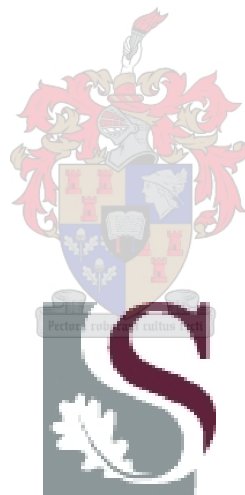# THE ENDOPOLYGALACTURONASES FROM *BOTRYTIS CINEREA* AND THEIR INTERACTION WITH AN INHIBITOR FROM GRAPEVINE

by

## Lizelle Wentzel

*Thesis presented in partial fulfilment of the requirements for the degree of Master of Sciences at Stellenbosch University.*

April 2004

*Supervisor:*
Prof. M.A. Vivier

# DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis 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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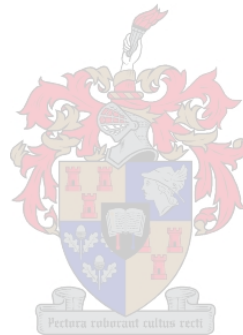
**Lizelle Wentzel**

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# SUMMARY

In the field of agriculture, plant pathogens are a major concern because of the severe damage these organisms cause to crops yearly. Fundamental studies regarding plant pathogens and their modes of action made it possible for researchers in the field of molecular biology to investigate pathogens further on a molecular level. *Botrytis cinerea*, has been used to great effect as a model system to investigate various aspects regarding pathogenesis, also on a molecular level.

Molecular research done on *B. cinerea* over the last few years has shown that the endopolygalacturonases (EPGs) of this fungus are key role players in pathogenesis. This hydrolytic enzyme family of six members, encoded by the *Bcpg1-6* genes, are important in breaking down the complex cell wall polymers of host plants, enabling the fungus to penetrate its host sufficiently. It has been shown that both BcPG1 and 2 are crucial for virulence of *B. cinerea*. A leucine-rich repeat inhibitor protein situated in the cell wall of various plant species, the polygalacturonase-inhibiting protein (PGIP), has been proven to interact with and inhibit EPGs, and thus the necrotic actions of *B. cinerea*. From literature it was clear that specific data regarding individual interactions of fungal EPGs with PGIPs are lacking currently. Furthermore, most experiments regarding the effects of EPG as well as interaction and inhibition studies of EPGs and PGIPs, rely on *in vitro* methods, without the possibility to contextualize the results on an *in vivo* or *in planta* level. The scope of this study was to specifically address the issues of individual EPG:PGIP interactions and the use of possible *in vivo* methodology by using EPGs from a highly virulent South African strain of *B. cinerea* and the grapevine VvPGIP1 that has been previously isolated in our laboratory. This PGIP, originally isolated from *Vitis vinifera* cv Pinotage, has been shown to inhibit a crude EPG extract from this strain with great efficiency. The approach taken relied on heterologous over-expression of the individual *Bcpg* genes and the isolation of pure and active enzymes to evaluate the inhibition of the EPGs with VvPGIP1. The genes were all successfully over-expressed in *Saccharomyces cerevisiae* with a strong and inducible promoter, but active enzyme preparations have been obtained only for the encoding *Bcpg2* gene, as measured with an agarose diffusion assay. The *in vitro* PGIP inhibition assay is also based on the agarose diffusion assay and relies on activity of the EPGs to visualize the inhibiting effect of the PGIP being tested. The active EPG2, however, was not inhibited by VvPGIP1 when tested with this assay.

The EPG encoding genes from *B. cinerea* were transiently over-expressed also in *Nicotiana benthamiana* by using the *Agrobacterium*-infiltration technique. Transgene expression was confirmed by Northern blot analysis and EPG-related symptoms were observed five to eight days post-infiltration. Differential symptoms appeared with the various EPGs, providing some evidence that the symptoms were not random events due to the infiltration or a hypersensitive response. Moreover, the symptoms observed for EPG2 was similar to those that were reported recently by another group on the same host. In spite of the expression data and the clear symptoms that developed, active preparations, as measured with the agarose diffusion plate asay, could only be obtained for EPG2 again.

In our search for a possible *in vivo* method to detect and quantify EPG activity and inhibition by PGIPs, we tested and evaluated a technique based on chlorophyll fluorescence to detect the effect of EPGs on the rate of photosynthesis. Our results showed that the over-expression of these genes reduced the rate of electrons flowing through photosystem II, indicating metabolic stress occurring in the plant. We used the same technique to evaluate possible interaction between VvPGIP1 respectively with BcPG1 and 2 and found that the co-expressing of the *Vvpgip1* gene caused protection of the infiltrated tissue, indicating inhibition of EPG1 and 2 by VvPGIP1. For EPG2, the observed interaction and possible inhibition by VvPGIP1 is the first report to our knowledge of an interaction between this specific EPG2 and a PGIP. Moreover, to further elucidate the *in planta* interaction between VvPGIP1 and the EPGs from the South African *B. cinerea* strain, we tested for possible interactions by making use of a plant two-hybrid fusion assay, but the results are inconclusive at this stage.

Previous studies in our laboratory have shown that several natural mutations exist between PGIP encoding genes from different *V. vinifera* cultivars. Based on this finding and the fact that these natural mutations could result in changes with regard to EPG inhibition and ultimately disease susceptibility, we isolated an additional 37 PGIP encoding genes from various grapevine genotypes, some of which are known for their resistance to pathogens.

Combined, these results make a valuable contribution to understand plant pathogen interactions, specifically in this case by modeling the interactions of pathogen and plant derived proteins. The possibility to use *in vivo* methods such as chlorophyll fluorescence to follow these interactions on an *in planta* level, provides exciting possibilities to strenghten and contextualize *in vitro* results.

# OPSOMMING

Plantpatogene organismes veroorsaak jaarliks erge skade aan landbougewasse en word dus as 'n ernstige probleem in die landbousektor beskou. Diepgaande studies wat handel oor plantpatogene en hul metodes van infeksie het dit vir molekulêre bioloë moontlik gemaak om patogene nou ook op molekulêre vlak verder te bestudeer. *Botrytis cinerea* is baie effektief as modelsisteem gebruik om verskeie aspekte van patogenese verder te bestudeer, ook op 'n molekulêre vlak.

Molekulêre navorsing op *B. cinerea*, het getoon dat die endopoligalakturonases (EPGs) van dié swam kernrolbelangrik in patogenese is. Hierdie sesledige hidrolitiese ensiemfamilie word gekodeer deur die *Bcpg1-6* gene en is belangrik vir die afbraak van die komplekse selwandpolimere van plantgashere, om suksesvolle gasheerpenetrasie te veroorsaak. Daar is aangetoon dat beide BcPG1 en 2 essensieël vir virulensie van die patogeen is. 'n Leusienryke-herhalings inhibitorproteïen wat in die selwand van verskeie plantspesies voorkom, die poligalakturonase-inhiberende proteïen (PGIP), het interaksie met en inhibeer EPGs en gevolglik ook die nekrotiserende aksies van *B. cinerea*. Uit die literatuur is dit duidelik dat spesifieke inligting aangaande individuele interaksies van fungiese EPGs met PGIPs tans nog ontbreek. Verder word daar op *in vitro* metodologie staatgemaak wannneer die effekte van EPGs asook die interaksie en inhibisie met PGIPs bestudeer word, sonder om die konteks van die *in vivo*- of *in planta*-omgewing in ag te neem. Die fokus van hierdie studie was om aspekte van individuele EPG:PGIP interaksies, asook die moontlike gebruik van *in vivo* metodologie te bestudeer deur EPGs, afkomstig van 'n hoogs virulente Suid-Afrikaanse ras van *B. cinerea* en die wingerd VvPGIP1, wat vroeër in ons laboratorium geïsoleer is, te gebrruik. Hierdie PGIP wat uit *Vitis vinifera* cv Pinotage geïsoleer is, inhibeer 'n kru EPG-ekstrak van bogenoemde ras baie effektief. Die benadering wat gevolg is het op die ooruitdrukking van die individuele *Bcpg*-gene in heteroloë sisteme staatgemaak en die gevolglike isolering van suiwer en aktiewe ensieme om EPG-inhibisie deur VvPGIP1 te beoordeel. Al die gene is suksesvol in *Saccharomyces cerevisiae* ooruitgedruk onder 'n sterk induseerbare promotor, maar volgens 'n agarose-diffundeerbare toets kon aktiewe ensiempreparate slegs vir die enkoderende *Bcpg2* verkry word. Die *in vitro* PGIP-inhibisie toets is ook op die gemelde toets gebasseer en vereis EPG-aktiwiteit om die inhiberende effek van die PGIP, te visualiseer. Die aktiewe EPG2 is egter nie deur VvPGIP1 geïnhibeer met die aanleg van hierdie toets nie.

Die EPG-enkoderende gene van *B. cinerea* is ook tydelik in *Nicotiana benthamiana* ooruitgedruk deur gebruik te maak van 'n *Agrobacterium*-infiltrasietegniek. Transgeenuitdrukking kon met die Noordelike kladtegniek bevestig word en EPG-verwante simptome is vyf tot agt dae na infiltrasie waargeneem. Verskillende simptome vir die verskillende EPGs is waargeneem, wat aanduidend is dat die simptome nie lukrake gevolge van die infiltrasies, of 'n hipersensitiewe respons is nie. Verder kon die simptome wat EPG2 vertoon het, gekorreleer word met dié wat onlangs deur 'n ander groep op dieselfde gasheer waargeneem is. Ten spyte van die ekspressiedata en die waargenome simptome, kon aktiewe ensiempreparate op die agarose-diffundeerbare toets, weereens slegs vir EPG2 waargeneem word.

'n Metode wat gebasseer is op chlorofilfluoressensie is getoets en geëvalueer as 'n moontlike *in vivo* metode om EPG aktiwiteit en inhibisie deur PGIPs waar te neem en te kwantifiseer. Die resultate het bevestig dat die ooruitdrukking van hierdie gene die elektronvloeitempo deur fotosisteem II verminder het wat 'n aanduiding is dat metaboliese stres in die plant heers. Dieselfde tegniek is gebruik om die moontlike interaksies tussen BcPG1 en 2 en VvPGIP1 te bestudeer en het aangetoon dat die mede-uitdrukking van die *Vvpgip1*-geen aanleiding gee tot 'n beskermende effek van die geinfiltreerde weefsel, wat aanduidend is van inhibisie van EPG1 en 2 deur VvPGIP1. In die geval van EPG2 is hierdie interaksie en moontlike inhibisie met 'n PGIP die eerste waarneming in die verband. In 'n verdere poging om die *in planta*-interaksie tussen VvPGIP1 en die EPGs van die Suid-Afrikaanse *B. cinerea* ras uit te klaar, is 'n plantgebasseerde twee-hibriede toets aangelê, maar geen klinkklare resultate kon verkry word nie.

Vorige werk het bevestig dat verskeie natuurlike mutasies in PGIP-enkoderende gene, afkomstig van verskillende *V. vinifera* kultivars, voorkom. Hierdie resultaat en die feit dat hierdie mutasies verskille in EPG inhibisie en uiteindelik vatbaarheid vir siektes kan beïnvloed, het aanleiding gegee tot die isolering van 'n verdere 37 PGIP-enkoderende gene uit 'n verskeidenheid druifplantgenotipes, sommige waarvan juis bekend vir hul weerstand teen patogene is.

Die gekombineerde resultate wat in dié studie verkry is, maak 'n waardevolle bydrae tot die verstaan van plant-patogeeninteraksies, spesifiek met die modelering van interaksies van patogeen- en plantgebasseerde proteïene. Die moontlikheid om *in vivo*-metodes soos chlorofilfluoressensie te gebruik in *in planta*-analises, is besonder bemoedigend om *in vitro*-resultate te versterk en ook in konteks te plaas.

This thesis is dedicated to my parents
Hierdie tesis is opgedra aan my ouers

# BIOGRAPHICAL SKETCH

Lizelle Wentzel was born in Stellenbosch, South Africa on the 25th of November 1978. She matriculated at Bloemhof Girls High School, Stellenbosch in 1996. Lizelle enrolled at Stellenbosch University in 1997 and completed her BSc degree majoring in Microbiology, Biochemistry and Genetics in 2001. The degree HonsBSc (Genetics, 2002) was subsequently awarded to her, whereafter she enrolled for an MSc degree in Wine Biotechnology.
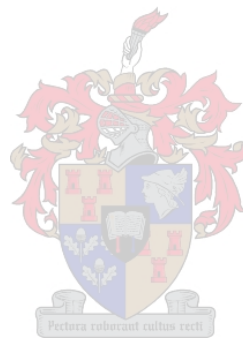
# ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

# PREFACE

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of the journal Plant Physiology. Chapters 3 and 4 forms part of a study that will be submitted for publication.

**Chapter 1**    **GENERAL INTRODUCTION AND PROJECT AIMS**

**Chapter 2**    **LITERATURE REVIEW**
Endopolygalacturonases (EPGs) and polygalacturonase-inhibiting proteins (PGIPs): two key role players in plant-pathogen interactions.

**Chapter 3**    **RESEARCH RESULTS**
Isolation, heterologous expression and *in vivo* analysis of the endopolygalacturonase gene family from a highly virulent strain of *Botrytis cinerea*

**Chapter 4**    **RESEARCH RESULTS**
The isolation of polygalacturonase-inhibiting protein (PGIP) encoding genes from *Vitis* and non-*Vitis* grapevine species

**Chapter 5**    **GENERAL DISCUSSION AND CONCLUSIONS**

# CONTENTS

## CHAPTER 5

# GENERAL INTRODUCTION AND PROJECT AIMS

## 1.1  INTRODUCTION

The necrotrophic fungus, *Botrytis cinerea*, causal agent of grey mould kills and lives on the dead tissue of dicotyledonous and non-graminaceous monocotyledonous plant cells (Jarvis, 1977).  *B. cinerea* has a broad host range and can cause great economic losses during the growth season of a variety of crops, as well as post-harvest decay of transported and stored crops (Berrie, 1994).  It is a well studied model organism within the field of plant pathology and a significant knowledge-base regarding the genetic diversity, host-range, epidemiology and mode of infection exists.  Research on disease prevention also continues to be on the forefront.  The last ten years have seen various advancements in the research on *Botrytis*, specifically in the field of molecular analysis.  The genome of *Botrytis* is also currently targeted for sequencing through a multinational collaborative endeavour.

The endopolygalacturonases (EPGs) of *B. cinerea* (encoded by the *Bcpg1-6* genes) play a vital role during the infection stage of this fungus (Ten Have, 2000).  The EPGs are some of the first enzymes to be secreted when *B. cinerea* invades its host and these enzymes are responsible for the degradation of the plant cell wall.  The polygalacturonase-inhibiting protein (PGIP) present in the cell walls of many plant species have been shown to specifically interact with and inhibit the hydrolytic activities of EPGs (De Lorenzo *et al.*, 2001; Esquerré-Tugayé *et al.*, 1999).  Powell *et al* (2000) also showed that transgenic tomato plants over-expressing a *pgip* gene from pear are less susceptible towards *B. cinerea* infection than the untransformed controls.

The fact that a well characterized interaction exists between fungal EPGs and plant PGIPs, make it an ideal model system to study and decipher some aspects of plant-pathogen interactions.  Advances in molecular biology techniques are enabling much more focused research into specific aspects of *B. cinerea* infection, such as the individual role of the various EPGs in pathogenesis and the *in planta* effect of these enzymes.  In this study the *in vitro* as well as *in vivo* interaction of the six EPGs of a hypervirulent *B. cinerea* strain with the *Vitis vinifera* L. cv Pinotage *Vvpgip1* encoding gene product (De Ascensao, 2001) was studied.  The following sections contain concise introductory remarks regarding the role-players in the interaction that will be studied (EPGs from *B. cinerea* and the grapevine VvPGIP1) to highlight the rationale behind the study.

## 1.2  THE ROLE OF FUNGAL EPGs ON THE PLANT CELL WALL DURING INFECTION BY *B. CINEREA*

The plant cell wall, a highly organized pectic-compound network, is the first barrier encountered by fungal pathogens and therefore plays a vital role in primary defence (De Lorenzo *et al.*, 2001; Esquerré-Tugayé *et al.*, 1999).  The pectic network confers the structural features of the plant cell wall and it is conceivable that any alteration in this structure will affect the physiological properties of the cell wall.  The various cell wall polymers may also serve as substrates to the numerous enzymes secreted by microbial pathogens, providing them with nutrients during the infection stage (Walton, 1994). *B. cinerea* encounters many cell wall components during the infection process and accordingly secretes a great number of cell wall degrading enzymes (CWDEs), including pectinases (Algishi and Favaron, 1995; Chen *et al.*, 1997).  Some of the first pectinases secreted by *B. cinerea* during the infection stage are EPGs that degrade the backbone of de-methylated pectin, i.e. polygalacturonic acid (PGA) (Ten Have, 2000).  The pectin-EPG interaction typically results in the release of oligogalacturonide (OG) fragments, which in turn can act as elicitors of plant defence responses (Esquerré-Tugayé *et al.*, 1999).  An increase of oligogalacturonides can also be found when the cell wall has been damaged by mechanical wounding (Bergey *et al.*, 1996).  Even at a very low concentration these OGs are able to induce the defence system of plants with the same efficiency as pathogens and their elicitors (Darvill *et al.*, 1992; Farmer *et al.*, 1991).

## 1.3  THE ROLE OF PGIPs IN DEFENCE

PGIPs are situated in the cell wall of various plant species and are encoded by a gene family whose expression is induced amongst others by injury or fungal infection (Bergey *et al.*, 1999; Torki *et al.*, 1999).  These glycoproteins share a basic common structure that contains leucine-rich repeat (LRR) sequences (De Lorenzo *et al.*, 2001; Di Matteo *et al.*, 2003).  A role of PGIP in plant defence is demonstrated by the reduction of disease symptoms in plants over-expressing *pgip* genes (Ferrari *et al.*, 2003; Powell *et al.*, 2000).

Plant PGIPs differ in their inhibition spectra towards fungal EPGs.  PGIP specificity against fungal EPGs has been reported by a number of researchers as reviewed by De Lorenzo *et al* (2001).  From these results it is clear that some PGIPs have broader inhibition spectra than others i.e., bean PGIP inhibits all the fungal EPGs assayed to date, including the EPG from *Fusarium moniliforme* (isolate FC-10), which in turn is not inhibited by PGIP from grape, pear, petunia and tomato (De Lorenzo *et al.*, 2001).  The interaction

between plant PGIP and fungal EPGs is of significant interest as part of the plant's defence system (De Lorenzo and Ferrari, 2002), since PGIP not only plays an important role in the inhibition of fungal EPGs, but is also suspected to function as a signalling molecule (Esquerré-Tugayé *et al.*, 1999).

## 1.4 PROJECT AIMS

The first PGIP-encoding gene has been isolated from grapevine by De Ascensao (2001). This gene, its promoter as well as the encoded protein have been studied extensively in our laboratory over the last four years (De Ascensao, 2001; Joubert, 2004). These previous studies have shown amongst other things that VvPGIP1 from *Vitis vinifera* can inhibit a crude extract of EPGs from a virulent South African *B. cinerea* strain, isolated from grapes in the Stellenbosch area, and that tobacco plants over-expressing the *Vvpgip1* gene are less susceptible to *B. cinerea* infection.

One of the most interesting aspects that still remains unclear, is whether the observed inhibition of *B. cinerea* EPGs are similar and equally effective against all six individual EPGs of the hypervirulent strain, or if some differentiation exists. The EPG:PGIP interaction studies that have been performed in the past have mostly relied on *in vitro* analyses and virtually no evidence and specific quantitative results exist for these interactions on the *in vivo* level. Given the fact that these interactions occur under natural conditions during pathogen invasion, it is quite important to have technologies available to test the suspected interactions also in a whole plant system.

Another aspect integrally linked to EPG:PGIP interactions is the specificity and efficacy of the PGIPs. Given the integrated role of PGIPs in plant defence, it is fair to hypothesize that the relative disease resistance of the plant's genotype might be correlated with the efficacy of the disease resistance proteins present, such as PGIPs. Amongst the grapevine genotypic material, the cultivated varieties mostly belong to *V. vinifera* spp. with virtually no natural resistance against most pathogens. Several of the other grapevine spp. does have significant resistance phenotypes against pathogens and specifically against the major fungal pathogens of grapevine. These resistant genotypes could thus be seen as genetic resources to potentially isolate PGIPs and other antifungal proteins with improved antifungal characteristics from.

The overriding goal of this study was to facilitate interaction studies between VvPGIP1 and the individual EPGs from *B. cinerea*. To this end the experimental outlay would be focussed on the cloning and heterologous over-expression of the *Bcpg1-6* genes to enable

*in vitro* activity and inhibition analysis. A transient over-expression system, facilitated by *Agrobacterium* infiltration of tobacco leaves will be used to study whole plant physiological interactions linked to EPG over-expression. Moreover, chlorophyll fluorescence as a method will be evaluated to detect *in planta* effects of EPGs as well as detect EPG:PGIP interactions on the *in vivo* level. To isolate additional grapevine PGIP encoding genes with possible increased antifungal efficacies, various *Vitis* and non-*Vitis* species will be used as source material for the amplification of different PGIP encoding genes.  These sequences will be analyzed and compared with the existing grapevine PGIP encoding genes and should represent a genetic resource for future interaction studies and even biotechnology approaches.

More specifically, the aims of the study were:

i. to isolate and clone the six EPG encoding genes (*Bcpg1-6*) from a highly virulent South African *B. cinerea* isolate and to compare the sequences with those present in the databases;

ii. to over-express the isolated *Bcpg1-6* genes in *Saccharomyces cerevisiae* to facilitate *in vitro* analysis of the encoded products and the grapevine VvPGIP1;

iii. to transiently over-express the *Bcpg1-6* genes in *Nicotiana benthamiana* with *Agrobacterium* infiltration to facilitate *in vitro* as well as *in vivo* analysis of EPG activity and possible inhibition by VvPGIP1;

iv. to evaluate chlorophyll fluorescence as a method to quantify or describe *in vivo* effects of EPGs and/or inhibition interactions between EPGs and PGIPs; and

v. to isolate additional PGIP encoding genes from various *Vitis* and non-*Vitis* genotypes to obtain PGIPs with possible enhanced antifungal activities.

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# LITERATURE REVIEW

Endopolygalacturonases (EPGs) and polygalacturonase-inhibiting proteins (PGIPs): two key role players in plant-pathogen interactions.

## 2.1 INTRODUCTION

The plant polysaccharide-rich cell wall is one of the first barriers against phytopathogenic fungi. To break this barrier and gain access to the plant cells, most fungi need to secrete cell wall degrading enzymes (CWDEs), capable of breaking down the polymers that make up the complex structure of the cell wall. CWDEs are essential for fungal pathogens that do not have specialized penetration structures as well as for necrotrophic pathogens during the late stages of the invasion process (De Lorenzo and Ferrari, 2002). Among these enzymes, endopolygalacturonases (EPGs) cause cell wall degradation as well as plant tissue maceration (Basham and Bateman, 1975; Bauer *et al.*, 1977). EPGs are the first enzymes to be secreted by pathogens when they encounter plant cell walls (De Lorenzo *et al.*, 1997; Idnurm and Howlet, 2001), and their contribution to the pathogenicity of some fungi and bacteria is well established (Shieh *et al.*, 1997). EPG primarily degrades the backbone of de-methylated pectin, i.e. polygalacturonic acid (PGA), or stretches of PGA embedded in pectin or rhamnogalacturonan I and in this process oligogalacturonide (OG) fragments are released from the plant cell walls. It has been shown that these oligogalacturonides serve as elicitors in various defence responses (Bergey *et al.*, 1996; Boudart *et al.*, 2003). It is hypothesized that the interaction between the polygalacturonase-inhibiting protein (PGIP) and PGs leads to the production of size dependent elicitor-active OGs (Cervone *et al.*, 1989; Cervone *et al.*, 1997; Ridley *et al.*, 2001). PGIPs are leucine-rich repeat (LRR) proteins situated in the cell wall of various plant species and have the potential of suppressing fungal colonization (De Lorenzo *et al.*, 2001) by acting as both an inhibitor as well as a regulator of PG activity. LRRs are defined by a consensus sequence that comprises the sequence xxLxLxx, predicted to form a β-strand/β-turn structure, in which the x-residues are solvent-exposed and involved in the interaction with EPGs (De Lorenzo *et al.*, 1994; Kobe and Deisenhofer, 1995a; Leckie *et al.*, 1999; Mattei *et al.*, 2001).

Although the recognition capabilities of PGIPs toward fungal PGs are constantly evolving in plants, recognition of PGs by PGIPs is an effective self-defence strategy, since parasitic fungi tend to maintain EPGs as pathogenicity factors (De Lorenzo and Ferrari, 2002).

## 2.2  PLANT PGIPs, KEY COMPONENTS OF DEFENCE

### 2.2.1  THE ROLE OF PGIPs IN DEFENCE

Similar to known defence- and pathogenesis-related genes, mechanisms regulating the expression of PGIP encoding genes include specific developmental cues, with stress- and pathogen-derived signals superimposed on them (Devoto *et al.*, 1998).  PGIPs share significant similarities in terms of structure and specificity with the *R* gene products (Stotz *et al.*, 2000).  It is strongly suggested that PGIP plays an important role in defence against pathogens, since their activity in restraining fungal invasion and thereby protecting the cell wall has been indicated (De Lorenzo *et al.*, 2001; Sella *et al.*, 2004).  It has been shown that PGIP from bean hypocotyls, protected bean cell walls against degradation by EPGs of *Colletotrichum lindemuthianum in vitro* (Lafitte *et al.*, 1984).  Similarly, PGIP from tomato protected tomato cell walls against EPGs from *Fusarium oxysporum* (Federici *et al.*, 2001), and PGIP from leek also protected leek tissue from EPG degradation (Favaron *et al.*, 1997).

In most cases increased levels of PGIP correlated with a decreased susceptibility in plants towards specific pathogenic fungi.  In bean hypocotyls infected with *Phaseolus vulgaris*, levels of PGIP increased during seedling growth along with increased resistance of the older bean hypocotyls (Salvi *et al.,* 1990).  Similarly, increasing susceptibility of ripening pear fruits to *Dithiorella gregaria* and *Botrytis cinerea* correlated with a reduction in the concentration of PGIP (Abu-Goukh *et al.,* 1983).  A transgenic tomato plant, over-expressing a *pgip* gene from pear also showed decreased susceptibility towards *B. cinerea* infection (Powell *et al.*, 2000).

### 2.2.1.1  THE RECOGNITION ABILITIES OF PGIPs

Most phytopathogenic fungi produce EPGs in various iso-enzymatic forms.  These enzymes vary in terms of stability, specific activity, optimum pH, substrate preference, mode of action as well as the types of oligosaccharides released (Cook *et al.*, 1999; De Lorenzo *et al.*, 2001).  EPGs have evolved over time to facilitate pathogenesis in various different conditions and on a variety of hosts (De Lorenzo *et al.*, 1997; Herron *et al.*, 2000; Walton, 1994).

Plants have adapted by evolving different PGIPs, which show specific recognition abilities against the many different EPGs produced by fungi.  PGIPs are quite effective against fungal EPGs from, for example, those of *Aspergillus niger, B. cinerea* and

*Fusarium moniliforme* (Cervone *et al.*, 1990; Cook *et al.*, 1999; Pressey, 1996), but ineffective against other pectic enzymes of either microbial or plant origin (Cervone *et al.*, 1990). PGIPs can inhibit a series of EPGs with an endo/exo mode of substrate degradation, but differentiate between EPGs with a classic endo mode of cleavage (Cook *et al.*, 1999). PGIPs from different plant sources differ in their inhibitory activities; also, PGIPs from a single plant source can inhibit EPGs from different fungi or different EPGs from the same fungus (De Lorenzo *et al.*, 2001). For example, bean PGIP is significantly more effective against an EPG from *C. lindemuthianum* than against an EPG of the related non-pathogen *Colletotrichum lagenarium,* suggesting that compatibility provides a selection pressure for more efficient PGIPs that can counteract fungal infection more efficiently (Lafitte *et al.,* 1984). It has been shown that a purified pear PGIP inhibits EPGs from *A. niger, B. cinerea* and *D. gregaria*, inhibits EPGs from *Penicillum expansum* to a lesser extent and does not inhibit EPGs from *F. oxysporum* (Abu-Goukh and Labavitch, 1983). On the other hand, purified soybean PGIP inhibits an EPG from *A. niger* as well as two EPGs from *Sclerotinia sclerotiorum* (Favaron *et al.*, 1994). A fruit-specific PGIP from apple shows different degrees of inhibition towards four EPGs from *B. cinerea* (Yao *et al.*, 1995).

The total PGIP activity in some plants is a mixture of different inhibitory activities, i.e. two bean PGIPs with nearly identical biochemical features, but with distinct inhibitory activities have been separated by differential affinity chromatography; one of the PGIPs inhibits an EPG from *A. niger,* but not the EPG from *F. moniliforme*, whereas the other PGIP inhibits both (Desiderio *et al.,* 1997).

The individual characterization of the products encoded by *pgip* genes isolated from either a single plant or different plants confirm that the apparent broad specificity of PGIPs may depend on the occurrence of different isoforms with narrow specificities. The ability of PGIPs to inhibit a wide spectrum of fungal EPGs may, therefore, be the sum of the abilities of various PGIPs present in the preparation, each contributing in part to confer a broad range of inhibitory activities (De Lorenzo *et al.,* 2001).

## 2.2.2 THE PRIMARY STRUCTURE OF PGIPs

PGIPs are glycoproteins associated with the cell wall of both mono- and dicotyledonous plants and have a molecular mass of approximately 40 kDa (De Lorenzo *et al.*, 2001; De Lorenzo and Ferrari, 2002). The predicted polypeptide of the *P. vulgaris* PGIP contains 342 residues, displaying several potential sites for glycosylation. Included in this mature

polypeptide is a 29-amino-acid signal peptide for translocation into the ER (Toubart *et al.*, 1992). Parts of the PGIP sequence reveal features of significant internal sequence identity. The internal sequence-identical domain spans 258 amino acids (residues 69-326) and consists of 10 modules characterized by the consensus sequence for extracytoplasmic leucine-rich repeats (LRRs) (De Lorenzo *et al.*, 1994; Jones and Jones, 1997).

## 2.2.2.1 PGIPs ARE LEUCINE-RICH REPEAT (LRR) PROTEINS

LRRs were discovered nineteen years ago in a leucine-rich α-2-glycoprotein, a protein with unknown function from human serum (Takahashi *et al.*, 1985). Today, this motif is found in over sixty classes of proteins with important cellular functions. It is unknown whether all LRRs share a common ancestor. LRRs are smaller than general protein domains, but large enough to question multiple independent evolutionary occurances (Kobe and Deisenhofer, 1995b). The variation in length and consensus sequence, however, does raise the possibility of at least a few independent occurrences of LRRs. Collagen contains an example of a LRR motif believed to have emerged independently several times during evolution (Kobe and Deisenhofer, 1995b).

The LRR is a sequence motif that contains a large proportion of repetitive sequence patterns (Wootton, 1994). Correspondingly, a substantial portion of known three-dimensional LRR structures shows internal symmetry, most likely as a result of gene duplication (Murzin, 1994). LRRs contain between 20 and 29 residues and are defined by a consensus sequence GxIPxxLGxLxxLxxLxLxxNxLT/S, where x represents any amino acid and L positions can be occupied by valine, isoleucine and phenylalanine (Krantz and Zipursky, 1990). Within each LRR the sequence xxLxLxx is predicted to form a β-strand/β-turn structure, in which the x-residues are solvent-exposed and involved in the interaction with EPGs (De Lorenzo *et al.*, 1994; Kobe and Deisenhofer, 1995a; Leckie *et al.*, 1999; Mattei *et* al., 2001).

LRR proteins participate in many biologically important processes, such as hormone-receptor interactions, enzyme inhibitions, cell adhesion and cellular trafficking (Kobe and Kajava, 2001). LRRs are not so common in the plant kingdom, whereas a number of studies confirmed the involvement of LRR proteins in early mammalian development, (Tong *et al.*, 2000), neural development (Mutai *et al.*, 2000), cell polarization (Bilder and Perrimon, 2000), regulation of gene expression (Linhoff *et al.*, 2001) and apoptosis signalling (Inohara *et al.*, 1999). In all these processes and in all living organisms, LRR

domains are specialized for interaction with protein ligands (Kobe and Kajava, 2001). Apart from the LRRs that provide an ideal structural framework for achieving protein-protein interactions, the repetitive structure may be valuable in processes where the rapid generation of new variants, such as plant disease resistance is required (Jones and Jones, 1997; Kobe and Kajava, 2000; Marcotte *et al.*, 1999).

PGIPs show a close relationship with a number of plant LRR proteins known to be involved in resistance to pathogens (Ellis *et al.*, 2000; Jones, 2001) and signal transduction pathways (Clark *et al.*, 1997; Gomez-Gomez and Boller, 2000; Jinn *et al.*, 2000; Li and Chory, 1997; Torii *et al.*, 1996). PGIPs also show similarities with decorins, small animal extracellular LRR proteins that belong to the leucine-rich proteoglycan (SLRP) class proteins (Iozzo, 1999). Decorins interact with a variety of proteins that are involved in matrix assembly, control of cell proliferation and tissue morphogenesis. Collagen, fibronectin, TGF-β and the epidermal growth factor receptor are all known ligands of decorin (Iozzo, 1999; Iozzo *et al.*, 1999). Similar to decorins, PGIPs also have the ability to bind to diverse ligands including EPGs, pectins and apoplastic lipoxygenases, but have been shown to be ineffective against other pectic enzymes, either of microbial or plant origin (Cervone *et al.*, 1990).

The majority of known resistance gene (*R*) products in plants are LRR proteins (Ellis *et al.*, 2000; Jones, 2001; Jones and Jones, 1997). The R-proteins Cf of tomato (Jones, 2001; Jones and Jones, 1997), Xa21 of rice (Ronald, 1997), the receptor kinase FLS2 for response to the bacterial elicitor flagellin (Gomez-Gomez *et al.*, 2001) and several receptor kinases that are involved in development or in hormone perception (Torii and Clark, 2002), contain extracytoplasmic LRRs similar to those found in PGIPs. Codon evolution analysis of the β-strand/β-turn region of *R*-genes supports the concept that this region is hypervariable and under selection for diversification (Meyers *et al.*, 1998; Noel *et al.*, 1999; Parniske *et al.*, 1997). Amino-acid changes in this region have been shown to influence the function of R-proteins (Dodds *et al.*, 2001; Van der Hoorn *et al.*, 2001; Warren *et al.*, 1998).

## 2.2.2.2  THE THREE DIMENSIONAL STRUCTURE OF PGIPs

The crystal structure of ribonuclease inhibitor (RI) yielded the first insight into the three dimensional (3D) structural arrangement of LRRs (Kobe and Deisenhofer, 1993). Crystal structures of RI complexed with its ligands provided the first structural views revealing how the LRR structure is used as a protein recognition motif (Kobe and Deisenhofer, 1995a;

Papageorgiou *et al.*, 1997). The structure of porcine RI, a protein containing 15 LRRs, showed that LRRs corresponded to structural units, each consisting of a β-strand and a α-helix connected by loops (Kobe and Deisenhofer, 1993). The structural units were arranged so that all the strands and helices were parallel to a common axis, resulting in a non-globular, horseshoe-shaped molecule with a curved parallel β-sheet lining the inner circumference of the horseshoe and the helices flanking the outer circumference (Fig 2.1a). The structure of RI explained the conservation of residues that constitute a LRR. The conserved pattern, LxxLxLxxN/CxL, correlated to the fragment surrounding the β-strands. According to available data on structure and sequence information, proteins containing LRRs could have structures related to RI, but considerable structural differences may exist in the regions between the β-strands. It was speculated that the helical area might be shorter or even substituted with an extended structure in certain cases (Kobe and Deisenhofer, 1994; Kajava, 1998), which led to the proposal that shorter LRRs may have structures that show more correlation towards the β-helix of pectate lyase (Yoder *et al.*, 1993) than that of the β/α-horseshoe of RI (Buchanan and Gay, 1996; Claudianos and Campbell, 1995; Heffron *et al.*, 1998; Kobe and Deisenhofer, 1995b). In Figure 2.1 and Table 2.I, three-dimensional structures of LRR proteins (that were published recently) are shown and compared. The structures reveal diversity in the lengths and sequences of the individual LRRs in these proteins, which make them exceedingly informative. Significant similarities are found among the structures. These include an overall curved shape with a parallel β-sheet on the concave side and predominantly helical elements on the convex side. Protein interaction involving LRRs occurs mostly on the concave domain together with the adjacent loops. The structure of the spliceosomal proteins U2B" (comprising a ribonuclease protein domain) and U2A' (containing a LRR), shows that the concave surface of the LRR domain is ideal for interaction with an α-helix and this may be a frequent trait of protein-protein interactions in LRR proteins (Price *et al.*, 1998).
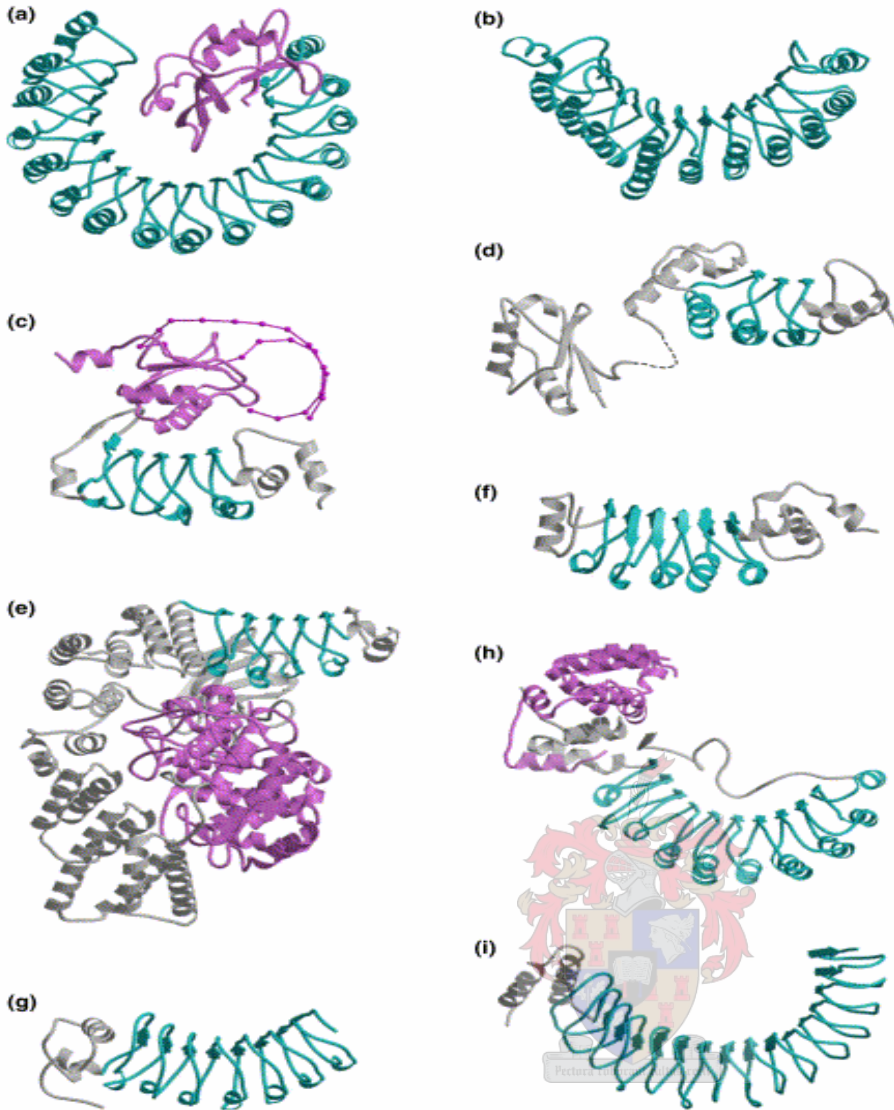
**Figure 1.** Three dimensional structures of LRR proteins. The LRR domains are shown in cyan, the flanking regions that are an integral part of the LRR domain, but do not correspond to LRR motifs, are shown in grey and the other domains/subunits in the structure are shown in magenta. Information in Table 2.1 is supplementary to the figure. (a) RI (Ribonuclease inhibitor) (Kobe and Deisenhofer, 1993); (b) rna1p (GTPase-activationprotein-rna1) (Hillig *et al.*, 1999); (c) U2B"-U2A' (Spliceosomal protein; RNA ternary complex) (Price *et al.*, 1998); (d) TAP (Nuclear export transport protein associated with antigen processing) (Liker *et al.*, 2000); (e) RabGGT (Rab geranylgeranyltransferase) (Zhang *et al.*, 2000) (f) dynein (Light chain 1) (Wu *et al.*, 2000); (g) InlB (Internalin B) (Marino *et al.*, 1999); (h) Skp2-Skp1 (Ubiquitin ligase; Cyclin A/Cdk2-associated protein p45 and p19) (Schulman *et al.*, 2000); and (i) YopM (Leucine-rich effector protein) (Evdokimov *et al.*, 2001).

**Table 2.1  Three dimensional structures of LRR proteins (continued).**

| LRR protein | Organism | Ligand present in structure | Function | Number of LRRs | LRR length (residues) | LRR subfamily | Secondary structure in interstrand segment | References |
|---|---|---|---|---|---|---|---|---|
| RI | Pig | - | Ribonuclease inhibitor | 15 | 28-29 | RI-like | α helix | Kobe and Deisenhofer, 1993 |
| RI | Pig | Ribonuclease A | Ribonuclease inhibitor | 15 | 28-29 | RI-like | α helix | Kobe and Deisenhofer, 1995a |
| RI | Human | Angiogenin | Ribonuclease inhibitor | 15 | 28-29 | RI-like | α helix | Papageorgiou *et al.*, 1997 |
| rna1p | *Schizosaccharomyces pombe* | - | GTPase-activating protein for Ran | 11 | 28-37 | RI-like | α helix | Hillig *et al.*, 1999 |
| U2A' | Human | U2B'' snRNA | Splicing | 5 | 22-26 | SDS22-like | $3_{10}$ helix, α helix, extended | Price *et al.*, 1998 |
| TAP | Human | - | RNA export from nucleus | 4 | 24-41 | SDS22-like | α helix | Liker *et al.*, 2000 |
| Rab GGT | Rat | - | Rab geranyl-geranyl transferase | 5 | 22-27 | SDS22-like | $3_{10}$ helix, α helix, | Zhang *et al.*, 2000 |
| LC1 (dynein) | *Chlamydomonas reinhardtii* | - | Protein-protein interactions in molecular motor complex | 6 | 22-25 | SDS22-like | α helix | Wu *et al.*, 2000 |
| InlB | *Listeria monocytogenes* | - | Phagocytosis | 7.5 | 22 | SDS22-like | $3_{10}$ helix | Marino *et al.*, 1999 |
| Skp2 | Human | Skp1 | Substrate binding in ubiquitination | 10 | 23-27 | Cysteine-containing | α helix | Schulman *et al.*, 2000 |
| YopM | *Yersinia pestis* | - | Virulence factor | 15 | 20-22 | Bacterial | Polyproline II | Evdokimov *et al.*, 2001 |

Sequence analyses revealed that several different LRR subfamilies exist (Buchanan and Gay, 1996; Claudianos and Campbell, 1995; Jones and Jones, 1997; Kajava, 1998; Kajava *et al.*, 1995). Published data distinguish seven subfamilies (Table 2.2), (Kajava, 1998). This classification suggests that repeats from different subfamilies do not occur simultaneously in the same protein and most likely have evolved independently. Three-dimensional structures of LRRs from the other subfamilies could be constructed based on the known structure of RI (Kajava, 1998; Kajava *et al.*, 1995).

**Table 2.2  Seven LRR subfamilies (Kajava, 1998).**

| *Subfamilies of LRR proteins* | | | | |
|---|---|---|---|---|
| **LRR subfamily** | **LRR length (range)** | **Organism origin** | **Cellular location** | **Structures available** |
| RI-like | 28-29 (28-29) | Animals | Intracellular | RI, rna1p |
| SDS22-like | 22 (21-23) | Animals, fungi | Intracellular | U2A', TAP, RabGGT, LC1, InlB |
| Cysteine-containing | 26 (25-27) | Animals, plants, fungi | Intracellular | Skp2 |
| Bacterial | 20 (20-22) | Gram-negative bacteria | Extracellular | YopM |
| Typical | 24 (20-27) | Animals, fungi | Extracellular | - |
| Plant-specific | 24 (23-25) | Plants, primary eukaryotes | Extracellular | - |
| TpLRR | 23 (23-25) | Bacteria | Extracellular | - |
| **Consensus sequence**[A] | | | | |
| RI-like | x x x L x x L x L x x N/C x L x x x g o x x L x x o L x - x | | | |
| SDS22-like | L x x L x x L x L x x N x I x x I x x L x - x | | | |
| Cysteine-containing | c x x L x x L x L x x c x - x I **T D** x x o x x L a x - x | | | |
| Bacterial | **P** x x L x x L x V x x N x L x x L **P e/d** L - | | | |
| Typical | L x x L x x L x L **x x** N x L **x x L p** x x o **F** x - x | | | |
| Plant-specific | L **x - x** L x x L x L x x N x L **t/s g - x** I **P** x x L G x | | | |
| TpLRR | C/N **x - x L x x** I x **L x - x x** L **x x** I **g** x x A F x x | | | |

[A]Residues identical or conservatively substituted in more than 50% and 30% of the repeats of a given protein are shown in uppercase and lowercase respectively.  Residues directed into the interior of the known protein structures or models are shown in bold.  "-" indicates a possible insertion site, "o" a non-polar residue and "x" indicates any residue.

The crystal structure of a plant LRR protein, *P. vulgaris* PGIP (PvPGIP2) was recently determined (Di Matteo *et al.*, 2003), providing the first structure of a plant LRR protein. PvPGIP2 displays a curved and extended shape, which is more twisted than other LRR proteins.  The concave inner side of the structure is occupied by a long parallel β-sheet, where the residues determining the affinity and specificity of PvPGIP2 are situated (Leckie *et al.*, 1999).  This corresponds to the β-sheet originally predicted by modelling studies.  An additional extended parallel β-sheet, absent in the majority of other LRR proteins, characterizes the structure and places the fold of PvPGIP2 between the typical LRR structure and the β-helical structural design found in pectate lyases and PGs (Jenkins and Pickersgill, 2001; Yoder and Jurnak, 1995a).  The second β-sheet may contribute to the formation of an additional surface for interactions with other ligands.  The recent finding that PGIP interacts with a membrane-associated lipoxygenase localized in the apoplastic space suggests that PGIP may take part in a multiprotein complex involved in signalling upon pathogen attack (D'Ovidio *et al.*, 2004).

The N-terminal region of PvPGIP2 (residues 1-52) consists of a 13-residue long α-helix and a short β-strand resembling the β-hairpin conformation observed in the N-terminal domains of the U2A' spliceosomal protein (Price *et al.*, 1998).  Four disulfide bridges flank the LRR domain of which two bridges are located in the N-terminal region (cys3-cys33, cys34-cys43) and the other two in the C-terminal region (cys281-cys303, cys305-312) (Price *et al.*, 1998).

A unique characteristic of the PvPGIP2 structure is the presence of two clusters of residues of opposite charge:  a negatively charged surface on the LRR concave face that is likely involved in binding EPGs, and a positively charged patch located between the two β-sheets.  Site-directed mutagenesis on residues of a EPG from the phytopathogenic fungus *F. moniliforme* (*Fm*PG) showed that the interaction with PvPGIP2 is mediated by at least two residues of the enzyme (Arg267 and Lys269), that are located at the edge of its active site and are presumably involved in substrate binding (Federici *et al.*, 2001).  The negative pocket of PvPGIP2, formed by three aspartic residues highly conserved in all PGIPs (De Lorenzo *et al.*, 2001), is thought to accommodate the positively charged residues Arg267 and Lys269 on the surface of the enzyme, thus covering its active site and preventing access to the substrate.  The interaction of PGIP with EPG residues, important for enzyme activity (Pages *et al.*, 2000), is an effective evolutionary strategy of the plant to decrease the possibilities of fungal EPGs escaping recognition.

The residue Gln224 of PvPGIP2, which is crucial for the specificity of the inhibitor towards FmEPG, is adjacent to the negative pocket putatively involved in EPG binding and may interact with an unidentified partner residue of FmEPG to correctly lock Arg267 and Lys269 into the negative pocket.  In PvPGIP1, which is unable to interact with FmEPG (Leckie *et al.*, 1999), this role may not be fulfilled by the corresponding Lys224.  The positively charged patch of PvPGIP2 consists of a cluster of regularly spaced Arg and Lys residues protruding into the solvent and creating a regular distribution of charges that resembles the prediction for the pectate-binding site in the apoplastic peroxidase APRX (Carpin *et al.*, 2001).  The proximity of this site to the region that interacts with EPG, suggests that upon binding the enzyme, PGIP is released from the pectic matrix (Carpin *et al.*, 2001).

## 2.3  THE EPGs OF *B. CINEREA*

*B. cinerea* is a necrotrophic pathogen that infects and kills dicotyledonous and non-graminaceous monocotyledonous tissues, and subsequently lives on the dead tissue

(Jarvis, 1977). The diseases caused by *Botrytis* species are generally referred to as "grey mold" since these pathogens produce a white, woolly mycelium on decayed tissue that will turn grey during sporulation. Sporulation can occur as soon as a few days after the start of infection (Hausbeck and Moorman, 1996; Kim and Cho, 1996). *Botrytis* species produce macro-conidia, but can also produce sclerotia on the surface of infected plant material (Honda and Mizumara, 1991). These latter structures serve to adapt the fungus to unfavourable conditions. Micro-conidia are also produced, but have not been implicated in disease formation. Furthermore, *B. cinerea* penetrates the epidermis preferably at the anticlinal position (Mansfield and Richardson, 1981), indicating a preference for cell walls. The broad host range of *B. cinerea* results in great economic losses not only during growth of the various crops, but also during storage and transport of the harvested products (Berrie, 1994). Research on *Botrytis* species, therefore, has mainly focused on understanding the disease cycle of the fungus, with specific focus on mechanisms for disease prevention.

### 2.3.1 THE ROLE OF CELL WALL DEGRADING ENZYMES (CWDEs) IN THE PATHOGENESIS OF *BOTRYTIS*

Cell walls play an important role in the architecture of the plant. They provide the cell with mechanical strength and maintain its shape. The intercellular space including cell walls is referred to as the apoplast (Holmes, 1979). The apoplast is a continuous, highly organized structure that stretches throughout the plant. The apoplast does not only serve as a major transport structure, but it also forms a barrier to harmful biotic and abiotic agents such as infection by *B. cinerea*.

During the different phases of infection, *B. cinerea* encounters different combinations of defence mechanisms. The apoplast does not only serve as a physical barrier for *B. cinerea*, but it also contains pre-formed components that can inhibit fungal growth and thus serve as a chemical barrier in the defence response (Mansfield and Richardson, 1981). In addition, the plant can respond upon pathogen invasion by producing various components that contribute to both the physical and the chemical barrier. These resistance mechanisms, however, are not always effective against pathogens, including *B. cinerea.*

*B. cinerea* encounters many cell wall components during the infection process and accordingly secretes a number of CWDEs, microbial enzymes that catalyze the degradation of cell wall components. A wide variety of enzymes have been identified.

Table 2.3 show examples of pectinases, the most important CWDEs during the infection process of *B. cinerea* (Ten Have, 2000).

**Table 2.3** **Pectinases secreted by *B. cinerea* classified according to the *Enzyme Commission* (Ten Have, 2000).**

| Name | E.C. Number | Abbreviation | Substrate[A] | Action | End-product[B] |
|---|---|---|---|---|---|
| Pectin lyase | 4.2.2.10 | PnL | Pectin | β-elimination | OGA-CH$_3$ |
| Pectin methylesterase | 3.1.1.11 | PME | Pectin | Hydrolysis | PGA |
| Exopectate lyase | 4.2.29 | exoPeL | PGA | β-elimination | GA |
| Endopectate lyase | 4.2.2.2 | endoPeL | PGA | β-elimination | OGA |
| Endopolygalacturonase | 3.2.1.15 | EPG | PGA | Hydrolysis | OGA |
| Exopolygalacturonase | 3.2.1.67 | exoPG | PGA | Hydrolysis | GA |

[A]Pectin indicates methylated polygalacturonic acid, PGA indicates non-methylated polygalacturonic acid.
[B]OGA indicates oligogalacturonic acid, GA indicates monogalacturonic acid, CH$_3$ indicates a methyl-group.

Components of the pectic compound network consist of various polysaccharide structures, containing a high content of galacturonides and rhamnoses. Three main types of polygalacturonans can be distinguished namely, homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II. Homogalacturonans are made up of α-1,4-linked chains of D-galacturonic acid (GA). Rhamnogalacturonan I contains an α-1,4-linked GA and α-1,2-linked rhamnose backbone that makes it a more complex molecule (Lau *et al.*, 1985). The complex structure of rhamnogalacturonan II is not yet completely identified, but this component plays a minor role in the pectic compound network (Lau *et al.*, 1985).

The residues of all galacturonans can either be methylated, acetylated or glycosylated. Pectate is the name generally used for homogalacturonan with a low degree of methylation, whereas pectin describes homogalacturonan with a high degree of methylation (Lau *et al.*, 1985). Xylogalacturonan is used to describe galacturonan rich in xylose side chains (Lau *et al.*, 1985). Apart from these three types of galacturonides, other polysaccharides can also be found in the pectic compound network. Arabinan, a highly branched molecule, contains an α-1,5-linked arabinose backbone with side chains that can either be α-1,2- or α-1,3-linked (Lau *et al.*, 1985). Galactan, present in the primary cell wall, is a β-1,4-linked galactose chain, containing a few β-1,6-linked galactose residues (Lau *et al.*, 1985). Arabinogalactan I consists of galactan with arabinan side chains (Lau *et al.*, 1985). All these non-galacturonan molecules are referred to as "pectic components", since they are all present in the pectic compound network.

Pectinases are enzymes that degrade pectins (Rombouts and Pilnik, 1980). All enzymes that degrade pectic components are named pectic enzymes or pectin complex enzymes (Ten Have, 2000; Table 2.3). *B. cinerea* secretes either one or multiple

isozymes of a variety of pectinases, as well as other pectic enzymes during the infection process (Ten Have, 2000) (Table 2.3). Pectin lyase (PnL) degrades only the backbone of pectin (Ten Have, 2000) (Figure 2), whereas polygalacturonases (PGs) and pectate lyase (PeL) degrade the backbone of de-methylated pectin (Ten Have, 2000) (Figure 2), i.e. polygalacturonic acid (PGA), or stretches of PGA embedded in pectin or rhamnogalacturonan I. Pectin methylesterase (PME) demethylates pectin to form pectate, which will consequently be degraded by PGs and PeLs (Ten Have, 2000) (Figure 2). The difference between PGs and lyases lies in their respective degradation products. PGs hydrolyze the α-1,4-glycosidic bond, which will result in the formation of GA (Rombouts and Pilnik, 1980), whereas pectin and pectate lyases catalyze a β-elimination leading to a α-4,5 unsaturated GA at the non-reducing end of the molecule (Ten Have, 2000; Figure 2.2). A further discrimination exists between pectin and pectate lyase, since the latter requires $Ca^{2+}$ for optimal functioning (Rombouts and Pilnik, 1980).
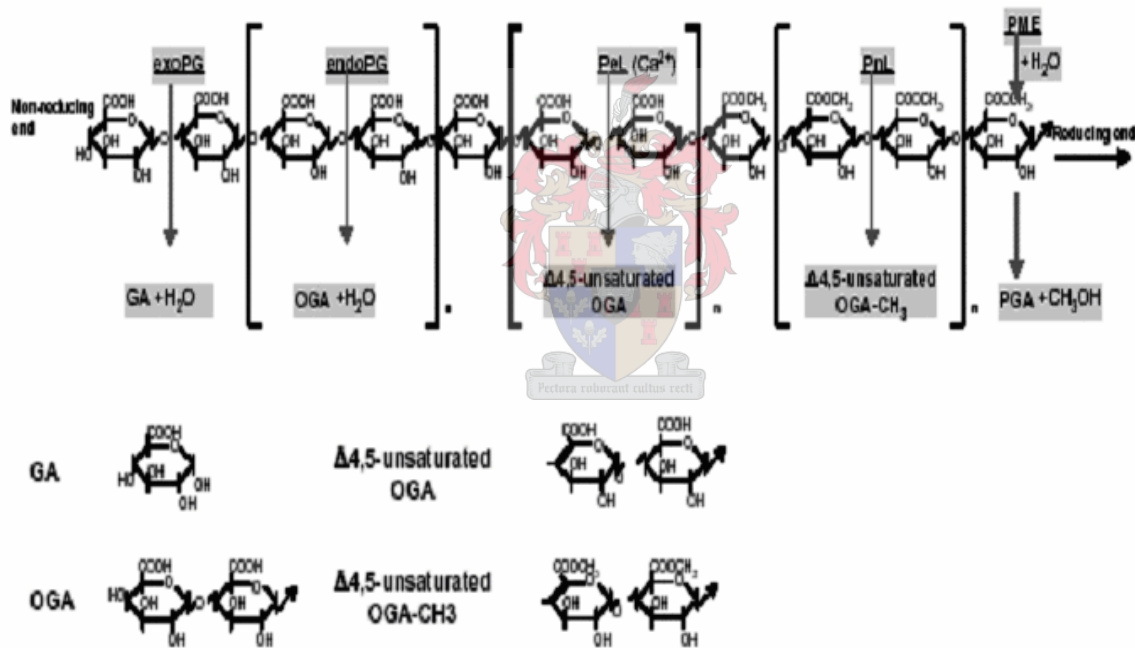


**Figure 2.2** Pectinase activities on a galacturonan-molecule with methylated and non-methylated stretches. Polygalacturonases (exoPG and EPG) hydrolyze polygalacturonic acid (PGA) at the α-1,4 glycosidic bond resulting in monogalacturonic acid (GA) and oligogalacturonic acid (OGA) respectively. Pectate and pectin lyase (PeL and PnL) perform a β-elimination, the latter on methylated galacturonan, resulting in OGA with a α-4,5 unsaturated bond at the non-reducing end. Pectin methylesterase (PME) demethylates pectin resulting in PGA (Ten Have, 2000).

## 2.3.2 THE STRUCTURE AND FUNCTION OF EPGs

PGs are encoded by multigene families and the members show a high degree of polymorphism (Annis and Goodwin, 1997; Markovic and Janecek, 2001). The activity of most EPGs is dependent on the esterification status of the $C_2$, $C_3$ or $C_6$ positions (Esquerré-Tugayé *et al.,* 1999). Most fungi produce multiple EPG isozymes that differ in their enzymatic properties, molecular weight and regulation (Annis and Goodwin, 1997; Markovic and Janecek, 2001). The size of the EPG multigene family has been shown to vary with the specificity of the interaction. Broad range pathogens, such as *Botrytis* and *Sclerotinia* spp. (Fraissinet-Tachet *et al.*, 1995; Ten Have *et al.*, 1998), contain more EPG encoding family members (up to six) than pathogens with a restricted host range, such as *C. lindemuthianum* that only infects bean plants, and contains only two EPG encoding genes (Centis *et al.*, 1997). This generalization, however, is not true for all species that have been analyzed (Gotesson *et al.*, 2002). The presence of an array of EPGs with a wide range of modes of action, specific activities, substrate specificities and pH optima does hold certain advantages for the fungal pathogen. The diversity of EPGs may give a higher adaptive ability to the pathogen by allowing invasion in a variety of different conditions and hosts, as well as protecting the fungus from loss of pathogenicity.

Multiple EPG isoforms may be the result of post-translational modifications of proteins and/or the presence of multiple genes. Glycosylation has been observed in many fungal EPGs, which can be crucial for the activity of the enzyme (Gotesson *et al.*, 2002; Ten Have *et al.*, 2001; Wubben *et al.*, 1999a). It has also been shown that glycosylation leads to higher enzyme stability (Stratilová *et al.*, 1998), as well as increased resistance to proteases (Rudd *et al.*, 2001) in many fungal EPGs. Another structural feature that influences the functional diversity of EPGs, is the presence/absence, as well as the type of N-terminal extension. This region plays a role in substrate specificity and interaction with specific areas of the pectin polymer (Gotesson *et al.*, 2002; Parenicova *et al.*, 2000).

All pectic enzymes share the same central core organization consisting of parallel β-strands forming a large right-handed helix defined as a parallel β-helix (Jenkins and Pickersgill, 2001). The parallel β-helix fold provides the pectic enzymes with stability, since these enzymes function in a variety of harsh extracellular environments. Structures of microbial pectic enzymes that were recently solved by X-ray crystallography include two PeLs of *Erwinia chrysanthemi* (Lietzke *et al.*, 1994; Yoder and Jurnak, 1995b) and a PeL of *Bacillus subtilis* (Pickersgill *et al.*, 1994); two PnLs of *A. niger* (Mayans *et al.*, 1997; Vitali

*et al.*, 1998); three PGs of *Erwinia carotovora* (Pickersgill *et al.*, 1998), *A. niger* (van Santen *et al.*, 1999) and *F. moniliforme* (Federici *et al.*, 2001); a rhamnogalacturonase of *Aspergillus aculeatus* (Petersen *et al.*, 1997) and a PME of *E. chrysanthemi* (Jenkins *et al.*, 2001).

Figure 2.3 gives an indication of the genomic organization of the *B. cinerea* EPG encoding gene family that consists of six genes (Wubben *et al.*, 1999a). The different *Bcpg* genes contain between one and four introns, except for *Bcpg*1 that contains no introns. The predicted EPGs of *B. cinerea* are between 371 and 515 amino acids in length, all containing a predicted signal sequence (Nielsen *et al.*, 1997). Monobasic (Arg) and dibasic (Lys-Arg) cleavage sites are present in most of the *Botrytis* EPGs (Arg for BcPG1 and BcPG2; Lys-Arg for BcPG4 and BcPG5) (Benen *et al.*, 1996). BcPG6 contains no apparent propeptide cleavage site, whereas the structure of BcPG3 differs completely from the other five genes. The structure of the BcPG3 protein is enlarged because of the presence of an N-terminal extension, comprising approximately 150 amino acids.
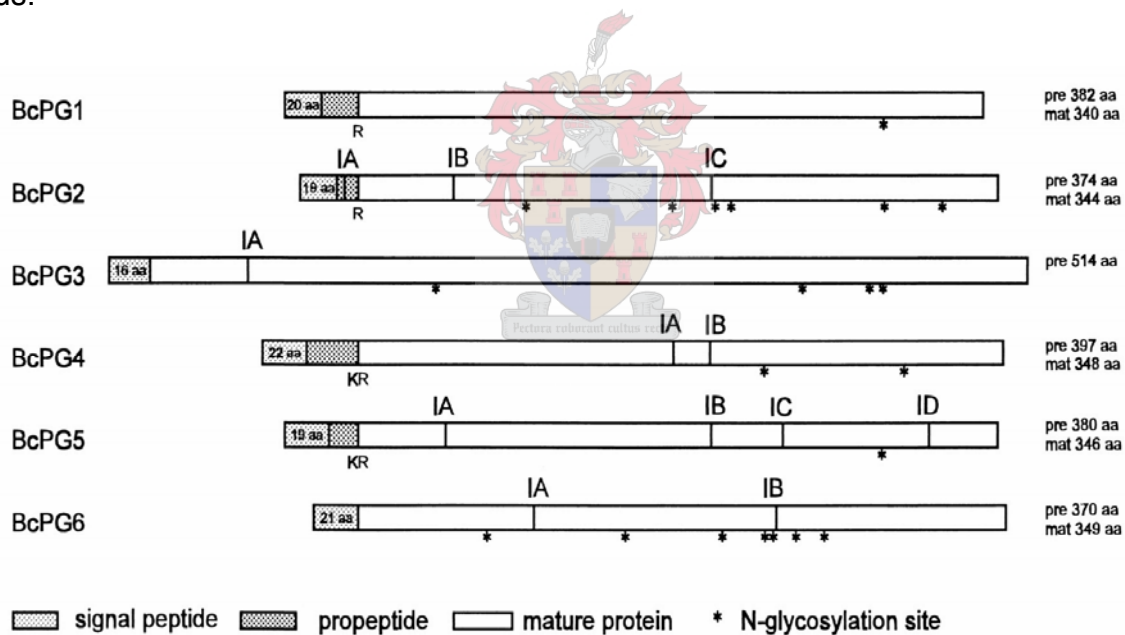


**Figure 2.3** Genomic organization of the endopolygalacturonase gene family of *Botrytis cinerea.* Indicated are the positions of the introns in the original DNA sequence (1A, 1B, 1C and 1D), the presence of a putative monobasic (R) or dibasic (KR) cleavage sites, and the presence of N-glycosylation signals (*). Also shown in the figure are the derived lengths of unprocessed proteins (pre) and mature processed proteins (mat). The lengths of predicted signal peptides for each of the proteins are indicated in the respective boxes (Wubben *et al.*, 1999a).

The predicted signal peptide of BcPG3 consists of 16 amino acids (Nielsen *et al.*, 1997), but no putative mono- or dibasic cleavage sites are present. The sequence identity at amino acid level within the EPG family of *B.* cinerea varies between 34 and 73% (Table 2.4; Wubben *et al.*, 1999a). Nine amino acid residues that are strictly conserved in all EPGs (Benen *et al.*, 1996; Ten Have *et al.*, 1998) are also present in each of the *Botrytis* EPGs. The presence of N-linked glycosylation sites in all of the EPGs of *B. cinerea* (Figure 2.3) indicates that they might be secreted as glycosylated enzymes (Wubben *et al.*, 1999a).

The expression of the complete *Bcpg* gene family is regulated in a sophisticated manner that enables the fungus to efficiently hydrolyze the heterogenous pectin substrate under various environmental conditions. It has been demonstrated that BcPG1 and BcPG2 contributes to the virulence of *B. cinerea* (Kars *et al.*, 2004; Ten Have *et al.*, 1998), but it is conceivable that other CWDEs can also play a part in the infection process of *B. cinerea*. All members of the EPG family are possible virulence factors, although most probably not under all circumstances, since expression patterns of the various genes differ greatly (Wubben *et al.*, 1999b). It is also possible that other pectolytic and non-pectolytic enzymes assist the EPGs in degrading the pectic compound network and other cell wall components respectively (Wubben *et al.*, 1999a). Of the EPG encoding genes, *Bcpg2* is the most likely candidate to encode a virulence factor (Ten Have *et al.*, 2001); the transient expression of this gene, during infection of tomato leaves, suggests a function early in pathogenesis, i.e. lesion expansion (Ten Have *et al.*, 2001). Recently Kars *et al.*, (2004) showed that deletion of this gene leads to reduced virulence in *B. cinerea*. *Bcpg3* is also probable to encode a virulence factor, based on expression data (Ten Have *et al.*, 2001). This gene is expressed at low pH in host tissues such as apple fruit. Furthermore, *B. cinerea* secretes acids during growth in liquid medium and *in planta* (Germeier *et al.*, 1994); this might result in the acidification and subsequent onset of *Bcpg3* gene expression. *Bcpg5* is less likely to encode a virulence factor, since general expression of this gene in tomato leaves is low (Ten Have *et al.*, 2001). There is no strict correlation between the level of expression of a gene and the activity of the resulting protein, but expression is at least a requirement for its involvement in pathogenesis. This gene however, may play a greater part in other host plants. Predictions for possible functions of *Bcpg4* and *Bcpg6* exist based on the regulation of their expression, suggesting a role in nutrient provision (Ten Have *et al.*, 2001), but no definite function has been assigned to these genes.

**Table 2.4 Sequence pair distance of the endoplygalacturonase (EPG) encoding gene family of *Botrytis cinerea* as determined by the CLUSTALW program (Wubben *et al.,* 1999a).**

| | Sequence pair distance (% identity) of EPG: | | | | |
|---|---|---|---|---|---|
| **EPG** | **BcPG2** | **BcPG3** | **BcPG4** | **BcPG5** | **BcPG6** |
| **BcPG1** | 72.0 | 38.7 | 65.8 | 72.7 | 55.0 |
| **BcPG2** | | 34.8 | 59.2 | 63.7 | 54.3 |
| **BcPG3** | | | 33.6 | 36.7 | 48.3 |
| **BcPG4** | | | | 67.7 | 48.5 |
| **BcPG5** | | | | | 55.2 |

To determine the contribution of CWDEs to the virulence of fungi, many infection systems have been studied. These studies revealed that several pectic enzymes act as virulence factors for pathogenic fungi, i.e. two inducible pectate lyases from *Nectria hematcocca* (Rogers *et al.*, 2000) encoded by *pelA* and *pelD*; pectin methylesterase encoded by *Bcpme1* from *B. cinerea* (Cimerman *et al.*, 2003); the EPG encoded by *pecA* from *Aspergillus flavus* (Shieh *et al.*, 1997); one of the six EPG genes from *B. cinerea* (Ten Have *et al.*, 1998) as well as the EPG from *Alternaria citri* (Isshiki *et al.*, 2001).

## 2.4 THE PGIP-EPG INTERACTION

## 2.4.1 THE PGIP:EPG COMPLEX RESULTS IN INCREASED CONCENTRATIONS OF OLIGOGALACTURONIDES THAT ACTIVATE PLANT DEFENCE RESPONSES

From the perspective of the plant, the plant needs to recognize an invader before the defence system can be triggered. Plants can induce a rapid defence response, referred to as the hypersensitive response (HR) that results in localized cell and tissue death at the site of infection to prevent spreading of the disease (Dixon *et al.,* 1994). The HR depends on the interaction between a dominant or semi-dominant *R* gene product in the plant and the corresponding dominant avirulence (*Avr*) gene product of the fungal pathogen (Flor, 1956). It has been hypothesized that fungal *Avr* products act as ligands whereas *R* gene products function as receptors in protein-protein interactions, which will lead to fungal resistance in plants (Gabriel and Rolfe, 1990; Jones and Jones, 1997). Plant *R-genes*, seem to encode receptors that interact, directly or indirectly via the LRR domain, with elicitors encoded by the fungal *Avr* genes (Gabriel and Rolfe, 1990; Jones and Jones, 1997).

An increase of OGs can be found when the cell wall has been damaged either by CWDEs or mechanical wounding (Bergey *et al.*, 1996). Even at a very low concentration these OGs are able to induce the defence system of plants, as efficiently as pathogens

and their elicitors (Darvill *et al.*, 1992; Farmer *et al.*, 1991). Galacturonides, products of pectinase activity, with a degree of polymerization (DP) between ten and thirteen in soybean (Nothnagel *et al.*, 1983) and between four and six in tomato (Simpson *et al.*, 1998) are quite effective as elicitors.

In general, EPGs exhibit *endo*, *exo* or a combination between *endo* and *exo* modes of action (Cook *et al.*, 1999). A convenient way of determining the mode of cleavage of the EPG is to compare the rate at which the enzyme reduces the viscosity of a substrate solution with the reducing rate of a known *exo* enzyme on the same substrate. EPGs acting via *endo* cleavage, hydrolize homogalacturonan by binding to the substrate at random sites along its length, catalyzing the hydrolysis of a glycosidic linkage and finally disassociating from the reaction products to be free to initiate another random cleavage of the substrate (Cook *et al.*, 1999). This mode of action produces a mixture of oligomers with a DP ranging from the monomer to the maximum DP of the starting polymer. Random cuts in homogalacturonan rapidly lower the DP and the solution viscosity and do so more rapidly than could be achieved by *exo* enzymes that remove monomers from one end of the molecule (Cook *et al.*, 1999). A combination of exoPGs and EPGs produce oligomers smaller than EPGs alone and also reduce the viscosity of a substrate medium at a rate less than that obtained from EPGs, but greater than that obtained with an exoPG (Cook *et al.*, 1999).

EPGs are mainly classified into two groups based on their mode of action on the pectic substrate. Single-attack enzymes generate long oligomers that are progressively converted to shorter fragments, whereas multiple-attack or processive enzymes will accumulate short oligomers already from the start of the reaction (Parenicova *et al.*, 2000). Fungal EPGs elicit plant defence responses i.e. the accumulation of phytoalexins, the synthesis of lignin, ethylene, proteinase inhibitor I as well as the production of β-1,3-glucanase (Annis and Goodwin, 1997; De Lorenzo *et al.*, 1997; Lang and Dornenburg, 2000). The plant cells detect the fungal EPGs directly by either soluble or membrane-bound "receptors" (Enkerli *et al.*, 1999; Furman-Matarasso *et al.*, 1999; Rouet-Mayer *et al.*, 1997) or indirectly via pectin-derived OG fragments. When a fungal pathogen attacks a plant, long OGs are produced in the plant tissue. This action is the cause of single-attack as well as multiple-attack enzymes and occurs in the presence of the plant-derived PGIPs. *In vitro* experiments have demonstrated that in the presence of PGIP, the activity of EPGs is reduced in such a way that the release of elicitor-active OGs is

favoured above the depolymerization of active OGs into inactive molecules (Cervone *et al.*, 1986).

It is still unknown how OGs activate defence responses in plants. Early responses thought to be part of the OG signal transduction pathways include membrane depolarization with $H^+$ influx and $K^+$ efflux (Mathieu *et al.*, 1998; Spiro *et al.*, 1998), elevation of cytosolic $Ca^{2+}$ concentration (Cessna and Low, 2001; Lecourieux *et al.*, 2002; Navazio *et al.*, 2002), activation of GTP binding proteins (Legendre *et al.*, 1992), stimulation of phospholipases C and $A_2$ (Legendre *et al.*, 1993; Narváez-Vásquez *et al.*, 1999), induction of receptor-like protein kinases (Montesano *et al.*, 2001), induction of protein phosphorylation and phosphatase activity (Droillard *et al.*, 1997) and the activation of mitogen-activated kinases (Droillard *et al.*, 2000; Stratmann and Ryan, 1997). OGs also induce the transient generation of active oxygen species (AOS). These include $O_2^-$, $H_2O_2$ and OH and they are produced by the action of plasma membrane-associated NAD(P)H oxidases and/or apoplastic-localized oxidases (Bolwell, 1999). Production of AOS in plants is thought to contribute to plant cell death, which is required for the hypersensitive response (HR), but OGs themselves have failed to induce cell death (Binet *et al.*, 2001; Mathieu *et al.*, 1991) suggesting that the production of AOS is not essential for the induction of the HR reaction (Dorey *et al.*, 1999).

Downstream, OGs are involved in the transcriptional activation of defence-related genes i.e. enzyme-encoding genes of the phenylpropanoid pathway as well as related pathways leading to the production of phytoalexins and also of genes involved in the metabolism and/or synthesis of jasmonic acid (Klarzynski *et al.*, 2000; Ridley *et al.*, 2001). In bean cells and seedlings of *Arabidopsis thaliana* it has been found that PGIP transcripts accumulate after the addition of OGs (Bergmann *et al.*, 1994; Ferrari *et al.*, 2003). An endogenous plant EPG in tomato leaves, with the possible role of inducing systemic acquired resistance, is induced by OGs (Bergey *et al.*, 1999). It is not clear if the production of OGs is necessary for the elicitor activity of fungal EPGs. OGs generated by fungal EPGs might mediate the elicitor activity of the enzyme (Boudart *et al.*, 2003), since it has been shown that AOS production and necrosis induced by a *C. lindemuthianum* EPG in tobacco plants, requires a functional catalytic site.

## 2.4.2 EPGs DIFFER IN THEIR SUSCEPTIBILITIES TO PGIPs

Previously published data have shown that different EPGs are not equally inhibited by different PGIPs (Stotz *et al.*, 1994). Table 2.4 shows data of the ability of four PGIPs to

inhibit the activity of seven EPGs. Based on this data, these EPGs can be subdivided into two subgroups relative to their susceptibilities to inhibition by the PGIPs. The first subgroup includes EPGs from *Cochliobus sativus, C. lindemuthianum, Cryphonectria parasitica* and *A. niger* EPGI, which are inhibited by all PGIPs tested. The second subgroup represents EPGs from *F. moniliforme, Postia placenta* and *A. niger* PGII. These EPGs are resistant to inhibition of at least one PGIP tested, although the PG from *P. placenta* was resistant to all the PGIPs tested. High performance anion exchange chromatography-pulsed amperimetric detection (HPAEC-PAD) profiles showed that the EPGs from subgroup 2 generated large amounts of OGs with DP 6-15 whereas EPGs from subgroup 1, excluding *A. niger* EPGI, generated OGs with DP 1-5, where mono- and digalacturonic acid dominated the products of these enzymes (Cook *et al.*, 1999). *A. niger* EPGI produced monogalacturonic acid and was found to be an exoPG. In further experiments the influence of PGIPs on the mode of action of EPGs were also tested. HPAEC-PAD profiles confirmed that PGIPs slow the rate of hydrolysis, but do not affect the profile of OG products (Cook *et al.*, 1999). A comparison of the amino acid sequences of exo-, E- and E/exoPGs showed that E/exoPGs are more closely related to EPGs than to exoPGs (Cook *et al.*, 1999). An alignment of the endo/exo and endo sequences indicates a few single amino acid differences between the two subgroups, which might be crucial in specificity of the PGIP-EPG interaction (Cook *et al.*, 1999).

**Table 2.5  Effect of PGIPs from different cultivars on the abilities of endolpygalacturonases (EPGs) to generate reducing sugars from PGA as assessed by a colorimetric assay[A] (Lever, 1972).**

| | EPGs and % reduction of reducing sugars | | | | | | |
|---|---|---|---|---|---|---|---|
| **PGIPs** | *F. moniliforme* | *A. niger II* | *P. placenta* | *A. niger* I | *C. lindemuthianum* | *C. sativus* | *C. parasitica* |
| **Bean (Pinto)** | >99 | 96 | 0 | 98 | 97 | 99 | 95 |
| **Bean (Blue Lake)** | 98 | 95 | 0 | 98 | 97 | 99 | 94 |
| **Tomato** | 0 | 10 | 0 | 90 | 97 | 95 | 91 |
| **Pear** | 0 | 0 | 0 | 93 | 96 | 97 | 94 |

[A]PAHBAH (p-hydroxy benzoic acid hydrazide). PGIPs were combined with EPGs, followed by introduction of substrate. Digestion of the substrate was allowed to proceed typically 1 hour before colorimetric determination of reducing sugar liberated during digestion. Values represent percent reduction of reducing sugars formed in the presence of sufficient PGIP to saturate the EPG, relative to the corresponding EPG control.

## 2.4.3 AMINO ACID SUBSTITUTIONS CHANGE THE SPECIFICITY OF PGIP-EPG INTERACTIONS

Because *R*-gene products are believed to function as receptors for pathogen-encoded avirulence (Avr) proteins, it has been hypothesized that sequence variation within LRRs influences recognition specificity (Leckie *et al.*, 1999). Comparison of members of the *Cf* family has identified the β-sheet/β-turn region as a hypervariable region, probably responsible for the ligand specificity in this class of proteins (Parniske *et al.*, 1997). In *P. vulgaris*, the *pgip* gene family consists of at least five members and perhaps as many as 15 (Frediani *et al.*, 1993). Previous data suggest that different members of the family encode PGIPs with nearly identical biochemical characteristics but distinct specificity, i.e. the ability to interact with different fungal PGs (Desiderio *et al.*, 1997). A comparative study was done between PGIP1 and PGIP2 of *P. vulgaris* based on their ability to recognize fungal PGs and the role of the single amino acids that distinguish PGIP1 and PGIP2 in the specific interactions with EPGs from *A. niger* and *F. moniliforme*. In this comparative study, Leckie *et al* (1999) showed that the residues that determine recognition specificity of PGIP reside in the region flanking the predicted β-sheet/β-turn structure of the protein and that a single amino acid variation in this motif can confer to PGIP a new recognition capability.

A total of 26 nucleotide changes, which result in 10 amino acid changes, are present between PGIP1 and PGIP2 (Leckie *et al.*, 1999). These changes were more frequent in the region encoding the C-terminal half of the LRR domain. A high number as well as specific distribution of non-synonymous or amino acid changing (11/26) substitutions compared with synonymous or silent (15/26) substitutions were also observed. Seven of the eleven non-synonymous substitutions lead to amino acid differences in the LRR domain. Five of these are internal to the xxLxLxx motif, predicted to form the solvent-exposed β-sheet/β-turn structure of the protein, whereas two other amino acid substitutions are very close to this region and their side chains most likely are also solvent exposed. The remaining three variant amino acids are outside the LRR domain: two are located in the signal peptide of the protein and therefore do not affect the structure of the mature protein and one residue in the C-terminal region of the protein. Instead, most synonymous nucleotide changes correspond to residues located outside the β-sheet/β-turn structural motif.

Caprari *et al* (1996) found that a single mutation in the His 234 position is critical for the enzymatic and macerating activities of the EPG from *F. moniliforme,* but not for the PGIP-binding capacity of the enzyme.

## 2.5  CONCLUDING REMARKS

The interaction between proteins and their respective ligands has drawn a lot of attention in the past few years.  A large proportion of studies have focussed on linking sequence data with protein structure and the role of these structures in protein:protein interactions. The knowledge obtained from these studies provided a large foundation for future studies, specifically protein-ligand interactions.  Also within the field of plant-pathogen interactions significant headway has been made to elucidate the role of specific protein-ligand interactions.  In many cases these interactions provide the basis for disease resistance and future, more focused studies undoubtebly will contribute to improving plant disease resistance.
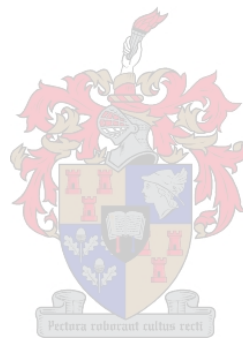
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# RESEARCH RESULTS

Isolation, heterologous expression and *in vivo* analysis of the polygalacturonase gene family from a highly virulent strain of *Botrytis cinerea*
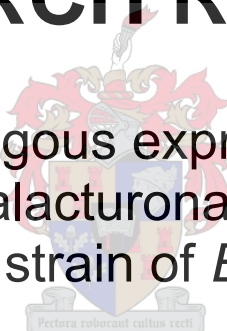
This manuscript forms a part of a study that will be submitted for publication in a journal to be decided

# ISOLATION, HETEROLOGOUS EXPRESSION AND *IN VIVO* ANALYSIS OF THE POLYGALACTURONASE GENE FAMILY FROM A HIGHLY VIRULENT STRAIN FROM *BOTRYTIS CINEREA*.

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## ABSTRACT

*Botrytis cinerea* has served as a model organism to study the mechanistic aspects of plant fungal diseases for many years. Recently, the endopolygalacturonases (EPGs) secreted by the fungus have received specific attention and especially the interaction of these fungal virulence factors with the polygalacturonase-inhibitor proteins (PGIPs) found in many plant species have sparked several studies. In this study we have isolated and cloned six EPG encoding genes from a highly virulent South African *B. cinerea* strain isolated from grapevine. Sequence comparison between these genes and those previously reported, yielded approximately 98% homology on amino acid level. The isolated genes from the South African isolate were over-expressed in both *Saccharomyces cerevisea* and *Nicotiana benthamiana*. Gene expression was confirmed for all the EPG-encoding genes in the yeast and the plant host, but *in vitro* activity, with the methods and conditions used, could only be established for EPG2 in both systems. However, severe phenotypical effects were observed in the tobacco leaves transiently expressing the EPG-encoding genes, suggesting *in vivo* EPG activity for all the heterologously produced proteins. To assess the physiological effects of the EPGs in the tobacco leaves, chlorophyll fluorescence was used as a diagnostic tool to monitor the variable fluorescence yield in the infiltrated tissues. Using the differences in $^{Fv}/_{Fm}$ ratios, we were able to use this technique to establish an *in vivo* system for analyzing the cellular and physiological effects of EPG on leaf tissue, without relying on macroscopic symptom development. This technique also proved useful to study the interactions between two of the EPGs (EPG1 and 2) and an inhibitor, the PGIP1 from grapevine, on an *in vivo* level. The preliminary chlorophyll fluorescence analysis of the EPG:PGIP *in vivo* interaction suggests that the VvPGIP1 inhibits the EPG1 and 2 from this *B. cinerea* strain. These two EPGs have also been shown previously to be essential for virulence and pathogenicity.

Moreover, the observed EPG2:VvPGIP1 interaction is the first interaction shown with this EPG and an inhibitor thus far.

## 3.1 INTRODUCTION

*Botrytis cinerea*, the causal agent of grey mould, attacks various agricultural crops and infection leads to important economic losses (Jarvis, 1977). The pathogen displays typical necrotrophic behaviour and destroys plant cells by penetrating the epidermis at the anticlinal position, where after it lives on the decaying tissue (Mansfield and Richardson, 1981). To facilitate the infection process, *B. cinerea* secretes several cell wall degrading enzymes (CWDEs) capable of breaking down the complex cell wall structure of the hosts. The first of these enzymes to be released, the endopolygalacturonases (EPGs), cause cell wall degradation as well as plant tissue maceration (Basham and Bateman, 1975; Bauer *et al.*, 1977; De Lorenzo *et al.*, 1997; Idnurm and Howlet, 2001). Plants typically respond when exposed to fungal EPGs with their own array of defence responses, one of which include the activity of the polygalacturonase-inhibiting proteins (PGIPs). PGIPs are leucine-rich repeat (LRR) proteins, located in the cell walls of many plant species and are capable of inhibiting the activity of fungal EPGs (Cervone *et al.*, 1989; De Lorenzo *et al.*, 1997; Powell *et al.*, 2000). It is hypothesized that PGIP protects the plant by slowing the hydrolytic activity of the EPGs on the cell wall (Cervone *et al.*, 1989), as well as prolonging the presence of signalling-active oligogalacturonides, which have been shown to be active during induced plant defence responses (Cessna and Low, 2001; Lecourieux *et al.*, 2002; Navazio *et al.*, 2002).

The EPGs in *Botrytis* is encoded by a six member *Bcpg* gene family which is regulated in a complex manner that enables the fungus to efficiently hydrolyze the heterogeneous pectin substrate in plant cell walls under various environmental conditions. It has been demonstrated that BcPG1 and BcPG2 are important virulence factors of *B. cinerea* (Kars *et al.*, 2004; Ten Have *et al.*, 1998). Recent work suggests that the EPGs as such can elicit plant defence responses, proposing that under certain conditions the EPGs act as signals in an activity-independent manner (Poinssot *et al.*, 2003). Unfortunately, most systems that are currently used to evaluate EPG activity and interaction with proteins such as the PGIPs, are based on *in vitro* systems that cannot assess the effect of EPG activity, and/or EPG inhibition on whole plant physiology.

A grapevine PGIP-encoding gene, *Vvpgip1* (Genbank Ac: AF499451), has recently been isolated in our laboratory and shown to inhibit crude extraxts containing a mixture of

EPGs from *B. cinerea.* In an attempt to study the nature of the interaction between the grapevine PGIP and the individual EPGs, we have cloned the six EPG-encoding genes, *BcPG1-6*, from a hypervirulent South African *B. cinerea* isolate. To obtain pure enzymes for subsequent analyses, the genes were over-expressed in two heterologous systems. The first system used was *Saccharomyces cerevisiae* and the genes were over-expressed under the control of a strong inducible promoter. Gene expression analyses were performed followed by crude protein analysis and *in vitro* EPG activity tests. The individual EPG encoding genes were also transiently over-expressed in *Nicotiana benthamiana* leaves using *Agrobacterium* infiltration (Yang *et al.*, 2000). The physiological effects of over-expressing the genes were assayed visually as well as by measuring the efficiency of photosynthesis. Measuring the efficiency of photosynthesis as a diagnostic tool was further evaluated by transiently over-expressing the grapevine PGIP1 encoding gene (*Vvpgip1*) either on its own, or in combination with *Bcpg1* or *Bcpg2* respectively. By comparing the variable yield of photosynthesis, we were able to show that VvPGIP1 affects the activity of BcPG2 *in vivo*, although no evidence of *in vitro* interaction between the two enzymes could be found. A modified version of the plant two-hybrid system described by Yang *et al.*, (2000) were also used to study possible EPG:PGIP interactions. Our results clearly show that current *in vitro* assaying systems are insufficient to study the interactions between PGIP en EPGs and we propose the inclusion of *in vivo* plant based systems in these experiments to provide a more holistic perspective.
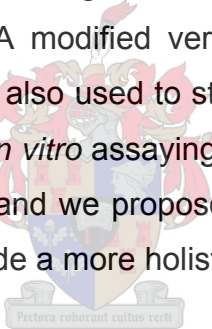
## 3.2  MATERIALS AND METHODS

### 3.2.1  Strains, culture and plant growth conditions

All bacterial and yeast strains are listed in Table 3.1. *Escherichia coli* strains were grown at 37°C in Luria Bertani (LB) media, supplemented with 100 μg/mL ampicillin or 50 μg/mL spectinomycin for the selection of transformants. *S. cerevisiae* strain YPH259 were grown at 30°C in yeast peptone dextrose broth (YPD) and transformants were selected from selective synthetic (SC) media lacking uracil. *Agrobacterium tumefaciens* strains were grown at 28°C in LB media, supplemented with 0.1% (w/v) glucose and 15 μg/mL rifampicin. Transformants were selected on the same media supplemented with 50 μg/mL kanamycin.

A highly virulent South African *Botrytis cinerea* isolate was obtained from the Department of Plant Pathology, Stellenbosch University (Coertze and Holz, 1999). Spores

were germinated and grown in MS medium (Murashige and Skoog, 1962), buffered with 10 mM phosphate buffer (KHPO$_4$), pH 7.0 and supplemented with 1% (w/v) glucose and 0.05% (w/v) yeast extract.  Cultures were grown on a rotary shaker at 180 rpm at room temperature for 72 h post inoculation, prior to harvesting the mycelium.

*N. benthamiana* seeds were germinated in peat pellets (Jiffy, Norway) under natural light conditions in a greenhouse.  Plants were maintained at 26°C and 60% relative humidity.

**Table 3.1**  Strains and plasmids used in this study

| Strains or Plasmids | Relevant features or insert | Source or reference |
|---|---|---|
| **Eschericia coli strain** | | |
| DH5α | supE44lacU169(φ80lacZM15hsdR17recA1gyrA96thi-1relA1) | Invitrogen Life Technologies, Carlsbad, USA |
| **Saccharomyces cerevisiae strain** | | |
| YPH259 | *ura3, his3, leu2, trp1* and Gal$^+$ | Sikorski and Hieter, 1989 |
| **Agrobacterium tumefaciens strain** | | |
| EHA105 | Disarmed, succinomopine strain | Hood *et al.*, 1993 |
| **Plasmids** | | |
| pGEM-T-Easy | pGEM5Zf(+) based PCR cloning vector | Promega,Madison, USA |
| pBI121 | Binary vector | Jefferson *et al.*, 1987 |
| pART7 | CaMV 35S promoter, transcriptional termination region of the octopine synthase gene | Gleave, 1992 |
| pART27 | RK2 minimal replicon, ColE1 origin of replication, Tn7 resistance gene (bacterial selectable marker), kanamycin resistance gene (T-DNA transfer) | Gleave, 1992 |
| pYES2 | *GAL1* promoter, *URA3* gene, 2μ origin | Invitrogen Life Technologies |
| pGAD | *GAL4* activation domain | James *et al.*, 1996 |
| pGBD | *GAL4* binding domain | James *et al.*, 1996 |
| GBS | *GAL4* binding site | Yang *et al.*, 2000 |
| pGEM(Bcpg1) | *Bcpg1* cloned into pGEM-T-Easy | This study |
| pGEM(Bcpg2) | *Bcpg2* cloned into pGEM-T-Easy | This study |
| pGEM(Bcpg3) | *Bcpg3* cloned into pGEM-T-Easy | This study |
| pGEM(Bcpg4) | *Bcpg4* cloned into pGEM-T-Easy | This study |
| pGEM(Bcpg5) | *Bcpg5* cloned into pGEM-T-Easy | This study |
| pGEM(Bcpg6) | *Bcpg6* cloned into pGEM-T-Easy | This study |
| pGAD(Bcpg1) | *Bcpg1* cloned into *Eco*RI and *Sal*I sites of pGAD | This study |
| pGAD(Bcpg2) | *Bcpg2* cloned into *Eco*RI and *Sal*I sites of pGAD | This study |
| pGAD(Bcpg3) | *Bcpg3* cloned into *Eco*RI and *Sal*I sites of pGAD | This study |
| pGAD(Bcpg4) | *Bcpg4* cloned into *Eco*RI and *Pst*I sites of pGAD | This study |
| pGAD(Bcpg5) | *Bcpg5* cloned into *Eco*RI and *Sal*I sites of pGAD | This study |
| pGAD(Bcpg6) | *Bcpg6* cloned into *Eco*RI and *Sal*I sites of pGAD | This study |
| pGEM(Bcpg1+AD) | *Bcpg1* containing the activation domain cloned into pGEM-T-Easy | This study |
| pGEM(Bcpg2+AD) | *Bcpg2* containing the activation domain cloned into pGEM-T-Easy | This study |
| pGEM(Bcpg5+AD) | *Bcpg5* containing the activation domain cloned into pGEM-T-Easy | This study |
| pGEM(Bcpg6+AD) | *Bcpg6* containing the activation domain cloned into pGEM-T-Easy | This study |
| pART7(Bcpg1) | *Bcpg1* cloned into *Eco*RI and *Xba*I sites of pART7 | This study |
| pART7(Bcpg2) | *Bcpg2* cloned into *Eco*RI and *Xba*I sites of pART7 | This study |
| pART7(Bcpg3) | *Bcpg3* cloned into *Eco*RI and *Xba*I sites of pART7 | This study |
| pART7(Bcpg4) | *Bcpg4* cloned into *Eco*RI and *Xba*I sites of pART7 | This study |
| pART7(Bcpg5) | *Bcpg5* cloned into *Eco*RI and *Xba*I sites of pART7 | This study |
| pART7(Bcpg6) | *Bcpg6* cloned into *Eco*RI and *Xba*I sites of pART7 | This study |
| pART7(Bcpg1+AD) | *Bcpg1* containing the activation domain cloned into *Xho*I site of pART7 | This study |
| pART7(Bcpg2+AD) | *Bcpg2* containing the activation domain cloned into *Xho*I site of pART7 | This study |
| pART7(Bcpg3+AD) | *Bcpg3* containing the activation domain cloned into *Bam*HI and *Hind*III sites of pART | This study |
| pART7(Bcpg4+AD) | *Bcpg4* containing the activation domain cloned into *Bam*HI and *Hind*III sites of pART | This study |
| pART7(Bcpg5+AD) | *Bcpg5* containing the activation domain cloned into *Xho*I | This study |

| | | |
|---|---|---|
| | site of pART7 | |
| pART7(Bcpg6+AD) | *Bcpg6* containing the activation domain cloned into *Xho*I site of pART7 | This study |
| pART27(Bcpg1) | *Bcpg1* cloned into *Not*I site of pART27 | This study |
| pART27(Bcpg2) | *Bcpg2* cloned into *Not*I site of pART27 | This study |
| pART27(Bcpg3) | *Bcpg3* cloned into *Not*I site of pART27 | This study |
| pART27(Bcpg4) | *Bcpg4* cloned into *Not*I site of pART27 | This study |
| pART27(Bcpg5) | *Bcpg5* cloned into *Not*I site of pART27 | This study |
| pART27(Bcpg6) | *Bcpg6* cloned into *Not*I site of pART27 | This study |
| pART27(Bcpg1+AD) | *Bcpg1* containing the activation domain cloned into *Not*I site of pART27 | This study |
| pART27(Bcpg2+AD) | *Bcpg2* containing the activation domain cloned into *Not*I site of pART27 | This study |
| pART27(Bcpg3+AD) | *Bcpg3* containing the activation domain cloned into *Not*I site of pART27 | This study |
| pART27(Bcpg4+AD) | *Bcpg4* containing the activation domain cloned into *Not*I site of pART27 | This study |
| pART27(Bcpg5+AD) | *Bcpg5* containing the activation domain cloned into *Not*I site of pART27 | This study |
| pART27(Bcpg6+AD) | *Bcpg6* containing the activation domain cloned into *Not*I site of pART27 | This study |
| pYES2(Bcpg1) | *Bcpg1* cloned into *Eco*RI and *Xba*I sites of pYES2 | This study |
| pYES2(Bcpg2) | *Bcpg2* cloned into *Eco*RI and *Xba*I sites of pYES2 | This study |
| pYES2(Bcpg4) | *Bcpg4* cloned into *Eco*RI and *Xba*I sites of pYES2 | This study |
| pYES2(Bcpg5) | *Bcpg5* cloned into *Eco*RI and *Xba*I sites of pYES2 | This study |
| pGEM(Vvpgip1) | *Vvpgip1* cloned into PGEM-T-easy | De Ascensao, 2001 |
| pBI121(Vvpgip1) | *Vvpgip1* cloned into *Sma*I and *Sac*I sites of pBI121 | De Ascensao, 2001 |
| pGEM(Vvpgip1-signal) | *Vvpgip1* without signal peptide cloned into pGEM-T-Easy | This study |
| pGBD(Vvpgip1-signal) | *Vvpgip1* without signal peptide cloned into *Bam*HI and *Sal*I sites of pGBD | This study |
| pGEM(Vvpgip1-signal+BD) | *Vvpgip1* without signal peptide containing the binding domain cloned into pGEM-T-Easy | This study |
| pART7(Vvpgip1-signal+BD) | *Vvpgip1* without signal peptide containing the binding domain cloned into *Xho*I site of pART7 | This study |
| pART27(Vvpgip1-signal+BD) | *Vvpgip1* without signal peptide containing the binding domain cloned into *Not*I site of pART27 | This study |

### 3.2.2 *B. cinerea* cultivation and BcPG induction

To facilitate the cloning of the six EPG encoding genes of *B. cinerea*, cell cultures were induced according to Wubben *et al* (1999). In short, *B. cinerea* spores were germinated as described and the mycelium harvested after 72 h with a 100 μm nylon cell strainer (Falcon) and washed thoroughly with buffered MS medium. Wet mycelium was transferred to fresh MS medium containing 1% (w/v) glucose or 1% (w/v) polygalacturonic acid (PGA), buffered to pH 7 with 10 mM phosphate buffer, or 1% (w/v) galacturonic acid (GA), buffered to pH 8 with 0.1 M Tris-HCl buffer. Following transfer, the cultures were incubated at room temperature on a rotary shaker and mycelium was harvested after 6 h, 12 h and 24 h as described. The harvested mycelium was blotted dry on filter paper, flash frozen in liquid nitrogen and stored at -80°C.

### 3.2.3 RNA isolation and Northern blot analysis

Total RNA was isolated from *S. cerevisiae* by vortexing cells in the presence of glassbeads in 1 mL extraction buffer consisting of 300 μL phenol (pH 8) and 700 μL extraction solution (200 mM Tris-HCl pH 8, 1.5% (w/v) SDS, 300 mM LiCl, 10 mM

Na$_2$EDTA pH 8, 1% (w/v) Na-deoxycholate, 1% (w/v) Igepal CA-630 with 5 mM Thiourea and 1% (w/v) Na-Metabisulfate added after autoclaving) (Joubert, 2004). *B. cinerea* and *N. benthamiana* tissue were ground in liquid nitrogen and added to 1 mL of extraction buffer. The homogenates were vortexed briefly, 200 μL of chloroform was added and the mixture was vortexed briefly again. The mixture was centrifuged subsequently for 10 min at 12 000 g and nucleic acids were precipitated from the supernatant with one volume of isopropanol. Precipitated genomic DNA was removed from the isopropanol solution by pipetting. The precipitated RNA was pelleted by centrifugation, washed with 70% ethanol and briefly dried at 65°C. RNA was denatured for 15 min at 95°C, separated on a 1.2% (w/v) formaldehyde gel and blotted onto Hybond N membranes (Amersham Biosciences, Buckinghamshire, UK) with 1× TAE buffer. *B. cinerea* EPG genes were PCR-labeled, using 10× DIG dNTP labeling mixture from Roche Diagnostics (Mannheim, Germany) with the following primers: BcPG1 5' and BcPG1 3', BcPG2 5' and BcPG2 3', BcPG3 5' and BcPG3 3', BcPG4 5' and BcPG4 3', BcPG5 5' and BcPG5 3', BcPG6 5' and BcPG6 3' (Table 3.2). All hybridizations and signal development were done according to the DIG application manual for filter hybridization from Roche Molecular Biochemicals.

### 3.2.4 DNA manipulations

All plasmids used are listed in Table 3.1. Unless otherwise stated, DNA manipulations were done according to Sambrook *et al* (1989). *Bcpg1* was amplified from *B. cinerea* genomic DNA with the BcPG1 5' and BcPG1 3' primers. The cDNA copies of the *Bcpg2-6* genes were obtained in a two step reverse transcriptase reaction. cDNA was synthesized from total RNA isolated from *B. cinerea* grown in glucose after 24 h and 12 h respectively for *Bcpg3* and *5*; *B. cinerea* grown for 6 h in PGA for both *Bcpg2* and *4* and *B. cinerea* grown for 6 h in GA for *Bcpg6*. All first strand cDNA reactions were done using SuperScript$^{TM}$ II RNase H$^-$ Reverse Transcriptase from Invitrogen according to the manufacturer's specifications. The *Bcpg2-6* genes were amplified from first strand cDNA using gene specific primers, BcPG2 5' and BcPG2 3', BcPG3 5' and BcPG3 3', BcPG4 5' and BcPG4 3', BcPG5 5' and BcPG5 3' as well as BcPG6 5' and BcPG6 3' (Table 3.2).

PCR amplifications were done using Expand high fidelity DNA polymerase from Roche Diagnostics in a reaction volume of 50 μL, consisting of 1× Expand high fidelity PCR buffer without MgCl$_2$, 200 μM dNTPs, 200 nM of each primer, approximately 5 ng template DNA. MgCl$_2$ concentration was adjusted optimally for each reaction. Amplification conditions included an initial DNA denaturation step at 94°C for 2 min, followed by 30 cycles of DNA

denaturation at 98°C for 15 sec, primer annealing at temperatures according to the specific primer melting temperatures and elongation at 72°C, allowing 1 min for each kb amplified. A final cycle of elongation was allowed at 72°C for 2 min. T4 DNA ligase and restriction enzymes were purchased from Roche Diagnostics (Mannheim, Germany) and used according to the manufacturer's specification. All sequencing was done by the DNA Sequencing Facility, Department of Genetics, US, using an ABI PRISM$^R$ 3100 automated DNA sequencer from Applied Biosystems. The gene products were cloned into pGEM-T-Easy and sequenced for verification. All sequence alignments were done using the AlignX algorithm as part of the Vector NTI 9 series from Informax. The genes were subsequently excised from pGEM-T-Easy with *Eco*RI and *Spe*I and sub-cloned into the *Eco*RI and *Xba*I sites of pART7 and pYES2 (Invitrogen, Carlsbad, USA), a yeast expression vector containing a galactose-inducible promoter.

Gene cassettes containing the CaMV 35S promoter and transcriptional termination region of the octopine synthase gene were excised from pART7 with *Not*I and sub-cloned into the corresponding site of the plant-expression vector pART27. The pART27-constructs were transformed via electroporation to *A. tumefaciens*. *S. cerevisae* was transformed with the pYES constructs according to the manufacturer's specifications.

The pBI121 construct containing *Vvpgip1* (Genbank Ac: AF499451) was obtained from De Ascensao (2001). In short, *Vvpgip1* was cloned into the *Sma*I and *Sac*I sites of the binary vector pBI121, replacing the β-glucuronidase gene to yield pBI121(*Vvpgip1*).

Two-hybrid constructs were prepared by amplifying the EPG- encoding genes from the pGEM-T-Easy constructs with the following primers: EcoRI-PG1 5' and SalI-PG1 3', EcoRI-PG2 5' and SalI-PG2 3', EcoRI-PG3 5' and SalI-PG3 3', EcoRI-PG4 5' and PstI-PG4 3', EcoRI-PG5 5' and SalI-PG5 3', EcoRI-PG6 5' and SalI-PG6 3' (Table 3.2). All amplification products were cloned into the pGEM-T-Easy vector. The products were excised from the pGEM-T-Easy vector with the primer specific restriction enzymes and sub-cloned into the corresponding sites of pGADc2, the vector containing the transcription activation domain sequence. The AD:*Bcpg3* and *4* fusions were excised from pGADc2 with *Bgl*II and *Hind*III and subcloned into the *Bam*HI and *Hind*III sites of pART7. The AD:*Bcpg1, 2, 5* and *6* fusions were amplified from pGADc2 with the SalI-AD 5' primer and the corresponding gene specific 3' primers (Table 3.2). The amplification products were cloned into the pGEM-T-Easy vector. Gene products containing the activation domain sequence were excised with *Sal*I and sub-cloned into the *Xho*I site of pART7. The fusions were subsequently excised from pART7 with *Not*I and sub-cloned into the corresponding

site of pART27. *Vvpgip1* was amplified with the BamHI-PGIP 5' and XbaI-PGIP 3' primers to obtain the sequence without the nucleotides encoding for the predicted signal peptide (Table 3.2). The amplification product was cloned into the pGEM-T-Easy vector and subsequently excised with *Bam*HI and *Xba*I and sub-cloned into the corresponding sites of pGBDc2. The BD:*Vvpgip1* fusion was amplified from pGBDc2 with the SalI-BD 5' and XbaI-PGIP 3' primers and cloned into the pGEM-T-Easy vector. The fusion was excised from pGEM-T-Easy with *Sal*I and *Xba*I and sub-cloned into the *Xho*I and *Xba*I sites of pART7. The pART7-construct was digested with *Not*I and the BD:*Vvpgip1* expression casette was sub-cloned into the *Not*I site of pART27. All final two-hybrid constructs were sequenced to verify their integrity. The Gal binding site (GBS) construct was obtained from Professor Yinong Yang, Department of Plant Pathology, University of Arkansas, Fayetteville.

### 3.2.5 Transient plant transformation

The leaves of eight week old *N. benthamiana* plants were infiltrated with *A. tumefaciens* according to Yang *et al* (2000). For the chlorophyll fluorescence experiments, five separate leaves per plant were infiltrated with pART27 and pART(*Bcpg1-6*). The two-hybrid infiltrations were done in triplicate and leaves were infiltrated with the GBS construct in combination with pART27(BD-*pgip*) and/or pART27(AD-*Bcpg1-6*). After infiltration, the plants were maintained as described. Leaf samples were taken 5 days post infiltration, flash-frozen in liquid nitrogen and stored at -80°C for subsequent extractions of RNA and proteins.

### 3.2.6 Chlorophyll fluorescence

The maximum quantum efficiency of photosystem II, the ratio of variable to maximum fluorescence ($^{Fv}/_{Fm}$), was measured with a Hansatech Plant Efficiency Analyzer (PEA). Prior to infiltration, *N. benthamiana* leaves were dark adapted for one hour using plastic leafclips with the shutter blades in the closed position. Measurements were performed by attaching the sensor unit to the clips and exposing the leaves for 5 s to a maximum light intensity of 3000 $\mu mol.m^{-2}.s^{-1}$. $\Delta^{Fv}/_{Fm}$ values were determined by deducting the $^{Fv}/_{Fm}$ value of a leaf 8 days post-infiltration from the pre-infiltration $^{Fv}/_{Fm}$ value of the same leaf.

**Table 3.2** Primers used in this study

| Primer | Sequence | Paired with | Template | Product |
|--------|----------|-------------|----------|---------|
| BcPG1 5' | ATGGTTCAACTTCTCTCAATGG | BcPG1 3' | *Botrytis* cDNA | *Bcpg1* |
| BcPG1 3' | TAAGATGTTTAACACTTGACACCAG | BcPG1 5' | *Botrytis* cDNA | *Bcpg1* |
| BcPG2 5' | AAAATGGTTCATATCACAAGCC | BcPG2 3' | *Botrytis* cDNA | *Bcpg2* |
| BcPG2 3' | ATTTAGCAAGAAGCTCCGGT | BcPG2 5' | *Botrytis* cDNA | *Bcpg2* |
| BcPG3 5' | ATGCAGTTGTCTTCAAGTAGCAA | BcPG3 3' | *Botrytis* cDNA | *Bcpg3* |
| BcPG3 3' | CATTTATGATGGGCATCCAG | BcPG3 5' | *Botrytis* cDNA | *Bcpg3* |
| BcPG4 5' | ATGCCTTCCACCAAGTCCAT | BcPG4 3' | *Botrytis* cDNA | *Bcpg4* |
| BcPG4 3' | CGCTTAAGAGCAAGAACCAA | BcPG4 5' | *Botrytis* cDNA | *Bcpg4* |
| BcPG5 5' | ATGGTTAAGTTTTCTGCCTGTCT | BcPG5 3' | *Botrytis* cDNA | *Bcpg5* |
| BcPG5 3' | GAAAGTGTCTACAAGGAACAAGAGA | BcPG5 5' | *Botrytis* cDNA | *Bcpg5* |
| BcPG6 5' | ATGCCTAAGAACTCTCAGATCTCTG | BcPG6 3' | *Botrytis* cDNA | *Bcpg6* |
| BcPG6 3' | TTTATGCGGGACAGCCAGTT | BcPG6 5' | *Botrytis* cDNA | *Bcpg6* |
| EcoRI-PG1 5' | GAATTCATGGTTCAACTTCTCTCAATGG | SalI-PG1 3' | pGEM(PG1) | *Bcpg1* |
| SalI-PG1 3' | GTCGACTAAGATGTTTAACACTTGACACCAG | EcoRI-PG1 5'/AD 5' | pGEM/pGAD(PG1) | *Bcpg1*/+AD |
| EcoRI-PG2 5' | GAATTCAAAATGGTTCATATCACAAGCC | SalI-PG2 3' | pGEM(PG2) | *Bcpg2* |
| SalI-PG2 3' | GTCGACATTTAGCAAGAAGCTCCGGT | EcoRI-PG2 5'/AD 5' | pGEM/pGAD(PG2) | *Bcpg2*/AD |
| EcoRI-PG3 5' | GAATTCATGCAGTTGTCTTCAAGTAGC | SalI-PG3 3' | pGEM(PG3) | *Bcpg3* |
| SalI-PG3 3' | GTCGACTTATGATGGGCATCCAGTAG | EcoRI-PG3 5'/AD 5' | pGEM/pGAD(PG3) | *Bcpg3*/+AD |
| EcoRI-PG4 5' | GAATTCATGCCTTCCACCAAGTCCAT | PstI-PG4 3' | pGEM(PG4) | *Bcpg4* |
| PstI-PG4 3' | CTGCAGTTAAGAGCAAGAACCAACAA | EcoRI-PG4 5'/AD 5' | pGEM/pGAD(PG4) | *Bcpg4*/+AD |
| EcoRI-PG5 5' | GAATTCATGGTTAAGTTTTCTGCCTG | SalI-PG5 3' | pGEM(PG5) | *Bcpg5* |
| SalI-PG5 3' | GTCGACCTACAAGGAACAAGAGACAC | EcoRI-PG5 5'/AD 5' | pGEM/pGAD(PG5) | *Bcpg5*/+AD |
| EcoRI-PG6 5' | GAATTCATGCCTAAGAACTCTCAGATCTCTG | SalI-PG6 3' | pGEM(PG6) | *Bcpg6* |
| SalI-PG6 3' | GTCGACTTATGCGGGACAGCCAGTTG | EcoRI-PG6 5'/AD 5' | pGEM/pGAD(PG6) | *Bcpg6*/+AD |
| SalI-AD 5' | GTCGACGCCAATTTTAATCAAAGTGG | See above | pGAD(PGs) | See above |
| SalI-BD 5' | GTCGACATGAAGCTACTGTCTTCTATCG | XbaI-PGIP 3' | pGBD(VvPGIP1-signal) | *Vvpgip1*-signal+BD |
| BamHI-PGIP 5' | GGATCCTTCGTTGCAACCCAAAAGACAAAAAG | PGIP 3' | pGEM(VvPGIP1) | *Vvpgip1*-signal |
| XbaI-PGIP 3' | TCTAGAACTTGCAGCTCTGGAGTGGAG | PGIP 5'/BD 5' | pGEM(VvPGIP1) | *Vvpgip1*-signal/+BD |

### 3.2.7 Two-hybrid analysis

Two-hybrid constructs were constructed as described. *N. benthamiana* leaves were infiltrated in triplicate with the two-hybrid fusion constructs. Each EPG-encoding gene fused to the AD sequence and *Vvpgip1* fused to the BD sequence was infiltrated individually with the GBS construct (Yang *et al.*, 2000), serving as negative controls. Each of the AD-fusions was also infiltrated together with the *Vvpgip1*-BD-fusion and the GBS construct. A slightly modified protocol of Yang *et al* (2000) was used to determine the levels of GUS-activity after the infiltration. A leaf disc from each infiltration site was collected 5 days post infiltration, grinded with liquid nitrogen and vortexed in 500 µL GUS extraction buffer (50 mM phosphate [NaHPO$_4$] buffer pH 7.0, 10 mM 2-mercaptoethanol, 0.1% (w/v) sodium laurylsarcosine and 0.1% (v/v) Triton X-100). Cell debris were sedimented by centrifugation for 10 min at 12 000 g and 50 µL of the supernatant was mixed with 250 µL of GUS assay solution (2 mM 4-methylumbelliferyl-D-glucuronide in extraction buffer) and 200 µL GUS extraction buffer. A 50 µL aliquot was immediately removed and added into 250 µL stop buffer (0.2 M sodium carbonate) to be used as

internal controls for normalization. The rest of the mixture was incubated at 37°C for 180 min. GUS activity was determined using a Bio-Tek FL600 Microplate fluorescence reader (Winooski, Vermont, USA). Protein concentration of the tissue homogenates was determined according to Bradford (1976), with Bradford reagent from Bio-Rad (Munich, Germany), using bovine serum albumin (BSA) as a standard.

### 3.2.8 Crude protein isolation

Crude proteins from *S. cerevisiae* were isolated with a commercial yeast protein extraction buffer from Pierce (Rockford, Illinois, USA) according to the manufacturer's specifications. The supernatant was used as extracellular protein fraction, while the yeast cell debris was lysed and used as intracellular protein fraction. Leaves infiltrated with *A. tumefaciens* were ground in liquid nitrogen with a mortar and pestle to a fine powder with subsequent addition of extraction buffer (20 mM sodium acetate [CH$_3$COONa] buffer [pH 6]) to a final ratio of 2 mL buffer per 1 g starting tissue. The mixture was centrifuged at 10 000 g for 10 min and the supernatant collected. Protein concentration was determined as described

### 3.2.9 Agarose diffusion plate assays

Crude protein isolations from transformed yeasts and *Agrobacterium*-infiltrated tobacco leaves were tested for EPG activity in an agarose diffusion plate assay (Taylor and Secor, 1988). Small holes were punched in the matrix consisting of 1% (w/v) Type II agarose (Sigma, St Louis, USA), 0.5% (w/v) PGA and 100 mM CH$_3$COONa, buffered to pH 4.5 or pH 6.0. The crude protein extracts from *S. cerevisiae* (0.5 µg for extracellular- and 5 µg for intracellular extracts) and *N. benthamiana* (5 µg) were pipetted into the wells and incubated for 16 h at 30°C. Zones were clarified with 6 N hydrochloric acid (HCl). A clear halo around the holes was indicative of EPG activity.

### 3.3 RESULTS

#### 3.3.1 Isolation and cloning of *B. cinerea* EPG encoding genes

A hypervirulent *B. cinerea* strain, isolated from a South African vineyard, was grown in liquid cultures with glucose, galacturonic acid or polygalacturonic acid as carbon source. The mycelium was harvested and RNA isolated at various time points. The RNA was used to synthesize the cDNA that subsequently served as template to amplify EPG

encoding genes 2 to 5 described by Wubben *et al.*, 1999 (Fig. 3.1). *Bcpg1* was isolated from the gDNA obtained from the RNA extraction (see section 3.2.3). The size of the amplification products corresponded well with the open reading frames (ORFs) of the genes described. Sequencing of the cDNA copies revealed 98% identity on amino acid level with the published open reading frames. None of the predicted introns could be detected in any of the cDNA copies and the intron splice sites corresponded 100% to that of the predicted introns.
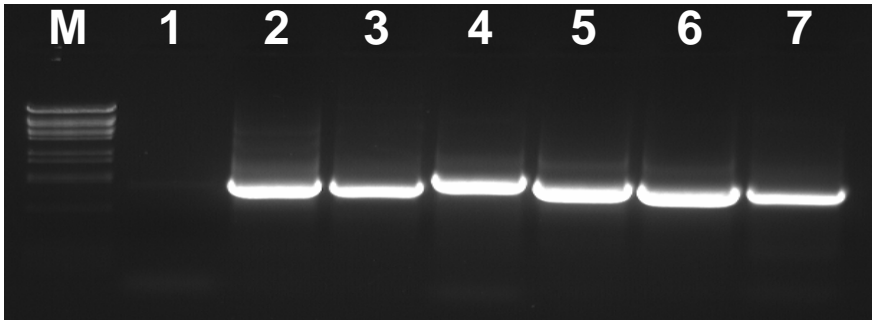


**Figure 3.1** *B. cinerea* endopolygalacturonase (EPG) encoding genes amplified from cDNA, synthesized from total RNA. λ-DNA digested with *BstE*II was used as the size marker (M). Lane 1 represents the negative control, amplified from total RNA. Lane 2 represents *Bcpg1* (1146 kb) amplified from *Botrytis* gDNA grown in glucose-containing MS media; lane 3, *Bcpg2* (1122 kb) amplified from cDNA synthesized from RNA of *Botrytis* grown in PGA-containing MS media after 6 h; lane 4, *Bcpg3* (1302 kb) amplified from cDNA synthesized from RNA of *Botrytis* grown in glucose-containing MS media after 24 h; lane 5, *Bcpg4* (1191 kb) amplified from cDNA synthesized from RNA of *Botrytis* grown in PGA-containing MS media after 6 h; lane 6, *Bcpg5* (1140 kb) amplified from cDNA synthesized from RNA of *Botrytis* grown in glucose-containing MS media after 12 h and lane 7, *Bcpg6* (1113 kb) amplified cDNA synthesized from RNA of *Botrytis* grown in GA-containing MS media after 6 h.

### 3.3.2 Heterologous expression of the *Bcpg1-6* genes

#### 3.3.2.1 *S. cerevisiae*

To confirm expression of the *Bcpg1-6* genes in *S. cerevisiae* YPH259, the transformed yeast strains were grown in liquid selective media for 48 h and subsequently induced with selective media containing galactose in stead of glucose. Total RNA, as well as intracellular- and extracellular proteins were extracted 48 h post-induction. Expression of the heterologous genes was confirmed with a Northern blot assay by using the coding sequences of the *Bcpg1-6* genes as probes (Fig. 3.2). The sizes of the observed transcripts corresponded well with the sizes of the respective ORFs. Transgene

expression levels for uninduced cells varied significantly and ranged from no transcript levels (*Bcpg1*) to transcript levels approximating that of the induced cells (*Bcpg5*). Induction by galactose resulted in elevated expression levels for all the transformants tested.



**Figure 3.2** Northern blot analysis of the *Botrytis cinerea* endopolygalacturonase-encoding genes, *Bcpg1-6*, that were heterologously expressed in *S. cerevisiae* YPH259. Expression of *Bcpg1-6* was induced with galactose and RNA was isolated from uninduced- as well as induced cells. Untransformed yeast cells served as negative controls. Total RNA from yeasts transformed with the *Bcpg1-6* genes was probed with the ORFs of the corresponding genes respectively. **A.** A high range RNA ladder from Fermentas was used as a size marker **(M)**. Lanes **1-2**, YPH259 uninduced and induced; Lanes **3-4**, *Bcpg1* uninduced and induced; Lanes **5-6**, *Bcpg2* uninduced and induced; Lanes **7-8**, *Bcpg3* uninduced and induced; Lanes **9-10**, *Bcpg4* uninduced and induced; Lanes **11-12**, *Bcpg5* uninduced and induced; Lanes **13-14**, *Bcpg6*; uninduced and induced **B.** Expression levels were normalized against 18S RNA using the AlphaEase V5.5 software package. Light blue bars indicate normalised expression levels for uninduced cells while dark blue bars indicate induced cells.

EPG activity was assayed in both extracellular and intracellular yeast extracts using an agarose diffusion plate assay as described by Taylor and Secor (1988). Equal amounts of proteins were loaded into small holes punched into the agarose plates containing PGA as substrate and assays were performed at pH 4.5 and pH 6.0. EPG activity is visualized as white or clear zones surrounding the holes. EPG activity could only be observed in the protein extracts from the yeasts transformed with *Bcpg2* (Fig. 3.3). Zones of roughly equal sizes were observed at both pH 4.5 and pH 6.0; this correlates well with the observed broad pH range of BcPG2 (Krooshof *et al.*, 2004). With this specific assay, as well as the conditions tested, no *in vitro* EPG activity could be detected for any of the other heterologously over-expressed *BcPG* genes in yeast.
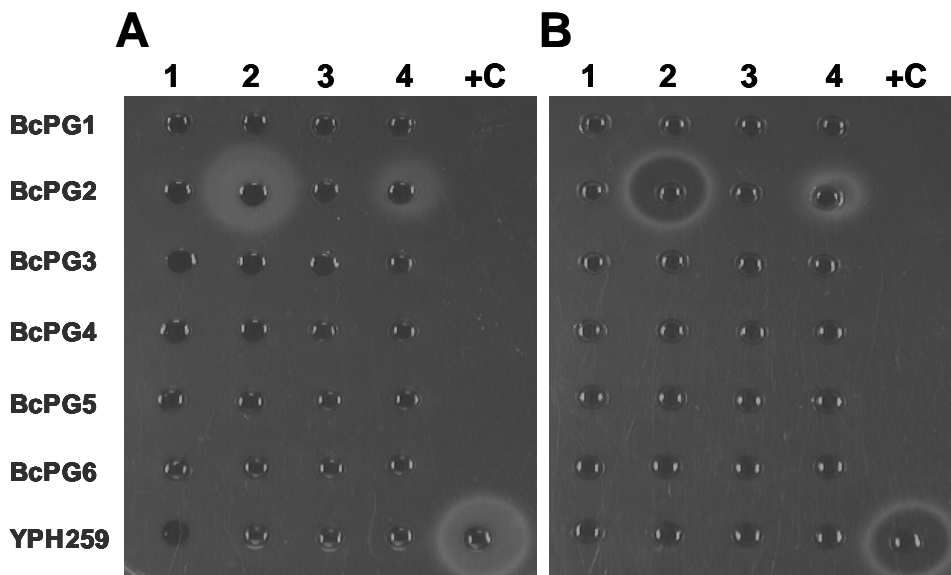
**Figure 3.3** Activity analysis of endopolygalcturonses (EPGs) from *B. cinerea* heterologously expressed in *S. cerevisiae* YPH259**.** The cDNA copies of the *Botrytis* EPG genes (*BcPG1-6*) were cloned into the pYES2 vector from Invitrogen under control of the *GAL1* galactose inducible promoter. Transformed yeasts were grown in selective media with glucose (lanes 1 and 3) for 48 h after which the *GAL1* promoter were induced in selective media containing galactose (lanes 2 and 4) for 48 h. Both the supernatant (lanes 1 and 2) and cytoplasmic extracts (lanes 3 and 4) of the transformants were assayed for EPG activity using an agarose diffusion assay with polygalacturonic acid (PGA) as substrate at pH 4.5 (A) and pH 6.0 (B) as described by Taylor and Secor (1988). Crude EPG extracts from *B. cinerea* and untransformed YPH259 yeast cells were used as positive (+C) and negative controls respectively.

### 3.3.2.2 Transient over-expression in tobacco

To confirm expression of the EPG-encoding genes in *N. benthamiana*, leaves transiently transformed with the *Bcpg1-6* genes were harvested 5 days post infiltration and total RNA was extracted. Expression of the transgenes was confirmed with a Northern blot assay using the coding sequences of the *Bcpg1-6* genes as probes (Fig. 3.4). Again the sizes of the observed transcripts corresponded well with the sizes of the respective ORFs. Transgene expression levels varied substantially between infiltrated constructs, the highest levels of expression were observed for *Bcpg1* and *Bcpg2*, whereas the expression levels for *Bcpg3-6* were very similar but between 4- and 5x lower than that of *Bcpg1*.
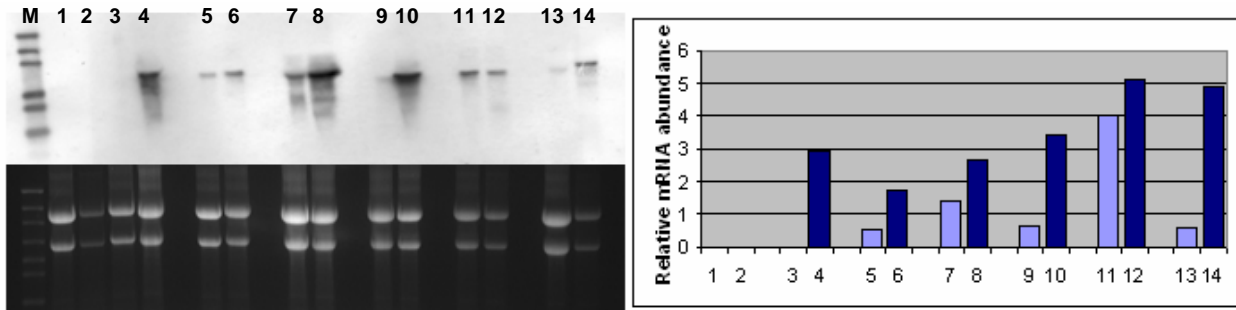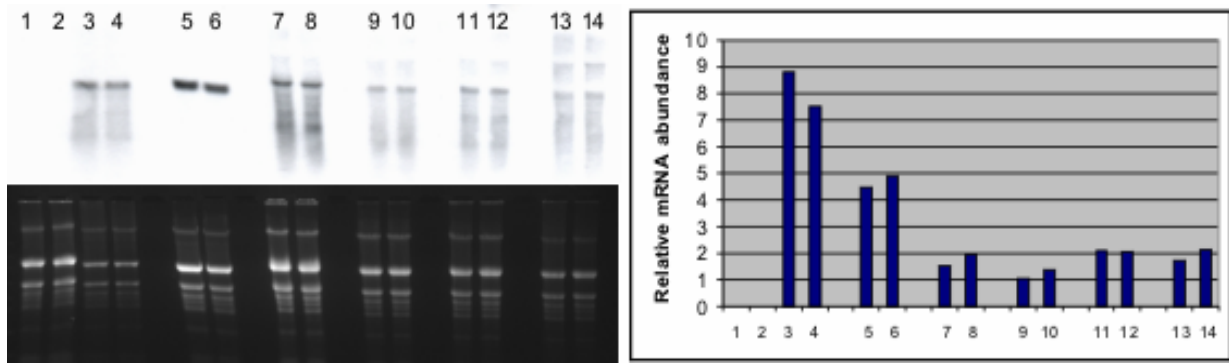
**Figure 3.4** Northern blot analysis of the *Botrytis cinerea* endopolygalacturonase-encoding genes, *Bcpg1-6*, that were heterologously expressed in in *N. benthamiana* using *Agrobacterium* infiltration as described by Yang et al. (2000). Total RNA was isolated in duplicate from infiltrated leaves 5 days post infiltration. Leaves infiltrated with the vector (pART27) alone served as negative controls. Total RNA from leaves infiltrated with the *Bcpg1-6* genes was probed with the ORFs of the corresponding genes respectively. **A.** Lanes **1-2**, Leaves infiltrated with pART27; Lanes **3-4**, pART(*Bcpg1*); Lanes **5-6**, pART(*Bcpg2*); Lanes **7-8**, pART(*Bcpg3*); Lanes **9-10**, pART(*Bcpg4*); Lanes **11-12**, pART(*Bcpg5*); Lanes **13-14**, pART(*Bcpg6*) **B.** Expression levels were normalized against 18S RNA using the AlphaEase V5.5 software package.

Phenotypical effects after transient over-expression of the different *Bcpg* genes in leaves from *N. benthamiana* could be observed from five to eight days post infiltration (Fig. 3.5). Leaves infiltrated with the empty vector showed no phenotypical changes, whereas leaves infiltrated with the *Bcpg* genes showed severe discoloration and/or necrotic lesions. Infiltration with *Bcpg1* resulted in a slight yellow discoloration with large white necrotic lesions appearing approximately eight days post infiltration. Leaves infiltrated with *Bcpg2* and *Bcpg5* did not show severe yellow discoloration, but whitish brown dry necrotic lesions appeared throughout the infiltrated areas. Leaves infiltrated with *Bcpg3, 4* and *6* showed an intense yellow discoloration approximately 5 days post infiltration with no necrotic spots except for small lesions that were visible at the (wounded) infiltration points.
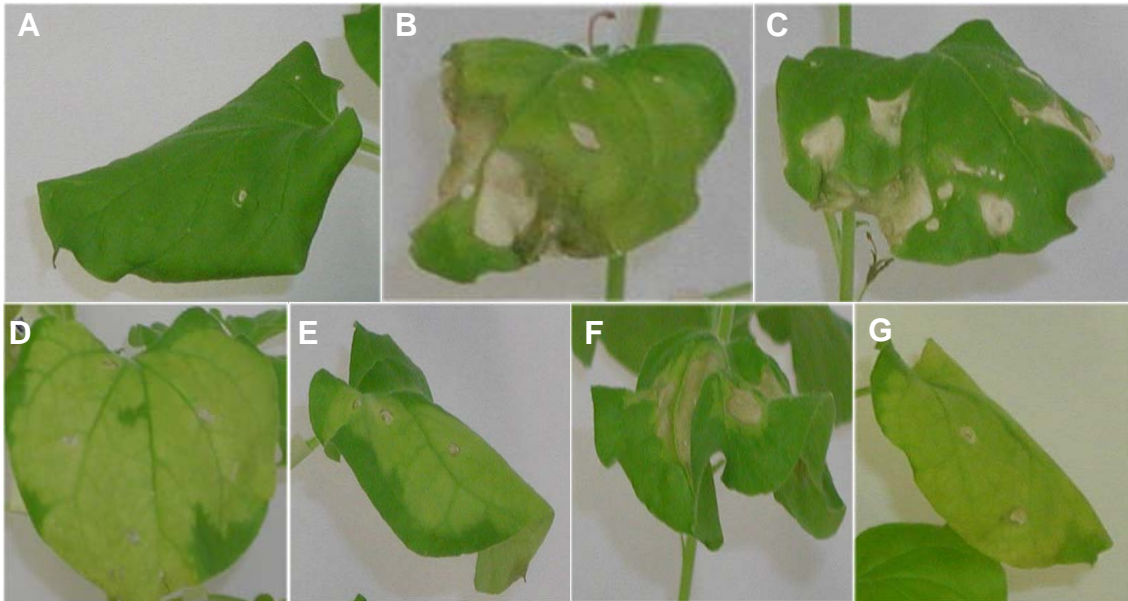
**Figure 3.5** Phenotypical effects displayed on leaves of *Nicotiana benthamiana* transiently over-expresing the *Botrytis cinerea* endopolygalacturonase encoding genes, *Bcpg1-6*. The leaves of eight-week old tobacco plants were infiltrated according to the method of Yang *et al* (2000). Severe leaf necrosis and discolouration were observed eight days post infiltration. **A.** No apparent phenotypical changes were observed in leaves infiltrated with the empty vector, pART27. **B.** Slight yellow discoloration was observed with white necrotic lesions visible in leaves Infiltration with pART(*Bcpg1*). **C.** For leaves infiltrated with pART(*Bcpg2*) no apparent yellow discoloration was observed, but white necrotic lesions appeared throughout the infiltrated areas. **D-G.** Leaves infiltrated with pART(*Bcpg3-6*) respectively. Strong yellow discoloration was observed in all these infiltrated leaves. The leaves infiltrated with pART(*Bcpg5*) (F), also showed irregular lesions.

The crude total protein extracts isolated from infiltrated leaves were tested with the agarose diffusion assay for EPG activity and only showed activity in the leaves infiltrated with the *Bcpg2* construct (Fig. 3.6A). As with the yeast expression system, no apparent difference could be observed at pH 4.5 and pH 6.0.
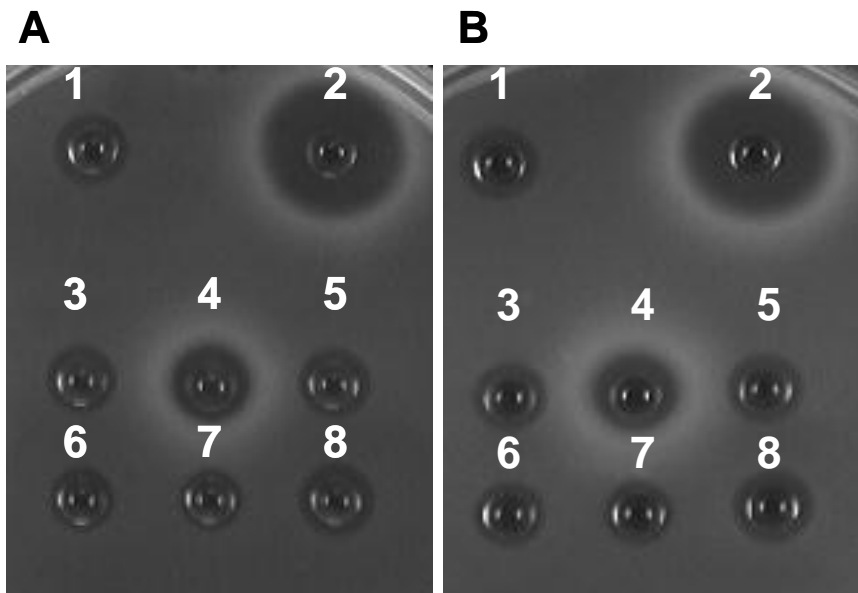
**Figure 3.6** Endopolygalacturonase (EPG) activity at pH 4.5 (A) and pH 6.0 (B) of crude total protein extracts from *Nicotiana benthamiana* leaves transiently over-expresing the *Botrytis cinerea Bcpg1-6* genes. Proteins were isolated 5 days post infiltration and equal amounts loaded into the wells. A clear halo surrounding a well denotes EPG activity and the level of activity is directly proportional to the size of the zones (Taylor and Secor, 1988). Leaves infiltrated with the empty vector (pART27) served as negative control (**1**) whereas a crude *B. cinerea* PG extract were used as positive control (**2**). Wells **3-8** represents crude EPGs encoded by *BcPG1-6* respectively.

The observed physiological effect of over-expressing the *Bcpg* genes in leaves of *N. benthamiana* was quantified by measuring the variable photosynthetic yield (expressed here as $^{Fv}/_{Fm}$) of the leaves before and after infiltration. The $^{Fv}/_{Fm}$ values of all the leaves before infiltration were within the 0.83 range as described for healthy leaves by Maxwell and Johnson, 2000. (Table 3.3). Post-infiltration $^{Fv}/_{Fm}$ values were lower for the leaves infiltrated with the EPG-encoding genes when compared to the respective pre-infiltration values, indicating a reduction in photosynthetic capacity (Table 3.3). The most dramatic change was observed for leaves infiltrated with *Bcpg1* where the infiltration resulted in an approximate 0.3 fold reduction in the $^{Fv}/_{Fm}$ ratio, whereas leaves infiltrated with the empty vector (pART27) showed virtually no change in physiological state as observed with this technique (Fig. 3.7). Infiltrations with buffer alone also did not influence the $^{Fv}/_{Fm}$ ratios (results not shown).

**Table 3.3** Average $^{Fv}/_{Fm}$ values of *N. benthamiana* leaves infiltrated with the *B. cinerea* endopolygalacturonase (EPG) encoding genes. Values were measured pre- and 8 days post-infiltration

| Construct | $^{Fv}/_{Fm}$ *pre-infiltration* | $^{Fv}/_{Fm}$ *post-infiltration* |
|---|---|---|
| pART27(*Bcpg1*) | 0.844±0.003 | 0.559±0.061 |
| pART27(*Bcpg2*) | 0.845±0.003 | 0.777±0.024 |
| pART27(*Bcpg3*) | 0.843±0.001 | 0.676±0.041 |
| pART27(*Bcpg4*) | 0.847±0.002 | 0.678±0.053 |
| pART27(*Bcpg5*) | 0.833±0.004 | 0.648±0.023 |
| pART27(*Bcpg6*) | 0.841±0.003 | 0.636±0.047 |
| pART27 | 0.834±0.002 | 0.808±0.006 |



**Figure 3.7** The effect of transient over-expression of the endopolygalacturonase (EPG)-encoding genes *(BcPG1-6)* from *Botrytis cinerea* on the variable chlorophyll fluorescence yield of *Nicotiana benthamiana* leaves. Five leaves were infiltrated for each construct and the $^{Fv}/_{Fm}$ value of each leaf was measured before infiltration as well as 8 days post-infiltration. $\Delta^{Fv}/_{Fm}$ values were obtained by subtracting the post-infiltration values from the pre-infiltration values. Higher $\Delta^{Fv}/_{Fm}$ values are indicative of greater physiological stress.

To assess the suitability of the tobacco infiltration technique combined with the chlorophyll fluorescence analysis as an *in vivo* system to investigate the interaction between fungal EPGs and plant PGIPs, a grapevine encoding PGIP gene (*Vvpgip1*) was co-infiltrated with *Bcpg1* or *Bcpg2*. Co-infiltration of *Vvpgip1* with both *Bcpg1* and *Bcpg2* resulted in significantly smaller $\Delta^{Fv}/_{Fm}$ (pre-infiltration $^{Fv}/_{Fm}$ − post-infiltration $^{Fv}/_{Fm}$) values, than the infiltrations with *Bcpg1* and *Bcpg2* alone (Fig. 3.8).
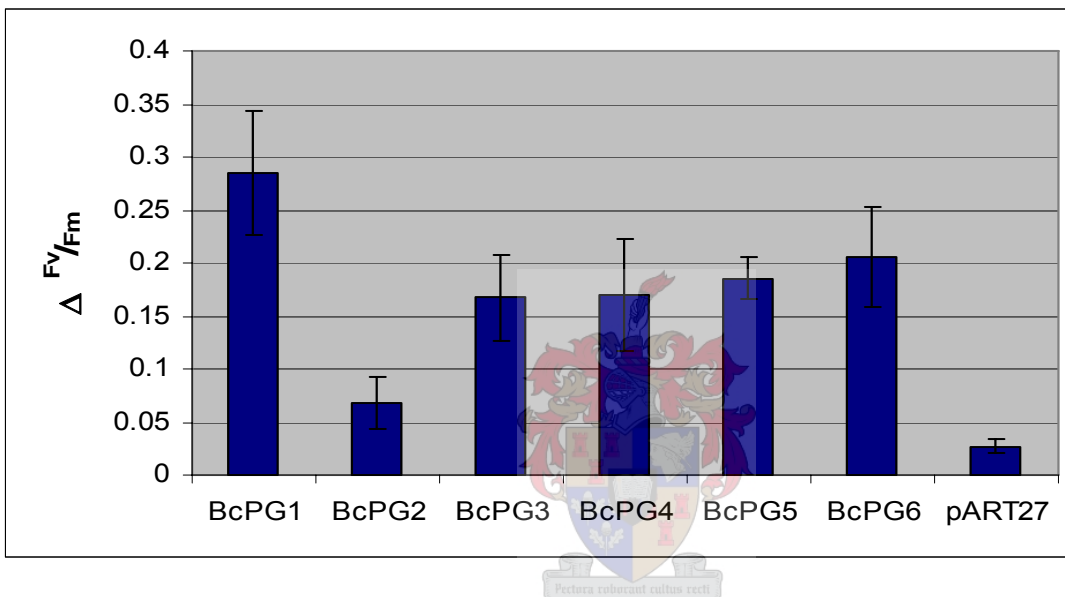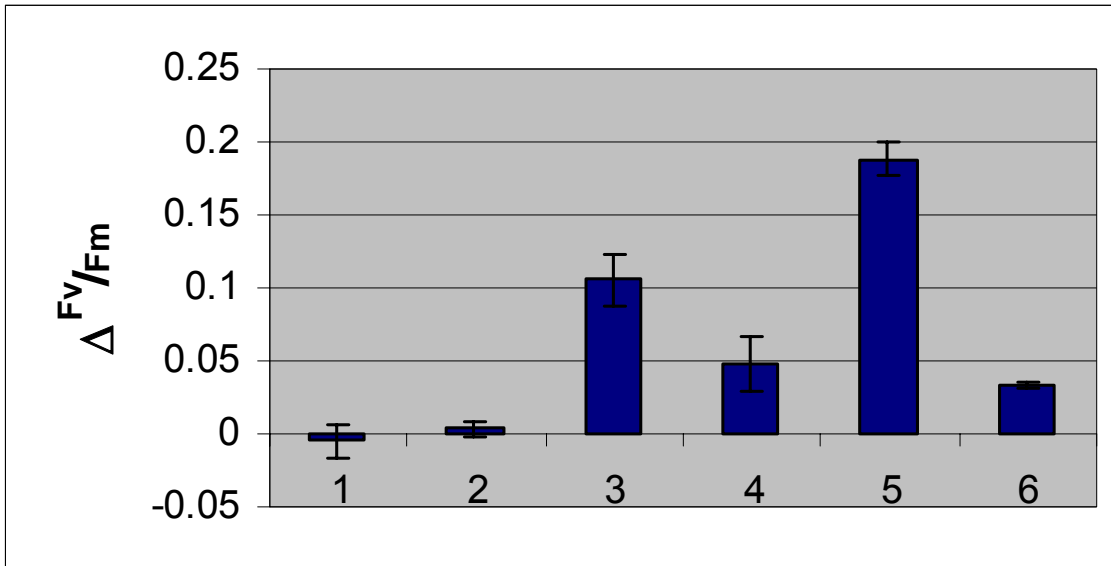
**Figure 3.8** The effect of transient over-expression of the endopolygalacturonase (EPG)-encoding genes *(Bcpg1-2)* from *Botrytis cinerea* alone or co-expressed with the polygalacturonase-inhibiting protein (PGIP)-encoding gene from grapevine (*Vvpgip1*), on the variable chlorophyll fluorescence yield of *Nicotiana benthamiana* leaves. The $^{Fv}/_{Fm}$ value of a leaf was measured before infiltration as well as 5 days post-infiltration and $\Delta^{Fv}/_{Fm}$ values were obtained by subtracting the post-infiltration values from the pre-infiltration values. Lane 1: Leaves infiltrated with the empty vector pART 27; lane 2; leaves infiltrated with *Vvpgip1* alone; lane 3: leaves infiltrated with *Bcpg1*; lane 4: leaves infiltrated with *Vvpgip1* and *Bcpg1*; lane 5: leaves infiltrated with *Bcpg2* and lane 6: leaves infiltrated with *Vvpgip1* and *Bcpg2*.

### 3.3.3 Two-hybrid analysis in *Nicotiana benthamiana*

Physical interaction between VvPGIP1 and the EPGs from *B. cinerea* was assessed using a modified two-hybrid system described by Yang *et al* (2000). The *BD:Vvpgip1* fusion was co-infiltrated with the GBS construct, with or without the *AD:Bcpg* fusions, the *AD:Bcpg* fusions were also co-infiltrated with the GBS construct without the *BD:Vvpgip1* fusion. Glucuronidase (GUS) activity was measured 5 days post infiltration (Fig. 3.9). For both the *AD:Bcpg1* and *AD:Bcpg2* fusions, co-infiltrated with the GBS construct, very high levels of GUS activity were observed (Fig. 3.9A), this was not observed for the *Bcpg3-6* fusions co-infiltrated with the GBS construct (Fig. 3.9B). The *BD:Bcpg3* fusion co-infiltrated with both the *BD:Vvpgip1* and GBS fusions also resulted in GUS acitivity levels above that of the individual (*Vvpgip1* + GBS and *Bcpg3* + GBS) infiltrations, suggesting possible interaction between VvPGIP1 and BcPG3.

**A**



**B**
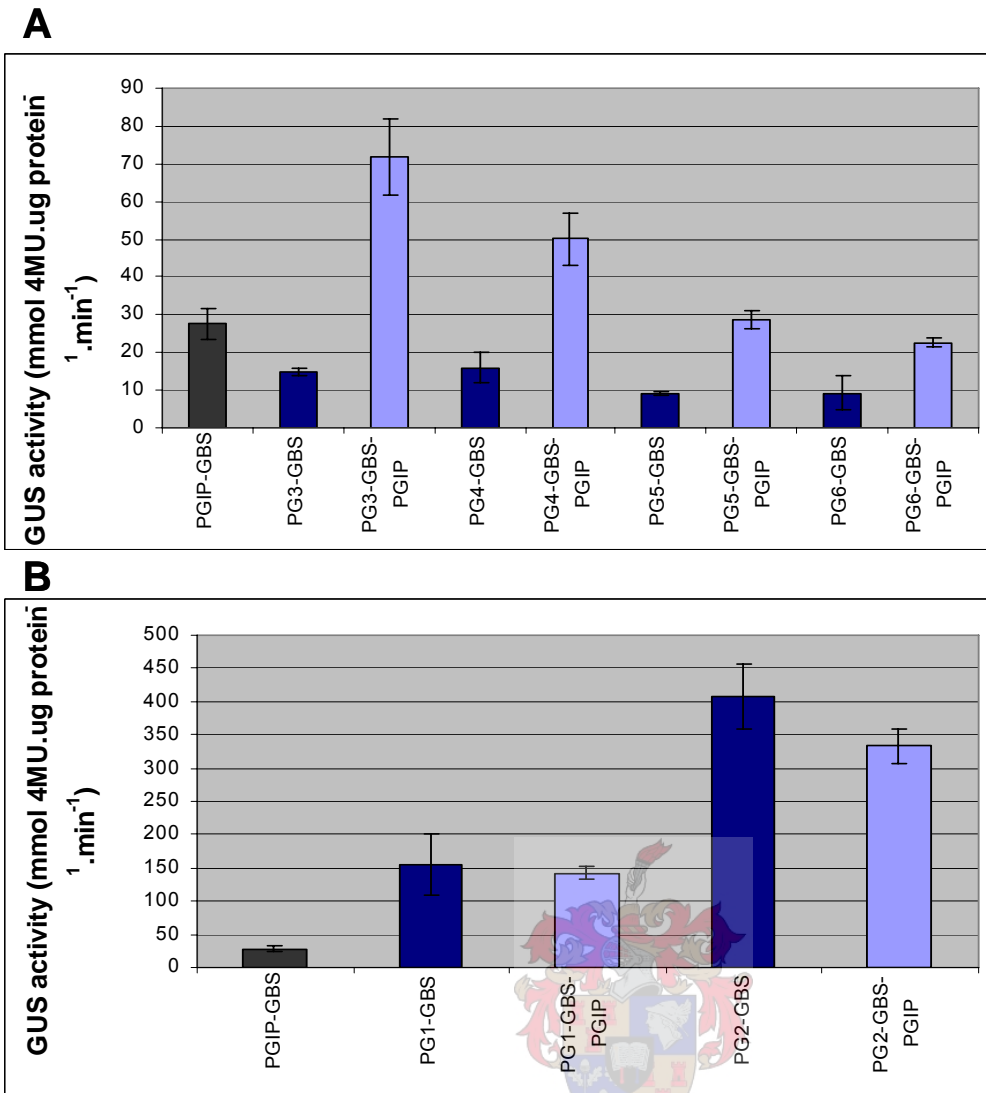


**Figure 3.9 A and B.** Two-hybrid analysis of the interactions between the endo-polygalacturonases (EPGs) from *Botrytis cinerea* (BcPG1-6) and the polygalacturonase-inhibiting protein from *Vitis vinifera* (VvPGIP1). Two-hybrid constructs as described in the text were co-infiltrated into leaves of eight week old *Nicotiana benthamiana* plants as described by Yang *et* al., (2000). The GBS construct was co-infiltrated with each infiltration. For each *Bcpg* construct, glucuronidase (GUS) activity was determined in leaves infiltrated only with the *Bcpg* construct and the GBS construct (dark blue bars) as well as for leaves con-infiltrated with *Vvpgip1* in combination with the *Bcpg-* and GBS constructs (light blue bars). GUS activity was also determined for the *Vvpgip1* construct co-infiltrated with the GBS construct (black bar). All GUS assays were performed 5 days post infiltration as described by Yang *et al.*, 2000. **A.** Co-infiltration of *Bcpg3-6* with and without *Vvpgip1*. **B**. Co-infiltration of *Bcpg1 & 2* with and without *Vvpgip1.*

## 3.4 DISCUSSION

The necrotrophic fungus *B. cinerea*, an important plant pathogen, has received a lot of attention due to its devastating effect on various plant crops. The six EPG-encoding genes of *B. cinerea* strain SAS56 have previously been cloned and at least two (BcPG1 and BcPG2) have been implicated in pathogenesis (Krooshof *et al.*, 2004; Ten Have *et al.*, 1998; Wubben *et al.*, 1999). EPGs are some of the first cell wall degrading enzymes to be secreted when *B. cinerea* encounters the polysaccharide rich cell wall of a plant host and also are important virulence factors (Ten Have *et al.*, 1998). *B. cinerea* also serves as a model organism in studies concerning disease resistance. From a molecular point of view the interaction between the EPGs from *B. cinerea* and PGIP, a LRR protein present in the cell walls of many plant species are of specific interest. PGIPs have been shown to interact and inhibit EPGs (also those from *B. cinerea*) *in vitro* (De Lorenzo and Ferrari 2002; Federici *et al.*, 2001) but to date, only indirect evidence derived from transgenic plants over-expressing PGIP genes (De Lorenzo *et al.*, 2001; Ferrari *et al.*, 2003; Powell *et al.*, 2000) exist to demonstrate that this interaction occurs *in vivo*.

The goal of this study was to provide the platform to facilitate an in depth investigation of the interactions between PGIP1, isolated from *Vitis vinifera* and the EPGs from a virulent South African *B. cinerea* strain. By mimicking the expression profile of the EPGs as described by Wubben *et al.*, 1999, we were able to successfully isolate the cDNA copies of six EPG encoding genes, suggesting that the EPGs of different *Botrytis* strains are induced in a similar fashion. Homology of the isolated EPG gene family correlated well with sequences in GenBank, isolated by Wubben *et al.*, 1999 from *B. cinerea* strains originating from Italy and the Netherlands and only very small sequence differences were observed between the strains.

The first attempt to heterologously express the genes was done in *S. cerevisae* and all EPG genes were expressed using their native signal peptides. For every transformation, both intra- and extracellular protein fractions were isolated from *S. cerevisiae* and tested for EPG activity. *In vitro* EPG activity could be found in both crude intra- and extracellular protein extracts from *S. cerevisiae* transformed with *Bcpg2*; this activity was not inhibited by VvPGIP1 in subsequent agarose diffusion assays, neither at pH 4.5 nor at pH 6.0. *S. cerevisiae*, however, is known to sometimes be a poor expressor of foreign proteins, mainly due to hyperglycosylation (Gellissen and Hollenberg, 1997). Recent work has also shown that full activity of the BcPGs is substrate dependant as well as pH sensitive

(Krooshof *et al.*, 2004). Sub-optimal expression conditions coupled with sub-optimal assay conditions, could provide an explanation for the observed lack of activity.

In a further attempt to obtain active protein, heterologous gene expression was done in *N. benthamiana*. *In vitro* results obtained from this system correlated precisely with that of the yeast expression system, although physiologically the expression of the EPG encoding genes showed a strong effect on the plants. These phenotypical effects were indicative of EPG activity and since differential phenotypes were observed with the various EPGs, it provides evidence for a true activity interaction and not non-related reactions due to the infiltration or the HR. Moreover, recent work by Kars *et al* (2004) also showed strong phenotypical symptoms in tobacco transiently over-expressing EPGs from another strain of *B. cinerea*, and for EPG2 they propose that this specific enzyme is very effective as a tissue maceration enzyme without involving the HR.

The results obtained in our study suggested that although the proteins are active *in vivo*, the *in vitro* assay system used was not appropriate. A more sensitive diagnostic technique, based on chlorophyll fluorescence, was therefore adopted. This technique is non-destructive and compares the variable fluorescence yield of healthy leaves vs. leaves expressing the *Bcpg* genes. Our results clearly indicate that leaves over-expressing the *Bcpg* genes resulted in a significant reduction of the $^{Fv}/_{Fm}$ ratio, a ratio commonly used to detect early stress in photosynthesizing plants (Maxwell and Johnson, 2000). This reduction was not due to the infiltration process or the metabolic load associated with foreign gene over-expression. The nature of this system also allowed us to investigate the *in vivo* interaction between VvPGIP1 and some of the BcPGs. We specifically looked at BcPG1 and BcPG2. Previous *in vitro* results in our laboratory clearly showed that BcPG2 is neither inhibited by VvPGIP1, nor does any physical interaction occur between the two proteins (results not shown). Yet, from the *in vivo* experiments, it seems as if VvPGIP1 does affect the activity of BcPG2. This is to our knowledge the first report of any interaction between BcPG2 and PGIP. Although the nature of this interaction must still be elucidated, it is clear that data obtained solely from *in vitro* systems is not always sufficient to clarify the relationships between EPGs and PGIPs.

Based on the *in vivo* results, interaction between VvPGIP1 and the individual BcPGs was investigated further using an *in planta* two-hybrid technique. This approach resulted in mixed success. Very high levels of background GUS activity were obtained with the *Bcpg1* and *Bcpg2* fusion constructs. This phenomenon was not observed for any of the other *Bcpg* fusions. It is interesting to note that *Bcpg1* and *Bcpg2* are both predicted to

have a basic pI, whereas the pI-values of all the other *Bcpgs* are in the acidic range. However, it is clear that the two-hybrid results are not conclusive and cannot be used to elucidate the interaction of VvPGIP1 with BcPG1 or BcPG2. Interaction results with the remaining EPGs seem to be easier to interpret. The *Bcpg3* and *Bcpg4* fusions co-infiltrated with the *Vvpgip1* fusion and the GBS-construct showed a significant increase in fluorescence when compared to *Bcpg3* and *Bcpg4* fusions co-infiltrated with only the GBS-construct. Also preliminary results obtained from *in vitro* interaction studies with VvPGIP1 and EPGs from *B. cinerea* with an acidic pI as well as a basic pI, suggest that VvPGIP1 inhibits EPGs from *B. cinerea* with an acidic pI. Results obtained from this study seem to corroborate this finding.

From the data obtained in this study, as well as results obtained from additional physical interaction studies (results not shown) an interesting albeit preliminary trend seems to emerge. All evidence to date suggests that VvPGIP only interacts with EPGs from *B. cinerea* with an acidic pI and although the data are very preliminary, this might hold some implications regarding requirements for *in planta* interactions between VvPGIP1 and the BcPGs.
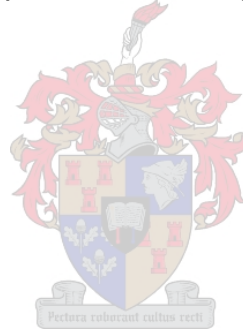
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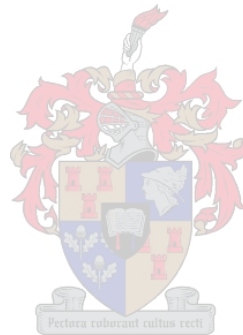
# RESEARCH RESULTS

## The isolation of polygalacturonase-inhibiting protein (PGIP) encoding genes from *Vitis* and non-*Vitis* grapevine species

To form part of a publication after incorporating additional results regarding interaction studies with fungal endopolygalcturonases

# THE ISOLATION OF POLYGALACTURONASE-INHIBITING PROTEIN ENCODING (PGIP) GENES FROM *VITIS* AND NON-*VITIS* GRAPEVINE SPECIES.

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## ABSTRACT

Polygalacturonase-inhibiting proteins (PGIPs) are defence related proteins found within the plant cell walls of most dicotyledonous plant species.  Their role in plant defence has been confirmed by several studies indicating that the over-expression of these genes can decrease fungal susceptibility.  Although significant sequence differences exist between the PGIP encoding genes from different plant species, the predicted structures of these proteins are all remarkably similar.  This can mainly be ascribed to the presence of conserved leucine rich repeat (LRR) domains forming the $\beta$-sheet/$\beta$-turn structure found in all described PGIPs to date.  Recent work also showed that single amino acid changes within these regions can change the endopolygalacturonase (EPG) inhibition specificity of PGIP and could therefore influence the effectiveness of the protein as an antifungal agent. PGIPs are usually encoded by multigene families where the encoded products differ in their specificity and inhibition profile. Results from various cultivars of grapevine suggest that instead of a true *pgip* multigene family, the *pgip* gene copies present in the genome of *Vitis* might instead be encoding isozymes of the isolated *Vvpgip1* gene product. It could be hypothesized therefore, that small amino acid changes within the PGIP encoding genes of different grapevine (*Vitis* and non-*Vitis*) species could be especially significant, an even more so if it occurs in the conserved regions that are known to impact on PGIP activity and specificity.  To this end, PGIP encoding sequences were amplified from 37 *Vitis* and non-*Vitis* species or cultivars by using the sequence information of the *Vvpgip1* gene. Sequence analysis showed between 0 and 11 amino acid differences, some which are situated within the predicted LRR regions of the proteins.  Preliminary structural data also indicated that some of these differences can lead to structural changes.  The genes will be

used as a basis for specificity analyses to determine whether the observed amino acid changes are significant or not.

## 4.1 INTRODUCTION

Polygalacturonase-inhibiting proteins (PGIP) are glycoproteins situated in the cell walls of many plant species. PGIPs are linked to the activated defence response of plants due to their inhibition of endoplygalacturonases (EPGs) that are secreted by invading fungi, certain structural features and their proposed role as signalling molecules (De Lorenzo *et al.*, 2001; Esquerré-Tugayé *et al.*, 1999). Apart from the sequence differences found between *pgip* genes from different plant species, the proteins all share a basic common structure, containing a leucine-rich repeat domain (De Lorenzo *et al.*, 2001; Di Matteo *et al.*, 2003).

The LRR domain of PGIP contains between 20 and 29 residues and is defined by a consensus sequence, GxIPxxLGxLxxLxxLxLxxNxLT/S, where x represents any amino acid and L positions can be occupied by valine, isoleucine and phenylalanine (Krantz and Zipursky, 1990). Within this sequence, xxLxLxx is predicted to form a β-strand/β-turn structure in which the x-residues are solvent-exposed and involved in the interaction with fungal PGs (De Lorenzo *et al.*, 1994; Kobe and Deisenhofer, 1995; Leckie *et al.*, 1999; Mattei *et* al., 2001). In all living organisms, LRR domains are specialized for interaction with protein ligands (Kobe and Kajava, 2001). Many resistance (*R*) genes also contain LRRs that share significant similarities in terms of structure and specificity with the LRR of PGIP, i.e. the R-proteins Cf of tomato (Jones and Jones, 1997; Jones, 2001) and Xa21 of rice (Ronald, 1997).

Sequence analysis of the predicted β-strand/β-turn region of R-proteins supports the concept that this region is hyper variable and under selection for diversification (Meyers *et* al., 1998; Noel *et al.*, 1999; Parniske *et* al., 1997). Amino acid changes in this region have been shown to influence the specificity and function of R-proteins (Van der Hoorn *et al.*, 2001; Warren *et al.*, 1998). Dodds *et al* (2001), for example, showed that only six amino acid changes in the LRR region determines the difference between P and P2 rust resistance specificities in flax.

Recently a PGIP encoding gene, *Vvpgip1*, was isolated in our lab from *Vitis vinifera* L. cv Pinotage (De Ascensao, 2001) (Genbank Ac: AF499451). Comparison of the *Vvpgip1* sequence with *pgip* sequences isolated from Chardonnay, Chenin blanc, Merlot, Shiraz, Dauphine, Red Globe and Sultana showed between 0 and 18 differences in nucleotide

sequence. These nucleotide changes resulted in between 0 and 9 non-synonymous and synonymous nucleotide changes (De Ascensao, 2001). It has also been shown that VvPGIP1, isolated from *véraison* grape berries, inhibits a total crude extract of EPGs isolated from a virulent South African *Botrytis cinerea* strain, originating from a vineyard in the Stellenbosch district, as well as EPGs from *Aspergillus niger* and *Colletotrichum lindemuthianum* (De Ascensao, 2001).

Most *V. vinifera* cultivars are susceptible towards a range of fungal diseases, whereas certain non-*Vitis* and American grape spp. have been shown to be quite resistant towards these pathogens. In this project we isolated and cloned PGIP encoding genes from the more resistant genotypes of several *Vitis* and non-*Vitis* cultivars, including rootstock material and American species. The genes were sequenced, analyzed and compared on the nucleotide and amino acid level with the corresponding sequence of the *Vvpgip1* gene from Pinotage. Preliminary modelling experiments were also initiated to compare the structures of the isolated genes with that of *Vvpgip1*. These isolated genes form a resource to further elucidate the inhibition spectrum and efficacy of the PGIP encoding genes in grapevine against the EPGs of fungal pathogens, specifically if it can be correlated with the various resistance levels of the genotypes it was isolated from.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Bacterial strains and culture conditions

*Eschericia coli* strain DH5α (Invitrogen Life Technologies, Carlsbad, USA), was grown at 37°C in LB media, supplemented with 100 μg/mL ampicillin or 50 μg/mL kanamycin for the selection of transformants. *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.*, 1993), was grown at 28°C in LB media, supplemented with 0.1% (w/v) glucose, 50 μg/mL kanamycin and 15 μg/mL rifampicin for the selection of transformants.

### 4.2.2 DNA manipulations

Grapevine material of various cultivars and species (Table 4.1) was collected from experimental vineyards in the Stellenbosch area and ground with a mortar and pestle to a fine powder in liquid nitrogen. Genomic DNA was isolated according to McGarvey and Kaper, 1991 and used as template in the amplification reactions. All DNA manipulations were done according to Sambrook *et al* (1989). PGIP encoding genes were amplified with 5' primer: AGTCGGTCGACATGGAGACTTCAAAACTTTTTCTAC, and 3' primer: GTC-

GACTCACTTGCAGCTCTG, designed from the ORF of *Vvpgip1* as template, using Expand high fidelity DNA polymerase from Roche Diagnostics (Mannheim, Germany). PCR reactions were done in a reaction volume of 50 μL, consisting of 1× Expand high fidelity PCR buffer without MgCl$_2$, 200 μM dNTPs, 200 nM of each primer, 5 ng template DNA. MgCl$_2$ concentration was adjusted optimally for each reaction. Amplification conditions included an initial DNA denaturation step at 94°C for 2 min, followed by 30 repetitive cycles of DNA denaturation at 98°C for 15 sec, primer annealing according to the specific primer melting temperatures for 30 sec and elongation at 72°C, allowing 1 min for each kb amplified. A final elongation step at 72°C for 2 min was performed at the end of the reaction. T4 DNA ligase and restriction enzymes were purchased from Roche Diagnostics (Mannheim, Germany) and used according to the recommendations of the supplier. Sequencing was done by the DNA Sequencing Facility, Department of Genetics, Stellenbosch University, using an ABI PRISM$^R$ 3100 automated DNA sequencer from Applied Biosystems.

The amplified *pgip* genes were cloned into the pGEM-T-Easy vector (Promega, Madison, USA) and electroporated into DH5α *E. coli* (Invitrogen Life Technologies, Carlsbad, USA). One positive clone of each gene was sequenced for verification. The *pgip* genes were excised from pGEM-T-Easy with *Sal*I and sub-cloned to the *Xho*I site of the plant expression vector, pCAMBIA1301 (CAMBIA, Canberra ACT, Australia). The pCAMBIA-constructs were transformed via electroporation to *A. tumefaciens* strain EHA105, (Hood *et al.*, 1993) for future heterologous expression of the genes.

Sequence alignments of the full PGIP amino acid sequences as well as the LRR domains were done with CLUSTALW software (Thompson *et al.*, 1994). Phylogenetic trees were assembled with PHYLIP (Phylogeny Inference Package) version 3.5c (Felsenstein, 1989) to highlight the homology between the genes compared to *Vvpgip1*. Both software packages were accessed through the Biology Workbench version 3.2 interface. Conserved domain predictions were done using the Reverse Position Specific BLAST function to query the NCBI conserved domain database (Marchler-Bauer *et al.*, 2003).

The *Vvpgip1* encoded sequence (Genbank Ac: AF499451) (De Ascensao, 2001) was threaded against the *P. vulgaris* PGIP2 (Di Matteo *et al.*, 2003) crystal structure using the Swiss-Model protein structure homology-modelling server. The threaded grape PGIP

structure was further optimized using the SYBYL molecular modelling package by Tripos. Secondary structural features were aligned including disulfide bonds and β-pleated sheets.

**Table 4.1** Grapevine material used for the isolation of the *pgip* genes.

| No | Code | Species/Cultivar | Origin | No | Code | Species/Cultivar | Origin |
|---|---|---|---|---|---|---|---|
| 1 | 1004 | *V. candicans Engelman* | France | 20 | 7090 | Herbemont black (*V. bourquiniana*) | RSA |
| 2 | 1012 | *V. doaniana Munson* | USA | 21 | 7102 | Isabella (*V. labrusca*) | RSA |
| 3 | 1018 | *V. riponia* (female) | RSA | 22 | 7180 | *V. doaniana Munson* | USA |
| 4 | 1024 | *V. solonis Hort* | RSA | 23 | 7182 | *V. Longii (V. solonis)* | RSA |
| 5 | 1030 | *V. flexuosa* | USA | 24 | 7194 | *V. amurensis Ruprecht* | USA |
| 6 | 1034 | *V. Bourquiniana* | Germany | 25 | 7198 | *V. doaniana Munson* | USA |
| 7 | 1038 | *V. caribaea* | USA | 26 | 7360 | *V. Treleaseii Munson* | USA |
| 8 | 1042 | *V. Popenoei* | USA | 27 | 7442 | *V. solonis Hort* | RSA |
| 9 | 1046 | *V. shutteworthii* | USA | 28 | 7536 | *V. Thunbergii* | Germany |
| 10 | 1048 | *V. smalliana* (female) | USA | 29 | 7538 | *V. Andersonii* | Germany |
| 11 | 1050 | *V. aestivalis* | USA | 30 | 7540 | *V. caucasica* | Germany |
| 12 | 1056 | *V. californica* Gold Hill 1 | USA | 31 | 7548 | *V. monticola* | USA |
| 13 | 1058 | *V. monticola* | USA | 32 | 7560 | *V. Coignetiae* | Germany |
| 14 | 7000 | *Ampelocissus atapulcensis* | Zimbabwe | 33 | 101-14 MGT | *V. riparia* x *V. rupestris* | RSA (rootstock) |
| 15 | 7010 | *Cissus cactiformis* ex *Ruacana* | Unknown | 34 | Ramsey | *V. champinii* | RSA (rootstock) |
| 16 | 7016 | *Cyphostemma currorii* | Unknown | 35 | Paulson | *V. berlandieri* x *V. rupestris* | RSA (rootstock) |
| 17 | 7048 | EVEX 13-5 (Berlandieri 13-5) | Spain | 36 | Richter 110 | *V. berlandieri* x *V. rupestris* | RSA (rootstock) |
| 18 | 7060 | Constantia Metallica (*V. rupestris*) | RSA | 37 | SO4 | *V. riparia* x *V. berlandieri* | RSA (rootstock) |
| 19 | 7074 | Dogridge (*V. champinii*) | RSA | | | | |

The classic leucine-rich repeat pattern within the β-sheet face causes the structure to be quite predictable. Subsequent grape PGIP sequences were threaded onto this first grape structure with high confidence, as the 37 grape PGIP sequences share greater than a 95% homology. The sequences were threaded without the predicted signalling peptide and all positions given assume that position one of the threaded sequences corresponds to position 28 of the pre-protein.

## 4.3 RESULTS

Thirty seven putative *pgip* sequences were amplified and cloned from various *Vitis* and non-*Vitis* species and genotypes as listed in Table 4.1. The nucleotide and deduced amino acid sequences of all the genes were confirmed to be PGIP encoding sequences and were subsequently compared to the sequence of the *Vvpgip1* gene previously isolated from Pinotage. Total nucleotide changes in the sequences relative to *Vvpgip1* ranged between 0 and 20, which resulted in amino acid changes ranging between 0 and 11 (Table 4.2).

**Table 4.2** Total nucleotide and amino acid changes in PGIP encoding genes isolated from various *Vitis* and Non-*Vitis* species. All sequences were compared to the nucleotide and amino acid sequences of *Vvpgip1*

| Grapevine species/cultivar code* | Nucleotide changes | Synonymous | Non-synonymous |
|---|---|---|---|
| 1004 | 14 | 11 | 3 |
| 1012 | 10 | 7 | 3 |
| 1018 | 15 | 7 | 8 |
| 1024 | 12 | 4 | 8 |
| 1030 | 13 | 4 | 9 |
| 1034 | 13 | 5 | 8 |
| 1038 | 14 | 7 | 7 |
| 1042 | 9 | 6 | 3 |
| 1046 | 11 | 7 | 4 |
| 1048 | 14 | 6 | 8 |
| 1050 | 14 | 10 | 4 |
| 1056 | 9 | 4 | 5 |
| 1058 | 13 | 6 | 7 |
| 7000 | 9 | 4 | 5 |
| 7010 | 12 | 4 | 8 |
| 7016 | 9 | 6 | 3 |
| 7048 | 10 | 7 | 3 |
| 7060 | 11 | 5 | 6 |
| 7074 | 9 | 5 | 4 |
| 7090 | 13 | 5 | 8 |
| 7102 | 9 | 5 | 4 |
| 7180 | 14 | 10 | 4 |
| 7182 | 12 | 4 | 8 |
| 7194 | 17 | 8 | 9 |
| 7198 | 11 | 7 | 4 |
| 7360 | 4 | 4 | 0 |
| 7442 | 10 | 6 | 4 |
| 7536 | 15 | 6 | 9 |
| 7538 | 17 | 9 | 8 |
| 7540 | 14 | 6 | 8 |
| 7548 | 15 | 10 | 5 |
| 7560 | 10 | 5 | 5 |
| Paulson | 13 | 5 | 8 |
| Ramsey | 14 | 6 | |
| Richter 110 | 11 | 6 | 5 |
| SO4 | 20 | 9 | 11 |
| 104-14 (MGT) | 18 | 8 | 10 |

\* Cultivar and/or species code are summarized in Table 4.1

The amino acid sequences of the genes were aligned with that of *Vvpgip1* (Fig. 4.1) and a rooted phylogenetic tree was constructed to illustrate the degree of diversification between the genes (Fig. 4.2). The deduced amino-acid sequences were found to share greater than 95% homology.

```
SO4            METSKLFLLSS-LLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
101-14_[MGT]   METSKLFLLSS-LLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
1030           METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
1024           MERSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7540           METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7090           METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7536           METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
Paulson        METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7182           METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7010           METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
1034           METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7538           METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7194           METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
1048           METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
1018           METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
Ramsey         METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7060           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
1038           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7548           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7180           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
1004           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7560           METSKLFLLSSSLLLLLLATRPCPSLSERCXPKDKKVLLQIKKYLDNPYILASWNPNTDC
1046           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7442           METSKLFLLSSSLLLLLLATRPCPSLSERCNPEDKKVLLQIKKALDNPYILASWNPNTDC
1012           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
1042           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7016           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7048           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7074           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
1050           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDDPYILASWNPNTDC
Richter_110    METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
1056           METSKLFLLSSSLLLLLLATRPCPSFSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7198           METSKLFLLSSSLLLLLLATRPCPSLSERGNPKDKKVLLQIKKALDNPYILASWNPNTDC
7000           METSKLFLLSSSLLLLLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7360           METSKLFLLSSSLLLVLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
Vvpgip1        METSKLFLLSSSLLLVLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
7102           METSKLFLLSSSLLLVLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
1058           METSKLFLLSPSLLLVLLATRPCPSLSERCNPKDKKVLLQIKKALDNPYILASWNPNTDC
               ** *******. ***:**********:***  *:********** **:************


SO4            CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
101-14_[MGT]   CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFHKLSNLTGQIPPAIAK
1030           CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
1024           CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7540           CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7090           CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7536           CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
Paulson        CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7182           CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7010           CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
1034           CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7538           CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7194           CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLTFRKLSNLTGQIPPAIAK
1048           CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
1018           CEWYCVECDLTTHRINSLTIFSGELSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
Ramsey         CEWYCVECDLTTHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7060           CEWYCVECDLTTHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
1038           CEWYCVECDLTSHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
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7548           CEWYCVECDLTTHRINSLTIFSGQLSGQLPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7180           CEWYCVECDLTTHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
1004           CEWYCVECDLTTHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7560           CEWSCVECDLTSHXINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLPNLTGQIPPAIAK
1046           CEWYCVECDLTSHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7442           CEWYCVECDLTSHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
1012           CEWYCVECDLTSHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
1042           CEWYCVECDLTSHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7016           CEWYCVECDLTSHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7048           CEWYCVECDLTSHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7074           CEWYCVECDLTTHRINSLTIFSGQLSGQIPNAVGDLPFLETLIFRKLSNLTGQIPPAIAK
1050           CEWYCVECDLTTHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
Richter_110    CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAISK
1056           CEWYCVECDLTTHRINSLTIFSGKLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7198           CEWYCVECDLTTHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7000           CEWYCVECDLTTHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7360           CGWYCVECDLTTHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
Vvpgip1        CGWYCVECDLTTHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
7102           CGWYCVECDLTTHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
1058           CGWYCVECDLTTHRINSLTIFSGQLSGQIPDAVGDLPFLETLIFRKLSNLTGQIPPAIAK
               * *  *******:* *********:****:*:********** *:**.**********:*


SO4            LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
101-14_[MGT]   LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
1030           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
1024           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7540           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7090           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7536           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
Paulson        LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7182           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7010           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
1034           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7538           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7194           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
1048           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
1018           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
Ramsey         LKHLKMVRLSWTNLSGPVPEFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7060           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLDALHLDRN
1038           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7548           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGRIPGSLSLLPNLGALHLDRN
7180           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGRIPGSLSLLPNLGALHLDRN
1004           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7560           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
1046           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7442           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
1012           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
1042           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7016           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7048           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7074           LKHLKMVRLSWTNISGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
1050           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDHSFNNLSGPIPGSLSLLPNLGALHLDRN
Richter_110    LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
1056           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7198           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7000           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLDALHLDRN
7360           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
Vvpgip1        LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
7102           LKHLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHLDRN
1058           LKRLKMVRLSWTNLSGPVPAFFSELKNLTYLDLSFNNLSGPIPGSLSLLPNLGALHIDRN
               **:**********:***** *********** ******* **********.***:***


SO4            HLTGPIPDSFGKFAGSTPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
101-14_[MGT]   HLTGPIPDSFGKFAGSTPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
1030           HLTGPIPDSFGKFAGSTPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
1024           HLTGPIPDSFGKFAGSTPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
7540           HLTGPIPDSFGKFAGSTPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
7090           HLTGPIPDSFGKFAGSTPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
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7536           HLTGPIPDSFGKFAGSTPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
Paulson        HLTGPIPDSFGKFAGSSPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
7182           HLTGPIPDSFGKFAGSSPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
7010           HLTGPIPDSFGKFAGSSPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
1034           HLTGPIPDSFGKFAGSSPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
7538           HLTGPIPDSFGKFAGSAPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
7194           HLTGPIPDSFGKFAGSAPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
1048           HLTGPIPDSFGKFAGSAPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
1018           HLTGPIPDSFGKFAGSAPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
Ramsey         HLTGPIPDSFGKFAGSPPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
7060           HLTGPIPDSFGKFAGSTPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
1038           HLTGPIPDSFGKFAGSTPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
7548           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
7180           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
1004           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNA
7560           DLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNA
1046           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNA
7442           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNA
1012           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNA
1042           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNA
7016           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNA
7048           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNA
7074           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNA
1050           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNA
Richter_110    HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNA
1056           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPIVMDLSRNKLEGDPSIFFNA
7198           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPIVMDLSRNKLEGDPSIFFNA
7000           HLTGPIPDSFGKFAGSTPGLYLSHNQLSGKIPYSFRGFDPIVMDLSRNKLEGDPSIFFNA
7360           HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNA
Vvpgip1        HLTGPIPDSFGKFAGSTPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNA
7102           HLTGPIPDSFGKFAGSTPGLHLSHNQLSGKIPYSFRGFDPNVMDLSRNKLEGDLSIFFNA
1058           HLTGPIPDSFGKFAGSTPGLHLSHNQLSGKIPYSFRGFDPNVMDLSRNKLEGDLSIFFNA
               .***************.* *:******************* *********** ******


SO4            KKSTQVVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
101-14_[MGT]   KKSTQVVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1030           KKSTQVVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRS
1024           KKSTQVVDFSRNLFXFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7540           KKSTQVVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7090           KKSTQVVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7536           KKSTQVVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
Paulson        NKSTQVVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7182           NKSTQVVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7010           NKSTQVVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1034           NKSTQVVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7538           KKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7194           KKSTQIVDFSRNSFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1048           KKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1018           KKSTQIVDFSRNLFQFDLSRMEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
Ramsey         KKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7060           NKSTQVVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1038           KKSTQVVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7548           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7180           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1004           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7560           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1046           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIARSLPEMMTSLDLQFLNVSYNRL
7442           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1012           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1042           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7016           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7048           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7074           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1050           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
Richter_110    NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1056           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7198           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7000           NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
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7360            NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
Vvpgip1         NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7102            NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1058            NKSTQIVDFSRNLFQFDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
                :****:****** * *****:***************** *******************


SO4             CGKIPVGGKLQSFDYDSYFHNRCLCCAPPQSCK
101-14_[MGT]    CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1030            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1024            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7540            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7090            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7536            RGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
Paulson         CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7182            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7010            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1034            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7538            CGKIPVGRKLQSFDYDSYFHNRCLCGAPLQSCK
7194            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1048            YGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1018            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
Ramsey          CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7060            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1038            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7548            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7180            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1004            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7560            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1046            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7442            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1012            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1042            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7016            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7048            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7074            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1050            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
Richter_110     CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1056            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7198            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7000            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7360            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
Vvpgip1         CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
7102            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
1058            CGKIPVGGKLQSFDYDSYFHNRCLCGAPLQSCK
                ****** **************** ** ****
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**Figure 4.1** The amino acid alignment between the PGIP encoding genes isolated from various *Vitis* and non-*Vitis* species. The predicted amino acid sequences were obtained by direct translation of the nucleotide sequences and the alignments were done using the CLUSTALW software (Thompson *et al.*, 1994). The predicted amino acid sequence of *Vvpgip1* (Genbank Ac: AF499451) was included in the alignment for comparative purposes
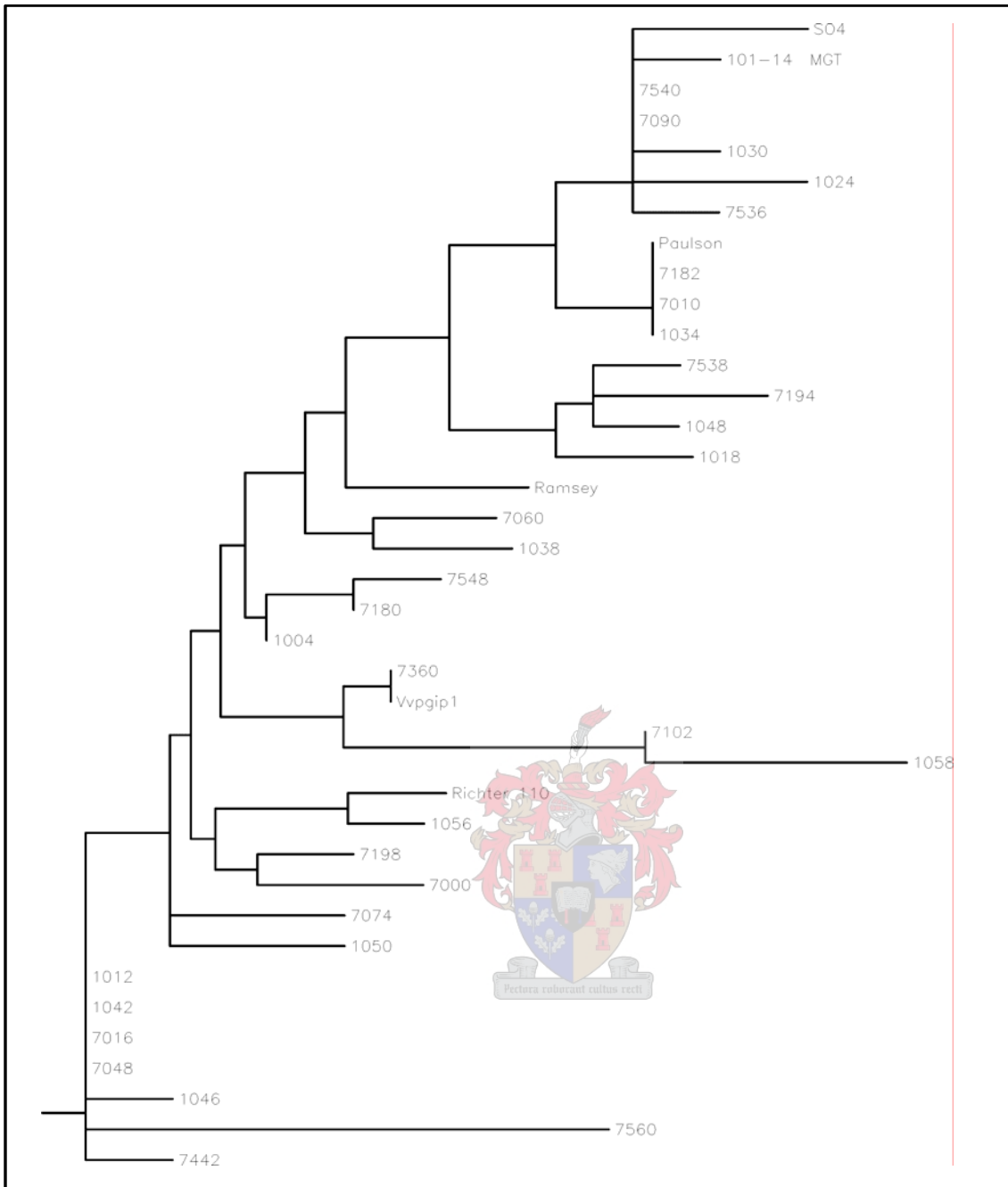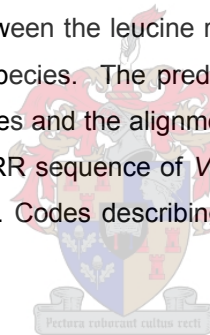
**Figure 4.2** Rooted phylogenetic tree of the amino acid alignment from PGIP encoding genes isolated from various *Vitis* and non-*Vitis* species. Codes describing the species and/or cultivars are explained in Table 4.1. The tree was constructed using PHYLIP (Phylogeny Inference Package) version 3.5c (Felsenstein, 1989), that was accessed through the Biology Workbench version 3.2. The amino acid sequence of *Vvpgip1* (Genbank Ac: AF499451) was included for comparative purposes.

To find conserved domains within the isolated sequences, the NCBI conserved domain database was queried with the coding regions from all of the isolated genes. LRR domains were predicted in all of the sequences and these domains are situated between amino acid residues 196 to 300. The predicted LRR domains were also aligned separately (Fig. 4.3) and a rooted phylogenetic tree constructed (Fig. 4.4). The LRR-domain sequences were found to share greater than 94% homology.

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7048LRR          STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
1046LRR          STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
1012LRR          STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
VvPGIP1LRR       STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
1042LRR          STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
1050LRR          STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
7016LRR          STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
7074LRR          STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
7360LRR          STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
7442LRR          STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
7560LRR          STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
Richter110LRR    STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
7198LRR          STPYLYLSHNQLSGKIPYSFRGFDPIVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
1056LRR          STPYLYLSHNQLSGKIPYSFRGFDPIVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
7000LRR          STPGLYLSHNQLSGKIPYSFRGFDPIVMDLSRNKLEGDPSIFFNANKSTQIVDFSRNLFQ
7548             STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNANKSTQIVDFSRNLFQ
7180LRR          STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNANKSTQIVDFSRNLFQ
1004LRR          STPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNANKSTQIVDFSRNLFQ
7538             SAPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQIVDFSRNLFQ
7194LRR          SAPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQIVDFSRNSFQ
1048LRR          SAPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQIVDFSRNLFQ
1018LRR          SAPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQIVDFSRNLFQ
RamseyLRR        SPPYLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQIVDFSRNLFQ
SO4LRR           STPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQVVDFSRNLFQ
1030LRR          STPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQVVDFSRNLFQ
7536             STPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQVVDFSRNLFQ
7540             STPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQVVDFSRNLFQ
1024LRR          STPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQVVDFSRNLFX
7090LRR          STPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQVVDFSRNLFQ
1038LRR          STPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQVVDFSRNLFQ
101-14_[MGT]     STPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNAKKSTQVVDFSRNLFQ
PaulsonLRR       SSPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNANKSTQVVDFSRNLFQ
7182LRR          SSPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNANKSTQVVDFSRNLFQ
7010LRR          SSPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNANKSTQVVDFSRNLFQ
1034LRR          SSPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNANKSTQVVDFSRNLFQ
7060LRR          STPGLYLSHNQLSGKIPYSFRGFDPTVMDLSRNKLEGDLSIFFNANKSTQVVDFSRNLFQ
7102LRR          STPGLHLSHNQLSGKIPYSFRGFDPNVMDLSRNKLEGDLSIFFNANKSTQIVDFSRNLFQ
1058LRR          STPGLHLSHNQLSGKIPYSFRGFDPNVMDLSRNKLEGDLSIFFNANKSTQIVDFSRNLFQ
                 *.* *:****************** *********** ******:****:****** *


7048LRR          FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1046LRR          FDLSRVEFPKSLTSLDLSHNKIARSLPEMMTSLDLQFLNVSYNRL
1012LRR          FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
VvPGIP1LRR       FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1042LRR          FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1050LRR          FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7016LRR          FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7074LRR          FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7360LRR          FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7442LRR          FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7560LRR          FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
Richter110LRR    FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7198LRR          FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
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1056LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7000LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7548                 FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7180LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1004LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7538                 FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7194LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1048LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1018LRR              FDLSRMEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
RamseyLRR            FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
SO4LRR               FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1030LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRS
7536                 FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7540                 FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1024LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7090LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1038LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
101-14_[MGT]         FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
PaulsonLRR           FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7182LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7010LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1034LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7060LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
7102LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
1058LRR              FDLSRVEFPKSLTSLDLSHNKIAGSLPEMMTSLDLQFLNVSYNRL
                     *****:**************** ******************
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**Figure 4.3** The amino acid alignment between the leucine rich repeats LRRs of the PGIP encoding genes isolated from various *Vitis* and non-*Vitis* species. The predicted amino acid sequences were obtained by direct translation of the nucleotide sequences and the alignments were done using the CLUSTALW software (Thompson *et al.*, 1994). The predicted LRR sequence of *Vvpgip1* (Genbank Ac: AF499451) was included in the alignment for comparative purposes. Codes describing the species and/or cultivars are explained in Table 4.1.

The high level of homology that exists between the isolated PGIP encoding sequences enabled the sequences to be threaded against the structure of the recently solved crystal structure of the bean PGIP2. The preliminary threading results indicates that some of the natural mutations found within the predicted LRR regions, specifically the conversion of the tyrosine at position 172 to glycine, results in structural differences situated within the cleft of the enzyme formed by the β-sheet/β-turn structure of the protein (Fig. 4.3).
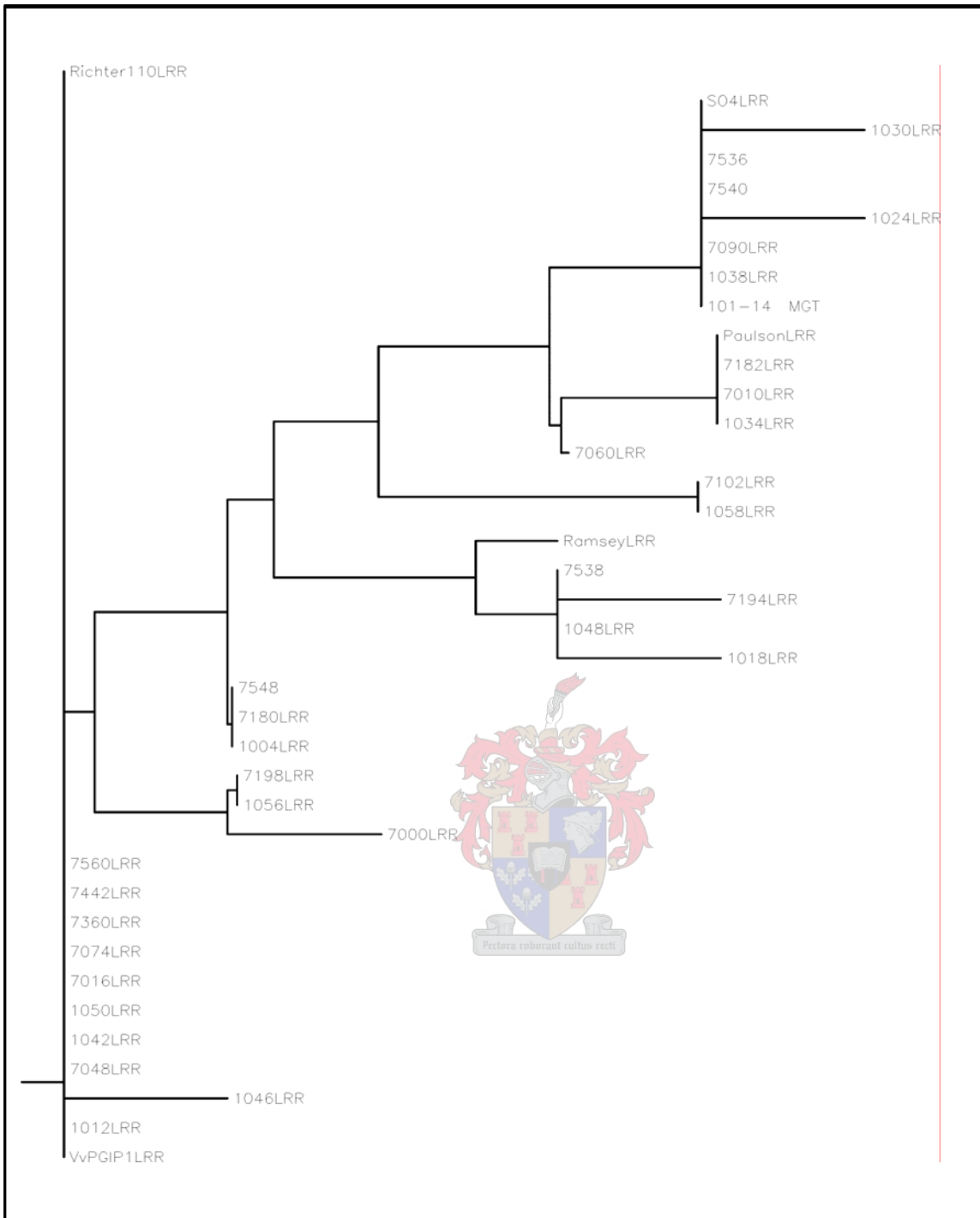
**Figure 4.4** Rooted phylogenetic tree of the amino acid alignment from the LRR region of PGIP encoding genes isolated from various *Vitis* and Non-*Vitis* species (Table 4.1). The tree was constructed using PHYLIP (Phylogeny Inference Package) version 3.5c (Felsenstein, 1989), that was accessed through the Biology Workbench vs. 3.2. The amino acid sequence of *Vvpgip1* was again included for comparative purposes.
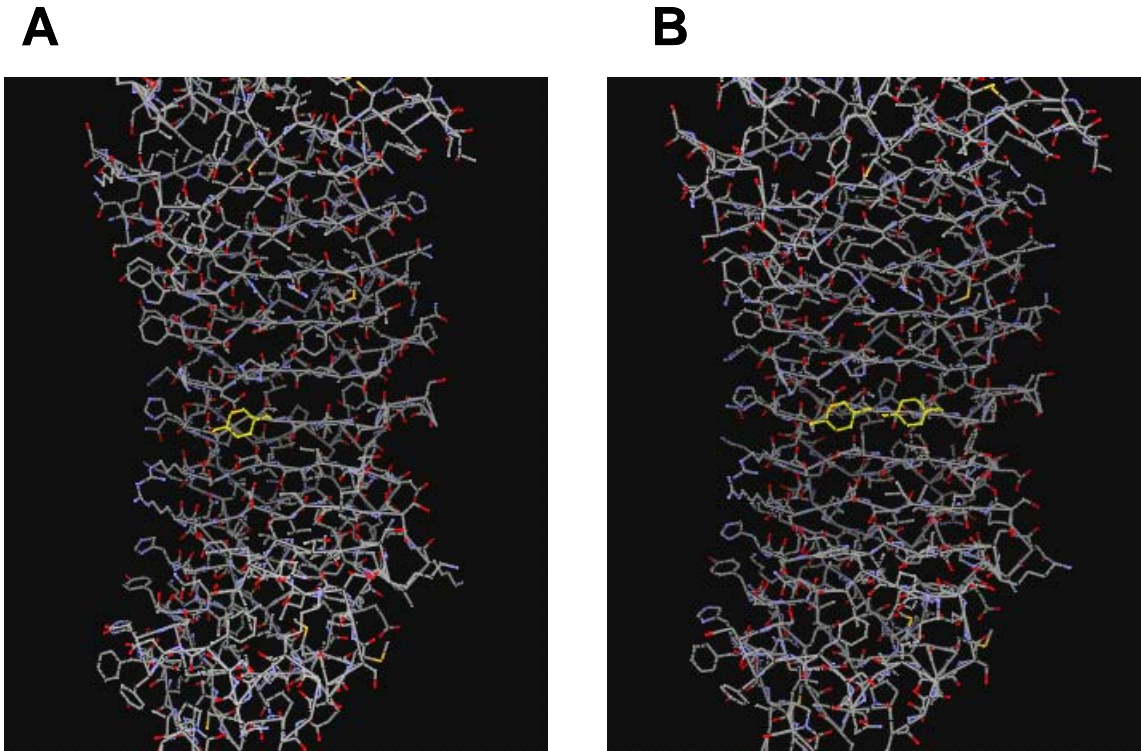
**Figure 4.5** Threaded structures of the PGIP encoding genes from **A**. 1034 and **B**. 1024. The grape PGIP sequence (De Ascensao, 2001 Genbank Ac: AF499451) was threaded against the *P. vulgaris* PGIP2 (Di Matteo et al., 2003) crystal structure (1ogq) using the Swiss-Model protein structure homology-modeling server. The threaded grape PGIP structure was further optimized using the SYBYL molecular modeling package by Tripos. Secondary structural features were aligned including disulfide bonds and β-pleated sheets. The sequences were threaded without the predicted signalling peptide and all positions given assume that position one of the threaded sequences corresponds to position 28 of the pre-protein. In **A** position 174 is highlighted in yellow while in **B**, both positions 174 and 172 is highlighted to emphasize differences.

## 4.4 DISCUSSION

In recent years a significant amount of research has been done to elucidate the structure-function relationships of PGIPs (De Lorenzo *et al.*, 2001; Di Matteo *et al.*, 2003; Federici *et al.*, 2001; Leckie et al., 1999). To this end the three-dimensional structure of the bean PGIP2 has been resolved (Di Matteo *et al.*, 2003) and several studies have been, and are currently conducted to elucidate the mechanistic features of the PGIP:EPG interaction (Federici *et al.*, 2001; Carl Bergmann, Complex Carbohydrate Research Centre, University of Georgia, personal communication; King *et al.*, 2002). Central to this research initiative are the LRR motifs found not only in PGIPs, but in most plant defence related genes studied to date. The LRR motifs found in PGIP encoding genes form the β-strand/β-turn region in the mature protein, and this area is especially important for ligand specificity. This area has been shown to be hyper variable (Parniske *et al.*, 1997) and Leckie *et al* (1999) furthermore showed that a single amino acid variation within the LRR region can confer a new recognition capability to PGIP.

Most plant species contain multigene PGIP encoding families, and the EPG inhibition spectrum of such a family are normally comprised of the differential specificities of its members (De Lorenzo *et al.*, 2001). Previous results obtained from various grapevine cultivars suggest that the *pgip* gene copies present in the genome of *Vitis* might instead of being a true multigene family, encode isozymes of the isolated *Vvpgip1* gene product (Joubert, 2004). Furthermore, preliminary studies indicated that various natural occurring mutations exist in *pgip* genes present in *V. vinifera* grapevine cultivars (De Ascensao, 2001). To further elucidate the inhibition spectrum and efficacy of the PGIP encoding genes in grapevine, 37 additional PGIP encoding genes were isolated from various *Vitis* and non-*Vitis* cultivars. Rootstock cultivars and American grapevine spp. were selected as source material. These genotypes are known for higher levels of resistance towards fungal pathogens when compared to traditional *V. vinifera* cultivars. The genes were sequenced and both the nucleotide and amino acid sequences were compared with that of the recently isolated *Vvpgip1* (De Ascensao, 2001). In a total nucleotide sequence alignment between *Vvpgip1* and the 37 isolated *pgip* genes, we found between 0 and 20 changes in nucleotides that resulted in between 0 and 11 changes in the resulting amino acid sequences. We furthermore found greater than 95% homology between the PGIP encoding genes. In an amino acid alignment between the LRR domains of the isolated *pgips* compared with *Vvpgip1*, homology of higher than 94% was observed.

Although *Vvpgip1* showed significant sequence differences compared with *pgips* from other plant species (De Ascensao, 2001), high homology between *Vitis* and non-*Vitis* cultivars made it possible to thread the deduced protein sequences against the crystal structure of bean PGIP2. Preliminary threading data revealed a structural difference in the cleft formed by the β-sheet/β-turn structure between the cultivars *V. bourquiniana* (1034) and *V. solonis hort* (1024) due to the substitution of tyrosine in position 172 with glycine. The sequence and structural data obtained from the additional 37 PGIP encoding genes serves as an important genetic and biotechnology resource. Experiments linking structure to function, such as inhibition and interaction assays, will furthermore provide valuable insights into the underlying mechanisms involved in PGIP mediated defence responses.
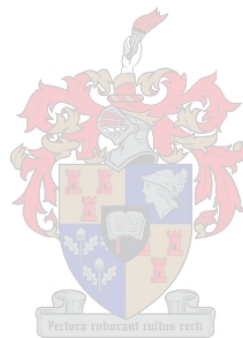
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# CHAPTER 5
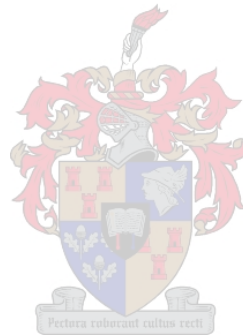
# GENERAL DISCUSSION AND CONCLUSION
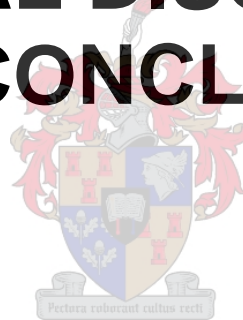
## 5.1 GENERAL DISCUSSION

The development and use of molecular biology as a scientific discipline and tool has opened possibilities for scientific study that was previously unimaginable. Especially within the agricultural fields, new technologies that compliment traditional crop improvement strategies have been implemented with great effect. Apart from the obvious benefits to applied science, the technologies also impacted positively on deciphering fundamental aspects and principles of science. The adoption and implementation of molecular biological tools by scientists to further elucidate the dynamic interaction between plants and pathogens are particularly important for this study, which was intended to extend our current knowledge of two classical role-players in plant-pathogen interactions. These role-players are the endopolygalacturonases (EPGs) secreted by fungal pathogens during infection and their inhibitors present in the cell walls of plants, the endopolygalacturonase-inhibiting proteins (PGIPs).

Over the years, several model organisms have been studied extensively, both from a plant and a pathogen perspective. *Botrytis cinerea* can be regarded as such a plant pathogenic model organism and a significant knowledge-base regarding the genetic diversity, host-range, epidemiology and mode of infection exists. The function of the EPGs of *B. cinerea* during the different stages of infection has been studied extensively (reviewed in Chapter 2 of this thesis), but the specific contribution of each individual EPG during pathogenesis was still unclear when this study commenced. The EPG encoding genes from *B. cinerea* have been cloned previously by Wubben *et al* (1999), providing a useful genetic resource that could for example be used to make knock-out mutants of these genes and assess their individual roles in pathogenesis. Very recently it was shown that EPG1 and 2 are essential for virulence and pathogenicity of *Botrytis.* (Krooshof *et al.*, 2004).

The specific interaction and/or inhibition of each of the EPGs with various inhibitor proteins are still poorly studied and published information in this regard is very limited. This aspect is of specific importance to our research group and was the main driving force behind this project where the EPGs from a hypervirulent strain of *B. cinerea* would be tested against the inhibitor from grapevine, VvPGIP1 that was previously isolated in our laboratory and shown to inhibit a crude extract of EPGs from this strain. Apart from developing the constructs and heterologous over-expression systems (as described in Chapters 1 and 3 of this thesis), another focus was to evaluate chlorophyll fluorescence

analyis as a tool to study the interactions between the EPGs and the PGIP on an *in vivo* level in plants.  Most current interaction analyses rely on *in vitro* systems that do not allow for analysis of whole plant physiology in reaction to the presence and activity of EPGs and PGIPs.

The last part of this study involves the isolation of more PGIP encoding genes from various grapevine genotypes. The fact that the grapevine PGIP encoding genes do not seem to be present in a true multigene family lead us to hypothesize that PGIPs isolated from grapevine genotypes with proven resistance phenotypes against fungal pathogens, might have structural and/or inhibitor features enabling them to be more effective against fungal EPGs.

The following sections will discuss the results obtained with the experimental layout as described in chapters 3 and 4 of this thesis and will specifically try to highlight the contributions made with the results obtained:

### 5.1.1  Cloning and heterologous expression of the six EPG encoding genes from *B. cinerea* in yeast

The sequence and expression data obtained by Wubben *et al.*, 1999 for the *B. cinerea* strain SAS56 were used to isolate the EPG encoding genes from the hypervirulent South African *B. cinerea* strain.  The relative ease with which the cDNA copies of the encoding genes were obtained, suggests that the described expression profile of the South African *B. cinerea* strain is very similar to that of strain SAS56.  A detailed comparison between the EPG encoding sequences of the South African and SAS56 strains, revealed only small differences between the strains.  To further investigate the effect of these sequence differences, specifically with regard to their interaction with VvPGIP1, the genes were heterologously expressed in *S. cerevisiae*.  This approach was largely unsuccessful and although all the genes were expressed efficiently, as confirmed with Northern blot analysis, *in vitro* plate assays (according to Taylor and Secor, 1988) of crude cell and supernatant yeast extracts showed that active preparations could only be obtained for BcPG2.  The possibility exist that the heterologous over-expression from *Saccharomyces* yielded enzymes that were not correctly processed and/or were hyperglycosylated, all aspects that could influence the activity and stability of the enzymes.  Given the lack of active EPGs for the rest of the over-expressed genes, it was impossible to proceed with the intended *in vitro* EPG:VvPGIP1 interaction and inhibition analyses, except for BcPG2.

Kars *et al* (2004) showed that BcPG2 is an important virulence factor for *B. cinerea*. The product would therefore be a logical target in defence responses employed by potential host plants, including grapevine. Using the same agarose diffusion assay to confirm EPG2 activity, we investigated whether the heterologously expressed EPG2 from the South African *B. cinerea* isolated was inhibited by VvPGIP1. No inhibition could be observed with this approach, and subsequent *in vitro* assays (not reported in this thesis) confirmed that the VvPGIP1 also does not interact or inhibit the EPG2 from the *B. cinerea* B05.10 isolate.

A similar approach as ours has been taken and recently reported on by Krooshof *et al* (2004). They over-expressed the *Bcpg1-6* genes in *Pichia pastoris* and could obtain active EPG preparations for all the BcPGs except BcPG5. Given the importance of the BcPGs within our selected scope of study, this approach will form part of the future initiative to purify the EPGs of the South African *B. cinerea* isolate and characterize their interaction with VvPGIP1.

## 5.1.2 Transient over-expression of the *BcPG1-6* genes in tobacco and subsequent *in vivo* interaction studies

The problems associated with obtaining active EPGs from *S. cerevisiae* prompted the investigation of alternative expression systems. Although more complex, plant-based expression systems have been used with great success to express and purify a variety of previously difficult to isolate proteins (Yoshida and Shinmyo, 2000). Recent advances in plant heterologous expression technology also provided the option to transiently over-express the genes instead of stably transforming plants (Yang *et al*., 2000). The six EPG encoding genes from the South African *B. cinerea* isolate were successfully over-expressed in leaves of *N. benthamiana* using *Agrobacterium* infiltration. Similar to the results with the yeast heterologous over-expression system, activity could only be found for BcPG2 with the *in vitro* agarose diffusion assays. The genes were expressed using the native *B. cinerea* signalling peptides and recent results from similar experiments showed that if these signalling peptides are replaced with a plant derived sequence, fungal EPG activity can be detected in the apolastic fluid of the leaves (Krooshof *et al*., 2004). However, it was clear that the over-expression of the EPGs caused phenotypical effects in the infiltrated leaves that resembled the effects of EPG activity. The phenotypical effects differed between the various EPG encoding genes providing some proof that the observed symptoms were linked to the activities of the EPGs and not other unrelated effects. Two of

the EPGs caused very characteristic necrotic lesions (EPG2 and 5) whereas EPG 3 and 4 caused significant yellowing of the leaves without the presence of spreading necrotic lesions. EPG1 caused yellowing as well as spreading necrotic lesions. Very recent work reported by Kars *et al* (2004) also described the same effects with BcPG2 from *B. cinerea* strain B05.10. The authors also put forward the theory that this enzyme has a very devastating and macerating effect on plant tissue and that this response is not linked to the HR response of plants.

The discrepancy between the typical EPG symptoms on the infiltrated leaves and the absence of any observed *in vitro* fungal EPG activity in leaf derived extracts, questioned whether the *in vitro* methods used to detect EPG activity were effective. A more sensitive detection method and more importantly an *in vivo*-based method, using chlorophyll fluorescence as an indication of metabolic stress, was tested. $^{Fv}/_{Fm}$ ratios, previously shown to be a reliable, early indicator of metabolic stress (Maxwell and Johnson, 2000), were taken before and after infiltration of each leaf with a plant efficiency analyzer (PEA). The pre- and post-infiltration differences of $^{Fv}/_{Fm}$ ratios clearly indicated that the over-expression of the EPG encoding genes resulted in a reduction in the photosynthetic rate of the leaves. This system provided an excellent platform to study the interaction of PGIP with the respective EPGs in an *in planta* environment.

The *Bcpg1* as well as *Bcpg2* genes were subsequently co-infiltrated with *Vvpgip1* and the $^{Fv}/_{Fm}$ ratios measured. Surprisingly, and in contrast to the *in vitro* data, $^{Fv}/_{Fm}$ ratios measured of leaves co-infiltrated with *Vvpgip1* and the *Bcpg1* and *Bcpg2* genes respectively*,* showed that VvPGIP1 might inhibit BcPG2 *in planta*. This is in fact the first report of a PGIP interacting with BcPG2 since it was recently reported that from a variety of PGIPs tested, none inhibited BcPG2 (Krooshof *et al.*, 2004). Moreover, the results obtained with the chorophyll fluorescence technique when it was combined with the co-infiltrations of EPG and inhibitor, provided a very exciting possibility to evaluate the probable interactions on an *in vivo* level and without having to rely on symptom development. These very promising preliminary results will be rigorously tested to further establish this technique as a quantitative *in planta* measurement of EPG:PGIP interactions.

In another approach to evaluate the interaction of the various EPGs with VvPGIP1, a modified two-hybrid approach, also based on *Agobacterium*-infiltration was used. This approach yielded very high levels of background GUS activity with the *Bcpg1* and *Bcpg2* fusion constructs. This phenomenon was not observed for any of the other *Bcpg* fusions

and the results seemed to indicate that VvPGIP1 might interact with BcPG3 and BcPG4. Further optimization of this system is needed before any definite conclusions regarding the physical *in vivo* interaction of VvPGIP1 with the respective EPGs from *B. cinerea* can be made.
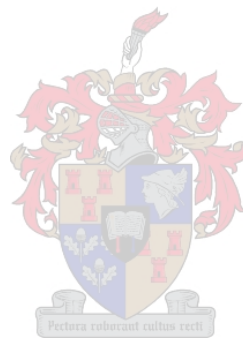
### 5.1.3 Isolation of 37 additional PGIP encoding genes from various grapevine genotypes.

Mulitigene PGIP encoding families are common among plant species and the EPG inhibition spectrum of such a family are normally comprised of the differential specificities of its members (De Lorenzo *et al.*, 2001). In grapevine no true PGIP multigene family exists, and the observed PGIP specificity profile can be attributed to the product of a single gene (Joubert, 2004). Leckie *et al.*, 1999 furthermore showed that a single change in amino acid can change the specificity and the resulting inhibition ability of PGIP significantly. Previous studies in our laboratory have shown that several natural mutations exist between *V. vinifera* cultivars (De Ascensao, 2001). Based on the findings of Leckie *et al.*, 1999 it is conceivable that the natural mutations within the PGIP encoding sequences of grapevine could result in changes with regard to EPG inhibition and ultimately disease susceptibility. To further investigate these possible correlations, 37 additional PGIP encoding genes were isolated from *Vitis* and non-*Vitis* spp, including rootstock material and American spp. Amino acid alignments between the complete protein sequences of the 37 isolated *pgips* showed greater than 95% homology between these proteins and greater than 94% homology between the LRR domains. The high homology between the PGIP encoding sequences also allowed for the threading of the sequences onto the existing crystal structure of bean PGIP2. Preliminary threading results showed that amino acid changes within the LRR could result in small structural changes between the PGIPs. The structural data obtained with these experiments will be used in conjunction with EPG interaction studies to try and correlate sequence and structural differences to specificity and ultimately disease resistance.

### 5.2 CONCLUSION

In this study, the initial goal to use heterologous expression systems to over-express and purify the individual EPG encoding genes from *B cinerea* for subsequent inhibition studies yielded successful gene expression in both systems used, but active proteins could only be detected for one EPG. This hampered the intended *in vitro* inhibition assays, since they

relied on enzyme activity. The *in vivo* analysis methods tested and the results obtained from them clearly highlighted the danger of only relying on cell free *in vitro* systems, since these artificial conditions could mask or disrupt interactions between protein complexes, possible co-factors and/or cell-associated factors necessary to observe true interactions. The preliminary positive results obtained with the chlorophyll fluorescence technique pave the way to develop this technique further to evaluate EPG effects and EPG:PGIP interactions on an *in vivo* level. These technologies and resources developed and obtained, together with the 37 additional PGIP encoding genes from grapevine genotypes, provide a strong basis to study these important role-players in host-pathogen interactions further.

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