68: 2043-2048

Wilkinson M, Doskow J, Lindsey S (1991) RNA blots: staining procedures and optimization of conditions. Nucleic Acids Res 19: 679

Woloshuk CP, Meulenhoff JS, Sela-Buurlage M, van den Elzen PJM, Cornelissen BJC (1991) Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell 3: 619-628

Wurms K, Long P, Greenwood D, Sharrock K, Ganesh S (1997) Endo- and exochitinase activity in kiwifruit infected with *Botrytis cinerea*. J Phytopathol 145: 145-151

Yalpani N, Enyedi AJ, Leon J, Raskin I (1994) Ultraviolet light and ozone stimulate accumulation of salicylic acid, pathogenesis-related proteins and virus resistance in tobacco. Planta 193: 372-376

Yalpani N, Leon J, Lawton MA, Raskin I (1993) Pathway of salicylic acid biosynthesis in healthy and virus-inoculated tobacco. Plant Physiol 103: 315-321

Yalpani Y, Silverman P, Wilson TMA, Kleier DA, Raskin I (1991) Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell 3: 809-818

Yeboah NA, Arahira M, Nong VH, Zhang DY, Kadokura K, Watanabe A, Fukazawa C (1998) A class III acidic endochitinase is specifically expressed in the developing seeds of soybean (*Glycine max* [L.] Merr.). Plant Mol Biol 36: 407-415

Yen HE, Edwards GE, Grimes HD (1994) Characterization of a salt-responsive 24-kilodalton glycoprotein in *Mesembryanthemum crystallinum*. Plant Physiol 105: 1179-1187

Yokotsuka K, Ebihara T, Sato T (1991) Comparison of soluble proteins in juice and wine from Koshu grapes. J Ferment Bioeng 71: 248-253

Yoshikawa M, Tsuda M, Takeuchi Y (1993) Resistance to fungal diseases in transgenic tobacco plants expressing the phytoalexin elicitor-releasing factor, β−1,3-endoglucanase, from soybean. Naturwissen 80: 417-420

Yun DJ, Ibeas JI, Lee H, Coca MA, Narasimhan ML, Uesono Y, Hasegawa PM, Pardo JM, Bressan RA (1998) Osmotin, a plant antifungal protein, subverts signal transduction to enhance fungal cell susceptibility. Molecular Cell 1: 807-817

Yun DJ, Paino D'Urzo M, Abad L, Takeda S, Salzman R, Chen ZT, Lee H, Hasegawa PM, Bressan RA (1996) Novel osmotically induced antifungal chitinases and bacterial expression of an active recombinant isoform. Plant Physiol 111: 1219-1225

Yun DJ, Zhao Y, Pardo JM, Narasimhan ML, Damsz B, Lee H, Abad LR, D'Urzo MP, Hasegawa PM, Bressan RA (1997) Stress proteins on the yeast cell surface determine resistance to osmotin, a plant antifungal protein. Proc Nat Acad Sci USA 94: 7082-7087

Zhu BL, Chen THH, Li PH (1993) Expression of an ABA-responsive osmotin-like gene during the induction of freezing tolerance in *Solanum commersonii*. Plant Mol Biol 21: 729-735

Zhu BL, Chen THH, Li PH (1995) Expression of three osmotin-like protein genes in response to osmotic stress and fungal infection in potato. Plant Mol Biol 28: 17-26

Zhu BL, Chen THH, Li PH (1996) Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin-like protein. Planta 198: 70-77

Zhu Q, Maher EA, Masoud S, Dixon RA, Lamb CJ (1994) Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Biotech 12: 807-812

THE UNIVERSITY OF ADELAIDE

Certification of Thesis for Examination

Candidate's Name: David Tattersall

Name of Principal Supervisor: Prof. Peter Høj

Department: Horticulture, Viticulture and Oenology

I have seen and read the final draft of the above candidate's thesis and, in accordance with the requirement of *Section 2.3.6* of the *Code of Practice*, certify that the thesis is, *prima facie*,

☑ worthy of examination.

☐ not worthy of examination and I attach a statement outlining my reasons.

24/5/99
Date

To be completed by Head of Department or Postgraduate Coordinator

☑ I endorse the Principal Supervisor's recommendation.

☐ I do not endorse the Principal Supervisor's recommendation and I attach a statement outlining my reasons.

*~~Head of Department~~/Postgraduate Coordinator

24.5.99
Date

(* *Please delete as appropriate.*)

et/20/01/97