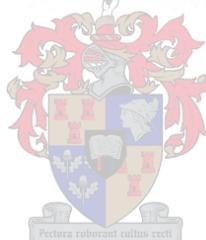


Isolation and characterisation of a polygalacturonase- inhibiting protein (PGIP) and its encoding gene from *Vitis vinifera* L.

by

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*Dissertation presented in partial fulfilment of the requirements for the
degree of Doctoral in Science at Stellenbosch University*

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any University for a degree.

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Date

SUMMARY

Polygalacturonase-inhibiting proteins (PGIPs) are present in the cell walls of a variety of plant species. These proteins have been shown to specifically inhibit endopolygalacturonases (endo-PGs) secreted by invading fungal pathogens as part of the induced disease resistance mechanism of plants. This is the first report on the isolation and characterisation of a *pgip* gene from *Vitis vinifera* L., designated grapevine *pgip1*. A single open reading frame encoding a deduced polypeptide of 333 amino acids with a predicted molecular mass of 37.1 kDa and a calculated isoelectric point of 8.61 was identified from a 5.6 kb subgenomic fragment of *V. vinifera* cv Pinotage. Nucleotide and derived amino acid sequence analysis of grapevine *pgip1* showed significant homology with other characterised PGIP encoding genes and revealed features characteristic of PGIPs found in several other plant families. Genomic DNA analysis showed that grapevine *pgip1* belongs to a small multigene family in *Vitis* cultivars. From Northern blot analysis it was evident that expression of the PGIP family is both tissue- and developmental stage specific. The grapevine *pgip1* was transiently expressed in *Nicotiana benthamiana* L. with potato virus X (PVX) as a vector. Grapevine PGIP1 isolated from crude protein extracts of PVX-infected *N. benthamiana* were tested and showed inhibitory activity against polygalacturonases (PGs) from *Botrytis cinerea*.

Grapevine PGIPs have not previously been purified and characterised. Molecular analyses have confirmed that PGIPs are typically encoded by multigene families and that the inhibitor specificities and kinetics of the isolated proteins differ within and among species. In this study, two PGIP isomers from *V. vinifera* berries were isolated. The one isomer, designated PGIP-A, was partially purified and had a molecular mass of 39 kDa, whereas the other PGIP, designated PGIP-B, was purified and had a molecular mass of 42 kDa as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Both proteins were cell wall-bound. Enzymatic deglycosylation confirmed that PGIP-B is a glycosylated protein. Grapevine PGIP-A showed strong inhibitory activity against a homogeneous PG from *Aspergillus niger* and to a lesser extent against PG from *Fusarium moniliforme*, but was unable to interact with a crude PG preparation from *B. cinerea*. Grapevine PGIP-B was able to strongly inhibit PGs from *B. cinerea* as well as from *Colletotrichum gleosporoides*, yet showed no inhibition towards PG from *A. niger*.

The grapevine *pgip1* gene was expressed under the control of the Cauliflower mosaic virus (CaMV) 35S promoter in tobacco plants via *Agrobacterium tumefaciens*-mediated transformation. Transgenic tobacco plants expressing the grapevine PGIP (gPGIP1) were used to demonstrate the effectiveness of this inhibitor against fungal PGs and to investigate whether gPGIP1 influences disease development. Northern blot analysis identified 19 transgenic plants expressing *pgip1* transcript levels. Crude

PGIP extracts from the transgenic tobacco plants inhibited PGs from *B. cinerea* and *C. gleosporoides*, but not PG from *A. niger*. Leaves from untransformed tobacco plants, from transgenic tobacco lines showing high and low PG inhibition, and from transgenic plants that did not express *pgip1*, were inoculated with *B. cinerea*. Transgenic leaves showed a reduction in the size of necrotic lesions of macerated tissues of approximately 45% relative to control and non-expressing transgenic leaves. The results from the heterologous expression of gPGIP1, together with the results from the protein purifications and inhibition studies, indicate that the isolated grapevine *pgip1* gene encodes the isolated PGIP-B isomer. This work has established a good model system to study certain aspects of plant-pathogen interactions in grapevine. Heterologous expression of gPGIP1 has demonstrated that PGIP inhibition of fungal PGs slows disease development of *B. cinerea in planta*.

OPSOMMING

Poligalakturonase-inhiberende proteïene (PGIPs) kom in die selwande van 'n verskeidenheid van plantspesies voor. Wanneer plante se siekteweerstandsmeganisme geïnduseer word, inhibeer hierdie PGIP-selfverdedigingsproteïene die endopoligalakturonases (endo-PGs) wat deur infekterende swampatogene uitgeskei word, op 'n baie gerigte wyse. Hierdie is die eerste melding van die isolasie en karakterisering van 'n *pgip*-geen uit *Vitis vinifera* L., die sogenaamde *pgip1*-druifgeen. Die afgeleide polipeptied van 333 aminosure, het 'n geskatte molekulêre gewig van 37.1 kDa en 'n berekende isoëlektriese punt van 8.61 en word deur 'n enkele oop leesraam wat vanuit 'n subgenomiese fragment van *V. vinifera* cv Pinotage, geïsoleer is, ge-encodeer. Nukleotied- en afgeleide aminosuursekwensanalises het bevestig dat die druifplant se *pgip1* betekenisvolle homologie met ander bekende PGIP-gene het en ook kenmerkende eienskappe, tipies van PGIPs in verskeie ander plantfamilies, besit. Genomiese DNA-analises het bewys dat die druif-*pgip1* deel van 'n klein multigeenfamilie in *Vitis*-kultivars vorm. Noordelike kladtegnieke het voorts getoon dat die uitdrukking van die druifplant se PGIP-familie, weefsel- en ontwikkelingstadiumspesifiek is. Die druif se *pgip1*-geen is ook tydelik in *Nicotiana benthamiana* L., met die hulp van aartappel virus X (PVX) as 'n vektor uitgedruk. Die druif-PGIP1 wat voorts uit kru proteïenekstrakte van die PVX-geïnfekteerde *N. benthamiana* plante geïsoleer is, het inhiberende aktiwiteit teen poligalakturonases (PGs) van *Botrytis cinerea* getoon.

Die druif-PGIPs is nog nooit voorheen gesuiwer en gekarakteriseer nie. Molekulêre analises het bevestig dat PGIPs tipies deur multigeen-families ge-encodeer word en dat die inhibitorspektra en kinetika van die bekende proteïene tussen spesies en selfs binne 'n spesiegroepering verskil. In hierdie studie is twee PGIP-isomere vanuit *V. vinifera*-korrels geïsoleer. Die een isomeer, genoem PGIP-A, is gedeeltelik gesuiwer en het 'n molekulêre massa van 39 kDa gehad. Dit is in teenstelling met die ander PGIP, genoem PGIP-B, wat volledig gesuiwer is en 'n molekulêre massa van 42 kDa, soos bepaal met natrium-dodesielsulfaat-poliakrielamielgelelektroforese (SDS-PAGE) en Westelike kladtegnieke, getoon het. Beide proteïene is selwand gebonde. Ensimatiese deglikosilering het bevestig dat PGIP-B 'n proteïen is wat aan suikerresidue gekoppel is. Druif-PGIP-A het sterk inhiberende aktiwiteit teen 'n homogene PG afkomstig van *Aspergillus niger* en tot 'n mindere mate teen 'n PG van *Fusarium moniliforme* getoon, maar het geen interaksie met kru PG-preparate van *B. cinerea* getoon nie. Druif-PGIP-B het PGs afkomstig van *B. cinerea* sowel as *Colletotrichum gleosporoides* sterk geïnhibeer, maar het geen inhibisie teen die PG van *A. niger* gehad nie.

Die druifplant se *pgip1*-geen is onder beheer van die blomkoolmosaïekvirus (CaMV) se 35S-promotor in tabakplante, na transformasie met *Agrobacterium tumefaciens*, uitgedruk. Transgeniese tabakplante, wat die druif-PGIP (gPGIP1)

uitdruk, is gebruik om die doeltreffendheid van die inhibitor teen fungiese PGs aan te toon en terselfdertyd te bepaal of gPGIP1 die proses van infeksie kan beïnvloed. Noordelike kladanalises het 19 transgeniese plante met *pgip1*-transkripvlakke geïdentifiseer. Kru PGIP-ekstrakte van dié transgeniese tabakplante inhibeer PGs van *B. cinerea* en *C. gleosporoides*, maar nie dié afkomstig van *A. niger* nie. Blare afkomstig van ongetransformeerde tabakplante, vanaf transgeniese tabaklyne met hoë en lae vlakke van PG-inhibisie en van transgeniese lyne wat nie *pgip1* uitdruk nie, is met *B. cinerea* spore ingeënt. In vergelyking met ongetransformeerde en transgeniese lyne wat nie dié geen uitdruk nie, het die transgeniese blare ongeveer 'n 45% afname in grootte van nekrotiese letsels vertoon. Die resultate wat met die heteroloë uitdrukking van gPGIP1 verkry is, tesame met dié afkomstig van die proteïensuiwering en -inhibisiestudies, dui daarop dat die gekloneerde druifplant se *pgip1*-geen, vir die PGIP-B isomeer wat gesuiwer is, kodeer. 'n Goeie modelsisteem om sekere aspekte van plant-patogeen interaksies in die druifplant te bestudeer, is dus ontwikkel. Heteroloë uitdrukking van gPGIP1 het verder bevestig dat die inhibisie van fungiese PGs deur PGIP, die *in planta* ontwikkeling van *B. cinerea*-siektesimptome negatief beïnvloed.

For my mother

BIOGRAPHICAL SKETCH

Ana De Ascensao was born in Lorenzo Marques, Mozambique, on 5 February 1973 and her family moved to South Africa when she was 18 months old. She attended Belgravia Convent (Johannesburg) and matriculated from Jeppe High School for Girls in 1990. Ana enrolled at the Rand Afrikaans University in 1991 and obtained the BSc degree in Chemistry and Biochemistry in 1993. In 1994 she received the BScHons degree in Biochemistry at the same university. In 1996 she received her MSc in Biochemistry *cum laude* at the same university. In 1997 she enrolled at the Stellenbosch University for her PhD in Biochemistry through the Institute for Wine Biotechnology.

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PREFACE

This thesis is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of the journal to which the manuscripts (Chapters 3, 4 and 5) were submitted.

- Chapter 1** **General Introduction and Project aims**
- Chapter 2** **Literature Review: Grapevines under fungal attack: Polygalacturonase-inhibiting proteins (PGIPs) and their role in plant defence mechanisms**
- Chapter 3** **Research Results: The isolation and characterisation of a gene encoding a polygalacturonase-inhibiting protein (PGIP) from *Vitis vinifera* L.**
- Chapter 4** **Research Results: The purification and characterisation of polygalacturonase-inhibiting proteins (PGIPs) with different specificities from *Vitis vinifera* L.**
- Chapter 5** **Research Results: Transgenic expression of the grapevine *pgip1* gene in tobacco increases resistance to *Botrytis cinerea***
- Chapter 6** **General Discussion and Conclusions**

I hereby declare that I was the primary contributor with respect to the experimental data presented on the multi-author manuscripts presented in Chapters 3, 4 and 5. My supervisors, Profs IS Pretorius and DU Bellstedt and Dr MA Vivier, were involved in the conceptual development, and continuous critical evaluation of this study. Dr Burger and Prof De Lorenzo collaborated with the author and her supervisors on the work outlined in Chapters 3 and 4, respectively.

Drs Caprari and Salvi assisted with the protein purification techniques outlined in Chapter 4.

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Plants are continually exposed to a vast array of potential fungal pathogens. In many cases, they resist attack by blocking fungal development soon after penetration. Vertebrates have evolved a circulatory immune system with antibodies, something which plants lack. Instead, plants have evolved a defence strategy by which each cell is capable of defending itself by means of a combination of constitutive mechanisms and induced responses. The understanding of how the plant senses or identifies a foreign microbe as a pathogen and relays this information is beneficial not only to our basic understanding of how these processes work, but has practical applications for the improvement of agriculture (Richter and Ronald, 2000).

If a plant detects an invasion, a set of inducible defence responses is deployed; these include programmed cell death, also referred to as the hypersensitive response (HR), tissue reinforcement at the infection site, production of anti-microbial metabolites and induction of defence-associated gene expression (Hammond-Kosack and Jones, 1996). Activation of local responses at the point of infection can be followed by the establishment of secondary resistance throughout the plant known as systemic acquired resistance (SAR), which results in non-specific plant immunity to a broad range of pathogens in the distal portions of the plant (Ryals *et al.*, 1996; Sticher *et al.*, 1997).

The activation of inducible defences is contingent upon recognition of an invasion. Surveillance in the plant is the collective duty of a complex array of constitutively expressed *R* genes (for resistance). Individual *R* genes have narrow recognition capabilities and they trigger resistance only when the invading pathogen expresses a corresponding *avr* gene (for avirulence) (Staskawicz *et al.*, 1995). These genetic attributes have inspired a molecular model in which *avr*-encoded proteins are delivered to the plant cell to facilitate invasion and there they are recognised by the corresponding *R* protein as a signal that invaders have arrived (Hammond-Kosack and Jones, 1997). Studies arising from molecular cloning of numerous *R* and *avr* genes have validated this 'receptor-elicitor' model of these respective genes (Van der Biezen and Jones, 1998).

For inducible defences to be effective they must be deployed rapidly to outpace the pathogen's counterattack. On the other hand, these defences cannot be unleashed in an uncontrolled manner, as they are resource-intensive and can inflict substantial collateral damage on host tissues. Thus, deployment must be confined to the proper place and time. These requirements suggest that a complex, highly integrated regulatory network triggers and controls defence responses (McDowell and Dangl, 2000).

The study of pathogenicity and defence has shown that the plant cell wall plays an important role in signalling phenomena underlying plant-pathogen interactions

(Esquerré-Tugayé *et al.*, 2000). As the first barrier encountered by most plant pathogens, the plant cell wall must be degraded in order to allow penetration and colonisation. The majority of fungi secrete a number of enzymes capable of degrading cell wall polymers. Of the enzymes involved in cell wall degradation, those that hydrolyse the pectic substances of plant cell walls have undergone the most intensive investigation. Amongst these pectinolytic enzymes, endopolygalacturonases (endo-PGs) have been implicated as pathogenicity factors of plant pathogenic fungi (Hahn *et al.*, 1989). The importance of endo-PGs has been confirmed with the observation that they are among the first enzymes to be secreted when fungi are grown on plant cell wall material *in vitro* (Jones *et al.*, 1972; Mankarios and Friend, 1980).

In plants, mechanisms exist to counteract the action of endo-PGs. Many dicotyledonous and some monocotyledonous plants have cell wall-localised polygalacturonase-inhibiting proteins (PGIPs) (De Lorenzo *et al.*, 1997) that play a major role in the plant defence model. PGIPs are specific, high-affinity receptor-like proteins that inhibit the activity of fungal endo-PGs. By inhibiting fungal PGs, PGIPs interfere directly with host cell wall degradation. Endo-PGs release elicitor-active oligogalacturonides from the plant cell wall, but also degrade them further into inactive oligomers. This implies that an extensive degradation of the cell wall homogalacturonan negatively affects the eliciting activity of the endo-PG. It has been hypothesised that PGIP modulates the endo-PG activity *in vitro* in such a way that the balance between the release of elicitor-active oligogalacturonides and the depolymerisation of the active oligogalacturonides into inactive molecules is altered and the accumulation of elicitor-active molecules is favoured (Cervone *et al.*, 1989), which in turn trigger strong plant defence responses.

PGIPs belong to a super-family of leucine-rich repeat (LRR) proteins. In plants, LRR proteins play an important role in both development and defence, where specificity of recognition is a fundamental prerequisite. PGIPs are evolutionarily related to several plant *R* genes, which participate in gene-for-gene resistance (Jones and Jones, 1997), and to several receptor kinases involved in plant development (Torii *et al.*, 1996; Clark *et al.*, 1997). All these proteins share LRRs of the extracellular or extracytoplasmic type, as well as homology in the regions outside the LRR domain (Bent, 1996).

PGIPs isolated from a number of plant species have differential inhibition spectra towards a range of PGs from phytopathogenic fungi (Cervone *et al.*, 1987; Johnston *et al.*, 1993; Stotz *et al.*, 1993; Stotz *et al.*, 1994). Furthermore, different inhibitory activities against PGs have also been observed in PGIPs from a single plant source (Desiderio *et al.*, 1997), indicating that *pgip* genes have undergone diversification during evolution. Likewise, this variability in PG specificity possibly reflects counter-adaptations between fungal PGs and plant PGIPs that lead to specialisation (Stotz *et al.*, 2000).

Previously, no *pgip* gene or encoded protein had been isolated from *V. vinifera*. Due to the widespread occurrence of PGIPs in dicotyledonous plant species, it was fair to assume that *Vitis* spp. would also contain copies of these genes. It has become clear that disease resistance in grapevine is strongly developmentally regulated. Typically, excellent resistance is observed until berry véraison is reached, whereafter the resistance collapses dramatically. Recent molecular analysis revealed that large groups of genes involved in disease resistance are upregulated during the early stages of berry development, as are a range of cell wall proteins until seed maturation (Davies and Robinson, 2000). It seems that the resistance mechanisms are intact until the progeny are secured in the seed, whereafter disease resistance breaks down. These interesting mechanisms, as well as the potential modelling capacity of PGIP proteins in disease resistance mechanisms, prompted our approach to isolate *pgip* gene(s) and encoded proteins from *V. vinifera* and to characterise them.

1.2 PROJECT AIMS

The specific aims and approaches of this study were as follows:

- i) To isolate and characterise a gene encoding a polygalacturonase-inhibiting protein from *V. vinifera*.
- ii) To purify and characterise polygalacturonase-inhibiting proteins from *V. vinifera* tissues.
- iii) To transform tobacco plants with the grapevine PGIP-encoding gene (*pgip1*) that was isolated by means of *Agrobacterium*-mediated transformation.
- iv) To use the transgenic tobacco plants expressing the grapevine *pgip1* gene to assess the effectiveness of this inhibitor *in planta* against fungal pathogens in disease development.

In this thesis, the first of these objectives was presented in a publication entitled "The isolation and characterisation of a gene encoding a polygalacturonase-inhibiting protein (PGIP) from *Vitis vinifera* L.", which was submitted to *Planta* (De Ascensao, A.R., Pretorius, I.S., Bellstedt, D.U., Burger, J.T., and Vivier, M.A. 2001). The paper describes (i) the isolation and sequencing of the first *pgip* encoded gene from grapevine, designated *pgip1* gene; (ii) a comparison of the grapevine *pgip* nucleotide- and deduced amino acid sequences with that of other *pgip* genes and PGIP proteins; (iii) the genomic organisation of the grapevine *pgip* genes; (iv) the expression pattern of grapevine PGIPs in different tissues; (v) the transient expression of the grapevine *pgip1* gene in *Nicotiana benthamiana* with potato virus (cloning) X (PVX) as a vector and (vi) the inhibitory activity and specificity of partially purified PGIP towards fungal PGs. **Chapter 3** is dedicated to these aspects of the study and contains the paper written in the style for the specific journal to which it was submitted. The chapter also includes Addendum A, containing a brief overview of the initial experimental approach, followed by additional results, which were not presented in the paper.

The second of these objectives was presented in a publication entitled "Purification and characterisation of polygalacturonase-inhibiting proteins (PGIPs) with different specificities from *Vitis vinifera* L.", which was submitted to *Planta* (De Ascensao, A.R., Pretorius I.S., Caprari, C., Salvi G., De Lorenzo, G., and Vivier, M.A. 2001). The paper describes (i) the isolation and purification of two PGIP isomers, namely PGIP-A and PGIP-B; (ii) immunoblotting of the two isomers; (iii) the inhibitory specificities of the two isomers as well as the crude protein extracts from PVX-infected *N. benthamiana* against a number of fungal PGs; and (iv) the characterisation of PGIP-B in terms of the type of inhibition it exhibits and inhibitor stability. This paper, written in the style according to the journal it was submitted to, is included in **Chapter 4**.

The final two objectives of this thesis are presented in **Chapter 5** in the form of an independent manuscript, complete with an introduction, material and methods, results, discussion and reference list. The paper describes (i) vector constructs and plant transformations; (ii) Southern and Northern blot analysis of transgenic lines; and (iii) PGIP activity assays and plant-pathogen interactions. The work presented in this chapter will be submitted to *Transgenic Research* and is written according to the style of this journal.

The work presented in this dissertation comprises the first report of the isolation, purification and characterisation of a polygalacturonase-inhibiting protein from *V. vinifera* and its encoding gene. It has laid the basis for further studies regarding the grapevine *pgip* gene family and has established a useful model system to study various aspects of plant-pathogen interactions and disease resistance mechanisms.

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CHAPTER 2

LITERATURE REVIEW

**Grapevines under fungal attack:
Polygalacturonase-inhibiting proteins (PGIPs)
and their role in plant defence mechanisms**

LITERATURE REVIEW

2.1 IN THE VINEYARD

When asked what the world's most economically important fruit crop might be, few people would answer "grapes". However, this fruit, which has been in existence since the tertiary period one to five million years ago, now holds this title. The grape is the most widely planted fruit crop in the world, covering an area of approximately nine million hectares of viticultural land in 1990 (Kanellis and Roubelakis-Angelakis, 1993). More than 5000 varieties of grapes are grown worldwide. The grape is a crop plant with many uses. It is used for wine, distilled liquors, juice, dried fruit (raisins), fresh consumption (table grapes) and concentrate.

Grapevines are woody perennial angiosperms that reach reproductive maturity in 4 to 5 years. Grapevine belongs to the genus *Vitis*, which was defined in 1700 by Tournefort. The word *Vitis* is derived from the Latin word *viere*, which means "to attach"; it was used by Virgil to describe the climbing habit of vines (Pongrácz, 1978). The grapevine in its natural state is a climber. Its natural home is the forest, hence the botanical name of *Vitis vinifera* subspecies *silvestris* - the woodland wine-bearing vine - for the wild vines that occur. Where and when the genus *Vitis* evolved is unclear, but it consists of two subgenera, *Euvitis* and *Muscadinia*, the former comprising the bulk of the cultivated varieties. The current distribution of *Euvitis* species includes northern South America, Central and North America, Asia and Europe. In contrast, *Muscadinia* species are restricted to the southeastern United States and northeastern Mexico (Jackson, 1994).

It is thought that *Vitis* spp. were first used to make wild wine in the foothills of the Caucasus region. Georgia has produced the earliest evidence of vine selection, leading to the emergence of the cultivated varieties of *V. vinifera* subspecies *sativa*. The domestication that led to this change probably occurred around 5000 BC (Jackson, 1994). The species *V. vinifera*, with its numerous varieties bearing both black and green or pale grapes, is the only species of European origin in commercial use (Antcliff, 1992).

Vines react to sunlight throughout the growing season, even in winter. Sunlight on the woody parts translates to a more fruitful vine (Coombe, 1987). At the base of each leaf is a bud, defined as the crop potential of the following year's vintage. The amount of sunlight on the vine when its new buds are forming acts as a signal, determining whether the buds become leafy shoots or primordial flowers for fruit. The yield of each plant is thus dependent initially on the amount of light reaching the vine up to 15 months before (Smart 1987). With this knowledge, the grower will manipulate the vine to achieve an appropriate balance between the production of leaves and fruit, principally avoiding a dense canopy of leaves that will shade the buds and also lead to favourable (humid) microclimates for the development of fungal diseases.

We are living in an era in which technological advancement (also in science) has made it possible to find innovative approaches for old problems based on knowledge extracted from underlying fundamental processes. One such process is the way plants defend themselves against pathogen attack. In the following sections the significance of disease in an economically important crop such as grapevine will be highlighted briefly, before the relevant literature on one group of proteins important in host plant disease resistance, the polygalacturonase inhibiting protein (PGIPs) and their substrates, fungal polygalacturonases (PGs), will be discussed.

2.2 SIGNIFICANCE OF DISEASES IN GRAPE PRODUCTION

2.2.1 Introduction

Diseases in grapes can result in substantial losses in production, harvesting, processing, marketing, and to the consumer. They lower quality, reduce yield and increase the costs of production and harvesting. Diseases cripple and kill vines, destroy vineyards and have rendered some land unfit for viticulture. Diseases in general are endemic and disease development, specifically fungal and bacterial infections, depends on weather. Epidemics may occur with losses ranging from 20 to 80% if the weather is favourable for the disease (Hewitt, 1988). Prolonged periods of wet weather favour *Botrytis* bunch rot, downy mildew and other fruit and leaf spot diseases. In contrast, powdery mildew is favoured by dry, relatively cool weather.

Some past epidemics illustrate the powers of pathogens and the consequences of diseases. The rich family of American grapevine species evolved over the millennia in temperate climates that encouraged the growth of downy and powdery mildew and in soils that harboured large colonies of the microscopic louse called phylloxera (Hewitt, 1988). In Europe, the American vines were introduced as novelties and it was inevitable they would bring with them the American diseases to which they had built up a near-total immunity. The European grapevine, *V. vinifera*, in contrast, had no such immunity and fell prey to these diseases with devastating rapidity (Galet and Morton, 1988). For this reason rootstock grafting was introduced to stop the destruction caused by phylloxera in Europe and elsewhere.

Although grapevine is prey to numerous pathogens, this review will focus mostly on the interaction of fungal pathogenicity factors with certain plant resistance mechanisms. To this end, grey mould of grapevine was chosen as an example of an economically important fungal disease.

2.2.2 Grey mould of grapevine

The causal agent of grey mould, *Botrytis cinerea* Pers., displays one of the widest host range specificities among fungal plant pathogens. This fungus infects a large range of plant genera and plant tissues, including many economically important crops (Jarvis, 1977). These include fruits such as tomatoes, apples, strawberries and raspberries, and flowers such as tulips, roses and orchids (Coley-Smith *et al.*, 1980).

Grey mould exists in all vineyards in the world and under a wide array of environmental conditions, attacking grapes at pre- and post-harvest stages (**Figure 2.1**). Cold, damp climates favour disease development (Bulit and Dubos, 1988). Under prolonged wet conditions, infection can progress rapidly into a bunch rot complex (**Figure 2.1C**), involving secondary invaders such as species of *Penicillium* (**Figure 2.1D**), *Aspergillus*, *Cladosporium* and *Rhizopus* (Fermaud and Le Menn, 1989). *B. cinerea* may also develop on the leaves and the woody stems of grapevine plants, even though this is less damaging than fruit loss.

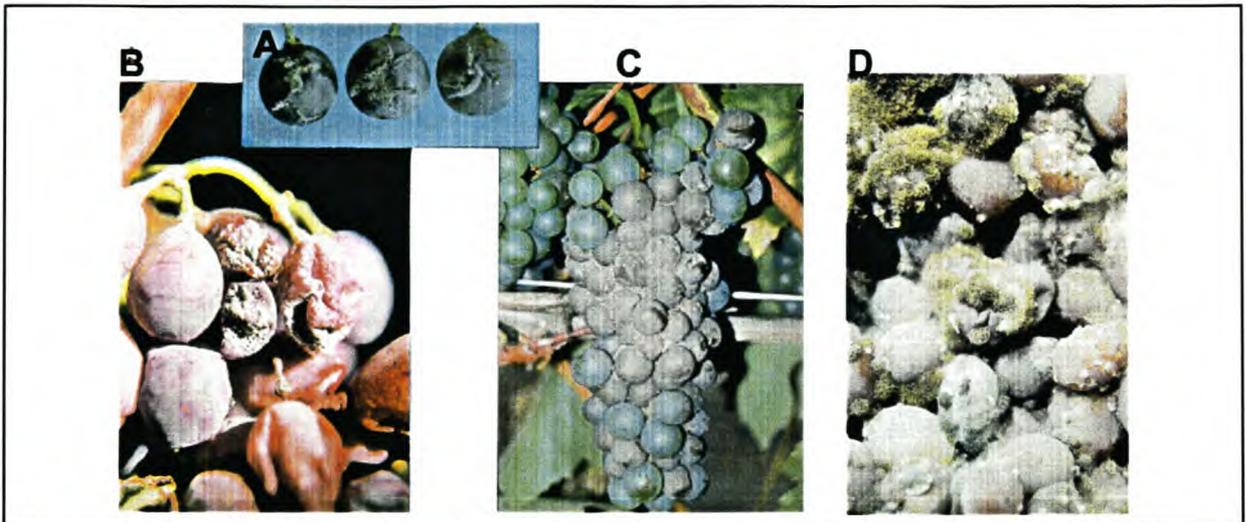


Figure 2.1 Typically, *Botrytis* invades cracks in the berries (A). Buff coloured spores of *Botrytis* present on rotten berries (B). Typical bunch rot (C). *Botrytis* and blue *Penicillium* on table grapes (D) (Emmett *et al.*, 1994).

Grey mould of grapevine is a serious disease; not only because it affects the quantity of the harvest but also, and probably more importantly, it affects the quality of the wine produced from grapes infected with *B. cinerea*. The reduction in yield is associated with the premature drop of bunches from stalk rot, the loss of juice and the desiccation of berries (Bulit and Dubos, 1988). If not controlled adequately, in table grape production, post-harvest decay caused by *Botrytis* can cause substantial loss in fruit quality during storage and export (Maude, 1980).

In wine production, the most serious damage is qualitative. This is due to the changes in the chemical composition of diseased berries (Farkas, 1988). It is known that laccase activity in the must of infected grapes is high and the activity of this enzyme deteriorates wine quality (Roudet *et al.*, 1992). The quality of the wine is also affected when the fungus converts glucose and fructose to glycerol and gluconic acid and produces enzymes that catalyse the oxidation of phenolic compounds, such as stilbene phytoalexins (Bavaresco *et al.*, 1997). In contrast to the polysaccharides of healthy grapes, β -glucans produced in *Botrytis*-infected grapes can cause severe problems. They hinder juice and wine clarification by inhibiting the precipitation of tannins and proteins (Ribéreau-Gayon *et al.*, 1980). Wines produced from rotten grapes have off-flavours and are prone to oxidation and bacterial contamination, making them unsuitable for ageing. *Botrytis*-infected grapes are always unwelcome

for red wine, as they have a pronounced effect on the colour, lightening it and causing premature browning (Riberéau-Gayon, 1988).

In certain cultivars and especially under certain climatic conditions, *Botrytis* infection of grape clusters takes on a form known as noble rot. Under ideal conditions the mould does its work gradually, over a prolonged period, and as the water evaporates from the grape, the sugar and acid in the juice will increase until they almost double their original concentration (Pucheu-Planté and Seguin, 1978). Under these circumstances, this rot is beneficial and contributes to the production of exceptionally sweet white wines, the most famous of which are the Tokays of Hungary, the Sauternes of France, and the *Auslese*, *Beerenauslese* and *Trockenbeerenauslese* of Germany (Donéche, 1992).

When an infection occurs in a plant, the invading pathogen is one of the key role-players in the process. Understanding the pathogen and its mode of infection is imperative to unravel the attack and resistance mechanisms of the pathogen and host, respectively. To illustrate this and to provide the relevant background to the coming sections on plant defence mechanisms, *B. cinerea* as the casual agent of grey mould will be discussed as a model pathogen.

2.3 BOTRYTIS CINEREA – ENEMY OF THE VINE AND A MODEL PATHOGEN TO UNRAVEL SOME ASPECTS OF FUNGAL PATHOGENICITY

2.3.1 The infection process of *Botrytis* in brief

Unravelling the key processes in host-pathogen interactions is a difficult task, as two organisms are involved, each with its own separate structure and metabolism. Each partner may influence the activity of the other by a dynamic process of signal and response, so that the properties of the host-pathogen complex are distinct from those of either partner alone (Lucas, 1998).

An infection process extends from the germination or multiplication of an infective propagule within or on the surface of a potential host to the time the pathogen establishes some form of parasitic relationship with its host. The infection process can be divided into three phases: pre-entry, entry and colonisation (Brown and Ogle, 1997).

B. cinerea is not a specialised grapevine parasite and infects an extensive variety of plants. Spores represent the main form of infection by this necrotrophic fungus. Spores of *B. cinerea* are dispersed in large numbers by air, water droplets and insects. As a consequence, spores may come from plant sources other than grapevines. Nevertheless, most early infections probably develop from spores produced on over-wintered fungal tissue in the vineyard (Jackson, 1994).

The interactions of *B. cinerea* with a susceptible host plant can be distinguished in three consecutive stages (Kamoen, 1992):

- i) germination of the conidia on the plant surface,
- ii) initial colonisation during penetration of the host tissue, and

iii) expansion of the lesion in the host tissue

Upon germination, spores produce one or more germ tubes that grow out through the spore wall (Epton and Richmond, 1980). Penetration occurs shortly thereafter, either through the cuticle or stomata or through wounds. Since physiological and anatomical changes increase the susceptibility of fruit to fungal attack during the growing season, penetration of the fruit often occurs either through microfissures, which develop around stomata, or through micropores, which form in the cuticle. Both provide sites for fungal penetration and the release of nutrients that can further aid spore germination (Ribéreau-Gayon *et al.*, 1980; Verhoeff, 1980). Subsequent ramification progresses parallel to the berry surface through the hypodermal tissue.

Senescent tissues, which remain attached to plants, also serve as an entry route for *B. cinerea* and facilitate the invasion of adjoining healthy tissues. This favours the fungus in two ways: it represents easy access to internal tissues, and it contains necrotic cells that may have a lower resistance to the fungus and provide a saprophytic growth base from which fungal enzymes and toxins may diffuse into adjacent healthy tissues (Kamoen, 1992).

2.3.2 The role of cell wall-degrading enzymes in *Botrytis* infection

Once the plant cuticle has been breached, or shortly after germination and germ tube extension in wounded or senescent tissues, the fungus secretes enzymes that degrade the cell walls of the host. Pathogen hydrolytic enzymes are crucial for infection by *B. cinerea* (Elad and Evensen, 1995). Cell wall degradation may be important to fungi not only for penetration and colonisation inside the plant tissue but also for releasing nutrients necessary for growth from the wall polysaccharides. Most fungi produce a wide array of enzymes capable of depolymerising the polysaccharides of the plant cell wall. Many of these enzymes are extracellularly targeted glycoproteins that are inducible upon exposure of the fungus to plant cell walls (De Lorenzo *et al.*, 1997).

Cell wall-degrading enzymes have been demonstrated in *B. cinerea*-infected tissues from many plants and in cultures of *Botrytis* spp. on media containing plant cell walls (Coley-Smith *et al.*, 1980). These include: cutinase, which hydrolyses secondary ester linkages of the cutin polymer (Salinas *et al.*, 1992); pectinase (exo- and endo-polygalacturonases, pectin lyase and pectin methyl esterase), which are secreted during the first phase of host-pathogen interactions (Collmer and Keen, 1986; Johnston and Williamson, 1992); cellulase and a trans-eliminase (Verhoeff and Warren, 1972); xylanases, arabinase, β -mannosidase, β -glucosidase, β -galactosidase, and α -galactosidase (Urbanek and Zalewska-Sobczak, 1984). The role of cell wall hydrolases in plant disease is well established, and some of the genes encoding these enzymes have been cloned. The evidence for the importance of hydrolases in grey mould disease is mostly correlative (Leone, 1992) or analogous with other plant pathogens (Collmer and Keen, 1986).

Fungal endo-PGs have important functions during the early stages of plant pathogenesis (Hahn *et al.*, 1989). These enzymes are the first detectable enzymes secreted by phytopathogenic fungi and the products of the enzyme's action are thought to be used as nourishment by the fungi (Jones *et al.*, 1972). Furthermore, pre-treatment of plant cell walls with endo-PGs appears to facilitate the ability of other fungus-secreted plant cell wall-degrading enzymes to attack their substrates (Karr and Albersheim, 1970). *B. cinerea* secretes a large number of PG isozymes (Johnston and Williamson, 1992; Van der Cruyssen *et al.*, 1994). Recent molecular genetic studies have identified a PG gene family consisting of six members (Wubben *et al.*, 1999). Mutation of one member of the PG family, *Bcpg1*, resulted in a significant reduction of virulence on tomato leaves and fruit, as well as on apple fruit (Ten Have *et al.*, 1998), thereby for the first time providing functional evidence that an endo-PG also contributes to virulence of a plant pathogen.

2.3.3 Reactions in the host plant upon infection

The resistance of unripe fruits to fungal decay has been associated with the presence of preformed antifungal compounds (Prusky and Keen, 1993; 1995). Pathogens often infect unripe fruits, but then remain quiescent. The onset of decay coincides with decreases in the concentrations of antifungal compounds to subtoxic levels as the fruit ripens (Morrissey and Osbourn, 1999). Thus, quiescence may represent a mechanism for avoiding toxic levels of antifungal plant compounds. Similarly, between bloom and véraison, grape berries are resistant to *B. cinerea*, although they can harbour the pathogen without any visible signs of disease development. Pezet and Pont (1984) have shown that crude extracts of healthy berries collected during this stage are able to strongly inhibit germination of *B. cinerea* conidia. Pathogen resistance in the quiescent stage is thought to involve both the active and passive defence of grapes. Some of the active defence systems involved include:

- i) The synthesis of phytoalexins (Langcake and Pryce, 1977),
- ii) The inhibition of exo-enzymes of *B. cinerea* by proanthocyanidins (Pezet and Pont, 1992),
- iii) The toxicity of glycolic acid, which enhances the biocidal activity of pterostilbene (Pezet and Pont, 1988),
- iv) The accumulation of anthocyanins and phenolic compounds (Nyerges *et al.*, 1975), and
- v) The production of the pathogenesis-related proteins, β -1,3-glucanase and chitinase (Renault *et al.*, 1996).

Passive defences that are also effective against infection include: synthesis of lignified barriers produced from oxidised phenols (Mansfield, 1980), the structure of the epidermis (Bernard, 1976) and the thickness and composition of the cuticle (Marois *et al.*, 1986).

The phytoalexin resveratrol has provoked intense interest due to its antifungal properties. Fungal challenge of the grapevine leaves and berries results in the production of the stilbene phytoalexin resveratrol (trans-3,5,4'-trihydroxystilbene), together with the biosynthetically related compounds viniferin and pterostilbene (Langcake, 1981; Jeandet *et al.*, 1995; Adrian *et al.*, 1997). There are a number of indications that resveratrol may contribute to disease resistance in grapevine. Resveratrol was shown to control *B. cinerea* growth, both *in vitro* (Hoos and Blaich, 1990) and *in planta*, after its accumulation in transgenic tobacco plants expressing the grapevine stilbene synthase gene (Hain *et al.*, 1993). In addition, a strong correlation has been found between the ability of grapevine cultivars to synthesise resveratrol in response to UV irradiation and their tolerance toward *B. cinerea* (Jeandet *et al.*, 1992). Although the resistance of grape berries to *B. cinerea* is the result of several conjugated processes, these processes are not yet understood completely (Goetz *et al.*, 1999; Davies and Robinson, 2000).

In grapevine, it was found that the resistance of different grape genotypes to powdery mildew was correlated directly with the levels of chitinase and β -1,3-glucanase enzymes (Giannakis *et al.*, 1998). Their antifungal properties were further confirmed when glucanase and chitinase enzymes purified from the leaves of a resistant cultivar showed inhibition to powdery mildew (Giannakis *et al.*, 1998). A number of chitinase and glucanase-encoding genes have been isolated from grapevine (Derckel *et al.*, 1996) and it is important to study their regulation and mode of action as a means of enhancing disease resistance (Robinson *et al.*, 1997).

The constitutive co-expression of the genes encoding chitinase and glucanase proteins in transgenic plants conferred higher levels of fungal resistance than either gene alone, indicating a synergistic interaction between the two enzymes (Zhu *et al.*, 1994; Jongedijk *et al.*, 1995). This approach has also been used in grapevine, and several transgenic lines expressing PR protein encoding genes are currently under evaluation (Kikkert *et al.*, 1996; Kikkert *et al.*, 2000).

2.4 PATHOGEN DEFENCE IN PLANTS – A MODEL OF BIOLOGICAL COMPLEXITY

2.4.1 Introduction

Plants, like animals, are continually challenged by a variety of potential pathogens. Unlike animals, however, plants lack a circulating somatically-adaptive immune system to protect themselves against pathogens. Instead they have developed sophisticated mechanisms to protect themselves from disease. Each plant cell is capable of defending itself by means of a combination of constitutive defences, providing built-in physical and chemical barriers that hinder infection, as well as a wide variety of defence responses induced only after pathogen attack (Kombrink and Somssich, 1995). When these induced responses are triggered rapidly and co-ordinately during a given plant-pathogen interaction, the plant is resistant to disease. A susceptible plant responds more slowly with an onset of defence mechanisms after

infection. Thus, the timely recognition of an invading micro organism and the rapid and effective induction of defence responses appear to make a key difference between resistant and susceptible plants (Yang *et al.*, 1997).

2.4.2 The hypersensitive response

The range of phytopathogenic organisms that attack plants is diverse and each has a unique mode of pathogenicity. Despite the vast array of potential phytopathogens, resistance is the rule and susceptibility is the exception. Plants have several inducible defence mechanisms that act to limit pathogen infection.

One of the most rapid and efficient mechanisms is the hypersensitive response (HR) (Hammond-Kosack and Jones, 1996). The HR is characterised by localised cell and tissue death at the site of infection and induction of metabolic changes in the cells surrounding necrotic lesions, resulting in confinement of the invading pathogen (Baker *et al.*, 1997). The HR has been proposed to play a key role in disease resistance. In interactions with biotrophic pathogens, plant cell death would deprive the pathogen of access to further nutrients. In interactions involving hemibiotrophic and necrotrophic pathogens, cell death may lead to the release of toxic preformed substances that are stored in the vacuole (Osbourn, 1996). Alternatively, the levels of induced phytoalexins may accumulate to inhibitory concentrations because they are no longer metabolised.

2.4.3 Gene-for-gene recognition

The first insight into the genetics of plant disease resistance involving the HR was the pioneering work done by Flor (Flor, 1956). Flor proposed a gene-for-gene model for the genetic interaction between plant and pathogen. This model states that a dominant gene from the host (*R* gene) interacts with a corresponding dominant avirulence (*avr*) gene from the pathogen. The interaction between the two corresponding genes elicits a HR, thus providing resistance.

The newly emerged tools of molecular biology have helped refine this model and have led to the hypothesis that, in gene-for-gene interactions (**Figure 2.2**), the induction of the plant defence response that leads to HR is initiated by the plant's recognition of specific signal molecules (elicitors) produced by the pathogen. These elicitors are encoded directly or indirectly by avirulence genes, and *R* genes are thought to encode receptors for these elicitors (Staskawicz *et al.*, 1995).

Elicitor recognition activates a cascade of defence responses (**Figure 2.3**) that lead to the HR and subsequent inhibition of pathogen growth. The defence responses include strengthening of the cell walls through increased synthesis and deposition of hydroxyproline-rich glycoproteins, callose, lignin and other phenolic compounds. Increased peroxidase activity, which is required for lignification and for cell wall protein cross-linking, is also observed. Additionally, phytoalexins, which are low molecular weight compounds with antimicrobial activity, and the phenylpropanoid pathway enzymes involved in phytoalexin synthesis are induced rapidly. A dramatic increase in the level of reactive oxygen species, known as an oxidative burst, also

occurs. Several other defence responses are not induced until many hours or even days after infection. These later responses include the induction of pathogenesis-related (PR) proteins, such as chitinases and β -1,3-glucanases. These events characterise a typical plant defence response, irrespective of the pathogen.

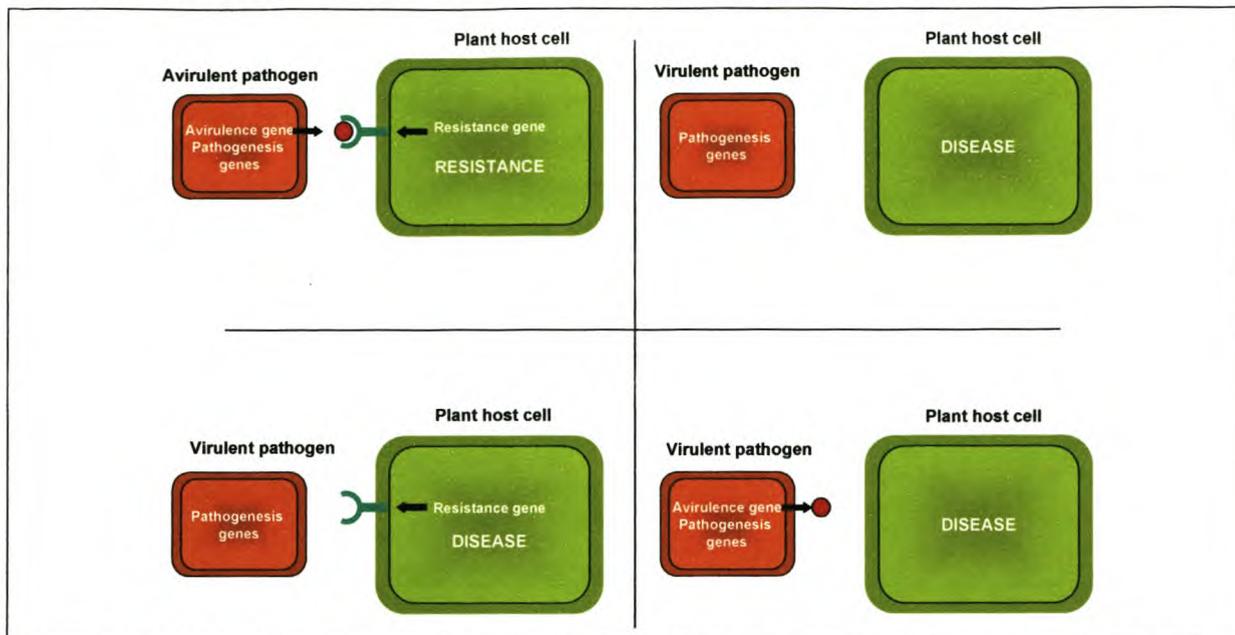


Figure 2.2 Gene-for-gene interactions specify plant disease resistance. Resistance is only expressed when a plant that contains a specific *R* gene recognises a pathogen that has the corresponding avirulence gene (upper left panel). All other combinations lead to lack of recognition by the host, and the result is disease (Staskawicz *et al.*, 1995).

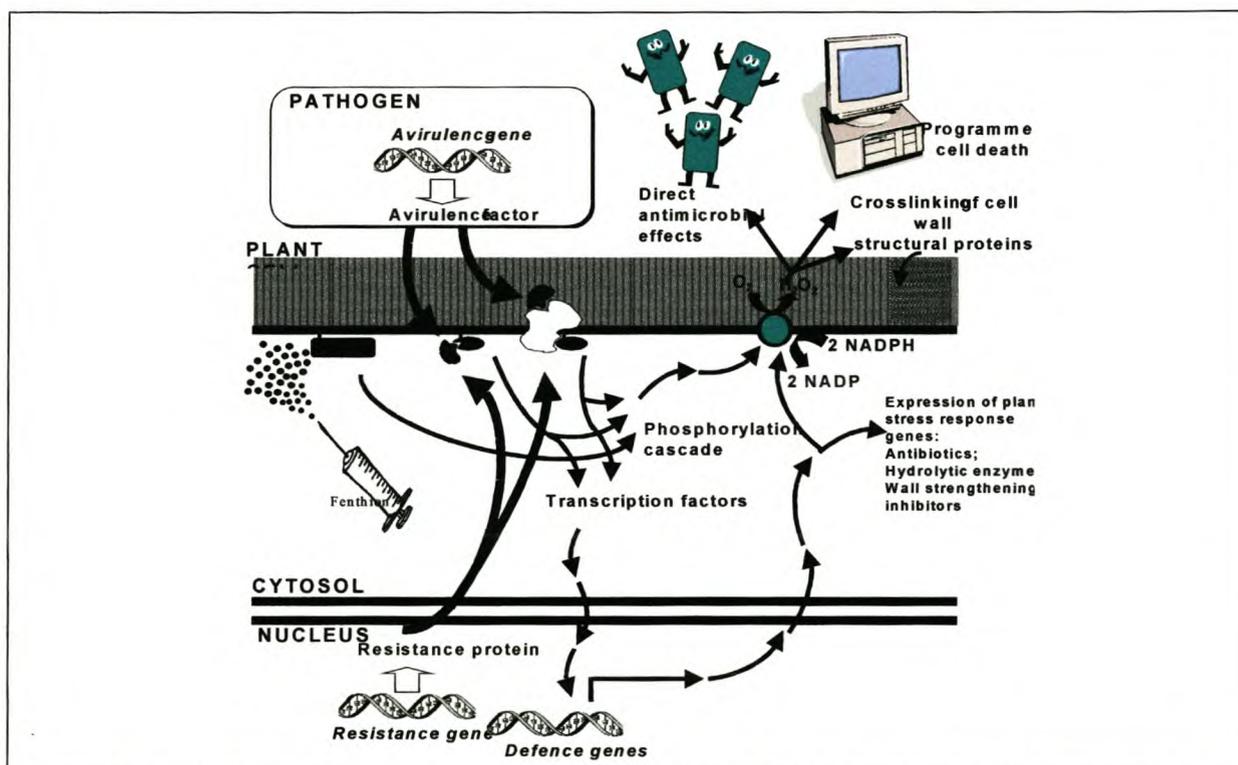


Figure 2.3 A speculative drawing of defence responses activated in a plant-pathogen interaction. A pathogen secretes elicitor molecules that are perceived via the plant resistance gene products. The resulting HR encompasses a number of defence responses (Lamb, 1994).

2.4.4 Structure and classes of resistance genes

The dominant nature of *R* and *avr* genes has led to the deduction that *R* genes encode proteins that can recognise *avr*-dependent ligands. Following pathogen recognition, the *R* protein is presumed to activate signalling cascade(s) that coordinate the initial plant defence responses to limit pathogen ingress. Implied in this view is the notion that *R* proteins would be expressed in healthy, unchallenged plants in readiness for the detection of attack. A third requirement of *R* proteins is the capacity for rapid evolution of specificity. New virulent races of pathogens frequently evolve that can evade specific *R* gene-mediated resistance (Keen, 1990; Michelmore, 1995; Hammond-Kosack and Jones, 1997). Thus a mechanism is required by which plants can rapidly evolve new *R* genes to resist virulent isolates.

R genes are organised in clusters that show varying levels of recombination between the component genes. Genes within a single cluster can determine resistance to very different pathogens. On the basis of their clustered distribution and by inference from other cell-to-cell recognition systems, *R* genes were hypothesised to encode functionally and evolutionarily related members of recognition systems (Michelmore and Meyers, 1998). Molecular data have supported this hypothesis. Most of the resistance genes that have been cloned and characterised all resemble components involved in signal transduction pathways (Baker *et al.*, 1997; Ellis and Jones, 1998). The genes can be classified into five general categories based on their predicted protein structures (**Table 2.1**).

R genes encode a number of protein motifs in a variety of combinations that are characteristic of receptors in yeast, *Drosophila* and vertebrates. The most prevalent class contains a nucleotide-binding site (NBS), a leucine-rich repeat (LRR) region and a leucine zipper (L Zip) motif. Some are composed predominantly of an LRR region and a trans-membrane (TM) domain. Another is comprised of a LRR region and a serine/threonine kinase domain (**Figure 2.4**).

The simplest interpretation of Flor's gene-for-gene hypothesis is that the *Avr*-gene dependent ligand binds directly to the *R* gene product, which then activates downstream signalling events to induce various defence responses (Gabriel and Rolfe, 1990). As the majority of the isolated *R* genes encode proteins that possess domains characteristic of authentic receptor proteins found in other species, a receptor-like function for plant *R* proteins appears likely. The most obvious candidate for providing the recognition specificity typical of a receptor is the LRR domain. LRRs are believed to mediate protein-protein interactions, or to determine specific recognition of ligands by the receptor molecules (Kobe and Deisenhofer, 1994). LRR domains of *R* gene products show similarity to diverse proteins controlling cell-cell communication in development and signalling, suggesting that these genes may have evolved through the duplication and divergence of common ancestors (Clark *et al.*, 1997; Li and Chory, 1997). A hydrophobic face could form for those *R* proteins predicted to be extracytoplasmic, to facilitate multiple interactions with other proteins or ligands. For the LRR proteins predicted to be cytoplasmically localised, the LRR

domain is accepted as the recognition domain, primarily because all the other motifs appear to have a signalling capacity. However, the role of the LRRs in these R proteins could be at the level of dimerisation or interaction with either upstream or downstream signalling components (Hammond-Kosack and Jones, 1997).

Table 2.1 The five classes of cloned plant disease resistance genes.

Class	Gene	Plant source	Associated pathogen	Infection type/ organ attacked	Predicted features of R protein
1.	<i>Hm1</i>	Maize	<i>Helminthosporium maydis</i> (race 1)	Fungal necrotroph/ leaf	Detoxifying enzyme HC-toxin reductase
2.	<i>Pto</i>	Tomato	<i>Pseudomonas syringae</i> pv. tomato (<i>avrPto</i>)	Extracellular bacteria/ leaf	Intracellular serine/ threonine protein kinase
3a.	<i>RSP2</i>	<i>Arabidopsis</i>	<i>Pseudomonas syringae</i> pv. tomato (<i>avrRpt2</i>)	Extracellular bacteria/ leaf	L. Zip/ NBS/ LRR
	<i>RPM1</i>	<i>Arabidopsis</i>	<i>Pseudomonas syringae</i> pv. <i>maculicola</i> (<i>avrRpm1/avrB</i>)	Extracellular bacteria/ leaf	Intracellular protein with amino terminal leucine zipper domain (L. Zip) and
	<i>I₂</i>	Tomato	<i>Fusarium oxysporium</i> f.sp. <i>lycopersicon</i>	Necrotrophic fungus/ root and vascular tissue	nucleotide binding site (NBS) and leucine rich repeat (LRR) domains
3b.	<i>N</i>	Tobacco	Mosaic virus	Intracellular virus/ leaf and phloem	Toll/ NBS/ LRR
	<i>L6, M</i>	Flax	<i>Melampsora lini</i> (<i>AL6, AM</i>)	Biotrophic fungal rust with haustoria/ leaf	Intracellular protein with amino terminal domain homology with
	<i>RPP5</i>	<i>Arabidopsis</i>	<i>Peronospora parasitica</i>	Biotrophic fungus with haustoria/ leaf	<i>Drosophila</i> Toll protein, NBS and LRR
4.	<i>Cf-9, Cf-2, Cf-4, Cf-5</i>	Tomato	<i>Cladosporium fulvum</i> (<i>Avr9, Avr2, Avr4, Avr5</i>)	Biotrophic extracellular fungus without haustoria/ leaf	Extracellular LRR protein with single membrane spanning region and short cytoplasmic carboxyl terminus
5.	<i>Xa-21</i>	Rice	<i>Xanthomonas oryzae</i> p.v. <i>oryzae</i> (all races)	Extracellular bacteria/ leaf	Extracellular LRR protein with single membrane spanning region and cytoplasmic kinase domain

In the NBS/LRR class of R genes, the nucleotide binding site and the leucine zipper domains are the most likely to be involved in signalling. The presence of the NBS, which is found in numerous ATP and GTP binding proteins, suggests that although these proteins do not possess intrinsic kinase activity, they could activate kinases of G proteins (Hammond-Kosack and Jones, 1997). The leucine zipper regions found in *RPM1* and *RPS2*, potentially could facilitate homodimerisation of the proteins themselves or heterodimerisation with other proteins (Landschulz *et al.*, 1988). R proteins could exist as monomers before pathogen challenge and then undergo dimerisation or oligomerisation upon activation. Alternatively, they could exist initially as dimers or multimers that disassociate upon activation.

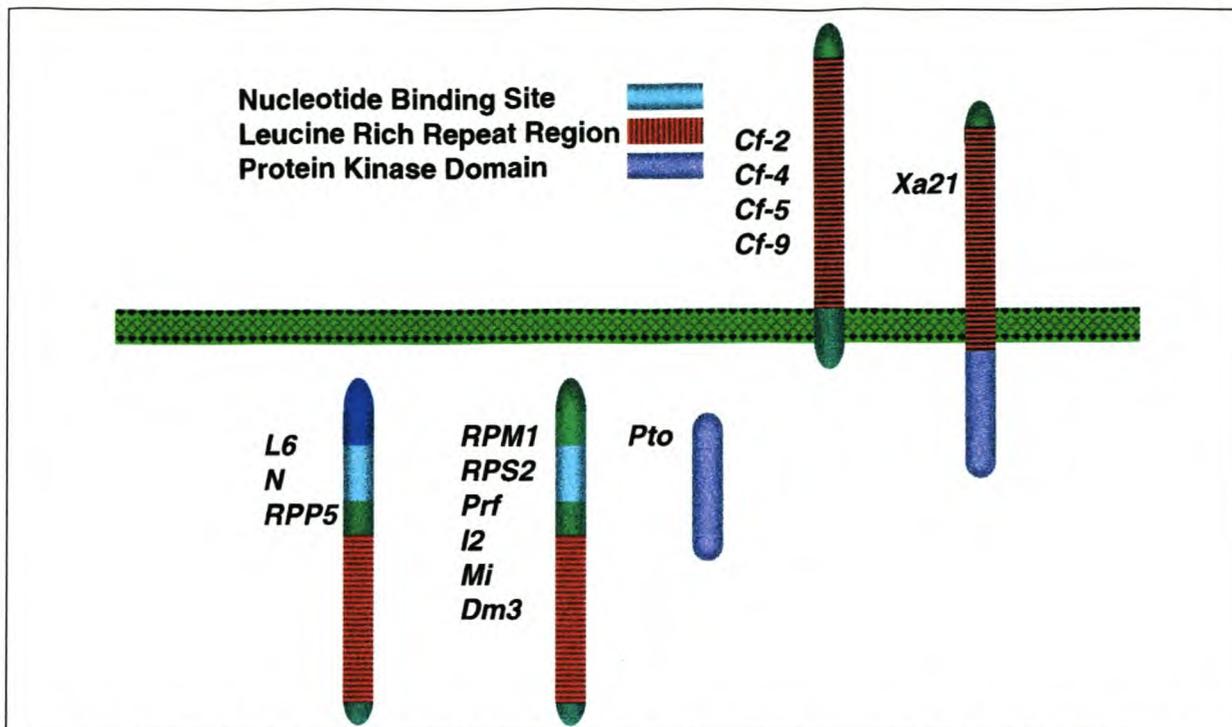


Figure 2.4 Schematic representation of the major protein motifs shared between the deduced products of cloned resistance genes (Michelmore and Meyers, 1998). *R* genes predicted to encode cytoplasmic proteins include: the *Arabidopsis* *RPS2* and *RPM1* genes, which confer resistance to strains of *Pseudomonas syringae* that carry the plasmid-borne *avrRpt2* gene and *avrB* or *avrRpm1* genes, respectively; the tobacco *N* gene, which confers resistance to tobacco mosaic virus; the flax *L6* gene, which confers resistance to *Melampsora lini*; the *Arabidopsis* *RPP5* gene which confers resistance to *Peronospora parasitica*, and the tomato *Pto* gene, which confers resistance to races of *P. syringae* pv. tomato that carry the *avrPto* gene. *R* genes predicted to encode proteins with extracytoplasmic domains include: the tomato *Cf-9*, *Cf-2*, *Cf-4* and *Cf-5* genes, which confer resistance to *Cladosporium fulvum* with *Avr* genes *Avr9*, *Avr2*, *Avr4* and *Avr5*, respectively, and the rice *Xa21*, which confers resistance to *Xanthomonas oryzae* pv. *oryzae*.

The serine/threonine kinase capacity possessed by *Pto* and *Xa21* could clearly facilitate downstream signalling. For many gene-mediated resistances, the addition of either kinase or phosphatase inhibitors significantly blocked the induction of rapid defence responses (Dunigan and Madlener, 1995). It therefore appears likely that both kinases and phosphatases are involved in downstream *R* protein-mediated signalling events.

It is interesting to note that the *R* proteins that are predicted to be cytoplasmic are the ones involved in the recognition of bacterial or viral AVR components whose activity requires a location inside the plant cell. Other *R* proteins have a membrane-spanning region and an extracellular LRR domain, suggesting that, in this case, the interaction occurs outside the cell (Hammond-Kosack and Jones, 1997).

2.4.5 Adaptive selection of pathogen recognition

The characterisation of nucleotide substitution patterns in resistance gene families has provided insight into the function and evolution of particular coding domains. The ratio of nucleotide substitution that result in changes in the amino acid sequence of the protein product (non-synonymous substitutions, K_A) and nucleotide substitutions

that do not result in changes at the amino acid level (synonymous substitutions, K_S) is particularly informative for the investigation of function (Richter and Ronald, 2000). Their relative ratio ($K_A:K_S$) is indicative of the selection pressure on the region. If there is no predominant selection on the region, $K_A:K_S$ will be ~ 1 . Amino acid changes tend to be deleterious to the conservation of protein structure and function, therefore most sequences are under purifying selection, which selects against deleterious mutations, and have a $K_A:K_S$ ratio of less than 1 (Michelmore and Meyers, 1998). Conversely, a $K_A:K_S$ ratio significantly greater than 1 indicates that there is a selective advantage for amino acid diversity between genes (Hughes and Nei, 1988; Messier and Stewart, 1997). Evidence of adaptive selection is rare but appears to be most common in gene regions that function in host and pathogen recognition (Endo *et al.*, 1996).

Analysis of 11 *Cladosporium fulvum* *R* gene family members revealed that the predicted solvent-exposed residues of the β -strand/ β -turn region of the LRR domain exhibit increased $K_A:K_S$ ratios relative to other residues in the LRR domain, suggesting that solvent-exposed residues play a role in ligand binding (Parniske *et al.*, 1997). Similarly, the LRR region encoded by *Xa21* and related paralogs show the same pattern (Wang *et al.*, 1998). This result indicates that this region has a functional importance and would be consistent with the LRR's putative role in ligand binding (Wang *et al.*, 1998). High $K_A:K_S$ ratios indicate that the LRR domain, which governs race-specific pathogen recognition, is subjected to adaptive evolution.

Although most models predict that resistance genes possessing novel ligand-binding capabilities arise from recombination events, another model predicted that novel ligand specificities would arise from single amino acid changes in the solvent-exposed surface of the LRR (Meyers *et al.*, 1998).

These results, and the compelling evidence that *R*-genes and their encoded proteins play a central role in the disease resistance mechanisms of plants and specifically in the recognition of pathogen elicitors, and the subsequent reliance of the disease signals throughout the plant, have established these and related proteins as models to study these processes. The polygalacturonase-inhibiting proteins (PGIPs), are one class of plant proteins that are described as receptor-like proteins. These proteins have been well established for their role in plant defence and will be discussed in further sections.

2.5 POLYGALACTURONASE-INHIBITING PROTEINS (PGIPs): THEIR ROLE IN SPECIFICITY AND DEFENCE AGAINST PATHOGENS

Polygalacturonase-inhibiting proteins and their substrates, endo-polygalacturonases (endo-PGs), are excellent examples of the dynamic interaction between pathogenicity and the defence of the pathogens and their hosts, respectively. To optimally understand the role of PGIPs in inhibiting PGs, it is imperative to understand the role of PGs in pathogenicity.

2.5.1 Endo-polygalacturonases (endo-PGs) involved in pathogenicity

Plant cell walls are at the forefront of plant-pathogen interactions. As the first barrier encountered by most plant pathogens, they must be degraded in order to allow penetration and colonisation. Plant cell walls contain various polymers that may serve as substrates for the numerous enzymes secreted by microbial pathogens, providing them with nutrients (Walton, 1994). Owing to their strategic location, plant cell walls are also involved primarily in plant defence. This is achieved through the increased deposition of structural polymers, particularly lignin, and the increase in several defence proteins (Bowles, 1990). The study of pathogenicity and defence has shown that the cell wall plays an important role in signalling phenomena underlying plant-pathogen interactions (Esquerré-Tugayé *et al.*, 2000).

It is generally accepted that cell wall degrading enzymes are essential for the pathogenic process (Howard *et al.*, 1990). Among the cell wall degrading enzymes, pectic enzymes capable of cleaving the 1,4-glycosidic bonds of homogalacturonans in an “endo” manner appear to play a key role. Endo-polygalacturonases (endo-PGs) are among the first enzymes to be secreted when fungi are grown on plant cell wall material *in vitro* (Jones *et al.*, 1972; Mankarios and Friend, 1980) and their action on cell walls is sometimes a prerequisite for wall degradation by other enzymes (Karr and Albersheim, 1970; Carpita and Gibeaut, 1993).

Endo-PGs hydrolyse homogalacturonan to mono-, di- and sometimes tri-galacturonic acid, assisting the colonisation of the plant tissues and providing nutrients for fungal growth during the early stages of pathogenesis (Hahn *et al.*, 1989). Fungal endo-PGs are also elicitors of plant defence responses. These enzymes may therefore play two contrary roles: as fungal aggression tools or as potential signaling molecules (elicitors). The early timing of endo-PG production is compatible with both functions. The ability of endo-PG to act as a signaling molecule raises the possibility that it may function as a determinant of specificity in plant-fungus interactions.

2.5.1.1 Oligogalacturonides as elicitors of plant defence responses

The available evidence indicates that fungal endo-PGs are not directly responsible for the induction of plant defence responses, but are rather pre-elicitors that release the true elicitors, namely oligogalacturonides, from the plant cell wall (Anderson, 1989). The first evidence that a galacturonide-rich fraction released from the walls induces accumulation of the phytoalexin glyceollin in soybean was provided by Hahn *et al.* (1981), who named the active component of the fraction an endogenous elicitor. The oligogalacturonide fragments and the more complex oligosaccharides, which are released from the cell wall homogalacturonan by the action of fungal endo-PGs, are endowed with biological activities (Darvill *et al.*, 1992).

Oligogalacturonides with a degree of polymerisation between 10 and 15 have been reported to strongly elicit a variety of defence mechanisms, including the accumulation of phytoalexins (Davis *et al.*, 1986), the synthesis of endo- β -1,3-

glucanase (Davis and Hahlbrock, 1987), chitinase (Broekaert and Peumans, 1988), lignin (Robertsen, 1986; Bruce and West, 1989), as well as the accumulation of PGIPs (Bergmann *et al.*, 1994). In carrot suspension-cultured cells, these oligogalacturonides also induced the transcriptional activation of several defence genes (Messiaen *et al.*, 1993). On the other hand, oligogalacturonides with a chain length shorter than 10 have been shown to possess little or no elicitor activity, confirming the importance of chain length for the elicitation potential (Hahn *et al.*, 1989; Darvill *et al.*, 1992).

2.5.1.2 Recognition and transduction of oligogalacturonide signals

The mechanism by which oligogalacturonides induce plant defence responses remains unknown. At present, little is known about the mechanism by which oligogalacturonide signals are perceived and subsequently imported into the plant cell. As the lipid bilayers of the plasma membrane are highly impermeable to these polar signalling molecules, it is very likely that they are recognised by cell surface receptors that specifically bind these extracellular signals, subsequently initiating a response in the target cell (John *et al.*, 1997). These responses at the plant cell surface include depolarisation of the plasma membrane, extracellular alkalinisation, stimulation of cytoplasmic Ca^{2+} influx, and K^{+} efflux across the plasma membrane of suspension-cultured tobacco cells (Mathieu *et al.*, 1991; Thain *et al.*, 1995). Furthermore, it has been reported that oligogalacturonides increase the level of free calcium in the cytosol of carrot protoplasts and induce an oxidative burst in soybean and cotton cells (Messiaen *et al.*, 1993). In plants, the production of reactive oxygen species (ROS) and associated processes, such as oxidative cross-linking of cell wall components and lipid peroxidation, have been proposed to contribute to both programmed cell death and rapid activation of defence responses (Levine *et al.*, 1994).

There are several lines of evidence suggesting that protein phosphorylation may be involved in oligogalacturonide-induced expression of defence genes. The *in vitro* phosphorylation of a threonine residue of a plasma membrane-associated protein from tomato and potato cells is strongly enhanced in the presence of oligogalacturonides (Farmer *et al.*, 1991). This suggests that a membrane-associated serine/threonine type protein kinase plays a role in transducing the message to the intracellular targets.

Oligogalacturonides have also been shown to control processes involved in plant growth and development (Campbell and Labavitch, 1991; Marfà *et al.*, 1991; Darvill *et al.*, 1992). Many of the development-related effects of oligogalacturonides appeared to be responsive to an auxin-antagonist activity of these oligosaccharins (Bellincampi *et al.*, 1993; 1996). Therefore, the same molecules appeared to act as signals for both plant development and plant defence responses, although the concentration at which oligogalacturonides affected development was one to two orders of magnitude lower than that required to induce plant defences (Bellincampi *et al.*, 1993). In healthy plants, it is likely that micromolar levels of oligogalacturonides

are released by developmentally regulated plant PGs (Peretto *et al.*, 1992), whereas much higher levels of oligogalacturonides are accumulated upon fungal attack. Different classes of receptors with different affinities for the same oligogalacturonides may discriminate between signalling for growth and morphogenesis and signalling for defence (De Lorenzo *et al.*, 1997).

2.5.2 Polygalacturonase-inhibiting proteins (PGIPs)

The first evidence of a proteinaceous inhibitor of fungal endo-PGs was obtained by Uritani and Stahmann (1961) in a water extract of sweet potato tissue. The isolation of a polygalacturonase inhibitor of *Phaseolus vulgaris* was reported in the early 1970s (Albersheim and Anderson, 1971). Since these initial reports, the occurrence of PGIPs have been reported in a variety of dicotyledonous plant spp., including bean, pea, green pepper, tomato, cucumber, apple, pear, orange, alfalfa (Hahn *et al.*, 1989 and references therein), soybean (Favaron *et al.*, 1994), raspberry (Johnston *et al.*, 1993), and more recently, potato (Machinandiarena *et al.*, 2001). PGIPs have also been identified in the pectin-rich monocotyledonous plant spp. *Allium cepa* (onion) and *A. porrum* (leek) (Favaron *et al.*, 1993). The characteristics of a few purified PGIPs are reported in **Table 2.2**.

Table 2.2 Characteristics of purified PGIPs.

Plant (tissue) isolated from	Molecular mass (kDa)	Isoelectric point	Mode of inhibition	Reference
Apple (fruits)	44 - 54	4.6	Mixed	Yao <i>et al.</i> (1995)
Pear (fruits)	43	6.6	Competitive	Abu-Goukh <i>et al.</i> (1983)
Bean (hypocotyls)	41	9.5	Non-competitive	Cervone <i>et al.</i> (1987)
Bean (flowers)	42	n.d. ¹	n.d. ¹	Salvi <i>et al.</i> (1990)
Bean (pods)				
PGIP-I	38	9.1	n.d.	Pressey (1996)
PGIP-II	38	9.2	n.d.	
Tomato (fruit)	35 - 41	9	Non-competitive	Stotz <i>et al.</i> (1994) Stotz <i>et al.</i> (2000)
Soybean (seedlings)	37 - 40	9 - 10	n.d.	Favaron <i>et al.</i> (1994)
Leek (stalk)				
PGIP-I	39	6.5	n.d.	Favaron <i>et al.</i> (1997)
PGIP-III	42	6.5	n.d.	
Raspberry (fruit)	38.5	>10	Non-competitive	Johnston <i>et al.</i> (1993)
Potato (leaves)	41	n.d.	n.d.	Machinandiarena <i>et al.</i> (2001)

¹n.d. = not determined

Some of these inhibitors are predominantly ionically bound to the plant cell wall, whereas others are readily extracted with dilute buffers. PGIPs are typically effective against the endo-PGs of fungi, with no effect on other pectic enzymes of either

microbial or plant origin (Cervone *et al.*, 1990). PGIP from bean hypocotyls protected bean cell walls against degradation by endo-PGs of *Colletotrichum lindemuthianum* *in vitro* (Lafitte *et al.*, 1984). Similarly, PGIPs prepared from tomato cell walls protected these walls from degradation by enzymes produced in culture by *Fusarium oxysporum* f.sp. *lycopersici* *in vitro* (Jones *et al.*, 1972).

Some observations suggest a correlation between the presence of PGIPs and the resistance of plants to fungal attack. In a study on the distribution of PGIP in various tissues of *P. vulgaris*, levels of PGIP in hypocotyls were shown to increase six- to nine-fold during seedling growth, leading to increased resistance of older bean hypocotyls (Salvi *et al.*, 1990; Bailey *et al.*, 1992). Increasing susceptibility of ripening pear fruits to *Dothiorella gregaria* and *B. cinerea* correlated with a decline in the concentration of PGIP present (Abu-Goukh *et al.*, 1983). In raspberry fruits, the level of PGIP was maximal in immature green fruits, which are more resistant to fungal attack, and decreased in mature, more susceptible fruits (Johnston *et al.*, 1993). Tissues of Chinese chestnut resistant to a virulent strain of *Cryphonectria parasitica* contained PGIP levels considerably higher than the susceptible American chestnut (Gao and Shain, 1995).

2.5.2.1 PGIPs favour the accumulation of elicitor-active oligogalacturonides

As discussed earlier, oligogalacturonides with a chain length of between 10 and 15 are produced transiently by the action of endo-PGs on homogalacturonan and act as elicitors of defence responses. The PGs eventually degrade the oligogalacturonides into inactive oligomers. This implies that a prolonged hydrolysis of homogalacturonan negatively affects the eliciting activity of endo-PGs. However, fungal endo-PGs and plant PGIPs form a specific, reversible, saturable and high affinity complex (Cervone *et al.*, 1986). PGIPs modulate the activity of endo-PGs in such a way that the balance between the release of elicitor-active oligogalacturonides and the depolymerisation of the active oligogalacturonides into inactive molecules is altered and the accumulation of elicitor-active molecules is favoured (Cervone *et al.*, 1989). Endo-PGs may function as signal molecules and PGIP as the receptor protein in the recognition that occurs between plants and their potential pathogens. A model of PGIP as a component of the cell surface signalling system that leads to the formation of elicitor-active oligogalacturonides is presented in **Figure 2.5**.

2.6 MOLECULAR AND BIOCHEMICAL CHARACTERISTICS OF PGIPs

2.6.1 Families of PGIP genes present in plants

Genes encoding PGIPs have been isolated from bean (Toubart *et al.*, 1992), soybean (Favaron *et al.*, 1994), tomato (Stotz *et al.*, 1994), pear (Stotz *et al.*, 1993), orange (unpublished, accession number Y08618), kiwifruit (Simpson *et al.*, 1995), apricot (unpublished, accession number AF020785), raspberry (Ramanathan *et al.*, 1997) and apple (Yao *et al.*, 1999). Genes cloned so far predict a hydrophobic N-terminal signal peptide that targets them to the endomembrane system for export

to the extracellular space (Toubart *et al.*, 1992; Stotz, *et al.*, 1993, 1994; Ramanathan *et al.*, 1997; Yao *et al.*, 1999).

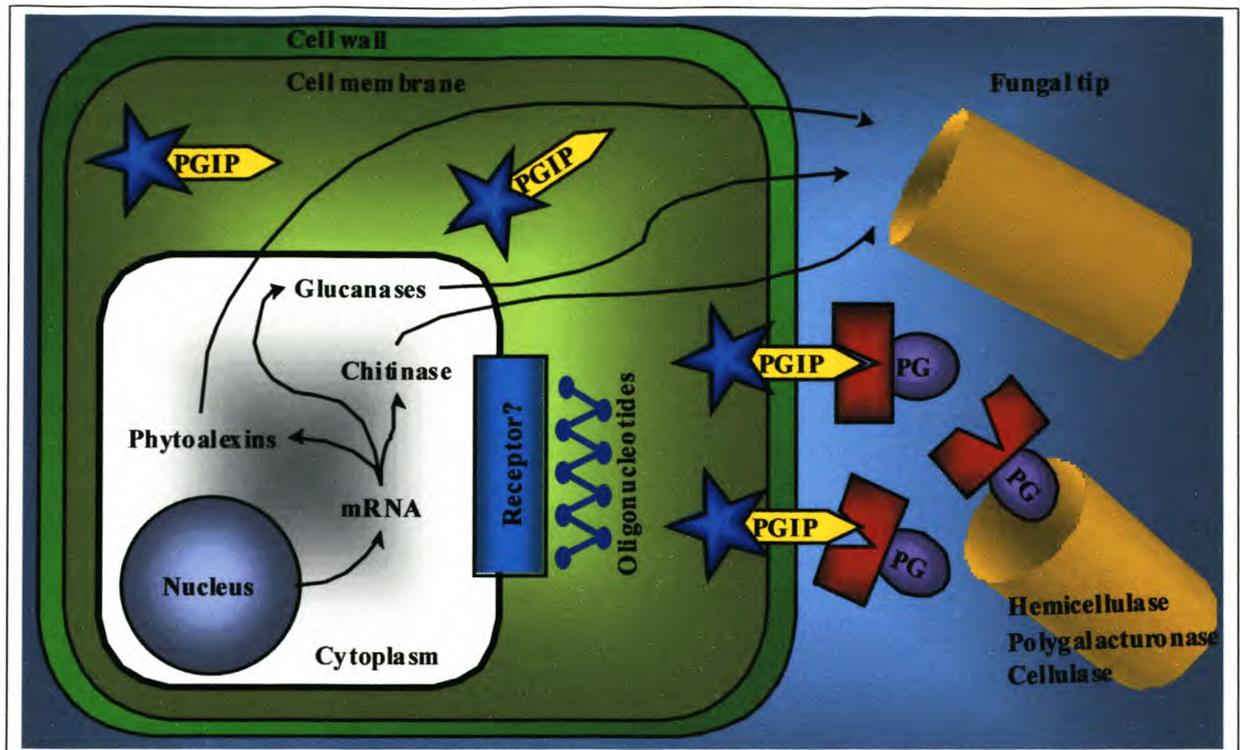


Figure 2.5 A schematic representation of the interaction of endopolygalacturonases (PGs), polygalacturonase-inhibiting proteins (PGIPs) and oligogalacturonides as a signalling system in communication between plants and fungi (adapted from De Lorenzo *et al.*, 1997).

Studies of the genomic organisation of the isolated *pgip* genes revealed their occurrence in multigene families. Two homologous genes have been found in pear by Southern blot analysis (Abu-Goukh *et al.*, 1983; Stotz *et al.*, 1993). Similarly, two homologous genes were also present in tomato (Stotz *et al.*, 1994), apple (Yao *et al.*, 1999) and raspberry (Ramanathan *et al.*, 1997). Genomic analysis of soybean revealed a single gene with other weakly hybridising bands, indicating the presence of related genes (Favaron *et al.*, 1994). A family of *pgip* genes with at least 5 members, and possibly as many as 15, is present in the genome of *P. vulgaris* (Frediani *et al.*, 1993). Characterisation of all *pgip* members and their regulation could contribute significantly to the understanding of the role of PGIP in resistance of plants to pathogens.

2.6.2 Regulation of *PGIP* expression

The organ-specific accumulation and expression of *pgip* transcripts differ among the isolated *pgip* genes. In bean, *pgip* transcripts were detected in suspension-cultured cells and at low levels in all tissues. Higher levels were found in the transition zone between the elongating and mature region of young hypocotyls, in the elongating region of etiolated hypocotyls, in the basal region of the stem of adult plants and in the pods (Devoto *et al.*, 1997). Expression of the pear *pgip* gene was also regulated in a tissue-specific manner; levels of *pgip* transcripts were much higher in fruits than in flowers or leaves (Stotz *et al.*, 1993). In contrast, *pgip* transcript levels in raspberry

remained high throughout fruit development (Ramanathan *et al.*, 1997). Similarly, *pgip* expression in tomato and apple, occurred in immature fruit right through to ripe fruit (Stotz *et al.*, 1994; Yao *et al.*, 1999).

Accumulation of *pgip* transcripts have been demonstrated in suspension-cultured bean cells following the addition of elicitor-active oligogalacturonides and fungal glucan, and in bean hypocotyls in response to wounding or salicylic acid (Bergmann *et al.*, 1994). Induction of *pgip* transcripts was detected in apple tissue adjacent to the inoculation sites of *Penicillium expansum* and *B. cinerea*, as well as in wounded apple fruit (Yao *et al.*, 1999). Rapid induction of *pgip* transcripts has also been associated with the establishment of an incompatible interaction manifested by the appearance of a hypersensitive reaction in bean infected with *C. lindemuthianum*, whereas a more delayed increase, coincident with the onset of lesion formation, occurs in compatible interactions (Nuss *et al.*, 1996). In incompatible interactions, the accumulation of *pgip* mRNA is higher in the epidermal cells proximal to the site of infection and within a few cell layers of parenchymal cells immediately below (Devoto *et al.*, 1997). These data indicate that PGIP expression is regulated upon the early race-specific recognition event in a manner similar to that observed for other known defence-related genes.

Most of the expression studies on *pgip* genes has been done by following the overall pattern of expression, presumably reflecting the bulk of PGIP activity. The importance of studying gene regulation and expression of single *pgip* genes became evident when a bean *pgip* promoter was analysed. A functional analysis of the promoter of the *P. vulgaris pgip-1* gene fused to a β -glucuronidase (GUS) reporter gene in transgenic tobacco plants showed developmentally-regulated GUS expression localised in vascular tissue and in the stigma. The *pgip-1* promoter responded to wounding, but not to infection, treatment with salicylic acid, elicitors such as oligogalacturonides or fungal glucan. This led to the conclusion that the activity of the *pgip-1* promoter did not mirror the pattern of expression of the whole gene family, suggesting that different members of the *pgip* gene family are regulated differentially (Devoto *et al.*, 1998).

The temporal and spatial pattern of the induction of PGIP expression during infection is indirect evidence for the importance of PGIP in plant defence and fungal endo-PGs in pathogenesis. Direct evidence may be provided by the analysis of transgenic plants that over-express PGIP-encoding genes. Transgenic tomato plants expressing the pear fruit PGIP (pPGIP) were used to demonstrate that the pPGIP influences disease development. The expressed pPGIP was active in both vegetative and fruit tissue, and showed inhibitory activity against endo-PGs from *B. cinerea*. The growth of *B. cinerea* on ripe tomato fruit expressing pPGIP was reduced and tissue breakdown was diminished. In transgenic leaves, the expression of pPGIP reduced lesions of macerated tissue (Powell *et al.*, 2000). Heterologous expression of pPGIP has demonstrated that PGIP inhibition of fungal PGs slows the expansion of disease lesions and the associated tissue maceration.

2.6.3 PGIP is a leucine-rich repeat (LRR) protein

A striking feature of PGIPs is their modular structure composed of leucine-rich repeats. In PGIP from *P. vulgaris*, the domain spanning amino acid 69 to 326 can be divided into a set of 10.5 tandemly repeating units, each derived from modifications of a 24-amino acid peptide; the repeat element matches the extracytoplasmic consensus LxxLxxLxxLxLxxNxLxGIPxx (De Lorenzo *et al.*, 1994). Proteins, from sources as diverse as bacteria to humans, contain leucine-rich tandem repeats similar to those found in PGIP (Kobe and Deisenhofer, 1993). In all described LRR proteins, consensus sequences for the repeating units are surprisingly similar considering the range of organisms and the widely divergent functions of the proteins (**Table 2.3**). The high degree of similarity in these motifs between these proteins indicates a strong selection pressure for the conservation of this structure. The similarity may indicate an evolutionary conservation between the proteins or reflect the convergent evolution of a protein domain (De Lorenzo *et al.*, 1997). Since a common feature among these proteins appears to be that of membrane association and protein binding, a domain comprised of tandem LRRs is likely to represent a structure specialised to achieve strong interactions between macromolecules.

Table 2.3 Comparison between the LRR motif of PGIP and those of other LRR proteins.

Protein	Organism	Consensus sequence	LRR repeats
PGIP	Bean	LxxLKxLxxLdLSxNxLxG----xIPxx	10
RLK5	<i>Arabidopsis</i>	LxxLxxLxxLxLxxNxLSG----xIPxx	21
<i>Cf-9</i>	Tomato	LxxLxxLxxLDLSSNNLxG----xIPSx	28
<i>Xa21</i>	Rice	LxxLxxLxxLDLSSNNLxG----xIPxx	23
LRG	Human	LxxLxxLxxLxLxxNxLxx----LPxxL	13
SLRP	Animal	axxLxxLxxLxLxxNxIxx----Ixxxx	7-12
<i>Toll</i>	Fly	FxHxxNLxxLxLxxNxLxx----LPxxP	15

x = any amino acid

a = an aliphatic amino acid residue

d = an acidic residue

In plants, LRR proteins play a relevant role in both development and defence, where specificity of recognition is a fundamental prerequisite. PGIPs are evolutionary related to several plant *R* genes that participate in gene-for-gene resistance mechanisms. These include the tomato genes *Cf-9* and *Cf-2*, the rice gene *Xa21* (Jones and Jones, 1997), as well as several orphan receptor kinases involved in *Arabidopsis* development, such as *ERECTA* (Torii *et al.*, 1996) and *CLAVATA1* (Clark *et al.*, 1997). All these proteins (including PGIPs) share LRRs of the extracellular or extracytoplasmic type and display a similarity not only in the LRR region, but also in the regions outside the LRR domain (Bent, 1996).

PGIPs also present intriguing similarities with a class of animal extracytoplasmic LRR proteoglycans designated SLRPs (small leucine-rich proteoglycans), which include five structurally related but genetically distinct members. These proteins are decorin, biglycan, fibromodulin, lumican and epiphycan (Iozzo and Murdoch, 1996). PGIPs and SLRPs share several properties: they are soluble proteins of the extracellular matrix, they exhibit similar molecular sizes, they have similar number of LRRs in the central domain, and they share the presence of cysteine-rich clusters flanking the central domain (Mattei *et al.*, 2001). SLRPs are involved in matrix assembly, orienting and ordering of collagen fibrils in the control of cell proliferation and in pathological processes such as wound healing and tissue repair. Some of the known interactions of SLRPs are with collagen and fibronectin. Binding to collagen type I is a common feature of all the SLRPs examined and is due to the LRR located between the fourth and sixth LRR in the LRR domain (Iozzo and Murdoch, 1996). By analogy with the function of animal SLRPs, involvement of the PGIPs in plant matrix assembly and tissue morphogenesis would not be surprising.

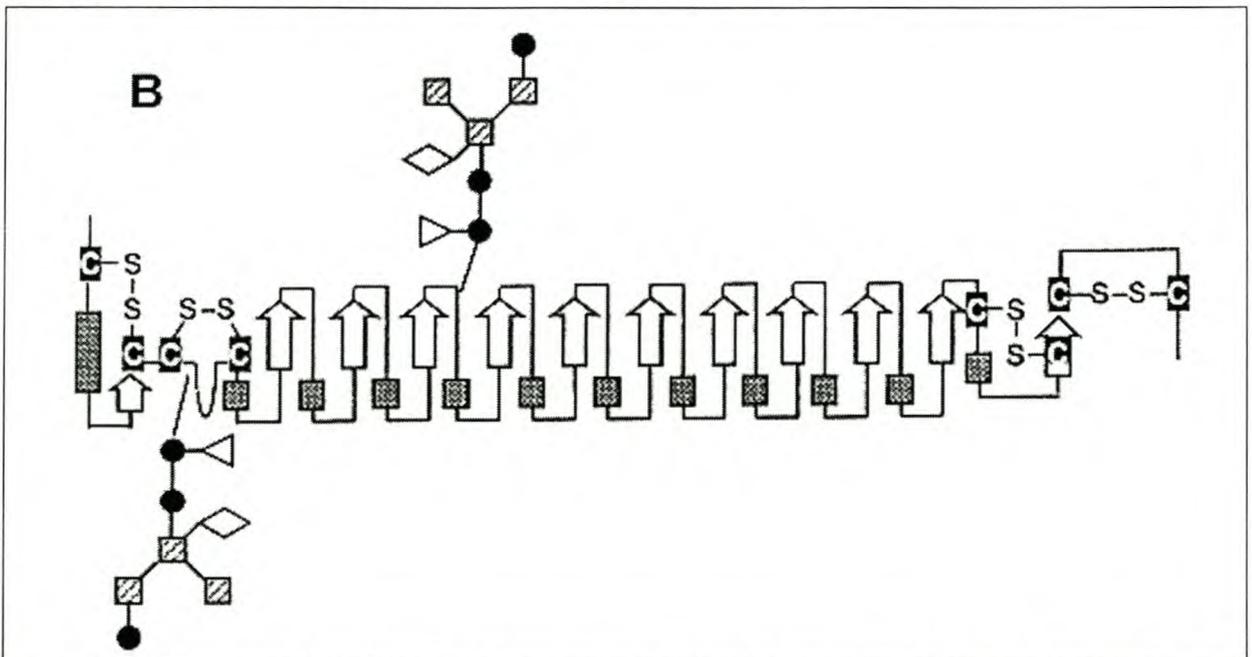


Figure 2.6 Schematic drawing of the secondary structure elements of PGIP. Arrows indicate β -strands and boxes indicate α -helices. Glycan structure has been indicated as follows: (●) N-acetylglucosamine, (striped \square) mannose, (∇) fucose, (\diamond) xylose (Mattei *et al.*, 2001).

There is no information available on the three-dimensional structure of plant LRR proteins; the difficulty is in applying generalisations from the structure of one member of the LRR superfamily to other distantly related members. This prompted Mattei *et al.* (2001) to perform a detailed analysis of the secondary structure of PGIP from *P. vulgaris* in order to gain more specific information on PGIP. The final picture indicated the presence of 12 α - and β -segments, allowing a schematic representation of three domains. These comprise the central LRR region and the two cysteine-rich flanking domains (**Figure 2.6**), four disulfide bonds and two N-linked oligosaccharides.

2.6.4 Is PGIP part of a cell surface signalling complex?

The hypothesis that PGIP may act as a secreted “receptor” involved in the recognition of fungi by plants has been proposed on the basis of the observed similarity between PGIP and the putative extracellular domain of a cloned *Arabidopsis* receptor-like protein kinase (RLK5) (De Lorenzo *et al.*, 1994). The catalytic domain of RLK5 shares homology with that present in the deduced proteins of other cloned genes (*ZmPK1*, RLK1, RLK4) (Walker, 1993), which in turn exhibit putative extracellular domains related to the products of the *Brassica* S locus glycoprotein (SLG) and S locus receptor kinase (SRK) genes (Nasrallah and Nasrallah, 1993). SRK and SLG are believed to act in combination to couple the recognition event at the pollen-stigma interface to a cytoplasmic phosphorylation cascade that leads to pollen rejection (Nasrallah and Nasrallah, 1993; Dickinson, 1994). The analogy between pollen-stigma interactions and plant-pathogen interactions at the cellular and genetic levels, the similarity between PGIP and the extracellular domain of RLK5, and the ability of PGIP to recognise molecules from microbial origin (endo-PGs), suggest that PGIP may act as a secreted “receptor”. This receptor has been proposed to be an LRR PGIP-related class of a two-component (secreted receptor/trans-membrane receptor kinase) surface signalling system active between plants and micro-organisms (De Lorenzo *et al.*, 1997).

2.6.5 Specificity of PGIPs

Several observations indicate that PGIPs from different plant sources have different inhibitory activities against PGs from a range of fungi. PGIPs isolated from bean (Cervone *et al.*, 1987), pear (Stotz *et al.*, 1993), raspberry (Johnston *et al.*, 1993), tomato (Stotz *et al.*, 1994), soybean (Favaron *et al.*, 1994), potato (Machinandiarena *et al.*, 2001) and apple (Yao *et al.*, 1995) showed differential inhibition spectra towards a range of PGs from phytopathogenic fungi. These results were confirmed with the analysis of transgenic plants harbouring various heterologous PGIPs. Ripe transgenic tomato plants expressing the pear PGIP that inhibits PGs from *B. cinerea* 20-fold more effectively than tomato PGIP showed a significant reduction in susceptibility to infection by *B. cinerea* (Powell *et al.*, 2000). In contrast, transgenic tomato plants over-expressing bean PGIP-1, which possesses a limited capacity to inhibit crude PG preparations from *F. oxysporum lycopersici*, *Alternaria solani* and *B. cinerea*, showed no increase in resistance to these fungi (Desiderio *et al.*, 1997). Apart from the differential inhibition spectra of the various PGIPs, different inhibitory activities against PGs have also been observed in PGIPs from a single plant source (Desiderio *et al.*, 1997), indicating that *pgip* genes have undergone diversification during evolution. This variability in target PG specificity could also reflect counter-adaptations between fungal PGs and plant PGIPs that lead to specialisation in the continually evolving host-pathogen interactions (Stotz *et al.*, 2000).

Since the ligand of PGIP is well known, it may serve as a model to study the evolution of specificity in LRR-containing proteins. Two members (*pgip-1* and *pgip-2*)

of the *pgip* gene family of *P. vulgaris* were isolated and shown to encode proteins with only eight amino acid variations in their mature form. Expression of the two genes in tobacco plants using the viral vector potato virus X (PVX) allowed the purification and characterisation of the single *pgip* gene products. The two proteins exhibited distinct specificities: PGIP-1 was not able to interact with the PG of *F. moniliforme*, while PGIP-2 completely inhibited this enzyme. Using site-directed mutagenesis, Leckie *et al.* (1999) identified a small number of amino acid substitutions responsible for most of the differences in inhibition. Structural models based on porcine ribonuclease inhibitor (RI) indicated that each of the 10.5 LRRs in PGIP possessed a segment that formed a solvent-exposed β -sheet/ β -turn structure and most of the amino acid substitutions separating the two bean PGIPs, including those responsible for differences in inhibition, resided in this solvent-exposed region. In a gain-of-function approach, amino acid K253 of PGIP1 was mutated into the corresponding amino acid of PGIP-2, a glutamine. With this single mutation, PGIP-1 acquired the ability to interact with *F. moniliforme* PG. (**Figure 2.7**). These structural and experimental analyses of PGIP have greatly advanced the understanding of LRR mediated protein-protein interactions.

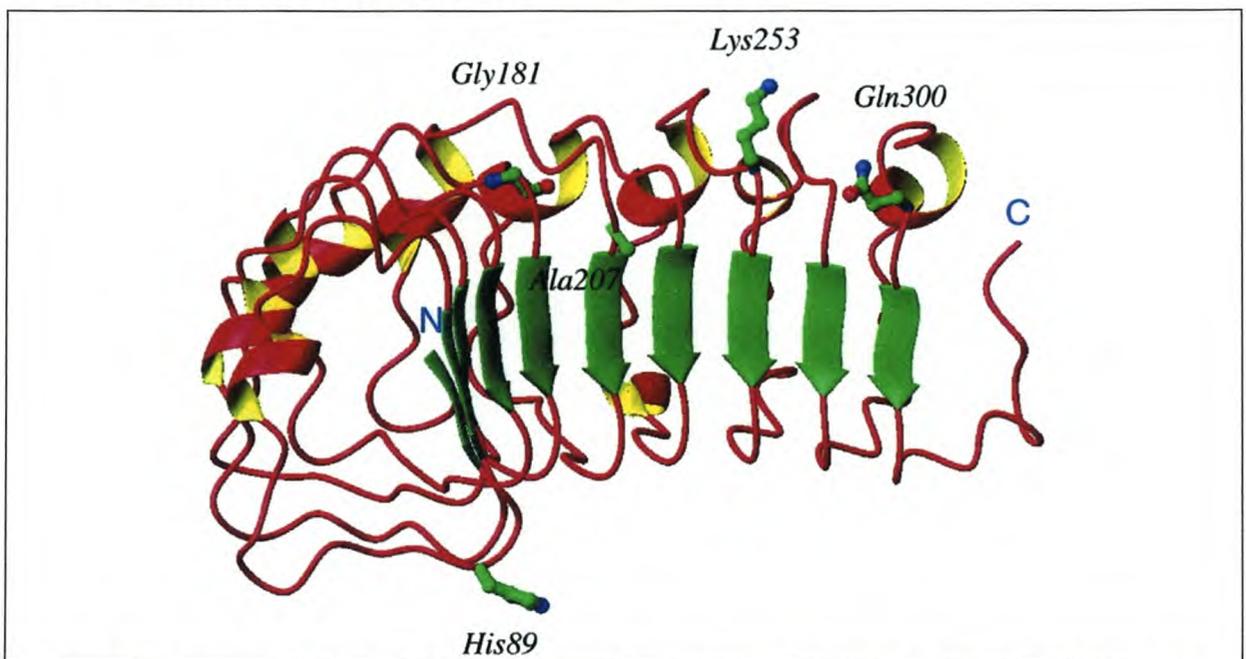


Figure 2.7 Three-dimensional folding of modelled bean PGIP-1. The positions of the five amino acid differences between PGIP-1 and PGIP-2 that lie in the LRR domain are shown. In a gain-of-function approach, amino acid K253 of PGIP-1 was mutated into the corresponding amino acid of PGIP-2, a glutamine. With this single mutation, PGIP-1 acquired the same inhibitory activity as that of PGIP-2 (Leckie *et al.*, 1999).

Successful pathogens, which decrease plant fitness, might select for amino acid substitutions that increase PGIP specificity or decrease the inhibition constant for a particular PG. Generally, most substitutions are not expected to have adaptive consequences, making it difficult to identify individual sites subjected to selection based on the ratio of synonymous and non-synonymous substitutions. The direct

mutagenesis gain of function approach provides a novel method of identifying residues important for PG or PGIP function and specificity (Nielsen and Yang, 1998).

Stotz *et al.* (2000) provided evolutionary analysis of 22 dicotyledonous PGIPs and 19 fungal PGs. Employing models of codon evolution, they indicated that advantageous substitutions dominated the molecular evolution of these genes and identified nine amino acid residues that are likely to evolve adaptively in response to natural selection. Many of these residues were within the β -strand/ β -turn region of the PGIP LRR, including the two sites known to alter inhibition specificities of bean PGIPs, whereas others were present outside this region. These results complemented existing molecular and biochemical studies of resistance specificity, and suggested new target amino acids for manipulating PG-inhibition.

2.7 CONCLUSION

Specificity in plant-pathogen interactions is likely to be determined by recognition steps involving pathogen-derived signals and complementary plant-derived receptor molecules. Both signals and receptors are thought to perform their roles at the contact surface between the two organisms, namely the plant cell wall. The interaction between fungal endo-PGs and PGIPs fits the requirements for functioning not only in defence mechanisms, but also in the perception that leads to incompatibility. Both molecules are synthesised early during an attempted infection and interact to give rise to the formation of oligogalacturonides that act as elicitors of several defence responses.

In plants, LRR proteins play a relevant role in both development and defence, where specificity of recognition is essential. Sequence analysis studies identified PGIPs as part of the LRR proteins. PGIPs are widespread cell wall proteins that recognise and bind fungal endo-PGs and are related structurally to several products of resistance genes that recently have been cloned in plants (Jones and Jones, 1997). Therefore, PGIPs belong to a super-family of proteins, which can be included in the resistance gene products, have become specialised for the recognition and rejection of pathogens. As PGIP is the only plant LRR protein whose interaction with its ligand has been demonstrated, the study of the structural basis of its interaction with endo-PGs can be used as a model to understand how plants recognise signals from pathogenic micro-organisms. More generally, the information gained on PGIP may open the way to a directed manipulation of those LRR receptor proteins that are structurally related to PGIP and are involved in both the development and resistance of plants.

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CHAPTER 3

RESEARCH RESULTS

The isolation and characterisation of a gene encoding a polygalacturonase-inhibiting protein (PGIP) from *Vitis vinifera* L.

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RESEARCH RESULTS

The Isolation and Characterisation of a Gene Encoding a Polygalacturonase-Inhibiting Protein (PGIP) from *Vitis vinifera* L.

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ABSTRACT

Polygalacturonase-inhibiting proteins (PGIPs) are present in the cell walls of a variety of plant species. These proteins have been shown to specifically inhibit polygalacturonases secreted by invading fungal pathogens as part of the induced disease resistance mechanism of plants. This is the first report on the isolation and characterisation of a *pgip* gene from *Vitis vinifera* L., designated grapevine *pgip1*. A single open reading frame encoding a deduced polypeptide of 333 amino acids with a predicted molecular mass of 37.1 kDa and a calculated isoelectric point of 8.61 was identified from a 5.6 kb subgenomic fragment of *V. vinifera* cv Pinotage. Nucleotide and derived amino acid sequence analysis of grapevine *pgip1* showed significant homology with other characterised PGIP-encoding genes and revealed features characteristic of PGIPs found in several other plant families. Genomic DNA analysis showed that the grapevine *pgip1* belongs to a small multigene family. From Northern blot analysis it was evident that expression of the PGIP family is both tissue- and developmental stage specific. Partially purified PGIPs from berries, representative of the bulk PGIP in the cultivar, strongly inhibited the activities of polygalacturonases (PGs) from *Botrytis cinerea*, *Aspergillus niger* and *Colletotrichum lindemuthianum*. The grapevine *pgip1* gene was also expressed transiently in *Nicotiana benthamiana* L. with potato virus X (PVX) as a vector. Crude protein extracts from the PVX-infected *N. benthamiana* were tested by Western blot analysis and agarose diffusion assays to indicate the presence, as well as the specificity of the overexpressed inhibitor, respectively. These results showed that the grapevine *pgip1*-encoding gene product was probably glycosylated and that it possibly contributed the inhibition against *B. cinerea* to the bulk of the PGIP activity observed. These preliminary results do not show any inhibition of PGs from *A. niger* for this protein product, confirming that other PGIPs with different specificities are present in the grapevine genome.

Key words: *Botrytis* – Endopolygalacturonases – Polygalacturonase-inhibiting protein – Plant-pathogen interaction – *Vitis*

3.1 INTRODUCTION

Fungal infection of plants usually occurs when the pathogen penetrates the polysaccharide-rich cell wall, after which colonisation of the tissues commences. Since the plant cell wall constitutes a major physical barrier to pathogens, most fungal pathogens produce a broad spectrum of cell wall-degrading enzymes to disrupt this barrier (Cooper 1984; Walton 1994; Alghisi and Favaron 1995). Of the enzymes involved in cell wall degradation, those that hydrolyse the pectic substances of plant cell walls have undergone the most intensive investigation. Amongst these pectinolytic enzymes, endopolygalacturonases (endo-PGs; EC 3.2.1.15) have been implicated as pathogenicity factors of plant pathogenic fungi (Karr and Albersheim 1970; Jones et al. 1972). Polygalacturonic acid, the preferential substrate for these enzymes, is the polymer that forms the 'backbone' of the pectin molecule in the intracellular matrix and primary walls of plant tissues (Jarvis 1984). In general, fungal PGs hydrolyse the glycosidic linkages of homogalacturonans in the plant cell wall, thereby facilitating fungal penetration and providing nourishment for fungal growth and development (Hahn et al. 1989). Oligogalacturonide fragments are released from the plant cell walls through this penetrative action of the fungal PGs which in turn may trigger characteristic active plant defence responses. These responses include a diverse array of actions in an attempt to localise and curb the pathogen attack.

Apart from the general active defence mechanisms that are triggered upon pathogen attack, some plant species also contain the so-called polygalacturonase-inhibiting proteins (PGIPs) in their cell walls that specifically and effectively inhibit fungal PGs (Cervone et al. 1997; De Lorenzo and Cervone 1997). The occurrence of PGIPs has been reported in a variety of dicotyledonous and monocotyledonous plant species. It has been reported that these proteins show differential inhibition spectra towards a range of PGs from pathogenic fungi, and PGIPs from a single plant source also show different inhibitory activities against various PGs (Desiderio et al. 1997). PGIPs are relatively heat stable glycoproteins that are targeted to the endomembrane system and exported to the extracellular space due to the presence of a hydrophobic N-terminal signal peptide (Toubart et al. 1992; Stotz et al. 1993, 1994).

Genes encoding PGIPs have been isolated from bean (Toubart et al. 1992), soybean (Favaron et al. 1994), tomato (Stotz et al. 1994), pear (Stotz et al. 1993), oranges, kiwifruit (Simpson et al. 1995), apricot, raspberry (Ramanathan et al. 1997) and apple (Yao et al. 1999). Sequence analysis studies identified these genes as part of a wider group of plant disease resistance genes encoding proteins that form part of a class of proteins that contain leucine rich repeats (LRRs). This is significant due to the fact that the LRR domain typically comprises approximately 80% of the mature PGIP peptide. Proteins containing LRRs have diverse functions and cellular locations. These LRRs are usually found in the primary structure of various proteins that function as receptors for extra- and intracellular ligands, enzyme inhibitors and protein-protein interactions (Kobe and Deisenhofer 1994). These sequence motifs also have roles in

cell-to-cell signalling (De Lorenzo et al. 1994), in signal transduction pathways as well as structural roles in stabilisation interactions between protein complexes (Dangl 1995; De Lorenzo and Cervone 1997). It has been found that many plant genes involved in gene-for-gene disease resistance contain LRRs, and may also play a role in plant development.

The isolation of a genomic clone from *V. vinifera* L. containing the open reading frame as well as the 5' and 3' flanking sequences of a PGIP-encoding gene is described here. Apart from sequence analyses on the isolated gene, the genomic organisation of the designated grapevine *pgip1* gene from *V. vinifera* was also studied and revealed the presence of a small family of PGIPs. Expression studies showed that grapevine *pgip* genes are both tissue-specific and developmentally regulated. Transient expression of the grapevine *pgip1* in *Nicotiana benthamiana* produced a protein that showed inhibition against PGs from *Botrytis cinerea*. From partial purification experiments performed with grapevine berries, it was evident that the bulk PGIP activities present in *V. vinifera* cv. Pinotage had activities against PGs from *B. cinerea*, *Colletotrichum lindemuthianum* and *Aspergillus niger*.

3.2 MATERIALS AND METHODS

Fungal material

A *B. cinerea* isolate was obtained from the Department of Plant Pathology, University of Stellenbosch. The fungal isolate was maintained on apricot medium [mixture of 50% (v/v) apricot pulp and 50% (v/v) water, adjusted to pH 4.0 and solidified with 2% (w/v) agar]. From this culture, spores were transferred to potato dextrose agar (Biolab) for 2 days at 20°C. Mycelium discs were subsequently taken from the edge of the culture and used for the preparations of PGs.

Plant material

Young leaves were harvested from *V. vinifera* L. cvs. Pinotage and Shiraz and from *Pyrus communis* L. cv. Bon Chretien for DNA isolations. Inflorescences, young leaves and véraison berries from Pinotage were collected and used for RNA isolations. Berries from several grapevine cvs. at different ripening stages were collected at 2-week intervals from December through to late February during the 1997/1998 growing season and used for RNA extractions and/or crude protein preparations. Immediately after picking, berries were frozen in liquid nitrogen and stored at -80°C until required.

Isolation and manipulation of nucleic acids

Genomic DNA was isolated from vine and pear leaves according to the methods used by Steenkamp et al. (1994), with the exception that 1% (w/v) insoluble polyvinylpyrrolidone (PVPP) was added to the extraction buffer. Total RNA was extracted from leaves, inflorescences and berry tissue, taken at different stages of ripening using the perchlorate method of Davies and Robinson (1996). The berry

tissues used were from pea-sized berries (4 weeks post-flowering), véraison berries (10 weeks post-flowering), post-véraison berries (12 weeks post-flowering), berries ripe for harvest (14 weeks post-flowering) and fully ripe berries (16 weeks post-flowering). Standard methods for plasmid DNA isolation, *E. coli* transformations, manipulation and cloning of DNA fragments, agarose gel electrophoresis and purification of DNA fragments were used as described by Sambrook et al. (1989).

Southern and Northern blot analyses

For Southern blot analysis, digested genomic DNA (10 µg) was separated on a 0.8% (w/v) agarose gel and transferred to positively charged Nylon membranes (Roche Molecular Biochemicals) according to Sambrook et al. (1989). Prehybridisation and hybridisation reactions were carried out at 42°C in DIG Easy Hyb (Roche Molecular Biochemicals). The membranes were prehybridised for 2 h and then hybridised for 16-20 h with a PCR-digoxigenin-labelled probe. Membranes were washed twice at room temperature in 2 × SSC and 0.1% SDS for 5 min each and twice at 68°C in 0.2 × SSC and 0.1% SDS for 15 min each. Chemiluminescence detection of nucleic acids was done using CSPD as substrate (Roche Molecular Biochemicals).

For colony blot analysis, colonies were lifted to Hybond-N nylon membranes (Amersham), fixed, prehybridised and hybridised at 42°C in a solution containing 50% (v/v) formamide, 2% (w/v) blocking reagent (Roche Molecular Biochemicals), 5 × SSC, 0.1% (w/v) N-lauroyl sarcosine and 0.02% (w/v) SDS. The membranes were prehybridised for 2 h and then hybridised for 16-20 h with a radioactively-labelled [³²P]dATP probe. Membranes were washed as described above.

For Northern blot analysis, total RNA was separated by electrophoresis on a 1% formaldehyde agarose gel and blotted to Hybond-N nylon membranes using standard techniques as described by Sambrook et al. (1989). The membranes were prehybridised at 50°C for 4 h in DIG Easy Hyb (Roche Molecular Biochemicals). The membranes were then hybridised in the same solution with the addition of a radioactively-labelled [³²P]dCTP probe. After hybridisation, membranes were washed twice in 2 × SSC and 0.1% SDS at room temperature for 15 min and twice in 0.5 × SSC and 0.1% SDS at 68°C for 15 min. Normalisation on Northern blots was done using a 18S rRNA probe from a highly conserved region of the human ribosomal RNA gene (Ambion).

Probe preparation: isolation and labelling of an internal fragment of the pear PGIP-encoding gene

Based on sequence homologies between PGIP-encoding genes from pear, apple, kiwifruit, tomato, orange, bean and soybean, primers pearPGIP-5' and pearPGIP-3' (listed in Table 1) were designed to amplify a 780 bp fragment from pear genomic DNA using PCR.

The amplified 780 bp PCR product was purified and cloned into the pGEM-T Easy vector (Promega) and designated pPGIP^P. The identity and integrity of the cloned PCR product was confirmed by sequencing pPGIP^P with plasmid-specific SP6 and T7 primers. This 780 bp pear PGIP fragment was used as a probe to detect putative grapevine PGIP fragments in Southern blot analysis as described in the following section. The probe was either PCR-digoxigenin labelled (according to the DIG System User's Guide - Roche Molecular Biochemicals) for use in Southern blot analysis, or radioactively labelled with [³²P]dATP (Amersham), using the randomly primed DNA labelling kit (Roche Molecular Biochemicals) for colony blots.

Construction and screening of a subgenomic library to isolate a PGIP-encoding gene from grapevine

In order to isolate a grapevine PGIP-encoding gene, genomic DNA from *V. vinifera* cv Pinotage was digested with a range of restriction enzymes (*Bgl*II, *Eco*RV and *Hind*III), separated on a 0.8% (w/v) agarose gel and hybridised with the digoxigenin-labelled pear PGIP probe. The *Eco*RV digest, which yielded a single hybridisation product of approximately 5.6 kb in size, was subsequently used to construct a subgenomic library for the isolation of a grapevine PGIP-encoding gene and promoter.

Genomic DNA digested with *Eco*RV was separated on a 0.8% agarose gel and fragments with a size between 5 and 6 kb were purified and cloned into the dephosphorylated *Eco*RV site of pBluescript SK(+) (Stratagene, La Jolla, CA). The ligation mixture was electroporated into *E. coli* DH10B. The resulting library of transformants was amplified, stored and plated for screening.

The radioactively labelled [³²P]dATP pear PGIP fragment was used to screen recombinant clones from the library using colony blots. Positive recombinant clones were confirmed by dot blots followed by Southern analysis. A positive clone containing a 5.6 kb insert was isolated. This clone was designated pPGIP^{G1}.

Subcloning and characterisation of the grapevine PGIP-encoding gene

PCR analysis performed on the pPGIP^{G1} clone using the pear PGIP-5' and 3' primers yielded a 820 bp PCR that was cloned into pBluescript SK (+) (Stratagene) to obtain pPGIP^{G2}. This 820 bp insert was sequenced using plasmid-specific T3 and T7 primers and shown to contain PGIP-encoding sequences. The complete sequence of the grapevine PGIP-encoding gene in pPGIP^{G1} was determined using gene-specific primers, grapePGIP-5' and grapePGIP-3' (Table 1).

Table 3.1 List of primers used in the study. Primers were designed to obtain sequences of the grapevine PGIP-encoding gene as well as a putative promoter area of the gene.

Primer name	Primer sequence	Primer position in 1002 kb gene fragment
pearPGIP-5'	5'-CTCTCTCCGAYCKCTGCAAC-3'	
pearPGIP-3'	5'-GGAATCTGACCACACAGCC-3'	
grapePGIP-5'	5'-AGGACAGAGAA ATGG GAGACTTCAAAC-3'	from nt +1 to +15, ATG indicated in boldface
grapePGIP-3'	5'-AGTCAGATCTGAGCCGTCACCTTGC-3'	from nt +994 to +1002
PVXgPGIP-5'	5'-ATCGATGGAGACTTCAA AACTT -3'	
PVXgPGIP-3'	5'-GTCGACTCACTTGCAGCTCTG-3'	

The gene-specific primers were subsequently used to also amplify the PGIP-encoding gene from genomic DNA isolated from *V. vinifera* cv. Shiraz using Expand DNA polymerase (Roche Molecular Biochemicals). The resulting 1002 bp PCR product was cloned into the pGEM-T Easy vector (Promega) to obtain pPGIP^S. Four individual clones were sequenced with plasmid-specific SP6 and T7 as well as gene-specific primers; the resulting sequences were compared with those from Pinotage.

Plant inoculation and expression analysis with the PVX system

The grapevine *pgip1* was amplified using primers PVXgPGIP-5' and PVXgPGIP-3' (Table 1) with *Clal* and *Sall* sites included at the 5' and 3' ends, respectively, to facilitate cloning. The PCR-generated fragment was cloned into the *Clal* and *Sall* sites of the pPVX201 expression vector to generate pPVXgPGIP1 (Baulcombe et al. 1995). Direct inoculation of *N. benthamiana* plants with DNA was done as described previously (Baulcombe et al. 1995). Leaf material was harvested upon appearance of viral symptoms (20 days after inoculation). To determine grapevine PGIP1 expression, total leaf protein extracts were prepared as described in Desiderio et al. (1997) and analysed by agarose diffusion assay according to Taylor and Secor (1988).

Protein concentration was determined according to Bradford (1976), using a Bio-Rad protein assay kit and bovine serum albumin (BSA) as a standard. SDS-PAGE was performed as described by Laemmli (1970). Gels were silver stained as described by Blum et al. (1987).

For Western blot analysis, the protein extracts were electrophoretically transferred to nitrocellulose membranes from a SDS-PAGE gel. A polyclonal antibody that recognises the total bean PGIP complex (kindly provided by G. De Lorenzo, Dipartimento di Biología Vegetale, Università' di Roma La Sapienza) was used to detect the grapevine PGIP1 with an ECL detection kit (Amersham).

Preparation of fungal PGs

The PG from *A. niger* that was used was a commercial preparation (Sigma) whereas PGs from *C. lindemuthianum* were provided by D. Oelofse (ARC-Roodeplaat, South Africa). Mycelium discs from *B. cinerea* (1 disc/25 ml of medium) were added to flasks containing 50 ml of a citrate phosphate buffer (pH 6.0) supplemented with 1% (w/v) citrus pectin. The medium also contained 2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6 μM $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 25 mM KNO_3 , 30 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.9 μM CuSO_4 and 65 μM FeSO_4 . The flasks were incubated with orbital shaking at 120 rpm in the dark at 22°C for 10 days. The mycelia were removed by filtration through Whatman No 1 filter paper. The filtrate was centrifuged at 5,000g for 8 min, filter sterilised (0.22 μm) and stored at 4°C. These filtrates were screened for PG activity using the agarose diffusion assay according to Taylor and Secor (1988) in the presence of the substrate polygalacturonic acid (0.01%). Zones, indicative of hydrolysis of the glycosidic bonds in the pectic substrate, were visualised by staining with 0.05% Ruthenium red (Sigma). The filtrates showing the highest PG activity were subjected to a 0–80% ammonium sulphate treatment to precipitate the PG proteins. The resulting pellet was resuspended in 40 mM sodium acetate buffer (pH 5.0) and checked for recovery of PG activity using the described agarose diffusion assay.

Isolation and partial purification of crude PGIP extract from grapevine

Finely crushed berry tissue (5 g) was homogenised in extraction buffer (2 ml per gram of tissue), consisting of 0.1 M sodium acetate buffer (pH 6.0), 10 mM β -mercaptoethanol and 1% (w/v) PVP-40. The homogenate was centrifuged at 10,000g for 15 min, followed by two more extractions in extraction buffer without PVP-40, each followed by centrifugation. The remaining insoluble tissue was resuspended in 2 volumes of 50 mM sodium acetate (pH 5.2) containing 1 M NaCl and stirred for 1 h at 4°C. The insoluble debris was removed by centrifugation at 10,000g for 20 min. Proteins precipitating at 80% (w/v) saturated ammonium sulphate were collected, resuspended in 20 mM sodium acetate (pH 5.2) and dialysed extensively at 4°C against deionised water. A partial purification step of the crude protein extract was performed by ion-exchange chromatography through a DEAE Sepharose fast flow column (Pharmacia), equilibrated with 20 mM ammonium acetate (pH 5.0). Proteins bound by the column were eluted with a stepwise gradient from 0.05 M to 1 M ammonium acetate (pH 5.0), with fractions eluting between 0.2 and 0.4 M ammonium acetate showing PGIP activity as measured by the agarose diffusion assay against PGs from *B. cinerea*. Even though no inhibitory activity was found for young berries, fractions eluting between 0.2 and 0.4 M ammonium acetate were still pooled. Fractions corresponding to the highest inhibitory activity were pooled, concentrated by freeze drying, dialysed as described above and subsequently tested against PGs from *B. cinerea*, *A. niger* and *C. lindemuthianum*. Equal amounts of total protein from each eluting fraction were used in the assay.

PGIP activity assay

Inhibition of PG activity by PGIPs was determined by the agarose diffusion assay (Taylor and Secor 1988). Clearing zones are indicative of PG activity, quantified by measuring the diameter of the zones. These were compared with clearing zones that developed when the inhibitor (PGIP extracts) was added to the PGs, causing a reduction in the diameter of the clearing zones. As a negative control, the protein extracts were heated at 95°C for 30 min.

3.3 RESULTS

Cloning and analysis of a grapevine PGIP-encoding gene

A subgenomic library was constructed from *V. vinifera* cv. Pinotage leaves and was screened for a grapevine *pgip* gene using a pear PGIP probe. A potential positive clone, designated pPGIP^{G1}, contained an insert of approximately 5.6 kb and was shown to contain a PGIP-encoding. The complete sequence of the grapevine PGIP-encoding gene in pPGIP^{G1}, designated grapevine *pgip1*, was determined and analysed.

Grapevine PGIP1 sequence relative to other characterised PGIPs

The isolated grapevine *pgip1* gene comprises a 1002 bp single open reading frame encoding a deduced polypeptide of 333 amino acid residues (Fig. 3.1) with a predicted molecular mass of 37,103 Da and a calculated isoelectric point of 8.61. The predicted molecular mass and pI of the mature protein are 34 kDa and 8.52, respectively. The N-terminus of the deduced polypeptide is preceded by a 27 amino acid signal peptide. The signal peptide cleavage site is conserved, with cleavage after the amino acid serine (S). The N-terminal amino acid of the deduced grapevine PGIP, glutamic acid (E), is identical to that of bean, soybean, raspberry and apricot, but contrasts with those of apple, kiwi, pear and orange, which contain aspartic acid (D), and tomato, which contain valine (V) in this position.

Computer analysis of the nucleotide and deduced amino acid sequence for the grapevine PGIP1 showed significant sequence similarities with other PGIP sequences available in the GenBank and SwissProt databases (Table 3.2).

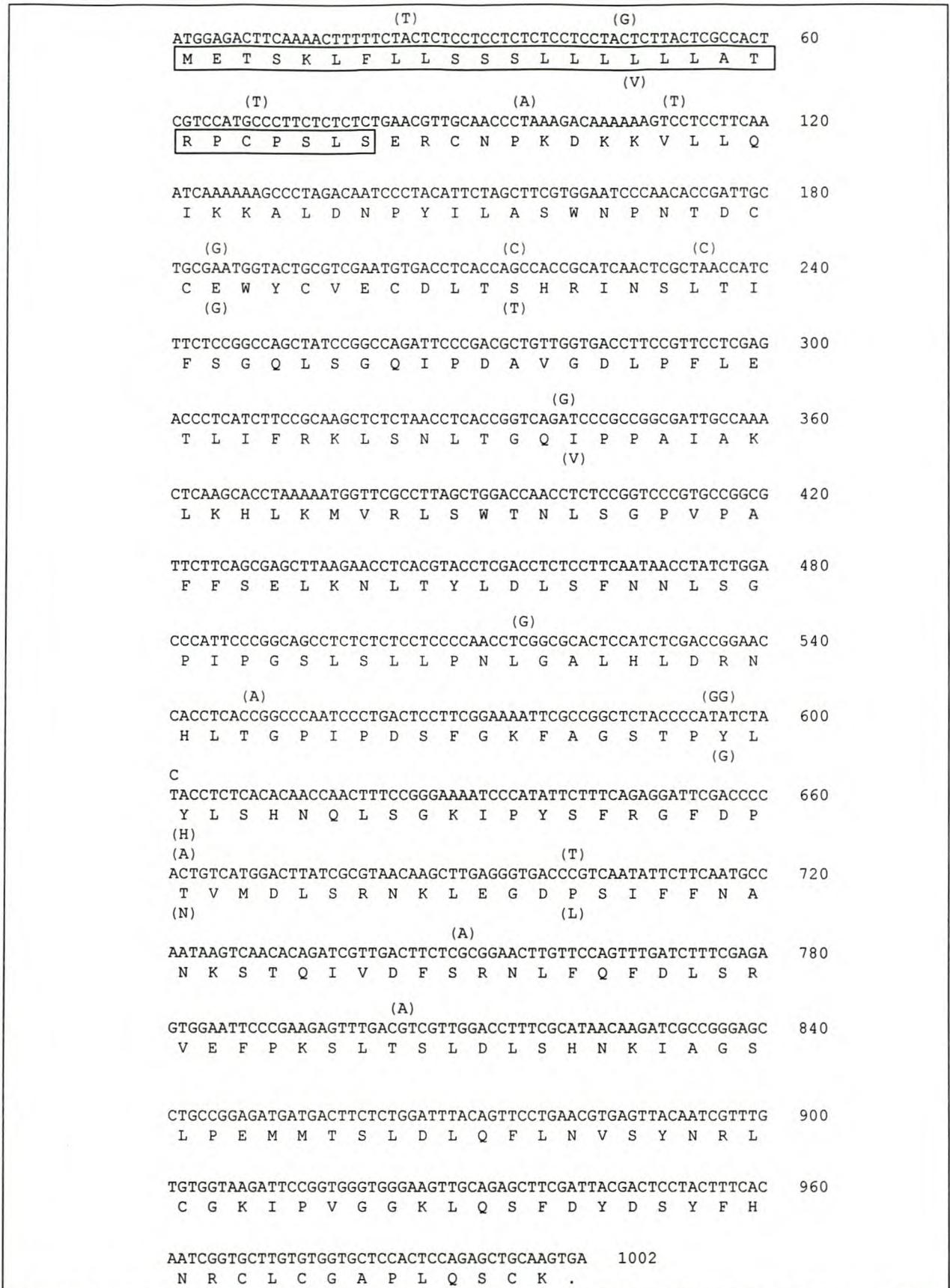


Fig. 3.1 Nucleotide sequence of the PGIP-encoding gene from *Vitis vinifera* L. cv Pinotage and the deduced amino acid sequence of the encoded protein. Amino acid sequences corresponding to a putative signal peptide are boxed. Above the nucleotide sequence, nucleotides for the PGIP-encoding gene from cv Shiraz that differ from that of Pinotage, as well as the corresponding changes in the deduced amino acid sequence (bottom), are shown between brackets.

Table 3.2 Comparison of grapevine PGIP with other published PGIP sequences available in the databases. Computer analysis, using the BLASTX and BLASTP programs, were used to identify significant sequence similarities with other known PGIP sequences.

Plant source	% similarity on nucleotide level	% similarity on amino acid level
Kiwifruit	69%	70%
Pear	68%	71%
Apple	68%	70%
Apricot	58%	68%
Raspberry	57%	66%
Orange	65%	68%
Tomato	61%	60%
Soybean	50%	43%
Bean	51%	45%

The alignment of characterised PGIP amino acid sequences indicated that the homology was scattered throughout the sequence, with smaller regions of higher homology, predominantly in the N- and C-terminal region of the sequence (Fig. 3.2A). The deduced grapevine PGIP1 polypeptide sequence showed the highest degree of homology to kiwifruit. Except for tomato, it is related more closely to the fruit PGIPs, namely those derived from pear, apple, apricot, raspberry and orange, than to those from the legumes, such as soybean and bean (Fig. 3.2B). The deduced grapevine PGIP1 contains six potential N-glycosylation sites (N-X-T/S), two of which are shared with the other fruit PGIPs. Two other sites, corresponding to residues 133-135 and 294-296 of the grapevine PGIP1, showed a conserved position among all inhibitors except for raspberry and soybean, respectively. Nine cysteine residues are found in the grapevine PGIP1 and are located in the N- and C-termini of the mature peptide, seven of which are highly conserved (Fig. 3.2A).

A characteristic of the PGIPs investigated thus far is their high leucine content (typically 15%). The deduced grapevine PGIP1 share homology with a broad range of proteins containing leucine-rich repeats (LRR). Among these LRR proteins, grapevine PGIP1 showed homology to those that are involved in plant disease resistance and signal transduction. These include the tomato disease resistance genes *Cf-9* (Jones et al. 1994) and *Cf-2* (Dixon et al. 1996), the tobacco *N* gene (Whitham et al. 1994), as well as receptor-like protein kinase genes from rice (Song et al. 1995) and *Arabidopsis thaliana* (Chang et al. 1992; Walker 1993). The deduced amino acid sequence of grapevine PGIP1 contained ten imperfect tandem repeats of leucine-rich sequence elements with an average length of 24 amino acids (Fig. 3.3). The LRR consensus sequence for the grapevine PGIP was identified as - L - - L - L S - N - L - G - I P - - - - - L -, which is shared with other PGIP protein families as well as gene products involved in the signalling processes in plant pathogen defence and in signal transduction (Kobe and Deisenhofer 1994). Moreover, it is also related to other LRR consensus

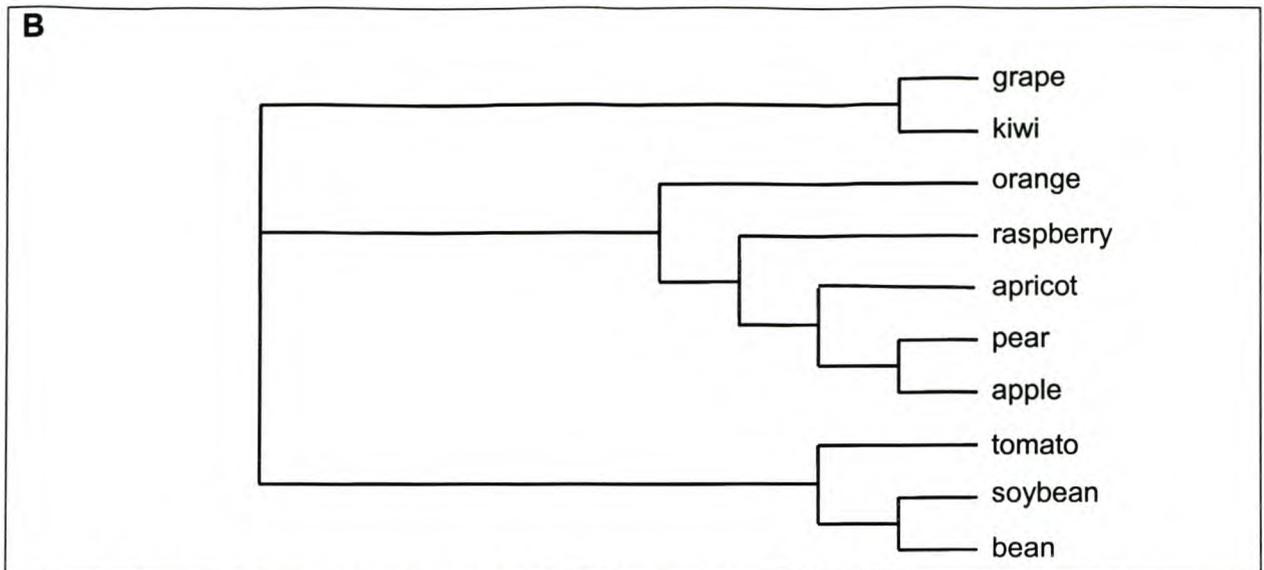


Fig. 3.2 Amino acid sequence alignment of all characterised PGIPs with grapevine PGIP1. Sequence alignments were performed with the CLUSTAL W multiple sequence alignment program (version 1.8, found on-line from the European Bioinformatics Institute) and are shown in A. Asterisks indicate identical amino acids, dots show the alignment of related amino acids (one dot indicating a greater evolutionary distance than two). The arrow indicates the potential signal peptide cleavage site; cysteine residues are in bold and underlined and potential N-glycosylation sites are shaded. PGIPs aligned were from *Vitis vinifera*, *Actinidia deliciosa*, *Pyrus communis*, *Malus domestica*, *Prunus armeniaca*, *Rubus idaeus*, *Citrus sinensis*, *Lycopersicon esculentum*, *Glycine max* and *Phaseolus vulgaris*. A phylogenetic tree representing deduced amino acid homology between the characterised PGIPs and that of grapevine is shown in B. The phylogenetic tree was created from the alignment using PAUP* (version 4.0b4).

Comparison of grapevine PGIP1 sequences from two V. vinifera cultivars

Using grape specific primers designed to amplify the entire PGIP-encoding gene from cv. Pinotage, a PGIP-encoding gene from cv. Shiraz was also amplified. Comparing the Shiraz PGIP sequence to that of Pinotage, as shown in Figures 3.1 and 3.3, five of the eight non-synonymous substitutions present lead to amino acid changes that are located in the LRR domain. Of these five, two are present in the predicted xxLxLxx motif to form the solvent-exposed β -sheet/ β -turn structure of the protein, while one is very close to this region. The non-synonymous substitutions on the β -strand region lead to amino acid changes from Y199G and from Y201H. The other two variants are present on the second and seventh LRR. The remaining three variants are outside the LRR domain; one is located in the signal peptide of the protein (Fig. 3.3A), and two reside in the N-terminal region of the protein (Fig. 3.3B). The most synonymous nucleotide changes correspond to residues located in the signal peptide, in the N-terminal region of the protein, while three correspond to those located on the LRR domain, two in the β -sheet/ β -turn structural motif and one outside this motif.

Genomic organisation of the grapevine pgip1 gene

Genomic DNA from *V. vinifera* cv. Pinotage was digested with a series of enzymes in Southern blot analysis to determine the organisation of the *pgip1* gene in the grapevine genome. From the hybridisation signals obtained (Fig. 3.4), it seems that

this cultivar may have at least two or a small family of homologous PGIP-encoding genes.

This was confirmed with some other cultivars of *V. vinifera* that were tested, which also yielded multiple hybridising restriction fragments, whereas a single fragment was observed for others (Fig. 3.5A-C). Length polymorphism of the hybridising restriction fragments was also observed (Fig. 3.5A-C).

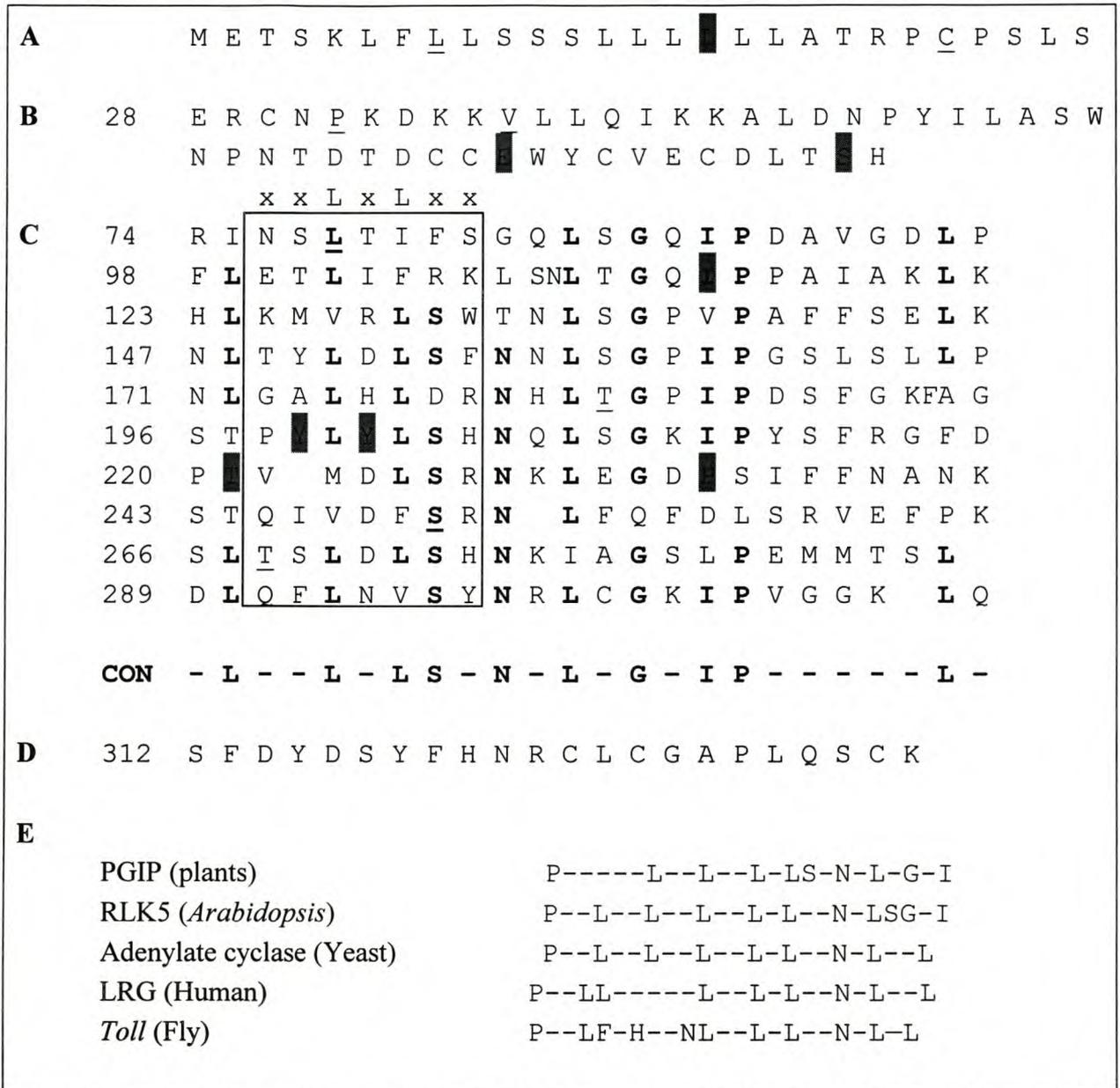


Fig. 3.3 Leucine-rich repeats present in the deduced grapevine PGIP1 extracted from the alignment of leucine-rich tandem repeats of grapevine PGIP1 from cv. Pinotage. (A), signal peptide; (B), N-terminal domain; (C), LRR domain; and (D), consensus sequence. Conserved amino acids are shown in bold. The sequence was condensed or expanded at seven different positions for an optimal alignment of the consensus sequence. The consensus sequence was derived from amino acids present in a specific position in at least 50% of the repeats. The box represents the area of the predicted solvent-exposed region of the β -strand/ β -turn structure as indicated by xxLxLxx. Based on the comparison between PGIP-encoding genes from cvs. Pinotage and Shiraz, amino acids corresponding to non-synonymous nucleotide changes are highlighted, while those corresponding to synonymous changes are underlined. (E) General comparison with consensus sequences of other proteins with leucine-rich repeats is shown.

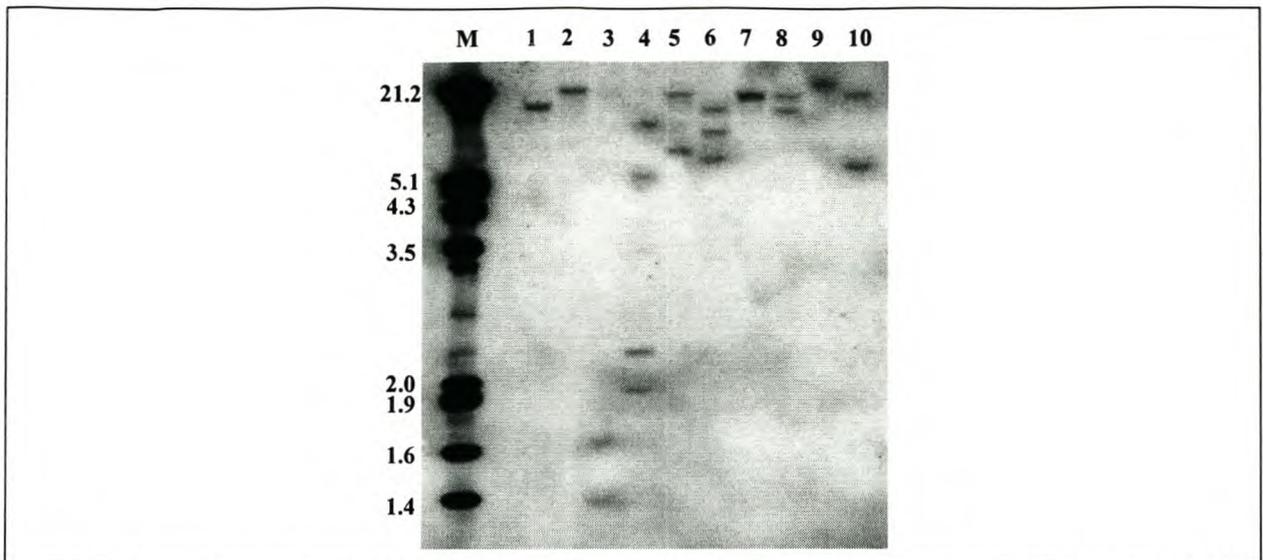


Fig. 3.4 Genomic organisation of the *pgip* gene family in *Vitis*. Southern blot analysis of genomic DNA from *Vitis vinifera* cv. Pinotage digested with *Bam*HI (lane 1), *Bgl*II (lane 2), *Bst*EII (lane 3), *Eco*RI (lane 4), *Eco*RV (lane 5), *Hind*III (lane 6), *Pst*I (lane 7), *Pvu*II (lane 8), *Sma*I (lane 9) and *Sph*I (lane 10) and hybridised with a digoxigenin-labelled 1002 bp fragment corresponding to the coding region of grapevine *pgip1*. The marker lane (lane M) contains *Eco*RI and *Hind*III-digested lambda DNA labelled with digoxigenin. Sizes of the standard DNA fragments are indicated in kb.

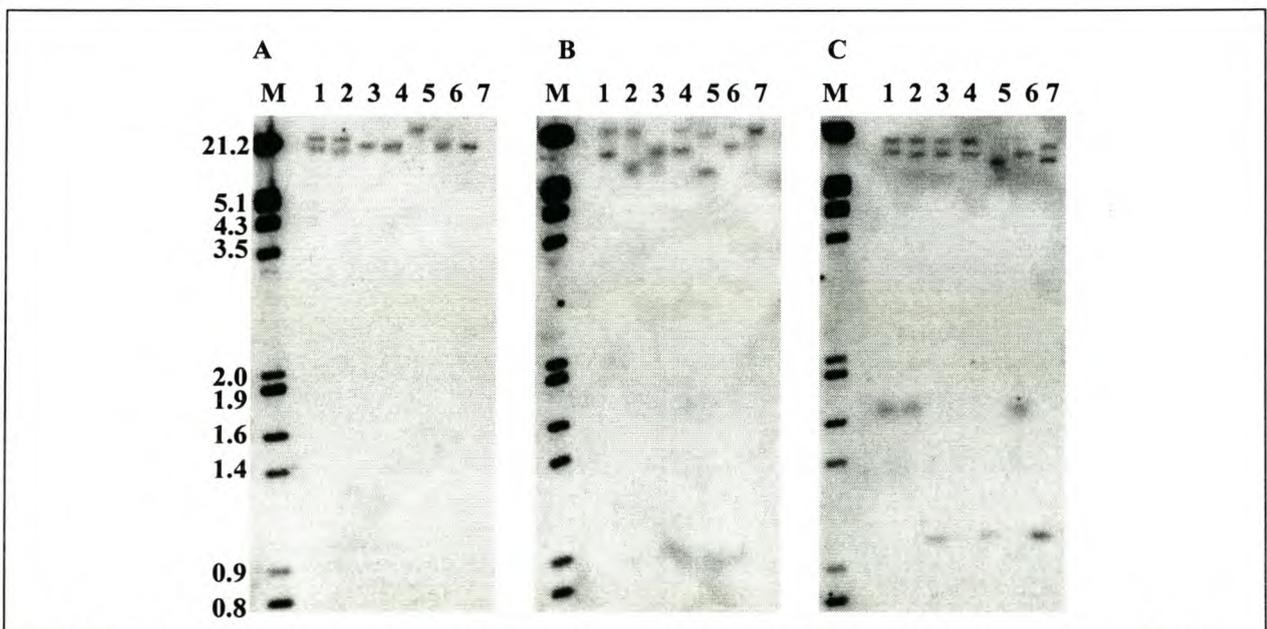


Fig. 3.5 Differences in genomic organisation of the *pgip* gene family in *Vitis vinifera* cultivars. Southern blot analysis of genomic DNA from different cultivars digested with *Bam*HI (A), *Eco*RV (B) and *Hind*III (C) and probed with a digoxigenin-labelled 1002 bp fragment corresponding to the coding region of grapevine *pgip1*. The cultivars used were Chardonnay (lane 1), Chenin blanc (lane 2), Merlot (lane 3), Shiraz (lane 4), Dauphine (lane 5), Red Globe (lane 6) and Sultana (lane 7). The marker lane (lane M) contains *Eco*RI and *Hind*III-digested lambda DNA labelled with digoxigenin. Sizes of the standard DNA fragments are indicated in kb.

Expression pattern of PGIP in grapevine tissues

Tissues from different organs of the grapevine were analysed for PGIP expression by Northern blot analysis, using the *pgip1* gene as a probe. A single mRNA transcript of approximately 1.3 kb was detected in véraison berries, but not in leaves or

inflorescences, suggesting that the expression of PGIPs in grapevine is berry-specific (Fig. 3.6).

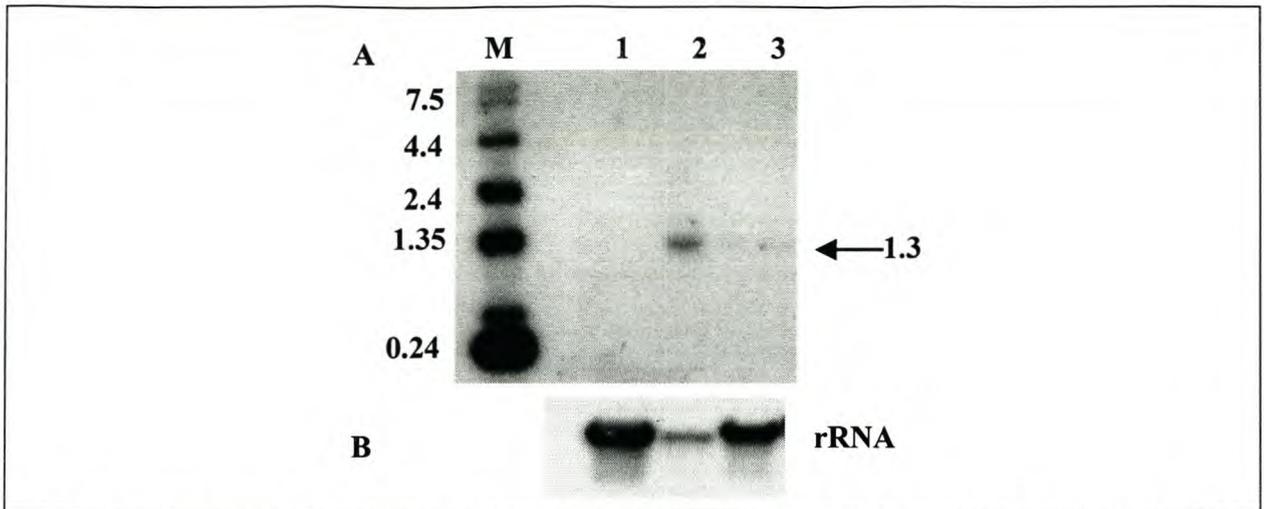


Fig. 3.6 Tissue specific expression studies of grapevine *pgip* genes. Northern blot analysis of mRNA expression in cv. Pinotage grapevine tissues. Total RNA was extracted from inflorescences (lane 1, 8 μ g), véraison berries (lane 2, 2 μ g) and leaves (lane 3, 6 μ g) and hybridised to a [32 P]-labelled 1002 bp grapevine *pgip1* probe as shown in A. The size of the PGIP mRNA is indicated. Lane M represents a RNA ladder (GibcoBRL). The membrane was then stripped and hybridised to a 18S rRNA as an internal standard (B). Sizes of the marker are indicated in kb.

To study possible developmental control of grapevine *pgip* gene expression, RNA extractions and Northern blot analysis proceeded from berries at different stages of ripening (Fig. 3.7A). The PGIP transcript levels were lowest in the early stage of berry growth, but increased significantly at véraison, corresponding with the onset of ripening (Fig. 3.7B). The levels of grapevine PGIP transcripts then declined steadily until, at 16 weeks post-flowering when the berries were ready for harvest, it was no longer detectable, suggesting that the PGIP-encoding gene is developmentally regulated (Fig. 3.7B). The hybridisation signals from grapevine PGIP transcripts were quantified and standardised with corresponding rRNA levels. The PGIP transcript level was the lowest at 4 weeks post-flowering, increasing 40-fold at véraison, after which levels decreased steadily as shown in Fig. 3.7C.

Inhibition specificity of the grapevine PGIP1 shown by expression of the V. vinifera pgip1 in N. benthamiana

In order to analyse the inhibition specificity of the isolated grapevine PGIP1, the complete coding sequence was introduced into the expression cassette of the potato virus X (PVX) vector system (Baulcombe et al. 1995). Crude protein extracts from symptomatic leaves inoculated with PVXgPGIP1 were subjected to SDS-PAGE (Fig. 3.8A). For transient expression in *N. benthamiana*, Western blot analysis on the crude protein extracts demonstrated the presence of a PGIP-specific signal with an approximate molecular mass of 42 kDa, which was absent from plants infected with only the vector (Fig. 3.8B). The crude protein extract from the infected plants showed inhibitory activity against PGs from *B. cinerea*, but no inhibitory activity against PG from *A. niger* as determined by the agarose diffusion assay (data not shown).

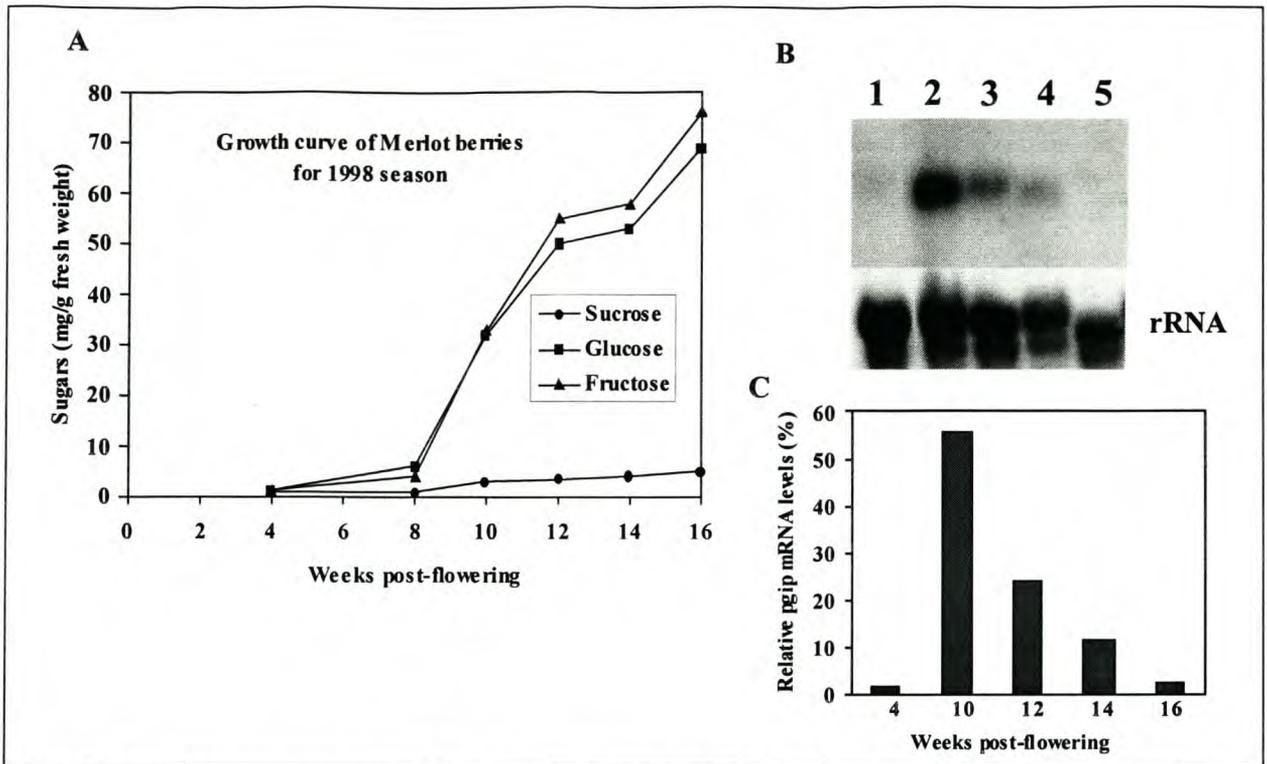


Fig. 3.7 Development stage-specific expression studies of grapevine *pgip* genes in cv. Merlot. A typical growth curve of berry development, based on the changes in sugar content, is shown in A. The Northern blot (B) was performed on total RNA extracted from pea-sized berries – 4 weeks post-flowering (lane 1), véraison berries – 10 weeks post-flowering (lane 2), post-véraison berries – 12 weeks post-flowering (lane 4), berries ripe for harvest – 14 weeks post-flowering (lane 5) and fully ripe berries collected just before harvest – 16 weeks post-flowering (lane 6). The membrane was first hybridised to a [³²P]-labelled 1002 bp grapevine PGIP1 probe (B), then stripped and hybridised to a 18S rRNA probe as an internal control. The quantification of the grapevine PGIP transcript level is shown in C, which is corrected with the rRNA level.

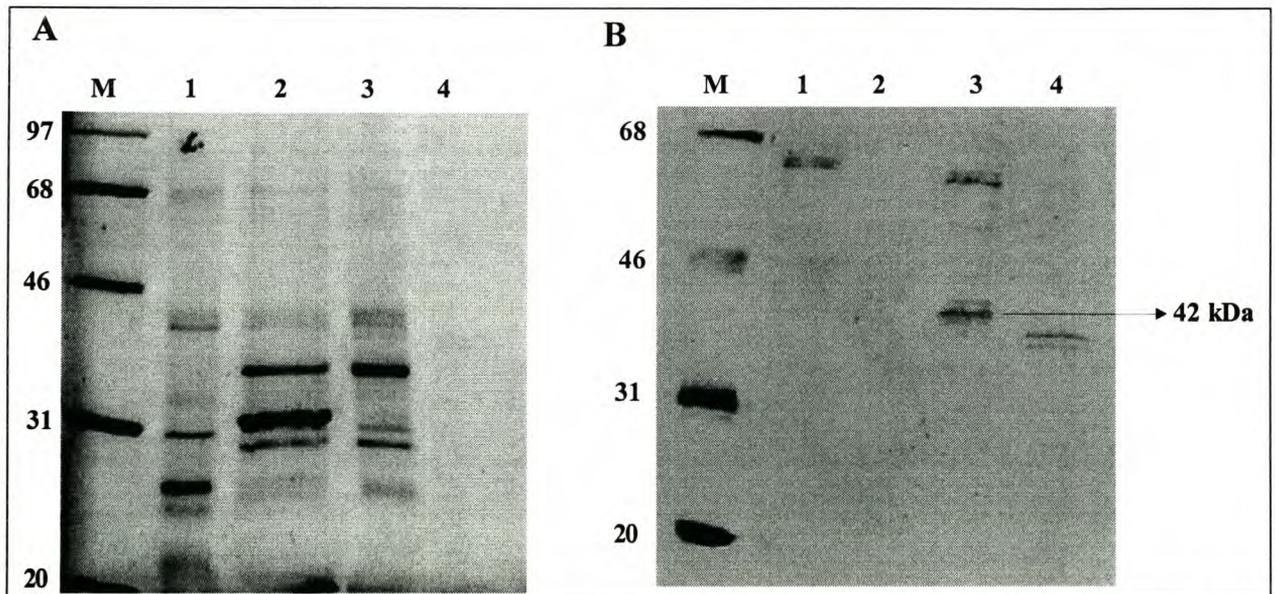


Fig. 3.8 Expression analysis using the PVX system. Crude protein extracts from uninfected *Nicotiana benthamiana* plants (lane 1), from plants infected with only the vector (lane 2), from plants infected with the grape *pgip1* (lane 3) and purified bulk PGIP purified from *Phaseolus vulgaris* pods (30 ng, lane 4) were subjected to 10% SDS-PAGE gel electrophoresis (A) and the corresponding Western blot analysis was done using a polyclonal antibody that recognises the total bean PGIP-1 protein (B). Lane M represents an ECL protein molecular weight marker. Sizes of the protein standards are given in kDa.

Inhibitory activity of partially purified grapevine PGIP1 against fungal PGs

A crude PGIP extraction was performed on grape berries at different stages of development. The extracts were partially purified by DEAE Sepharose anion exchange chromatography. Fractions were pooled, concentrated, dialysed and subsequently used in agarose diffusion assays (Fig. 3.9). Inhibitory PGIP activity against PGs from *B. cinerea* was not detected in young berries (Fig. 3.9A), but there was a significant increase in inhibitory activity in proteins isolated from véraison berries (Fig. 3.9B) that continued to increase slightly in ripening berries (Fig. 3.9C). Active fractions from véraison berries were also tested against PGs from *C. lindemuthianum* and *A. niger* PG and showed an 80% and 75% inhibitory activity, respectively (Fig. 3.9D).

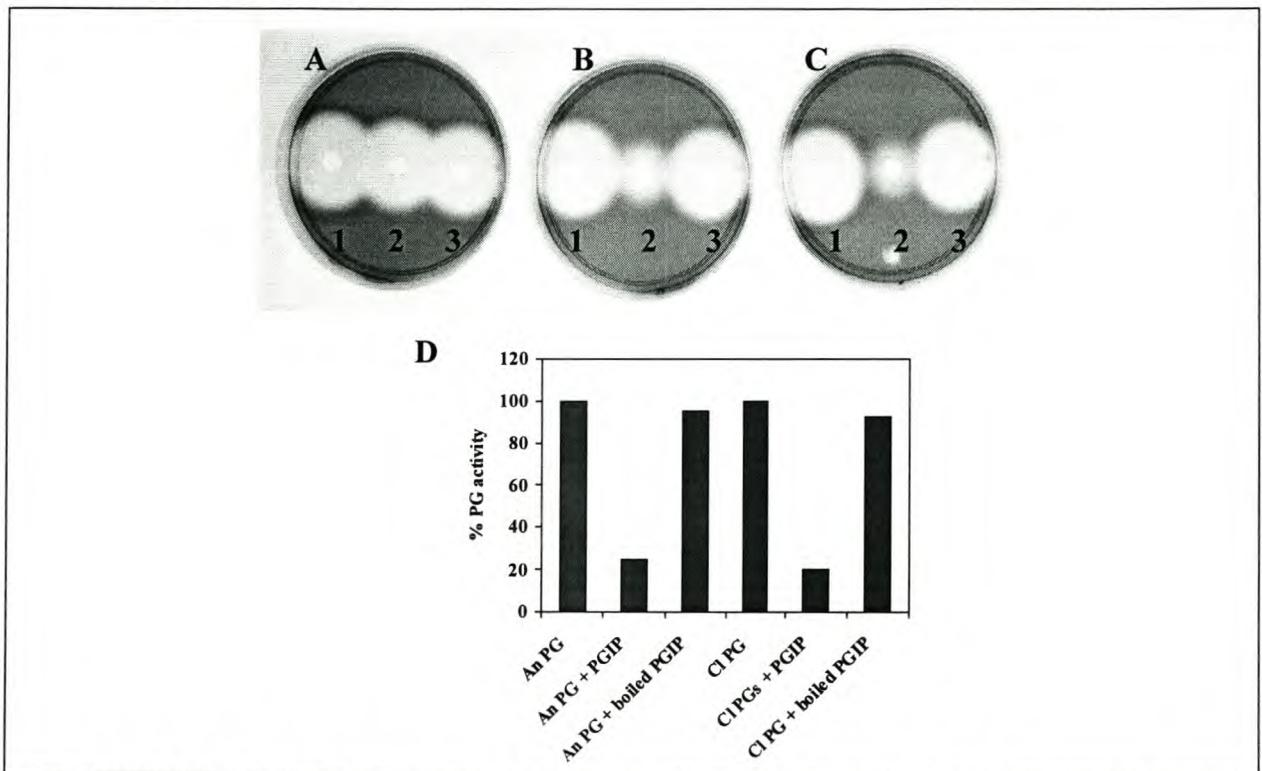


Fig. 3.9 Inhibition studies of grapevine PGIPs against fungal PGs. Inhibition studies were done using the agarose diffusion assay. Partially purified grapevine PGIPs (100 ng) from young berries (4 weeks post-flowering) (A), véraison berries (10 weeks post-flowering) (B) and ripe berries (14 weeks post-flowering) (C) were tested against PGs isolated from *B. cinerea*. A clear zone is indicative of PGs degrading the pectic substrate in the medium (well 1). Partially purified PGIP extract inhibited PG activity on the pectic substrate, resulting in a reduction in the zone size (well 2). As a negative control, partially purified PGIP extract was inactivated by heating. This resulted in no inhibition of the PG activity, causing a clear zone (well 3). (D) Inhibitory PG activity of véraison berries was also tested against PGs from *Colletotrichum lindemuthianum* (Cl PGs) and *Aspergillus niger* PG (An PG).

3.4 DISCUSSION

Polygalacturonase-inhibiting proteins have generated wide interest in the study of plant pathogen interactions. In this study, a genomic clone encoding a grapevine PGIP gene, designated *pgip1*, was isolated from *V. vinifera* cv. Pinotage and shown to be part of a small multigene family in *Vitis*. The detection of only one species of mRNA by Northern blot analysis, however, probably indicates that the different *pgip* genes have

similar transcript sizes, not separable with the conditions used, or that only a single grapevine *pgip* gene is expressed under the conditions examined.

The identity of the isolated gene as a grapevine PGIP was confirmed by a high degree of similarity of the deduced amino acid sequence with other characterised PGIPs. Unlike the raspberry and apricot PGIP-encoding genes, grapevine *pgip1* was not interrupted by introns. The deduced structural features of the grapevine PGIP1 are very similar to those observed from previously isolated PGIP genes. It shows a high content of leucine residues and six potential glycosylation sites that are shared with other PGIPs. Moreover, seven out of the nine cysteine residues found in the N- and C-termini of the protein were highly conserved, suggesting that they may function to stabilise the three-dimensional structure of the protein (Neame et al. 1989). The stretch of 27 amino acids at the N-terminus of the grapevine PGIP peptide is highly hydrophobic and could correspond to a putative signal peptide, which is likely to direct the grapevine PGIP to the extracellular space (Bergmann et al. 1994; Steinmayr et al. 1994).

Highly significant also is the large degree of conservation in the LRR motifs present in grapevine PGIP1 when compared to other LRR-containing proteins. Sequence variability within the structure of the LRR domain in PGIPs has been shown to affect ligand binding and determines recognition specificity (Leckie et al. 1999). The residues of PGIP that determine specificity and affinity for fungal PGs are present in the region corresponding to the predicted β -strand/ β -turn structure of the protein, indicated by the xxLxLxx motif (Kobe and Deisenhofer 1993, 1995). A single amino acid variation in this motif can render a PGIP with a new recognition capability (Leckie et al. 1999). It was evident that in the two grapevine *pgip* genes isolated from two different cvs. in this study, a number of non-synonymous nucleotide substitutions occurred that led to amino acid variations specifically within the predicted xxLxLxx motif. The possibility of PCR-generated changes induced due to the isolation methodology employed can be effectively ruled out, since several individual clones were sequenced to confirm the nucleotide changes. The same trend was also observed when *pgip1* genes were amplified from more cvs. of grapevine and analysed by sequence analysis (results not shown). These amino acid variations in the area responsible for recognition specificity of fungal PGs might prove to be responsible for the differential inhibition specificities against a range of fungal PGs characteristic of the different *pgip* gene family members present in the *V. vinifera* cvs.

Different inhibitory activities against PGs have been observed in PGIPs from different plant species (Cervone et al. 1987; Johnston et al. 1993; Stotz et al. 1993, 1994; Yao et al. 1995) and even from a single plant source (Desiderio et al. 1997). This could be an indication that PGIP-encoding genes have undergone diversification during evolution. Similar to many plant resistance genes, PGIP-encoding genes are organised into multigene families and different members of these families encode PGIPs with nearly identical biochemical characteristics, but distinct specificities (Desiderio et al. 1997). From partial PGIP purifications it was evident that the bulk

grapevine PGIPs are active against PGs from *B. cinerea*, *C. lindemuthianum* and *A. niger*. In an attempt to specifically evaluate the inhibitory activities of grapevine *pgip1*-encoded proteins, the gene was transiently expressed in *N. benthamiana*. Inhibition studies performed with the crude extracts that were recovered, showed that the expressed grapevine *pgip1* had strong inhibitory activities against PGs from *B. cinerea*, but not those from *A. niger*. This suggests that within the grapevine PGIP multigene family, the grapevine *pgip1* gene encodes a PGIP with specificity against PGs from *B. cinerea* and possibly other fungal PGs, whereas other *pgip* genes encode for a PGIP with the ability to interact and inhibit PGs from at least *A. niger*.

Western blot analysis of the extracts from *N. benthamiana* revealed the presence of a PGIP-specific signal with an approximate molecular mass of 42 kDa. This differed from the predicted mature grapevine PGIP1 molecular mass of 34 kDa. Similar results have previously been obtained with apple and pear PGIPs (Stotz et al. 1993; Yao et al. 1995). Deglycosylation of purified pear PGIP, for example, led to a decrease in molecular mass from 43 kDa to 34 kDa, which closely corresponded to the predicted molecular mass of 33.9 kDa (Stotz et al. 1993).

Grapevine *pgip* transcripts have been shown to be strictly tissue-specific, which is in contrast to the situation found in bean, pear tomato and raspberry. Grapevine PGIP mRNA was detected only in berries, but not in inflorescences and leaves. Unlike any of the findings on PGIP expression, grapevine PGIP transcription levels were shown to be developmentally regulated, increasing dramatically at véraison, which usually signals the onset of berry ripening. This developmental phase is characterised by a decrease in organic acids, significant sugar accumulation, colour change and an alteration in the physiological and biochemical characteristics of the grape berry. During the progressive ripening stages of the grape berry, the grape *pgip* transcript levels decreased to very low levels.

It has been well established that several grape berry proteins possess antifungal activities (Salzman et al. 1998) and their abundance and developmentally regulated, controlled accumulation in berries imply a potential role for these proteins in host-plant defence against pathogens. Ripe fruits are particularly susceptible to fungal attack because of increased levels of sugars and other nutrients, as well as physical changes that facilitate fungal colonisation and ultimately infection. However, it has been known for some years that grape berries acquire resistance to several pathogens during ripening (Delp 1954; Lafon and Clerjeau 1988; Pearson 1988). Recently it has been shown that mRNA accumulation patterns are upregulated for several groups of proteins, including antifungal and structural proteins with possible roles in disease resistance at véraison and later stages of berry development (Davies and Robinson, 2000). This is also the time during berry development when seed formation is complete and the seed coats are hardening to become resistant structures. It therefore seems that a range of defence-related genes, including PGIPs, are upregulated to provide a pathogen-free environment for the seed containing the progeny to develop and mature. Even though reproductive organs are an exception to

induced acquired resistance, the importance of flowers and ovaries to the maintenance of the species mandate that reproductive organs acquire pathogen resistance during development (Salzman et al. 1998).

The very specific and dynamic interactions between PGIPs and their PG substrates provide an excellent model to study certain aspects of disease resistance and fungal pathogenicity. The study of the development and the diversity of the grapevine *pgip* genes and encoding proteins in this ancient genotype, with all its varieties, undoubtedly will lead to more insight into the intricate control mechanisms imparted on gene expression in a woody perennial fruit species and some of the evolutionary adaptations with regard to disease resistance towards fungal pathogens.

3.5 ACKNOWLEDGEMENTS

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A.2 CONSTRUCTION AND SCREENING OF A SUBGENOMIC LIBRARY

The second approach to isolate a grapevine *pgip* gene involved a subgenomic library. Genomic DNA from *V. vinifera* cv. Pinotage was digested with a range of restriction enzymes (*Bgl*II, *Eco*RV and *Hind*III) and hybridised with the digoxigenin-labelled pear PGIP probe. Only the *Eco*RV digest yielded a single hybridisation product of approximately 5.6 kb in size and was subsequently used to construct a subgenomic library for the isolation of a grapevine PGIP-encoding gene and promoter (**Figure A.2A**).

Genomic DNA digested with *Eco*RV was separated on an agarose gel and a fragment of the gel, corresponding to the area where the pear PGIP probe hybridised, was excised (**Figure A.2B**). The DNA was purified and cloned into the dephosphorylated *Eco*RV site of pBluescript (+). The ligation mixture was electroporated into *Escherichia coli* DH10B. The resulting library of transformants was amplified, stored and plated for screening.

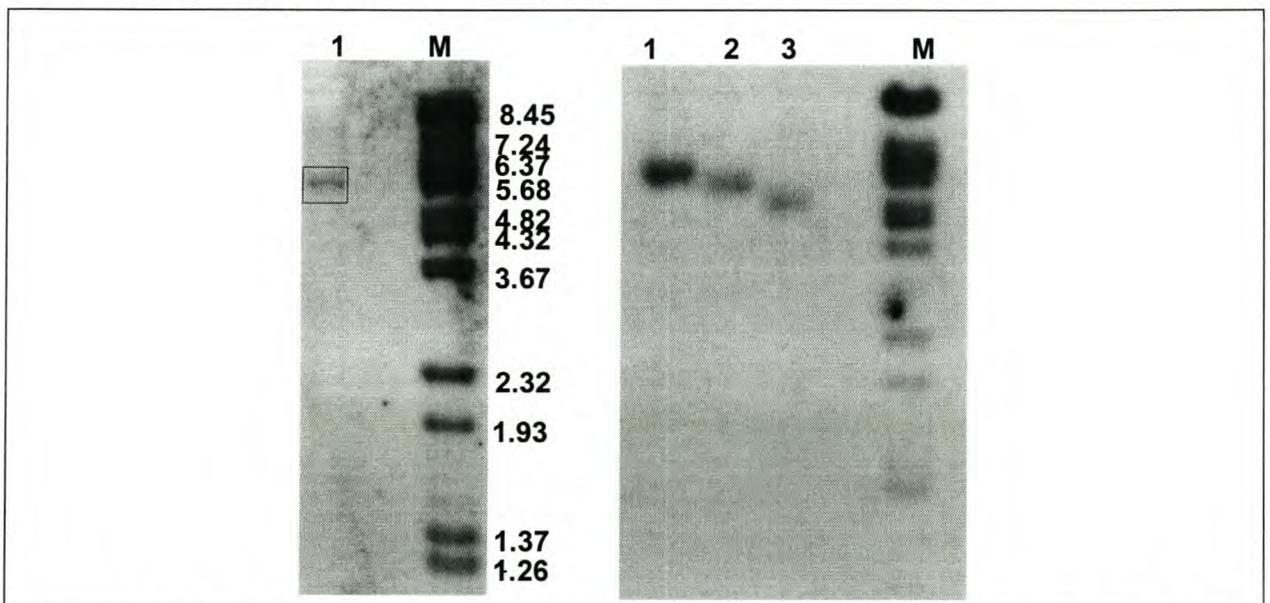


Figure A.2 Southern blot analysis of genomic DNA from *Vitis vinifera* cv Pinotage digested with *Eco*RV (lane 1) and hybridised with a digoxigenin-labelled 780 bp pear PGIP fragment is shown in A. A hybridising product of approximately 5.6 kb is indicated. Three fragments of the gel corresponding to the hybridised area were excised, purified and another Southern blot was done on the corresponding fragments (B). All three fragments were pooled (lanes 1-3) and used for the construction of a subgenomic library. The marker lane (lane M) contains *Bst*EII-digested lambda DNA labelled with digoxigenin. Sizes of the standard DNA fragments are indicated in kb.

The radioactively labelled [32 P]dATP pear PGIP fragment was used to screen recombinant clones from the library using colony blots. A potential positive clone was identified from 13 000 screened colonies and isolated for further analysis. The clone, designated pPGIP^{G1} (**Table A.1**), contained an insert of approximately 5.6 kb and was shown to contain a putative PGIP-encoding gene by Southern blot analyses when probed with the pear PGIP probe (**Figure A.3**).

Table A.2 Bacterial strains and plasmids used in this study. A description of the strains and plasmids as well as new plasmids constructed in this study, are shown in the table below.

Strains and plasmids	Genotypes and characteristics	Source or reference
<u>Bacterial strains</u>		
<i>Escherichia coli</i> DH 5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook <i>et al.</i> (1989)
<i>Escherichia coli</i> DH 10B	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>deoR recA1 endA1 araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galK</i> λ ⁻ <i>rpsL nupG</i>	Life Technologies
<u>Plasmids</u>		
pGEM-T Easy vector	Cloning vector containing T7 & SP6 transcription initiation sites, <i>lacZ</i> start codon, β -lactamase (Amp ^r) coding region, phage F1 region	Promega
pPGIP ^P	pGEM-T Easy vector with PCR amplified 780 bp pear PGIP fragment	This study
pBluescript SK (+)	Cloning vector containing T7 & T3 transcription initiation sites, <i>lacZ</i> start codon, β -lactamase (Amp ^r) coding region, phage F1 region, ColE1 origin	Stratagene
pPGIP ^{G1}	pBluescript SK (+) with 5.6 kb Pinotage genomic DNA fragment	This study
pPGIP ^{G2}	pBluescript SK (+) with PCR amplified 820 bp grapevine PGIP fragment	This study
pPGIP ^{G3}	pBluescript SK (+) with PCR amplified 1002 bp Pinotage PGIP-encoding gene	This study
pPGIP ^C	pGEM-T Easy vector with PCR amplified 1002 bp Chardonay PGIP-encoding gene	This study
pPGIP ^{CB}	pGEM-T Easy vector with PCR amplified 1002 bp Chenin Blanc PGIP-encoding gene	This study
pPGIP ^M	pGEM-T Easy vector with PCR amplified 1002 bp Merlot PGIP-encoding gene	This study
pPGIP ^S	pGEM-T Easy vector with PCR amplified 1002 bp Shiraz PGIP-encoding gene	This study
pPGIP ^D	pGEM-T Easy vector with PCR amplified 1002 bp Dauphine PGIP-encoding gene	This study
pPGIP ^{RG}	pGEM-T Easy vector with PCR amplified 1002 bp Red Globe PGIP-encoding gene	This study
pPGIP ^{Su}	pGEM-T Easy vector with PCR amplified 1002 bp Sultana PGIP-encoding gene	This study

A.3 SUBCLONING AND CHARACTERISATION OF THE GRAPEVINE PGIP-ENCODING GENE

PCR analysis of pPGIP^{G1} using pearPGIP-5' and 3' primers resulted in an amplification product of approximately 820 bp. This cloned product, designated pPGIP^{G2}, was sequenced, which revealed sequence similarities to other cloned PGIP-encoding genes (Stotz *et al.*, 1993; Favaron *et al.*, 1994, Stotz *et al.*, 1994; Ramanathan *et al.*, 1997; Yao *et al.*, 1999). Based on the sequence of this product, gene-specific sequencing primers, seqPGIP-R and seqPGIP-L (Table A.2) were designed to obtain the complete sequence for the grapevine PGIP-encoding gene.

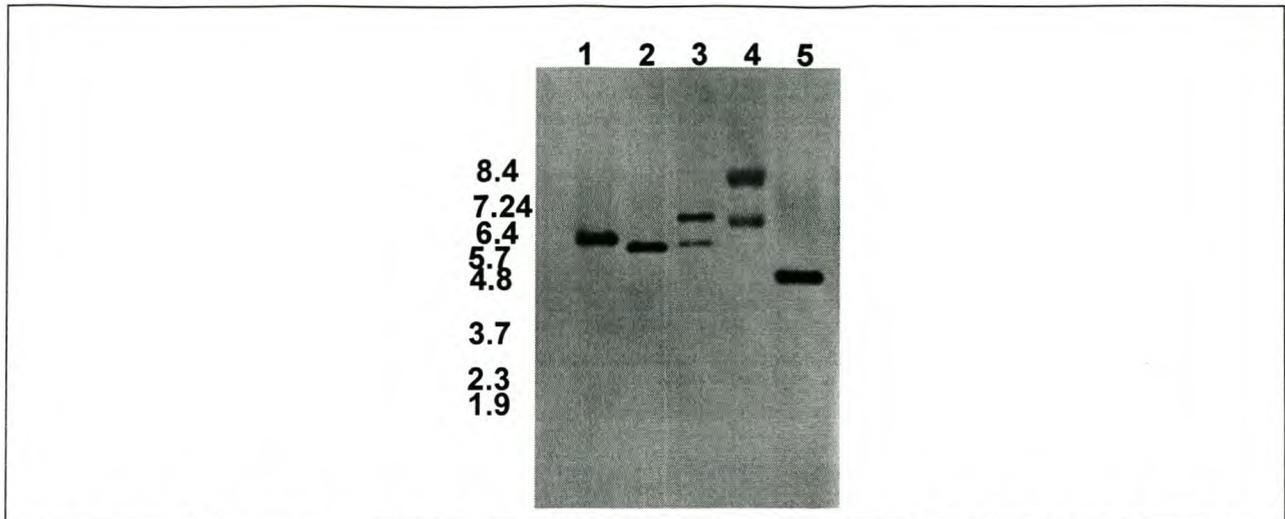


Figure A.3 Southern blot analysis of pPGIP^{G1} digested with *EcoRV* (lane 1), *EclXI* and *HindIII* (lane 2), *SpeI* and *Sall* (lane 3), *NotI* (lane 4) and *PvuII* (lane 5) and hybridised with a digoxigenin-labelled 780 bp pear PGIP fragment. Sizes of the *BstEII*-digested lambda DNA fragments are indicated in kb.

Table A.2 List of primers used in the study. Primers were designed to obtain sequences of the grapevine PGIP-encoding gene as well as a putative promoter area of the gene.

Primer name	Primer sequence	Primer position in 5.6 kb fragment
pearPGIP-5'	5'-CTCTCTCCGAYCKCTGCAAC-3'	
pearPGIP-3'	5'-GGAATCTGACCACACAGCC-3'	
seqPGIP-R	5'-GGTTAGCGAGTTGATGC-3'	from nt +221 to +237
seqPGIP-L	5'-TCGAGAGTGG AATTCCC-3'	from nt +776 to +793
seqPGIP-R2	5'-CCTTGAGGGCTTATTTGG-3'	from nt -129 to -112
seqPGIP-R3	5'-CTCAAATGATTTAATTCATAGG-3'	from nt -745 to -724
grapePGIP-5'	5'-AGGACAGAGAAATGGAGACTTCAAAC-3'	from nt +1 to +15
grapePGIP-3'	5'-AGTCAGATCTGAGCCGTC ACTTGC-3'	from nt +994 to +1002

Sequencing results using these primers on pPGIP^{G1} confirmed that the 5.6 kb subgenomic fragment contained the open reading frame as well as a putative promoter area of the grapevine PGIP-encoding gene (**Figure A.4**). To obtain the sequence of the putative promoter area, primers seqPGIP-R2 and seqPGIP-R3 (**Table A.2**) were designed and the corresponding sequenced regions of the putative promoter are shown in **Figure A.5**. Gene-specific primers, grapePGIP-5' and grapePGIP-3', were designed and used to amplify the entire putative PGIP coding region, and PCR reactions carried out on grapevine genomic DNA yielded an amplification product of 1002 bp. This product was cloned and designated pPGIP^{G3}, and subsequently sequenced. The sequencing results confirmed that the product amplified from genomic DNA yielded the identical nucleotide sequence to that of the pPGIP^{G1} clone. This was confirmed to be a grapevine *pgip* gene and was designated grapevine *pgip1*.

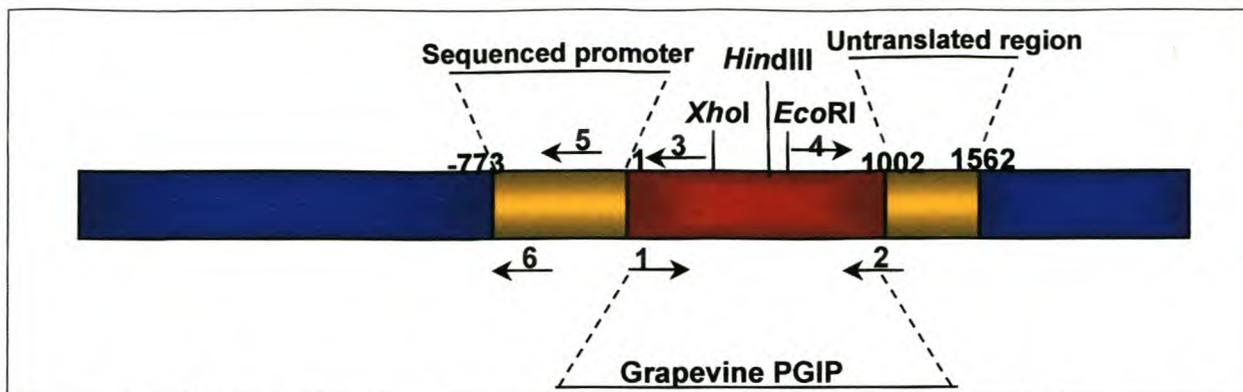


Figure A.4 Schematic representation of a 5.6 kb Pinotage genomic DNA fragment containing a *pgip* gene from grapevine. Positions of the primers described in the text and used in this study are indicated by numbered arrows. Primers 1 and 2 represent grapePGIP-5' and 3', primers 3 and 4 represent seqPGIP-R and seqPGIP-L, primers 5 and 6 represent seqPGIP-R2 and seqPGIP-R3, respectively. Restriction sites are shown.

GTTATTTTAAATTTATTTATTTATTTTAAACCTATGATTAAATCATTTGAG	50
AATGTATAAATATTTTATTAATTTTGATTATATTTTATTTTCTTAATGT	100
TTTTTAAGACAATCAAATGATAGAATAATTTTTTAAAATGTTTTTCGATA	150
ATCAAATATAACTTTAATATAGAGAAAATGATGGTTGAATTTATATTTTT	200
AGAATTTTTTTAATAATATAGAAAAAAAGGTAAGGTTATCTAATTTGTG	250
GTATTAATGGAAATAATTATTTTTTAATAATGTTGTTGAGTTTATCTATT	300
TCTAAATTAGTATTTTGAAAATGTCTTTTAAATTTAAAATAACTTTTAAG	350
ATTTTAAATTTTGTATCCCTATTTTTTATTTTTTAAAACTAAAAAGTAGT	400
AAATCCTATATTCTGATTTATTTTTTTTATGAAAATATTATATATTTATA	450
TTTTTAATAAAAAACAATTTTGTAGTATTTTTCATTTTTTTCAATTATTCT	500
CATAATGTTTAAAAAAAATCTTAAACCACTTTAAATTTATTTTAAAA	550
ATAAAATATTTTTTAAATAAATTTTCAGAAAATATTTTTAATCCAAATTT	600
GTAAAATATATTTTACTAAAGAAACAAAAATGGAAGGAACTCCAAT	650
AAGCCCTCAAGGCCGCCCTCAGCTGGAAGCCCATTTACCATATTCGTGTT	700
CAATTTGGCCACGCGAGCAAGTGCTATAAATTCAAGATTTGGTCTAGGC	750
ATTACGTAGCCAAATTCAGAGAA	773

Figure A.5 Nucleotide sequence of the grapevine *pgip1* putative promoter. The 773 fragment immediately to the 5' side of the coding region of the *pgip1* gene from *V. vinifera* revealed the presence of a putative CAAT box at position -73 and a putative TATA box at position -48 as highlighted.

A.4 GRAPEVINE PGIP1 SEQUENCES FROM DIFFERENT *V. VINIFERA* CULTIVARS

Gene-specific primers were subsequently used to amplify a PGIP-encoding gene from genomic DNA isolated from the following wine grape cultivars: Chardonnay, Chenin Blanc, Merlot and Shiraz, as well as from the table grape cultivars Dauphine, Red Globe and Sultana. The amplified 1002 bp PCR products from all seven cultivars were

cloned into the pGEM-T Easy vector (Promega) and designated pPGIP^C, pPGIP^{CB}, pPGIP^M, pPGIP^S, pPGIP^D, pPGIP^{RG} and pPGIP^{Su}, respectively.

Sequence analyses revealed nucleotide changes in six of the tested grapevine cultivars relative to cv Pinotage, whereas cv. Chenin Blanc showed no nucleotide changes. A high number of non-synonymous substitutions (nucleotide substitutions that lead to amino acid replacements) compared with synonymous substitutions (nucleotide substitutions that do not alter amino acids) were observed (**Table A.4**).

Table A.4 Number of non-synonymous and synonymous nucleotide substitutions observed for the seven sequenced grapevine cultivars. All the non-synonymous and synonymous nucleotide substitutions were compared to *Vitis vinifera* L. cv. Pinotage.

<i>Vitis vinifera</i> L. cultivars	Total nucleotide changes	Non-synonymous nucleotide changes	Synonymous nucleotide changes
Chardonnay	18	9	9
Chenin Blanc	0	0	0
Merlot	10	4	6
Shiraz	16	8	7
Dauphine	11	8	3
Red Globe	12	6	6
Sultana	15	9	6

Non-synonymous substitutions for the six grapevine cultivars were compared to that of cv Pinotage (**Figure A.6**). Non-synonymous substitutions were not found for cv Chenin Blanc relative to cv Pinotage. Of all the non-synonymous substitutions for the six cultivars, only three, corresponding to residues 199, 201 and 221 of the grapevine PGIP, showed a conserved position among all the grapevine inhibitors. All three substitutions were present in the predicted xxLxLxx motif to form the solvent-exposed β -sheet/ β -turn structure of the protein. The non-synonymous substitutions on the β -strand region lead to amino acid changes from Y199G, from Y201H and from T221N for all six cultivars, with the exception of cv Merlot, whose non-synonymous substitutions at those positions, were the same as that of cv Pinotage.

One could speculate that changes in residues in this region found in different cultivars of *V. vinifera*, could lead to differential activities in PGIPs from different cultivars to interact with and inhibit PGs from *B. cinerea* as well as other fungal PGs. Applying a maximum likelihood model of codon evolution, Stotz *et al.*, identified a number of residues that are likely to evolve adaptively in response to natural selection. Many of these residues are within the β -sheet/ β -turn region of the PGIP LRR, but others lie outside this region (Stotz, H.U., Bishop, J.G., Bergmann, C.W., Koch, M., Albersheim, P., Darvill, A.G., and Labavitch, J.M. (2000). Non-synonymous substitutions found outside the predicted β -sheet/ β -turn region in the six grapevine cultivars could therefore be significant and lead to differential activities in PGIPs to interact with and inhibit fungal PGs.

Pinotage	METSKLFLSSSSLLLLLALTRPCPSLSERCNPDKKVLQIKKALDNPYILASWN	55
ChardonnayV.....	
MerlotL.....D.....	
ShirazV.....	
DauphineL.....	
Red GlobeV.....	
SultanaV.....	
Pinotage	PNTDCCEWYCVCEDLTSHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNL	110
ChardonnayG.....T.....	
MerlotE.....T.....	
ShirazG.....T.....	
DauphineG.....T.....	
Red GlobeG.....T.....	
SultanaG.....T.....T.....	
Pinotage	TGQIPPAIAKLKHLKMWRLSWTNLSGPVPAFFSELKNLTYLDLSFNLSGPIPGS	165
Chardonnay	...V.....	
Merlot	...I.....	
Shiraz	...V.....	
Dauphine	...I.....I.....	
Red Globe	...I.....	
Sultana	...I.....	
Pinotage	LSLLPNLGAHLDRNHLTGPIPDSEFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDP	220
ChardonnayI.....G.H.....	
MerlotG.H.....	
ShirazG.H.....	
DauphineN.....G.H.....	
Red GlobeG.H.....	
SultanaG.H.....	
Pinotage	TVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHN	275
Chardonnay	N.....L.....	
Merlot	...G.....P.....	
Shiraz	N.....L.....	
Dauphine	N.....L.....	
Red Globe	N.....P.....	
Sultana	N.....L.....	
Pinotage	KIAGSLPEMMSLDLQFLNVSYNRLCGKIPVGGKLSFDYDSYFHNRCLECGAPLQSCK	330
ChardonnayG.....	
MerlotG.....	
ShirazG.....	
DauphineG.....	
Red GlobeG.....	
SultanaG.....	

Figure A.6 Alignment of grapevine PGIP1 amino acid sequences for seven grapevine cultivars. For PGIP1 from cultivars Chardonnay, Merlot, Shiraz, Dauphine, Red Globe and Sultana, only non-synonymous substitutions that lead to amino acid changes that differ from cultivar Pinotage are indicated. Dots indicate identity. Boxes indicate the area of the protein predicted to form the β -sheet/ β -turn structural motifs.

CHAPTER 4

RESEARCH RESULTS

The purification and characterisation of polygalacturonase-inhibiting proteins (PGIPs) with different specificities from *Vitis vinifera* L.

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RESEARCH RESULTS

PURIFICATION AND CHARACTERISATION OF POLYGALACTURONASE-INHIBITING PROTEINS (PGIPs) WITH DIFFERENT SPECIFICITIES FROM *VITIS VINIFERA* L.

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ABSTRACT

Plant polygalacturonase-inhibiting proteins (PGIPs) have been shown to specifically bind and inhibit fungal polygalacturonases (PGs). Molecular analyses have confirmed that PGIPs are typically encoded by multigene families and that the inhibitor specificities and kinetics of the isolated proteins differ within and among species. The first isolated PGIP-encoding gene from *Vitis vinifera* L. was recently reported and shown to be part of a small gene family (A. De Ascensao, I. Pretorius, D. Bellstedt, J. Burger, and M. Vivier, submitted to *Planta*, 2001). The isolation of two PGIP isomers from *V. vinifera* berries is described here. The one isomer, designated PGIP-A, was partially purified and had a molecular mass of 39 kDa, whereas the other, designated PGIP-B, was purified and had a molecular mass of 42 kDa as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses. Both proteins were cell wall-bound. Enzymatic deglycosylation confirmed that PGIP-B is glycosylated. Grapevine PGIP-A showed strong inhibitory activity against a homogeneous PG from *A. niger* and to a lesser extent against PGs from *Fusarium moniliforme*, but was unable to interact with a crude PG preparation from *Botrytis cinerea*. Grapevine PGIP-B, on the other hand, was able to strongly inhibit PGs from both *B. cinerea* and *Colletotrichum gleosporoides*, yet showed no inhibition towards PG from *Aspergillus niger*. Kinetic studies of PGIP-B suggested a non-competitive form of inhibition. Comparison of the inhibition spectra of PGIP-B and a crude PGIP extract from *Nicotiana benthamiana* infected with the PVX vector, transiently overexpressing the grapevine *pgip1* gene, yielded similar inhibition specificities. This and other characteristics provide a good indication that the grapevine *pgip1* gene encodes the purified PGIP-B isomer.

Key words: leucine rich repeat (LRR) proteins – phytopathogens – oligogalacturonides – plant defences

4.1 INTRODUCTION

During pathogenesis, plant cell walls are the first line of defence that plant-pathogenic bacteria and fungi must overcome in order to establish colonisation sites and obtain nutrients (Elad and Evensen 1995). Many fungal pathogens secrete a range of hydrolytic enzymes to degrade the pectin polymers that constitute the plant cell wall. Among the pectinases produced by phytopathogens during infection, polygalacturonases (PGs), especially the endo-cleaving form (endo-PGs), are thought to play a principal role during the early stages of plant pathogenesis (reviewed in Hahn et al. 1989). Similarly, endo-PGs of *Botrytis cinerea* appear to contribute to fungal invasion and degradation of host tissues and *Botrytis* has been shown to secrete a large number of PG isozymes (Johnston and Williamson 1992). Functional evidence that an endo-PG contributed to the virulence of *Botrytis* was shown when mutation of one member of the PG gene family, *Bcpg1*, resulted in a significant decrease of virulence on tomato leaves and fruits, as well as on apple fruit (Ten Have et al. 1998).

In plants, mechanisms exist to counteract the action of endo-PGs. Many dicotyledonous plants have cell wall-localised polygalacturonase-inhibiting proteins (PGIPs) (Cervone et al. 1997), which play a major role in the known plant defence mechanisms. PGIPs are specific, high-affinity receptor-like proteins that inhibit the activity of fungal endo-PGs. Through this inhibition, PGIPs interfere directly with host cell wall degradation. It is hypothesised that the formation of the PGIP-PG complexes benefits the plant by slowing down the hydrolytic activity of endo-PGs. This results in a change in the balance between the release of elicitor-active oligogalacturonides and the breakdown of active oligogalacturonides into inactive molecules, favouring accumulation of the former (Cervone et al. 1989).

A study of the structure of PGIP peptides, based on predicted sequences, reveals that these genes form part of a larger class of plant disease resistance genes. PGIPs belong to the large family of leucine-rich repeat (LRR) proteins. This protein family includes several resistance gene products (Bent 1996), PGIPs, as well as several receptor kinases. It has been proposed that PGIP may act as a secreted receptor involved in the surface signalling system between plants and fungi (De Lorenzo and Cervone 1997).

Kinetic studies of PGIPs showed that inhibition of fungal PGs occurs by either a mixed-type- (Yao et al. 1995), a competitive-, or a non-competitive mechanism (Abu-Goukh et al. 1983; Lafitte et al. 1984; Johnston et al. 1993). Furthermore, different inhibitory activities against PGs have been observed in PGIPs from a single plant source (Desiderio et al. 1997). In addition, PGIPs isolated from different plants (Cervone et al. 1987; Johnston et al. 1993; Stotz et al. 1993, 1994; Favaron et al. 1994) show differential inhibition spectra towards a range of PGs from phytopathogenic fungi, indicating that *pgip* genes have undergone diversification during evolution. Likewise, this variability in PG specificity possibly reflects counter-adaptations between fungal PGs and plant PGIPs that lead to specialisation.

A *pgip* gene from *V. vinifera* has been cloned, characterised and designated grapevine *pgip1* (De Ascensao et al. 2001, in press). Genomic DNA analysis showed that grapevine *pgip1* belongs to a multigene family, indicating a probability that different grapevine *pgip* genes encode PGIPs with the ability to interact with and inhibit PGs from different fungal sources. The grapevine *pgip1* gene was transiently expressed in *Nicotiana benthamiana* L. with potato virus X (PVX), yielding crude protein extracts that showed inhibitory activity against PGs from *B. cinerea*.

Grapevine PGIPs have not previously been purified or characterised. In this study we describe the purification and characterisation of two PGIP isomers, PGIP-A and PGIP-B, from *V. vinifera* cv. Pinotage berries. PGIP-A was partially purified, but was characterised in terms of its ability to inhibit various fungal PGs. PGIP-B was purified and was characterised in terms of the type of inhibition it exhibits, inhibitor stability and specificity. The inhibitory activity of both PGIP-A and B was examined against homogeneous PGs from *Aspergillus niger* and *Fusarium moniliforme*, as well as crude preparations of PGs from *B. cinerea* and other pre- and post-harvest causing pathogenic fungi. A crude PGIP extract from PVX-vector-infected tobacco plants, transiently expressing the grapevine *pgip1* gene, was also analysed for inhibition specificities.

4.2 MATERIALS AND METHODS

Preparation of fungal PGs

Cultures of *Alternaria alternata*, *B. cinerea*, *Colletotrichum gleosporoides*, *Monilinia laxa*, *Mycor* spp., *Penicillium expansum* and *Rhizopus stolonifer* were obtained from the Department of Plant Pathology, Stellenbosch University and were maintained on potato dextrose agar (PDA) medium at 23°C. Crude PG preparations from the fungal strains were obtained by growing the mycelia in a citrate phosphate buffer (pH 6.0) supplemented with 1% (w/v) citrus pectin. The medium contained 2 mM MgSO₄·7H₂O, 0.6 μM MnSO₄·H₂O, 25 mM KNO₃, 30 μM ZnSO₄·7H₂O, 0.9 μM CuSO₄ and 65 μM FeSO₄. Cultures were incubated on a rotary shaker at 22°C for 10 days. Culture media were filtered through Whatman No 1 filter paper and precipitated overnight with 80% ammonium sulphate (NH₄)₂SO₄ at 4°C. The resulting pellet obtained after centrifugation at 10,000 x g for 20 min was resuspended in 40 mM Na acetate (pH 5.0) and dialysed against 20 mM Na acetate (pH 5.0). The PG from *A. niger* that was used was a commercial preparation (Sigma), whereas PG from *F. moniliforme* was prepared according to De Lorenzo et al. (1987). PG activity was determined by the agarose diffusion assay according to Taylor and Secor (1988) and by the p-hydroxybenzoic acid hydrazide (PAHBAH) assay (Cervone et al. 1989).

Plant material

Véraison grape berries from *V. vinifera* L. cv. Pinotage were collected during the 2000 growing season from vines grown at a commercial vineyard in Stellenbosch,

Western Cape and used for protein extractions. Immediately after picking, berries were frozen in liquid nitrogen and ultimately finely crushed using an industrial grinder and stored at -80°C .

Extraction and purification of grapevine PGIP isomers

Finely crushed berry tissue (1 kg) was homogenised in extraction buffer (2 ml per g fresh weight), 0.1 M Na acetate (pH 6.0), 10 mM β -mercaptoethanol and 1% (w/v) PVP-40. The homogenate was centrifuged at $10,000 \times g$ for 15 min, after which the pellet was subjected to two more extractions in extraction buffer without PVP-40. The remaining insoluble tissue was resuspended in 2 volumes of 50 mM Na acetate (pH 5.2) containing 1 M NaCl and stirred for 1 h at 4°C . After centrifugation ($10,000 \times g$ for 20 min) at 4°C , the protein precipitating at 80% saturated $(\text{NH}_4)_2\text{SO}_4$ was collected, resuspended in 20 mM Na acetate (pH 5.2) and dialysed extensively at 4°C against 20 mM Na acetate (pH 5.0).

The dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction was applied to a diethylaminoethyl (DEAE) Sepharose fast flow column (Amersham, Pharmacia Biotechnology) equilibrated with 20 mM ammonium acetate (pH 5.0). Proteins bound by the column were eluted with a stepwise gradient from 0.05 M to 1 M ammonium acetate (pH 5.0). The fractions were assayed for PG inhibition by the agarose diffusion assay using PGs from *A. niger* and *B. cinerea*. Fractions corresponding to the highest inhibitory activity were pooled, concentrated by freeze drying and dialysed as above.

Grapevine PGIPs were further purified by an affinity-based procedure on an *A. niger* PG-Sepharose column as described previously (Cervone et al. 1989). The PGIPs absorbed to the column and were recovered by eluting with phosphate buffered saline (PBS) that favoured the dissociation of the PG-PGIP complex (Cervone et al. 1987). Fractions (0.5 ml) were collected and assayed for inhibition with the agarose diffusion assay against PGs from *A. niger* and *B. cinerea*. Inhibitory activity against *B. cinerea*, but not against *A. niger*, was found in the flow-through. The flow-through was then purified by fast protein liquid chromatography (FPLC), using a Mono S HR 5/5 column (Pharmacia, Milan, Italy) run at a flow rate of 0.5 ml/min. The column was equilibrated with 20 mM Na acetate (pH 5.0) and eluted with a linear gradient of 0 to 0.5 M NaCl in 20 mM Na acetate (pH 5.0). Fractions of 1 ml were collected and assayed for inhibitory activity against PGs from *A. niger* PG and *B. cinerea*.

PGIP activity assay

Inhibition of PG activity was determined by the agarose diffusion assay as previously described (Taylor and Secor 1988). Alternatively, for kinetic studies, inhibitor specificities and stability, PG activity was determined by measuring reducing groups released from sodium polypectate using the PABA-H colorimetric assay (Cervone et al. 1989). Activity was expressed as reducing group units (RGU). One activity unit was defined as the amount of enzyme producing 1 microequivalent of reducing

groups per minute at 30°C with 1% (w/v) polygalacturonic acid as substrate. The same approach was used to assay PGIP activity. One unit of PGIP was defined as the amount of inhibitor required to reduce the activity of 0.01 RGU of PG by 50%. The inhibition specificities were determined for the PGIP isomers isolated in this study, as well as for a crude PGIP extract from *N. benthamiana* infected with the PVX vector harbouring the grapevine *pgip1* gene that was isolated previously (De Ascensao et al., 2001 *in press*).

Kinetics of the grapevine inhibitor

The relationship between the grapevine PGIP concentration and the degree of inhibition was studied by incubating fixed quantities of *B. cinerea* PG of known activity with varying quantities of the purified PGIP-B. The mode of inhibition was determined using double reciprocal Lineweaver-Burk plots.

Inhibitor stability

The temperature stability of the grapevine PGIP-B was tested over a range of temperatures (30–95°C). Aliquots of the inhibitor were incubated at various temperatures for 15, 30, 45 and 60 min, respectively. Subsequently, each preparation was cooled to room temperature and tested against PGs from *B. cinerea* by the PABA assay. Inhibition values were calculated as a percentage of the activity of *B. cinerea* PGs in the presence and absence of purified PGIP-B.

The effect of pH on inhibitor activity was tested by investigating the ability of the purified PGIP to inhibit PGs from *B. cinerea* in sodium acetate buffer varying in pH-values from 4.0 to 6.5, as quantified by the PABA assay. Inhibition values were calculated as a percentage of the activity of a sample of *B. cinerea* PGs in sodium acetate buffer at pH values ranging from 4.0 to 6.5 in the presence and absence of purified PGIP-B.

Preparative isoelectric focussing (IEF)

An aliquot of the crude sample before application to the DEAE Sepharose column was dialysed against 0.5% glycine, and subjected to IEF at 4°C with an LKB 8100 ampholyte electrofocussing column (Pharmacia, Milan, Italy) containing 4.5 ml of 40% ampholine, pH 3 to 10.5, in a linear sucrose gradient (0 to 40% w/v). Inhibitory activity of the fractions was tested against *B. cinerea* PGs by the agarose diffusion assay.

Protein concentration determinations, gel electrophoresis and Western blot analysis of grapevine PGIP isomers

Protein concentrations were determined by the method of Bradford (1976) using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as the standard. SDS-PAGE was performed as described by Laemmli (1970). The 10% sodium dodecyl

sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were silver stained as described by Blum et al. (1987).

For Western blot analyses, proteins were electrophoretically transferred to nitrocellulose from a 10% SDS-PAGE gel according to Towbin et al. (1979). A polyclonal antibody that recognises the total bean PGIP complex (kindly provided by G. De Lorenzo) (Bergmann et al., 1994) was used to detect the antigen-antibody complex with the ECL detection kit (Amersham).

Deglycosylation of PGIP-B

Aliquots of samples containing 100 ng of PGIP-B were used for enzymatic deglycosylation analyses. To remove N-linked or O-linked glycans, aliquots were incubated with 2.5 and 0.2 units of N-glycosidase F (Roche Molecular Biochemicals) and 0.5 units of O-glycosidase (Prozyme, San Leandro, CA) for 12, 4 and 6 h, respectively. Following incubation, the mixtures were analysed on a 10 % SDS-PAGE gel.

4.3 RESULTS

Extraction and purification of grapevine PGIP isomers

The purification procedures aimed at isolating *Vitis* PGIP isomers used véraison berries as starting material, since previous expression and activity studies of bulk grapevine PGIPs yielded high transcript levels as well as PGIP activity at this stage. Analysis of a NaCl extract of berry tissue confirmed that the PGIPs were cell wall bound, since this fraction contained approximately 76% and 81% inhibitory activity against PGs from *A. niger* and *B. cinerea*, respectively. Since most of the PGIP activity was present in this extract, it was used further for PGIP purification. IEF showed that the fraction with the highest inhibitory activity against *B. cinerea* PGs had a pI of 8.78. Two chromatographic steps and an affinity-based purification procedure, as summarised in Table 4.1, were undertaken to purify grapevine PGIP isomers. The grapevine extract was subsequently passed through a (DEAE) Sepharose column. Fractions eluting between 0.2 and 0.6 M ammonium acetate showed PGIP activity. Inhibitory activity varied from 86% in the 0.2 M fraction to 79% in the 0.4 M fraction and 48% in the 0.6 M fraction when tested against PGs from *B. cinerea*. Inhibitory activity ranged from 73%, 70% and 35% inhibition in the 0.2, 0.4 and 0.6 M fractions, respectively, when tested against *A. niger* PG.

The active fractions were further purified by affinity chromatography through an *A. niger* PG-Sepharose column. Two fractions were obtained. Fraction A, the flow-through, contained proteins not bound to the column. The second fraction, which was eluted with phosphate-buffered saline (PBS), contained proteins absorbed by *A. niger* PG and was designated PGIP-A (Fig. 4.1A). Fraction A and PGIP-A were tested separately for inhibitory activity against PGs from *A. niger* and *B. cinerea* and it was

clear that fraction A inhibited PGs from *B. cinerea* but not from *A. niger*. In contrast, PGIP-A inhibited PGs from *A. niger* PG, but not from *B. cinerea*.

Table 4.1 Purification of polygalacturonase-inhibiting protein (PGIP) isomer, PGIP-B, from *Vitis vinifera* L cv. Pinotage^a

Fraction	Volume (ml)	Protein (mg)	Total activity ^b (units)	Specific activity (units/mg)	Yield (%)
Crude extract	250	198	12 630	64	100
Diethylaminoethyl-Sepharose	100	110	7810	71	62
PG-Sepharose	20	05	3500	7000	44
Mono-S	5	004	1040	26000	29

^a PGIP-B was purified from véraison grape berries

^b One unit of PGIP is the amount of protein required to reduce the activity of 001 RGU (reducing group units) of *Botrytis cinerea* PGs by 50%

Fraction A was then applied to a Mono S HR 5/5 column. Inhibitory activity against *B. cinerea* PGs was only found in fractions that eluted between 0.25 and 0.3 M NaCl (Fig. 4.1B). Fractions from the Mono S column containing PGIP activity were pooled and designated PGIP-B.

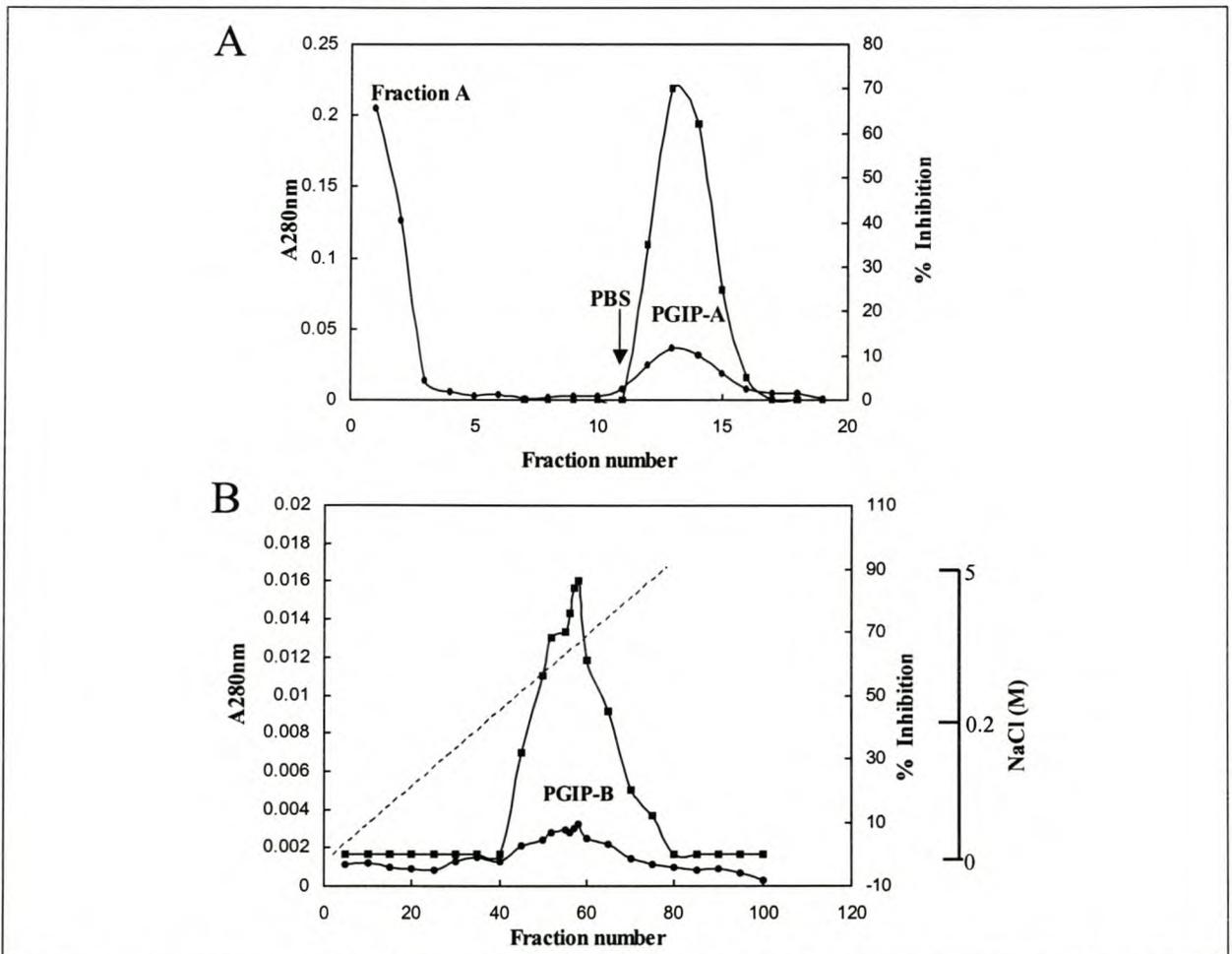


Fig. 4.1 Separation of PGIP-A and PGIP-B from extracts of *Vitis vinifera* berries by column chromatography. **(A)** Elution profile of extract passed through an *Aspergillus niger* PG-Sepharose affinity column. Two fractions were obtained: Fraction A, the flow-through, and a second fraction eluted with phosphate-buffered saline (PBS), which showed inhibitory activity against *A. niger*, designated PGIP-A. **(B)** Fraction A was in turn passed through a Mono-S column and found to contain active fractions against *Botrytis cinerea*. It was designated PGIP-B.

Molecular mass determination of grapevine PGIP isomers and deglycosylation of PGIP-B

When the active fractions from the *A. niger* PG-Sepharose column were subjected to SDS-PAGE, a diffuse protein band with a molecular mass of approximately 39 kDa and a doublet protein band of approximately 41 kDa were detected after silver staining (Fig. 4.2A). The fractions from the Mono S column with inhibitory activity against *B. cinerea* PGs were also analysed by SDS-PAGE. The results indicated two polypeptides with a molecular mass of approximately 44.5 kDa and 42 kDa (Fig. 4.2B).

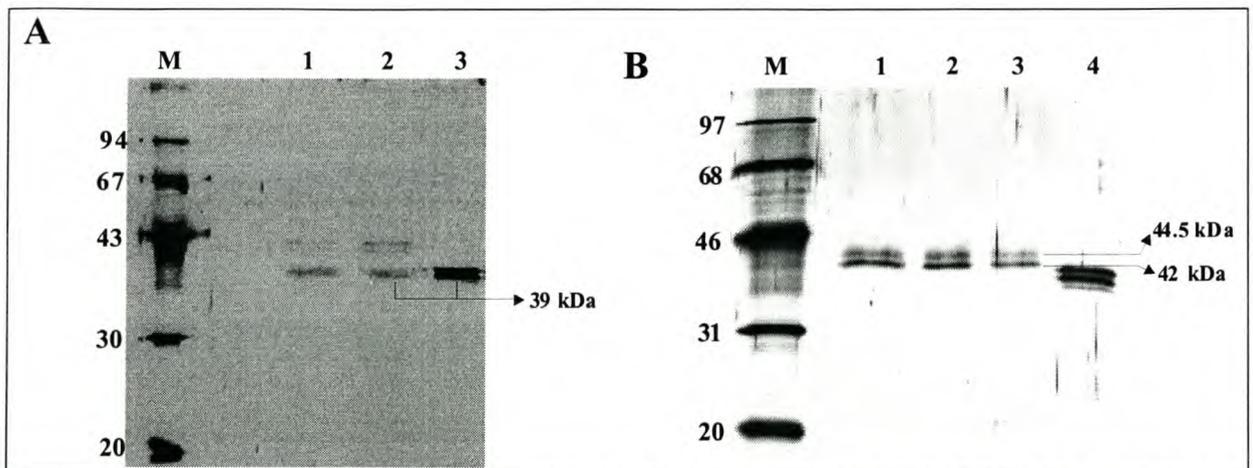


Fig. 4.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of grapevine PGIP-A and PGIP-B after anion exchange chromatography. **(A)** PGIP-A, approximately 30 ng of protein corresponding to peak fractions 13 (lane 1) and 14 (lane 2), respectively, after affinity chromatography. Band sizes are indicated. 70 ng of purified PGIP from bean pods was loaded in lane 3. **(B)** SDS-PAGE analysis of purified grapevine PGIP-B, approximately 50 ng of purified inhibitor corresponding to peak fractions 57 (lane 1), 58 (lane 2) and 59 (lane 3), respectively, from the Mono-S column were loaded. Molecular masses of individual bands are indicated. Purified PGIP from bean pods (70 ng) was loaded in lane 4. Molecular mass standards, in kDa, are shown in lane M. Gels were stained with silver staining.

When purified grapevine PGIP-B was N-deglycosylated, three polypeptides with molecular masses of approximately 41, 39 and 37 kDa, respectively were observed after SDS-PAGE analysis (Fig. 4.3A). The same pattern was observed even after an excess of enzymes and long incubation times were used, ruling out the possibility of a partial deglycosylation reaction (data not shown). Western blot analysis of glycosylated PGIP-B showed that the bean PGIP antibody cross-reacted with all three polypeptides detected by SDS-PAGE (Fig. 4.3B). No change in molecular mass was observed after incubation with O-glycosidase.

Western blot (immunoblot) analyses of PGIP-A and PGIP-B

Western blot analyses performed on electrophoresed samples of PGIP-A and PGIP-B demonstrated the presence of a PGIP-specific signal. Polyclonal antibodies against the native bean PGIP cross-reacted with the 39 kDa protein band present in the PGIP-A fraction (Fig. 4.4A), corresponding to the eluted fraction with the highest activity. Western blot analyses of increasing amounts of the eluted fraction from the

Mono S column that corresponded to the highest inhibitory activity (as shown in Fig. 4.1B), resulted in the presence of two PGIP-specific signals but with an increasing signal intensity corresponding to a 42 kDa band (Fig. 4.4B, lane 4).

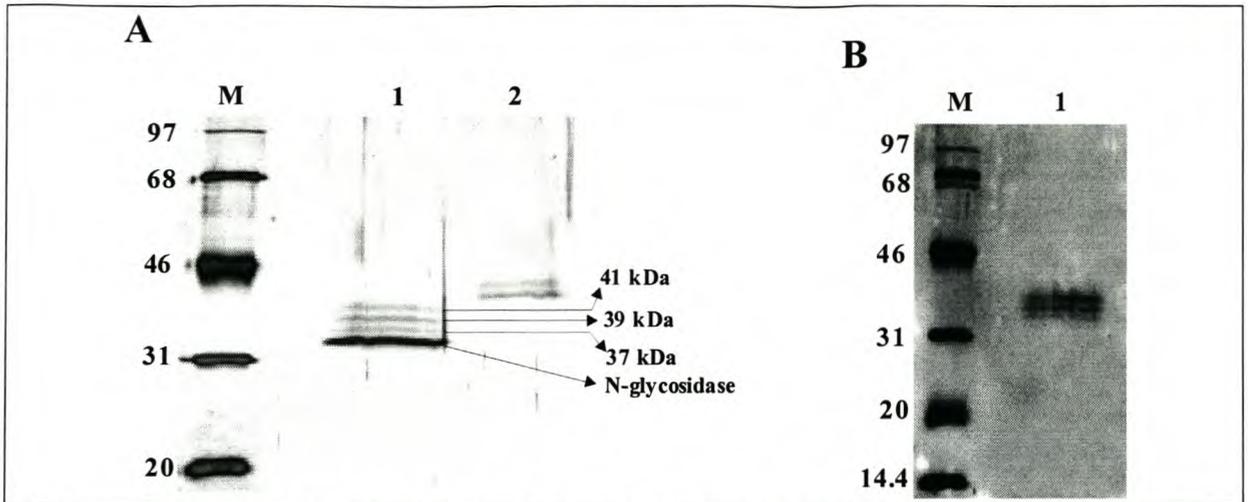


Fig. 4.3 Deglycosylation of PGIP-B **A:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of PGIP-B after enzymatic deglycosylation using N-glycosidase F (lane 1, 100 ng) and before deglycosylation (lane 2, 100 ng). Molecular masses of deglycosylated polypeptides are indicated. **B:** Western blot (immunoblot) analysis of deglycosylated PGIP-B using a polyclonal antibody that recognised the total bean PGIP-1 protein (lane 1). ECL molecular mass standards, in kDa, are shown in lane M.

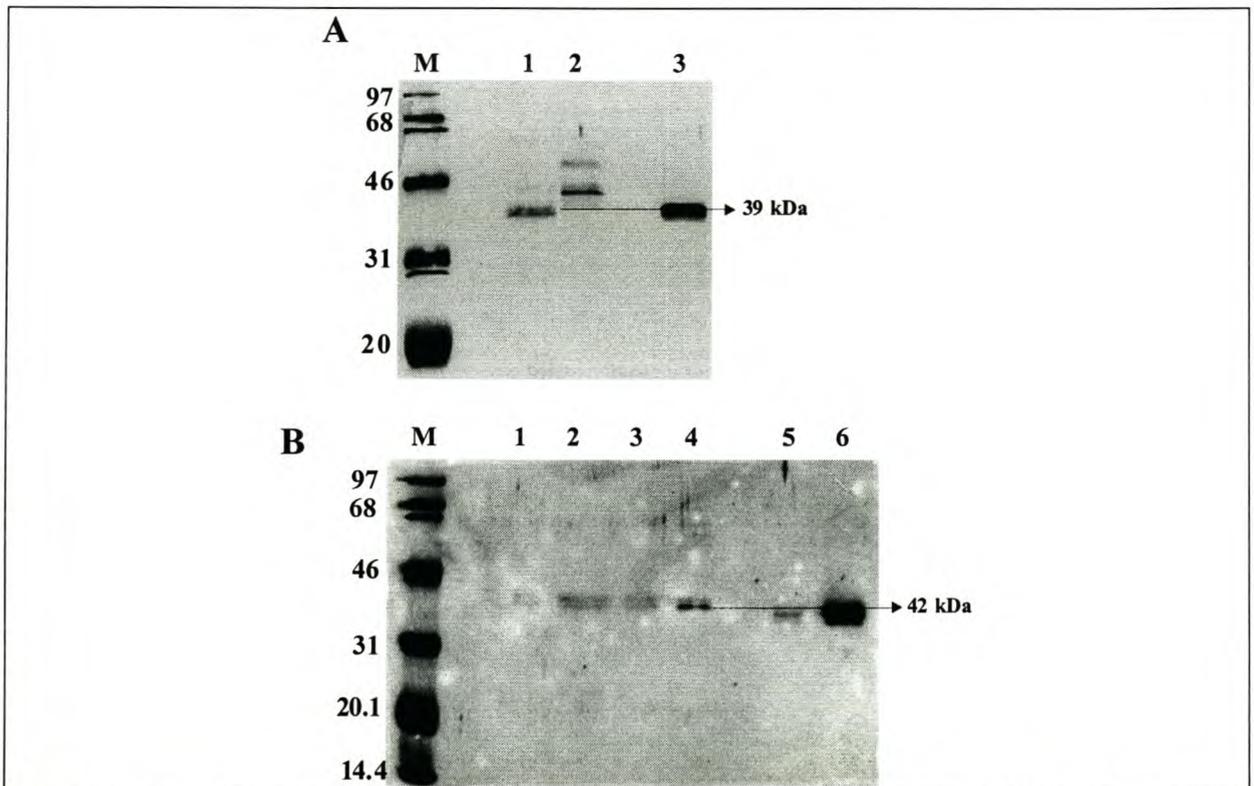


Fig. 4.4 Western blot (immunoblot) analysis of protein extracts from PGIP-A and PGIP-B. A polyclonal antibody that recognised the total bean PGIP-1 protein was used as probe. **A:** Protein extract (30 ng) from PGIP-A after PG-Sepharose chromatography (lane 1) and before affinity chromatography (lane 2) is shown. 70 ng of purified PGIP from bean pods was loaded in lane 3. **B:** Increasing amounts of protein extracts from purified PGIP-B after eluting from Mono-S column are shown; lane 1, 10 ng; lane 2, 20 ng; lane 3, 30 ng and lane 4, 50 ng. Approximately 4 ng of purified bean PGIP-2 (lane 5) and 70 ng of purified PGIP from bean pods were loaded in lane 6. ECL molecular mass standards, in kDa, are shown in lane M.

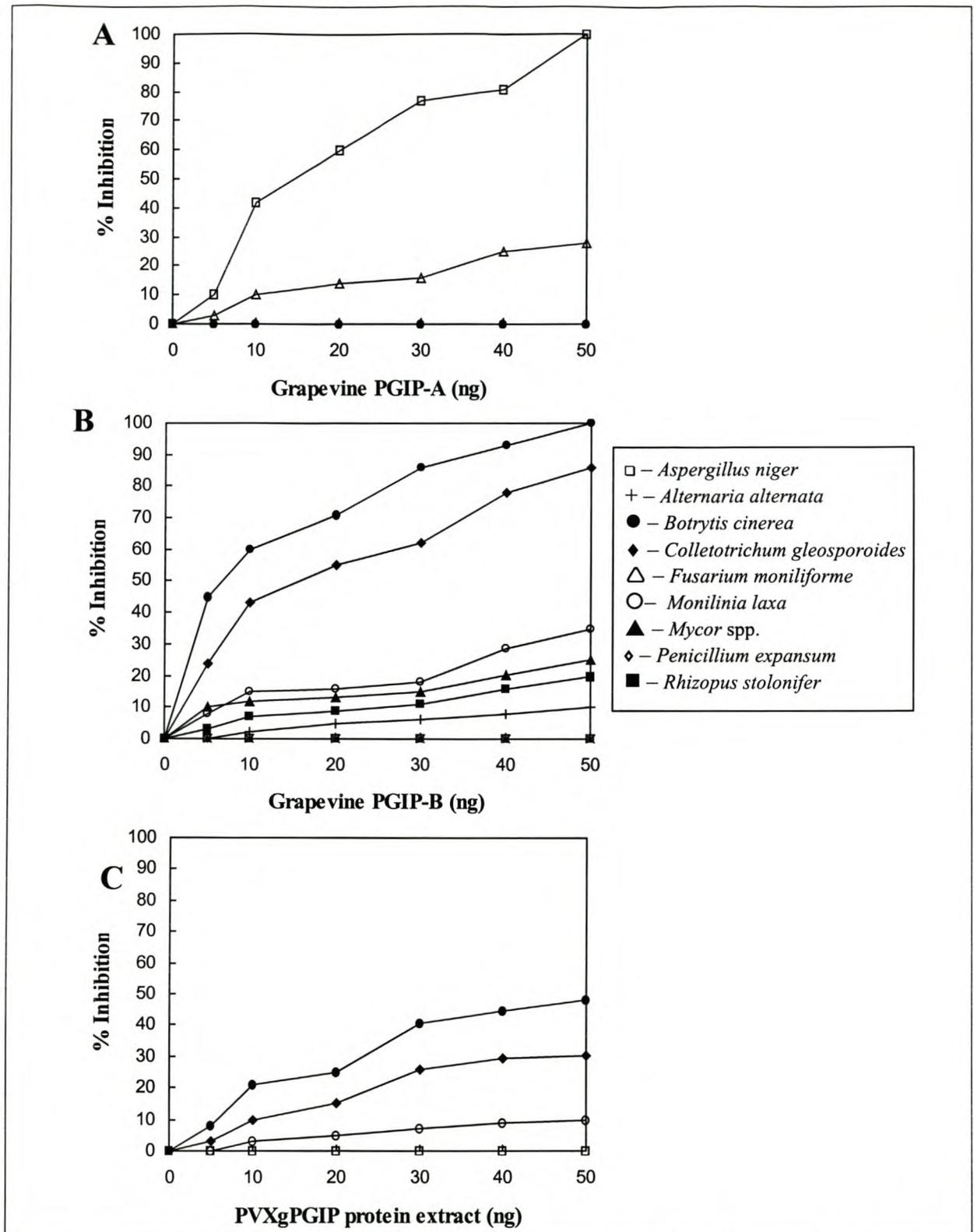


Fig. 4.5 Inhibitory PG activity of increasing amounts of grapevine PGIP-A (**A**), grapevine PGIP-B (**B**) and crude potato virus X-infected *Nicotiana benthamiana* plants (**C**), against a number of polygalacturonases (PGs) from fungal pathogens. Enzymes used: 0011 units of homogeneous *Aspergillus niger* PG, 0009 units of a crude PG preparation from *Alternaria alternata*, 0014 units of a crude PG preparation from *Botrytis cinerea*, 0012 units of a crude PG preparation from *Colletotrichum gleosporoides*, 0015 units of PG purified from *Fusarium moniliforme*, 0008 units of a crude PG preparation from *Monilinia laxa*, 0005 units of a crude PG preparation from *Mycor spp.*, 0004 units of a crude PG preparation from *Penicillium expansum* and 0007 units of a crude PG preparation from *Rhizopus stolonifer*. One unit of PG is the amount of enzyme producing 1 microequivalent of reducing groups per min at 30°C. Each data point is the average of three determinations. Standard deviations (not indicated for clarity) were always lower than 5%.

Inhibition specificities of PGIP-A and PGIP-B

The ability of PGIP-A and PGIP-B to inhibit PGs from different fungal pathogens was investigated to determine differences in target PG selectivity (Fig. 4.5). In contrast to the purified PG from *A. niger* and *F. moniliforme*, the crude protein extract from *B. cinerea* contained several PG isozymes as well as various unidentified proteins. Similarly, the crude protein extract from *A. alternata*, *C. gleosporoides*, *M. laxa*, *Mycor* spp., *P. expansum* and *R. stolonifer* also contained several PG isozymes and a number of unidentified proteins. PGIP-A (50 ng) was able to completely inhibit PG from *A. niger*, partially inhibit PG from *F. moniliforme* by 25% and showed no inhibition towards PGs from *B. cinerea* (Fig. 4.5A). PGIP-A showed no inhibition towards the other six PGs (data not shown). PGIP-B (50 ng) was able to completely inhibit PGs from *B. cinerea*, show 85% inhibition towards PGs from *C. gleosporoides*, but did not inhibit PGs from *A. niger*, *F. moniliforme* or *P. expansum*. PGIP-B was able to partially inhibit PG preparations from *A. alternata* (10% inhibition), *M. laxa* (35% inhibition), *Mycor* spp. (25% inhibition) and *R. stolonifer* (20% inhibition) (Fig. 4.5B). These data demonstrate the differences in PG-inhibition specificities between PGIPs from the same plant species.

The specificity of the crude grapevine PVXgPGIP1 extract from infected *N. benthamiana* plants, revealed a similar specificity to that of PGIP-B. No inhibition of PGs from *A. niger*, *F. moniliforme*, *Mycor* spp., *P. expansum* or *R. stolonifer* was observed, whereas a 50%, 30% and 10% inhibition was shown towards *B. cinerea*, *C. gleosporoides* and *M. laxa*, respectively (Fig. 4.5C).

Kinetics of inhibition of PGIP-B isomers

The interaction between specific plant PGIPs and fungal PGs differs in terms of their inhibition kinetics due to different molecular associations. A crude PG preparation from *B. cinerea* was assayed in the absence and presence of varying concentrations of purified PGIP-B over a range of polygalacturonic acid (PGA) concentrations. Lineweaver-Burk double reciprocal plot analysis showed that an increase in inhibitor resulted in a corresponding decrease in V_{max} , but did not alter the K_m , indicating a non-competitive inhibition of *B. cinerea* PGs by grapevine PGIP-B with a K_i of 0.033 (Fig. 4.6).

Inhibitory stability of PGIP-B

Aliquots of purified PGIP-B were incubated for 15, 30, 45 and 60 min at various temperatures in order to test the stability of its activity against *B. cinerea* PGs. The inhibitory activity of PGIP-B decreased slightly when treated at 65°C for 15 and 30 min, but dropped drastically to 30% inhibition after 45 min and completely lost activity after 60 min at 65°C (Fig. 4.7A). There was no inhibitory activity following heating at 95°C for 15, 30, 45 and 60 min, respectively.

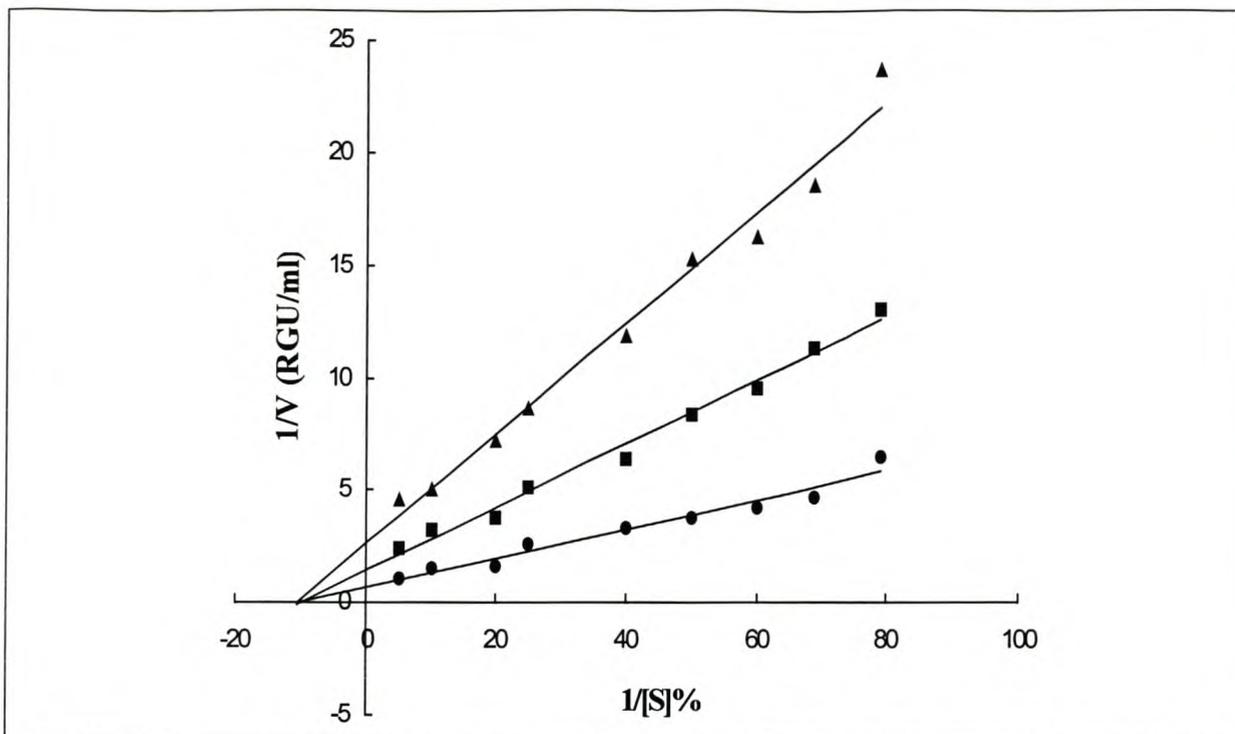


Fig. 4.6 Lineweaver-Burk double reciprocal plot analysis of the reaction rates at different substrate concentrations was used to determine the kinetics of inhibition of *Botrytis cinerea* PGs by grapevine PGIP-B. Assay analysis were done at different substrate concentrations in the absence (●) and presence of 0.005 mg ml⁻¹ (■) and 0.01 mg ml⁻¹ (▲) of grapevine PGIP-B.

The ability of PGIP to inhibit *B. cinerea* PGs at various pHs was measured in sodium acetate buffer (Fig. 4.7B). The results indicate that the highest inhibition of PG activity occurred between pH 4.5 to 6.0. Inhibitory activity decreased to 15% inhibition above pH 6.0.

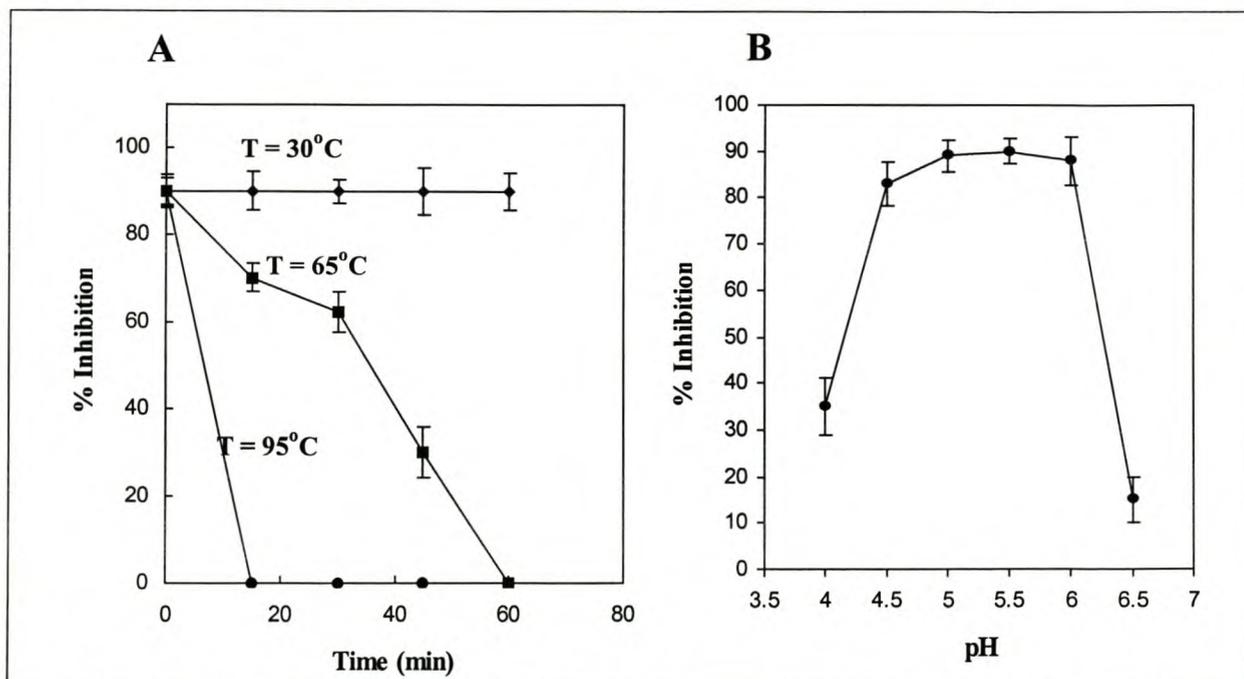


Fig. 4.7 The relative stability of grapevine PGIP-B with respect to temperature (A) and pH (B) was examined. Aliquots of PGIP-B were incubated at various temperatures for the indicated times. The effect of pH on the PGIP activity was tested over a pH range from 4 to 6.5. Error bars represent standard errors of the means. Each data point is the mean of three replicates.

4.4 DISCUSSION

The isolation and characterisation of the *pgip1* gene from grapevine (De Ascensao et al. 2001) indicated the presence of PGIPs in this species. In order to gain more insight into their inhibitory abilities and to further investigate their *in vivo* roles, it was essential to purify and characterise PGIPs from grapevine. In this study, the purification procedures used resulted in the isolation of two grapevine PGIP isomers, designated PGIP-A and PGIP-B. These isomers were characterised further and also compared to crude PGIP extracts from *N. benthamiana* which were infected with the overexpressed grapevine *pgip1* gene on a PVX vector.

Grapevine PGIP-A, with a molecular mass of 39 kDa, was purified partially and confirmed as a PGIP by Western blot analysis (Fig. 4.4A). Grapevine PGIP-B was shown to contain two polypeptides with molecular masses of 44.5 and 42 kDa (Fig. 4.2A). The latter, however, yielded a more intense PGIP-specific signal in Western blot analysis (Fig. 4.4B). Similarly, Western blot analysis of the PVXgPGIP1 extract from the infected *N. benthamiana* plants demonstrated the presence of a PGIP-specific signal with a molecular mass of approximately 42 kDa (De Ascensao et al. 2001).

Enzymatic N-deglycosylation of PGIP-B gave rise to three polypeptides ranging from 41 to 37 kDa in molecular mass. This is very similar to the predicted molecular mass of the isolated *pgip1* gene product, suggesting that differential glycosylation accounted for the heterogeneity in molecular mass. The molecular masses are also in good agreement with the molecular masses of purified PGIPs from a number of other plant genera (Cervone et al. 1987; Johnston et al. 1993; Stotz et al. 1993, 1994; Favaron et al. 1994; Yao et al. 1995). O-linked deglycosylation did not yield a further reduction in size of any of the polypeptides, suggesting that PGIP-B does not contain O-linked oligosaccharides or, if they are present, that they were not accessible to O-glycosidase. Furthermore, the predicted pI of the mature protein was calculated to be 8.52 and is in agreement with the basic nature of grapevine PGIP-B (pI 8.78). Other PGIPs also have basic pIs, with the exception of pear PGIP which has isoforms with pIs of 4.5, 6.6 and 7.7, respectively (Abu-Goukh et al. 1983), and apple PGIP, which has an acidic pI of 4.6 (Yao et al. 1995).

A low inhibitory activity against PGs from *A. alternata*, *Mycor* spp., *P. expansum*, *R. stolonifer* and to some extent that of *M. laxa*, was observed and could be due to a limited capacity of grapevine PGIPs to interact with PGs in those preparations. The proportion of activity in the culture medium due to endoPGs or exoPGs could not be established, as the patterns of pectin-degrading enzymes of these fungi were not investigated. Taking into account that PGIPs are typically effective against endoPGs, the possibility that a high proportion of exoPGs was present in those extracts cannot be excluded. Evolutionary adaptations of PGIPs to different forms of PG could be attributed to deviations in PG-PGIP interactions. These deviations may be encountered due to spatial and temporal variation in superiority of different fungal

pathogens, or due to evolutionary response by PGs to effective inhibition (Stotz et al. 2000).

PGIPs differ in PG-target selectivity within and among plant species. It was found that even though pear, bean and tomato PGIPs are relatively divergent on the basis of phylogenetic comparisons, all three inhibited PG activity from *B. cinerea*. However, they differ in their ability to inhibit PGs from *A. niger* and *F. moniliforme* (Stotz et al. 2000). Individual plant PGIPs can also selectively inhibit particular fungal PG isoforms, as was shown by substrate overlays where pear PGIP preferentially inhibited certain PG isozymes from culture filtrates of *B. cinerea* (Sharrock and Labavitch 1994). Both grapevine PGIP isomers isolated and characterised differed in their ability to inhibit fungal PGs. Most notably, PGIP-B strongly inhibited PGs from *B. cinerea* and a few others, but not PGs from *A. niger*. PGIP-A, however, exhibited strong inhibition against *A. niger*, but not against *B. cinerea*. Furthermore, the transiently expressed grapevine *pgip1* gene in *N. benthamiana* yielded PGIP activities similar to that shown by the purified PGIP-B isomer.

Grapevine PGIP-B showed a non-competitive form of inhibition to *B. cinerea* PGs. Non-competitive inhibition requires binding of the inhibitor to a site on the PG molecule different from the active site. Since the PGIP-B shows inhibitory activity against PGs from more than one fungus, this could contribute to the observed differential inhibitory specificity of PGIP-B. PGIPs from tomato (Stotz et al. 2000), bean (Lafitte et al. 1984) and raspberry (Johnston et al. 1993) also act through a non-competitive form of inhibition, whereas pear PGIP shows a competitive form of inhibition (Abu-Goukh et al. 1983). Apple PGIP, on the other hand, exhibits a mixed-type inhibition (Yao et al. 1995). Since these studies used distinct PGs, differences in inhibition kinetics may depend on the target PG rather than on PGIP properties.

The molecular mass and pI of the native PGIP-B are similar to that of the predicted grapevine PGIP1, which is encoded by the grapevine *pgip1* gene. The molecular mass of glycosylated PGIP-B is identical to that of the PVX-expressed *pgip1* crude extract, and the presence of a PGIP-specific signal of 42 kDa was identified by Western blot analysis for both protein extracts. Furthermore, since both PGIP-B and the PVX extract show similar inhibitory specificities, one could conclude that the previously isolated *pgip1* gene encodes the 42 kDa polypeptide found in PGIP-B.

Genomic DNA analysis indicated that *V. vinifera* may have at least two or a small family of homologous PGIP-encoding genes, with the isolated *pgip1* gene being part of this family (De Ascensao et al. 2001). The total grapevine PGIP activity may therefore comprise polypeptides encoded by different *pgip* genes. Our data showed that the purified PGIP-B could correspond to the isolated *pgip1* gene, and that a second, as yet unidentified, *pgip* gene probably encodes PGIP-A.

In conclusion, plant PGIPs are defence-related proteins that have evolved different specificities and expression profiles to inhibit PG activity from different fungal pathogens. A purification procedure based on ion exchange, affinity and FPLC

resulted in the identification of two grapevine PGIPs (PGIP-A and PGIP-B) with different specificities. The inhibitory activity of total grapevine PGIP in *V. vinifera* may be due to the presence of different molecules with narrow specificities, as shown with PGIP-A and PGIP-B. From the results obtained with the purified peptides and the crude PGIP1 extracts from PVX-infected tobacco, it seems that PGIP-B is probably encoded by the *pgip1* gene. It would now be of importance to further purify PGIP-A and to isolate other members of the *V. vinifera* PGIP gene family. Similarly, it would be of significant importance to further separate, purify and identify the two polypeptides present in PGIP-B. These studies will undoubtedly provide information about the structural requirement of PG-PGIP interactions.

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CHAPTER 5

RESEARCH RESULTS

Transgenic expression of the grapevine *pgip1* gene in tobacco increases resistance to *Botrytis cinerea*

To be submitted to *TRANSGENIC RESEARCH*

RESEARCH RESULTS

TRANSGENIC EXPRESSION OF THE GRAPEVINE *PGIP1* GENE IN TOBACCO INCREASES RESISTANCE TO *BOTRYTIS CINEREA*

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ABSTRACT

The grapevine *pgip1* gene from *Vitis vinifera* L., encoding a polygalacturonase-inhibiting protein (PGIP), was expressed under the control of the Cauliflower mosaic virus (CaMV) 35S promoter in tobacco plants via *Agrobacterium tumefaciens*-mediated transformation. Transgenic tobacco plants expressing the grapevine PGIP (gPGIP1) were used to demonstrate the effectiveness of this inhibitor against fungal polygalacturonases (PGs) and to investigate whether gPGIP1 influences disease development. The polymerase chain reaction (PCR) and Southern blot analysis were used to identify those independent transgenic lines that had successfully integrated the grapevine *pgip1* gene and to determine the transgene copy number. Northern blot analysis identified 19 primary transgenic plants expressing *pgip1*. Crude PGIP extracts from the transgenic tobacco lines inhibited PGs from *Botrytis cinerea* and *Colletotrichum gleosporoides*, but not PG from *Aspergillus niger*. Leaves from either untransformed control plants, transgenic lines showing high and low PG inhibition, or from transgenic plants, where *pgip1* expression was silenced, were inoculated with *B. cinerea* and scored for disease symptoms. An approximated reduction of 45% in the size of necrotic lesions on the leaves of transgenic plants relative to the control and the non-expressing transgenic leaves was observed. The levels of inhibition activity could be correlated with the various lesion sizes obtained. Heterologous expression of gPGIP1 has demonstrated that PGIP inhibition of fungal PGs slows disease development of *B. cinerea in planta* and that the previously purified grapevine PGIP-B isomer corresponds to the inhibition profile of the gPGIP1.

Key words: *Botrytis cinerea* – endopolygalacturonases – polygalacturonase-inhibiting proteins – *Vitis vinifera*

5.1 INTRODUCTION

An important part of the implementation of strategies to increase host plant resistance by genetic transformation is the discovery and characterisation of plant antifungal proteins and the isolation of their encoding genes. Available evidence indicates that many of these proteins are induced by the presence of fungal pathogens and thereby

function as a natural plant defence mechanism against pathogens (reviewed in Yun et al. 1997). In addition, several experiments have been reported where transgenic plants that overproduce these proteins exhibited enhanced resistance to fungal diseases (Alexander et al. 1993; Zhu et al. 1994; Jach et al. 1995; Powell et al. 2000).

Plant cell walls are at the forefront of plant-pathogen interactions. As the first barrier encountered by most plant pathogens, they must be degraded in order to allow penetration and colonisation. Plant cell walls contain various polymers that may serve as substrates to the numerous enzymes secreted by microbial pathogens providing them with nutrients (Walton 1994). Endo- and exo-polygalacturonases (PGs), pectate lyases and pectin esterases are among the cell wall-degrading enzymes produced by phytopathogens. They are also among the first enzymes secreted during pathogenesis, thereby indicating a central role.

PG-encoding genes are organised into complex multigene families. The number of genes present within these families varies with the specificity of the interaction, being higher in pathogens such as *Botrytis cinerea*, which is pathogenic on numerous plant species and plant tissues. In pathogens with a restricted host range, such as *Colletotrichum lindemuthianum* that only attacks bean plants, only two PG-encoding genes are present (Centis et al. 1997; Wubben et al. 1999).

Inhibitors of cell wall-degrading enzymes have been proposed to be part of the plant defence system that limits the development of disease symptoms caused by microbial pathogens (Cervone et al. 1989; Stotz et al. 1993; 1994). These polygalacturonase-inhibiting proteins (PGIPs) are glycoproteins located in plant cell walls that specifically inhibit fungal PGs. PGIPs have been identified in all dicotyledonous plants analysed thus far (Hahn et al. 1989), as well as in some monocotyledonous plants (Favaron et al. 1993; 1997; Machinandiaarena et al. 2001), and genes that encode these proteins have been cloned from several species (Toubart et al. 1992; Stotz et al. 1993; 1994; Favaron et al., 1994; Ramanathan et al. 1997; Yao et al. 1999; De Ascensao et al. 2001a, submitted).

The role for PGIPs in plant defence against fungal pathogens has been shown (Cervone et al. 1997; De Lorenzo 1997). Formation of the PG-PGIP complexes *in vitro* resulted in a change in the balance between the release of elicitor-active oligogalacturonides and the depolymerisation of the active oligogalacturonides into inactive molecules favouring the accumulation of elicitor-active molecules (Cervone et al. 1989). Furthermore, PGIPs are structurally related to several products of resistance genes (Jones and Jones 1997) and belong to a super-family of leucine-rich repeat (LRR) proteins specialised for the recognition of foreign molecules and the rejection of pathogens (Cervone et al. 1997). Also, transgenic tomato plants expressing the pear PGIP demonstrated that PGIP inhibition of fungal PGs slows the expansion of disease lesions and associated tissue maceration (Powell et al. 2000), confirming the contribution of PGIP to plant defence mechanisms.

A *pgip* gene from *Vitis vinifera* L. has recently been cloned, characterised and designated grapevine *pgip1* (De Ascensao et al. 2001a, submitted). It was shown to be part of a multigene family that encode proteins with strong inhibitory activities against PGs from *B. cinerea*, amongst others. This was also confirmed when the isolated grapevine *pgip1* gene was expressed transiently in *Nicotiana benthamiana* with potato virus X (PVX) as a vector. Crude protein extracts of the infected plants showed inhibitory activity against *B. cinerea* PGs. From purification and characterisation experiments, two PGIP isomers were isolated from grapevine berries and shown to have different inhibitory specificities. PGIP-A showed strong inhibitory activity against *Aspergillus niger* PG, but was unable to inhibit *B. cinerea* PGs. PGIP-B was able to strongly inhibit PGs from both *B. cinerea* and *C. gleosporoides*, yet showed no inhibition towards *A. niger* PG (De Ascensao et al. 2001b, submitted).

To assess the effectiveness of the grapevine PGIP1 (gPGIP1) *in planta* against *B. cinerea* during disease development, tobacco plants were transformed with a plant expression vector harbouring the grapevine *pgip1* gene under the transcriptional regulation of a constitutive Cauliflower mosaic virus (CaMV) 35S promoter. Leaves of independent transgenic lines showing *pgip* mRNA transcript levels were inoculated with the fungal pathogen *B. cinerea*. Differences in the expansion of disease lesions and the associated tissue maceration were investigated on transgenic and control plants. Results showed that initial leaf necrosis occurred in tissues of transgenic plants, but that the expanding area of tissue maceration was reduced in comparison with the infections of control plants.

5.2 MATERIALS AND METHODS

Fungal material

Cultures of *C. gleosporoides* and *B. cinerea* were obtained from the Department of Plant Pathology, University of Stellenbosch and were maintained on potato dextrose agar (PDA) medium at 23°C. Mycelium discs were subsequently taken from the edge of the culture and used for the preparation of PGs.

Vector constructs and plant transformation

Plasmid pPGIP^{G3}, containing the genomic clone of the grapevine *pgip1* gene (De Ascensao et al. 2001a, submitted), was used as template in a polymerase chain reaction (PCR) to amplify a DNA fragment corresponding to the entire open reading frame of the gene. Oligonucleotide primers 5'-AGGACAGAGAAATGG-AGACTTCAAAC-3' and 5'-AGTCAGATCTGAGCCGCTCACTTGC-3' (*Bgl*II site underlined) were used to amplify the 1002 bp fragment. The fragment was cloned into pGEM-T Easy vector (Promega, Madison, WI) and confirmed by sequencing. The grapevine *pgip1* insert was subcloned into the *Pst*I and *Bam*HI site of pBluescript

SK (+) (Stratagene, La Jolla, CA) for mobilisation into a plant expression vector. The binary vector pBI121 (Jefferson et al. 1987) was prepared to receive the grapevine *pgip1* fragment by digestion with *Sma*I and *Sac*I to liberate the β -glucuronidase gene (GUS). The *pgip1* fragment was excised from pBluescript SK (+) with *Eco*RV and *Sac*I and subcloned into the *Sma*I-*Sac*I sites of pBI121. The recombinant clone was designated pBlgPGIP1. *Escherichia coli* DH5 α , containing pBlgPGIP1, was used in triparental mating with *Agrobacterium tumefaciens* strain EHA105 and *E. coli* HB101 containing the helper plasmid pRK2013 (Armitage, 1988).

Tobacco plants (*Nicotiana tabacum* cv Petit Havanna SR1) were transformed with *A. tumefaciens* EHA105 harbouring the pBlgPGIP1 construct by the leaf disc method, with regeneration under kanamycin selection (100 mg/L) (Gallois and Marinho, 1995), and several primary transgenic lines were recovered and acclimatised for growth in a glasshouse.

PCR and Southern blot analyses of transgenic lines

Transgene integration was confirmed by PCR analysis, with the grapevine *pgip1* gene-specific primers used for amplification of the open reading frame from the genomic clone. All PCR analyses were conducted in a Biometra Trio-thermoblock automated temperature cycler. Genomic DNA (1 μ g) was combined with 0.25 μ M of each primer, 10 mM dNTPs and 0.5 U of Taq DNA polymerase (Roche Molecular Biochemicals) in 1 \times PCR buffer adjusted to a final volume of 50 μ l. PCR cycle conditions were as follows: denaturation at 95°C for 3 min, annealing at 58°C for 40 sec and elongation at 72°C for 40 sec; a total of 30 cycles were used followed by a final elongation time of 5 min at 72°C. PCR products were separated by agarose gel electrophoresis and visualised by ethidium bromide staining under UV light.

Genomic DNA was extracted from 0.1 g of tobacco leaves according to McGarvey and Kaper (1991). For Southern blot analysis, genomic DNA from the transgenic lines (10 μ g) was digested with *Eco*RV, separated on a 0.8% (w/v) agarose gel and transferred to positively charged Nylon membranes according to Sambrook et al. (1989). Prehybridisation and hybridisation reactions were carried out at 42°C in DIG Easy Hyb (Roche Molecular Biochemicals). The membranes were prehybridised for 2 h and then hybridised for 16-20 h. The grapevine *pgip1* gene was used as a probe and PCR-digoxigenin-labelled according to the DIG System User's Guide (Roche Molecular Biochemicals). Membranes were washed twice at room temperature in 2 \times SSC and 0.1% SDS for 5 min each and twice at 68°C in 0.5 \times SSC and 0.1% SDS for 15 min each. Chemiluminescence detection of nucleic acids was done using CSPD as substrate (Roche Molecular Biochemicals).

Northern blot analyses of pgip1 transcript levels

Total RNA was extracted from leaves from glasshouse-kept transgenic plants using Trizol Reagent (GibcoBRL, Life Technologies) according to the manufacturer's specificities. Total RNA (10 µg per lane) was size fractionated by electrophoresis on a 1% formaldehyde agarose gel and blotted to Hybond-N nylon membranes using standard techniques as described by Sambrook et al. (1989). The membranes were prehybridised at 50°C for 4 h in DIG Easy Hyb (Roche Molecular Biochemicals). The membranes were then hybridised in the same solution with the addition of the PCR digoxigenin-labelled grapevine *pgip1* probe. After hybridisation, membranes were washed twice in 2 × SSC and 0.1% SDS at room temperature for 15 min and twice in 0.5 × SSC and 0.1% SDS at 68°C for 15 min. Chemiluminescence detection of nucleic acids was done using CSPD as substrate (Roche Molecular Biochemicals).

Preparation of fungal PGs

The PG from *A. niger* that was used was a commercial preparation (Sigma). Crude PG preparations from *C. gleosporoides* and *B. cinerea* were obtained by growing the fungus in a citrate phosphate buffer (pH 6.0) supplemented with 1% (w/v) citrus pectin. The medium contained 2 mM MgSO₄·7H₂O, 0.6 µM MnSO₄·H₂O, 25 mM KNO₃, 30 µM ZnSO₄·7H₂O, 0.9 µM CuSO₄ and 65 µM FeSO₄. Cultures were incubated on a rotary shaker in the dark at 23°C for 10 days. One flask was harvested each day from day 3 to 14 by suction filtration through a Whatman No. 1 filter paper. The filtrates that contained the extracellular polygalacturonase (PG) enzymes were passed through 0.22 µm filter units and stored at 4°C before screening for PG activity using the agarose diffusion assay (Taylor and Secor 1988). Filtrates that showed the highest PG activity were subjected to a 0-80% ammonium sulphate treatment to precipitate the PG proteins, which were subsequently resuspended in 40 mM ammonium acetate buffer (pH 5.0). The PG extracts were checked for recovery of PG activity using the agarose diffusion assay.

Preparation of crude gPGIP1 extracts from transgenic plants

Independent transgenic tobacco lines and untransformed control tobacco plants were analysed for the presence of PGIP by inhibition of PG activity by the agarose diffusion assay. Finely crushed leaf tissue (0.4 g) was homogenised in extraction buffer, 0.1 M sodium acetate buffer (pH 6.0), 10 mM β-mercaptoethanol and 1% (w/v) PVP-40. The homogenate was centrifuged at 10,000 g for 15 min, followed by two more extractions in extraction buffer without PVP-40, each followed by centrifugation. The remaining insoluble tissue was resuspended in 2 volumes of 50 mM sodium acetate buffer (pH 5.2) also containing 1 M NaCl and stirred for 1 h at 4°C. The insoluble debris was removed by centrifugation at 10,000 g for 20 min. The proteins precipitating at 80% (w/v) saturated ammonium sulphate were collected, resuspended in 20 mM sodium

acetate (pH 5.2) and dialysed extensively at 4°C against 20 mM sodium acetate (pH 5.2). The protein concentration was determined according to Bradford (1976), using a Bio-Rad protein assay kit and bovine serum albumin (BSA) as a standard. The dialysed samples were assayed for PG inhibition by the agarose diffusion assay using PGs from *A. niger*, *B. cinerea* and *C. gleosporoides*. Equal amounts of total protein were used in the assay.

PGIP activity assay

Inhibition of PG activity was determined by the agarose diffusion assay (Taylor and Secor 1988) in the presence of the substrate polygalacturonic acid (0.01%). The diameter of clearing zones as a result of PG hydrolysis of the glycosidic bonds in the pectic substrate was compared with those formed when inhibitor was added, typically resulting in a reduction in the zones. Zones were visualised by staining with 0.05% Ruthenium red (Sigma).

Inoculation of detached leaves and disease assessment

Pathogenic cultures of *B. cinerea* were maintained on apricot medium [mixture of 50% (v/v) apricot pulp and 50% (v/v) water, adjusted to pH 4.0 and solidified with 2% (w/v) agar] in a dark growth chamber at 23°C until sporulation occurred. Spore inocula were harvested from fungal mycelia incubated on apricot medium plates for 12 days by rinsing with sterile distilled water. Spore viability was evaluated by plating a serial dilution of the spore suspension on PDA and 1% (w/v) water agar (Phytagar, Difco). Plates were placed in a dark growth chamber at 23°C for 24 h, after which the viability of the spores was quantified by the number of spores that had germinated.

Fully developed leaves of 6- to 8-week-old glasshouse plants from transgenic and untransformed control plants were used for inoculation. Leaves were detached with a scalpel blade and placed in a Magenta box, with the cut end embedded in 0.8% (w/v) water agar. Two 2 µl droplets containing 5×10^3 spores were applied at the adaxial side of the leaves without wounding the surface. The Magenta box was sealed to maintain 100% humidity and placed in a growth room at 23°C, where disease symptoms were scored 3 days after inoculation by measuring the diameter of the lesions as well as evaluation of the lesions compared to those on the untransformed tobacco plants. The inoculations were repeated on three separate occasions. Statistical analysis of the results was carried out with a two-way ANOVA.

5.3 RESULTS

Construction of a plant expression cassette and subsequent tobacco transformations

The complete coding region of the grapevine *pgip1* gene isolated by De Ascensao et al. (2001a) was inserted downstream of the CaMV 35S promoter in a plant expression

vector. The construct (Figure 5.1) was mobilised into *A. tumefaciens* and subsequently into tobacco via leaf disk infection.

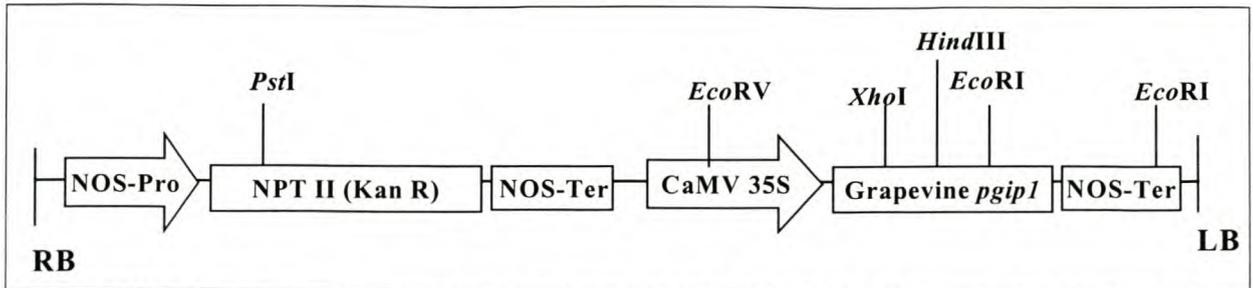


Figure 5.1 Schematic representation of the binary plasmid pBIgPGIP1. The binary vector pBI121 was digested with *Sma*I and *Sac*I to liberate the β -glucuronidase (GUS) gene. The grapevine *pgip1* gene was subsequently cloned into the corresponding *Sma*I-*Sac*I sites of pBI121. The resulting recombinant plasmid was used for *Agrobacterium tumefaciens* transformation. Restriction sites are shown. Abbreviations: RB/LB = right/left T-DNA border sequence; NOS-Pro/Ter = nopaline synthase promoter/terminator; CaMV 35S = cauliflower mosaic 35S promoter.

Analyses of transgenic plants by PCR and Southern blot

PCR and Southern blot analyses (Figure 5.2) were used to identify independent transgenic tobacco lines. PCR analyses performed on 46 independent primary transgenic tobacco lines yielded a 1002 bp fragment corresponding to the size of the grapevine *pgip1* gene in all 46 transgenic plants (Figure 5.2A). PCR analysis on untransformed tobacco plants did not amplify any DNA (Figure 5.2A, lane C).

The transgenic status and the number of T-DNA copies inserted into the genome of the plants were confirmed by Southern blot analysis (Figure 2B). The transgene copy number as estimated by Southern blot ranged from 1 to 5 copies (Figure 2B).

Expression of grapevine pgip1 gene in transgenic tobacco

Expression of *pgip1* in the transgenic lines was analysed by Northern blot analysis using grapevine *pgip1* gene as a probe. Hybridisation analysis of total RNA from leaf tissue demonstrated that grapevine *pgip* mRNA was expressed in 19 of the transgenic lines, whereas no grapevine *pgip1* transcript was detected in untransformed plants (Figure 5.3).

Inhibitory activities of gPGIP1 produced in transgenic tobacco lines

The ability of the gPGIP1 to inhibit PGs from different fungi was investigated. Crude PGIP extractions from leaves of the untransformed and the 19 transgenic tobacco lines expressing the grapevine *pgip1* were performed. Agarose diffusion assays showed that extracts from the transgenic tobacco plants tested contained an active PGIP that inhibited the PG activity from *B. cinerea* (Figure 5.4A) and from *C. gleosporoides* (Figure 5.4B), but not from *A. niger* (Figure 5.4C). An extract containing the same amount of total protein from leaves of an untransformed control tobacco plant did not show any inhibitory activity against the tested PGs. Different

levels of PG inhibition were found in protein extracts from the 19 transgenic tobacco plants against PGs from *B. cinerea* and *C. gleosporoides* (Table 5.1).

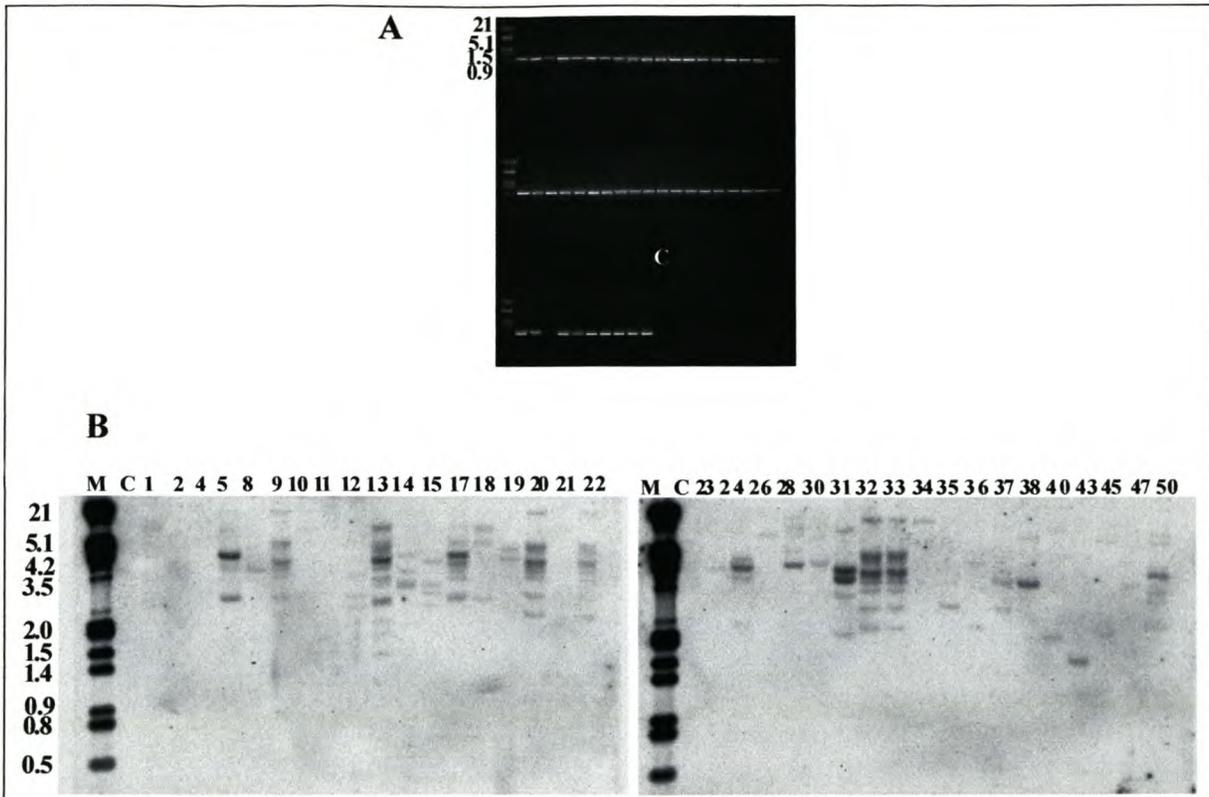


Figure 5.2 PCR and Southern blot analyses of primary transgenic tobacco plants. **A:** PCR analysis done on 46 independent transgenic tobacco lines using grapevine *pgip1* gene-specific primers led to 1002 bp fragment corresponding to the size of the grapevine *pgip1* coding region. PCR analysis done on untransformed tobacco plants did not result in any fragment (lane C). **B:** Southern blot analysis of genomic DNA from tobacco plants digested with *EcoRV* and hybridised with a digoxigenin-labelled 1002 bp fragment corresponding to the coding region of the grapevine *pgip1* gene. The numbers identify each independent transgenic plant tested. The marker lane (lane M) contains *EcoRI* and *HindIII* digested lambda DNA. Sizes of the standard DNA fragments are indicated in kb.

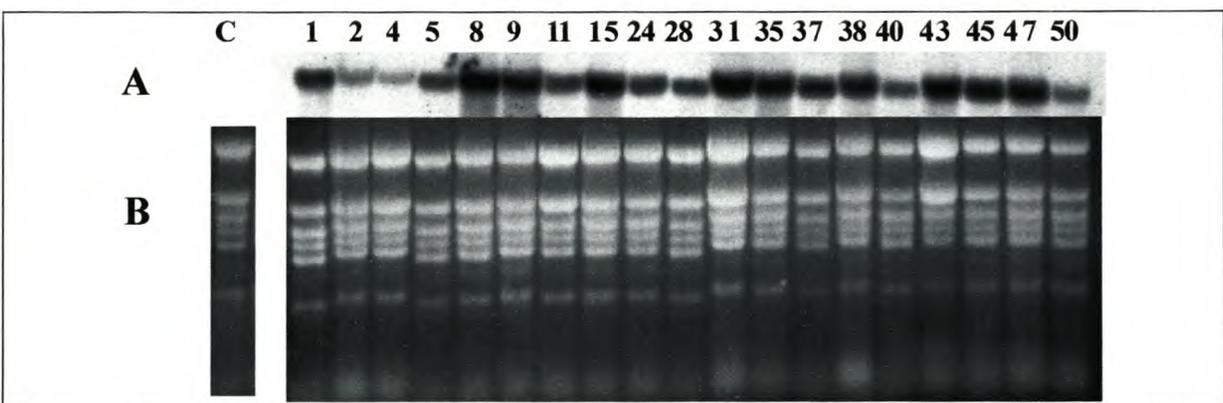


Figure 5.3 Northern blot analysis of the expression of grapevine *pgip1* transgene in independent tobacco lines. Total RNA was extracted from leaf tissue and probed with a digoxigenin-labelled 1002 bp fragment corresponding to the coding region of the grapevine *pgip1* gene as shown in A. Ethidium bromide staining of the formaldehyde agarose gel is shown in B. Numbers identify each transgenic plant that showed mRNA *pgip1* expression. Total RNA extracted from an untransformed tobacco plant is shown in lane C.

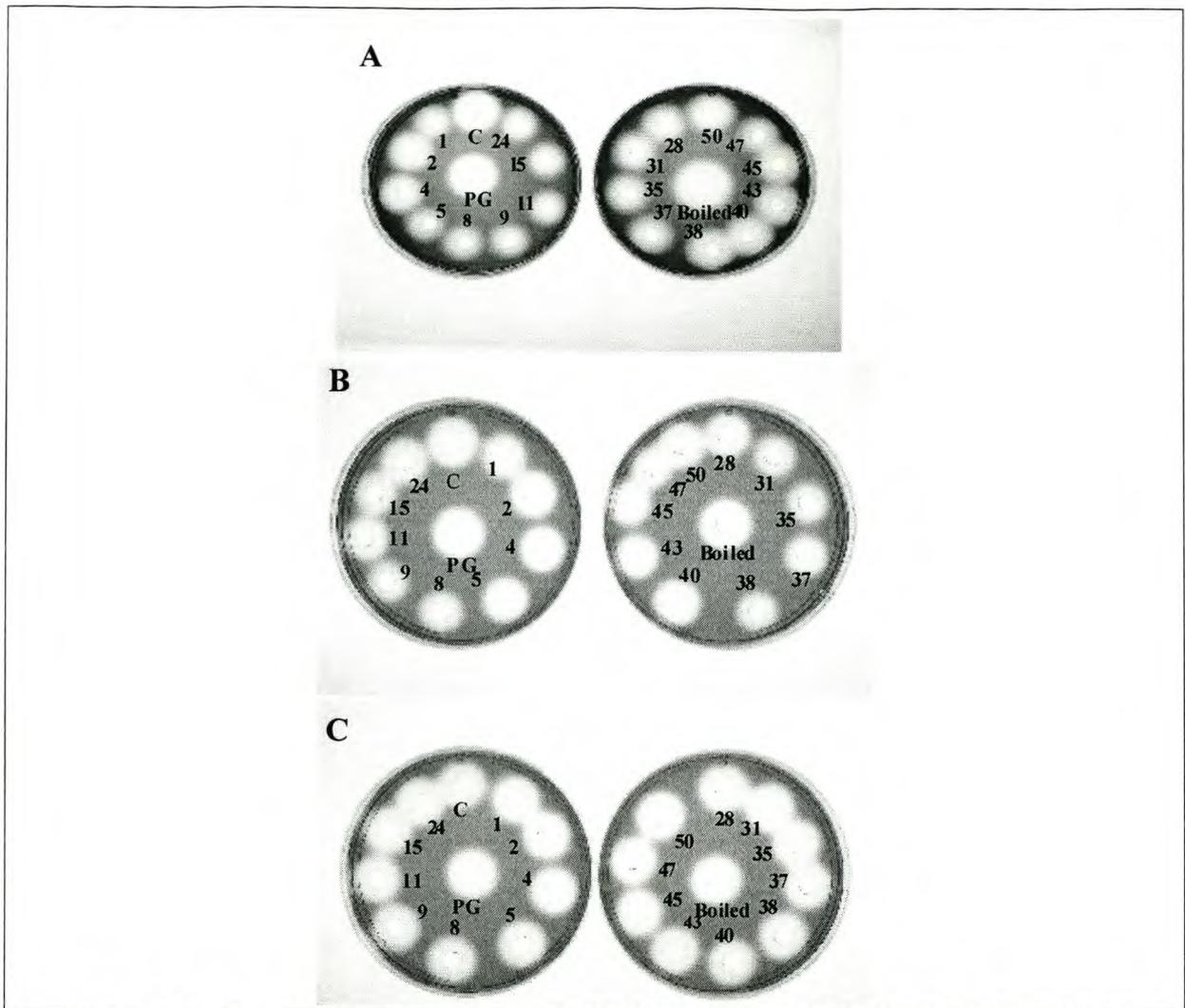


Figure 5.4 Inhibition studies of the expressed gPGIP1 against PGs from *Botrytis cinerea* (A), *Colletotrichum gleosporoides* (B) and *Aspergillus niger* PG (C). Inhibition studies were done using the agarose diffusion assay. Crude PGIP extraction (100 ng) from leaves of the untransformed (well C) and from the independent transgenic tobacco lines expressing the grapevine *pgip1* as determined by Northern blot analysis are identified by their respected numbers. A clear zone is indicative of PGs degrading the glycosidic bonds present in the polygalacturonic substrate (well PG). As a negative control, crude PGIP extraction from transgenic tobacco plant number 9 was inactivated by heating at 95°C for 15 min. This resulted in no inhibition of PG activity, causing a clear zone (Boiled).

Table 5.1 Levels of PG inhibition in protein extracts from the 19 transgenic tobacco plants^a against PGs from *Botrytis cinerea* and *C.olleotrichum gleosporoides*.

Plant lines ^b	Inhibition of PGs from <i>Botrytis cinerea</i> (%) ^c	Inhibition of PGs from <i>Colletotrichum gleosporoides</i> (%)
Untransformed	3	3
2, 4, 50	25 to 33	22 to 30
24, 28, 40, 43	45 to 59	40 to 55
1,5, 8, 11, 15, 37, 45, 47	60 to 68	60 to 68
9, 31, 35, , 38	70 to 82	70 to 79

^aCrude extracts were prepared from leaves.

^bNumbers identify each independent transgenic line tested.

^cPG inhibition was determined by the agarose diffusion assay. 100 ng of extract was used for each sample.

Plant infection studies

The 19 independent tobacco lines expressing grapevine *pgip1* were analysed for resistance to *B. cinerea*. Detached leaves inoculated with a spore suspension developed primary lesions at each inoculation site after overnight incubation, followed by secondary lesion formation that was measured after 3 days. The area of tissue necrosis (lesion diameter) was reduced on average by as much as 45%, compared to that of the untransformed control plants and tobacco plants that showed no *pgip1* expression (Figure 5.5).

In several transgenic lines, the observed area of chlorosis surrounding the necrotic zone appeared to be greatly reduced (Figure 5.6A) compared to that of the untransformed plant tissue (Figure 5.6B) and lines where gene silencing occurred (Figure 5.6C). The lesions on the transgenic lines were also less water-soaked and spreading than those on the control plants. Moreover, correlations were obtained between the observed lesion sizes and the measured PGIP inhibitory activities as reported in Table 5.1. A reduction in tissue necrosis was not so prominent in the independent transgenic tobacco lines showing inhibitory PGIP activities ranging from 25 to 45% (Figure 5.6D and E), whereas a good reduction in lesion expansion was seen in those tobacco lines showing inhibitory PGIP activities of 60% (Figure 5.6F). Inoculated lines that had the highest inhibitory activities also resulted in the strongest reduction in lesion sizes as well as reduced disease symptoms (Figure 5.7A-C). These results constitute the confirmed results from three independent repeats of the infections. The same tendencies were also observed when the inoculations were repeated and analysed at 7 days post inoculation (results not shown).

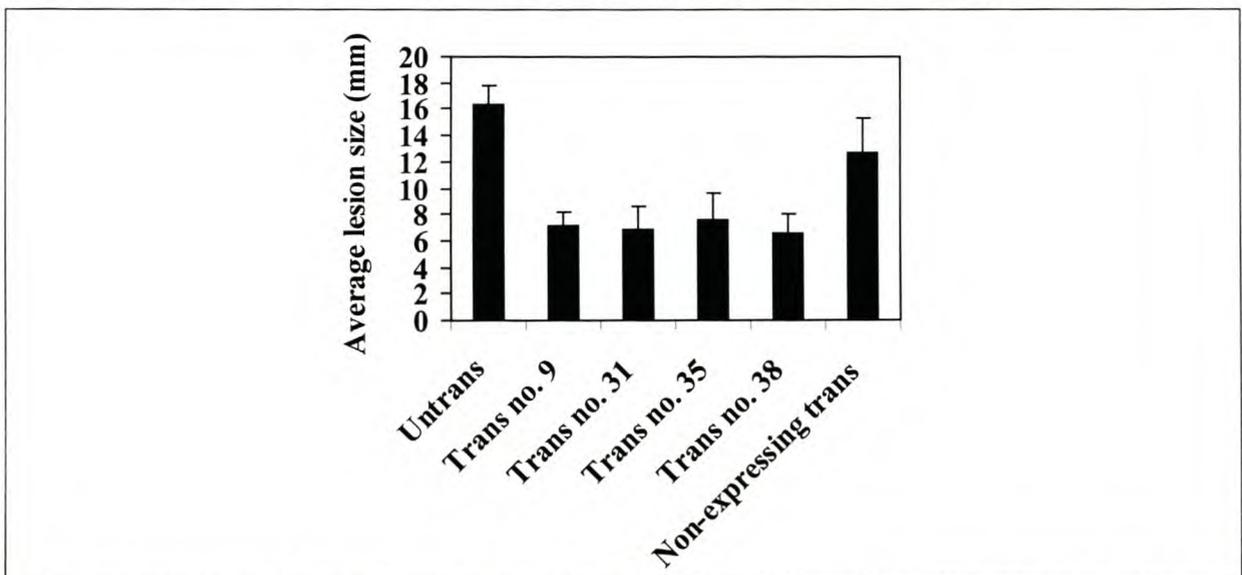


Figure 5.5 Analysis of untransformed, transgenic and non-expressing transformant plants for disease resistance. Shown is a quantitative analysis of the tolerance to *Botrytis cinerea* infection of four independent grapevine *pgip1*-expressing tobacco lines, no. 9, 31, 35 and 38 (Trans), relative to untransformed control plants (Untras) and transformed plants not showing grapevine *pgip1* expression (Non-expressing trans). The average lesion was determined 3 days after inoculation with 2- μ l drops containing 5^6 CFU/ml. Error bars represent standard deviation of the mean.

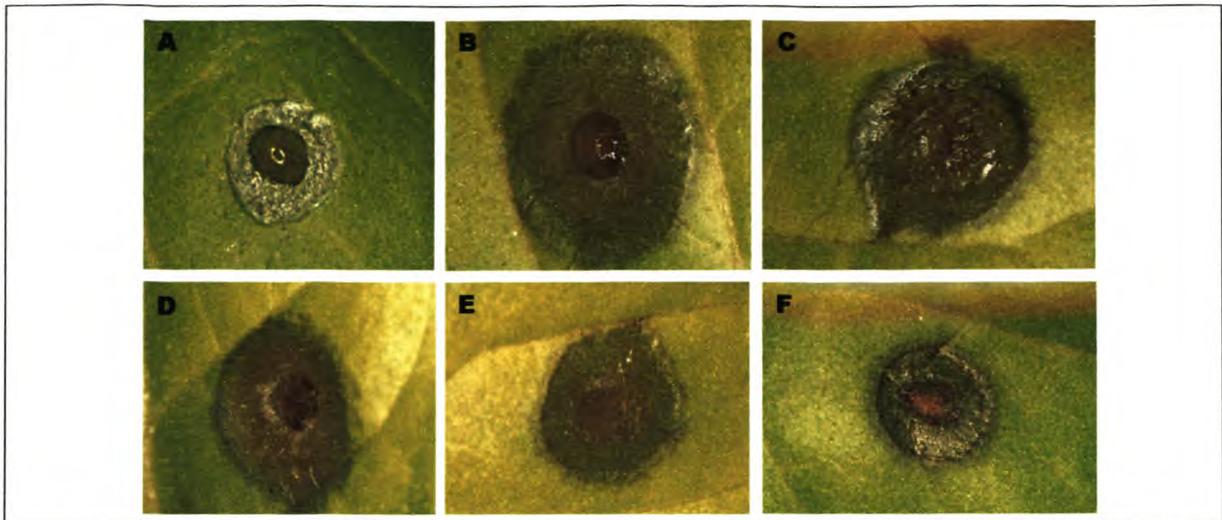


Figure 5.6 *Botrytis cinerea* colonisation of untransformed, transgenic and non-grapevine *pgip1*-expressing tobacco leaves. A tobacco leaf from a grapevine *pgip1* transformed line no. 9 (A) that showed high PG inhibition was compared to that of an untransformed control (B) and a non-expressing transformed tobacco line (C) showing disease symptoms development after 3 days post-inoculation. A tobacco leaf from three independent transgenic lines, no. 2 (D), no. 28 (E) and no. 45 (F) that showed 25%, 45% and 60% inhibition, respectively, displayed more prominent disease symptoms than that of transformed line no. 9 (A).

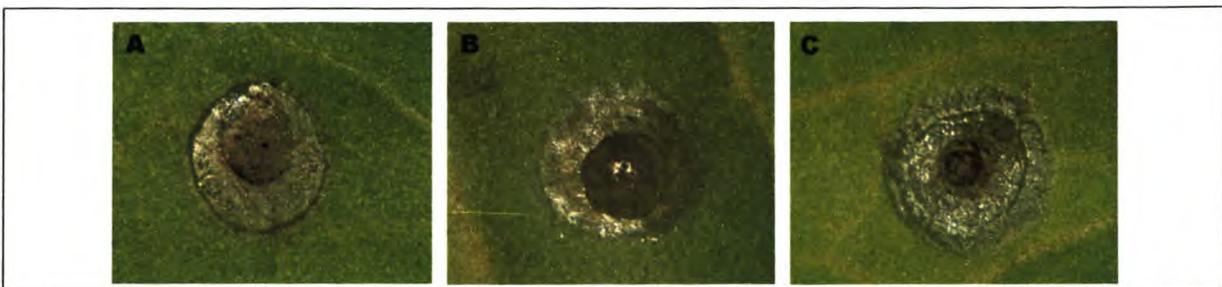


Figure 5.7 *Botrytis cinerea* colonisation of those independent transgenic lines that showed a high PG inhibition. A tobacco leaf from transgenic line no. 31 (A), 35 (B) and 38 (C) showed reduced disease symptoms after inoculation with *Botrytis cinerea* spores.

5.4 DISCUSSION

In this study, the grapevine *pgip1* gene from *V. vinifera* (De Ascensao et al. 2001a) was expressed at high levels in tobacco plants, resulting in the accumulation of gPGIP1 in several transgenic tobacco lines. The transgenic tobacco lines harbouring gPGIP1 were used to assess the effectiveness of this inhibitor against fungal pathogens, such as *B. cinerea*, in disease development. Powell et al. (2000) were the first to report that the heterologous expression of pear PGIP in tomato successfully conferred resistance against *B. cinerea* by targeting and inhibiting the tissue-macerating functions of the fungus.

The expression of grapevine *pgip1* was controlled by a constitutive rather than a pathogen-inducible promoter in tobacco plants to determine whether the heterologous gPGIP1 that accumulated prior to infection could contribute to pathogen defence. Expression of grapevine *pgip1* in leaf tissues of the transgenic tobacco lines resulted

in the production of heterologous gPGIP1. It was shown previously that grapevine *pgip1* belongs to a multigene family, indicating a probability that different grapevine *pgip* genes encode PGIPs with the ability to interact with and inhibit PGs from different fungal sources (De Ascensao et al. 2001a,b).

Purification of PGIPs from *V. vinifera* berries resulted in the presence of two PGIP isomers with different specificities, as described earlier. From previous data, we concluded that the isolated *pgip1* gene encodes for PGIP-B and that a second, as yet unidentified, *pgip* gene may encode PGIP-A. The heterologous gPGIP1 extracted from transgenic tobacco lines generated in this study showed similar inhibitory specificities to those of PGIP-B, namely that it was able to inhibit PGs from *B. cinerea* and *C. gleosporoides*, but not PG from *A. niger*. This further reinforces the possibility that the isolated *pgip1* gene encodes for PGIP-B. The observed differences in the levels of gPGIP1 that were found in the 19 independent transgenic tobacco lines probably constitute differences in processing or post-translation modifications of the gPGIP1 polypeptide in the heterologous hosts.

Following inoculation, it was found that the independent lines transformed with the grapevine *pgip1* gene exhibit reduced grey mould symptoms caused by *B. cinerea* grown on tissues of transgenic plants expressing gPGIP1, compared to those that develop on inoculated control plant tissues. This reduction in disease symptoms indicates that PGIPs can reduce fungal maceration of plant tissues. Even though leaves developed primary lesions at each inoculation site after overnight incubation, a difference was observed in the proportion of expanding lesions between the control plants and the transgenic plants. This suggests that inhibition of fungal PGs does not prevent the establishment of the initial plant-pathogen interaction, but does influence the subsequent expansion of fungal mass. Since fungal lesion expansion is reduced by gPGIP1 expressed in tobacco plants, this provides evidence for the relevance of fungal PGs in the virulence of *B. cinerea* and the fact that PGIPs in plant tissues can regulate fungal PG activity during infection. The difference in the proportion of expanding lesions between the control plants and transgenic plant tissue showing a low, medium or high PGIP activity correlates the relative absence/presence of the gPGIP1 to disease symptoms.

The ability of PGIP to inhibit fungal PGs *in vitro* suggests that the *in planta* role of PGIPs includes the hinderance of pathogen degradation of the plant cell walls. PGIP expression can be induced by several pathogen and wound-related processes, as seen in the vegetative tissues of bean, soybean and apple fruit (Bergmann et al. 1994; Favaron et al. 1994; Devoto et al. 1997; Yao et al. 1999). However, in tomato flowers, bean pods, pear fruit and raspberry and grape berries, PGIPs are expressed in the absence of disease symptoms (Johnston et al. 1993; Stotz et al. 1993, 1994; De Ascensao et al. 2001a). The expression of grapevine PGIP in developing grape berries suggests that the expression of grapevine PGIP may not require a signal arising from pathogen infections. Before bloom and after véraison, grape clusters can

be infected and destroyed by *B. cinerea*. Between these two developmental stages, young clusters are resistant to *B. cinerea*. Previous studies have shown a marked increase in the transcript levels of grape PGIPs and inhibition of PGs from *B. cinerea* at véraison (De Ascensao et al. 2001a). In developing grape berries, pre-existing grapevine PGIP could limit the tissue colonisation process by inhibiting fungal PGs. Thus, in developing fruit, PGIP, together with other mechanical and chemical processes may contribute to the defence mechanism to restrict fungal pathogen infection as seeds are maturing in ripening berries. This mandates the importance for the reproductive organs to acquire pathogen resistance during their development for the maintenance of the species.

The modification of certain key amino acids of PGIPs, thereby modulating their recognition abilities and the transgenic expression of these modified PGIPs, as well as native PGIPs from other plant sources, may result in an improved source of fungal inhibitors that can specifically reduce tissue degradation by pathogens. The expression of gPGIP1 in transgenic tobacco plants demonstrates the effectiveness of this inhibitor to reduce disease symptoms caused by *B. cinerea*.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

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6.1 GENERAL DISCUSSION AND CONCLUSIONS

In this studies, the first gene from *Vitis vinifera* L. encoding a polygalacturonase-inhibiting protein (PGIP), designated grapevine *pgip1*, was isolated and characterised. The isolated genomic clone comprises a 1002 bp single open reading frame corresponding to a deduced polypeptide of 333 amino acid residues with a predicted molecular mass of 37.1 kDa and a pI of 8.52. Nucleotide and derived amino acid sequences analysis of grapevine *pgip1* showed significant homology with other characterised PGIP encoding genes and revealed features characteristic of PGIPs found in several other plant species.

A characteristic of the PGIPs investigated thus far is their high leucine content. Grapevine PGIP1 shared this homology with a broad range of proteins containing leucine-rich repeats (LRR). In PGIPs, sequence variability within the structure of the LRR domain affects ligand binding and determines recognition specificity (Leckie *et al.*, 1999). It has been found that the residues of PGIPs that determine specificity and affinity for fungal polygalacturonases (PGs) are present in the conserved xxLxLxx motif (Kobe and Deisenhofer, 1993; 1995). A single amino acid variation in this motif can change the recognition capability of the PGIP (Leckie *et al.*, 1999). Comparing the grapevine PGIP1-encoding gene from the grape cultivars Pinotage and Shiraz, it was found that non-synonymous nucleotide substitutions leading to amino acid variations occurred within this predicted xxLxLxx motif. One could speculate that significant changes in residues in this region in the various cultivars of *V. vinifera* and other *Vitis* species are indicative of differential activities of the encoded PGIPs towards a range of fungal PGs. It would be important to isolate and analyse *pgip* genes from grapevine species highly resistant to fungal attack to compare the structure of the important ligand binding and recognition specificity motifs. Hereby it could be possible to find and/or manipulate a range of extremely efficient PGIPs in grapevine.

Genomic DNA analysis showed that the grapevine *pgip1* gene is part of a small family of PGIP-encoding genes in the *V. vinifera* genome. From Northern blot analysis it was evident that expression of the grapevine *pgip1* gene is berry specific and developmentally regulated. Like many plant resistance genes, PGIP-encoding genes have been organised into multigene families and different members of these families encode PGIPs with nearly identical biochemical characteristics, but distinct specificities (Desiderio *et al.*, 1997). When the grapevine *pgip1* gene was transiently expressed in *Nicotiana benthamiana*, crude protein extracts from the PVX-infected plants showed inhibitory activity against PGs from *B. cinerea*, but not against those from *Aspergillus niger*. From partial purifications from grapevine tissues, it was evident that the bulk of grapevine PGIPs are active against PGs from *B. cinerea*, *Colletotrichum lindemuthianum* and *A. niger*, suggesting that within the grapevine

PGIP multigene family, the grapevine *pgip1* gene encodes a PGIP with specificity against PGs from *B. cinerea*, but not against those from *A. niger*. It is likely that another *pgip* gene(s) encodes for a PGIP(s) with the ability to interact and inhibit PGs from *A. niger* and other fungal pathogens. There was a marked increase in PGIP inhibitory activity towards PGs from *B. cinerea* from green to véraison berries. This could indicate that PGIP accumulation forms part of a developmentally-regulated defence mechanism to restrict fungal pathogen infection as the seeds are maturing in ripening berries.

Two PGIP isomers, designated PGIP-A and PGIP-B, were purified and characterised from grape berries. PGIP-A was partially purified and had a molecular mass of 39 kDa, whereas grapevine PGIP-B contained two polypeptides with molecular masses of 44.5 and 42 kDa, respectively. The 42 kDa polypeptide, however, yielded a more intense PGIP-specific signal on a Western blot. Both grapevine PGIP isomers differed in their ability to inhibit fungal PGs. PGIP-A showed strong inhibition against *A. niger* PG and to a lesser extent against PGs from *Fusarium moniliforme*. It showed no inhibitory activity towards *B. cinerea* PGs. In contrast, PGIP-B inhibited PGs from *B. cinerea* almost completely and strongly inhibited PGs from *C. gleosporoides*, but was unable to inhibit PGs from *A. niger*. The inhibition profile of the PVX-infected tobacco lines harbouring the grapevine *pgip1* was similar to that of the PGIP-B isomer.

Enzymatic deglycosylation of PGIP-B decreased the molecular mass and gave rise to three polypeptides with molecular masses of 41, 39 and 37 kDa, respectively. This is very similar to the molecular masses of the predicted PGIP1 with and without the putative signal sequence (37 and 34 kDa, respectively), suggesting that differential glycosylation accounted for the heterogeneity in molecular mass. The pI of the grapevine PGIP-B mature protein was calculated to be 8.78.

Powell *et al.* (2000) were the first to report that the heterologous expression of a PGIP (pear PGIP in tomato) successfully conferred resistance against *B. cinerea* by targeting the tissue-macerating functions of the fungus. The *pgip1* gene from *V. vinifera* was expressed constitutively at high levels in tobacco plants, specifically to assess the effectiveness of this inhibitor against *B. cinerea* and other fungal pathogens in disease development. Expression of the grapevine *pgip1* gene was found in 19 transgenic plants. The heterologous gPGIP1 extracted from transgenic lines showed similar inhibitory specificities to those of PGIP-B, namely that it was able to inhibit PGs from *B. cinerea* and *C. gleosporoides*, but not PGs from *A. niger*.

Independent transgenic tobacco lines transformed with the grapevine *pgip1* gene exhibited reduced grey mould symptoms when compared to the untransformed inoculated control plants. Even though leaves developed primary lesions at each inoculation site after overnight incubation, a difference was observed in the proportion of expanding lesions between the control plants and the transgenic plants. This suggests that the inhibition of fungal PGs does not prevent the establishment of the initial plant-pathogen interaction, but that it does influence the subsequent

expansion of fungal mass. Since fungal lesion expansion is reduced by gPGIP1 expressed in tobacco plants, this provides evidence for the relevance of fungal PGs regarding the virulence of *B. cinerea*. Moreover, it indicates that PGIPs in plant tissues can regulate fungal PG activity during infection leading to a reduction in disease symptoms.

As previously mentioned, genomic analysis indicated that *V. vinifera* may have at least two or a small family of homologous PGIP-encoding genes, with the isolated *pgip1* gene being part of this family. The total grapevine PGIP activity therefore may comprise of polypeptides encoded by different *pgip* genes. The combined set of data from the various experiments seems to indicate that the isolated grapevine *pgip1* gene encodes the isolated PGIP-B isomer. The following pertinent results are applicable:

- i) the molecular mass and pI of the native PGIP-B are similar to that of the predicted grapevine PGIP1 encoded by grapevine *pgip1*;
- ii) the molecular mass of the glycosylated PGIP-B is identical to that of the PVX-expressed *pgip1* crude extract (PVXPGIP1), and the presence of a PGIP-specific signal of 42 kDa was identified by Western blot analysis for both protein extracts;
- iii) both PGIP-B and the PVXgPGIP1 extract show similar inhibitory specificities towards the same PGs;
- iv) overexpression of the grapevine *pgip1* in tobacco yielded transgenic proteins with similar inhibitory specificities to those of PGIP-B.

In conclusion, this work has provided insight in and access to a group of genes that have been proven over and over again to be involved in fungal resistance in plants and now also in grapevine. These results, together with other initiatives such as the isolation and characterisation of more grapevine *pgip* genes and promoter elements, may lead to an excellent model system to study various aspects of grapevine resistance versus fungal attack. Strategic modifications of the amino acids of PGIPs, the transgenic expression of the modified PGIPs as well as native PGIPs from other plant sources may also prove to be a productive source of fungal inhibitors that can specifically reduce tissue degradation by pathogens. The more information that can be extracted through multidisciplinary approaches on PGIPs and their PG ligands, the more light might be shed on the intricate host defence mechanisms of pathogen recognition and subsequent signalling events in the plant. The PGIP-PG interaction truly fits the mould of an excellent tool to study plant-pathogen interactions, a fact that will be exploited well in the future study of grapevine disease resistance.

6.1.1 Literature cited

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