The functional analysis of *Vitaceae* polygalacturonase-inhibiting protein (PGIP) encoding genes overexpressed in tobacco

by

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Declaration

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Summary

Agriculture worldwide is under great pressure to produce enough food in order to sustain the ever-growing world population. Among the many challenges faced by food producers, crop losses and damage caused by fungal plant pathogens is a major problem. The study of fungal pathogens and the interaction between plants and fungi is therefore essential, and has been carried out for many years. Much has been learned in this time, but the full mechanisms of the various modes of fungal attack and plant defence have still not been elucidated.

Many fungi rely on the action of cell-wall degrading enzymes (CWDEs) to breach the plant cell wall and facilitate access to the nutrients within. CWDEs are among the very first enzymes to be secreted at the start of fungal attack, and many of them are considered to be essential pathogenesis factors. Endopolygalacturonases (ePGs) are CWDEs that cleave the homogalacturonan stretches of the plant cell wall and are vital virulence factors for a number of fungi, including *Botrytis cinerea*. An important defence mechanism of plants involves the inhibition of CWDEs in order to halt or slow down the fungal attack. Plant polygalacturonase-inhibiting proteins (PGIPs) are cell wall associated CWDE-inhibiting proteins that specifically act on fungal ePGs. Many different PGIPs from a number of diverse plant species have been described to date. They are known to have differential inhibition capabilities that often result from only a few key amino acid changes within the leucine-rich repeat (LRR) active domains.

Previously, the first grapevine PGIP was isolated and characterised from *Vitis vinifera* cultivar Pinotage (*Vvpgip1*). This *Vvpgip1* gene was overexpressed in the tobacco species *Nicotiana tabacum*, and was shown to be very effective in reducing the susceptibility of tobacco towards *B. cinerea*. The combined results confirmed transgene overexpression, increased PGIP activity and a strong resistance response against *Botrytis*, leading to the characterisation of these lines as having PGIP-specific resistance phenotypes. In a subsequent transcriptomic analysis of these lines it was found that they display differential expression of cell wall metabolism genes and biochemical characteristics that might indicate possible cell wall strengthening compared to wild-type tobacco under uninfecting conditions.

The *V. vinifera* cultivars are all very susceptible to fungal attack, whereas other grapevine species, specifically the North American *Vitis* species, are known for their strong resistance and even immunity against many fungal pathogens. Thirty seven PGIPs have previously been isolated from these more resistant species. The amino acid sequences of the active domains of these PGIPs were previously aligned with that of VvPGIP1, and the proteins were found to be highly homologous with each other and with VvPGIP1. The different non-*vinifera* PGIPs separated into 14 subgroups based on their active domain sequences. For this study, one PGIP from each group was selected for functional analysis in tobacco.

The selected PGIP-encoding genes were transformed into tobacco by means of *Agrobacterium tumefaciens*. Analyses of the putatively transformed plantlets were performed to test for transgene presence, transgene expression, and PGIP activity: final transgenic tobacco populations consisting of three to twelve individually transformed lines of nine different non-*vinifera* PGIPs were obtained. A subset of the resultant transgenic lines was infected with *B. cinerea* in two independent whole plant infections over 11-14 days in order to investigate the disease resistance afforded by the various PGIPs towards this fungus. A line from the previously characterised VvPGIP1 population was included as reference; all the infections were contrasted to the WT tobacco. All the infected lines overexpressing the non-*vinifera* PGIPs displayed very strong disease reduction in comparison to the WT control: after initial primary lesion formation, the spread of fungal infection was contained and halted in these lines, while wild-type tobacco plants were severely affected. Although the VvPGIP1 line displayed the

characteristic PGIP-defense response, the non-*vinifera* PGIP plants displayed smaller lesions, indicating very strong resistance phenotypes.

The characterised non-*vinifera* PGIP overexpressing lines, together with the VvPGIP1 line and the WT control were also used to further evaluate the previous observation that overexpression might lead to changes in expression of cell wall genes. Analysis of the expression of a *xyloglucan endotransglycosylase* (*xth*) gene in the transgenic population showed that this gene was down-regulated in healthy uninfected tissue from all the transgenic lines tested. This confirmed previous results and have confirmed in all grapevine PGIP overexpressing lines tested so far that this gene is downregulated. XTH is typically involved in cell wall metabolism and specifically in controlling the strength and elasticity of the plant cell wall. From previous work it is known that downregulation of this gene leads to strengthening of the wall.

The results obtained in this study showed that the PGIP-specific resistance phenotype seen for VvPGIP1-overexpressing tobacco could be confirmed in transgenic tobacco overexpressing non-*vinifera* PGIPs from more resistant grapevine species as well. The fact that these PGIPs lines all performed even better than the VvPGIP1 lines in conferring resistance towards *B. cinerea* provides an interesting angle for further investigation into the structural differences between the non-*vinifera* PGIPs and VvPGIP1. The transgenic lines are also excellent material to study the *in vivo* functions of PGIPs further in the context of plant-pathogen interactions.

Opsomming

Die landboubedryf is wêreldwyd onder groot druk om genoeg voedsel te produseer vir die groeiende wêreldbevolking. Een van die grootste probleme wat die bedryf ondervind, is die groot skade wat aan gewasse aangerig word deur patogeniese swamme. Dit is dus noodsaaklik om swamme en die interaksie tussen plante en swamme te bestudeer, en dit word al vir jare gedoen. Hoewel daar al baie geleer is in hierdie tydperk, is die volle meganismes van die verskeie maniere hoe swamme aanval en hoe plante hulleself verdedig, nog nie bekend nie.

Verskeie swamme maak staat op die aktiwiteit van selwand-afbrekende ensieme (SWAEe) om deur die plantselwand te breek en sodoende toegang tot voedingstowwe in die plantsel te fasiliteer. SWAEe is van die eerste ensieme wat tydens die begin van patogeniese aanval deur swamme afgeskei word en verskeie SWAEe word as noodsaaklike patogeniese faktore beskou. Endopoligalakturonases (ePGs) is SWAEe wat die homogalakturoniese dele van die plantselwand verteer en is noodsaaklike virulensie faktore vir ‘n aantal swamme, onder andere *Botrytis cinerea*. ‘n Belangrike weerstandsmeganisme van plante behels die inhibering van swam SWAEe om sodoende die patogeen-aanval te stop of te vertraag. Die poligalakturonase-inhiberende proteïne (PGIPs) van plante is selwand-geassosieerde SWAE-inhiberende proteïne wat spesifiek teen swam ePGs optree. Verskeie verskillende PGIPs vanuit verskillende plantspesies is tot dusver beskryf. Dit is bekend dat hulle differensiële inhiberende vermoëns het wat dikwels toegeskryf kan word aan slegs ‘n paar belangrike aminosuurvolgordeverskille in die leusien-ryke herhalende (LRH) aktiewe areas.

Die eerste wingerd PGIP is vantevore geïsoleer vanuit *Vitis vinifera* kultivar Pinotage (*Vvpgip1*) en gekarakteriseer. Hierdie *Vvpgip1* geen is ooruitgedruk in die tabakspesie *Nicotiana tabacum* en was baie effektief om die weerstand van tabak teen die swam *Botrytis cinerea* te verhoog. Die ooruitdrukking van die transgeen, verhoogde PGIP aktiwiteit en goeie weerstand teen *Botrytis cinerea* is bevestig, en het gelei daartoe dat die transgeniese VvPGIP1 plantlyne geklassifiseer is as lyne met PGIP-spesifieke weerstandsfenotipes. ‘n Daaropvolgende transkriptomiese analise van die plantlyne het gewys dat hulle differensiële uitdrukking van selwand-geassosieerde gene het, asook biochemiese eienskappe, wat ‘n moontlike selwandversterking aandui in vergelyking met wilde-tipe tabak in die afwesigheid van infeksie.

Die *V. vinifera* kultivars is hoogs vatbaar vir swamme, terwyl ander wingerdspesies, spesifiek die Noord-Amerikaanse spesies, bekend is vir hoë weerstand en selfs immuniteit teenoor verskeie patogeniese swamme. Sewe-en-dertig PGIPs is vantevore geïsoleer vanuit hierdie meer weerstandbiedende spesies. Die aminosuurvolgordes van die aktiewe areas van hierdie PGIPs is vantevore vergelyk met die van VvPGIP1 en dit is gevind dat hierdie proteïne hoogs homoloog is aan mekaar, sowel as aan VvPGIP1. Die verskillende nie-*vinifera* PGIPs het in 14 groepe verdeel na aanleiding van die homologie van hulle aktiewe areas. Vir hierdie studie is een PGIP vanuit elkeen van hierdie groepe gekies vir verdere funksionele analise in tabak.

Die 14 nie-*vinifera* PGIP-koderende gene is stabiel oorgedra na tabak deur middel van *Agrobacterium tumefaciens*. Die vermeende transgeniese plante is geanaliseer vir die teenwoordigheid van die transgeen, die uitdrukking daarvan en PGIP aktiwiteit: bevestigde transgeniese tabak populasies wat wissel van drie tot 12 individuele getransformeerde lyne kon verkry word vir nege van die verskillende nie-*vinifera* PGIPs. ‘n Aantal van die transgeniese lyne is geïnfekteer met *B. cinerea* in twee onafhanklike heelplantinfeksies vir 11-14 dae om die siekteweerstand van hierdie PGIPs teenoor die swam te evalueer. ‘n Plantlyn van die VvPGIP1-populasie is as ‘n verwysing ingesluit en al die infeksies is vergelyk met die wilde-tipe tabak. Al die geïnfekteerde lyne wat die nie-*vinifera* PGIPs ooruitdruk het ‘n baie sterk afname

in siektesimptome getoon in vergelyking met die wilde-tipe kontrole: na aanvanklikle primêre lesies gevorm het, is die verspreiding van die infeksie ingeperk en gestop in hierdie lyne, terwyl die wilde-tipe plante baie erg geaffekteer is. Terwyl die VvPGIP1 lyn ook die tipiese PGIP-weerstandsrespons getoon het, het die nie-*vinifera* PGIPe kleiner lesies ontwikkel, wat dui op baie sterk weerstandsfenotipes.

Die gekarakteriseerde nie-*vinifera* PGIP ooruitdrukkende lyne, asook die VvPGIP1 lyn en die wilde-tipe kontrole, is gebruik om die vorige waarneming dat die ooruitdrukking kan lei tot veranderinge in selwandgeen-uitdrukking verder te ondersoek. Analise van die uitdrukking van ‘n *xiloglukaan-endotransglikosilase* (*xth*) geen in die transgeniese populasie het getoon dat hierdie geen afgereguleer is in gesonde, oninfekteerde weefsel van al die transgeniese lyne wat getoets is. Dit het vorige resultate bevestig en het ook bevestig dat hierdie geen afgereguleer is in alle wingerd PGIP-ooruitdrukkende lyne wat tot dusver getoets is. XTH is tipies betrokke by selwandmetabolisme, spesifiek by die beheer van selwandsterkte en selwandelastisiteit. Dit is uit vorige werk bekend dat die afregulering van hierdie geen lei tot versterking van die plantselwand.

Die resultate verkry tydens hierdie studie het gewys dat die PGIP-spesifieke weerstand fenotipe van VvPGIP1-ooruitdrukkende tabak ook bevestig kon word in transgeniese tabak wat nie-*vinifera* PGIPs vanuit meer weerstandbiedende wingerdspesies ooruitdruk. Die feit dat hierdie PGIP lyne almal selfs beter weerstand teen *B. cinerea* bied as VvPGIP1 lyne is ‘n interessante invalshoek vir opvolgende ondersoeke na die belang van strukturele verskille tussen die nie-*vinifera* PGIPs en VvPGIP1. Hierdie transgeniese lyne is ook uitstekende hulpbronne om die *in vivo* funksies van PGIPs verder te bestudeer in die konteks van plant-patogeen interaksies.

This thesis is dedicated to

My whole family

Biographical sketch

Alida was born in Johannesburg, South Africa on the 8th of January 1982. After attending several different schools in several different towns (and countries) she matriculated in 2000 at Vredenburg High School. She completed her BSc-degree in 2004 at Stellenbosch University, majoring in Genetics and Chemistry. In 2005 she was awarded the degree of HonsBSc (Wine Biotechnology) at the same university, and subsequently enrolled for an MSc degree in Wine Biotechnology.

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Preface

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately. Chapter 3 will be submitted for publication in a journal to be determined at a later date.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
Polygalacturonase-inhibiting proteins (PGIPs) in plant defence: a Review

Chapter 3 **Research results**
The functional analysis of Vitaceae polygalacturonase-inhibiting protein (PGIP) encoding genes overexpressed in tobacco

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Contents

1: General Introduction and Project Aims

1.1 Introduction

Plants face a unique challenge in surviving environmental stress and pathogen attack for two reasons. Firstly, they are anchored in place by their roots and cannot physically move in order to evade a pathogen or any other abiotic stress factor. Secondly, they do not have a circulatory system that can transport resistance molecules or other appropriate anti-stress factors to endangered sites, meaning every cell has to be able to defend itself and respond to signals from neighbouring cells.

Pathogens of plants cause major agricultural damage worldwide by lowering the yield and quality of crops. Fungi, bacteria, insects, herbivores and viruses can infect or damage plants, with some of the most severe damage being caused by phytopathogenic fungi. Pesticide management of fungi is costly, labour-intensive, detrimental to the environment and human health and is not always effective since pathogens develop resistance to the various products over time. Therefore, studying and utilising the biological defence mechanisms of plants in order to improve their resistance to fungi is very important.

One of the most important defence mechanisms of plants is the maintenance of cell wall integrity by the inhibition of fungal degradation enzymes. Phytopathogenic fungi secrete a number of cell wall degrading enzymes (CWDEs) in order to penetrate the plant cell wall and gain access to the nutrients within (Cooper, 1984; Walton, 1994; Alghisi and Favaron, 1995; De Lorenzo *et al.*, 1997; Idnurm and Howlett, 2001; Ten Have *et al.*, 2002). Endopolygalacturonases (ePGs) are CWDEs that break down pectin in the cell walls of plants and are vital pathogenesis factors for a number of fungi (Shieh *et al.*, 1997; Ten Have *et al.*, 1998; Wubben *et al.*, 1999; Huang and Allen, 2000; Wubben *et al.*, 2000; Isshiki *et al.*, 2001; Oeser *et al.*, 2002; Kars *et al.*, 2005). Some bacterial pathogens also rely on ePG activity for pathogenic activity (Huang and Allen, 2000).

Polygalacturonase-inhibiting proteins (PGIPs) are found in many mono- and dicotyledonous plant species. They specifically inhibit fungal ePGs and help to protect plants from attack.

1.2 The role of polygalacturonase-inhibiting proteins (PGIPs) in plant defence

PGIPs have been studied extensively for the better part of forty years (Albersheim and Anderson, 1971; Cheng *et al.*, 2008), and much has been learned about their inhibitory properties and the role they have in plant defence against pathogenic fungi (De Lorenzo and Ferrari, 2002; D'Ovidio *et al.*, 2004a; Di Matteo *et al.*, 2006). Yet the exact mechanism of PGIP-mediated disease resistance and the exact role of PGIP in plant defence have not been completely elucidated.

The most direct and important role that PGIP has is the direct inhibition of fungal ePGs that degrade the pectic plant cell walls. PGIPs selectively bind to and inhibit ePGs *in vitro.* PGIPs from different plants, or from within the same species show differential affinity, inhibition kinetics and specific activity towards ePGs (Johnston *et al.*, 1993; Favaron *et al.*, 1994; Yao *et al.*, 1995; Desiderio *et al.*, 1997). For example, the inhibition kinetics between a specific PGIP:ePG pair can be competitive, non-competitive or of a mixed mode (Abu-Goukh *et al.*, 1983; De Ascensao, 2001; James and Dubery, 2001; Sicilia *et al.*, 2005). It is also known that different PGIP isoforms from the

same species inhibit different ePGs and to different degrees (Leckie *et al.*, 1999; Ferrari *et al.*, 2003; D'Ovidio *et al.*, 2004b; Manfredini *et al.*, 2005). In spite of the large differences between the specific activities of PGIPs, the protein structure and amino acid sequence of PGIPs are highly conserved. PGIPs are leucine-rich repeat (LRR) proteins (Stotz *et al.*, 1994; De Lorenzo *et al.*, 2001; Di Matteo *et al.*, 2003), a large group of proteins that is found across the plant and animal kingdoms and are involved specifically in ligand binding and protein:protein interactions (Kobe and Deisenhoffer, 1994; Gomez-Gomez and Boller, 2000; Jones, 2001; Kobe and Kajava, 2001; Becraft, 2002; Kistner and Parniske, 2002). It has been shown that the specific inhibition profiles of PGIPs are determined by specific amino acid residues at locations within the LRR domain that are important for protein:protein binding (Warren *et al.*, 1998; Leckie *et al.*, 1999; Dodds *et al.*, 2001; Van der Hoorn *et al.*, 2001).

Another consequence of ePG inhibition by PGIP is that the depolymerisation of the pectic cell wall is slowed down (Cervone *et al.*, 1989; Ridley *et al.*, 2001). ePGs randomly cleave non-methylated stretches of homogalacturonan in pectin into smaller oligogalacturonic acid (OGA) chains that can be utilised by the fungus as nutrients (De Lorenzo *et al.*, 2001). OGA molecules with a degree of polymerisation of 10 to 15 have been shown to have elicitor activity and play a role in triggering further downstream plant defence responses, like the hypersensitive response (Simpson *et al.*, 1998; Ridley *et al.*, 2001; Aziz *et al.*, 2004; Federici *et al.*, 2006). Based on mostly *in vitro* studies, PGIP inhibition of ePG is hypothesised to prolong the lifetime of the elicitor-active OGA chains (Cervone *et al.*, 1989).

Overexpression of PGIPs from a number of sources in various heterologous hosts has confirmed the role of PGIP in reducing disease susceptibility towards fungi. Transgenic PGIP-overexpressing plants showed reduced lesion size and slower spread of lesions when infected with the necrotroph *Botrytis cinerea* compared with untransformed wild-type control plants (Powell *et al.*, 2000; Ferrari *et al.*, 2003; Agüero *et al.*, 2005; Manfredini *et al.*, 2005; Joubert *et al.*, 2006). Silencing of PGIP has resulted in increased disease susceptibility, thus verifying the importance of PGIP in plant defence reactions (Ferrari *et al.*, 2006).

Previous work in our laboratory has shown that grapevine has a single PGIP-encoding gene (*Vvpgip1*) that is under developmental and tissue-specific control (De Ascensao, 2001). *Vvpgip1* expression is however upregulated in the presence of pathogens and other inducing agents. This grapevine PGIP was overexpressed in tobacco, leading to a significant decrease in disease susceptibility against *B. cinerea* (Joubert *et al.*, 2006). The level of disease susceptibility/resistance was measured with a time-course whole-plant infection assay over 15 days. This assay, in combination with genetic analyses and protein activity assays confirmed that the transgenic tobacco population displayed PGIP-specific resistance phenotypes (Joubert *et al.*, 2006). These phenotypes were analysed with transcriptomic analyses leading to new evidence suggesting that PGIP overexpression might prepare the plant for possible infection by strengthening the plant cell walls even before pathogen attack (Becker, 2007).

The cultivars of *Vitis vinifera* (the European grape) are typically weakly resistant to fungal pathogens, whereas other *Vitis* species (typically the North American varieties) are known for their high levels of disease resistance against fungal pathogens. Based on this attribute, and the significant decrease in disease susceptibility caused by the characterised grapevine PGIP, additional grapevine PGIPs isolated from non-*vinifera Vitis* species were targeted for comparative analyses with regards to their antifungal capacities.

1.3 Rationale and specific project aims

The variable inhibition specificity and inhibition profiles of different PGIPs towards ePGs has been shown to be caused by small amino acid changes at key positions of the protein (Leckie *et al.*, 1999). For instance, bean PvPGIP1 does not inhibit crude *Fusarium monoliforme* ePGs at all, while bean PvPGIP2 is able to completely inhibit these ePGs. In a mutation study that changed each variable amino acid of PvPGIP1 to correspond with that of PvPGIP2 one by one, it was found that one specific mutation in the active domain of PvPGIP1 enabled it to completely inhibit the *F. monoliforme* ePGs (Leckie *et al.*, 1999).

The approach of this study is to functionally analyse and compare a set of grapevine PGIPs for their ability to decrease disease susceptibility against *B. cinerea*. The PGIP encoding genes that would be used in this study were previously isolated from *Vitaceae* species. The sequence variation between the *Vitaceae pgips* and *Vvpgip1* is low and amino acid changes in the LRR motifs of the PGIPs would be used to categorise the PGIPs in order to select candidates for *in planta* functional characterisation. The approach would be similar to the functional characterisation of VvPGIP1, as described in Joubert *et al.* (2006), and will lead to transgenic tobacco populations overexpressing the different PGIP-encoding genes. The populations will be evaluated for their transgenic status and PGIP activity before the resistance phenotypes of the transgenic lines would be determined.

The aim of this study: Functional characterisation and comparison of a selection of *Vitaceae* PGIPs in transgenic tobacco. The specific aims include:

(i) Selection of a subset of *Vitaceae pgip* genes based on amino acid comparisons of the LRR active domain motifs;
(ii) Subcloning of the selected genes into suitable plant expression vectors, mobilisation of these vectors into *Agrobacterium*-transformation strains and subsequent transformation of *Nicotiana tabacum* (tobacco) leaf discs to generate putative transgenic populations;
(iii) Evaluation of the putative transgenic populations to confirm transgene integration and copy number, transgene expression as well as PGIP activity in the transgenic populations. A characterised VvPGIP1 transgenic line will be used as control and for comparative purposes;
(iv) Determining the antifungal effect of the *Vitaceae* PGIPs with whole-plant infections of the transgenic plants with the necrotrophic fungus *B. cinerea*, using a VvPGIP1 overexpressing line as a comparative reference;
(v) Evaluation of xyloglucan endotransglycosylase expression patterns in a subset of the transgenic lines to further evaluate a possible cell wall strengthening phenotype in PGIP overexpressing lines.

1.4 References

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2: Polygalacturonase-Inhibiting Proteins (PGIPs) in Plant Defence: a Review

2.1 Introduction

Plants and their various pathogens have been waging war on each other for thousands of years. Fungal pathogens in particular cause devastating losses to commercial crops, yet in spite of this, most fungal-plant interactions do not lead to establishment of disease and are deemed incompatible interactions. Therefore, the successful plant pathogen is one that can escape recognition by the plant long enough to be able to launch a successful assault against the physical barriers of the plant, convert the plant material into smaller components that can be utilised for growth and reproduction, and effectively withstand the defence response launched by the plant in retaliation.

The first line of defence that the pathogen encounters during attack is the plant cell wall. It is a complex pectin-rich physical matrix that forms an effective barrier against invaders. In order to efficiently colonise the plant host, the pathogen needs to be able to breach the plant cell wall and gain access to nutrients within the plant cells. Cell wall degrading enzymes (CWDEs) are the 'weapons of choice' for many plant pathogens and are among the first enzymes to be secreted during the start of an infection attempt (Cooper, 1984; Idnurm and Howlett, 2001; Ten Have *et al.*, 2002). Polygalacturonases (PGs) are a family of CWDEs produced by a wide range of pathogens, including bacteria, fungi, insects and nematodes, and they are essential pathogenesis factors for many fungi, including *Botrytis cinerea* (Shieh *et al.*, 1997; Ten Have *et al.*, 2002; Kars *et al.*, 2005). Many pathogens possess a range of PGs with varying activities in order to broaden the range of possible hosts that can be infected *(Caprari et al.*, 1993; Wubben *et al.*, 1999; Daroda *et al.*, 2001; De Lorenzo *et al.*, 2001). PGs are involved in cell wall degradation as well as tissue maceration, as they depolymerise the homogalacturonan component of pectin by cleaving the bonds between galacturonic acid units (Hahn *et al.*, 1989; De Lorenzo *et al.*, 2001). During this enzymatic cleavage process, oligogalacturonic acid (OGA) fragments are released that have been shown to be involved in eliciting plant defence mechanisms, depending on the length of the OGA chain (Cervone *et al.*, 1989; Ridley *et al.*, 2001; Aziz *et al.*, 2004).

Plants that can resist the initial pathogen attack as long as possible and hamper the spread of the pathogen by vigorously defending itself, will overcome the infection. A variety of strategies are used by plants to achieve this: cell-wall strengthening genes are upregulated following pathogen attack; antimicrobial compounds are produced (such as toxic secondary metabolites and hydrolytic enzymes); and the hypersensitive response (HR) is launched (this is characterised by a rapid and localised cell death at the point of pathogen recognition and is triggered by specific signals during pathogen attack) (Hammond-Kosack and Jones, 1996, 1997; Sticher *et al.*, 1997; Maleck and Lawton, 1998; Somssich and Hahlbrock, 1998; Heath, 2000; Mur *et al.*, 2008; Bolton, 2009).

Many plants possess polygalacturonase-inhibiting proteins (PGIPs) that specifically interact with and inhibit a group of fungal polygalacturonases called endopolygalacturonases (ePGs) (Powell *et al.*, 2000; De Lorenzo *et al.*, 2001). PGIPs are associated with the plant cell wall and belong to a large family of leucine-rich repeat (LRR) proteins (Stotz *et al.*, 1994; De Lorenzo *et al.*, 2001) that are primarily involved in protein-protein binding interactions (Kobe and Deisenhoffer, 1994; Jones and Jones, 1997; Jones, 2001; Kobe and Kajava, 2001). PGIPs are usually encoded by small gene families (Frediani *et al.*, 1993; Stotz *et al.*, 1993; Favaron *et al.*,

1994; Stotz *et al.*, 1994; Desiderio *et al.*, 1997), and their LRR active domain sequences are highly conserved between genes, even between genes from diverse plant species. Yet, despite the high active domain homology, PGIPs vary greatly in their modes of inhibition (Abu-Goukh *et al.*, 1983; Johnston *et al.*, 1993; De Ascensao, 2001; James and Dubery, 2001; Sicilia *et al.*, 2005), as well as the range of pathogens and individual ePGs that they can inhibit *(*Cervone *et al*., 1987; Johnston *et al.*, 1993; Stotz *et al.*, 1994; Yao *et al.*, 1995; De Ascensao, 2001). This variability has been linked to small amino acid changes in the active domains between different PGIPs, and it has been shown that even one amino acid change can change the inhibition specificity of PGIP towards ePGs (Leckie *et al.*, 1999). Different PGIP-encoding genes are differentially regulated (D'Ovidio *et al.*, 2002; Ferrari *et al.*, 2003; D'Ovidio *et al.*, 2004b; Cheng *et al.*, 2008), enabling defence against ePG attack under many different conditions. It has been shown in a range of heterologous hosts that overexpression of various PGIPs leads to decreased susceptibility to fungi and a bacterium (Powell *et al.*, 2000; Ferrari *et al.*, 2003; Agüero *et al.*, 2005; Manfredini *et al.*, 2005; Joubert *et al.*, 2006). Recent work has also implicated overexpression of PGIP in promoting cell wall strengthening, even before any pathogen attack takes place (Becker, 2007). There is also evidence for strong *in vivo* inhibition of ePG2 from *B. cinerea* by VvPGIP1 from grapevine, without any detection of a physical protein-protein interaction (Joubert *et al.*, 2007), raising the possibility that a third component might be involved in ePG inhibition by PGIP. This third role player is currently hypothesised to be a pectin-derived component (Joubert *et al*., 2007).

This overview will summarise and discuss important aspects of PGIPs and their role in plant disease resistance. Firstly, the inhibitory function of PGIP is discussed, focussing on the interaction between ePGs and PGIP; the overexpression of PGIP in heterologous hosts; and the *in planta* role of PGIPs in mediating disease resistance. Secondly, the physical structure of PGIP is discussed, specifically with reference to inhibition of ePGs. Lastly, there is a summary of research done on VvPGIP1 from *Vitis vinifera* cultivar Pinotage at the Institute for Wine Biotechnology (IWBT).

2.2 PGIPs: Important Plant Defence Proteins

Albersheim and Anderson first described the presence of an ePG inhibitor in plants more than thirty years ago (Albersheim and Anderson, 1971). In that study the inhibitory protein was found in three different plant species (bean, *Phaseolus vulgaris*; tomato, *Lycopersicon esculentum*; and sycamore, *Acer pseudoplatanus*) and it has subsequently been found in almost every plant species analysed for PGIP activity (Powell *et al.*, 2000). Table 2.1 summarises all the plant species for which *pgip*-encoding sequences have been entered into the GenBank nucleotide database to date (www.ncbi.nlm.nih.gov/Genbank). From the initial *in vitro* analyses of the first extracted PGIPs, it was found that: i) PGIPs are very specific in their inhibition of ePGs (they had no inhibitory effect on other cell-wall degrading enzymes such as cellulases and xylanases); ii) different PGIPs have differential inhibitory activities (different amounts of the three PGIP extracts were needed to achieve the same level of inhibition towards specific ePGs); and iii) there may be more than one PGIP present in a specific plant (the purified bean PGIP was a much poorer inhibitor than an impure extract from the same plant) (Albersheim and Anderson, 1971).

2.2.1 The Inhibitory Function of PGIPs

PGIPs are associated with the plant cell wall and they counteract the action of ePGs (De Lorenzo *et al.*, 2001). They directly bind to ePGs and inhibit them in a one-on-one protein:protein interaction, as has been found in a number of *in vitro* studies (Federici *et al.*, 2001; D'Ovidio *et al.*, 2004a; Di Matteo *et al.*, 2006).

Table 2.1 *List of all plant species for which a pgip-encoding gene sequence has been entered into the GenBank nucleotide database to date* (www.ncbi.nlm.nih.gov/Genbank).

Species	Common name	Species	Common name
Actinidia deliciosa	Chinese gooseberry	*Musa acuminata*	Dessert banana
Adenostoma fasciculatum	Chamise	*Nicotiana tabacum*	Tobacco
Arabidopsis thaliana	Thale cress	*Neviusia alabamensis*	Alabama snow-wreath
Aruncus dioicus	Goat's beard	*Oryza sativa*	Rice
Brassica rapa subsp. pekinensis	Chinese cabbage	*Oryza sativa* Japonica Group	Japanese rice
Capsicum annuum	Chilli pepper	*Phaseolus vulgaris*	French bean
Cercocarpus ledifolius	Curl-leaf mountain mahogany	*Photinia serratifolia*	Chinese photinia
Chaenomeles speciosa	Japanese quince	*Physocarpus capitatus*	Pacific Ninebark
Chamaebatia foliolosa	Mountain misery	*Physocarpus opulifolius*	Ninebark
Chamaebatiaria millefolium	Fernbush	*Pisum sativum*	Pea
Chorispora bungeana	Common name not known	*Poncirus trifoliata*	Hardy orange
Citrus aurantiifolia	Lime	*Potentilla anserina*	Silverweed
Citrus hystrix	Thai lime	*Potentilla fruticosa*	Shrubby cinque
Citrus iyo	Orange	*Prinsepia sinensis*	Chinese prinsepia
Citrus jambhiri	Rough lemon	*Prunus americana*	Goose plum
Citrus latipes	Tanaka	*Prunus armeniaca*	Apricot
Citrus unshiu	Satsuma orange	*Prunus dulcis*	Almond
Crataegus monogyna	Hawthorn	*Prunus emarginata*	Bitter cherry
Daucus carota	Carrot	*Prunus mahaleb*	Mahaleb cherry
Duchesnea indica	Indian strawberry	*Prunus mume*	Japanese apricot
Eucalyptus camaldulensis	Murray red gum	*Prunus persica*	Peach
Eucalyptus grandis	Rose Gum	*Prunus salicina*	Korean cherry
Eucalyptus nitens	Ribbon Gum	*Purshia tridentata*	Bitterbrush
Eucalyptus saligna	Sydney blue gum	*Pyracantha fortuneana*	Chinese firethorn
Eucalyptus urophylla	Timor mountain gum	*Pyrus communis*	Pear
Exochorda racemosa	Pearlbush	*Pyrus hybrid cultivar*	Pear hybrid
Fortunella margarita	Nagami kumquat	*Pyrus pyrifolia*	Asian pear
Fragaria x ananassa	Strawberry	*Pyrus ussuriensis*	Chinese pear
Fragaria iinumae	Asian strawberry	*Rhodotypos scandens*	Jetbead
Fragaria vesca	Woodland strawberry	*Solanum torvum*	Devil's fig
Frangula californica	California buckthorn	*Solanum tuberosum*	Potato
Gillenia stipulata	Indian physic	*Sorbaria sorbifolia*	False spiraea
Gillenia trifoliata	Mountain Indian physic	*Spiraea cantoniensis*	Reeves' meadowsweet
Glycine max	Soybean	*Spiraea densiflora*	Dense-Flowered Spiraea
Gossypium barbadense	Sea-island cotton	*Stephanandra chinensis*	Chinese Rose
Helianthus annuus	Common sunflower	*Triticum aestivum*	Bread wheat
Heteromeles arbutifolia	Toyon	*Triticum militinae*	Wheat
Holodiscus microphyllus	Small-leaved Creambush	*Triticum turgidum subsp. dicoccoides*	Wild emmer wheat
Horkelia cuneata	Wedge-leaved Horkelia	*Triticum turgidum subsp. durum*	Durum wheat
Kageneckia oblonga	Bollèn	*Triticum urartu*	Thumanian ex Gandilyan wheat
Kerria japonica	Japanese rose	*Ulmus americana*	American elm
Lyonothamnus floribundus	Ironwood	*Ulmus pumila*	Siberian elm
Malus x domestica	Cultivated apple	*Vauquelinia californica*	Arizona rosewood
Microcitrus sp. Citruspark	Common name not known	*Vitis vinifera*	Wine grape

The outcome of the inhibition interaction is hypothesised to lead to the prolonged presence of elicitor-active molecules that could act as signals to activate defence responses in the plant (Cervone *et al.*, 1989). Overexpression of PGIPs from a number of different sources in different heterologous hosts has shown that PGIP overexpression can dramatically reduce the susceptibility of the host plant to fungal infection (Powell *et al.*, 2000; Ferrari *et al.*, 2003; Agüero *et al.*, 2005; Manfredini *et al.*, 2005; Joubert *et al.*, 2006). However, the specific *in planta* mechanism of PGIP-mediated disease resistance is not yet fully understood. These aspects are discussed in further detail below.

2.2.1.1 The ePG:PGIP inhibition interaction

Both biotrophic and necrotrophic fungi sequentially produce a broad range of enzymes that degrade the plant cell wall, often starting with ePGs, followed by pectin lyases, proteases, cellulases and others *(*Cooper*,* 1984; Walton, 1994; Alghisi and Favaron, 1995; De Lorenzo *et al.*, 1997; Idnurm and Howlett, 2001; Ten Have *et al.*, 2002). ePGs are the most extensively studied cell wall-degrading enzymes and are produced by many different organisms, including fungi, bacteria, insects and plants themselves (Girard and Jouanin, 1999; De Lorenzo and Ferrari, 2002; Jaubert *et al.*, 2002). ePGs randomly cleave the α-1-4 linkages between galacturonic acid units in the non-methylated homogalacturonan stretches of pectic cell walls, resulting in separation of cells and maceration of tissue (Hahn *et al.*, 1989; De Lorenzo *et al.*, 2001). ePGs therefore facilitate fungal penetration and provide the fungus with nourishment.

ePGs are also vital pathogenesis factors for various fungi. For example, an *Aspergillus flavus* strain with a deleted *pecA* ePG gene showed reduced lesion development in cotton (Shieh *et al.*, 1997). Expression of this same ePG gene in an *A. flavus* strain without ePG activity led to increased lesion sizes. *B. cinerea* also requires two specific ePG genes (*BcPG1* and *BcPG2*) for full virulence on various host plants (Ten Have *et al.*, 1998; Kars *et al.*, 2005) and *Alternaria citri* requires a specific ePG gene for efficient invasion of citrus fruit (Isshiki *et al.*, 2001). A strain of *Claviceps purpurea* with both *cppg1* and *cppg2* ePG genes deleted is nearly non-pathogenic on rye (Oeser *et al.*, 2002) and the bacterium *Ralstonia solanacearum* also depends on ePG for infection of tomato (Huang and Allen, 2000).

In order to be able to colonise a broad range of hosts under various different conditions, pathogens produce a number of different ePG isozymes with variable optimum enzymatic conditions, which are often polymorphic between different races or isolates (Caprari *et al.*, 1993; Wubben *et al.*, 1999; Wubben *et al.*, 2000; Daroda *et al.*, 2001; Poinssot *et al.*, 2003; Favaron *et al.*, 2004). ePGs can differ with respect to primary structure, specific activity, pH optimum, substrate preference and mode of action.

The ePGs of *Botrytis* are the most well-known and best characterised. The *B. cinerea* genome contains at least six different ePG-encoding genes (Wubben *et al.*, 1999) that have differential enzymatic properties (Kars *et al.*, 2005) and gene regulation patterns (Wubben *et al.*, 1999; Wubben *et al.*, 2000; Ten Have *et al.*, 2001). BcPGs 1,2,3,4, and 6 were heterologously expressed in *Pichia pastoris* (Kars *et al.*, 2005), and the purified recombinant enzymes were found to differ in specific activity, protein stability, substrate preference and end-products. These six BcPG-encoding genes are differentially regulated *in vitro* (Wubben *et al.*, 1999; Wubben *et al.*, 2000). When the fungus was grown on four different carbon sources (glucose, polygalacturonic acid (PGA), apple pectin and D(+)galacturonic acid (GA)), BcPG1, 2 and 6 were expressed on all the carbon sources, BcPG3 and 5 were expressed only on glucose and pectin, and BcPG4 was expressed on OGA, GA and weakly on pectin at a later stage of growth. Differential BcPG expression was confirmed in tomato leaves, broad bean leaves, apple fruit

and zucchini fruit in an *in planta* assay (Ten Have *et al.*, 2001). The expression of the individual BcPGs also differed depending on the host tissue, temperature and the stage of infection.

Just as ePGs vary with regards to their specific enzymatic action and properties, PGIPs also possess different specific inhibition spectra, mode of actions and gene expression patterns in order to counter the different ePGs. PGIPs from different plant sources show differential inhibition spectra towards a range of fungal ePGs (Cervone *et al.*, 1987; Johnston *et al.*, 1993; Stotz *et al*., 1993; Favaron *et al.*, 1994; Stotz *et al.*, 1994; Yao *et al.*, 1995). There are often a number of different PGIP isoforms present in a plant, with total PGIP activity for that plant being a combination of all the individual proteins that can also have differential inhibition spectra (Favaron *et al.*, 1994; Stotz *et al.*, 1994; Desiderio *et al.*, 1997). Bean PvPGIP1, 2, 3 and 4 all inhibit *B. cinerea* and *Colletotrichum acutatum* ePGs with different efficiencies, while *A. niger* PGII is inhibited by PvPGIP1 and 2, but not 3 or 4 (D'Ovidio *et al.*, 2004b). *F. monoliforme* FmPG is inhibited only by PvPGIP2 (Leckie *et al.*, 1999). *Arabidopsis thaliana* PGIPs inhibit *C. gleosporoides*, *Stenocarpella maydis* and *B. cinerea* ePGs, but not those from *F. monoliforme* or *A. niger* (Ferrari *et al.*, 2003; D'Ovidio *et al.*, 2004b; Manfredini *et al.*, 2005).

The different PGIP isoforms in plants are typically encoded for by small, highly homologous *pgip* gene families (Frediani *et al.*, 1993; Stotz *et al.*, 1993; Stotz *et al.*, 1994). PGIP-encoding genes from different plant species or members of the same *pgip* family can also be differentially regulated (D'Ovidio *et al.*, 2002). For example, both *A. thaliana* AtPGIP1 and AtPGIP2 are induced by *B. cinerea* infection and wounding; neither one of them is regulated by salicylic acid (SA), but AtPGIP2 is induced after methyl jasmonate (MeJA) treatment, whereas only AtPGIP1 is induced by OGA and cold treatment (Ferrari *et al.*, 2003). In the bean *P. vulgaris* PGIP family of four members, PvPGIP2 is the only one induced after SA treatment, whereas PvPGIP1, 2 and 3 are induced by wounding and OGA treatments, but not PvPGIP4 (D'Ovidio *et al.*, 2004b). Soybean GmPGIP1 and GmPGIP3 are upregulated after wounding and *Sclerotinia sclerotiorum* infection, but GmPGIP2 is not expressed after wounding and only expressed at a late stage after infection (D'Ovidio *et al.*, 2002). In *Populus deltoides*, PdPGIP2 and PdPGIP4 is upregulated to similar levels in response to SA, MeJA and H_2O_2 treatment (Cheng *et al.*, 2008), and although PdPGIP2 and PdPGIP4 are both upregulated following inoculation with the fungus *Marssonina brunnea*, PdPGIP4 expression was 5 times more than PdPGIP2 expression (10.9 times and 2.3 times more than uninoculated plants, respectively).

The possession of multiple *pgip* isoforms can therefore afford the plant a two-fold advantage, as the proteins can differ in their specific ePG inhibition, and the genes can also differ in their expression patterns.

2.2.1.2 The role of PGIPs in the activation of plant defence responses against pathogens

In addition to the direct protein-protein interaction with ePG that can result in the inhibition of the enzyme, it is thought that PGIPs could also promote the plant defence reaction by prolonging the lifetime of elicitor-active OGA fragments that are released to the apoplast during the first stages of pathogen attack (Cervone *et al.*, 1989; De Lorenzo *et al.*, 2001; Ridley *et al.*, 2001; De Lorenzo and Ferrari, 2002).

OGA fragments are derived from enzymatic cleaving of β-1,3 glucans or chitin by fungal ePGs and other CWDEs. Plant chitinases and β-1,3 glucanases can also generate similar elicitors from the fungal cell wall. OGA molecules with a degree of polymerisation of 10 to 15 molecules have elicitor activity and are involved in triggering a number of defence responses, such as phytoalexin accumulation, lignin synthesis, ethylene synthesis, β-1-3-glucanase and proteinase inhibitor expression, and production of reactive oxygen species (Simpson *et al.*,

1998; Ridley *et al.*, 2001; Aziz *et al.*, 2004; D'Ovidio *et al.*, 2004a; Federici *et al.*, 2006). Treatment of detached grapevine leaves with OGAs prior to infection with *B. cinerea* resulted in a 50-65% reduction in lesion sizes after five days compared to untreated leaves (Aziz *et al.*, 2004).

Since the short-chain elicitor active OGAs are intermediates formed from the degradation of pectin by ePGs, the inhibition of ePGs by PGIP could possibly result in the prolonging of the lifetime of the active OGAs. This has been confirmed in *in vitro* analyses (Cervone *et al.*, 1989), but no *in planta* evidence exists currently that can confirm that PGIP defence signalling occurs through OGAs.

2.2.1.3 PGIP overexpression

The potential of PGIPs to reduce susceptibility to fungal pathogens, as described above, has been investigated by overexpression strategies of different PGIPs in various heterologous host plants. A number of examples are discussed in this section.

Resistance towards infection by *B. cinerea* has been afforded by overexpression of a number of different PGIPs in a range of heterologous hosts, including pear PGIP overexpressed in both tomato and grapevine (Powell *et al.*, 2000; Agüero *et al.*, 2005), bean PGIP overexpressed in tobacco (Manfredini *et al.*, 2005), grapevine PGIP overexpressed in tobacco (Joubert *et al.*, 2006), and two endogenous *Arabidopsis* PGIP genes separately overexpressed in *Arabidopsis* (Ferrari *et al.*, 2003). In all these cases, initial infection was established by the pathogen, but the spread of lesions were contained and prevented from developing significantly, whereas wild-type control plants formed large necrotic lesions that spread rapidly. The overexpression of bean PvPGIP2 in wheat led to transgenic plants that were more resistant towards the fungus *Bipolaris sorokiniana* (Janni *et al.*, 2008). Antisense expression of one of the two *Arabidopsis* PGIPs resulted in plants that were more susceptible to *B. cinerea*, and those plants also showed reduced inhibitory activity in response to other biotic and abiotic stimuli (Ferrari *et al.*, 2006).

There is also some evidence suggesting that PGIP might have an inhibitory effect on bacterial pathogens. The bacterium *Xylella fastidiosa* causes Pierce's Disease (PD) in grapevine. Its genome indicates the presence of a putative PG gene that could contribute to the virulence of the organism. Transgenic grapevine overexpressing a pear PGIP was infected with *X. fastidiosa*, and some of the transgenic plants showed less severe PD symptoms than the wild-type controls and the concentration of bacteria in the transgenic stem tissue was lower than in the controls (Agüero *et al.*, 2005). This inhibition of *X. fastidiosa* was very slight and not effective enough to be of significant benefit, but it opens up the possibility of PGIP overexpression being useful against bacterial pathogens that rely on ePG activity for pathogenesis.

There are also cases in which PGIP overexpression did not have an effect on the disease resistance of the heterologous host plant. For example, PvPGIP1 from bean (*P. vulgaris*) was constitutively overexpressed in tomato (Desiderio *et al.*, 1997) and the transgenic plants were used in infection studies with the fungi *F. oxysporum* f. sp. *lycopersici*, *B. cinerea* and *A. solani*. No enhanced resistance was observed toward any of the three fungi.

In an interesting approach, scions of untransformed grapevine (specifically Chardonnay and Thompson Seedless) were grafted onto grapevine rootstocks that were transformed with pear PGIP under constitutive expression (Agüero *et al.*, 2005). Xylem sap from the untransformed scions showed 100% inhibition of ePGs, showing that the overexpressed PGIP was transported via the xylem through the graft union into the wild type tissue. It was not tested

whether the PGIP molecules were transported into leaves, berries or other structures. This result opens up the possibility of conferring fungal resistance to many different cultivated grapevine cultivars without having to go through the laborious process of stable transformation for each cultivar. If PGIP is indeed transported to leaves or berries, only the desired rootstock species needs to be transformed.

2.2.2 The Protein Structure of PGIP

2.2.2.1 PGIP is an LRR-protein

Leucine-rich repeat (LRR) domains are found throughout the life kingdoms and are present in many plant proteins, specifically ones that are involved in protein-protein interactions and the recognition of non-self molecules (Kobe and Deisenhoffer, 1994; Jones, 2001). The LRR-domain is a versatile structure that is specialised for interaction with protein ligands and can interact with diverse molecules (Kobe and Kajava, 2001). It is often fused with other functional domains and they have been found in proteins involved in hormone perception and development (Becraft, 2002), elicitor perception (Gomez-Gomez and Boller, 2000), defence responses against insects and bacterial and fungal symbiosis (Kistner and Parniske, 2002). Two plant LRR proteins from *Arabidopsis* have also recently been shown to be involved in signalling in cell wall biosynthesis and define a novel cell wall regulatory signalling pathway (Xu *et al.*, 2008). The majority of plant resistance gene products are LRR proteins (Jones and Jones, 1997; Ellis *et al.*, 2000; Jones, 2001).

All extracytoplasmic plant LRR proteins (eLRRs) are characterised by a specific 24-residue tandem repeat sequence: xxLxLxxNxLt/sGxIPxxLxxLxxL (Kobe and Kajava, 2001). This repeat differs from those found in cytoplasmic plant LRRs as well as other LRR families. The xxLxLxx sequence within the eLRR forms a β-strand/β-turn region in which the L residues form a hydrophobic core and the x residues are solvent-exposed and involved in ligand interaction (Kobe and Deisenhoffer, 1995; Leckie *et al.*, 1999; Mattei *et al.*, 2001a). This β-strand/β-turn structural motif is responsible for the diverse recognition specificity seen amongst eLRR proteins (Kobe and Deisenhoffer, 1994). Work done on the tomato *Cf*-protein family and other *R* genes has identified the β-strand/β-turn region as a hypervariable region under diversifying selection that is responsible for the ligand binding specificity of these proteins (Parniske *et al.*, 1997; Meyers *et al.*, 1998; Bishop *et al.*, 2000; Stotz *et al.*, 2000). Amino acid changes in this region can alter the function of *R* proteins (Warren *et al.*, 1998; Leckie *et al.*, 1999; Dodds *et al.*, 2001; Van der Hoorn *et al.*, 2001; Van der Hoorn *et al.*, 2005).

All known PGIPs are ~40 kDa LRR glycoproteins with similar primary structures and contain the 24-residue eLRR tandem repeat sequence (Stotz *et al.*, 1994; De Lorenzo *et al.*, 2001; Mattei *et al.*, 2001a). The main PGIP protein body consists of a central LRR-containing region flanked by two adjacent cysteine-rich domains that stabilise the PGIP molecule and determines its secondary structure (Protsenko *et al.*, 2008). The structural units within the molecule are orientated in such a way that the β-sheets and α-helices are parallel to the general protein axis. This results in a saddle-shaped molecule that has bent β-sheets covering the internal fold of the saddle and α-helices surrounding the external surface.

The crystal structure of bean PvPGIP2 has been determined (Figure 2.1) (Di Matteo *et al*., 2003). The structure is that of the typical curved and elongated LRR protein, although its scaffold is more twisted. Ten tandemly repeating units made up of the 24-residue consensus sequence (xxLxLxxNxLt/sGxIPxxLxxLxxL) are folded into a right-handed superhelix (residues 53-289) that makes up the central LRR structure. It is characterised by three main elements: (1)

a parallel β-sheet, B1 that occupies the concave face of the protein; (2) an array of 3_{10} helices that form the convex face; and (3) a second β-sheet, B2 that is located at the interface between the two faces of the protein. This second β-sheet is absent in most other LRR proteins.

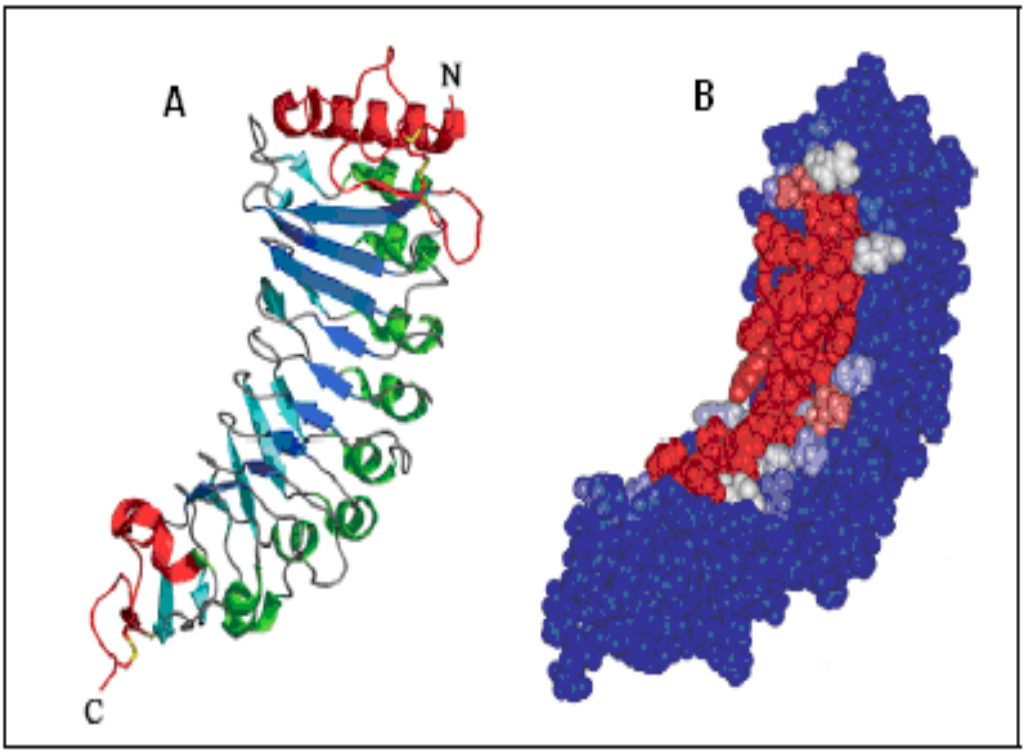

Figure 2.1 *The structure of bean PvPGIP2*. **A**: Ribbon representation (Kobe and Kajava, 2001). The central LRR domain is made up of ten tandem repeat units that form three main structures. The β-sheet B1 is shown in **blue**, and occupies the concave side of the protein. An array of 3_{10} helices make up the convex side (**green**) and a second atypical β-sheet B2 is present between the two faces (**cyan**). The LRR region is capped by N-terminal and C-terminal cysteine rich areas (**red**) that each contains two disulfide bonds that cap the hydrophobic core of the protein (sulphurs shown in **yellow**). **B**: Docking energy calculations showing the interaction propensity of the protein (Fernandez-Recio, 2004, 2005). **Red** areas have a high propensity value (0.4) that indicates a high probability of protein-protein interaction. **Blue** areas have values below 0, and intermediate values are scaled from red to blue. The concave face's surface shows a high probability of being involved in protein-protein interactions. Figure obtained from Federici *et al.* (2006).

Sheet B1 is conserved in all known LRR protein structures (Kobe and Kajava, 2001) and occupies the inner concave side of PvPGIP2. The residues that determine the affinity and specificity of PvPGIP2 are located in B1 (Leckie *et al.*, 1999; Sicilia *et al.*, 2005). The second β-sheet, B2, of PvPGIP2 is not found in most other LRR proteins. The variable length of the β-strands in B2 and the twisted shape of the molecule cause B2 to be distorted.

Specific residues within the ten 24-residue tandem repeat sequences (xxLxLxxNxLt/sGxIPxxLxxLxxL) of PvPGIP2 are occupied by hydrophobic amino acid residues that are orientated towards the interior of the protein structure and stabilize the fold through van der Waals interactions (specifically residues 3, 5, 10, 18, 21 and 24) (Di Matteo *et al.*, 2006). The asparagine residue that usually occupies position 8 forms hydrogen bonds with the main-chain residues and the conserved serine or threonine residues at position 17 provide an additional stabilization across the LRR domain. The residues at positions 12, 14 and 15 (glycine, isoleucine and proline, respectively) are conserved in plant eLRR proteins. The stereochemistry of glycine is unique, and this largely determines the peculiar bending of B2.

The PvPGIP2 LRR domain is flanked by two cysteine-rich domains that are conserved in plant LRR proteins (Van der Hoorn *et al.*, 2005). The N-terminal domain consists of an α-helix followed by a short β-strand that form a hydrophobic signal peptide for transport to the extracellular matrix (Toubart *et al.*, 1992; Stotz *et al.*, 1993; Stotz *et al.*, 1994). The C-terminal region is not homologous to any other known structures and consists of two 3_{10} helices, the last strand of B2 and a short loop. The protein solenoid has a hydrophobic core that is capped by two disulfide-bonds at either end.

2.2.2.2 The structure of PGIP in relation to function

Structural and functional analyses of PGIPs show that they have evolved a wide interacting surface on the concave side of the LRR domain. This interaction surface is under pressure for evolutionary diversification (Bishop, 2005) and is occupied by diverse chemical residues that are hydrophobic as well as hydrophilic. PGIPs seem to be capable both of polar and apolar interactions with structurally similar enzymes.

Specific amino acid residues in the PGIP sequence are vital for binding specificity, as demonstrated by bean PvPGIP1 and PvPGIP2 (Leckie *et al.*, 1999). PvPGIP1 inhibits *A. niger* ePG at ~100%, has reduced capability to inhibit ePG from *F. oxysporum* f. sp. l*ycopersici* and *B. cinerea*, and does not inhibit *F. monoliforme* ePG at all. The same concentration of PvPGIP2 inhibited all the ePGs almost completely, except for that of *F. oxysporum* f. sp. *lycopersici* which was partially inhibited. Despite these differences in their interaction specificities, the two *pgips* differ by only 26 nucleotides in their coding regions that are responsible for 10 amino acid changes (Figure 2.2). Seven of these amino acid changes occur in the LRR domain (five are located in the xxLxLxx structure of the β-sheet/β-turn domain of the protein, and two are peripheral to this area and possibly solvent-exposed). The other three amino acid changes are not located in the LRR domain. Two of these do not affect the mature protein structure because they are in the signal peptide, and the other is in the C-terminal region. The other non-synonymous nucleotide changes are outside the β-sheet/β-turn structure of the protein.

Since PvPGIP2 is able to inhibit an ePG that is not inhibited by PvPGIP1 at all, this was exploited in order to identify which of the variant amino acids are involved in the interaction with *F. monoliforme* ePG (Leckie *et al.*, 1999). Site directed mutation was used to change each of the variant amino acids of PvPGIP2 into the corresponding amino acid of PvPGIP1 (excluding the two variant amino acids that are located in the signal peptide). Eight mutated *Pvpgip2* genes were created and expressed in tobacco. The proteins were purified and used in surface plasmon resonance (SPR) studies with FmPG as the analyte in order to determine their interaction with the ePG. The mutation Q253K had a strong influence on reducing the ability to interact with FmPG. A326S also decreased the affinity for FmPG, though not as much as Q253K. V181G resulted in a slight decrease in affinity, L89H and Q320K caused very little change, whereas H300Q and A340S had almost no effect on the FmPG:PvPGIP2 interaction. Inhibition studies were also done, and the only mutant that was completely unable to inhibit FmPG was the mutant Q253K. Double-mutant proteins were also created and tested in inhibition studies, and the mutants V181G/Q253K and Q253K/A326S did not inhibit FmPG. V181G/A326S was able to inhibit FmPG, demonstrating the importance of the mutation Q253K in the FmPG:PvPGIP2 interaction.

To confirm the importance of the mutation at position 253, PvPGIP1 was mutated at that point to correspond to PvPGIP2 (K253Q) (Leckie *et al.*, 1999). The resultant mutant protein was able to both interact with FmPG (SPR analysis) and inhibit FmPG (inhibition assay). This study thus showed that certain key amino acid residues are important for the binding capability of

PGIP, and that even one amino acid change can allow PGIP to change its inhibition specificity. However, AtPGIP1 and AtPGIP2 from *Arabidopsis* differ by 79 amino acids, but the only detected difference in activity is a slight change in affinity (Ferrari *et al*., 2003). It seems that the exact positions and chemical properties of the variable residues are important in determining PGIP activity.

10	M	S	S	S	L	S	I	I	L	V	I	L	V	S	L	R	T	A	H	S									
30	E	L	C	N	P	Q	D	K	Q	A	L	L	Q	I	K	K	D	L	G	N	P	T	T	L	S	S	W		
57						L	P	T	T	D	C	C	N	R	T	W	L													
69	G	V	L	C	D	T	D	T	Q	T		Y	R	V	N	N	L	D	L	S	G		L	N	L	P	K	P	
95	Y	P	I	P	S	S	L	A	N	L		P	Y	L	N	F	L	Y	I	G	G	I	N	N	L	V			
120	G	P	I	P	P	A	I	A	K	L		T	Q	L	H	Y	L	Y	I	T	H		T	N	V	S			
144	G	A	I	P	D	F	L	S	Q	I		K	T	L	V	T	L	D	F	S	Y		N	A	L	S			
168	G	T	L	P	P	S	I	S	S	L		P	N	I	V	G	I	T	F	D	G		N	R	I	S			
192	G	A	I	P	D	S	Y	G	S	F	S	K	L	F	T	S	M	T	I	S	R		N	R	L	T			
217	G	K	I	P	P	T	F	A	N	L			N	L	A	F	V	D	L	S	R		N	M	L	E			
240	G	D	A	S	V	L	F	G	S	D		K	N	T	Q	K	I	H	L	A	K		N	S	L	A			
264	F	D	L	G	K	V	G	L	S			K	N	I	N	G	L	D	L	R	N		N	R	I	Y			
287	G	T	L	P	Q	G	L	T	Q	L		K	F	L	H	S	L	N	V	S	F		N	N	L	C			
311	G	E	I	P	Q	G		G	N	L		Q	R				F	D	V	S	A								
327	Y	A	N	N	K	C	L	C	G	S	P	L	P	A	C	T													

Figure 2.2 *The amino acid sequence of PvPGIP2 with the differences in comparison with PvPGIP1 indicated.* Residue **10-29**: Signal peptide. Residue **30-68**: N-terminus region. Residue **69-326**: LRR domain with 10.5 LRR repeats. The β-strand/β-turn structure important for binding specificity is shown in the block. The consensus sequence is xxLxLxx. Residue **327-342**: C-terminus region. The amino acids that correspond to synonymous nucleotide changes are shown in **red**. The residues that differ between PvPGIP2 and PvPGIP1 due to non-synonymous nucleotide changes are highlighted in **green** (Leckie *et al.*, 1999).

The interaction energy of PGIP can be analysed and used to calculate the tendency of surface residues to be involved in protein-protein binding (Fernandez-Recio, 2004, 2005). Bean PvPGIP2 has a wide interaction area on the concave side of the LRR domain (Figure 2.1 B) that corresponds to the xxLxLxx sequence of the domain (Federici *et al.*, 2006). The concave side of the LRR domain is also involved in ligand recognition in other proteins (Kobe and Deisenhoffer, 1995; Papageorgiou *et al.*, 1997; Schubert *et al.*, 2002). PGIPs have been shown to inhibit PGs both in competitive, non-competitive and mixed-type mechanisms (Abu-Goukh *et al.*, 1983; Lafitte *et al.*, 1984; Johnston *et al.*, 1993; Yao *et al.*, 1995; Stotz *et al.*, 2000; De Ascensao, 2001; James and Dubery, 2001; Deo and Shastri, 2003; Sicilia *et al.*, 2005). For instance: bean PvPGIP2 inhibits *F. monoliforme* PG (FmPG) competitively (Federici *et al.*, 2001; Mattei *et al.*, 2001b) and *A. niger* PG (AnPG) non-competitively (King *et al.*, 2002). This mixture of inhibition mode suggests that the orientation of PGIP in the PGIP:PG complex might differ depending on the PG ligand. FmPG and AnPG have different, but overlapping high propensity interaction areas in the complex with PvPGIP2 (Federici *et al.*, 2006). Fungal ePGs generally have the same fold but they are variable with regards to shape and distribution of hydrophilic and hydrophobic surface residues, as illustrated by FmPG and AnPG that only share 43% identity, but have an extremely similar three-dimensional structure (Van Santen *et al.*, 1999; Herron *et al*., 2000; Federici *et al.*, 2001). Docking geometry and energetics analysis for the

FmPG:PvPGIP2 and AnPG:PvPGIP2 complexes suggested that the enzymes are differently orientated in the complexes (Figure 2.3) (Fernandez-Recio, 2004). For FmPG:PvPGIP2, the active cleft of the enzyme is completely covered and blocked by PvPGIP2, resulting in competitive inhibition of the enzyme. In contrast, in AnPG:PvPGIP2 the active cleft is still accessible and capable of binding substrate, leading to a non-competitive interaction. This hypothesis was further supported by data showing that three aspartic residues that form a negatively charged pocket in the active domain of PvPGIP2 are vital for the interaction of PvPGIP2 with FmPG, but not for interaction with AnPG (Spinelli *et al.*, 2008).

It is shown by the research reviewed above that direct binding of ePG by PGIP is an important factor in inhibiting the enzymatic degradation caused by ePG. However, direct protein:protein inhibition is not always observed in all PGIP:ePG pairs. For example, it was shown that a grapevine PGIP completely inhibits ePG2 from *B. cinerea in planta*, despite there being absolutely no *in vitro* evidence of a direct protein:protein interaction (Joubert *et al.*, 2007). The specific *in planta* mechanism of PGIP-specific disease resistance is therefore poorly understood. The last section of this review will discuss work done by our group at the IWBT on grapevine PGIPs.

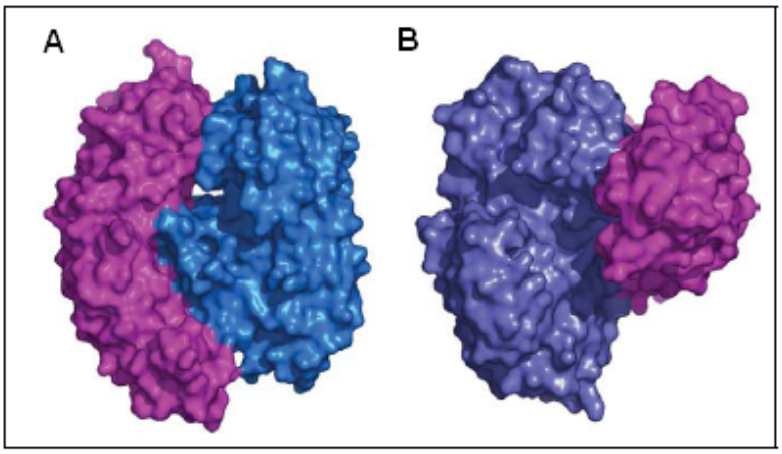

Figure 2.3 *The energetics and docking geometry of the PG:PGIP complex* (Fernandez-Recio, 2004). **A**: Model of *Phaseolus vulgaris* PGIP2 (**purple**) and *Fusarium monoliforme* ePG (**blue**). The orientation of the molecules leaves the active site of ePG (middle left indentation) blocked and unavailable to bind with substrate (competitive inhibition). **B**: Model of *Phaseolus vulgaris* PGIP2 (**purple**) and *Aspergillus niger* ePG (**violet**). PGIP binds in a different orientation, leaving the active site of the ePG (middle left indentation) available for substrate binding (non-competitive inhibition). Figure from Federici *et al*. (2006).

2.3 The first grapevine PGIP: VvPGIP1

Grapevine is one of the most important and widely cultivated fruit crops in the world. There is a large market for fresh grapes as well as grape-derived products such as raisins, wine, vinegar, grape juice and even jams and jellies. Grapevines belong to the *Vitis* genera, which consists of the *Euvitis* and *Muscadinia* sub-genera. The single *Vitis* species that originated in Europe, *Vitis vinifera*, is the most cultivated species worldwide and comprises of thousands of different cultivars that are used in the grape industry. The popularity of this species can be attributed to the quality of its fruits. Unfortunately, *V. vinifera* is also extremely susceptible to disease, especially fungal infection. Due to the high nutrient content of grape berries, they are ideal

environments for fungal growth and proliferation, and diseases such as powdery mildew, downy mildew and bunch rot are common in vineyards over the world. Although it is true that there are some situations in which fungal infections can be beneficial (specifically in the case of Noble Late Harvest wines that are made from grapes infected with *B. cinerea* under very specific conditions), the majority of fungal infections are extremely detrimental and negatively affect the quality as well as the yield of fruit. The other *Vitis* species, from Asian and American origins, are much more resistant towards fungal and other pathogens, but are not commercially popular due to inferior fruit quality, smaller yields and other detrimental factors. Some of these species are commonly used as rootstocks, onto which *V. vinifera* cultivars are grafted, specifically because of their good resistance towards a number of pathogens and better drought and stress tolerance. These non-*vinifera* species are therefore good potential sources for disease resistance genes in general, and perhaps also for PGIPs with potentially wide inhibitory activities.

2.3.1 The isolation and characterisation of *Vvpgip1* and VvPGIP1

The first PGIP from grapevine was isolated from *Vitis vinifera* cultivar Pinotage (De Ascensao, 2001) by screening, with a pear PGIP probe, a subgenomic library constructed from Pinotage leaves. A 1002 bp PGIP-encoding ORF was isolated, analysed and designated *Vvpgip1*. The ORF had no introns and encoded a deduced peptide of 333 amino acids, that had a calculated iso-electric point of 8.61 and a predicted molecular mass of 37 103 Da. A 27-amino acid signal peptide with a conserved peptide cleavage site precedes the N-terminus of the deduced protein. Genomic analysis of the sequenced *V. vinifera* genome showed that grapevine does not have a multigene PGIP family like most other plant species, but that there are rather multiple copies of the same gene present in the genome (Joubert *et al.*, 2006).

Grapevine PGIP expression has been shown to be tissue-specific, as mRNA transcripts were only detected in grape berries at véraison stage, and not in leaves or inflorescences (De Ascensao, 2001). The expression is also developmentally controlled: mRNA levels in berries were very low at the early stages of berry growth, increased significantly (up to 40-fold) at the onset of ripening, and then decreased until PGIP was no longer detected at 16 weeks post-flowering. Under inducing conditions (wounding, infection, elicitors and oxidative stress), expression was strongly induced in all tissues (Joubert, 2004).

Initial analysis and purification of VvPGIP1 from véraison berries revealed that VvPGIP1 is cell-wall associated and has a pI of 8.78 (De Ascensao, 2001). In *in vitro* assays, 50 ng of purified VvPGIP1 completely inhibited a crude ePG isolate from *B. cinerea*, inhibited ePGs from *C. gleosporoides* 85%, partially inhibited PGs from *A. alternata*, *M. laxa*, *Mycor* spp. and *R. stolonifer*, and was unable to inhibit PGs from *A. niger*, *F. monoliforme* and *P. expansum*. Enzyme kinetic studies showed that VvPGIP1 inhibited *B. cinerea* ePGs non-competitively.

VvPGIP1, purified from overexpresssing transgenic tobacco, was used in *in vitro* inhibition assays with seven different fungal ePGs (Joubert *et al.*, 2006). Purified VvPGIP1 successfully inhibited AnPGA and AnPGB from *A. niger*, but not AnPGII. *B. cinerea* BcPG1 and BcPG6 were also significantly inhibited, but the activity of BcPG3 was not inhibited at all. BcPG4 was only inhibited at low pH levels.

An interesting result comes from the study of the interaction of VvPGIP1 with BcPG2 from *B. cinerea* (Joubert *et al.*, 2007). Infiltration of *Nicotiana benthamiana* (tobacco) leaves with *Agrobacterium tumefaciens* that expresses BcPG2 results in necrosis and wilting of tissue in the infiltrated area. The damaging effect of transient BcPG2 expression could be measured by calculating the variable fluorescence yield before and after infiltration. When VvPGIP1 was co-

infiltrated into *N. benthamiana* along with BcPG2, the tobacco leaves visually showed much less wilting and necrosis than observed for BcPG2 infiltrated alone. The variable fluorescence was also significantly less in the co-infiltrated tissue, indicating less damage to the tissue. This *in vivo* inhibition reaction confirmed that expression of VvPGIP1 in tobacco inhibited the cell wall degradation caused by BcPG2. It is thus logically expected that VvPGIP1 would also inhibit BcPG2 directly *in vitro*. However, absolutely no evidence of a physical *in vitro* protein:protein interaction could be observed between the two molecules, either by analysing PG inhibition (reducing sugar assay), physical interaction (SPR spectroscopy) or substrate profiling (anion exchange chromatography) (Joubert *et al.*, 2007). The strong *in planta* inhibitory effect of VvPGIP1 on BcPG2 could thus not be established *in vitro*. It is known that PGIP can also interact with and bind to pectin (Spadoni *et al.*, 2006), so it is possible that PGIP could physically protect the parts of the plant cell wall that it is bound to. Such a mechanism was proposed for the fungus *Cladosporum fulvum* protein AVR4 that can bind to chitin in the fungal cell wall and so protect the fungal cell wall from chitinase degradation (Van den Burg *et al.*, 2006). The result of a strong *in planta* inhibition that does not take place *in vitro* (Joubert *et al.*, 2007) indicated that an absence of *in vitro* inhibition of any PG by a specific PGIP does not necessarily imply that there is no inhibition *in planta*. PGIP candidates for stable overexpression should thus not necessarily be chosen on the basis of *in vitro* inhibition studies alone.

2.3.2 Overexpression of VvPGIP1 in *Nicotiana tabacum* resulted in a PGIP-specific resistance response against *B. cinerea*

Vvpgip1, under control of the constitutive CaMV35S promoter, was overexpressed in tobacco via *Agrobacterium*-mediated transformation (Joubert *et al.*, 2006). The resultant transgenic plant lines were analysed for the presence and expression of the transgene, as well as PGIP activity against *Botrytis* ePGs. Detached leaves were furthermore infected with *Botrytis* and PGIP activity could be correlated with resistance phenotypes. To confirm the antifungal effect of the grapevine PGIP further, a whole plant infection was conducted over a 15 day period (Joubert *et al*., 2006).

2.3.2.1 Whole-plant *Botrytis cinerea* infection of transgenic VvPGIP1-expressing tobacco

Transgenic lines that overexpressed VvPGIP1 were infected with *B. cinerea* in a whole-plant time-course infection assay in order to determine the susceptibility of the VvPGIP1-overexpressing plants to this fungus (Joubert *et al*., 2006). The diameter of lesions formed by the fungal infection was measured over 15 days and analysed statistically. The lesions that formed on the untransformed wild-type plants were significantly bigger than those of the PGIP overexpressing plantlines; they developed faster and the appearances of the lesions were wet and translucent (Fig 2.4 A). The lesions on the transgenic plants became dry and necrotic, did not develop to the same size and effectively stopped expanding (Figure 2.4B). Statistical analysis of the measurements confirmed that most of the PGIP-overexpressing lines were more resistant to *B. cinerea* infection than the untransformed control plants, with an average reduction in lesion size of between 47 and 69% (Figure 2.5).

Based on the genetic analysis of the transgenic population, the PGIP activities and the infection studies, it was concluded that in these transgenic lines we demonstrated a PGIP-specific resistance phenotype against *B. cinerea*. These plants could therefore be useful tools to further investigate the specific *in planta* mechanism(s) used by PGIP to combat fungal infection,

as well as the specific mechanism(s) triggered by PGIP that make plants better able to withstand pathogenic attack.

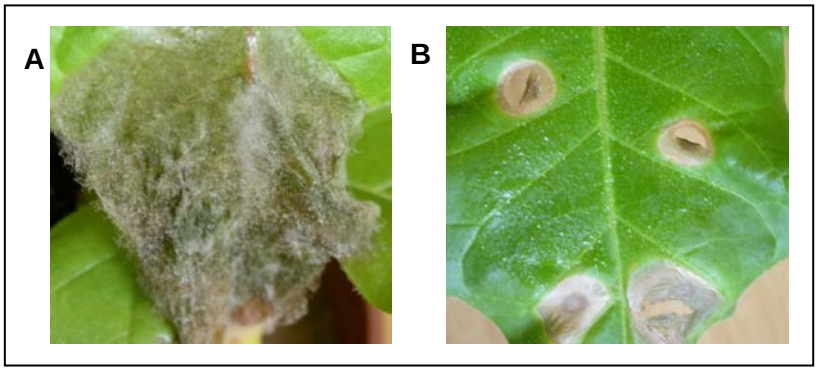

Figure 2.4. *Appearance of lesions on wild-type and VvPGIP1-overexpressing tobacco after 13 days of infection with Botrytis cinerea.* **A**: Untransformed tobacco leaf showing severe symptoms and spread of disease. **B**: VvPGIP1-overexpressing tobacco line 37 showing dry and confined lesions. From Joubert et al. (2006) and Becker (2007).

In order to further characterise the PGIP-specific resistance against *B. cinerea*, infected tissue from two VvPGIP1-overexpressing lines (37 and 45) was used in microarray, Real-time qRT-PCR and phytohormone analyses in comparison with infected wild-type tobacco (Becker, 2007) in a time-course experiment. In the early stages of defence (24-48 hours post infection) and at the site of infection (local response), a divinyl ether synthase (*des1*) gene that is involved in antifungal oxylipin formation was found to be significantly upregulated in the transgenic lines. Hormone analyses showed that jasmonic acid levels in the transgenic lines were also significantly more upregulated, and appeared earlier than in the wild-type tissues. These very interesting findings are currently being studied further in our laboratory.

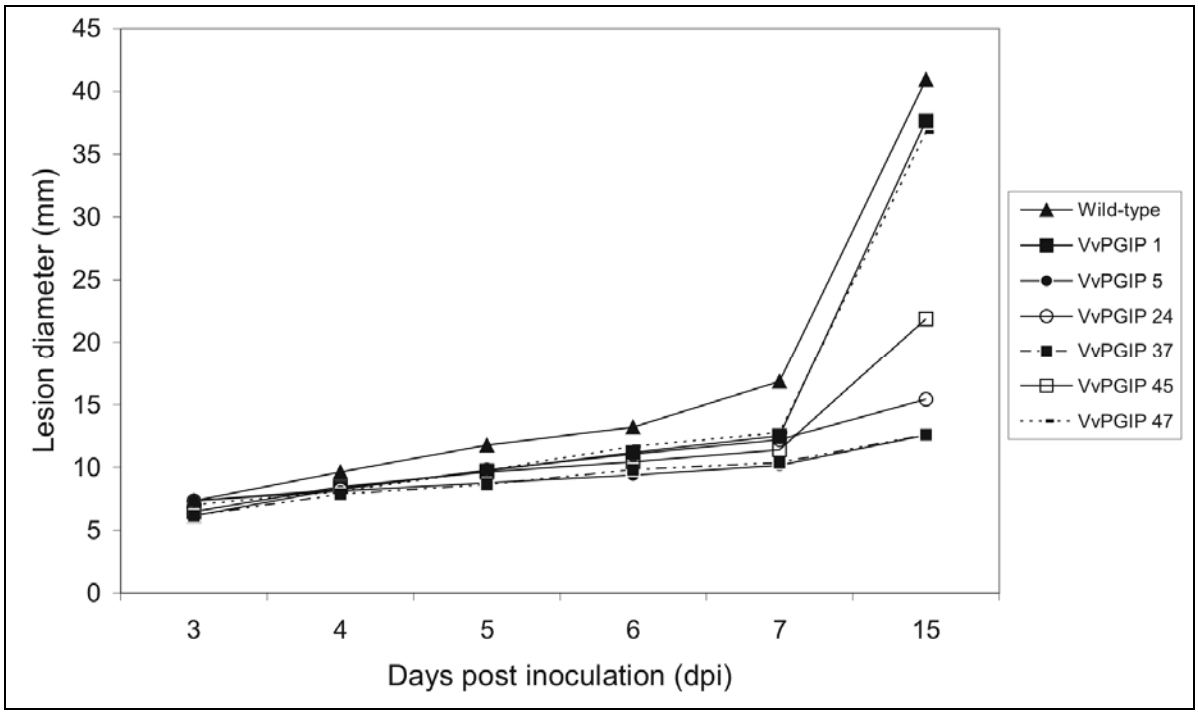

Figure 2.5 *Progression of infection of VvPGIP1-overexpressing tobacco lines with Botrytis cinerea over fifteen days*. The diameters of lesions formed are presented per day they were measured over the course of the infection assay. The untransformed wild-type tobacco developed the largest lesions that also developed fastest. From Joubert *et al*. (2006).

2.3.2.2 The effect of overexpression of VvPGIP1 on the cell wall metabolism in healthy tobacco

As part of the microarray analysis conducted by Becker (2007) , uninfected transgenic and wild-type material was also compared. Although intended as controls initially, this analysis yielded very interesting findings, as outlined below.

Microarray analysis of VvPGIP1 lines 37 and 45, and WT tobacco (uninfected), identified 52 genes that were differentially regulated in the transgenic versus the control plant lines. Many of the genes that were downregulated in the transgenic plants encode for xyloglucan endotransglucosylase/hydrolases (XTHs). XTHs are involved in cell wall strength regulation and tissue integrity in plants, and it has been shown that XTH downregulation leads to xyloglucan of a higher molecular weight in tobacco leaves (Herbers *et al.*, 2001). Primers were designed for the cDNA with the highest degree of downregulation, which was homologous to the *Nicotiana tabacum XTH*-encoding gene (GenBank Acc AB017025.1 and D86730), in order to confirm the differential expression in a larger set of transgenic VvPGIP1 plants with quantitative Real-Time PCR (qRT-PCR). Statistical analysis of the qRT-PCR data showed the same levels of XTH downregulation in the two VvPGIP1-overexpressing tobacco lines than the microarray analyses, as well as in additional VvPGIP1 lines.

From the microarray data it was also found that both plant lines showed differential expression in genes involved in lignin biosynthesis, but with different specific genes that were affected in the two lines. In line 37, a cinnamyl alcohol dehydrogenase (CAD) encoding gene was 1.5 times upregulated and in line 45 a lignin-forming peroxidase (LfPOD) encoding gene was 2 times upregulated. The CAD and LfPOD proteins are involved in the last two reactions in lignin formation. An increased deposition of lignin was also observed in the two transgenic VvPGIP1 lines (37 and 45) by staining of leaf and stem sections (Figure 2.6) (Becker, 2007).

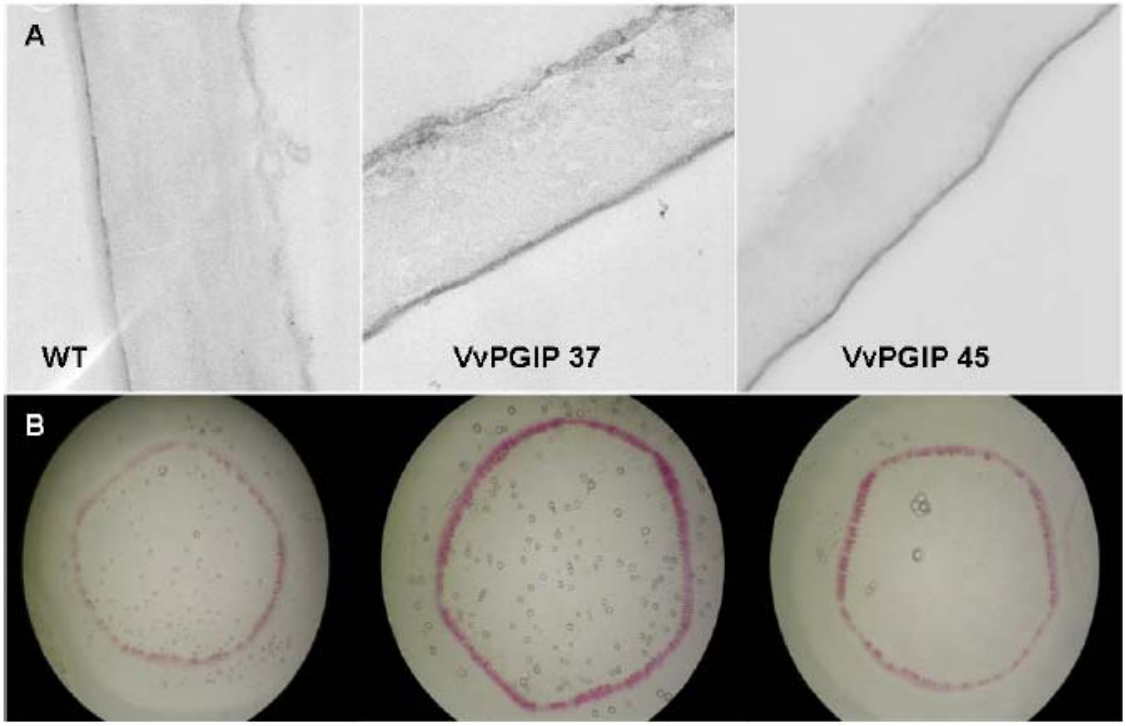

Figure 2.6 *Increased lignin deposition in two VvPGIP1 expressing plantlines as compared to untransformed WT tobacco*. **A**: Leaf sections stained with potassium permanganate. VvPGIP1 line 37 and 45 showed a darker stain than the untransformed plant tissue due to the higher lignin content. **B**: Stem sections stained with phloroglucinol. A darker pink colour corresponds to higher lignin content in VvPGIP1 line 37 and 45 (Becker, 2007).

In situ lignin deposition is generally induced in defence responses towards several plant pathogens and acts to strengthen the physical barrier against pathogen invasion (Mitchell *et al.*, 1994; Dixon and Paiva, 1995; Hano *et al.*, 2008). Lignin, lignans and neolignans can be formed *de novo* in response to fungal attack from monolignol precursors (Gang et al., 1999) and there is evidence that suggests that different biosynthetic enzymes are involved in comparison with "normal" vegetative lignification (Vermerris and Nicholson, 2008). In wheat, resistant cultivars accumulate more lignin than susceptible ones in response to attack by the leaf rust pathogen (*Puccinia recondite* f. sp. *tritici*) and the head blight pathogen (*Fusarium graminearum*) (Southerton and Deverall, 1990; Kang and Buchenauer, 2000). In wheat cultivars highly resistant to stem rust (*Puccinia graminis* Pers F. sp. *tritici*) that were treated with lignification-enzyme inhibitors (specifically CAD inhibitors and PAL inhibitors), *de novo* lignification was decreased and fungal development increased (Moerschbacher *et al.*, 1990).

It has been shown that lignin content and composition can be altered by up- or downregulating several of the lignin biosynthesis enzymes, including LfPOD and CAD. Moreover, these studies revealed that even when the total lignin levels were unaffected by the engineered changes, the lignin composition was typically altered, including changes to the ratio of monolignol components and the incorporation of other precursor molecules (See Table 2.2) (Vanholme *et al.*, 2008).

Plants appear to cope quite well with changes in lignin composition and can incorporate many other molecules into lignin polymers, such as incomplete products from monolignol biosynthesis or derivatives of the common monolignols (Ralph *et al.*, 2004; Vanholme *et al*., 2008).

This background information and the observation that in healthy, uninfected tobacco plants genes involved in cell wall strengthening are differentially expressed when grapevine PGIP1 is overexpressed (from Becker 2007) suggest a possible new role for PGIP. The current working hypothesis is that the high levels of PGIP in the cell wall might trigger specific changes to the cell wall to promote cell wall strengthening in anticipation of infection (Figure 2.7).

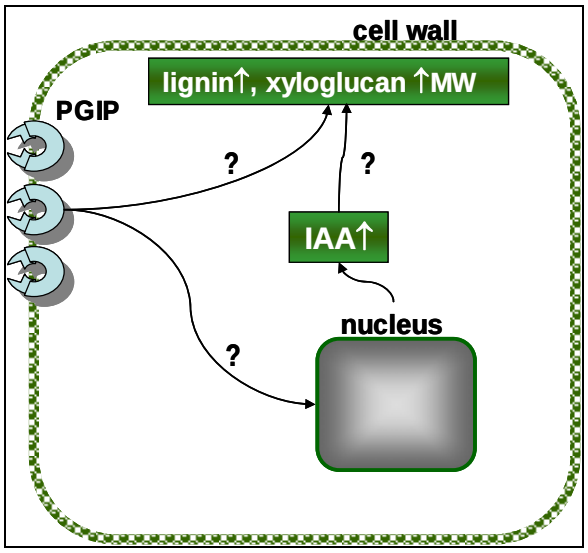

Figure 2.7 *The current working model of a possible cell wall strengthening mechanism facilitated by PGIP overexpression*. PGIP causes altered cell wall metabolism in transgenic plants in the absence of a fungal pathogen by an unknown signalling mechanism, possibly involving indole acetic acid (IAA). From Becker (2007).

Table 2.2 *Effects on lignin content and lignin monomer content in various mutant and transgenic plants with altered monolignol biosynthesis*. (Table obtained from Vanholme *et al*., 2008.)

Gene(s)[a]	Total lignin	H[b]	G[b]	S[b]	S/G[b]	References[c]
PAL ↓	↓	↓	↓	↓	↓/↑	Baucher *et al.*, 2003
						Chen *et al.*, 2006
						Rohde *et al.*, 2004
PAL ↑	↑	n.a.	↑/No changes	↓/No changes	↓/No changes	Baucher *et al.*, 2003
C4H ↓	↓	↓	↓	↓	↓	Baucher *et al.*, 2003
						Chen *et al.*, 2006
C4H ↑	No changes	n.a.	No changes	No changes	No changes	Baucher *et al.*, 2003
4CL ↓	↓	↑	↓	↓	No changes	Baucher *et al.*, 2003
HCT ↓	↓	↑	↓	↓	↑	Chen *et al.*, 2006
						Hoffmann *et al.*, 2004
						Besseau *et al.*, 2007
C3H ↓	↓	↑	↓	↓	n.a.	Baucher *et al.*, 2003
						Abdulrazzak *et al.*, 2006
CCoAOMT ↓	↓	↑	↓	↓/No changes	↓/No	Baucher *et al.*, 2003
					changes/↑	Chen *et al.*, 2006
						Do *et al.*, 2007
CCR ↓	↓	↓	↓	↓	↓/↑	Baucher *et al.*, 2003
						Do *et al.*, 2007
						Leplè *et a*l., 2007
F5H ↓	↓/No changes	n.a.	↑	↓	↓	Baucher *et al.*, 2003
						Chen *et al.*, 2006
F5H ↑	↓/No changes	n.a.	↓	↑	↑	Baucher *et al.*, 2003
COMT ↓	↓/No changes/↑	n.a.	↓/↑	↓	↓	Baucher *et al.*, 2003
						Chen *et al.*, 2006
						Do *et al.*, 2007
COMT ↑	No changes	n.a.	No changes	No changes	No changes	Baucher *et al.*, 2003
CAD ↓	↓/No changes	n.a.	↑/No changes	↓/No changes	↓/No changes	Baucher *et al.*, 2003
						Sibout *et al.*, 2005
4CL ↓ F5H ↓	↓	n.a.	n.a.	n.a.	↑	Baucher *et al.*, 2003
CCoAOMT ↓ COMT ↓	↓/No changes	n.a.	↓	↓	↓	Baucher *et al.*, 2003
						Do *et al.*, 2007
CCR ↓ COMT ↓	↓	n.a.	n.a.	n.a.	↑	Baucher *et al.*, 2003
CCR ↓ CAD ↓	↓	n.a.	↓	↓	↑	Baucher *et al.*, 2003
COMT ↓ CCR ↓ CAD ↓	↓	n.a.	n.a.	n.a.	n.a.	Baucher *et al.*, 2003

[a]Genes abbreviated as follows: PAL = phenylalanine ammonia-lyase; C4H = cinnamate 4-hydroxylase; 4CL = 4-coumarate: CoA ligase; HCT = *p*-hydroxycinnamoyl-CoA: quinate shikimate *p*-hydroxycinnamoyltransferase; C3H = *p*-coumarate 3-hydroxylase; CCoAOMT = caffeoyl-CoA *O*-methyltransferase; CCR = cinnamoyl-CoA reductase; F5H = ferulate 5-hydroxylase; COMT = caffeic acid/5-hydroxyconiferaldehyde *O*-methyltransferase; CAD = cinnamyl alcohol dehydrogenase
[b]H, G and S are the three main monolignols that lignin is composed of (H = *p*-coumaryl alcohol; G = coniferyl alcohol; S = sinapyl alcohol)

2.3.3 Isolation of additional PGIP genes from non-*vinifera* grapevine species

The evidence that a small amount of amino acid differences between PGIPs could have a big impact on ePG inhibition (Leckie *et al.*, 1999), coupled with the strong inhibition of *B. cinerea* by

V. vinifera PGIP1 from cultivar Pinotage (Joubert *et al*., 2006), led to the isolation of a set of additional PGIP-encoding genes from a number of grapevine species that are traditionally more resistant towards fungal pathogens (Wentzel, 2005). Material used for *pgip* gene isolation was obtained from 37 different grapevine species as listed in Table 2.3.

Table 2.3 *Grapevine material used for the isolation of additional grapevine pgip genes, with total nucleotide changes as well as non-synonymous nucleotide changes with regards to V. vinifera pgip1 indicated.* Table modified from Wentzel (2005).

No	Code	Species/Cultivar	Origin	Nucleotide changes*	Non-synonymous changes*
1	1004	*V. candicans Engelman*	France	14	3
2	1012	*V. doaniana Munson*	USA	10	3
3	1018	*V. riponia* (female)	RSA	15	8
4	1024	*V. solonis Hort*	RSA	12	8
5	1030	*V. flexuosa*	USA	13	9
6	1034	*V. Bourquiniana*	Germany	13	8
7	1038	*V. caribaea*	USA	14	7
8	1042	*V. Popenoei*	USA	9	3
9	1046	*V. shutteworthii*	USA	11	4
10	1048	*V. smalliana* (female)	USA	14	8
11	1050	*V. aestivalis*	USA	14	4
12	1056	*V. californica* Gold Hill 1	USA	9	5
13	1058	*V. monticola*	USA	13	7
14	7000	*Ampelocissus atapulcensis*	Zimbabwe	9	5
15	7010	*Cissus cactiformis ex Ruacana*	Unknown	12	8
16	7016	*Cyphostemma currorii*	Unknown	9	3
17	7048	EVEX 13-5 (Berlandieri 13-5)	Spain	10	3
18	7060	*V. rupestris* (Constantia Metallica)	RSA	11	6
19	7074	*V. champinii* (Dogridge)	RSA	9	4
20	7090	*V. bourquiniana* (Herbemont black)	RSA	13	8
21	7102	*V. labrusca* (Isabella)	RSA	9	4
22	7180	*V. doaniana Munson*	USA	14	4
23	7182	*V. Longii* (V. solonis)	RSA	12	8
24	7194	*V. amurensis Ruprecht*	USA	17	9
25	7198	*V. doaniana Munson*	USA	11	4
26	7360	*V. Treleaseii Munson*	USA	4	0
27	7442	*V. solonis Hort*	RSA	10	4
28	7536	*V. Thunbergii*	Germany	15	9
29	7538	*V. Andersonii*	Germany	17	8
30	7540	*V. caucasica*	Germany	14	8
31	7548	*V. monticola*	USA	15	5
32	7560	*V. Coignetiae*	Germany	10	5
33	101-14 MGT	*V. riparia x V. rupestris*	RSA (rootstock)	13	8
34	Ramsey	*V. champinii*	RSA (rootstock)	14	8
35	Paulson	*V. berlandieri x V. rupestris*	RSA (rootstock)	11	5
36	Richter 110	*V. berlandieri x V. rupestris*	RSA (rootstock)	20	11
37	SO4	*V. riparia x V. berlandieri*	RSA (rootstock)	18	10

* Nucleotide sequences of the isolated genes were compared with the nucleotide sequence of *Vvpgip1*

A PCR-based gene isolation strategy was followed using *Vvpgip1*-specific primers. The nucleotide and deduced amino acid sequences of all the isolated genes were confirmed to be PGIP-encoding sequences, and were compared to the nucleotide sequence of *Vvpgip1*. In total,

the nucleotide changes ranged between 0 and 20, with predicted amino acid changes ranging between 0 and 11 (Table 2.3).

The deduced amino acid sequences of the 37 isolated *pgip* genes were aligned with the amino acid sequence of VvPGIP1, and it was found that the sequences shared greater than 95% homology. All the sequences were predicted to contain an LRR active domain between residues 196 and 300. Alignment of the active LRR domain sequences found a greater than 94% homology shared between them. These additional sequences are valuable resources and were used to evaluate the antifungal potential of the genes isolated from the non-*vinifera* grapevine species (this study).

2.4 Summary

The role and mechanism of cell wall associated PGIPs in protecting plants from fungal invasion has been the subject of many studies to date. One function of PGIPs is to specifically and effectively inhibit fungal cell wall degrading ePGs by direct protein:protein interaction. PGIPs from different plant species and also different PGIPs from within the same plant species are known to have differential inhibition spectra towards a range of fungal ePGs, as well as differential kinetic inhibition properties. This differential inhibition capability of PGIP is linked to the structure of the protein, as it has been shown that as little as one single amino acid exchange in the active domain can alter the inhibition profile of PGIP. Since a number of bacteria, insects and nematodes also secrete ePGs, it is possible that PGIP can also play a role in inhibiting ePGs from organisms other than fungi.

PGIP encoding genes seem to occur in small gene families that are under evolutionary pressure for diversification. The members of a *pgip* gene family can be under control of different signal transduction pathways, enabling the different family members to be expressed under different cellular conditions and in response to different signals. This gives the plant an advantage, as there is a good chance that at least one of its *pgip* genes will be induced during attack.

PGIPs could possibly play a role in the activation of further plant defence responses that are triggered following pathogen attack. OGA molecules are released from the pectic plant cell wall by fungal cell wall degrading enzymes such as ePGs. OGA chains with a degree of polymerisation between 10 and 15 are elicitor-active molecules, and they play an important role in activating downstream defence responses. It has been shown that the inhibition of ePG by PGIP prolongs the lifetime of the elicitor-active OGAs *in vitro* by preventing or slowing their breakdown into chains that are too short and thus no longer have elicitor ability. *In planta* evidence for this prolonging of elicitor-active molecules is still lacking.

The overexpression of a number of different PGIPs from a number of different plant species in heterologous hosts has resulted in decreased susceptibility of the transgenic plants to a number of fungal pathogens. Specifically, work on the first PGIP from grapevine (VvPGIP1) has revealed it to be an effective antifungal gene, causing a significant decrease in disease susceptibility against the fungus *B. cinerea* in PGIP-specific resistant phenotypes of tobacco. Understanding the mode of action of PGIPs in affording plants protection against invading pathogens need to also be conducted *in planta* and is the current focus of our group.

Moreover, additional PGIP-encoding genes have been isolated from a range of *Vitaceae* species, providing the possibility to further evaluate the natural diversity of PGIP present in grapevine species.

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3: Research Results

The functional analysis of *Vitaceae* polygalacturonase-inhibiting protein (PGIP) encoding genes overexpressed in tobacco

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3.1 Abstract

Plant polygalacturonase-inhibiting proteins (PGIPs) specifically interact with and inhibit endopolygalacturonases (ePGs) secreted by invading fungal pathogens. PGIPs are known for their wide variable range of inhibitory properties towards different pathogens and the ePGs they secrete. This variability has been linked to small amino acid changes in the active sites of PGIPs that were most likely brought about by a long process of co-evolution, adaptation and counter-adaptation between ePGs and PGIPs. Previous work done on the first PGIP from grapevine, *Vitis vinifera* PGIP1 (VvPGIP1), has shown that the overexpression of this protein increases the disease resistance of transgenic tobacco against *Botrytis cinerea*. A PGIP-specific resistance phenotype could be confirmed for these transgenic tobacco lines. In this study, the previous work on grapevine PGIPs has been extended by functionally analysing additional PGIPs isolated from *Vitaceae* accessions. None of these PGIPs are from the cultivated *V*. *vinifera* species, but form part of a group of 37 grapevine PGIPs previously isolated from *Vitaceae* species known for their high levels of resistance against fungal pathogens and abiotic stress. The overall sequence homology between the additional PGIPs (hereafter referred to as the non-*vinifera* PGIPs) and VvPGIP1 is high. In this study, PGIP-encoding genes were selected for the functional analyses based on their amino acid differences occurring in the LRR motif; this conserved motif contains the active site of PGIPs and specifies the inhibition activity and spectrum against ePG ligands. The 37 non-*vinifera* PGIPs were previously clustered into 14 groups based on sequence variation in their LRR motifs. The approach followed was to overexpress a selection of these PGIPs, one from each of the 14 classes identified, in tobacco to evaluate their effectiveness as antifungal proteins. Transgenic populations were obtained for nine of the selected PGIP-encoding genes; the lines were analysed for gene integration, PGIP activity, as well as disease resistance, the latter through the use of a previously optimised whole plant infection assay with *Botrytis*. In combination, these results confirmed PGIP-specific resistance phenotypes for all the transgenic lines tested. Statistical analysis of the infection assays grouped all the PGIP overexpressing lines clearly separated from the wild type control. Moreover, the *Vitaceae* PGIP overexpressing lines statistically separated from the VvPGIP1 transgenics, indicating that the nine non-*vinifera* PGIPs were all more effective against *Botrytis* than the VvPGIP1 in these assays. The specific amino acid changes in the active regions of these PGIPs might be at the base of the enhanced capabilities of the PGIPs and should be systematically studied further. Furthermore, a selection of the characterised PGIP-overexpressing lines with specific resistance phenotypes were evaluated for the expression of XTH, an enzyme involved in cell wall metabolism. All the lines tested showed downregulation of the gene under non-infecting conditions, confirming previous results that showed that high levels of PGIPs might promote cell wall strengthening in anticipation of disease.

3.2 Introduction

The plant cell wall is targeted by plant pathogens in their different colonisation and infection strategies. Fungi and other pathogens secrete a number of cell wall degrading enzymes (CWDEs) that break down the complex plant cell wall structures during infection and colonisation (Cooper, 1984; Walton, 1994; Alghisi and Favaron, 1995; Ten Have *et al.*, 2002). Among these CWDEs, endopolygalacturonases (ePGs) are some of the first enzymes secreted. Apart from providing access to the plant host cells and nutrients to the colonising pathogen, elicitor-active oligogalacturonic acid (OGA) molecules have been shown to form from the enzymatic cell wall digestion by ePGs.

Plants use various mechanisms to defend themselves against invading pathogens, including cell wall associated inhibitors of ePGs. Albersheim and Anderson first reported on a polygalacturonase inhibitor from plants in 1971 (Albersheim and Anderson, 1971). Eventually named polygalacturonase-inhibiting proteins (PGIPs), these inhibitors have been found in almost all plant species examined so far (Powell *et al.*, 2000), and many studies confirmed that PGIPs differ widely in their inhibition specificities towards ePGs (Cervone *et al.*, 1987; Johnston *et al.*, 1993; Stotz *et al.*, 1993; Favaron *et al.*, 1994; Yao *et al.*, 1995; Leckie *et al.*, 1999; De Ascensao, 2001; D'Ovidio *et al.*, 2004b). The overexpression of several PGIPs in different host plants resulted in increased resistance to fungi, specifically against *Botrytis cinerea* (Powell *et al.*, 2000; Ferrari *et al.*, 2003; Agüero *et al.*, 2005; Manfredini *et al.*, 2005; Ferrari *et al.*, 2006; Joubert *et al.*, 2006; Janni *et al.*, 2008), although not all PGIP overexpressions have led to increased disease resistance (Desiderio *et al.*, 1997). PGIPs form part of a larger family of leucine-rich repeat (LRR) proteins (Stotz *et al.*, 1994; De Lorenzo *et al.*, 2001; Di Matteo *et al.*, 2003) known for very specific ligand binding capabilities (Kobe and Deisenhoffer, 1994; Jones and Jones, 1997).

Studies into the mechanisms of action of PGIPs showed that the inhibitors physically interact with ePGs, leading to inhibition of the polygalacturonase activities on the cell walls. Inhibition of ePG by PGIP is further thought to prolong the presence of elicitor-active OGAs, thus resulting in a PGIP-specific trigger of downstream defence responses (Cervone *et al.*, 1989; Simpson *et al.*, 1998; De Lorenzo *et al.*, 2001; Ridley *et al.*, 2001; Aziz *et al.*, 2004). The ePG inhibition interaction has been linked to the LRR active domain in PGIPs. Differences in the amino acids in the active domain have been shown to alter the specific binding and inhibition capacities of the proteins (Leckie *et al.*, 1999; Spinelli *et al.*, 2008). These differences probably arise from the extensive co-evolution of ePGs with PGIPs that drives constant adaptation and counter-adaptation of both proteins (Bishop *et al.*, 2000; Bishop, 2005). This domain has also been shown to be under selective pressure.

The direct PGIP:ePG protein-protein interaction, although crucial for resistance to fungal degradation of plant tissue, does not account for the full resistance effect seen in *in planta* studies. In a surprising result that strongly suggests that there are other role players and mechanisms involved in PGIP-mediated fungal resistance, the *Vitis vinifera* PGIP1 (VvPGIP1) has been shown to inhibit BcPG2 from *Botrytis cinerea* strongly *in planta* (Joubert *et al.*, 2007), although no indication of a PGIP:PG interaction or inhibition could be found *in vitro*. The PGIP-encoding gene from grapevine (*Vitis vinifera pgip1*, *Vvpgip1*) has been isolated and characterised previously (De Ascensao, 2001). It has been confirmed to be an effective antifungal gene (Joubert *et al.*, 2006; Joubert *et al.*, 2007) and has been used to study activated defense mechanisms in grapevine. When overexpressed in tobacco, it leads to strong PGIP-specific resistance phenotypes against *Botrytis* (Joubert *et al.*, 2006). A transcriptome analysis of these phenotypes showed that constitutive overexpression of VvPGIP1 in tobacco led to

altered expression of genes that would favour cell wall strengthening, without any fungal infection (Becker, 2007). This suggested a possible novel role for PGIP in preparing a plant for fungal invasion before any fungal ePGs are present.

Commercial grapevine production primarily relies on a few cultivars of the European grape, *V. vinifera*. The *Vitaceae* family, however, contains numerous additional species native to North America, Asia and Europe. Many of the non-vinifera *Vitis* species are known for their excellent resistance to fungal and other pathogens, as well as their adaptations to adverse soil-conditions, in contrast to the highly susceptible *V. vinifera* cultivars. The non-*vinifera* species and varieties are increasingly studied to evaluate the natural variation in the *Vitis* genus with regards to specific traits. Following on the isolation and characterisation of the first grapevine PGIP (VvPGIP1) (Joubert *et al.*, 2006; Joubert *et al.,* 2007), 37 additional (non-*vinifera*) grapevine PGIP-encoding genes were isolated from *Vitaceae* species (Wentzel, 2005). These PGIPs share high sequence homology with VvPGIP1, but based on amino acid changes in the conserved LRR motif, the PGIPs could be clustered into 14 different groupings. In this study representative genes from each cluster have been targeted for constitutive overexpression in tobacco. Transgenic tobacco populations could be recovered for nine of the non-*vinifera* PGIP-encoding genes. Transgenic lines overexpressing the non-*vinifera* grapeving PGIP encoding genes exhibited enhanced resistance against *Botrytis cinerea* in comparison with VvPGIP1 plants, and a subset of non-*vinifera* lines also show a downregulation of a *xyloglucan endotransglycosylase* cell wall metabolism gene compared to wild-type tobacco.

3.3 Materials and methods

3.3.1 Selection of *pgip* genes used in this study

In a previous study done in our lab, 37 *Vitaceae pgip*-encoding genes were isolated and sequenced (Wentzel, 2005). Their deduced amino acid sequences were shown to be 95% homologous. They were grouped into a phylogenetic distance tree (Figure 3.1) based on the amino acid sequences of their Leucine Rich Repeat (LRR) active domains in comparison with that of the previously isolated and characterised *Vvpgip1* from Pinotage (De Ascensao, 2001; Joubert *et al.*, 2006). Using this distance tree, 14 different groups or subclades with identical LRR amino acid sequences were identified, of which one *pgip* per group was selected for use in this study (indicated in red on Figure 3.1). Table 3.1 shows the extent of the nucleotide and amino acid differentiation (also in the LRR domains) in comparison with the *Vvpgip1* gene.

3.3.2 Construction of PGIP-plant expression vectors

Bacterial strains used in this study, as well as all plasmids used and generated are listed in Table 3.2. Two *pgip*-specific primers were used to amplify the different *pgip* genes (1002 bp ORFs) from previously constructed clones (Wentzel, 2005). The following primer sequences were used to amplify the PGIP sequences: forward - 5'-GTCGACATGGAGACTTCAAAAC-3' (*Sal*I restriction site underlined), reverse - 5'-TCTAGAACTTGCAGCTCTGGAGTGGAG-3' (*Xba*I restriction site underlined). The amplified fragments were subcloned into the pGEM-T-Easy vector (Promega Corporation, Madison, USA), transformed into *Escherichia coli* and sequenced. The confirmed *pgip* sequences were then excised from pGEM-T-Easy with *Sal*I and *Xba*I , and cloned into the *Xho*I (isoschizomer of *Sal*I) and *Xba*I sites of the pART7 plant expression vector's multiple cloning site. The *Not*I cassettes of the pART7 vectors, each containing a *pgip* ORF, were sub-cloned into the pART27 expression vector (Table 3.2).

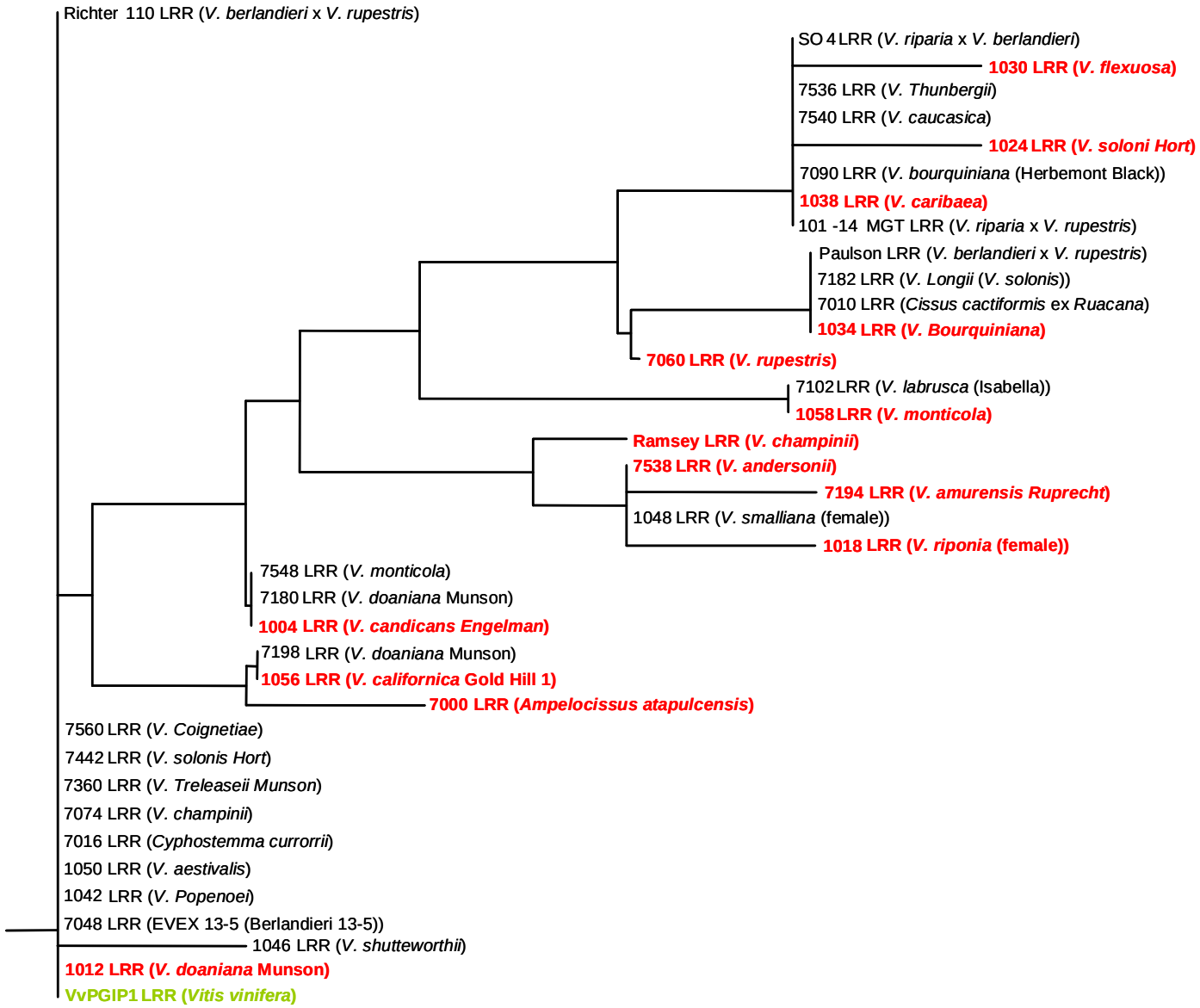


Figure 3.1 *Phylogenetic distance tree of the amino acid alignment of the LRR regions of PGIP encoding genes isolated from various Vitaceae accessions.* The amino acid sequences of the LRR active domains of each non-*vinifera* PGIP was aligned with that of VvPGIP1, and every other non-*vinifera* PGIP, resulting in fourteen homologous groups. The red font indicates the *pgips* chosen from this distance tree for use in this study, with the reference VvPGIP1 shown in green. The species to which the PGIP LRR sequence refers are shown in Table 3.1 (Figure modified from Wentzel, 2005).

Table 3.1 *Sources of Vitaceae pgip genes used in this study and the number of total, synonymous and non-synonymous nucleotide changes between each gene and Vvpgip1, as well between the active LRR domains.* Each gene has an assigned code obtained from the Nietvoorbij grapevine database (Table modified from Wentzel, 2005).

Grapevine species/cultivar code	*Species/Cultivar*	*Nucleotide Changes (total gene sequence)*	*Synonymous (total gene sequence)*	*Non-synonymous (total gene sequence)*	*Non-synonymous (LRR active domain)*
1004	*V. candicans Engelman*	14	11	3	1
1012	*V. doaniana Munson*	10	7	3	0
1018	*V. riponia* (female)	15	7	8	4
1024	*V. colonist Hort*	12	4	8	5
1030	*V. flexuosa*	13	4	9	5
1034	*V. bourquiniana*	13	5	8	3
1038	*V. caribaea*	14	7	7	4
1056	*V. californica* Gold Hill 1	9	4	5	1
1058	*V. monticola*	13	6	7	4
7000	*Ampelocissus atapulcensis*	9	4	5	2
7060	*V. rupestris*	11	5	6	3
7194	*V. amurensis Ruprecht*	17	8	9	4
7538	*V. Andersonii*	17	9	8	3
Ramsey	*V. champinii*	14	6	8	3

3.3.3 Plant growth conditions and tobacco transformations

The tobacco species *Nicotiana tabacum* SR1 (Petit Havana) was used as the heterologous plant host for the grapevine PGIPs. Wild-type tobacco plantlets were cultivated on MS medium in tissue culture (Murashige and Skoog, 1962) and maintained at 26^{o}C with a 16 h/8 h light/dark cycle.

The final plant expression vectors, each containing one of the 14 selected *pgip* genes, as described in 3.3.2 and listed in Table 3.2, were transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation. The *A. tumefaciens* strains were subsequently used to transform *N. tabacum* leaf disks according to the method of Gallois and Marinho (1995). Transformed tobacco leaf disks were grown on MS media supplemented with 0.5 μg/ml 6-benzyl-aminopurine (BAP) to induce shoot formation, and 100 μg/ml kanamycin to select for transformed plant tissue. Shoots were excised and transferred to MS media containing 0.1 μg/ml naphthalenacetic acid (NAA) to induce root formation, and the same concentration kanamycin to select for transgenic tissue. The putative transgenic *in vitro* plantlets were maintained on MS medium and eventually hardened off and transferred to peat soil (Jiffy International AS, Kristiansand, Norway) and maintained under natural light conditions at 26^{o}C and 65% humidity in a greenhouse.

Table 3.2 *Strains and plasmids used in this study.*

Strains or Plasmids	*Relevant features or insert*	*Source or reference*
Eschericia coli strain		
DH5α	supE44lacU169(ϕ*80lac*ZM15hsdR17recA1gyrA96*thi*-1relA1)	Invitrogen Life Technologies, Carlsbad, USA
Agrobacterium tumefaciens strain		
EHA105	Disarmed, succinomopine strain	Hood *et al*., 1993
Plasmids		
pGEM-T-Easy	pGEM5Zf(+) based PCR cloning vector	Promega,Madison, USA
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
pGEM(pgip1004)	*Pgip 1004* from *V. candicans Engelman* cloned into pGEM-T-Easy	This study
pGEM(pgip1012)	*Pgip 1012* from *V. doaniana Munson* cloned into pGEM-T-Easy	This study
pGEM(pgip1018)	*Pgip 1018* from *V. riponia* (female) cloned into pGEM-T-Easy	This study
pGEM(pgip1024)	*Pgip 1024* from *V. solonis Hort* cloned into pGEM-T-Easy	This study
pGEM(pgip1030)	*Pgip 1030* from *V. flexuosa* cloned into pGEM-T-Easy	This study
pGEM(pgip1034)	*Pgip 1034* from *V. Bourquiniana* cloned into pGEM-T-Easy	This study
pGEM(pgip1038)	*Pgip 1038* from *V. caribaea* cloned into pGEM-T-Easy	This study
pGEM(pgip1056)	*Pgip 1056* from *V. californica* Gold Hill 1 cloned into pGEM-T-Easy	This study
pGEM(pgip1058)	*Pgip 1058* from *V. monticola* cloned into pGEM-T-Easy	This study
pGEM(pgip7000)	*Pgip 7000* from *Ampelocissus atapulcensis* cloned into pGEM-T-Easy	This study
pGEM(pgip7060)	*Pgip 7060* from *V. rupestris* cloned into pGEM-T-Easy	This study
pGEM(pgip7194)	*Pgip 7194* from *V. amurensis Ruprecht* cloned into pGEM-T-Easy	This study
pGEM(pgip7538)	*Pgip 7538* from *V. andersonii* cloned into pGEM-T-Easy	This study
pGEM(pgipRamsey)	*Pgip Ramsey* from *V. champinii* cloned into pGEM-T-Easy	This study
pART7(pgip1004)	*Pgip 1004* from *V. candicans Engelman* cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgip1012)	*Pgip 1012* from *V. doaniana Munson* cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgip1018)	*Pgip 1018* from *V. riponia* (female) cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgip1024)	*Pgip 1024* from *V. solonis Hort* cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgip1030)	*Pgip 1030* from *V. flexuosa* cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgip1034)	*Pgip 1034* from *V. Bourquiniana* cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgip1038)	*Pgip 1038* from *V. caribaea* cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgip1056)	*Pgip 1056* from *V. californica* Gold Hill 1 cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgip1058)	*Pgip 1058* from *V. monticola* cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgip7000)	*Pgip 7000* from *Ampelocissus atapulcensis* cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgip7060)	*Pgip 7060* from *V. rupestris* cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgip7194)	*Pgip 7194* from *V. amurensis Ruprecht* cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgip7538)	*Pgip 7538* from *V. andersonii* cloned into *Xho*I and *Xba*I sites of pART7	This study
pART7(pgipRamsey)	*Pgip Ramsey* from *V. champinii* cloned into *Xho*I and *Xba*I sites of pART7	This study
pART27(pgip1004)	*Pgip 1004* from *V. candicans Engelman* cloned into *Not*I site of pART27	This study
pART27(pgip1012)	*Pgip 1012* from *V. doaniana Munson* cloned into *Not*I site of pART27	This study
pART27(pgip1018)	*Pgip 1018* from *V. riponia* (female) cloned into *Not*I site of pART27	This study
pART27(pgip1024)	*Pgip 1024* from *V. solonis Hort* cloned into *Not*I site of pART27	This study
pART27(pgip1030)	*Pgip 1030* from *V. flexuosa* cloned into *Not*I site of pART27	This study
pART27(pgip1034)	*Pgip 1034* from *V. Bourquiniana* cloned into *Not*I site of pART27	This study
pART27(pgip1038)	*Pgip 1038* from *V. caribaea* cloned into *Not*I site of pART27	This study
pART27(pgip1056)	*Pgip 1056* from *V. californica* Gold Hill 1 cloned into *Not*I site of pART27	This study

Table 3.2 (cont.)

Strains or Plasmids	*Relevant features or insert*	*Source or reference*
pART27(pgip1058)	*Pgip 1058* from *V. monticola* cloned into *Not*I site of pART27	This study
pART27(pgip7000)	*Pgip 7000* from *Ampelocissus atapulcensis* cloned into *Not*I site of pART27	This study
pART27(pgip7060)	*Pgip 7060* from *V. rupestris* cloned into *Not*I site of pART27	This study
pART27(pgip7194)	*Pgip 7194* from *V. amurensis Ruprecht* cloned into *Not*I site of pART27	This study
pART27(pgip7538)	*Pgip 7538* from *V. andersonii* cloned into *Not*I site of pART27	This study
pART27(pgipRamsey)	*Pgip Ramsey* from *V. champinii* cloned into *Not*I site of pART27	This study

The plantlets were supplemented with Chemicult fertiliser (Chemicult Products, Camps bay, Cape Town, South Africa) every 14 days. The plants were allowed to flower under controlled conditions that ensured self-pollination in order to establish a T1 generation of transgenic tobacco. To establish populations of the various T1-transgenic lines, seeds were sown on MS media supplemented with 100 μg/ml Kanamycin. When seedlings had four expanding leaves, they were hardened off and transferred to a greenhouse as described above and used for subsequent genetic and phenotypical analyses.

3.3.4 Analyses of transgenic plant lines

3.3.4.1 PCR and Southern Blot analyses

Tobacco leaves from greenhouse grown T1 lines were harvested and frozen in liquid nitrogen. Genomic DNA extraction was done according to the method of McGarvey and Kaper (1991) for use in PCR screening and Southern blot analyses. Tissue from untransformed wild-type tobacco, and from VvPGIP1 (line 37, as described in Joubert *et al.*, 2006) were included as a negative and positive control, respectively. PCR screening was performed to confirm the presence of the *pgip* genes in the tobacco plantlets. The *pgip*-specific primers described in 3.3.2 were used to amplify the transgenes using 100 ng of genomic DNA as template. The reaction mixtures contained 1 x PCR buffer (containing $MgCl_2$), 0.25 μM primers, 0.25 μM dNTPs, 100 ng of template and 0.5 U of Taq polymerase in 25 μl reactions. The reaction cycle consisted of an initial denaturation step at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s and elongation at 72°C for 60 s, and ending with a final elongation step of 72°C for 10 min.

For Southern blot analyses, extracted gDNA was further purified from contaminating substances by binding a DEAE Sepharose matrix to the gDNA, washing the bound DNA, and recovering it from the matrix by high-salt denaturation (Sharma *et al*., 2000). Thirty micrograms of purified genomic DNA was restricted with *Xba*I. This enzyme digests once just outside the 5' end of the *pgip* gene, but not in the gene itself, thus excising a minimum band of ~ 1000 bp. Every hybridisation signal thus represents one copy of the gene. The digested DNA was separated on a 0.8% (w/v) agarose gel and subsequently transferred to positively charged nylon membranes according to Sambrook *et al*. (1989). A *pgip*-specific probe was labelled with digoxigenin in a PCR labelling reaction using the PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's specifications and using the *pgip*-specific primers described in 3.3.2. After cross-linking the transferred DNA to the membrane, the DIG-labelled *pgip*-specific probe was hybridised to the DNA according to the DIG System User's Guide (Roche Diagnostics GmbH, Mannheim, Germany). Pre-hybridisation and hybridisation steps were carried out at 42°C in DIG Easy Hyb (Roche Diagnostics GmbH, Mannheim, Germany) for 2 h and 20 h, respectively. Membranes were then washed twice for 15

min each at 68°C with 2xSSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0 and 0.1% SDS w/v), followed by two washes of 15 min each with 0.2xSSC at 68°C. Blocking and antibody binding (Anti-DIG AP Fab Fragments, Roche Diagnostics GmbH, Mannheim, Germany) were carried out in casein blocking buffer (1% w/v casein dissolved in Maleic acid buffer) for 1 h and 30 min respectively. Detection proceeded with CSPD chemiluminescent substrate (Roche Diagnostics GmbH, Mannheim, Germany) after the membranes were thoroughly washed with DIG washing buffer (Maleic acid buffer containing 3 g/l Tween 20).

3.3.4.2 Northern blot analysis

Tobacco leaves from greenhouse-grown putative transgenic plants were harvested and frozen in liquid nitrogen. Total RNA extraction was done according to the method of Chang *et al*. (1993) for use in northern blot analysis. Tissue from untransformed wild-type tobacco as well as from VvPGIP1 line 37 was included as controls. RNA was denatured at 68°C and then run on a 1.2% (w/v) agarose gel containing 0.6% formaldehyde. The RNA was then transferred and cross-linked to positively charged nylon membranes according to Sambrook *et al*. (1989). The same probe that was created for Southern Blotting was used to hybridise to the RNA at 50°C after pre-hybridisation at the same temperature. The subsequent wash steps, blocking, antibody application and detection steps were the same as described for Southern analysis, section 3.3.4.1.

3.3.4.3 Detection of PGIP activity

An agarose diffusion assay (Taylor and Secor, 1988) was used to confirm PGIP inhibitory activity of crude protein extracts from the transgenic tobacco lines. Polygalacturonic acid (PGA) was used as substrate and incorporated into the agarose plate. Crude extracts of *B. cinerea* ePGs alone, or mixed with crude protein extract from the transgenic tobacco to test for PGIP activity was added to the plates in small wells. ePGs activity on the substrate would result in clearing zones on the agarose plate when treated with 10 N HCl after incubation at 30°C for 16 h. Co-incubation with active PGIP added to the wells would result in a reduction of zone diameters as a result of ePG inhibition.

Crude PGIP extracts were prepared by homogenising finely ground leaf tissue in 1 ml sodium acetate extraction buffer (50 mM NaOAc, pH 5.0, 1 M NaCl) per gram tobacco tissue. The tissue was agitated on ice for 60 minutes in the sodium acetate buffer and left at 4°C for 16-20 h. Extracts were then centrifuged at 4°C for 30 min at 10 000 rpm and the supernatants were collected.

Crude *Botrytis* ePGs were prepared by cultivating *B. cinerea* spores in citrate phosphate buffer (pH 6.0, with 1% w/v citrus pectin, 2 mM $MgSO_4.7H_2O$, 0.6 μM $MnSO_4.H_2O$, 25 mM KNO_3, 30 μM $ZnSO_4.7H_2O$, 0.9 μM $CuSO_4$, 65 μM $FeSO_4$) for 10 days at 25°C. The culture was filtered through Whatman filter paper and precipitated with 80% $(NH_4)_2SO_4$ at 4°C overnight. The culture was then centrifuged at 4°C for 20 min at 10 000 g to harvest the precipitated proteins, and the pellet was resuspended in 40 mM sodium acetate, pH 5.0.

The crude protein extracts were quantified according to Bradford (1976). Equal amounts of the crude PGIP extracts were used in the agarose diffusion assay with crude ePGs from *B. cinerea*. All diffusion assays were done in triplicate. The clearance zones were measured and expressed as a percentage of zone reduction as compared with a zone formed by ePG without any PGIP added.

3.3.5 Whole-plant infection of transgenic tobacco with *Botrytis cinerea*

A whole-plant infection was carried out on a subset of 6 to 8 week-old T1 transgenic plant lines. The lines selected for the infection assay tested positive with the PCR, Southern, and northern analyses, as well as the protein activity assay data. The infection assay was repeated twice and the lines and the number of individuals per line used for the infections are indicated in Table 3.3.

Table 3.3 *Sub-population of plant lines infected with B. cinerea in two separate infection assays.* Each individual plant line infected is listed, as well as the number of individual plants of each line. The control lines (wild-type tobacco and VvPGIP1 line 37, described in Joubert *et al*. (2006)) are shown in italics.

Infection experiment 1			Infection experiment 2		
PGIP	**Line #**	**# of individuals**	**PGIP**	**Line #**	**# of individuals**
1004	5	4	n.d.*	n.d.	n.d.
1012	14	4	n.d.	n.d.	n.d.
	16	4	n.d.	n.d.	n.d.
	17	3	n.d.	n.d.	n.d.
1018	2	3	n.d.	n.d.	n.d.
	4	1	n.d.	n.d.	n.d.
1024	2	2	1024	2	4
n.d.	n.d.	n.d.		7	4
1030	7	1	1030	7	4
n.d.	n.d.	n.d.		8	4
n.d.	n.d.	n.d.	1038	5	4
n.d.	n.d.	n.d.		6	4
n.d.	n.d.	n.d.		8	4
1056	1	1	n.d.	n.d.	n.d.
7000	2	1	7000	2	4
7060	2	1	7060	7	4
n.d.	n.d.	n.d.	Ramsey	1	4
n.d.	n.d.	n.d.		2	4
n.d.	n.d.	n.d.		4	4
Untransformed wild-type control	*n.a.***	*4*	*Untransformed wild-type control*	*n.a.*	*8*
VvPGIP1	*37*	*4*	*VvPGIP1*	*37*	*8*

*not determined

**not applicable

The *B. cinerea* strain used in the infection assays was maintained on sterile apricot halves in a dark growth chamber at 25^{o}C until sporulation occurred. Spores were collected with sterile water after 12 days of incubation and filtered to remove debris. Viability and germination potential was evaluated by plating the spores out on 0.8% (w/v) water agar.

The infection assays were carried out according to the method described in Joubert *et al* (2006). For the infections, 5 μl of a 1 x 10^3 spore suspension in 50% grape juice was spotted on the adaxial side of the leaves. Three leaves per plant (position 3, 4 and 5) were inoculated with four spots per leaf (Figure 3.2). Plants were kept in Perspex humidity chambers at high relative humidity levels (85-100%) at ambient room temperature. The infections were allowed to progress for 11-15 days. The disease susceptibility of the plants were determined by measuring the expansion of the lesions (diameter, in mm) every second day. The disease progression was compared to that of the untransformed wild-type control, as well as the previously characterised VvPGIP1 line 37. The data of the various lines per PGIP per leaf was recorded and analysed separately, as well as combining the data for the final analysis. The combined data is presented as average lesion diameters for each PGIP per leaf, as well as all the lesion sizes from all three leaves of each individual plant of each PGIP line to calculate the average lesion diameter for

each PGIP. Statistical analysis of the combined data was done by performing a one-way Analysis of Variance using the Statistica software package (Statsoft Inc., Tulsa, USA) at 95% confidence levels.

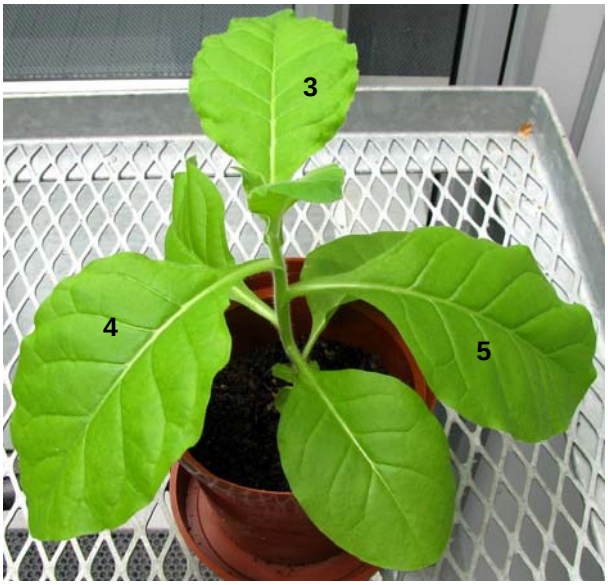


Figure 3.2 *An example of the size and condition of tobacco plants before infection with B. cinerea*. The three leaf positions that were used to inoculate with spores are indicated as leaf positions 3, 4 and 5. Four infection spots per leaf were made.

3.3.6 Preliminary quantitative Real-Time (qRT)-PCR screen of a selection of lines to study the expression level of the *XTH* gene in the transgenic and control tobacco lines

Previously, it was found that all tobacco lines with a VvPGIP1-specific resistance phenotype displayed downregulation of the *XTH* gene (Becker, 2007). A selection of lines from this study was screened with qRT-PCR analysis for expression patterns of this gene, compared to the untransformed control and a VvPGIP1 line (line 37, characterised in Joubert *et al*., 2006). For the qRT-PCR analysis, two PGIPs that were highly resistant to *B. cinerea* infection (Section 3.3.5) were selected: PGIP1012 and PGIP1038. For PGIP1012, the same three lines that were used in infection studies were used (lines 14, 16 and 17) and for PGIP1038 two of the three lines used in the infection assay were used again (lines 6 and 8). Wild-type untransformed tobacco and VvPGIP1 (line 37) plants were included as control plants. For each plant line in the assay, three individual plants were analysed, except for PGIP1012 line 17, of which there was only two plants available (see Table 3.4 for an outline of the lines used).

Table 3.4 *Sub-population of plant lines used to evaluate xth expression in the PGIP transgenic lines and the controls (in italics).*

PGIP	Transgenic line tested	# of individual plants tested
1012	14	3
	16	3
	17	2
1038	6	3
	8	3
Untransformed wild-type control	*n.a.**	*3*
VvPGIP1	*37*	*3*

*n.a. not applicable

The leaves at position 3 of 8-week old transgenic tobacco plants were harvested (similar to figure 3.2) and the tissue homogenised for RNA extraction in the same manner as described above for northern blots. Total RNA, treated with DNase enzyme to remove residual gDNA, was used for cDNA synthesis with the SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Invitrogen, California USA) according to the manufacturer's specifications. The ABI 7500 Real-Time PCR Instrument (Applied Biosystems, California USA) was used to amplify the cDNA samples using SYBR Green Fluorescent Dye as a detector (KAPA, Massachusetts USA).

The tobacco genes amplified were xyloglucan endotransglycosylase (*XTH*, Genbank Acc AB017025.1) and the actin gene as normalisation control. The *pgip* gene was also included in order to confirm transgene expression and to compare the relative expression levels between different transgenic plant lines. The cDNA amplification reaction conditions were: denaturation for 10 min at 95^{o}C, followed by 40 cycles of denaturation at 95^{o}C for 15 s and annealing and elongation at 58^{o}C for 1 min with a single data acquisition point per cycle. At the end of the reaction, a melting curve analysis was performed by raising the temperature from 58 to 95^{o}C by 0.1^{o}C per second. LinRegPCR software was used to calculate PCR efficiencies (Ramakers *et al*., 2003). The PCR efficiencies and threshold cycles were used to calculate relative expression with a mathematical model (Pfaffl, 2001).

For the genes, relative expression levels were calculated for each of the individual plants in the population, and there was also an independent biological repeat (separate RNA extraction and cDNA synthesis) done of one individual plant from each line. The data is presented as average values of the two biological repeats of each plant line, and also as average levels of expression within each PGIP population.

3.4 Results

3.4.1 Generating transgenic tobacco populations overexpressing a range of different grapevine PGIPs

Fourteen grapevine *pgip* genes were selected from a larger set of *pgip* genes previously isolated (Wentzel, 2004) from *Vitaceae* accessions that are known for their good resistance towards many fungal pathogens (including *B. cinerea* and several grapevine mildews). These fourteen genes were each cloned into the plant expression vector pART27 (see Table 3.2) and transformed into *A. tumefaciens* in order to create strains with which to transform tobacco. *N. tabacum* explants were subsequently transformed with each of the fourteen *pgips*, and putative transgenic populations were regenerated for nine of the *pgips*. No putative transgenic plants were obtained from *pgips* 1034, 1058, 7194 and 7538 and most lines of *pgip* 1056 were lost due to fungal contamination during regeneration. As a result, the final T1 tobacco population consisted of lines from nine of the *pgips* (minimum four independent transformants per *pgip*), along with one line from *pgip* 1056.

3.4.2 Analyses of the transgenic populations

The putative transgenic lines did not display any visible phenotype and grew normally vegetatively and reproductively. All transgenic and control lines were analysed for transgene presence (PCR screen) and PGIP activity (PGIP activity plates) to screen the putative populations for transgenic lines. For the PCR analysis, *pgip*-specific primers (described in 3.3.2) were used for amplification that yielded a 1000 bp fragment in transformed lines. Tissue from VvPGIP1 line 37 was used as positive control and untransformed tobacco tissue was used as negative control, the latter not yielding any PCR product. Northern blot analysis was performed to confirm transgene expression. Lines were selected from the northern positive lines for further

analysis (*B. cinerea* infection and/or qRT-PCR); these lines were finally subjected to Southern blot analysis to confirm gene integration events (see Table 3.5 for a summary of these results, as well as Addendum A to this chapter for the documented PCR, Northern blot, PGIP activity assays and Southern blot analysis per line).

Table 3.5 *Summary of transgenic population analyses* (transgene integration, PGIP activity assay, Northern and Southern analyses, as well as subsequent analyses performed of each line).

PLANT LINE	TRANSGENE PRESENCE (PCR)	PGIP ACTIVITY	TRANSGENE EXPRESSION (NORTHERN)	TRANSGENE COPY NUMBER (SOUTHERN)	SUBSEQUENT ANALYSIS PERFORMED
WT	-	-	-	-	*B. cinerea* infection *XTH* gene expresssion
VvPGIP1					
Line 37	+	+	+	1	*B. cinerea* infection *XTH* gene expresssion
PGIP 1004					
Line 1	-	-	-	n.d.[a]	
Line 2	+	+	+	n.d.	
Line 3	+	+	+	>4	
Line 4	+	+	+	n.d.	
Line 5	+	+	+	2	*B. cinerea* infection
Line 6	+	+	+	2	
Line 7	-	-	-	n.d.	
PGIP 1012					
Line 2	+	-	-	n.d.	
Line 3	+	+	+	n.d.	
Line 4	+	+	+	n.d.	
Line 5	+	+	+	n.d.	
Line 6	-	-	-	n.d.	
Line 7	+	-	-	n.d.	
Line 8	+	+	+	n.d.	
Line 9	+	+	+	n.d.	
Line 10	+	-	-	n.d.	
Line 11	-	-	-	n.d.	
Line 12	+	-	-	n.d.	
Line 13	+	+	+	n.d.	
Line 14	+	+	+	1	*B. cinerea* infection *XTH* gene expresssion
Line 15	-	-	-	n.d.	
Line 16	+	+	+	1	*B. cinerea* infection *XTH* gene expresssion
Line 17	+	+	+	3	*B. cinerea* infection *XTH* gene expresssion
PGIP 1018					
Line 2	+	+	+	>4	*B. cinerea* infection
Line 3	+	+	+	1	
Line 4	+	+	+	3	*B. cinerea* infection
Line 5	-	-	-	n.d.	
Line 6	-	-	-	n.d.	
PGIP 1024					
Line 1	+	+	+	n.d.	
Line 2	+	+	+	2	*B. cinerea* infection
Line 3	-	-	n.d.	n.d.	
Line 4	+	+	+	n.d.	
Line 5	+	+	+	n.d.	
Line 6	-	-	n.d.	n.d.	
Line 7	+	+	+	>4	*B. cinerea* infection
Line 8	+	+	+	2	
Line 9	+	+	+	n.d.	
Line 10	+	+	+	n.d.	
Line 14	+	+	+	n.d.	
Line 16	-	-	-	n.d.	
Line 17	+	+	+	n.d.	

[a] not determined

Table 3.5 (cont.)

PLANT LINE	TRANSGENE PRESENCE (PCR)	PGIP ACTIVITY	TRANSGENE EXPRESSION (NORTHERN)	TRANSGENE COPY NUMBER (SOUTHERN)	SUBSEQUENT ANALYSIS PERFORMED
PGIP 1030					
Line 1	-	-	-	n.d.	
Line 2	+	+	+	2	
Line 3	+	-	-	n.d.	
Line 5	-	-	-	n.d.	
Line 6	+	-	-	n.d.	
Line 7	+	+	+	>4	*B. cinerea* infection
Line 8	+	+	+	>4	*B. cinerea* infection
Line 9	-	-	-	n.d.	
Line 10	-	-	-	n.d.	
Line 11	-	-	-	n.d.	
PGIP 1038					
Line 1	+	+	+	n.d.	
Line 4	-	-	-	n.d.	
Line 5	+	+	+	n.d	*B. cinerea* infection
Line 6	+	+	+	3	*B. cinerea* infection *XTH* gene expresssion
Line 8	+	+	+	>4	*B. cinerea* infection *XTH* gene expresssion
Line 9	+	-	-	n.d.	
PGIP 1056					
Line 1	+	+	+	2	*B. cinerea* infection
PGIP 7000					
Line 1	-	-	-	n.d.	
Line 2	+	+	+	3	*B. cinerea* infection
Line 3	+	+	+	3	
Line 4	+	+	+	2	
PGIP 7060					
Line 1	-	-	-	n.d.	
Line 2	+	+	+	2	*B. cinerea* infection
Line 3	+	+	+	2	*B. cinerea* infection
Line 4	+	+	+	2	
PGIP Ramsey					
Line 1	+	+	+	2	*B. cinerea* infection
Line 2	+	+	+	2	*B. cinerea* infection
Line 3	-	-	-	n.d.	
Line 4	+	+	+	2	*B. cinerea* infection
Line 5	-	-	-	n.d.	
Line 6	-	-	-	n.d.	
Line 7	-	-	-	n.d.	
Line 8	+	+	+	n.d.	
Line 9	-	-	-	n.d.	
Line 10	-	-	-	n.d.	
Line 11	-	-	-	n.d.	

[a] not determined

3.4.3 Whole-plant infection assay with *Botrytis cinerea*

A comprehensive time-course infection assay of whole plants was previously optimised and used to define PGIP-specific resistance phenotypes in transgenic tobacco populations overexpressing VvPGIP1 (Joubert *et al.*, 2006). From that study, VvPGIP1 line 37 was used as a positive control for PGIP activity and as a PGIP-specific resistance phenotype. In this study, the transgenic populations overexpressing the nine non-*vinifera* grapevine PGIPs were analysed with this optimised infection assay and compared with the WT and VvPGIP1 line 37. The specific plant lines used for each infection assay are listed in Table 3.3. The results from the two separate tobacco infection experiments are shown in Figure 3.3: the lesion sizes were measured and data from all the infection spots on all the leaves per plant, as well as the data for

all the repeats were averaged per PGIP. Also see Figure 3.4 for a subset of data showing the data for each leaf position separately.

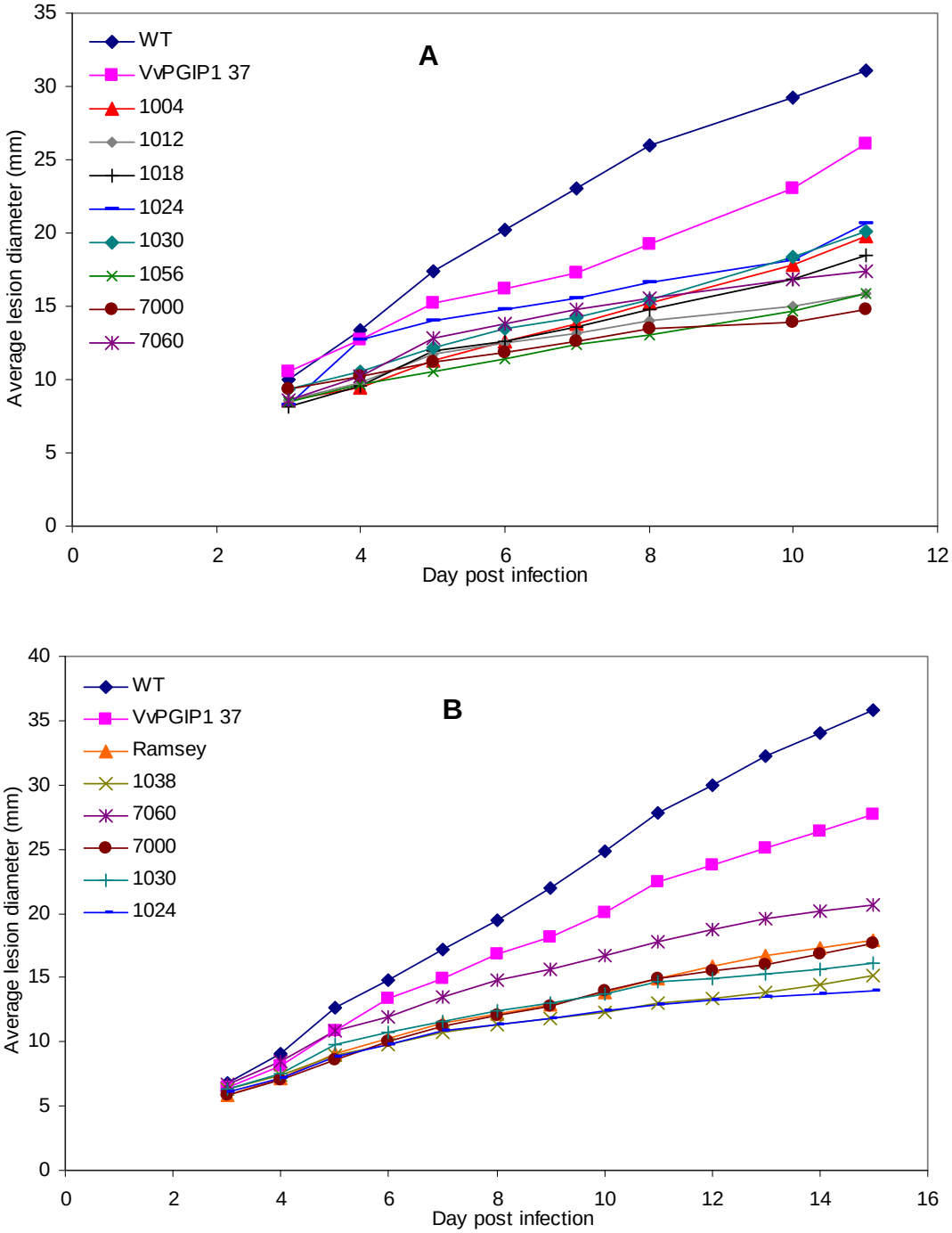
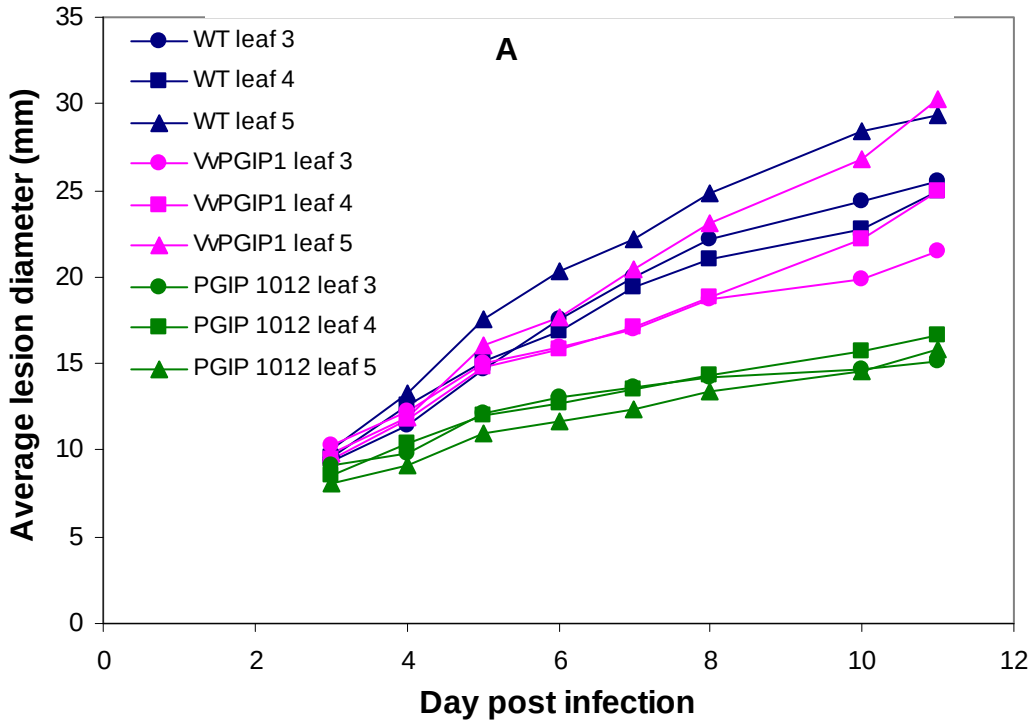


Figure 3.3 *The development of lesions formed over time by the fungus Botrytis cinerea on tobacco leaves from untransformed wild-type and transgenic PGIP-overexpressing plant lines.* The infection was followed until the lesions on the untransformed leaves were no longer measurable (11 days, first group of infections (**A**) or 15 days, second group of infections (**B**)).



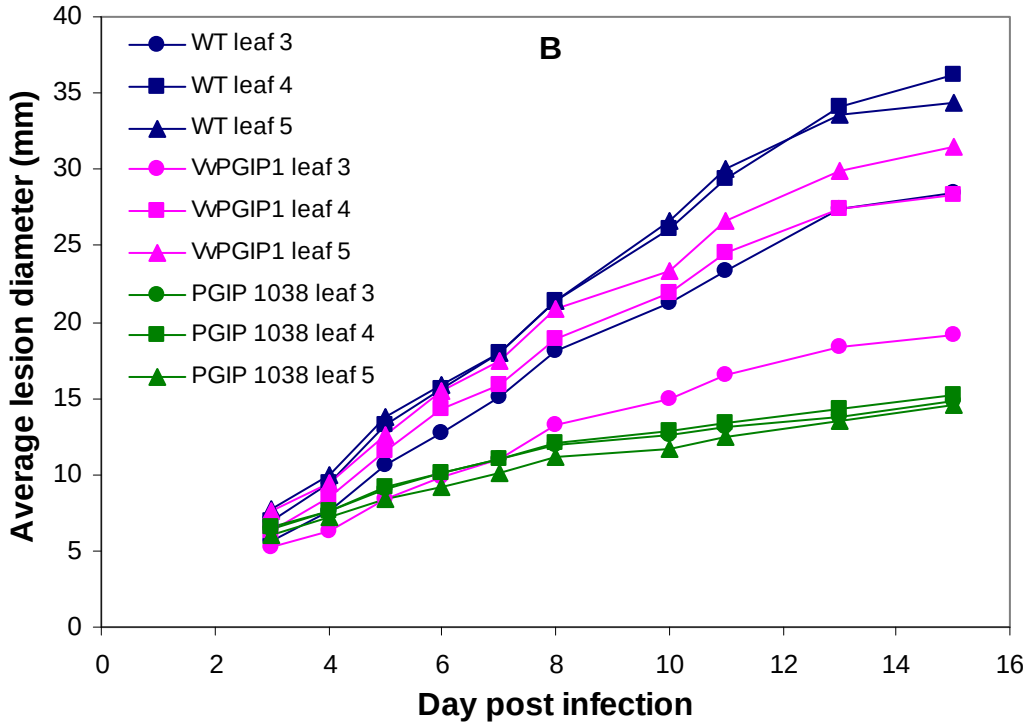


Figure 3.4 *Data for a subset of PGIP lines showing the development of B. cinerea infection separately for each of the three leaf positions used in the two infection assays.* The infection was followed until the lesions on the untransformed leaves were no longer measurable (11 days, first group of infections (**A**) or 15 days, second group of infections (**B**)).

One-way ANOVA statistical analysis of the data was performed, and showed that the PGIP overexpressing lines were significantly less susceptible to *Botrytis cinerea* infection than the wild-type tobacco plants (Table 3.6). The wild-type control, the VvPGIP1 line and the non-*vinifera* PGIPs separated in four separate homologous groups. The members of the non-*vinifera* PGIPs clearly separated from the wild-type control and the VvPGIP1 line (Table 3.6). Plants overexpressing VvPGIP1 developed lesions 16-22% smaller than the wild-type plants, and all the lines overexpressing the PGIPs from non-*vinifera* species developed lesions 33-60% smaller than the wild type.

Table 3.6 *Analysis of lesion development after whole-plant Botrytis cinerea infection of transgenic tobacco overexpressing various grapevine PGIPs.* Statistical analysis divided the plant lines into significant homogenous groups (p = 0.05). **A** shows the analyses of the first infection assay, and **B** the data of the second assay.

A PGIP	Average lesion diameter in mm on the last measured day	% decrease in lesion size compared to WT	Group 1[a]	Group 2	Group 3	Group 4
WT	31.09	n/a	*****			
VvPGIP1 (line 37)	26.09	16.1		*****		
1004	19.76	36.5			*****	
1012	15.85	49.03				*****
1018	18.43	40.1			*****	*****
1024	20.61	33.7			*****	
1030	20.06	35.5			*****	
1056	15.90	48.9				*****
7000	14.75	52.6				*****
7060	17.43	43.9			*****	*****

n/a = not applicable

[a]Statistical one-way ANOVA analysis of the lesion size data separated the PGIPs into homogenous groups (p = 0.05). Four separate groups were formed. PGIPs 1018 and 7060 could belong to either group 3 or 4.

B PGIP	Average lesion diameter in mm on the last measured day	% decrease in lesion size compared to WT	Group 1[b]	Group 2	Group 3	Group 4
WT	35.82	n/a	*****			
VvPGIP1 (line 37)	27.72	22.6		*****		
1024	14.02	60.8				*****
1030	16.11	54.2				*****
1038	15.16	57.6				*****
7000	17.12	47.8			*****	*****
7060	19.02	43.15			*****	
Ramsey	17.94	50.0			*****	*****

n/a = not applicable

[b]Statistical one-way ANOVA analysis of the lesion size data separated the PGIPs into homogenous groups (p = 0.05). Four separate groups were formed. PGIPs 7000 and Ramsey could belong to either group 3 or 4.

During both infection experiments, the inoculated lesions yielded actively spreading lesions and the formation of reproductive structures on the WT tobacco controls. The infection efficiency of the applied spots were calculated to be 98 – 100%, indicating very high infection rates and very favorable conditions for pathogen colonisation and infection. In all cases, the primary lesions started developing within two to three days post infection (dpi), whereas the secondary spreading lesions and the resistance phenotypes developed in the remaining time of the assay. In the wild type tobacco, the formed lesions were very wet and spread rapidly until the whole leaf was completely infected, with reproductive organs of the fungi becoming visible as the disease progressed (Figure 3.4, WT tobacco from four dpi to 13 dpi). In the PGIP-overexpressing tobacco lines, the initially spreading lesions progressed slower and were ultimately contained in a necrotic and dry lesion without fungal reproductive structures on the tobacco leaves (Figure 3.4, VvPGIP1 line 37 and PGIP 1038, four to 13 dpi).

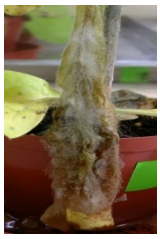
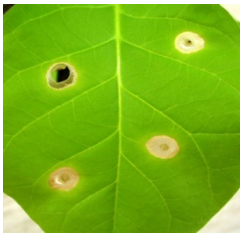
Plant Line	4 dpi	6 dpi	10 dpi	13 dpi
WT				
VvPGIP 1 line 37				
PGIP 1038				

Figure 3.5 *Progression of Botrytis infection of one of the non-*vinifera *PGIPs and the WT and VvPGIP1 control plants from four to 13 dpi.*

The variable amino acids between the different PGIP sequences are shown in Table 3.7, relative to the sequence of VvPGIP1. No single amino acid difference in the active domain of the protein distinguishes VvPGIP1 from the other PGIPs, although there are a number of variable amino acids in that region. There is one amino acid in the signal peptide of the protein (position 16) that is a valine in VvPGIP1 but a leucine in all the other PGIPs, and there is also one amino acid in the N-terminal region (position 62) that is a glycine in VvPGIP1, but a glutamate in the other PGIPs.

Table 3.7 *Variable amino acids between the PGIPs used in the whole-plant B. cinerea infection assay.* The sequence of VvPGIP1 (last row) is used as the reference sequence to which the other PGIPs are compared. The amino acids shown in yellow blocks form part of the leucine-rich repeat (LRR) active domain of the proteins. Residues 1-29 are part of the signal peptide, while 30-68 are part of the N-terminus region (pink blocks).

	3	16	26	62	72	84	140	173	197	199	221	234	241	246	255	261	300
1004	T	L	L	E	T	Q	A	G	T	Y	T	L	N	I	Q	V	L
1012	T	L	L	E	S	Q	A	G	T	Y	T	P	N	I	Q	V	L
1018	T	L	F	E	T	E	A	G	A	Y	T	L	K	I	Q	M	L
1024	R	L	F	E	T	K	A	G	T	G	T	L	K	V	X	V	L
1030	T	L	F	E	T	K	A	G	T	G	T	L	K	V	Q	V	S
1038	T	L	L	E	S	Q	A	G	T	G	T	L	K	V	Q	V	L
1056	T	L	F	E	T	K	A	G	T	Y	I	P	N	I	Q	V	L
7000	T	L	L	E	T	Q	A	D	T	G	I	P	N	I	Q	V	L
7060	T	L	L	E	T	Q	A	D	T	G	T	L	N	V	Q	V	L
Ramsey	T	L	L	E	T	Q	E	G	P	Y	T	L	K	I	Q	V	L
VvPGIP1	T	V	L	G	T	Q	A	G	T	Y	T	P	N	I	Q	V	L

3.4.4 qRT-PCR of cell wall biosynthesis gene expression within the transgenic PGIP population

The gene expression pattern of the xyloglucan endotransglycosylase (*xth;* Genbank Acc AB017025.1) gene was evaluated in a subset of the non-*vinifera* lines that showed strong resistance phenotypes. A housekeeping gene, actin, was also amplified, as well as *pgip* to confirm the PGIP-expression of the individual transgenic plants and to determine the relative PGIP expression levels between the different transgenic lines. The expression levels of these genes in the transgenic lines were compared with the levels in wild-type tobacco, as well as one VvPGIP1 overexpressing line (line 37).

The data shown below (Figure 3.6) is the average expression levels of two independent biological repeats of one individual plant from each plant line analysed in this experiment. Also see Addendum B to this chapter for the individual datapoints per repeat line, i.e. the non-averaged data.

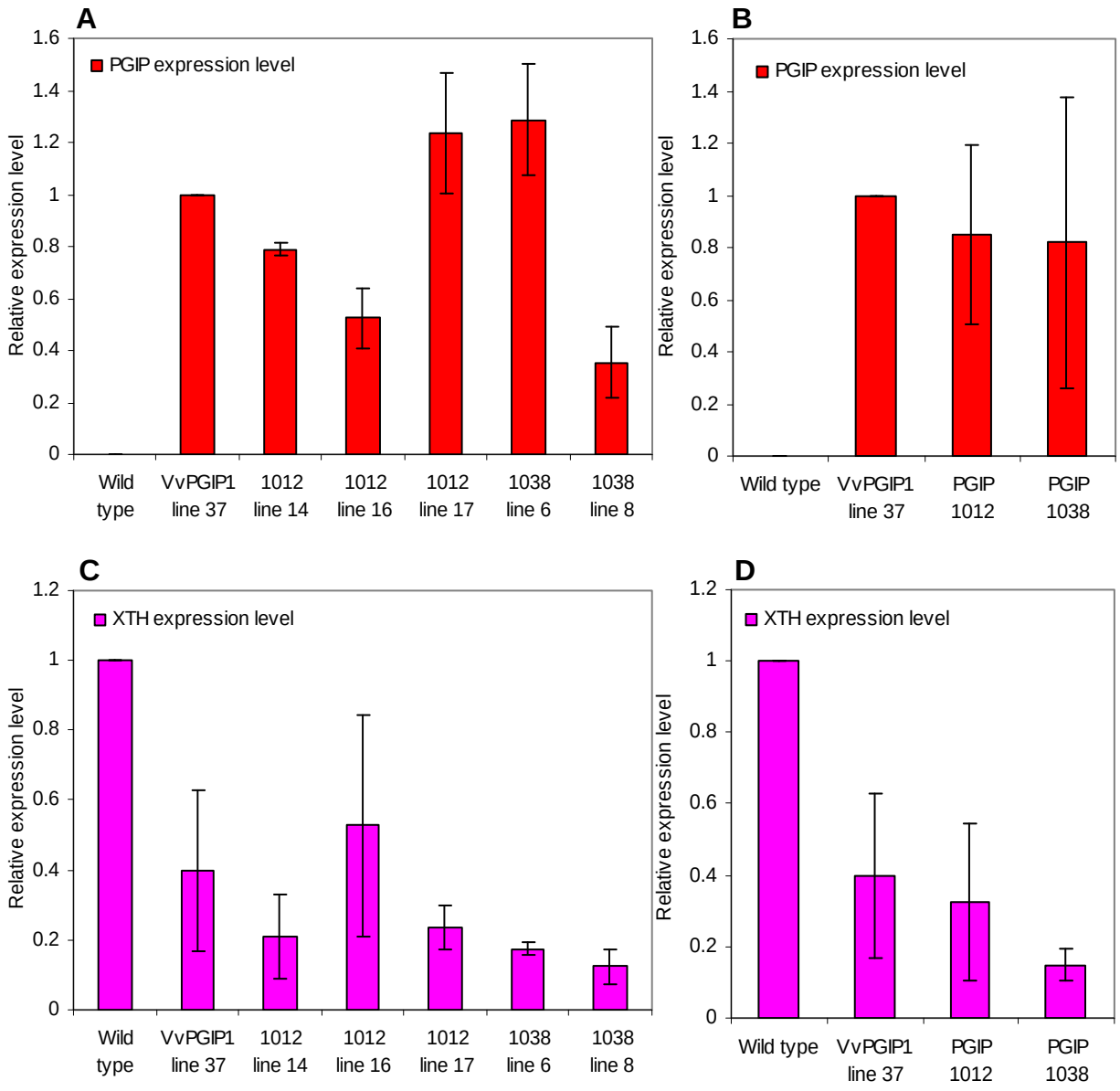


Figure 3.6 *Comparison of the relative expression levels of the xth gene in a population of healthy (uninfected) transgenic tobacco overexpressing different grapevine PGIP encoding genes (qRT-PCR analysis).* **A** and **B** show the levels of PGIP expression relative to that of VvPGIP1 line 37. The values in A are the averages of two biological repeats per plant line, with standard deviation shown. The values in B are the averages of the different *pgip* genes (for *pgip* 1012, it is the average of three independent lines with two biological repeats each; for *pgip* 1038 it is the average of two independent lines with two biological repeats each). **C** and **D** show the levels of *xth* expression relative to that of untransformed wild-type tobacco. As with A and B, C shows the average values of two biological repeats of one individual plant of each line, and D shows the average values of the different grapevine PGIPs. Addendum B to this chapter contains the non-averaged data for the individual datapoints of each plant line.

3.5 Discussion

Overexpression of non-*vinifera* grapevine PGIPs results in increased disease resistance towards *Botrytis cinerea* in transgenic tobacco

The PGIPs in plant species are considered one of the primary defence mechanisms of plants to protect against invading pathogens, specifically those that secrete ePGs in their infection strategy. The previous and current work on grapevine PGIPs are providing interesting insights

into the PGIP:ePG inhibition interaction (Joubert *et al.*, 2006; Joubert *et al.*, 2007). The first grapevine PGIP, VvPGIP1, was isolated from *V. vinifera*, the cultivated grape with known susceptibility to most fungal pathogens. VvPGIP1 was shown to be strongly developmentally regulated and tissue-specific in grapevine, but when overexpressed in tobacco, the high levels of PGIP protected the plants against *B. cinerea* in a whole plant infection assay that favoured disease development (Joubert *et al.*, 2006). The grapevine PGIP was purified from the transgenic tobacco and tested for inhibition interactions with the individual ePGs from *Botrytis* and *Aspergillus* (Joubert *et al.*, 2006). In a subsequent study, it became clear that BcPG2, one of the most potent ePGs from *Botrytis* that also acts as a virulence factor, was strongly and convincingly inhibited by VvPGIP1 in an *in vivo* co-infiltration experiment (Joubert *et al*., 2007). This result was pertinent since the previous *in vitro* analyses could not detect any inhibition interaction between VvPGIP1 and BcPG2, indicating that the *in planta* environment in the plant cell wall, provided a context for these two proteins to interact which were not replicated in the *in vitro* experiments (Joubert *et al.*, 2007).

Our approach here relies on *in vivo* analysis of PGIPs and their response by identifying PGIP-specific resistance responses. This approach was very successful for VvPGIP1 (Joubert *et al*., 2006) and was extended to include more grapevine PGIPs, specifically targeting the non-*vinifera Vitaceae* accessions that are known for high levels of disease resistance against pathogens. Previously 37 non-*vinifera* PGIPs were isolated and grouped according to the amino acid sequences in their active sites (Wentzel, 2005). Fourteen of these PGIPs were targeted for overexpression in tobacco (Figure 3.1). The non-*vinifera* PGIPs had greater than 95% sequence homology overall, but displayed amino acid differences (compared to VvPGIP1) in the LRR domains that might influence PGIP activity and specificity towards ePGs (Leckie *et al.*, 1999). Transgenic populations of a sufficient size were only obtained for nine of the fourteen PGIPs and these lines, in comparison with VvPGIP1 line 37 (from Joubert *et al.*, 2006) and the WT, were analysed further in this study. Genetic analysis, protein activity assays and infection assays confirmed PGIP-specific resistance phenotypes against *Botrytis* for all the PGIPs tested (Figures 3.3, 3.4 and 3.5).

The two whole plant infection assays clearly separated the WT, VvPGIP1 line 37 and the non-*vinifera* PGIPs in their disease susceptibility (Figure 3.5 and Table 3.6). The latter group was consistently more effective in protecting the plants against the disease progression, leading to strong resistance phenotypes. After the infection assays were completed and measurement of lesions was stopped, the infected plants were left in the high humidity infection chambers for an additional week. After this time, the lesions on the transgenic PGIP plants did not appear to have spread any further (visual inspection, no measurements were made). From the appearance of the lesions that developed, it was evident that the VvPGIP1 resistance response was very similar to those of the non-*vinifera* PGIPs, but that the latter group was more efficient at curbing the pathogen, leading to smaller lesions on these lines at the end of the infection assays. The diameter of each lesion was measured daily from three dpi onwards during the infection. For each PGIP, all the lesions that formed were used to determine the average lesion diameter for that PGIP per day, i.e. the measurements of all the lesions on every individual plant from all the individual lines of each PGIP were used together to determine the average sizes. This pooling of data was deemed correct for the following reasons: Firstly, data obtained from all the leaves on each plant were grouped together, because although there was a slight age effect (older leaves developed slightly larger lesions than young leaves), this effect was constant across the population (Figure 3.4). Thus comparing separate leaves of the same age would lead to the same relative differences in lesion sizes as determined when pooling the leaves together. Secondly, all the individual plants from the different independent lines for each PGIP

were grouped together, because there was a very similar response seen across the different lines and individual plants.

The results of the two infection assays were similar, but the first experiment progressed faster than the second (11 days until WT leaves were completely infected compared to 15 days for the second experiment). The fast progression of the infection in the first experiment probably reflected the slightly higher humidity levels that were obtained compared to the second experiment. Both data sets and the progression of the disease development were, however, typical of this assay, as optimised and reported on in Joubert *et al*., (2006).

The stronger resistance response of the non-*vinifera* PGIPs and how this relates to the amino acid differences in the LRR domains remains to be elucidated. It would be very useful to analyse the non-*vinifera* PGIPs against a wider range of ePGs and specifically also individual ePGs of *Botrytis* to compare the inhibition interactions and profiles of these PGIPs to the characterised VvPGIP1. Specific studies to understand the structure-function relationship of these PGIPs are also foreseen.

The non-*vinifera* PGIP-lines show down-regulation of a gene involved in cell wall architechture, similar to a pattern observed for VvPGIP1 under non-infecting conditions

PGIPs have been proven to interact with and inhibit ePGs, a process that will protect the plant cell wall, as well as generate elicitor-active molecules that could trigger downstream defence responses in activated response mechanisms (Cervone *et al.*, 1989; Esquerré-Tugayé *et al.*, 2000; De Lorenzo *et al.*, 2001; Aziz *et al.*, 2004; D'Ovidio *et al.*, 2004a). Some of these would include classical mechanisms such as deposition of lignin to strengthen cell walls, increase in hydrolytic enzymes and production of toxic substances (Hammond-Kosack and Jones, 1996; Sticher *et al.*, 1997; Simpson *et al.*, 1998; Federici *et al.*, 2006). All the known PGIP functions have been described in interaction with ePGs and/or in response to wounding or pathogen attack. When we analysed the VvPGIP1 lines that were shown to have a PGIP-specific resistance phenotype, a microarray analysis indicated the surprising result that specific genes linked to cell wall strengthening were differentially regulated between the WT and the transgenic lines tested under uninfecting conditions (Becker, 2007). This would suggest that the presence of PGIP in higher concentration due to the constitutive overexpression influenced a process whereby one of the classical activated defence responses were initiated, perhaps preparing the plants for possible attack.

In this study, the non-*vinifera* PGIPs delivered transgenic tobacco lines with very strong resistance phenotypes against *Botrytis*. A subset of lines was used to evaluate whether these lines exhibited the same down-regulation of the *xth* gene in grapevine PGIP overexpressing lines. As found with the VvPGIP1 lines tested, the tobacco XTH was always downregulated in all transgenic lines tested (Figure 3.6). It has been shown that down-regulation of this gene leads to cell-wall strengthening (Herbers *et al.*, 2001). It was also shown that a reduction of XTH activity in the leaves lead to a shift towards xyloglucan with a higher molecular weight, resembling stronger cell walls found in older leaves (Herbers *et al.*, 2001). Saladié *et al.* (2006) also showed that XTHs are important for the regulatory mechanisms controlling cell wall strength, extensibility and tissue integrity. The biological relevance of this observation is currently investigated further, but it has been confirmed now in all transgenic tobacco lines overexpressing grapevine PGIPs. Moreover, the same effect was seen with three different PGIPs (VvPGIP1, PGIP from *V. doaniana Munson* and *V. caribeae*).

In combination, these findings significantly support grapevine PGIPs as potent antifungal proteins and also highlight the importance of investigating the natural variation in PGIP structure

that might be present in the grapevine genepool and how it relates to the functions of these genes. The whole plant infection assays, in combination with the confirmation that the PGIP-specific resistance phenotype is probably linked to changes in the cell wall, provide significant momentum towards understanding a possible additional role of PGIPs in preparing plant cells for pathogen attach when present at high levels. Further work includes enzymatic activity analyses of the enzymes involved in cell wall metabolism in the PGIP-specific resistance phenotypes and cell wall profiling in comparison with the WT controls.

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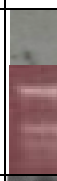
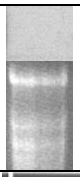
Addendum A to Chapter 3

This Addendum contains the results of the genetic analyses of all putative PGIP-overexpressing lines generated during this study. Wild type tobacco without any PGIP activity has been used as a negative control throughout the analyses, and VvPGIP1 line 37 has been used as a positive control with known active PGIP overexpression. Included in this Addendum are the PCR screens (to test for transgene presence), Southern blots (to determine transgene copy number and possible clonality of plant lines), Northern blots (to confirm transgene expression) and an agarose diffusion PGIP activity assay (Figure A1)

The PCR screen and Southern blot analyses were carried out as described in Section 3.3.4.1 of Chapter 3. The size of the PCR products is 1000 bp. The genomic DNA digestion for Southern hybridisation was performed in such a way that each single band on the Southern blot corresponds to one copy of the *pgip* transgene, i.e. a blot with two bands indicates that that plantline contains two copies of the transgene within its genome. The minimum size of each band is 1000 bp.

The northern blot analyses are described in Section 3.3.4.2 of Chapter 3, and the size of the RNA transcripts on the positive northern blots is 1000 bp.

The agarose diffusion assay used to confirm PGIP activity is described in Section 3.3.4.3. A clearance zone around the well in the agarose matrix indicates that there is no PGIP activity in the crude protein extract of that plant line, while no clearance zone or a significantly smaller zone is indicative of PGIP activity.

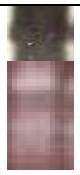
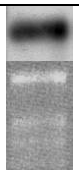
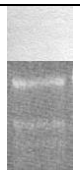
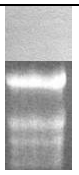
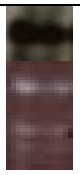
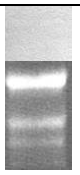
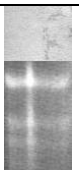
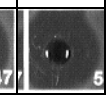
Plant line	WT	VvPGIP1 line 37	PGIP 1004 line 1	PGIP 1004 line 2	PGIP 1004 line 3	PGIP 1004 line 4	PGIP 1004 line 5	PGIP 1004 line 6	PGIP 1004 line 7	PGIP 1012 line 2	PGIP 1012 line 3	PGIP 1012 line 4
PCR screen												
Southern Blot			n.d.	n.d.		n.d.			n.d.	n.d.	n.d.	n.d.
Integration	0	1			>4		2	2				
Northern Blot												
RNA												
PGIP activity assay												

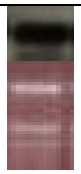
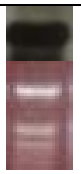
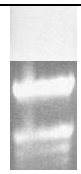
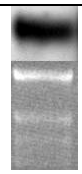
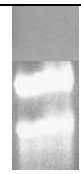
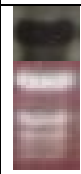
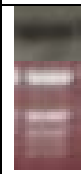
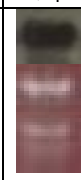
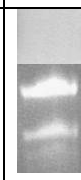
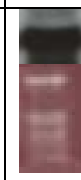
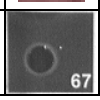
Plant line	PGIP 1012 line 5	PGIP 1012 line 6	PGIP 1012 line 7	PGIP 1012 line 8	PGIP 1012 line 9	PGIP 1012 line 10	PGIP 1012 line 11	PGIP 1012 line 12	PGIP 1012 line 13	PGIP 1012 line 14	PGIP 1012 line 15	PGIP 1012 line 16
PCR screen												
Southern Blot	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	

Integration											1		1

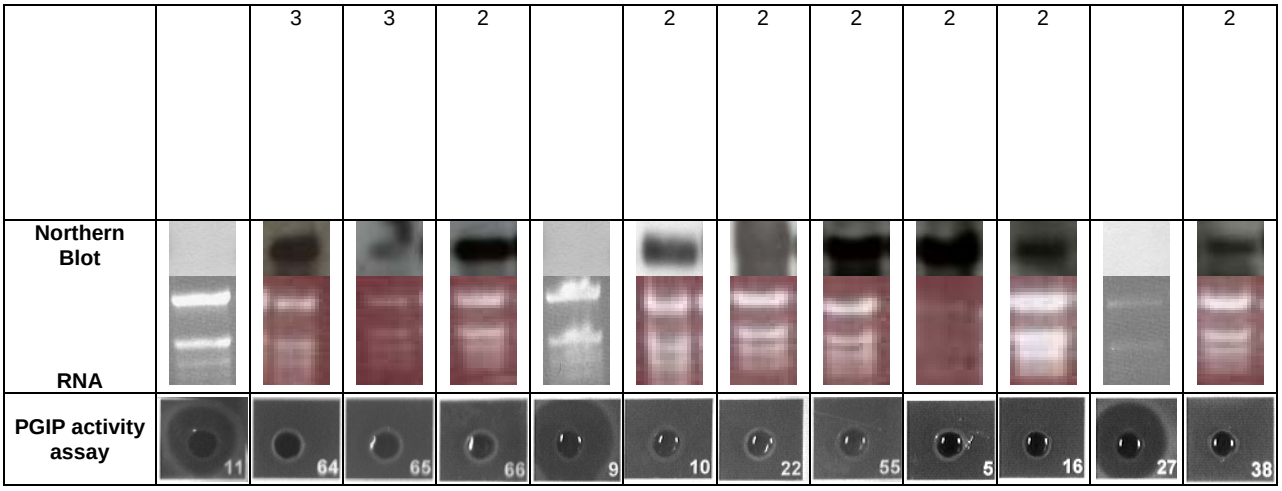
Plant line	**PGIP 1012 line 5**	**PGIP 1012 line 6**	**PGIP 1012 line 7**	**PGIP 1012 line 8**	**PGIP 1012 line 9**	**PGIP 1012 line 10**	**PGIP 1012 line 11**	**PGIP 1012 line 12**	**PGIP 1012 line 13**	**PGIP 1012 line 14**	**PGIP 1012 line 15**	**PGIP 1012 line 16**
Northern Blot **RNA**												
PGIP activity assay												
Plant line	PGIP 1012 line 17	PGIP 1018 line 2	PGIP 1018 line 3	PGIP 1018 line 4	PGIP 1018 line 5	PGIP 1018 line 6	PGIP 1024 line 1	PGIP 1024 line 2	PGIP 1024 line 3	PGIP 1024 line 4	PGIP 1024 line 5	PGIP 1024 line 6
PCR screen **Southern Blot**					n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.
Integration	3	>4	1	3				2				
Northern Blot **RNA**												
PGIP activity assay												

Plant line	**PGIP 1024 line 7**	**PGIP 1024 line 8**	**PGIP 1024 line 9**	**PGIP 1024 line 10**	**PGIP 1024 line 14**	**PGIP 1024 line 16**	**PGIP 1024 line 17**	**PGIP 1030 line 1**	**PGIP 1030 line 2**	**PGIP 1030 line 3**	**PGIP 1030 line 5**	**PGIP 1030 line 6**
PCR screen												
Southern Blot			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.

Integration	 >4	 2							 2			
Northern Blot												
RNA												
PGIP activity assay	 16	 27	 38	 49	 60	 71	 82	 14	 23	 36	 47	 5

Plant line	**PGIP 1030 line 7**	**PGIP 1030 line 8**	**PGIP 1030 line 9**	**PGIP 1030 line 10**	**PGIP 1030 line 11**	**PGIP 1038 line 1**	**PGIP 1038 line 4**	**PGIP 1038 line 5**	**PGIP 1038 line 6**	**PGIP 1038 line 8**	**PGIP 1038 line 9**	**PGIP 1056 line 1**
PCR screen												
Southern Blot			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.			n.d.	
Integration	>4	>4							3	>4		2
Northern Blot												
RNA												
PGIP activity assay	 67	78	9	102	11	24	79	68	94	106	18	51

Plant line	**PGIP 7000 line 1**	**PGIP 7000 line 2**	**PGIP 7000 line 3**	**PGIP 7000 line 4**	**PGIP 7060 line 1**	**PGIP 7060 line 2**	**PGIP 7060 line 3**	**PGIP 7060 line 4**	**PGIP Ramsey line 1**	**PGIP Ramsey line 2**	**PGIP Ramsey line 3**	**PGIP Ramsey line 4**
PCR screen												
Southern Blot	n.d.				n.d.						n.d.	
Integration												



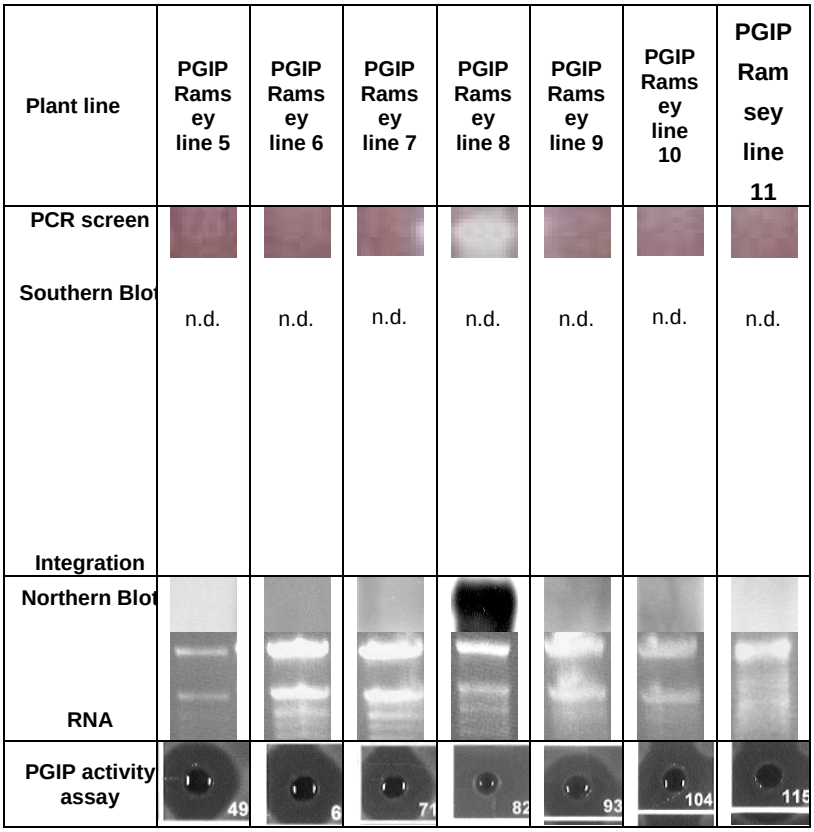


Figure A1 *Analysis of putative transgenic PGIP-overexpressing tobacco population.* The PCR screen was carried out with Vvpgip1-specific primers. Southern blot analysis was carried out only on plantlines used for subsequent functional analyses of the PGIP genes. Plantlines for which Southerns were not determined are indicated n.d. Integration copy number is indicated as either 0, 1, 2, 3 or >4 copies. Northern analysis was carried out on all the plant lines with ‘n VvPGIP1-specific probe. Total RNA extracted is also shown. PGIP activity was determined with an agarose diffusion assay using PGA as a substrate for crude *B. cinerea* PGs that were added to the agarose matrix along with crude extracted PGIP. A clear zone indicated enzymatic digestion of the PGA substrate by PG. The activity of PGIP on the PGs results in a reduced clearance zone, or no zone at all being formed.

Addendum B to Chapter 3

This Addendum contains all the data collected by real-time qRT-PCR of relative expression of cell wall synthesis genes in transgenic tobacco. The assay is described in Section 3.3.6 of Chapter 3. A sub-population of PGIP-overexpressing tobacco lines that displayed PGIP-specific resistance phenotypes were analysed by qRT-PCR for the expression of a *xyloglucan-endotransglycosylase* (*xth*) gene as well as the *pgip* transgene. Seven different plant lines were used in this experiment: one line overexpressing VvPGIP1 (line 37), three lines overexpressing PGIP 1012 (line 14, 16 and 17), two lines overexpressing PGIP 1038 (line 6 and 8) and wild-type untransformed tobacco. Three individual plants from each plant line were used, designated either A, B or C (for PGIP 1012 line 17, there were only two individual plants available: A and B). A complete biological repeat of all the A plants was included (i.e. a second RNA extraction and cDNA synthesis) and a technical repeat of the B plants were included for the *xth* gene (i.e. a second PCR reaction from the same cDNA pool). Calculation of relative expression levels was done as described in Section 3.3.6. The results presented here show the relative gene expression levels for each individual plant used in this assay, either relative to the wild-type plants (for *xth*) or relative to the Vv*pgip*1-overexpressing control line (for *pgip*) (Figure B1).

Figure B1 (A) shows that all the transgenic lines showed high levels of PGIP expression that were comparable to the level of overexpression in the VvPGIP1-overexpressing control line (line # 37), while in the wild-type plants no significant PGIP expression was detected. The variation of relative expression levels was quite pronounced within each group of plants, for example the A, B and C individual plants of each PGIP line did not display the same expression levels. This is probably due to small differences in the growth conditions of the individual plants. Although the plants were kept in the same greenhouse, ambient light and humidity conditions within the room were not always constant, for example some positions within the chamber received more direct sunlight than others. Care was taken to rotate the plants on a regular basis, but the precise conditions for each plant might have differed slightly. For the plants designated A , a biological repeat consisting of another mRNA extraction and subsequent cDNA synthesis process from the same stored tissue sample was done. The resultant expression levels for the biologic repeats were comparable to that of the original analysis.

Figure B1 (B) shows that the *xth* expression levels of the transgenic population were consistently lower than that of the wild-type plants. The variation of expression levels within each group was quite varied between the A, B and C individual plants, as was also the case with the pgip-expression analysis. It appears as if the expression level variation within the two PGIP 1038 lines was less than that within the PGIP 1012 lines. As for the *pgip*-expression analysis, a biological repeat was done for the plant lines designated A, consisting of another mRNA extraction and subsequent cDNA synthesis process from the same stored tissue sample. The *xth*-expression levels detected for the A plants varied considerably form that of the original analysis, for reasons not known. It might be possible that the stored tissue samples was not completely homogenously mixed. Two technical repeats were also done, one for the B plants and one for the C plants, in which the same cDNA samples that were synthesized previously were used in another qRT-PCR reaction. The results of the two technical repeats were comparable to the original analysis.

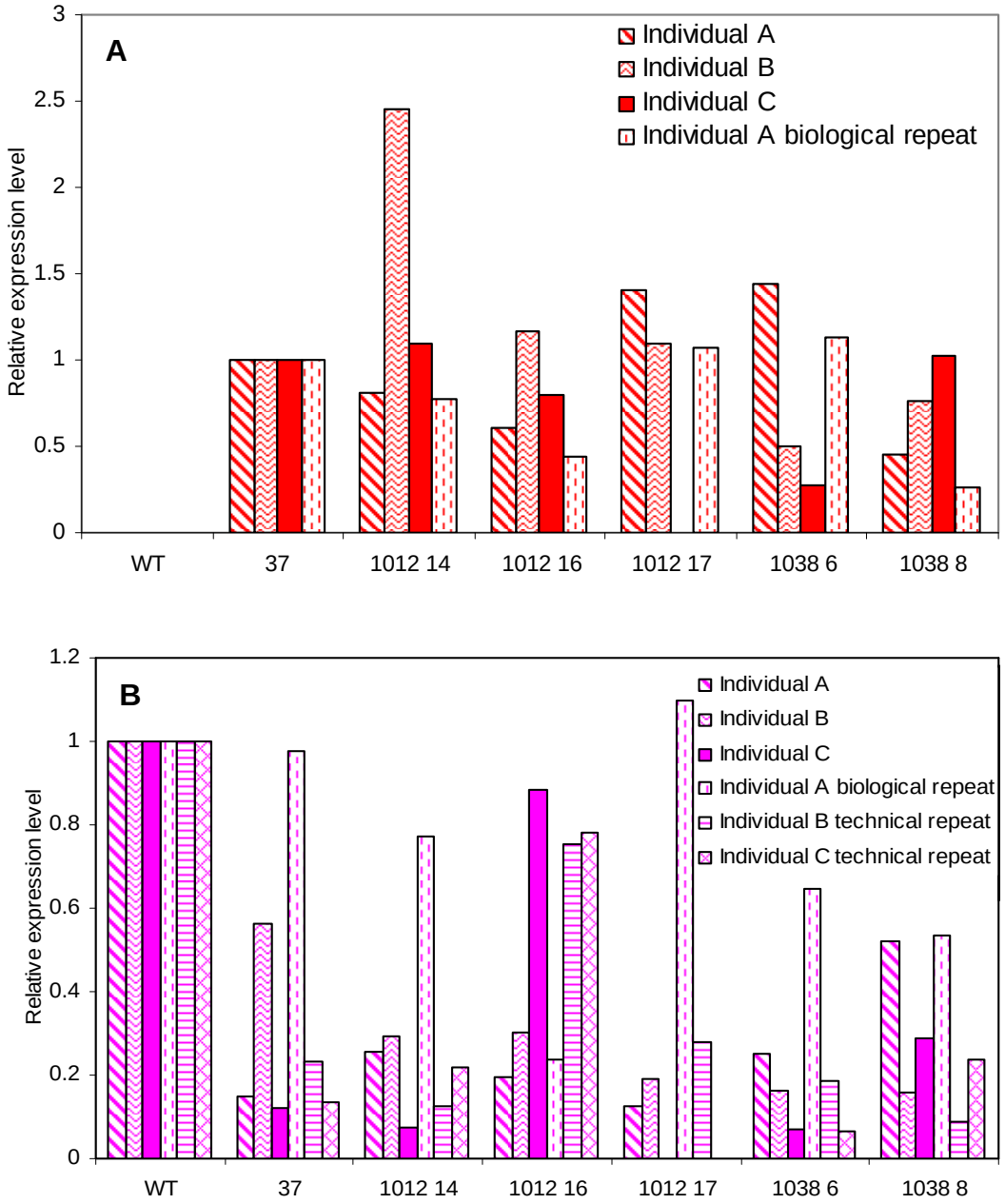


Figure B1 *Relative expression levels of all individual plants assayed*. **A** is the expression of *pgip* relative to that of VvPGIP1 line 37. The VvPGIP1 line 37 was used as a reference and thus received an expression level of 1; all other data was expressed relative to this reference value. **B** is the expression of *xth* relative to that of the wild-type plants. The VvPGIP1 line 37 was used as a reference and thus received an expression level of 1; all other data was expressed relative to this reference value.

4: General Discussion and Conclusion

4.1 General Discussion

With the global population expanding rapidly, the earth will soon be filled to capacity and every acre of fertile soil will be enormously valuable. Producing enough food to feed the multitudes is becoming more of a challenge with each passing year, and factors like climate change due to global warming are also putting extra strain on agriculture. Enormous losses to crops are also caused by pathogens that destroy or severely damage produce before and after harvest. Strategies to enhance the yield, nutritional value and disease resistance of agriculturally important plants are consequently essential.

Genetic engineering as a plant improvement strategy has great potential. It allows for very specific and targeted changes to plant species that would otherwise take very long to achieve with traditional breeding and crossing strategies. In traditional breeding it is also very difficult to improve a species for a specific trait in such a way that other, unwanted traits are not incorporated as well. There is still a 'Frankenscience' stigma clinging to genetic enhancement in many societies, but despite this genetically modified crops are becoming widespread and the field of Biotechnology is expanding rapidly. One of the important focuses of genetic engineering is to reduce the susceptibility of plants to pathogens that cause losses of yield in agriculture.

Fungal pathogens in particular cause great damage to crops and resistance to fungal pathogens would be a great advantage. Fungi rely heavily on cell wall degrading enzymes (Cooper, 1984; Idnurm and Howlett, 2001; Ten Have *et al.*, 2002), and especially endopolygalacturonases (ePGs) (Shieh *et al.*, 1997; Ten Have *et al.*, 2002; Kars *et al.*, 2005) for their pathogenesis, and plants in turn rely on inhibitors of cell wall degrading enzymes to combat pathogenic CWDE attack (Hammond-Kosack and Jones, 1996, 1997; Maleck and Lawton, 1998). Among these inhibitors, polygalacturonase-inhibiting protein (PGIP) has been shown to be instrumental in plant resistance strategies against fungi (Powell *et al.*, 2000; De Lorenzo *et al.*, 2001; De Lorenzo and Ferrari, 2002).

PGIPs facilitate plant resistance to pathogenic fungi in a number of different ways. Firstly and most importantly, they directly bind to and inhibit fungal ePGs (Federici *et al.*, 2001; D'Ovidio *et al.*, 2004; Di Matteo *et al.*, 2006), thus preventing the ePGs from degrading the plant cell wall. This direct inhibition interaction between PGIP and ePG has been shown to be differential with regards to specificity, mode of binding and kinetics (Abu-Goukh *et al.*, 1983; Lafitte *et al.*, 1984; Cervone *et al.*, 1987; Johnston *et al.*, 1993; Yao *et al.*, 1995; Stotz *et al.*, 2000; De Ascensao, 2001; James and Dubery, 2001; Mattei *et al.*, 2001; King *et al.*, 2002; Deo and Shastri, 2003; Sicilia *et al.*, 2005), and depends on specific key amino acid residues in the PGIP active domain (Leckie *et al.*, 1999; Spinelli *et al.*, 2008). *In vitro* studies have shown that PGIPs prolong the lifetime of elicitor active oligogalacturonic acid fragments (OGAs) that are released from the plant cell wall by ePGs during attack (Cervone *et al.*, 1989; Ridley *et al.*, 2001; De Lorenzo and Ferrari, 2002). There is not yet any evidence to confirm that this effect is also evident *in planta*. PGIP has also been implicated to possibly 'prime' the plant cell before pathogen attack takes place, by facilitating strengthening of the cell wall (Becker, 2007) when PGIP is present at high levels (Becker, 2007).

PGIP overexpression has resulted in enhanced disease resistance towards the necrotrophic fungus *Botrytis cinerea* in a variety of heterologous host plants (Powell *et al.*, 2000; Ferrari *et al.*, 2003; Agüero *et al.*, 2005; Manfredini *et al.*, 2005; Joubert *et al.*, 2006, 2007; Janni *et al.*, 2008). Finding a PGIP with a very broad inhibition spectrum and very strong inhibitory effects, or being able to engineer such a PGIP if amino acid residues important to ePG inhibition can be identified, and overexpressing it in economically important crops can possibly lead to great reductions in crop losses due to fungal infection. A better understanding of the role that PGIPs play in plant defence will also contribute to better strategies against fungal attack.

To that end, the aim of this study was to functionally analyse and compare a set of grapevine PGIPs for their ability to decrease disease susceptibility against *Botrytis cinerea*. These non-*vinifera* grapevine PGIPs were previously isolated (from grapevine species highly resistant towards fungal pathogens) and compared on amino acid sequence level, and were shown to be very homologous to each other and to VvPGIP1 (De Ascensao, 2001), but to differ from one another at a number of amino acid residues within their leucine-rich repeat active domains. These non-*vinifera* pgip genes were overexpressed in *Nicotiana tabacum* in order to create a resource for the *in planta* study of the role of these PGIPs in plants defence and their effectiveness as resistance molecules. Whole-plant infections of a sub-population of transgenic PGIP overexpressing plant lines were carried out to evaluate the disease resistance capabilities of these non-*vinifera* PGIPs, and analysis of the expression levels of a xyloglucan endotransglycosylase gene that is involved in cell wall metabolism was also done in uninfected transgenic tobacco to investigate the role of PGIP in cell wall strengthening.

4.1.1 The overexpression of a number of grapevine PGIP-encoding genes from resistant species resulted in transgenic plant lines that all show enhanced resistance against the fungus *Botrytis cinerea*

In Joubert *et al*. (2006) a whole-plant time-course infection assay was developed for infecting tobacco plants overexpressing VvPGIP1 with *Botrytis cinerea* and using lesion size measurements as an indication of the level of resistance/susceptibility of a plant. This same assay was employed in this study to measure the levels of resistance towards *B. cinerea* of transgenic tobacco lines overexpressing nine different *pgip*-encoding genes from grapevine species highly resistant to fungal pathogens. One plant line overexpressing VvPGIP1 from the susceptible *Vitis vinifera* grapevine species was infected as well as a reference plant line. The VvPGIP1 transgenic line showed a significant reduction of infection lesions and symptoms compared to untransformed wild-type plants, as was expected according to previous experimental results. In addition, all nine PGIPs from the more resistant grapevine sources also showed a significant decrease in symptom development and lesion size. Interestingly, and surprisingly, *all* of the other PGIPs performed better than VvPGIP1 in reducing *B. cinerea* infection. This result was not expected, and cannot be readily explained simply by studying the amino acid changes in the active domains of the PGIPs. All nine PGIPs differ from VvPGIP1 by between three and nine amino acid residues, but no two PGIPs differ from VvPGIP1 in the same positions. Also, PGIP 1012, which showed good resistance against *B. cinerea,* does not differ from VvPGIP1 in the sequence of its active domain at all. Since all the PGIPs were overexpressed under control of the same promoter, including the VvPGIP1 reference line, the differential disease resistance cannot be attributed to differential *pgip* gene

expression in the transgenic plants. Transgene copy number varied within the transgenic population, with some lines containing only single copies of the transgene, and others containing more than 4 copies. The transgene copy number could however not be correlated to level of disease resistance, with single-copy plant lines performing on average just as well as lines with multiple copies. Further downstream effects that PGIP has on the plant, like altered cell wall metabolism (see 4.1.2 below) may be responsible for the enhanced resistance conferred by these alternative PGIPs. It is also known that PGIP binds to pectin (Spadoni *et al.*, 2006), so it is possible that the alternative PGIPs all share a common structural change that facilitates better binding to the cell wall, and thereby causing a physical blockage so that ePG cannot readily reach the cell wall.

4.1.2 The cell wall metabolism of healthy PGIP-overexpressing transgenic plant lines is altered from that of the wild-type

Two of the set of more resistant PGIPs (1012 and 1038), VvPGIP1 and wild-type tobacco plants were analysed for differential xyloglucan endotransglycosylase gene expression levels – healthy plants that were not infected with a pathogen were analysed. A uniform down-regulation of a gene encoding xyloglucan endotransglycosylase (XTH) was observed in the transgenic plant lines. Expression levels were on average 0.4 times that of the wild-type in VvPGIP1 plants, and 0.38 times and 0.18 times that of the wild-type in the two alternative PGIP plants. XTH activity increases the elasticity of the plant cell wall (Herbers *et al.*, 2001), as the enzyme cleaves the bonds between lignin polymers and hemicellulose. Therefore, a downregulation of XTH means that the cell wall is stronger and more rigid. It appears that the overexpression of PGIP in tobacco could lead to a cell-wall strengthening phenotype that would also enhance the resistance of these plants towards CWDEs.

4.2 Conclusions and Future prospects

In this study, the successful overexpression of *pgip* genes from grapevine cultivars highly resistant to fungal pathogens, the determination of disease resistance of the resultant transgenic lines against the fungus *B. cinerea*, and the determination of down-regulated expression levels of an XTH enzyme involved in cell wall strengthening in the uninfected transgenic lines was achieved. In so doing, a valuable resource for the further investigation of the *in vivo* role of PGIPs in plant defence was created.

The high levels of resistance against *B. cinerea* observed within a subset of the transgenic population should be further investigated by determining the interaction of these PGIPs with the individual ePGs of *B. cinerea* as well. Different ways to achieve this could include transient infiltration of the transgenic PGIP-overexpressing plants with *Agrobacterium* strains expressing the individual ePG enzymes, co-infiltration of *Agrobacterium* strains expressing one of the ePGs and one of the PGIPs, or isolation of the PGIPs from transgenic plant tissue followed by *in vitro* enzyme-inhibition assays with the individual *B. cinerea* ePGs.

The range of fungal resistance of the transgenic population could be further investigated by additional infection assays with a number of different fungal plant pathogens, and specifically fungal grapevine pathogens, that rely on cell-wall degradation as their attack strategies. The usefulness of

PGIPs as antifungal agents in agriculture could be investigated by selecting one or more of the non-*vinifera pgip*-encoding genes for overexpression in *Vitis vinifera* cultivars.

The down-regulation of a cell-wall strengthening XTH enzyme observed in a subset of the transgenic lines and the differential regulation of cell-wall metabolism genes detected by Becker (2007) indicate that it would be useful to further analyse the cell wall structure and/or metabolism within these lines. For instance, a more extensive qRT-PCR analysis that includes more cell-wall metabolism target genes could be done, quantitative lignin assays could be performed (as was done with VvPGIP1-overexpressing lines in Becker (2007)) to determine differences in the amount of lignin in the cell walls of the transgenic plants, or the activities of other cell-wall metabolism enzymes could be determined with specific *in vitro* activity assays.

There are many bioinformatical tools available that could also aid the investigation of the specific ePG:PGIP inhibition interaction, for example software could be used to predict the interaction surfaces of the PGIPs, the binding or inhibition with individual ePG enzymes, or the binding or interaction with the plant cell wall components, based on the amino acid sequences of the proteins.

The intricate involvement of PGIP in plant defence, and the successes obtained from a range of studies with heterologous overexpression of *pgips* (including this study), merits a continuous focus on these proteins as defence targets in plant-pathogen interactions.

4.3 References

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